Workshop in Diagnostic Immunohistochemistry

Oud St. Jan/ Old St. John – Brugge (Bruges), Belgium June 13th – 15nd 2018

The technical test approach

Pre-Analytical - Analytical (I & II) - Post Analytical phase

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The technical test approach – Analytical phase

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 factors (excentric to the tissue block) can be modified and controlled within the immunohistology 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

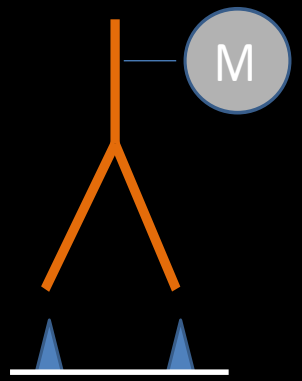
Post-analytic phase

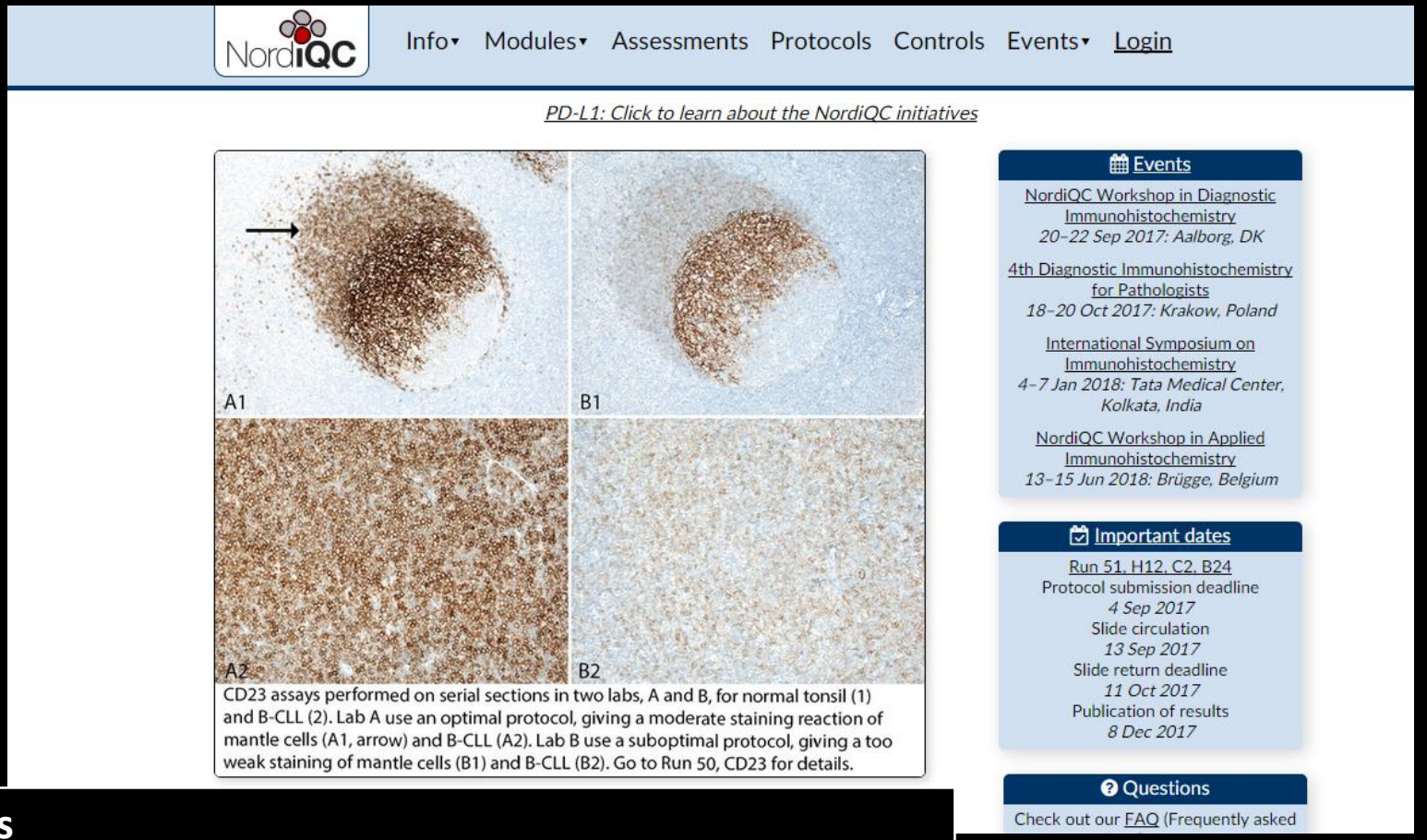
Design of controls
Critical stain indicators
Internal/External control
Interpretation
Positive/Negative
Localization
Quantification
Cutt-of levels
Reporting

The total test paradigm
Key elements in the immunohistochemical procedure

The technical test approach – Analytical phase

Immunohistochemistry – A simple technique ?





External Quality Assurance programs

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

The technical test approach – Analytical phase

Optimization of the IHC assay – issues to be addressed

- Purpose and/or "fit-for-purpose" of the IHC test
- How to establish "best practice protocol" of the IHC test (Calibration of the IHC assay with focus clone, antigen retrieval, titer & detection system)
- How to validate (technical) the IHC-test
  - Is the IHC test reproducible/robust (preanalytic 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 these processes (calibration, validation and controls)

The technical test approach – Analytical phase

Purpose

What do we want to detect and what is the intended use of the assay ?

"Fit-for-purpose"

Describes an assay that has been successfully validated for the intended use at the time the assay was developed combining both laboratory and clinical definitions.

In other words: An assay that is "fit-for-purpose" is good enough to do the job it was designed to do

Expectations of the biomarkers/assays:

It may or will improve diagnosis
It may or will define disease subsets that may differ in response to therapy.
It may or will provide early clues regarding response to therapy.
It may or will define individual variability in the drug's molecular target

The technical test approach – Analytical phase

Immunohistochemistry: Calibration of a biomarker/antibody may vary depending on IHC-type (1&2)

IHC-type 1 markers (Diagnostic)

Often calibrated to produced the highest 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 …….)

IHC: Technical considerations to intended use and “fit-for-purpose” approach

Do we have the right antibody (IHC type markers 1 & 2) – can it provide appropriate sensitivity and specificity

Does the antibody work on the chosen automatic platform(s)

Does the automatic platform come with appropriate reagents fulfilling purpose and intended use of the IHC assay

- **Appropriate Antigen Retrieval solutions (enzymes and HIER buffers)**
- **Appropriate antibody diluents and wash buffers**
- **Appropriate detection and visualization products**
- **Appropriate protocol library**

Do we have access to appropriate tissue, reflecting the range of different antigen expression levels, both for the optimization process but also for the laboratory`s internal quality assurance program (control tissue) – monitoring specificity and sensitivity of the assays

The technical test approach – Analytical phase

An assay should be calibrated so it "fit-for-purpose"

MART1/Melan A (MLA)

Malignant melanoma → Optimal results (NQC run 49)

mAb A103
mAb BS52
mAb M2-7C10
rmAb EP43

Steroid producing tumors (e.g. Granulosa cell tumor of the ovary) → Optimal results (NQC run 49)

mAb A103

MART1/Melan A NQC results (Run 49) – conclusions and challenges

Melan A clone A103: Optimal result is difficult to obtain on the platforms Dako Omnis or Ventana Benchmark (HRP conjugated detection systems) ?

RTU product mAb A103 (IS/IR633,Dako) developed for the Autostainer was used on the Omnis - 13 % suff. (2 of15)

mAb A103 MLA RTU system (790-2990. Ventana):

- **UltraView-AP as detection system = pass rate of 7% (recommended protocol settings by the vendor)**
- **UltraView-AP with amplification = pass rate of 100%.**

The recently introduced rmAb clone EP43 showed promising performance as optimal results were seen on both the Ventana Benchmark and Dako Omnis platforms – steroid producing tumors ?

Control material

- **mAb A103 versus rmAb EP43, mAb BS52 & M2-7C10 ?**

Other melanocytic lesions (e.g. ……..) ?

RTU IR/IS633 (Autostainer)

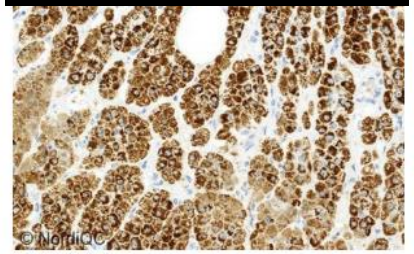


Fig. 1a (x200)
Optimal MLA staining of the adrenal gland using the mAb clone A103 in a RTU format (Dako IR633/IS633) with an incubation time of 20 min., HIER in TRS High pH 9 for 10 min., 2-step polymer based detection kit (EnVision Flex) and performed on Autostainer Link, Dako. Virtually all cortical epithelial cells show a moderate to strong, distinct, granular cytoplasmic staining reaction. No background reaction is seen.
Also compare with Figs. 2a – 4a, same protocol.

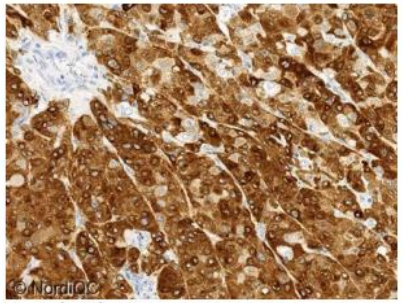


Fig. 2a (x200)
Optimal MLA staining of the malignant melanoma, tissue core no. 4 (high-level expressor), using same protocol as in Fig. 1a. All the neoplastic cells show a moderate to strong cytoplasmic staining reaction.
No background reaction is seen.

RTU IR/IS633 (Omnis)

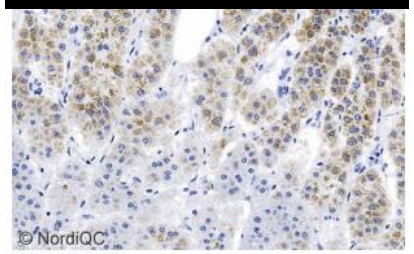


Fig. 1b (x200)
MLA staining of the adrenal gland using an insufficient protocol. Using the mAb clone A103 in a RTU format (Dako IR633/IS633) in similar settings as in Fig. 1a, but on the Dako OMNIS instrument. The majority of cortical epithelial cells are demonstrated, but the intensity is significantly reduced. Compare with Fig. 1a. – same field.
Also compare with Figs. 2b - 4b – same protocol.

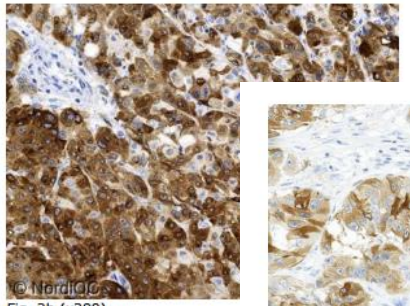


Fig. 2b (x200)
MLA staining of the malignant me
4 (high-level expressor), using sa
1b – same field as in Fig. 2b. The
cells are demonstrated, but the ir
reduced compared to Fig. 2a.
However, compare with Fig. 3b a

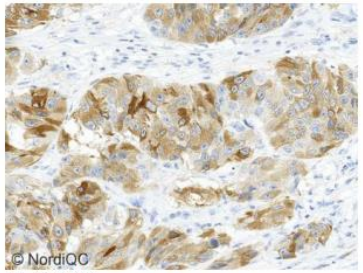


Fig. 3a (x200)
Optimal MLA staining of the malignant melanoma, tissue core no. 5 (low-level expressor), using same protocol as in Figs. 1a and 2a.
The majority of neoplastic cells show a weak to moderate cytoplasmic staining reaction.
No background reaction is seen.

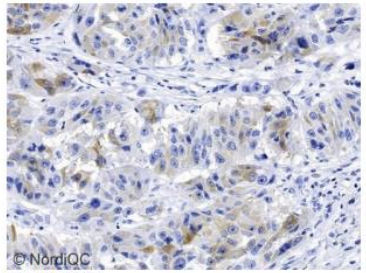


Fig. 3b (x200)
Insufficient MLA staining of the malignant melanoma, tissue core no. 5 (low-level expressor), using the same protocol as in Figs. 1b and 2b.
Only a few scattered neoplastic cells display a very faint staining reaction. Compare with Fig. 3a – same field.

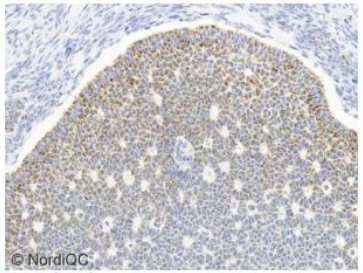


Fig. 4a (x200)
Optimal MLA staining of the granulosa cell tumor using same protocol as in Figs. 1a - 3a.
The majority of the neoplastic cells show a weak to moderate granular cytoplasmic staining reaction.
No background reaction is seen.

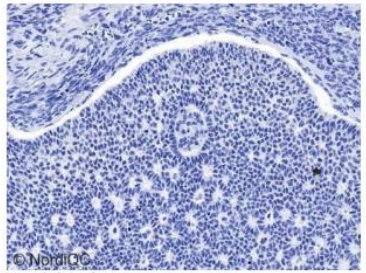


Fig. 4b (x200)
Insufficient MLA staining of the granulosa cell tumor using same protocol as in Figs. 1b - 3b - same field as in Fig. 4a.
All the neoplastic cells are negative.

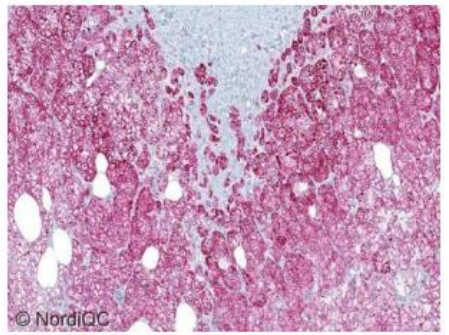


Fig. 5a (x100)
Optimal MLA staining of the adrenal gland using the mAb clone A103 in a RTU format (790-2990) with an incubation time of 32 min., HIER in **CC1 for 64 min.**, and **UltraView AP-RED (760-501) with amplification** as detection system and performed on the BenchMark Ultra. Virtually all cortical epithelial cells show a moderate to strong, distinct, granular cytoplasmic staining reaction.

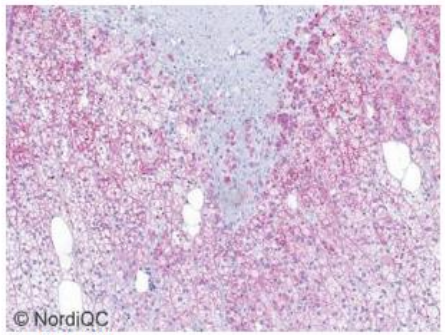


Fig. 5b (x100)
Insufficient MLA staining of the adrenal gland using the mAb clone A103 in a RTU format (790-2990) with an incubation time of 32 min., HIER in **CC1 for 36 min.**, and **UltraView AP-RED (760-501)** as detection system and performed at the BenchMark Ultra. The combination of relative short HIER and a detection system without amplification results in a significantly reduced intensity and proportion of cortical epithelial cells demonstrated.
Compare with Fig. 5a (same field)

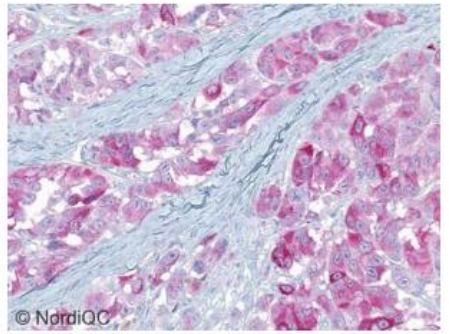


Fig. 6a (x200)
Optimal MLA staining of the malignant melanoma, tissue core no. 5 (low-level expressor), using same protocol as in Fig. 5a.
The majority of neoplastic cells show a weak to moderate cytoplasmic staining reaction.
No background reaction is seen.

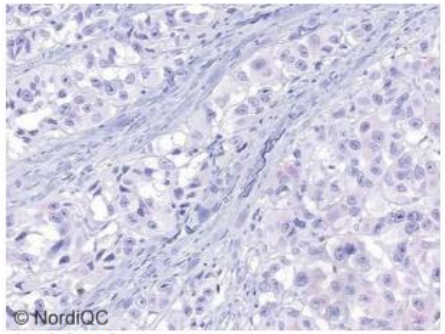


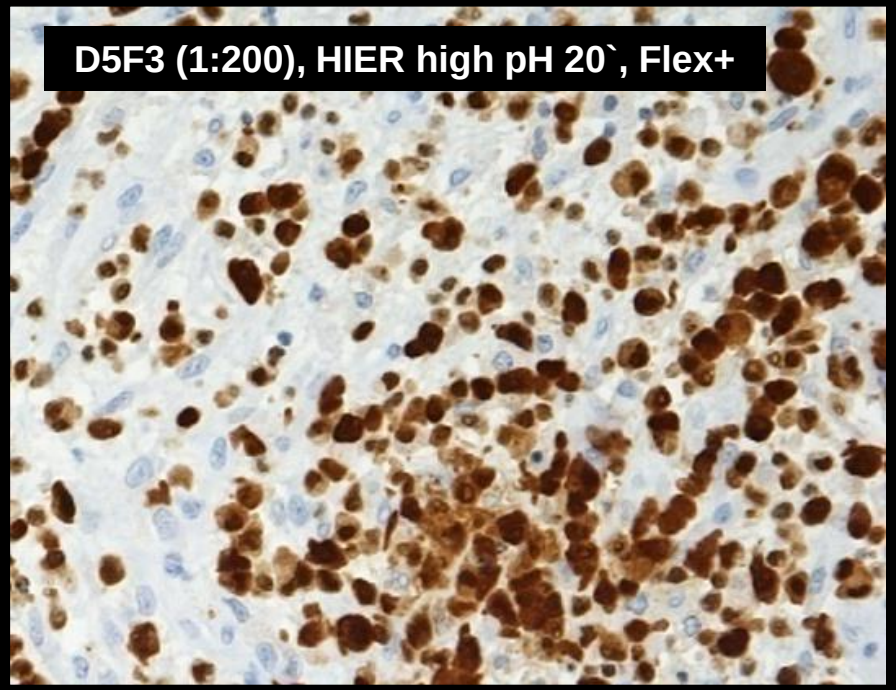
Fig. 6b (x200)
Insufficient MLA staining of the malignant melanoma, tissue core no. 5 (low-level expressor), using the same protocol as in Figs 5b.
The neoplastic cells are false negative.
Compare with Fig. 6a – same field.

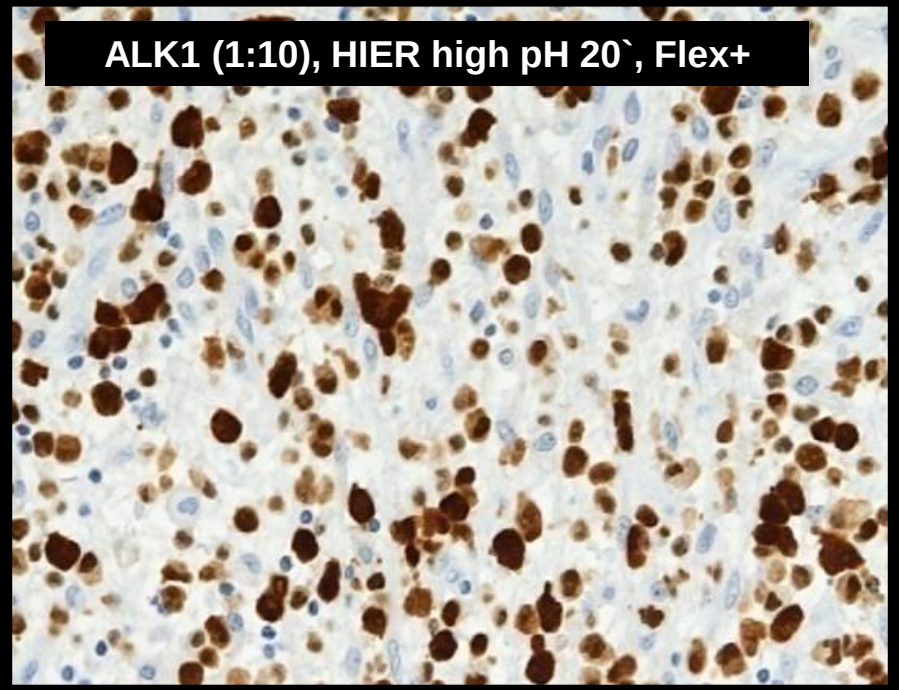
Vendor recommend protocol settings

The technical test approach – Analytical phase

Anaplastic lymphoma kinase (ALK)

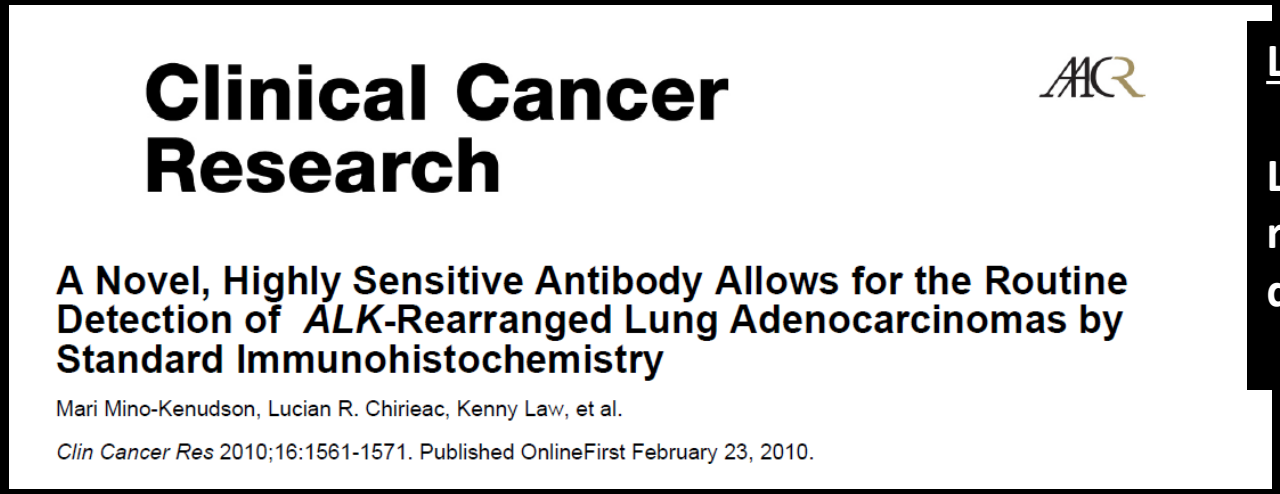
Anaplastic large cell lymphoma (ALCL) (ALK-NPM rearrangement)





Anything wrong ?

The technical test approach – Analytical phase



Clinical Cancer Research

AACR

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

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

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

Lung tumors

Low concentration of fused protein = require a high 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

The technical test approach – 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 1 1	Leica/Novocastra Thermo/NeoMarkers Monosan Abcam Biocare Zytomed	24	16	13	1	74%	81%
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 1	Cell Signaling PrimeBioMed	18	2	1	1	91%	95%
rmAb clone **SP8**	2	Thermo/NeoMarkers	0	0	1	1	-	-
Ready-To-Use antibodies								
mAb clone **5A4 PA0306**	3	Leica/Novocatra	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.

Don`t use clone ALK1 to detect ALK rearranged lung adenocarcinomas

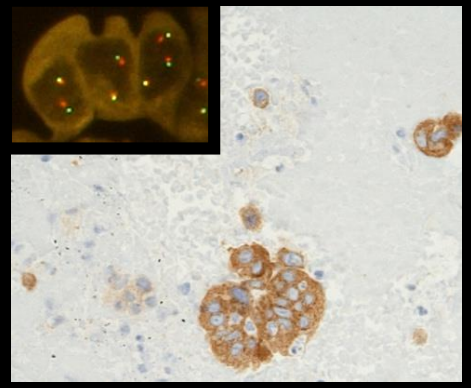
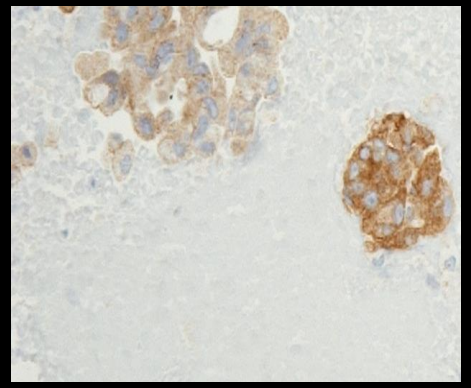
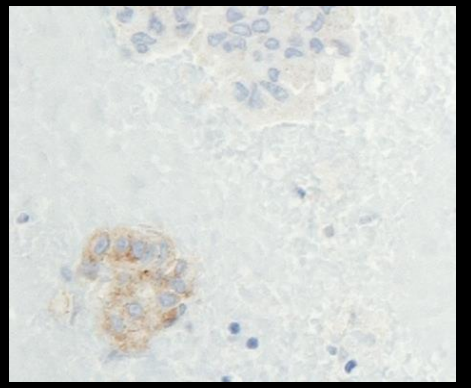
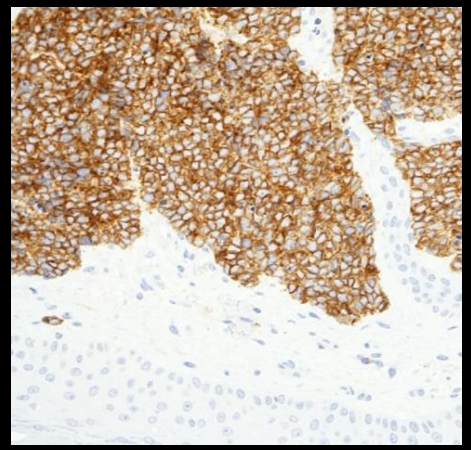
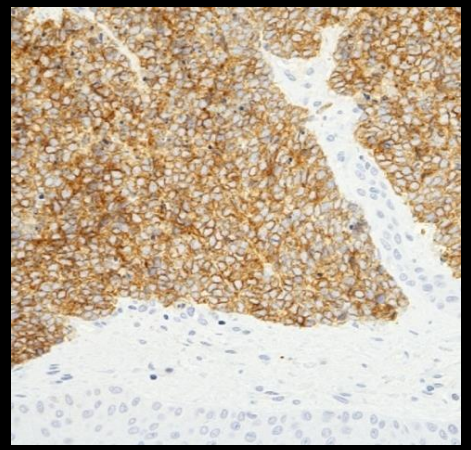
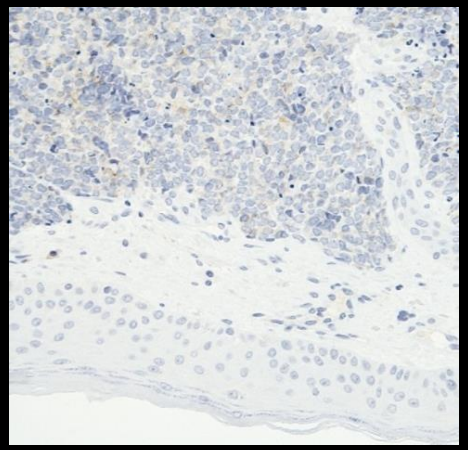
It doesn`t ”fit-for-purpose”

D5F3, 5A4, OTI1A4

35 protocols were based on ALK1:

Only one protocol (3%) were assessed as sufficent, none were optimal

The technical test approach – Analytical phase

	ALK, D5F3 (1:200)	ALK, 5A4 (1:50)	ALK, ALK1 (1:10)
Adenocarcinoma Lung ALK-EML4			
Merkel cell carcinoma Skin			

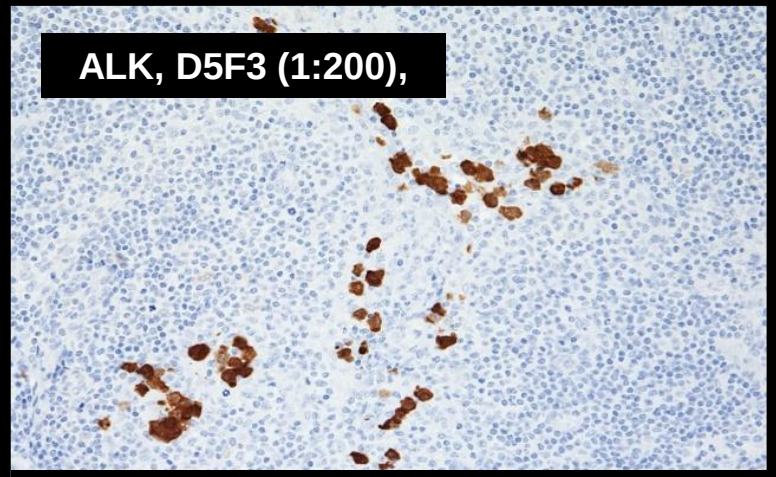
HIER in high pH buffer 20`, Flex+

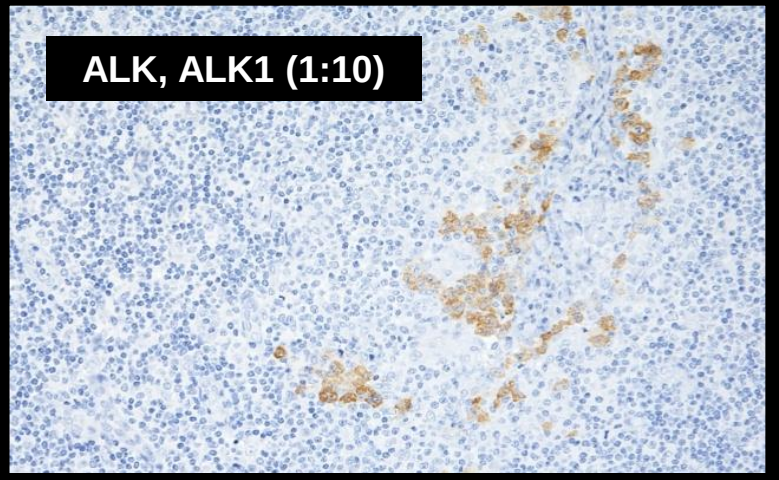
Clone ALK1 provides low sensitivity

The technical test approach – Analytical phase

HIER in high pH buffer, Flex+

ALCL

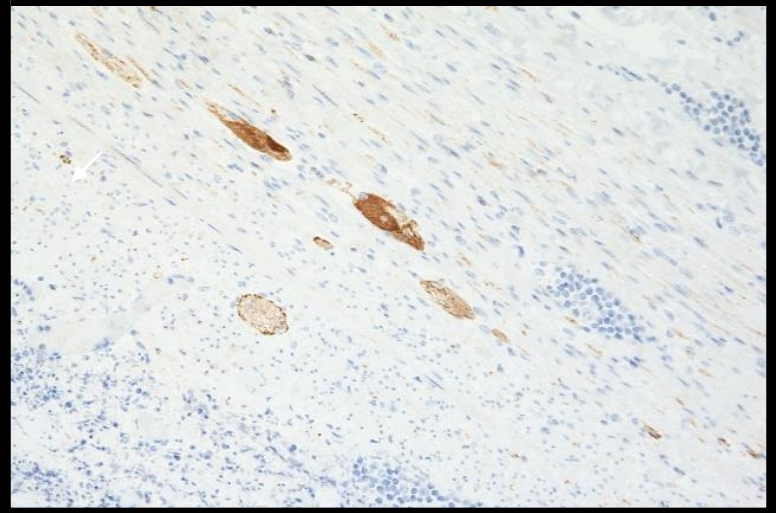


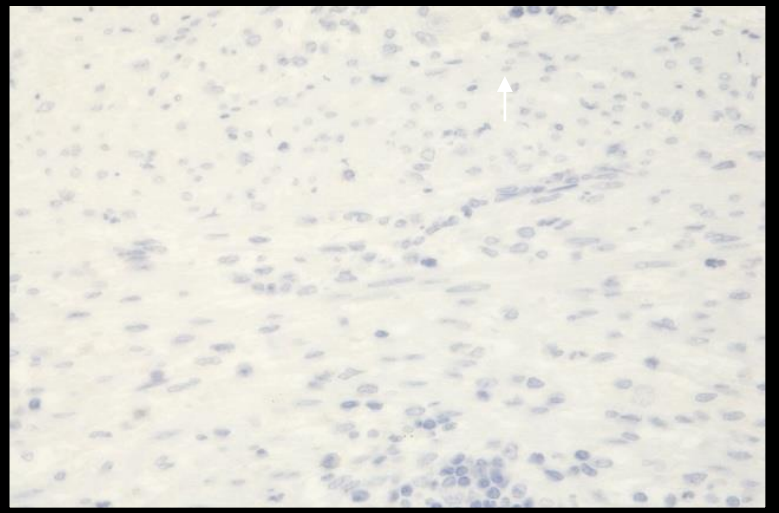


Clone ALK1 provides low sensitivity

”iCAPS” : Ganglion and peripheral nerve cells ?

Appendix





The technical test approach – Analytical phase

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- Purpose and/or "fit-for-purpose" of the IHC test
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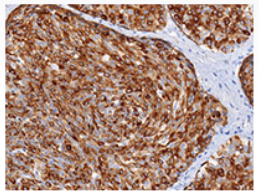
- How to validate (technical) the IHC-test
  - Is the IHC test reproducible/robust (preanalytic conditions)
  - Evaluation of the analytical sensitivity and specificity
- Identification of most robust controls providing information that the established level of detection is obtained in each test performed in daily practice.

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

Calcitonin optimization (data sheets ?)

anti-Calcitonin (SP17), Rabbit Monoclonal Primary Antibody

Ventana/ Cell Marque



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

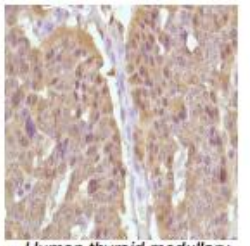
This antibody is intended for in vitro diagnostic (IVD) use. Calcitonin (SP17) antibody is intended for qualified laboratories to qualitatively identify by light microscopy the presence of associated antigens in sections of formalin-fixed, para test methods. This antibody is used as an aid in the id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

Recommended staining protocol with *ultra*View	
Procedure Type	Method
Deparaffinization	Selected
Cell Conditioning (Antigen Unmasking)	Cell Conditioning 1, Mild
Enzyme (Protease)	Not required
Antibody (Primary)	BenchMark ULTRA instrument: 16 minutes, 36°C BenchMark XT instrument: 16 minutes, 37°C BenchMark GX instrument: 16 minutes, 37°C
Amplification	Not selected
Counterstain	Hematoxylin II, 8 minutes
Post Counterstain	Bluing, 4 minutes

SPRING BIOSCIENCE

Rabbit Anti-Human Calcitonin Monoclonal Antibody (Clone SP17)

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



Human thyroid medullary carcinoma stained with anti-calcitonin antibody

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

CLONE: SP17

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

IG ISOTYPE: Rabbbit IgG

EPITOPE: Not determined

MOLECULAR WEIGHT 15kDa

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

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

APPLICATIONS: Immunohistochemistry (IHC)

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

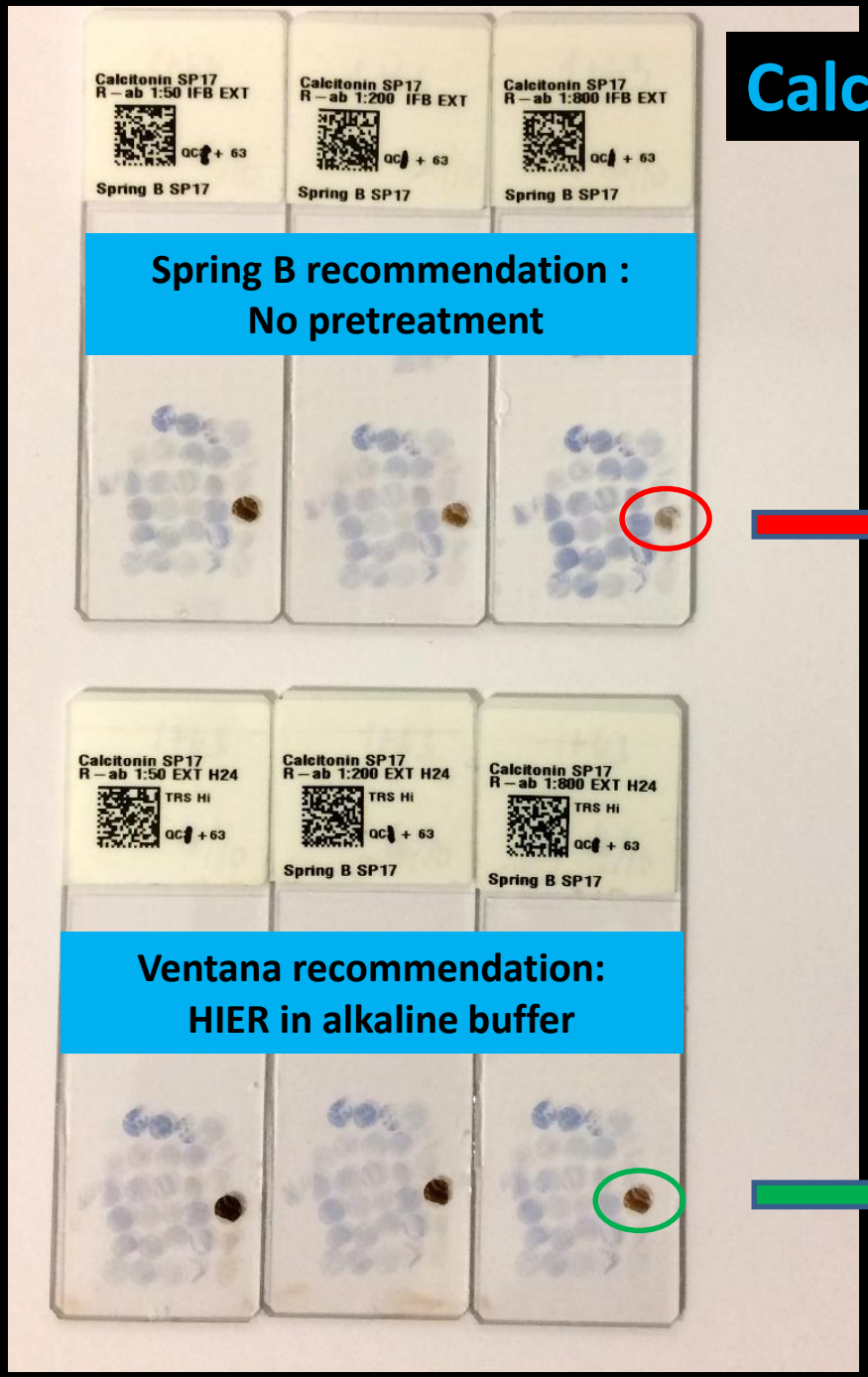
POSITIVE CONTROL: Thyroid medullary carcinoma

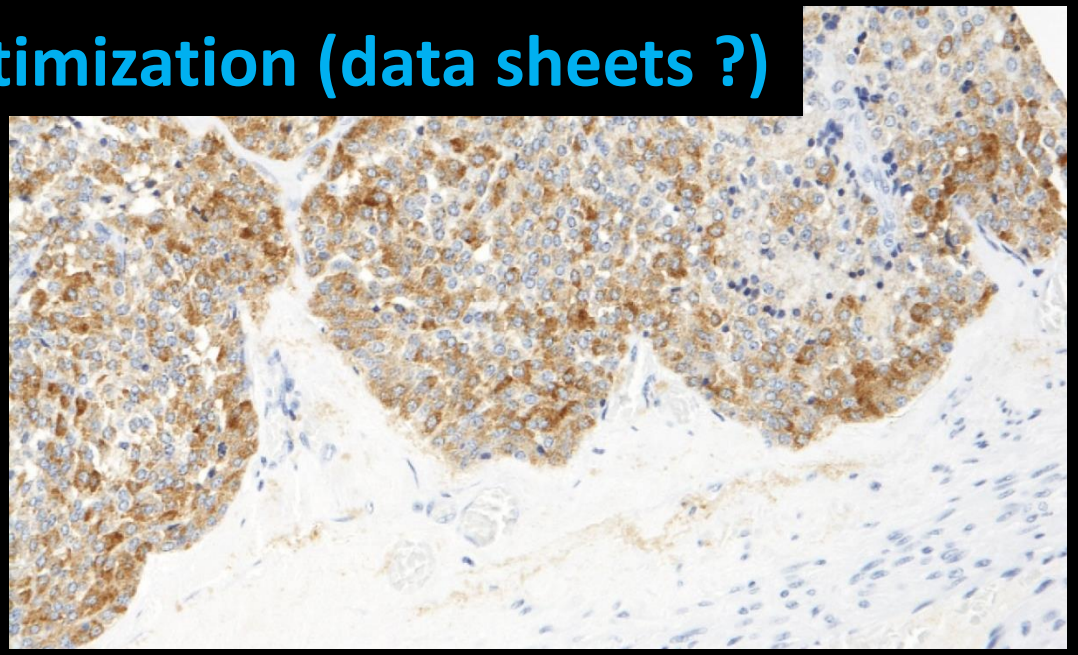
CELLULAR LOCALIZATION: Cytoplasm

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

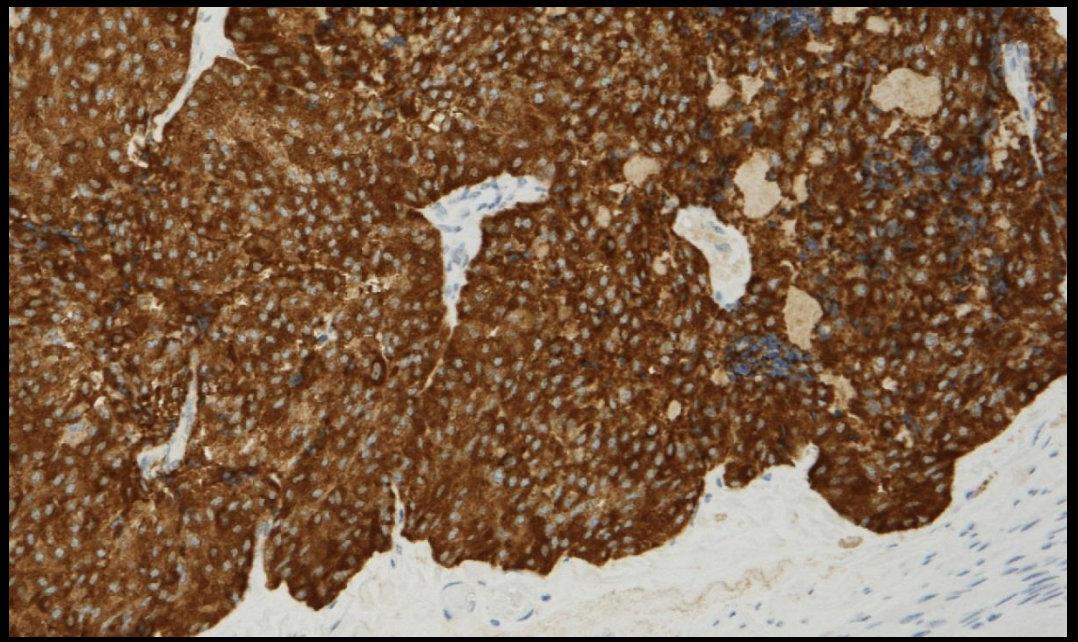
Omnis, Flex+

Calcitonin optimization (data sheets ?)





Thyroid medullary carcinoma



The technical test approach – Analytical phase

How to establish "best practice protocol" of the IHC test - parameters to consider

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

Test more than one antibody clone against 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 • Vol13, Number 5, September 2006

Technical aspects of IHC and pitfalls– Analytical phase

Concentrated antibodies - Dept. of Surgical Pathology, Region Zealand, Denmark – Omnis (app. 240 Abs)

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 (48`)	HIER TRS High pH 9.0 (48`)	HIER TRS High pH 9.0 (48`)
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

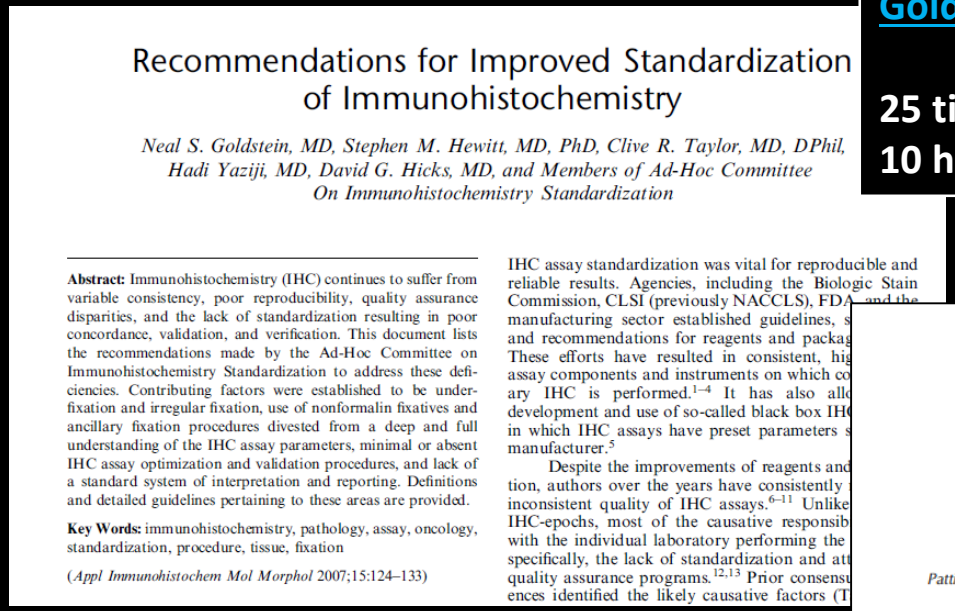
Protocol A:	0.5 %
Protocol B:	2.0 %
Protocol C:	10.0 %
Protocol D:	83.5 %
Protocol E:	1.0 %
Protocol F:	3.0 %
Protocol G:	0 %
Protocol H:	0 %

* 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 IHC and pitfalls– Analytical phase

Analytical Validation - Evaluation of sensitivity and specificity - Tissue is the key element



Recommendations for Improved Standardization of Immunohistochemistry

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

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

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

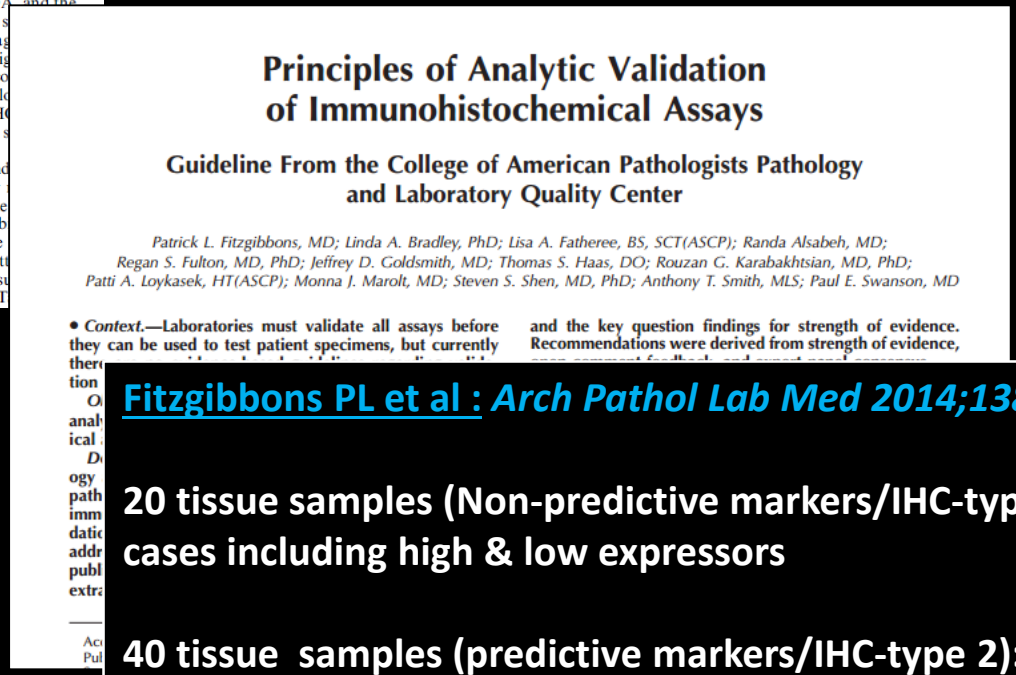
(*Appl Immunohistochem Mol Morphol* 2007;15:124–133)

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

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

Goldstein NS et al : ***Appl Immunohistochem Mol Morphol 2007 Mar; 15 : 124-133***

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



Principles of Analytic Validation of Immunohistochemical Assays

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

Patrick L. Fitzgibbons, MD; Linda A. Bradley, PhD; Lisa A. Fatheree, BS, SCT(ASCP); Randa Alsabeh, MD; Regan S. Fulton, MD, PhD; Jeffrey D. Goldsmith, MD; Thomas S. Haas, DO; Rouzan G. Karabakhtsian, MD, PhD; Patti A. Loykasek, HT(ASCP); Monna J. Marolt, MD; Steven S. Shen, MD, PhD; Anthony T. Smith, MLS; Paul E. Swanson, MD

• *Context.*—Laboratories must validate all assays before they can be used to test patient specimens, but currently

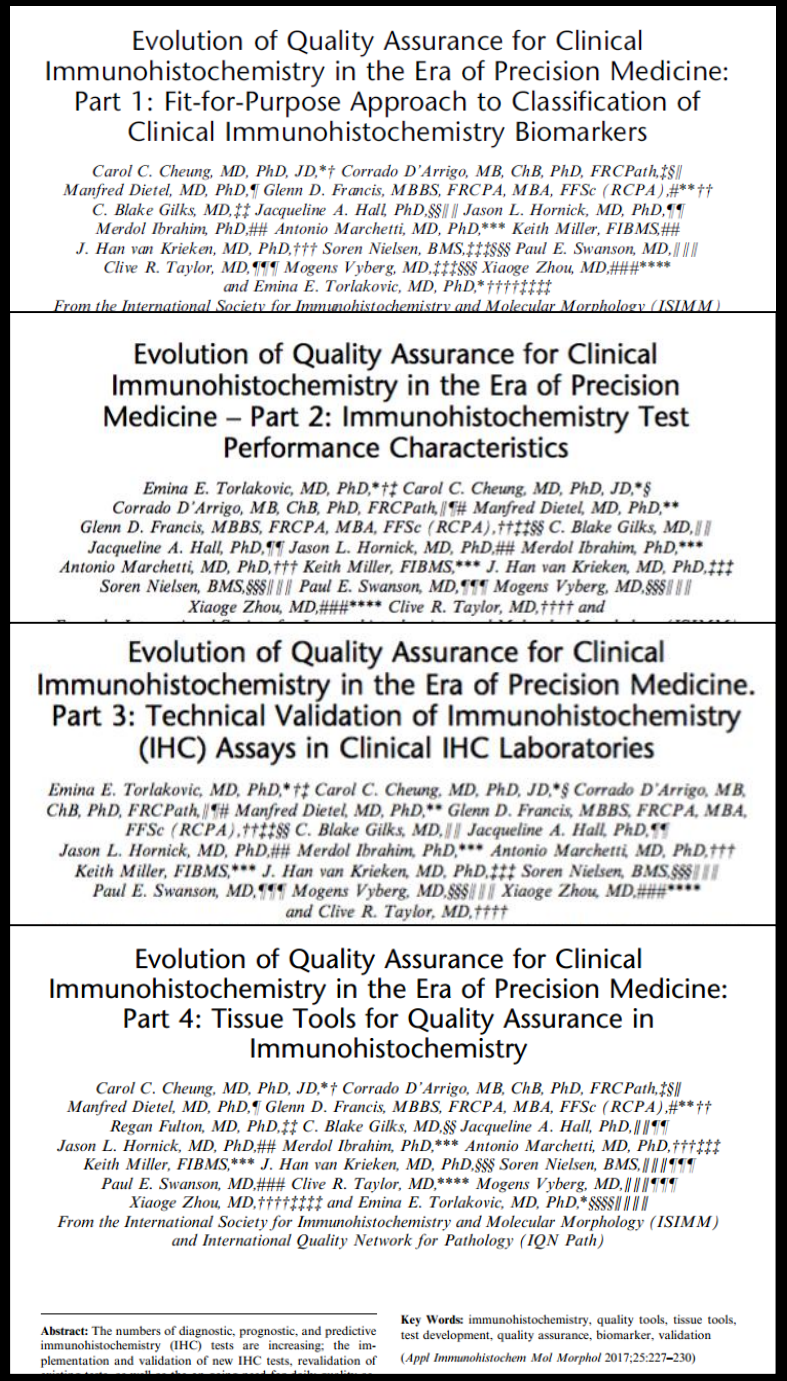
and the key question findings for strength of evidence. Recommendations were derived from strength of evidence,

Fitzgibbons PL et al : ***Arch Pathol Lab Med 2014;138:1432-1443***

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

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

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



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

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

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

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

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

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

Abstract: The numbers of diagnostic, prognostic, and predictive immunohistochemistry (IHC) tests are increasing; the implementation and validation of new IHC tests, revalidation of

Key Words: immunohistochemistry, quality tools, tissue tools, test development, quality assurance, biomarker, validation

(*Appl Immunohistochem Mol Morphol* 2017;25:227–230)

Article sequence (part 1-4) published in Appl Immunohistochem Mod Morphol (2017) systematically describing/defining all aspects of the IHC test from purpose (fit-for-purpose) of a test, through test performance characteristics (analytical sensitivity, analytical specificity, preanalytical reproducibility…….).

Importance of validation with focus on the technical part and the use of tissue tools for Quality assurance in immunohistochemistry.

Full technical validation

The technical test approach – Analytical phase

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.)

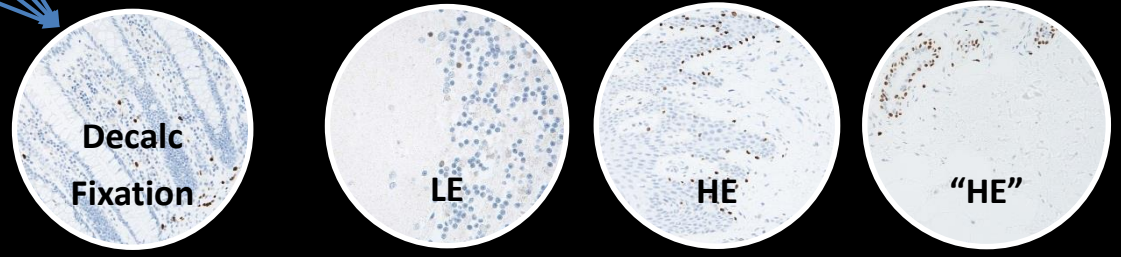
SOX10, BS7; HIER High pH 24`; 1:350 RR; Flex+Mouse linker

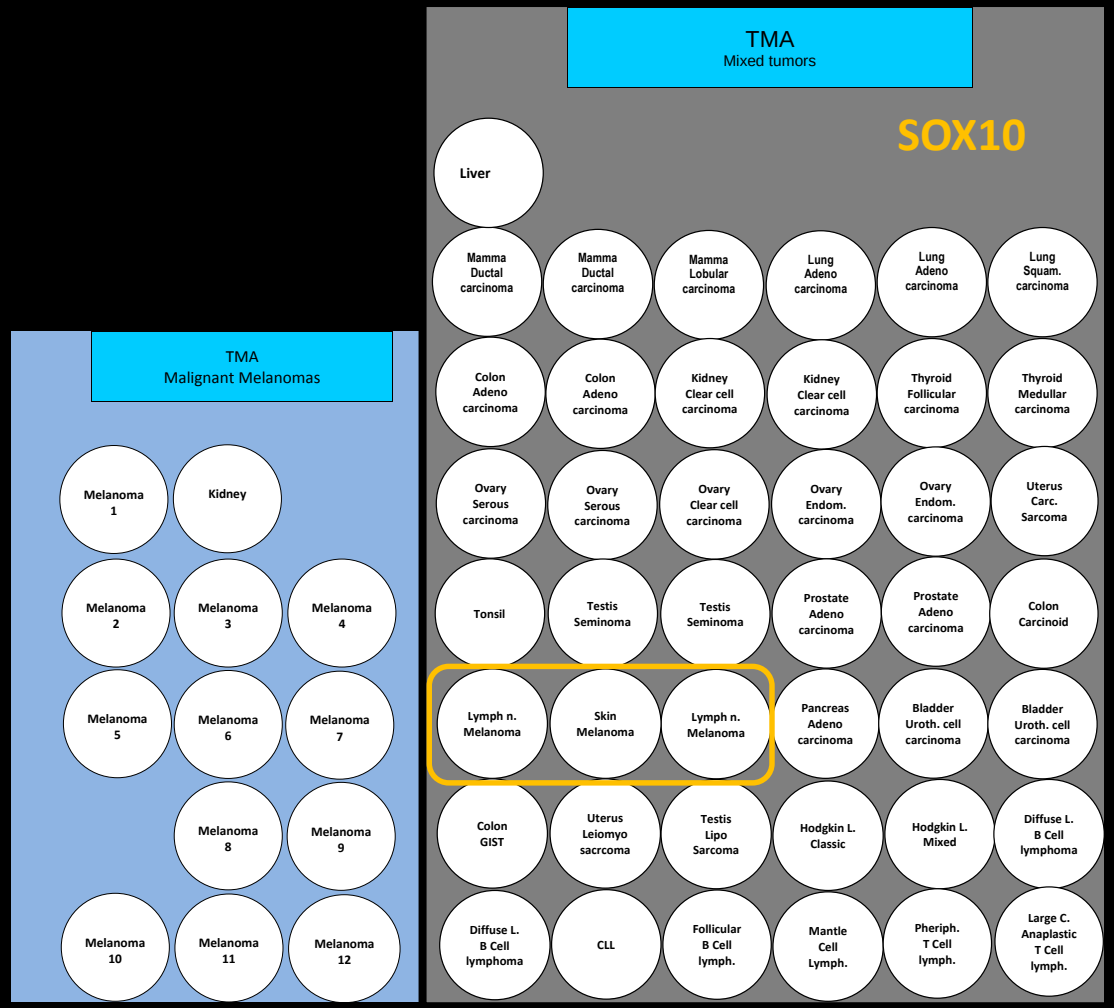
Establishing robustness of the IHC assay / pre-analytic parameter`s ?

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

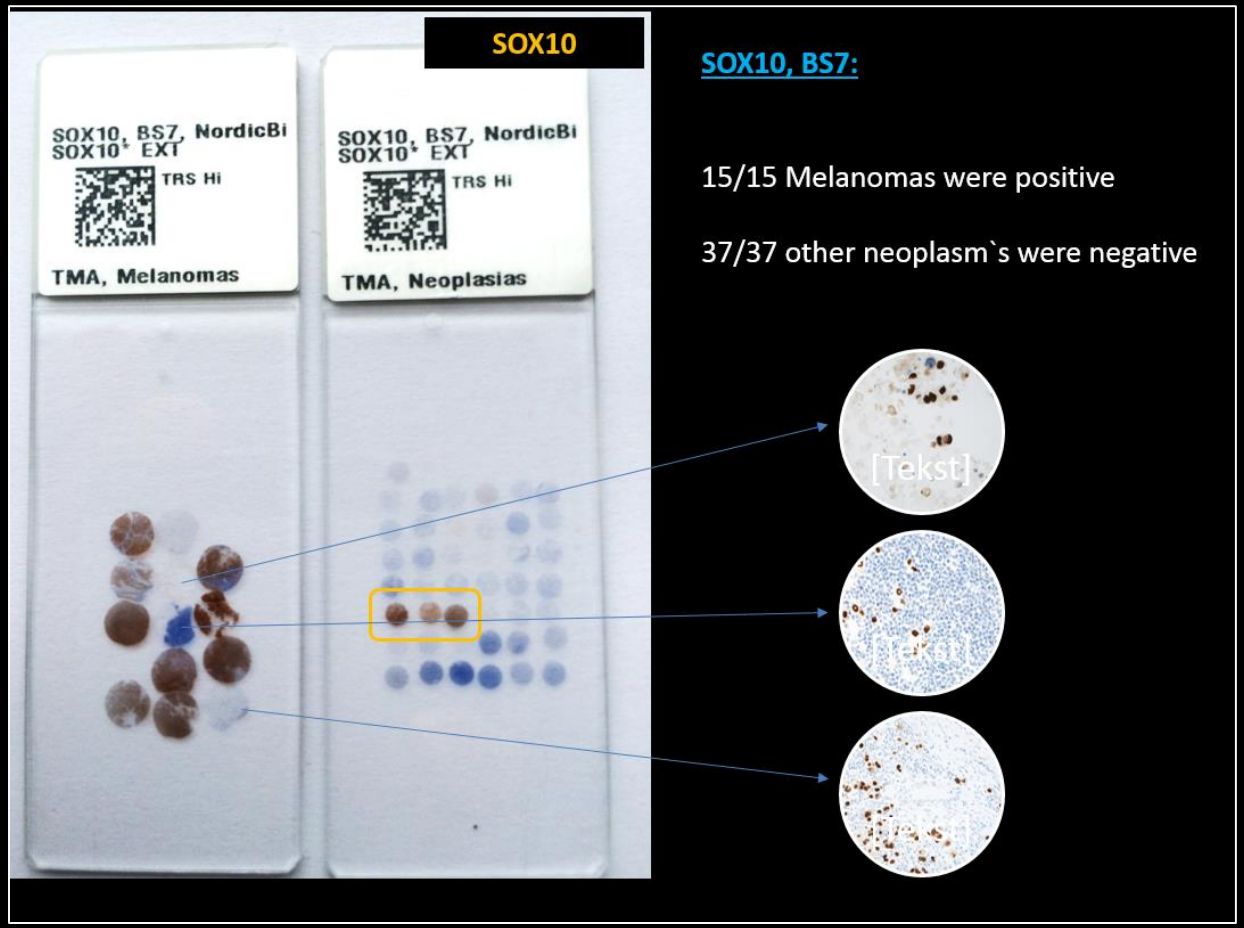
Identification of robust controls

SOX10, BS7; High, low & non-expressors ?

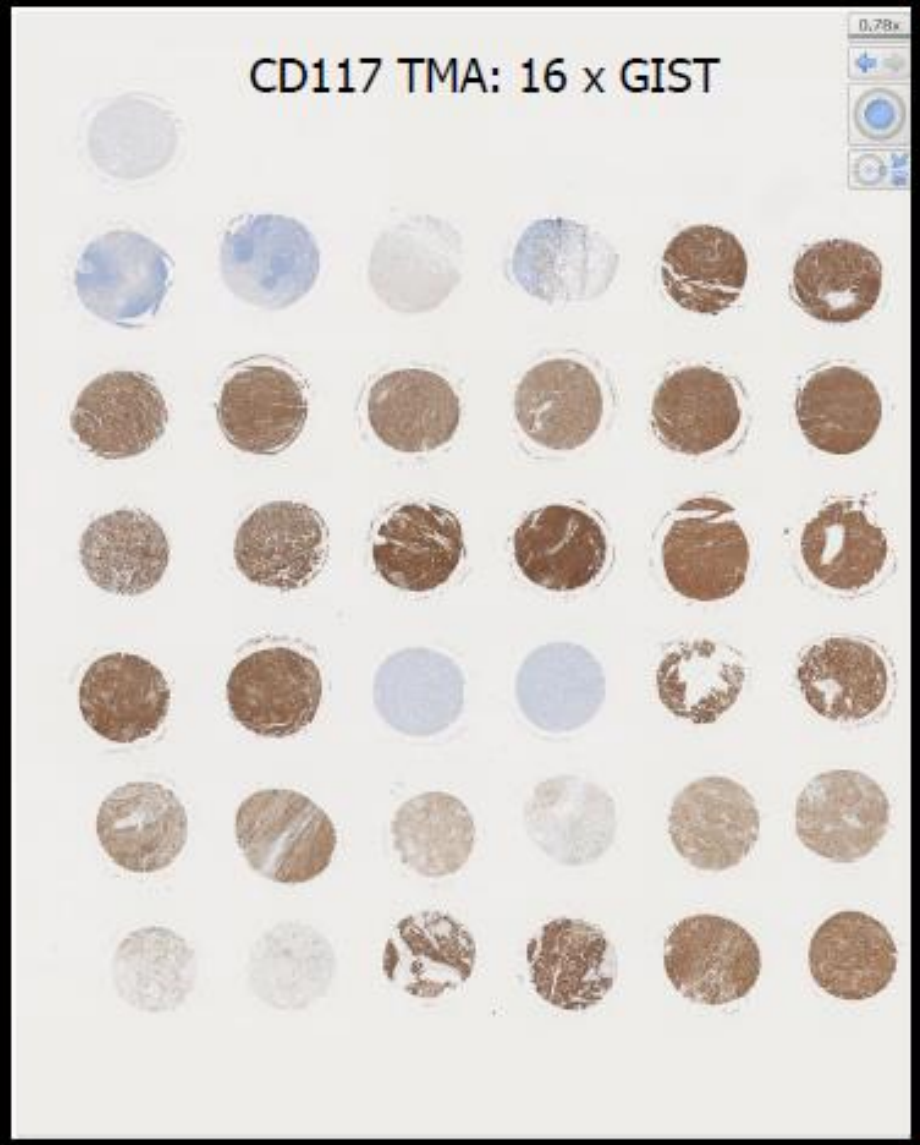




Diagnostic potential & Analytical validation



IHC – The Technical Test Approach



Slide (modified) and information kindly provided by Søren Nielsen, Aalborg, DK

Virchows Arch (2016) 468:19–29
DOI 10.1007/s00428-015-1829-1

ANNUAL REVIEW ISSUE

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

Mogens Vyberg[1,2] · Søren Nielsen[1]

Major 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 antibody dilutions
  - b) Stainer platform dependent antibodies
- The choice of detection system

83 % of insufficient results

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

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

Virchows Arch (2016) 468:19–29
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27%

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Problems related to the choice of antigen retrieval method :

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

False positive or false negative results

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

The technical test approach – 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)**

The technical test approach – Analytical phase

0022-1554/91/$3.30
The Journal of Histochemistry and Cytochemistry
Copyright © 1991 by The Histochemical Society, Inc.
Vol. 39, No. 6, pp. 741–748, 1991
Printed in U.S.A.

Rapid Communication

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

SHAN-RONG SHI, MARC E. KEY,[1] and KRISHAN L. KALRA
BioGenex Laboratories, San Ramon, California 94583.

Received for publication Ja

We describe a new appr
formalin-fixed, paraffin-

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 where typically unreactive with formalin-fixed tissue gave excellent staining.**

The technical test approach – Analytical phase

The mechanism of HIER ?

Several hypothesis in regard of the mechanism of HIER has been proposed, but the mechanism of action of HIER is not completely understood.

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

- ❑ **Hydrolysis 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 ?**

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

The technical test approach – Analytical phase

Efficient HIER depends on:

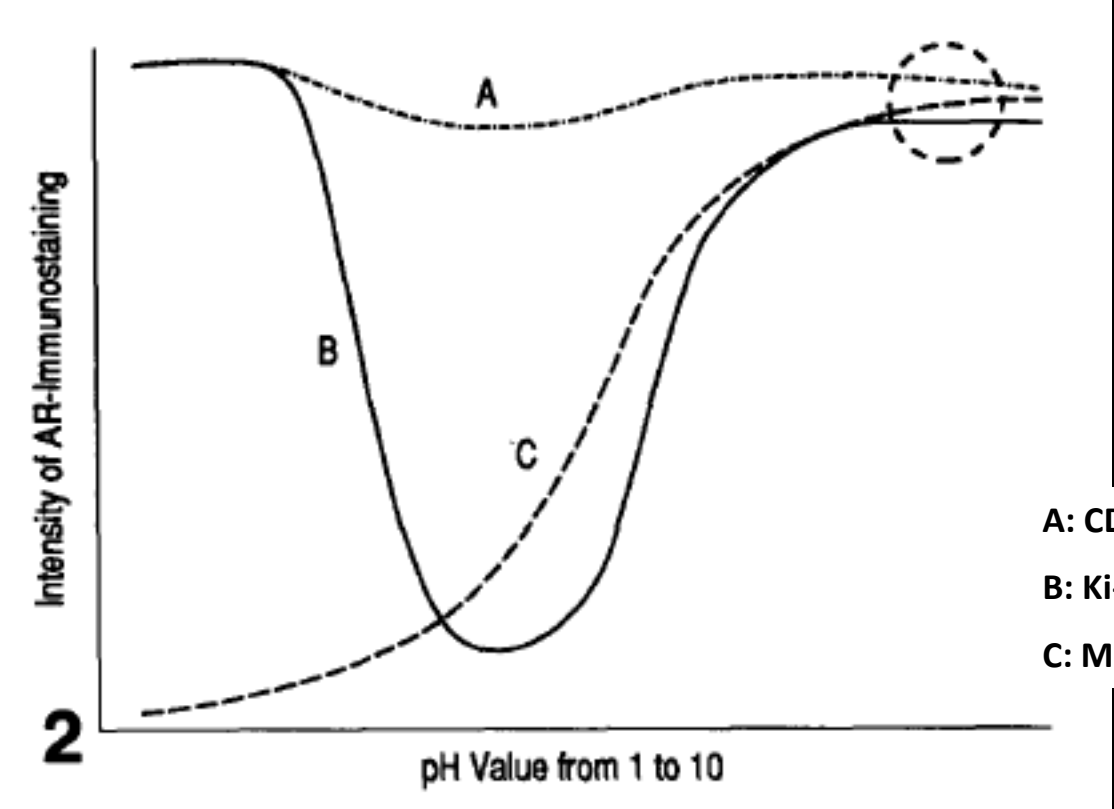
- ❑ pH of the HIER buffer
- ❑ Temperature
- ❑ Time
- ❑ Elementary nature of the HIER buffer (e.g. Citrate; TRIS; EDTA; TE)
- ❑ “Fixation time in formalin”

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

> 95% of all commonly used antibodies require HIER

The technical test approach – Analytical phase

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



Efficient HIER - Influence of pH

A: CD20 (clone L26)

B: Ki-67 (clone MIB1)

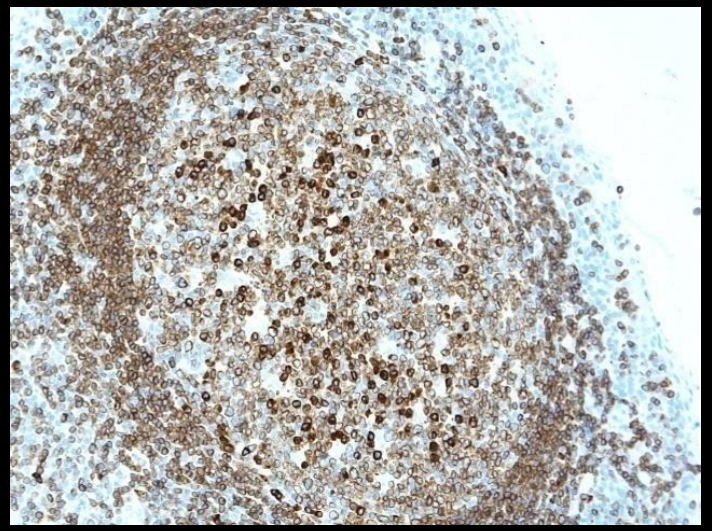
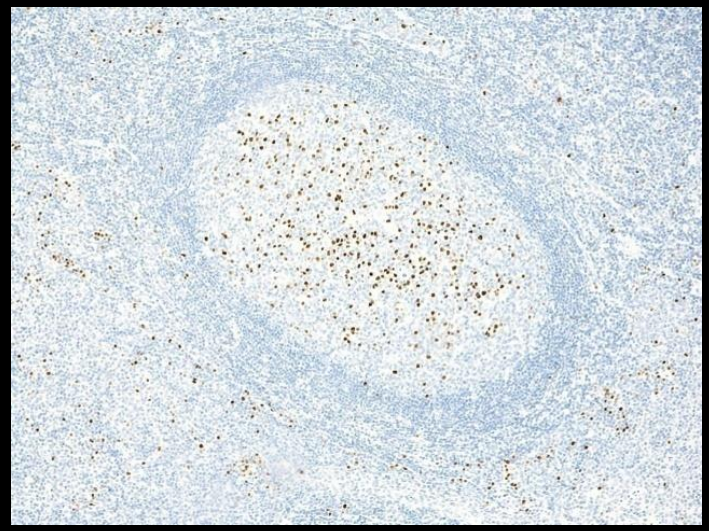
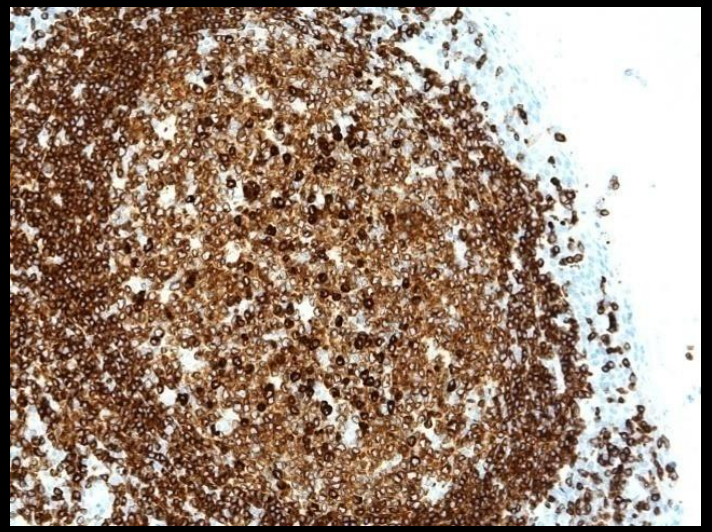
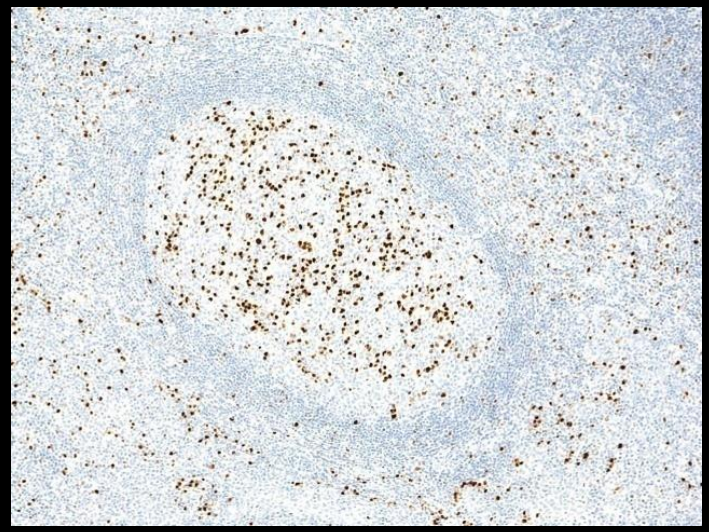
C: MSA (clone HMB45)

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

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

The technical test approach – Analytical phase

Efficient HIER - Influence of pH

	CD79, JCB117 (1:300)	MUM-1, MUM1p (1:400)
HIER in TRS pH 6.1 (20 min at 97°C)		
HIER in TRS pH 9 (20 min at 97°C)		

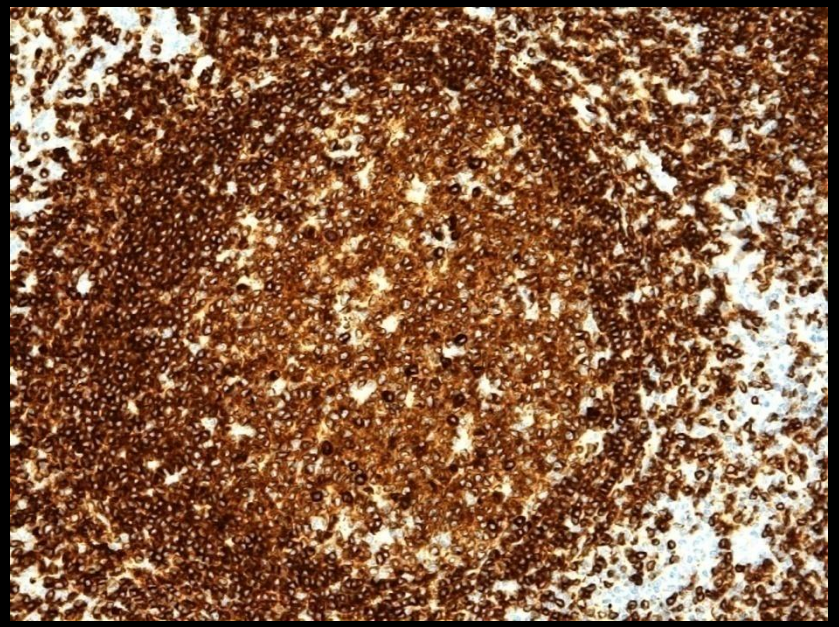
Tonsillar tissue fixed in 10% formalin (48h).

The technical test approach – Analytical phase

Efficient HIER - Influence of pH

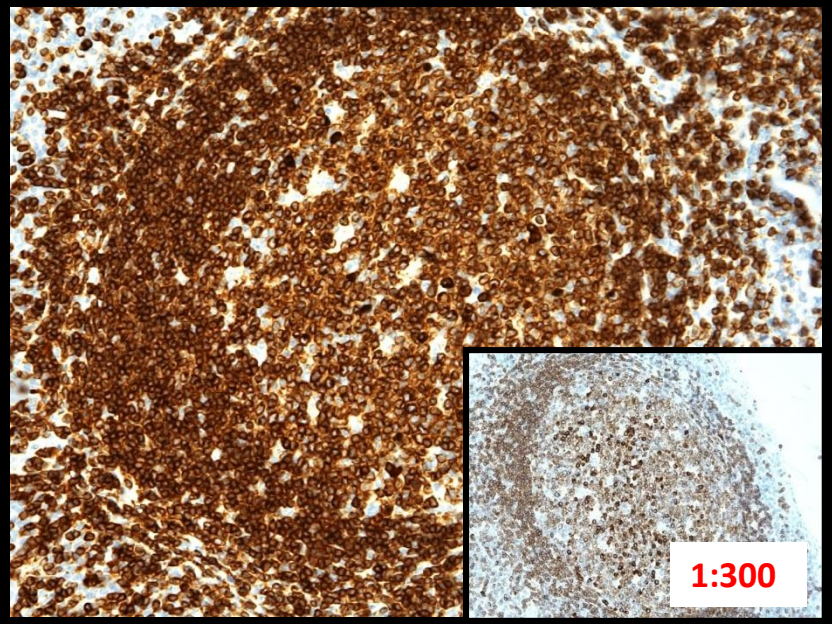
HIER in TRS pH 9

CD79, JCB117 (1:300)



HIER in TRS pH 6.1

CD79, JCB117 (1:50)



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

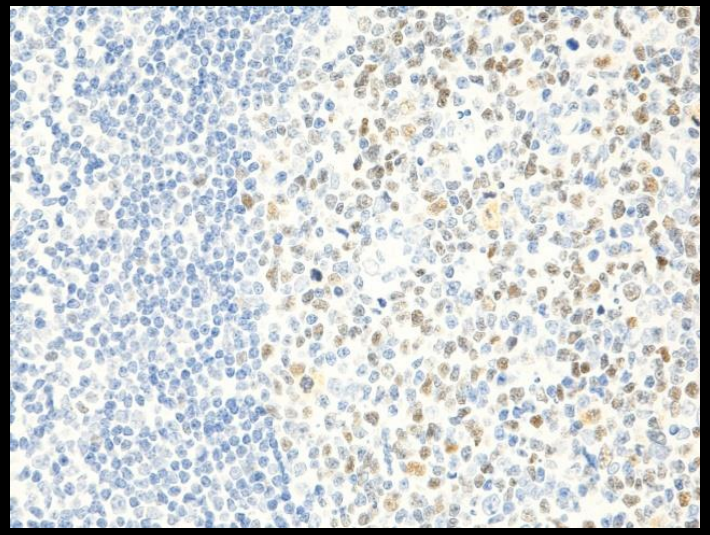
The technical test approach – Analytical phase

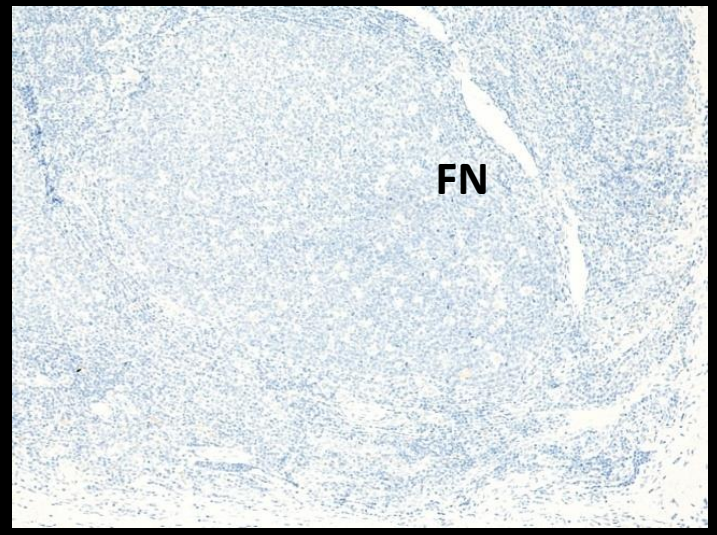
Efficient HIER - Influence of pH

BCL-6, LN22 (1:100)

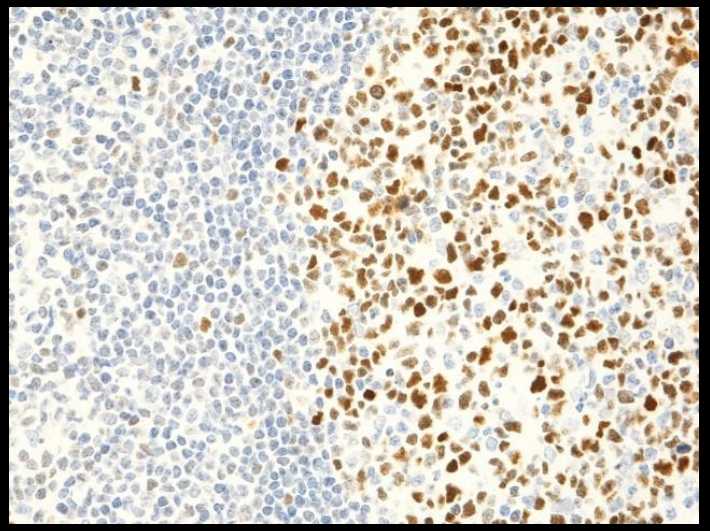
CD163, MRQ-26 (1:200)

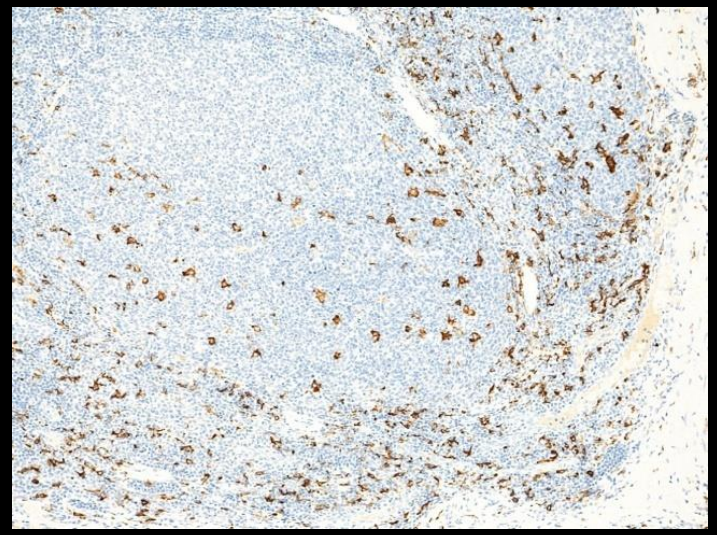
HIER in TRS pH 6.1





HIER in TRS pH 9





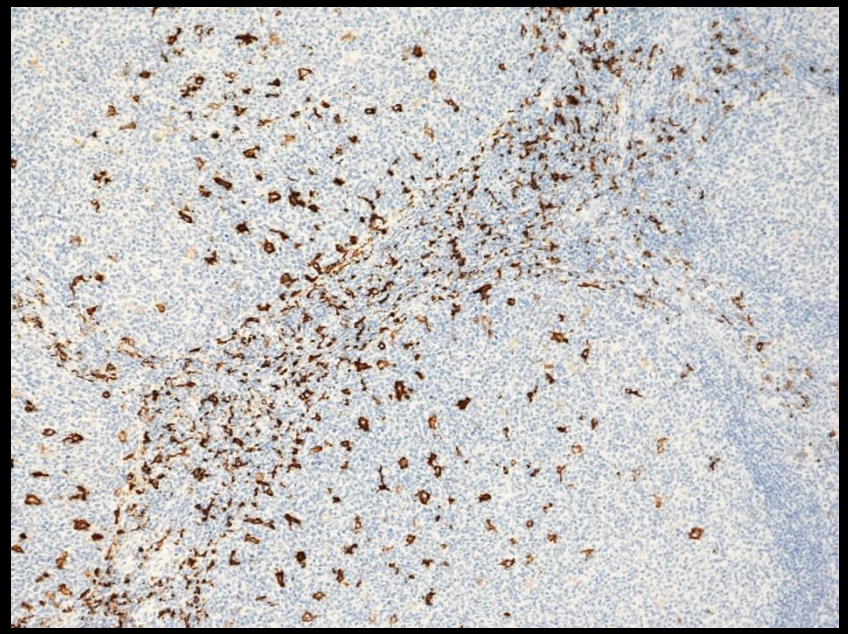
Tonsillar tissue fixed in 10% formalin (48h).

The technical test approach – Analytical phase

Efficient HIER - Influence of pH

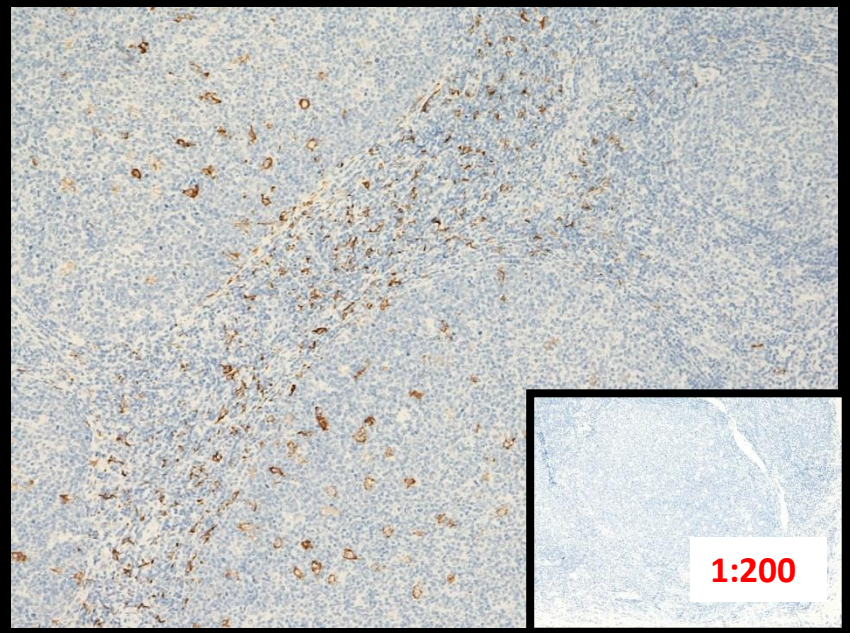
HIER in TRS pH 9

CD163, MRQ-26 (1:200)



HIER in TRS pH 6.1

CD163, MRQ-26 (1:25)



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

For app. 90-95% of the epitopes, HIER in buffers at pH 8-9 is preferable to pH6

The technical test approach – 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**

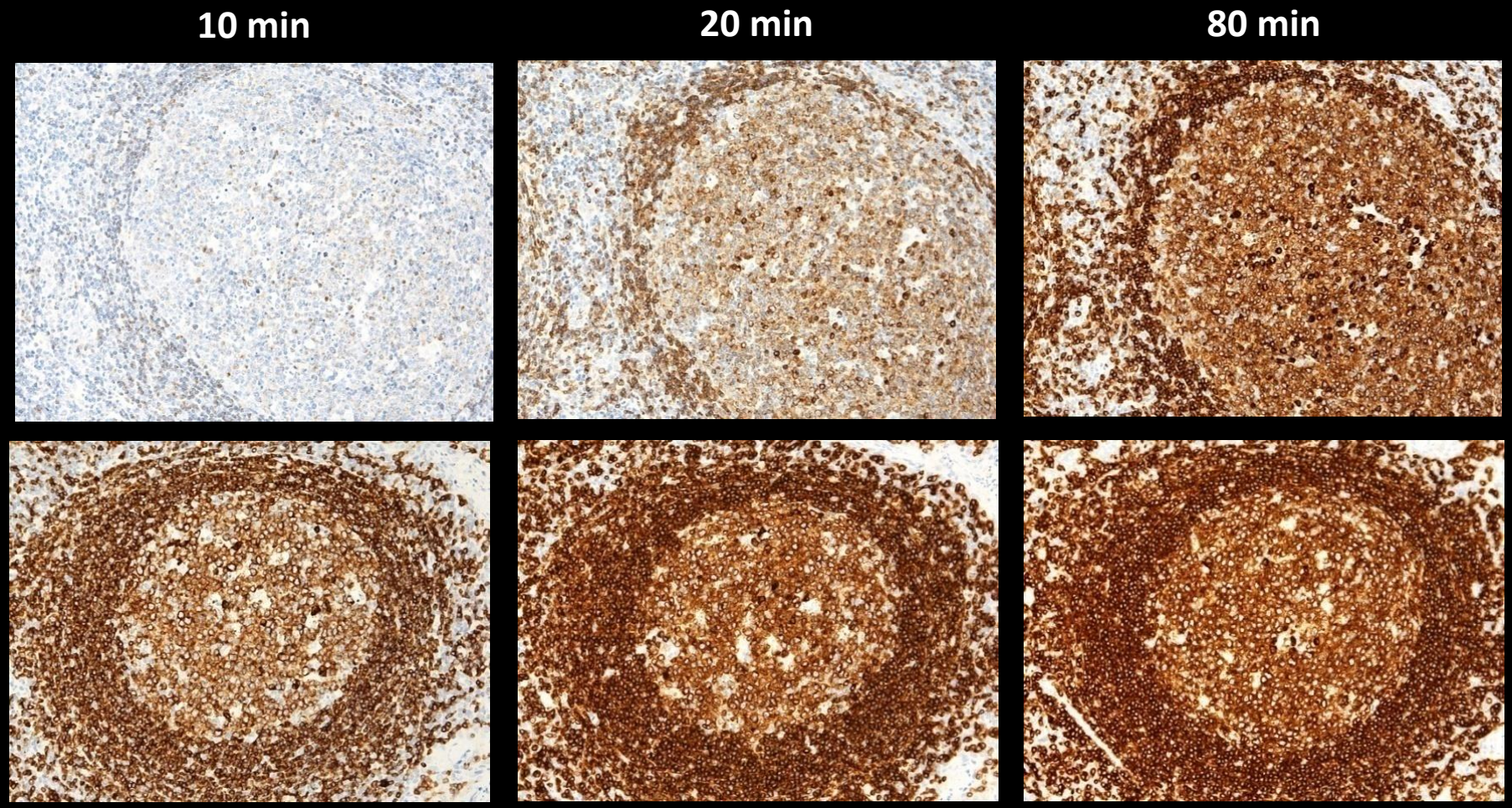
Optimal staining intensity could be generated by the following heating conditions:

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

The technical test approach – Analytical phase

Efficient HIER - Influence of time and temperature

CD79, JCB117 (1:300)

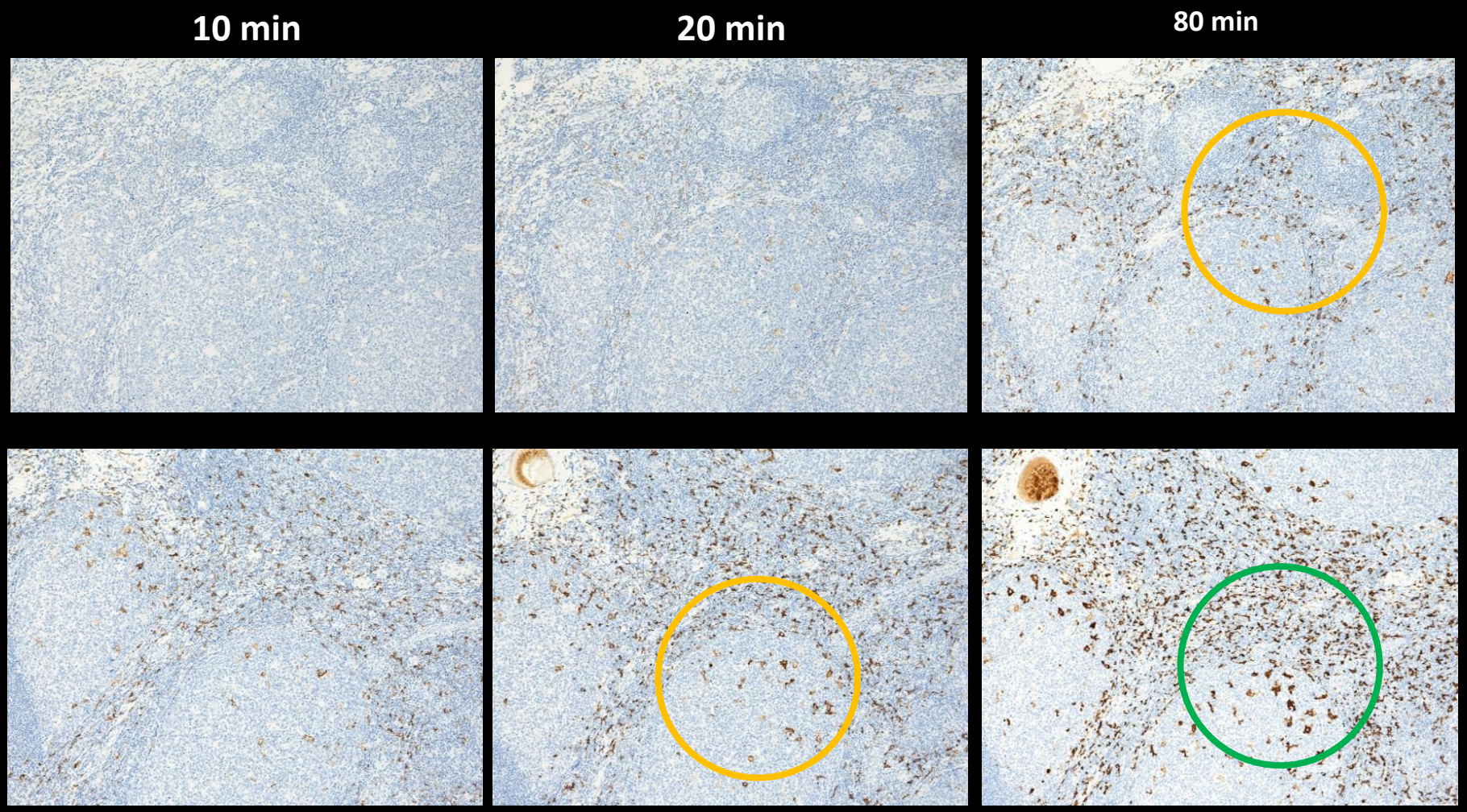


Tonsillar tissue fixed in 10% formalin (48h).

The technical test approach – Analytical phase

HIER buffer - Influence of time and temperature

CD163, MRQ-26 (1:200)



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

JOURNAL OF PATHOLOGY, VOL. 183: 116–123 (1997)

ANTIGEN RETRIEVAL TECHNIQUES IN IMMUNOHISTOCHEMISTRY: COMPARISON OF DIFFERENT METHODS

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

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

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

Chemical composition of the HIER buffer`s

Standard low pH buffer`s (citrate pH 6.0)

Standard high pH buffer`s (Tris-EDTA pH9, Tris-HCL pH 8-10, EDTA 8.0)

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

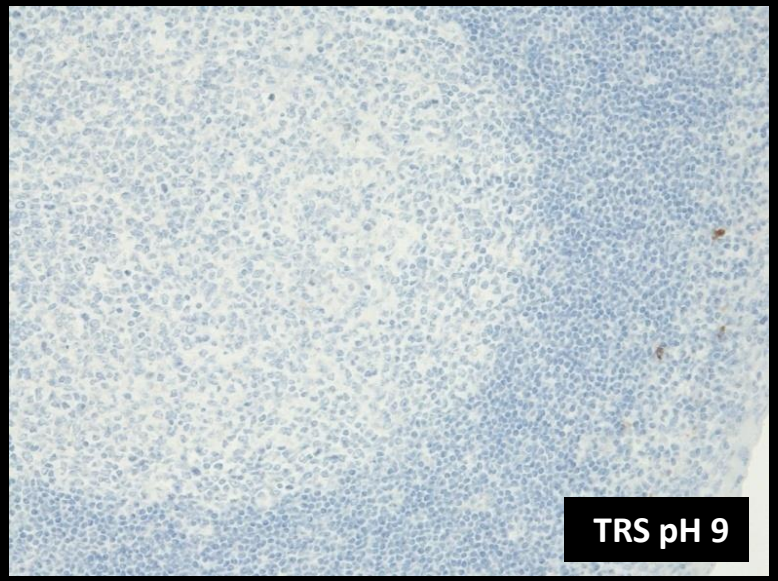
Overall best performance:

HIER in EDTA pH 8.0 (compare with Tris-HCL pH8.0)

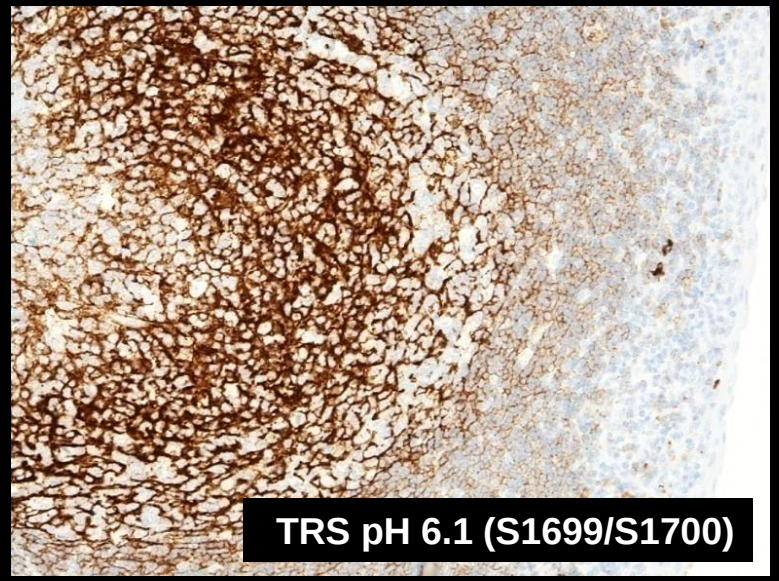
The technical test approach – Analytical phase

Modified low pH buffers

CD21, clone 1F8



CD21, clone 1F8



CD21, clone 2G9

HIER time 20` / Flex+

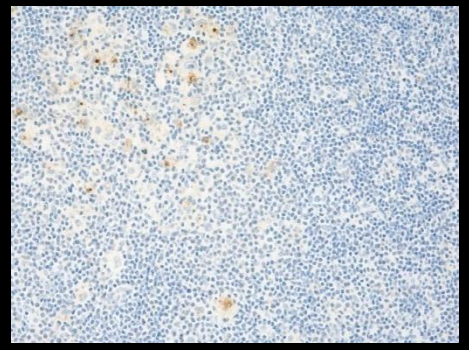
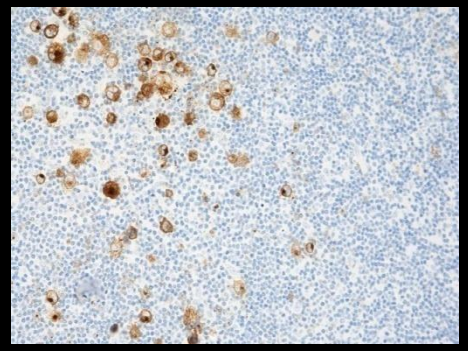
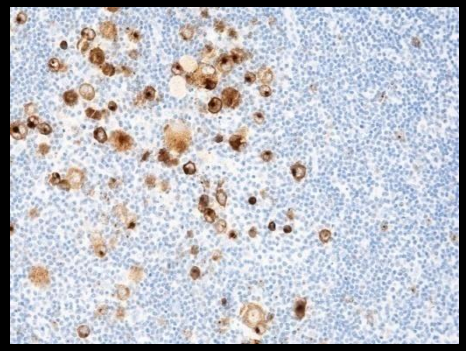
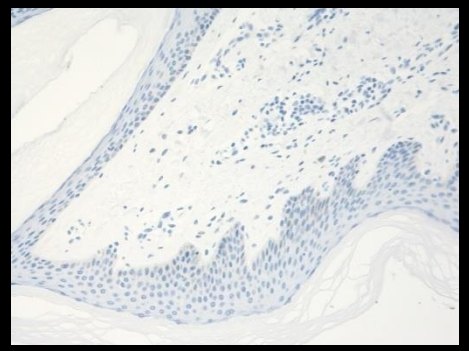
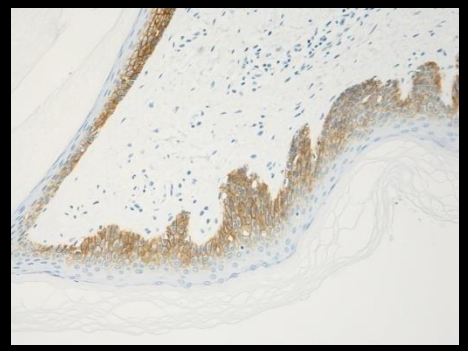
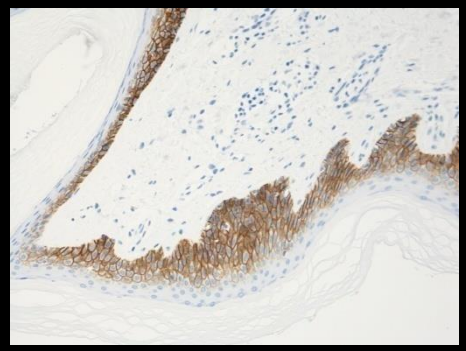
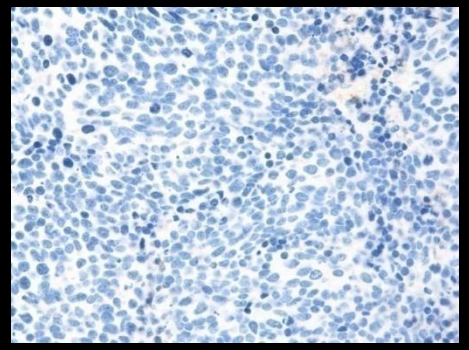
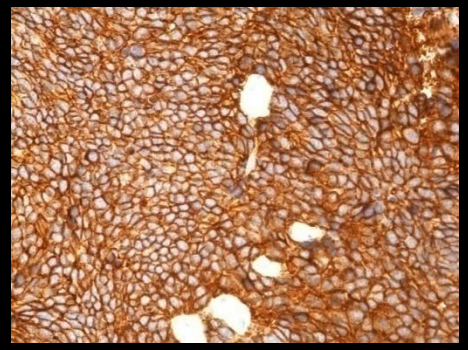
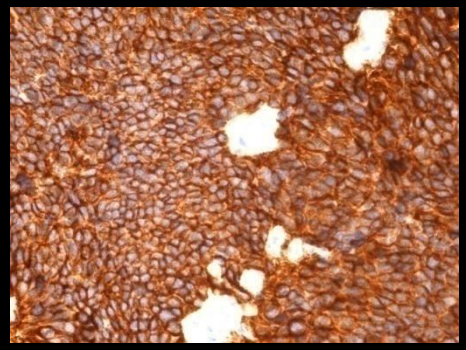
Markers requiring the TRS Low pH 6.1 (Dako, S1699/S1700) or Diva Decloaker pH 6.2 (Biocare, DV2004) :

EP-CAM (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

The technical test approach – Analytical phase

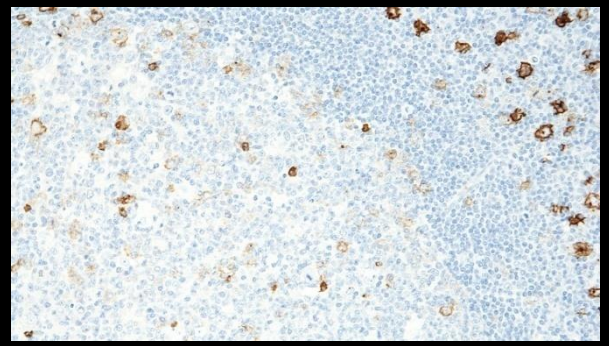
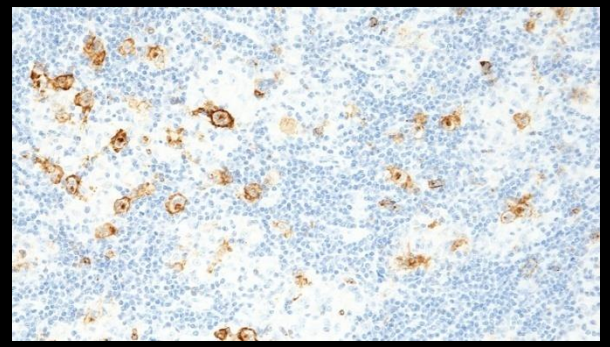
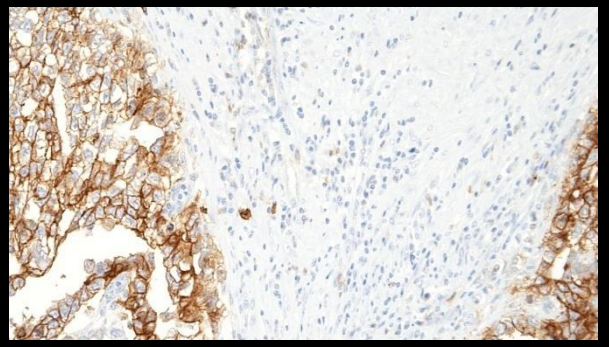
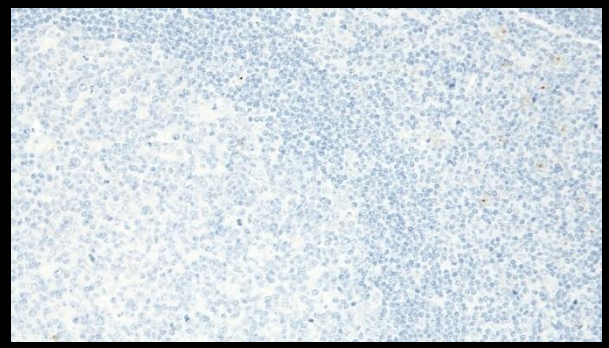
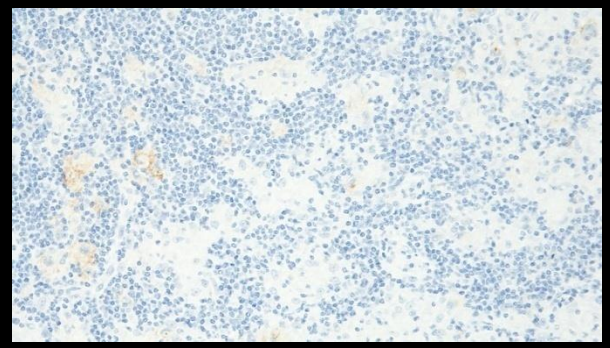
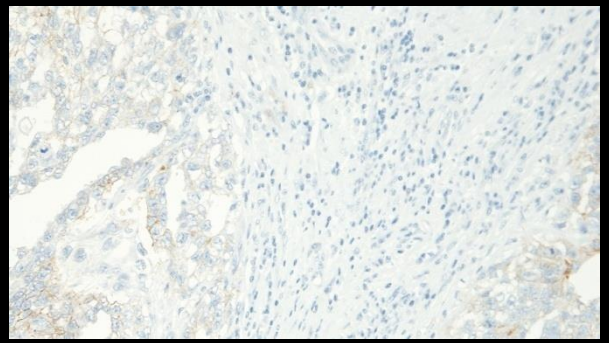
Modified low pH buffers

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

The technical test approach – 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)			

The technical test approach – Analytical phase

Length of formalin fixation and HIER time

TABLE 1. Staining Results of 30 Tonsil Antigens Following Formalin Fixation (FF) of 12 Hours to 3 Months and Heat-Induced Antigen Retrieval for 20 (60) Minutes in 0.01 M Citrate Buffer, PH 6.1

Antigen	Antibody Clone & Dilution	Length of FF					
		12 h	1 d	2 d	4 d	8 d	3 mo
B cell, 33kD	L26, 1:200	4	4	4	4	4	2
BAG-1	KS-6C8, 1:200	2	2	2	2	2(3)	1
BLA.36	A27-42, 1:50	2	3	3	3	3	+/−
CD1a	010, 1:50	3	3	3	3	3	2
CD8	C8/144B, 1:50	4	4	4	4	4	3
CD15	C3D1, 1:50	4	4	3	4	2(2)	0
CD21	1F8, 1:50	4	4	4	4	4	1
CD30	Ber H2, 1:50	3	4	4	4	4	1
CD31	JC70A, 1:50	4	4	4	4	4	2
CD34	QBEnd 10, 1:50	4	4	4	4	4	2
CD43	DF-T1, 1:100	4	4	4	4	4	+/−
CD45RA	4KB5, 1:200	4	4	4	4	4	2
CD45RO	UCHL1, 1:200	4	4	4	4	4	3
CD74	LN2, 1:50	4	4	4	4	4	3
CDw75	LN1, 1:100	3	3	3	3	3	2
CD79α	JCB117, 1:50	4	4	4	4	4	2
CD79α	HM57, 1:50	4	4	4	3	4	2
CD95	DX-2, 1:50	1	1	1	1	1(3)	0
CD95	DX-3, 1:200	2	2	2	2	1(2)	0
CD117 (c-Kit) (Mast cells)	PolyAb	3	3	3	3	3	3
Cytokeratin	AE1/AE3, 1:100	4	4	4	4	4	2
Cytokeratin 8	35BH11, 1:200	3	2	2	3	1(0)	0
Cytokeratin 1,5,10,14	34BE12, 1:50	4	4	4	4	4	+/−
Cytokeratin 5,6,8,17,19	MNF116, 1:100	4	4	4	4	4	1
HLA-DR	TAL. 1B5, 1:200	4	4	4	4	4	1
Kappa LC	A8B5, 1:100	4	4	4	4	4	1
Ki-1	BerH2, 1:50	4	4	4	4	4	1
Ki-67	Ki-67, 1:50	4	4	4	4	4	2
Ki-67	KiS5, 1:50	4	4	4	4	4	1
Ki-67	MIB-1, 1:100	4	4	4	4	4	1
Lambda LC	N10/2, 1:200	4	4	4	2	1(4)	0
p53	DO-7, 1:50	3	2	3	3	+/− (3)	1
PCNA	PC10, 1:800	4	4	4	4	4	1
Vimentin	V9, 1:800	4	4	4	4	4	0

Scores in parentheses are the results of AR for 60 mins.

Boenisch T : *Applied Immunohistochemistry 2005; 13(3) : 283-286*

Effect of Heat-Induced Antigen Retrieval Following Inconsistent Formalin Fixation

TABLE 2. Staining Intensities of Several Tissue Antigens Following 3 Months of Formalin Fixation and Heat-Induced Antigen Retrieval (AR) at 121°C

Antigen	Antibody Clone	AR	
		121°C	97°C
B-cell, 33 kD	L26, 1:200	4	4
CDw75	LN-1, 1:100	4	3
CD43	DF-T1, 1:200	3	+/−
HLA-DRα	TAL.1B5, 1:100	+/−	1
Ki-67	KiS5, 1:50	4	1
Ki-1	BerH2, 1:50	2	1
Lambda	N10/2, 1:100	4	0

Staining intensities after retrieval at 97°C for 20 minutes are listed for comparison.

121°C/ 5`

Demonstrated that:

Consistent optimal staining of 26 of the 30 antigens was achieved despite the variable length of fixation (up to 8 days of fixation).

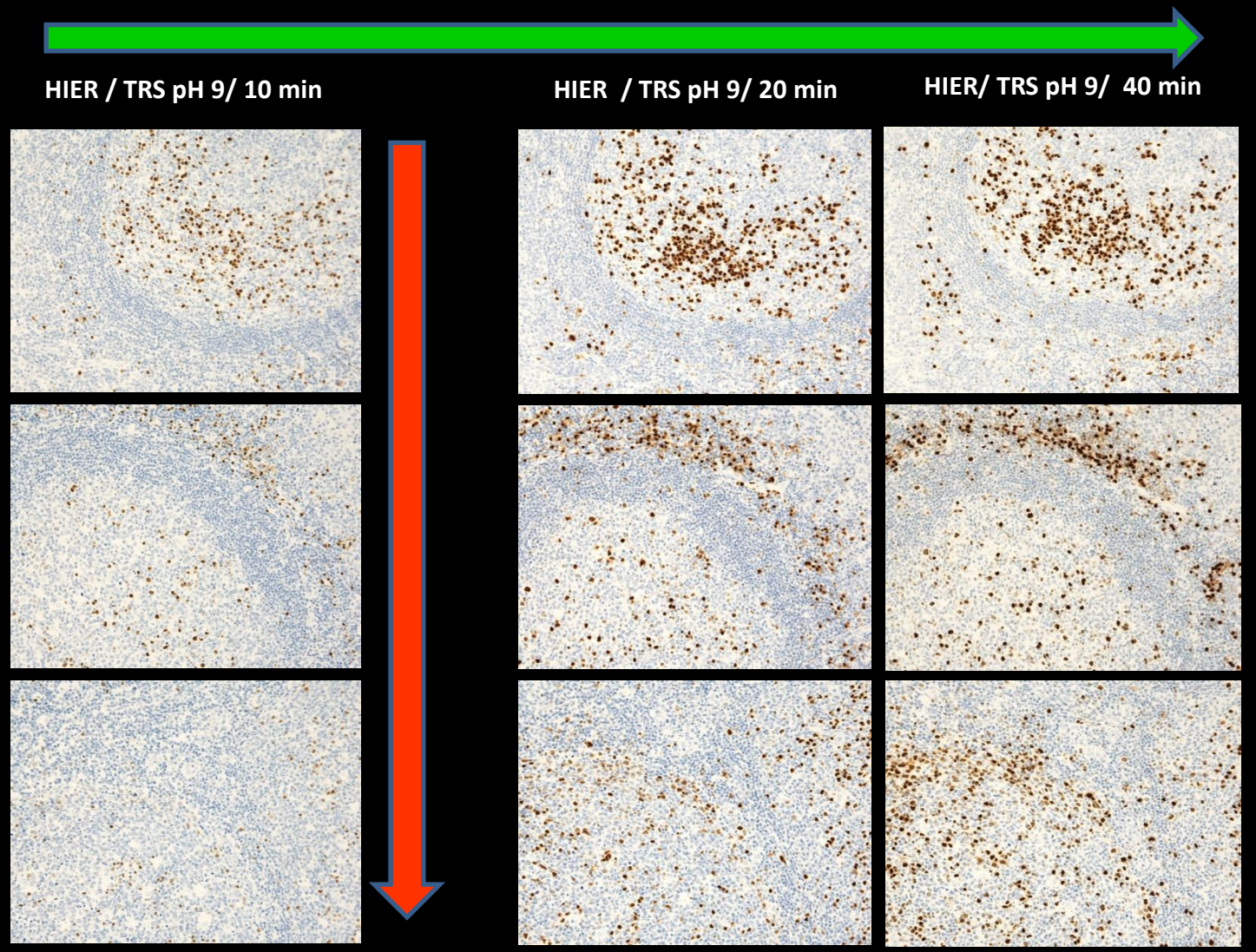
Prolonging HIER time or increasing HIER temperature could restore antigen determinants more efficiently compared to standard HIER protocols in “over fixed” tissue

The technical test approach – Analytical phase

Length of formalin fixation and HIER time

MUM-1, MUM1p

HIER in TRS High pH 9 at 97°C



Best performance: Efficient HIER time ~ 20-40 min at 97-99°C

The technical test approach – 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 immunodominant conformation of epitopes of interest.

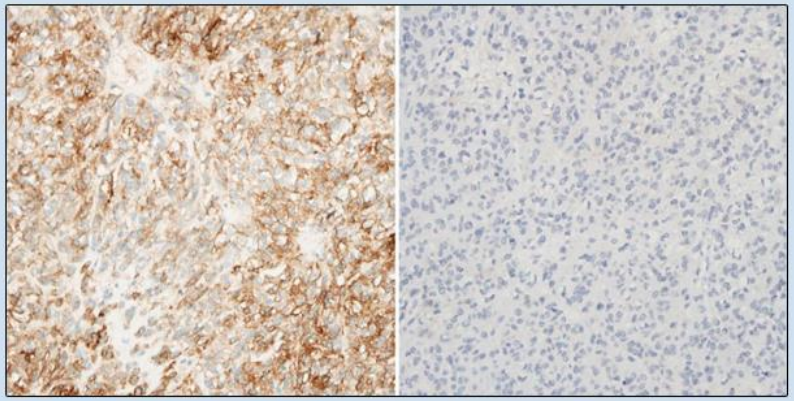
NordiQC Info Modules Assessments Protocols Controls Events Login

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 as the solitary pre-treatment procedure for routine purpose

The technical test approach – 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

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

Markers requiring enzymatic pretreatment :

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

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

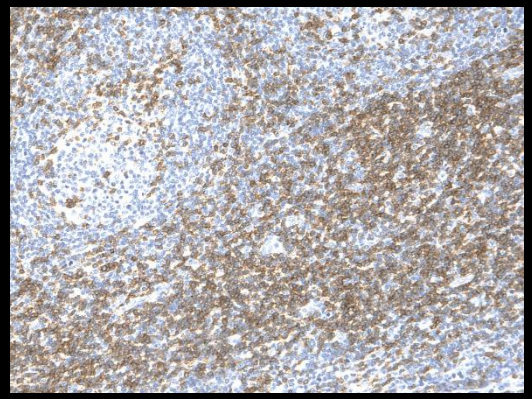
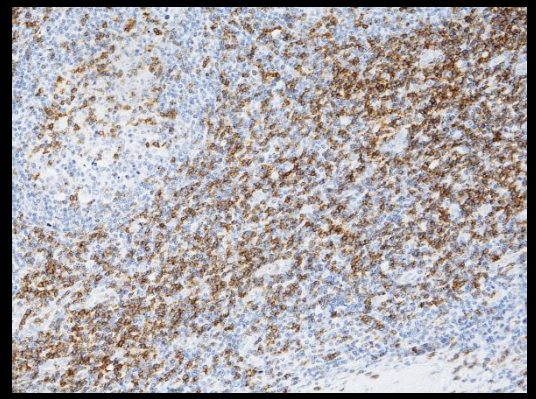
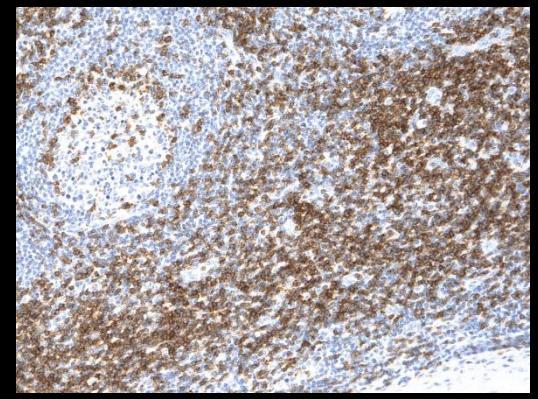
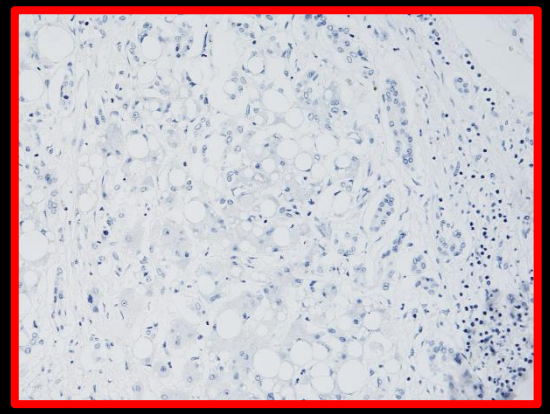
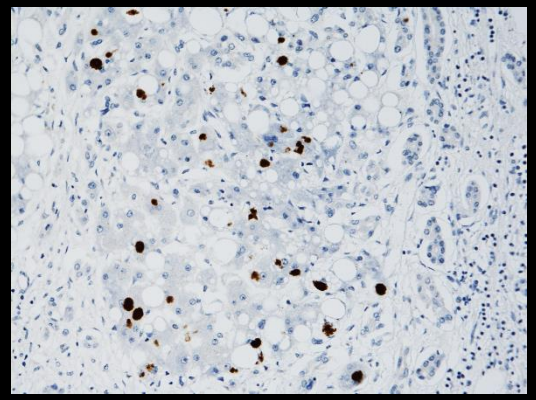
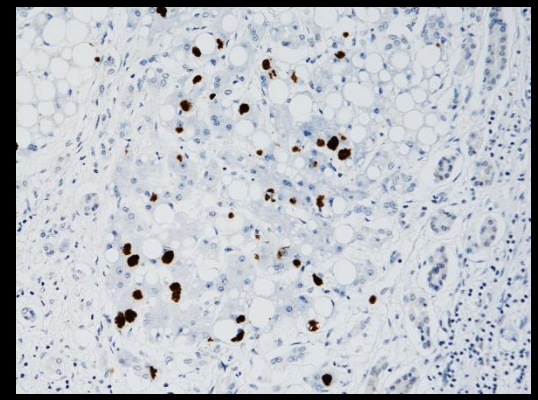
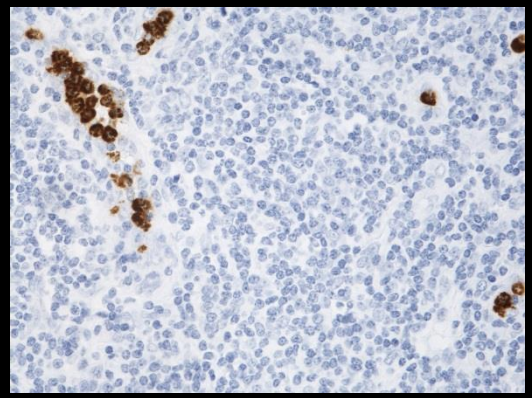
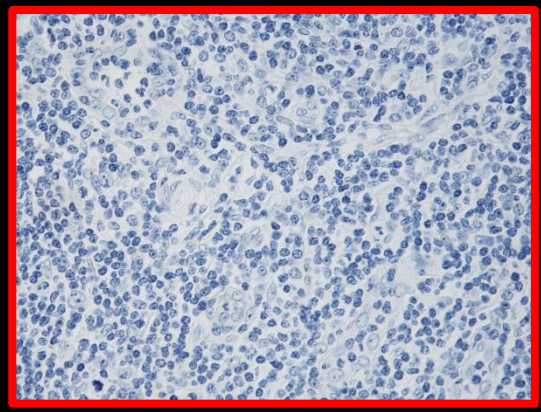
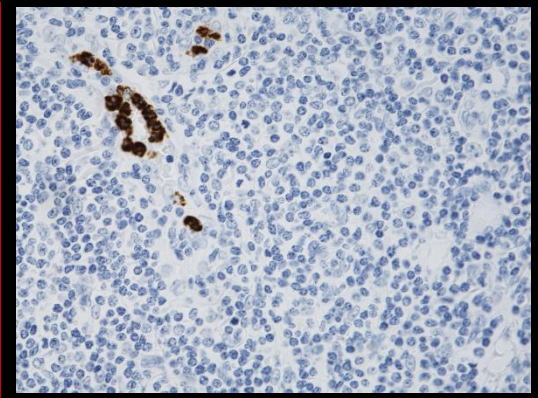
≤ 2% of all commonly used antibodies require enzymatic (or no) pre-treatment

The technical test approach – Analytical phase

Most common enzymes used in IHC:

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

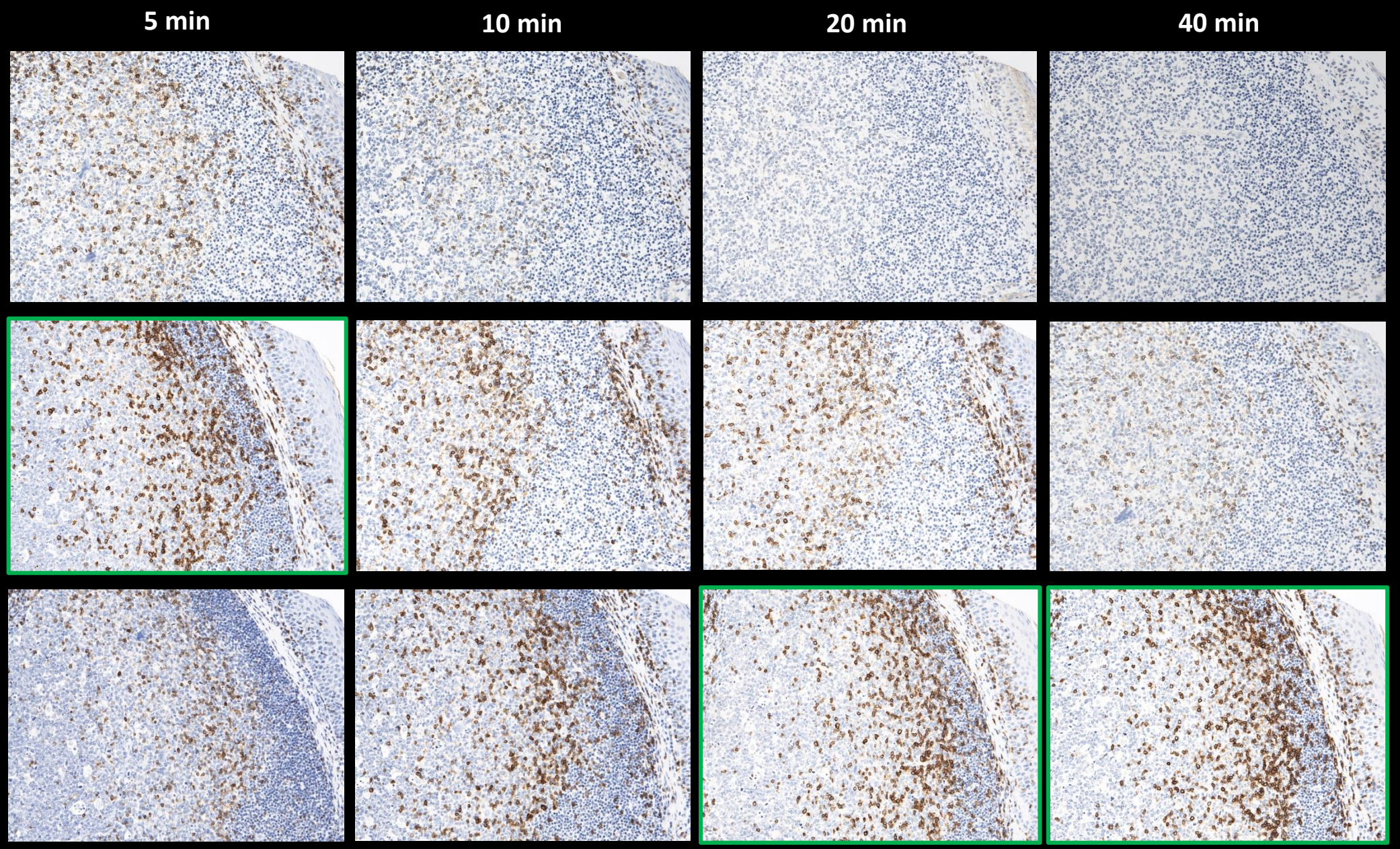
Choice of proteolytic enzyme

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

Proteolytic enzyme & digestion time ?

Tonsil NBF 48h

Digestion temp. 32°C



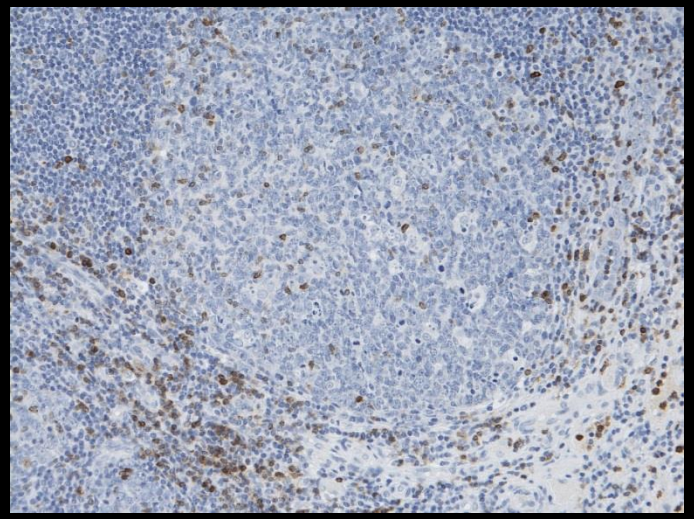
TCR β clone 8A3 (1:200 RR) / Flex+ (Omnis)

Proteolytic enzyme & digestion temperature ?

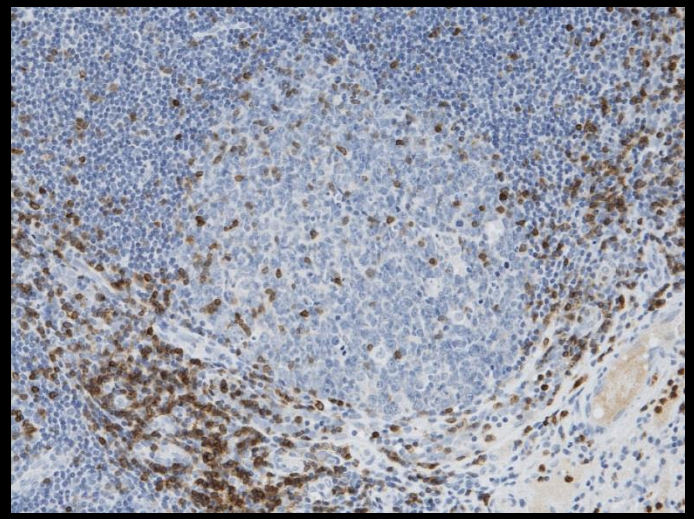
Tonsil NBF 48h

TCR β clone 8A3 (1:200 RR) / Flex+

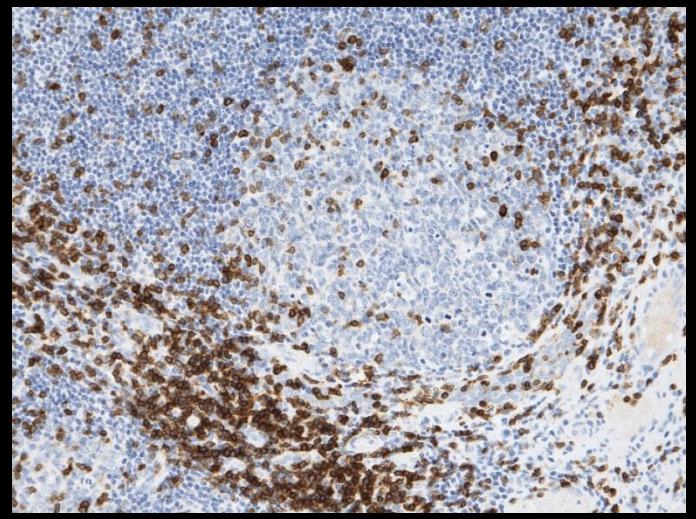
Trypsin Digestion temp. 4°C (10 `)



Trypsin Digestion temp. 24°C (10`)



Trypsin Digestion temp. 37°C (10`)



Increased intensity of TCR β positive T-cells

The technical test approach – Analytical phase

Enzymatic digestion (Influence of fixation time)

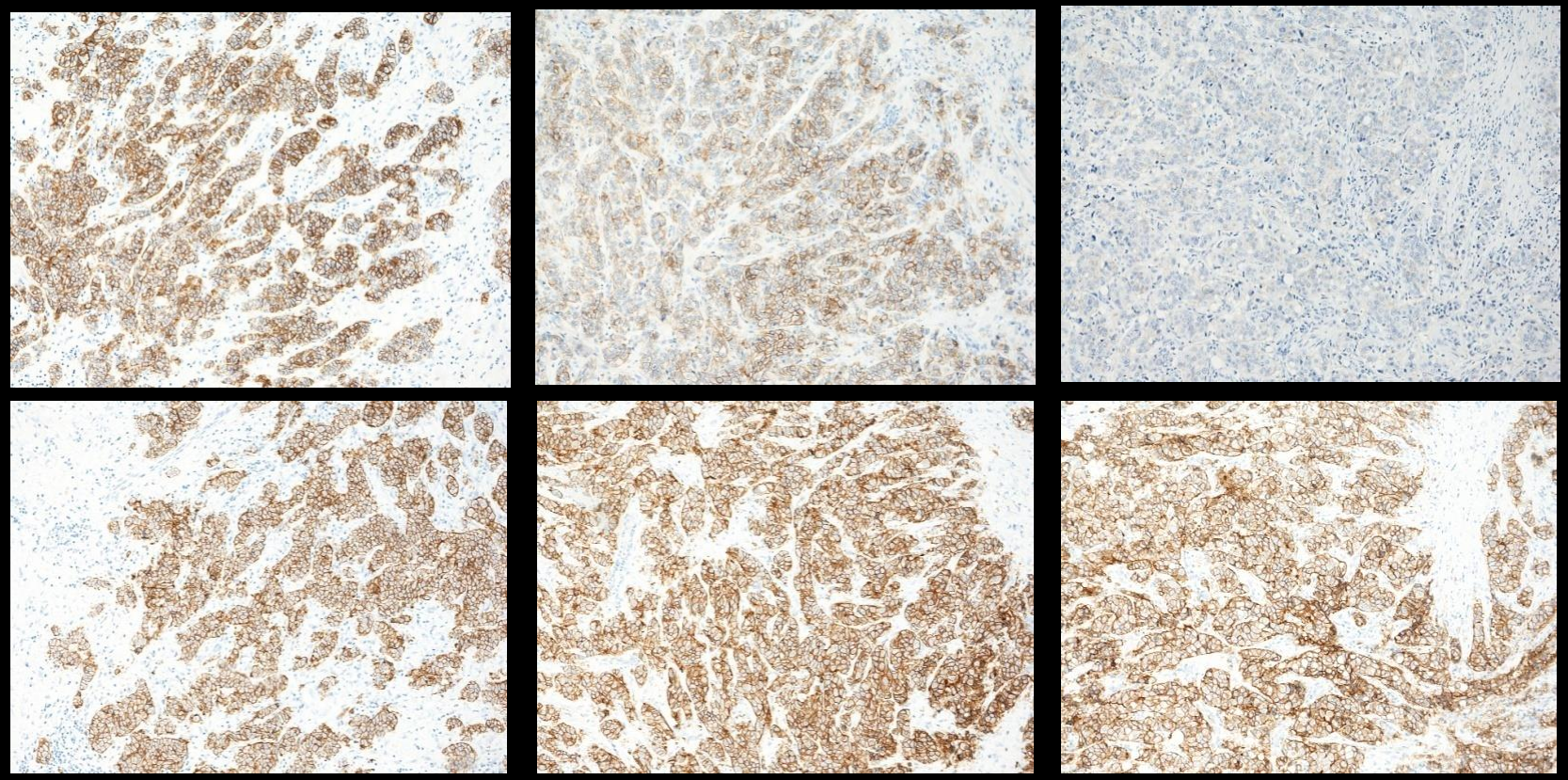
EP-CAM, clone MOC-31, dilution 1:20

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

Virchows Arch (2016) 468:19–29
DOI 10.1007/s00428-015-1829-1

ANNUAL REVIEW ISSUE

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

Mogens Vyberg [1,2] · Søren Nielsen [1]

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 :

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

False positive or false negative results

The technical test approach – Analytical phase

Excessive retrieval:

- **Proteolytic pretreatment - over digestion (not calibrated to the fixation time in NBF)**
- **HIER using too high temperature for too long time (especially in alkaline retrieval buffers)**
- **Antigen Retrieval using standard HIER procedures on fragile tissue/cell material (cell pellets)**

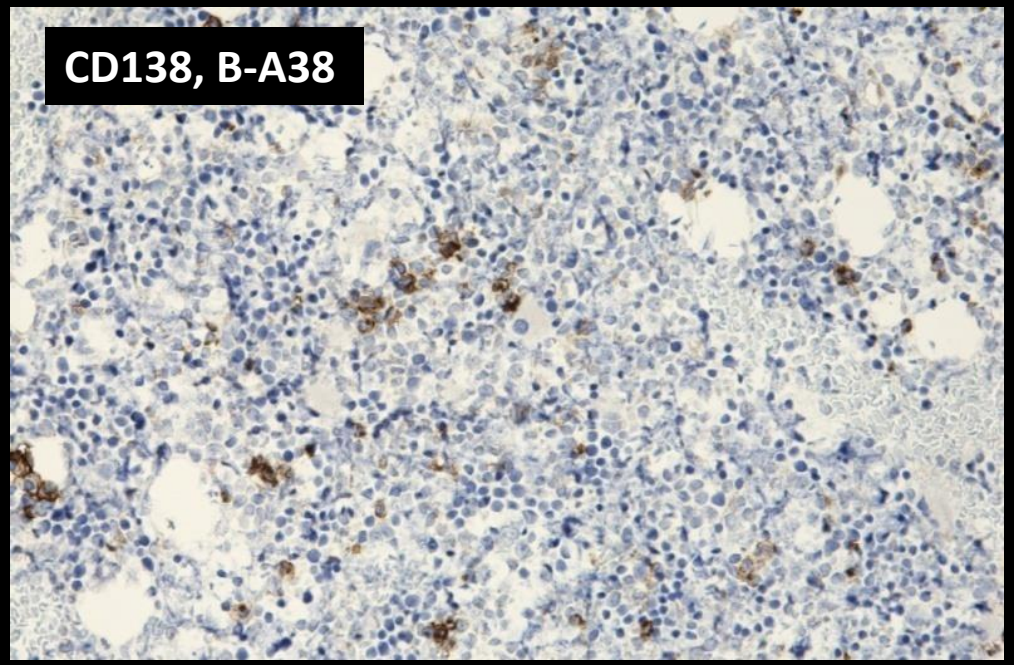
HIER settings: Recommendations given by the manufacturer`s

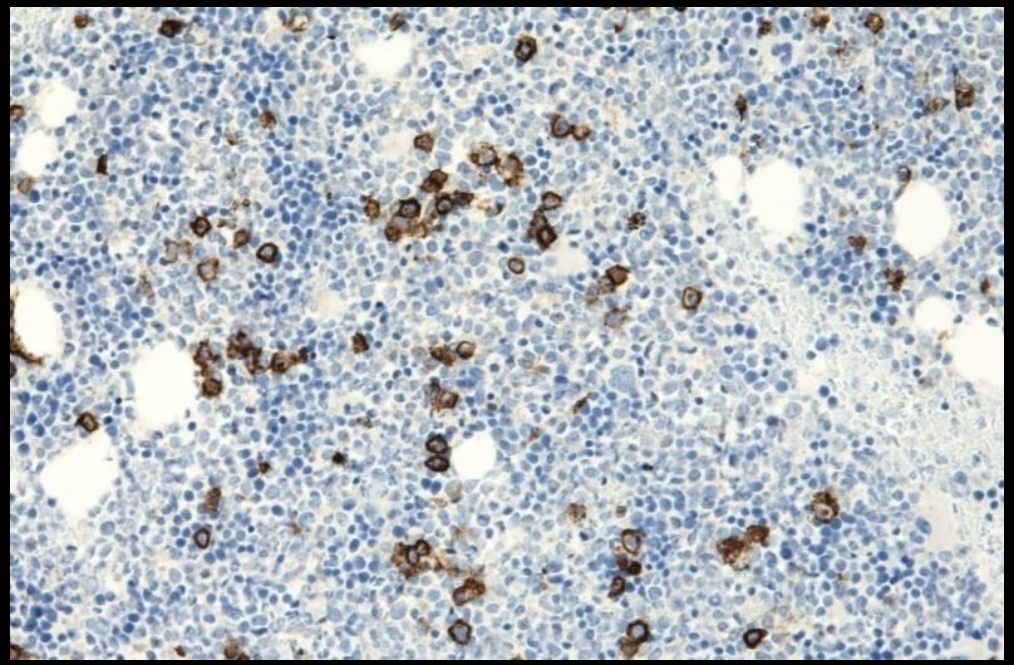
Excessive antigen retrieval related to the PT-module (Dako)

Influence of pre-heat temperature (65°C versus 85°C)

P/E 65ºC

P/E 85ºC





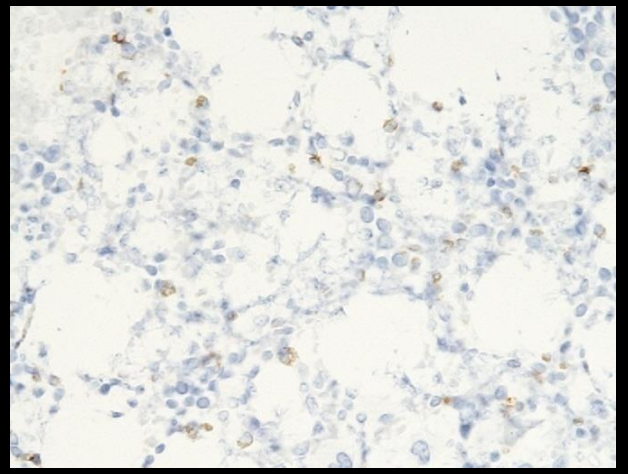
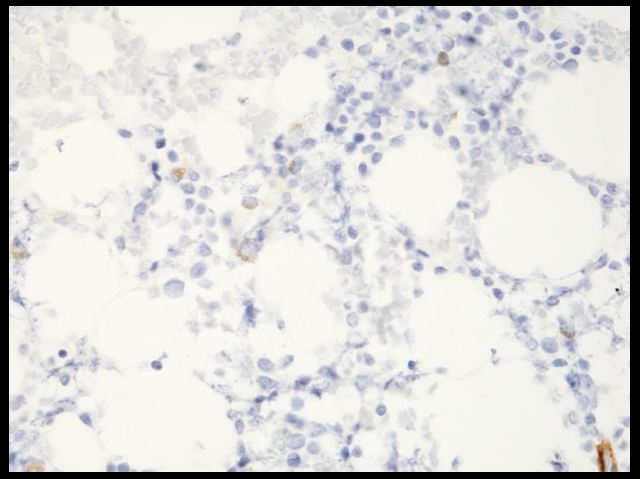
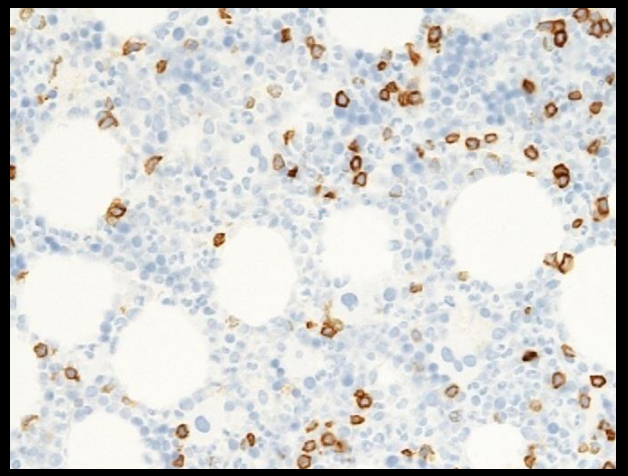
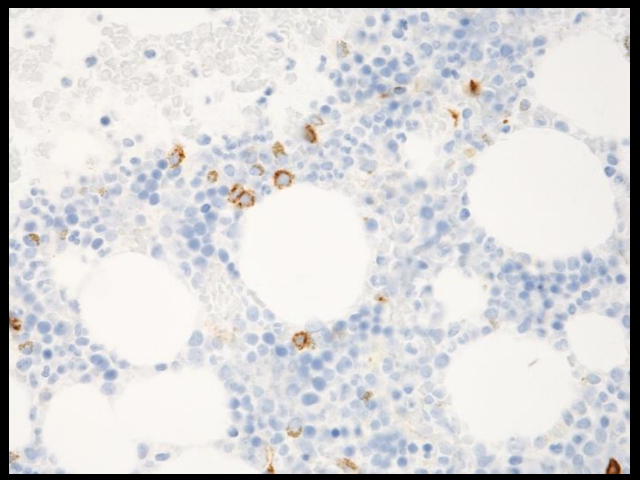
Bone marrow aspirate clot, NBF 96h

PT, High pH (3-1) 95ºC, / 20 min

The technical test approach – Analytical phase

Bone marrow aspirate clot

AS: PT-Link, High pH buffer´s at 97ºC / 20`

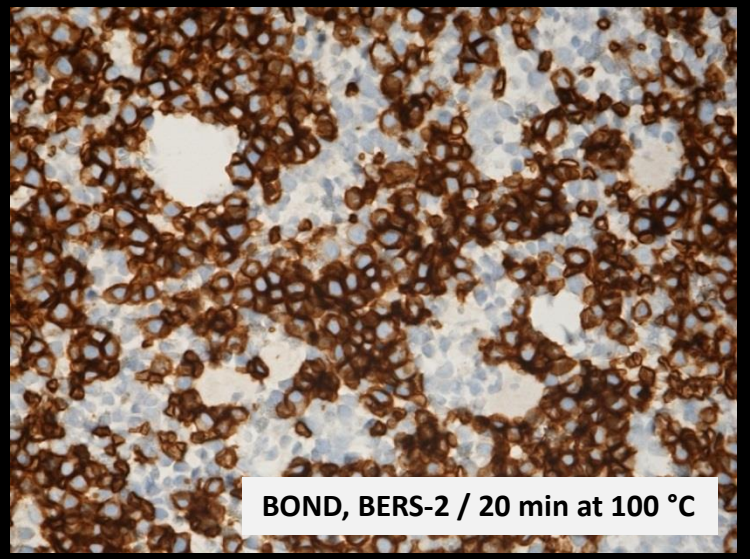
	CD5 clone SP19	CD34 clone QBEND-10
High pH (3-1) (Dako) Recommended settings: 65ºC		
HIER buffer H (LabVision) Recommended settings: 85ºC		

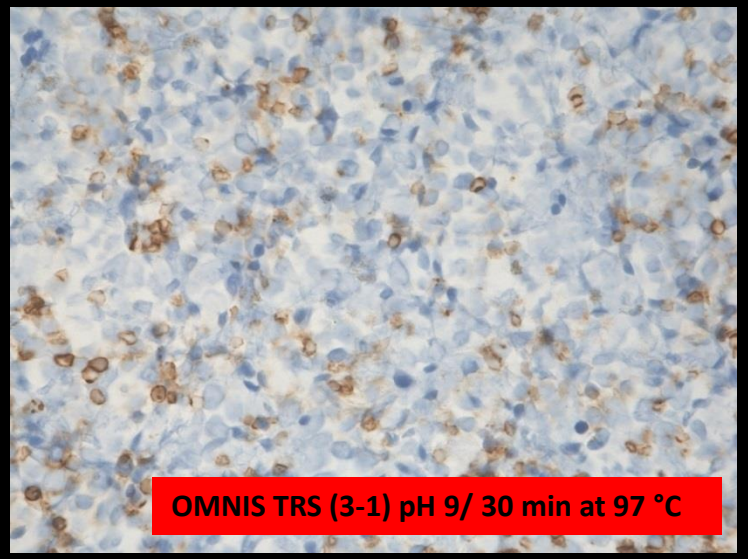
The technical test approach – Analytical phase

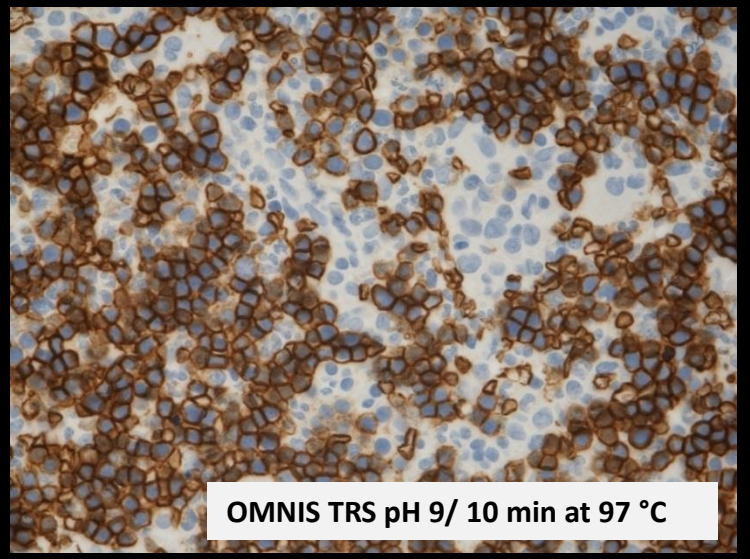
Bone marrow aspirate clot (fixed for 24h in 10% formaldehyde)

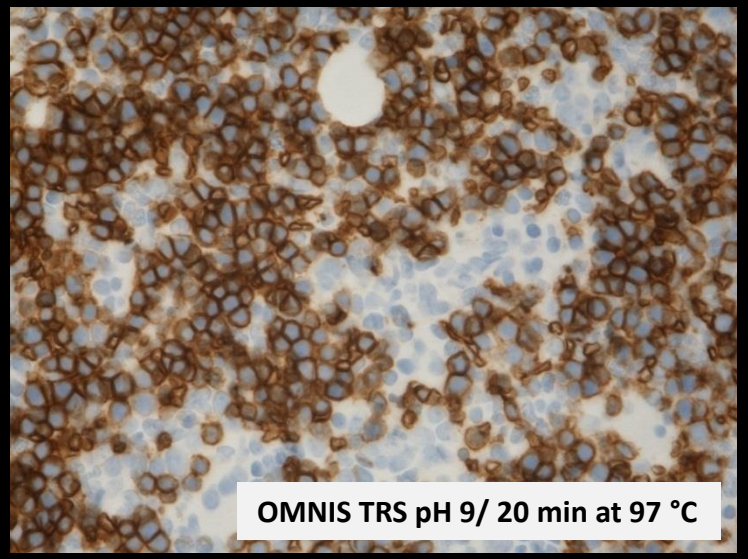
Glycophorin A clone JC159 (1:500)

Flex+









Morphology ?

Bone Marrow cloth fixed for 24 h in 10% formalin

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

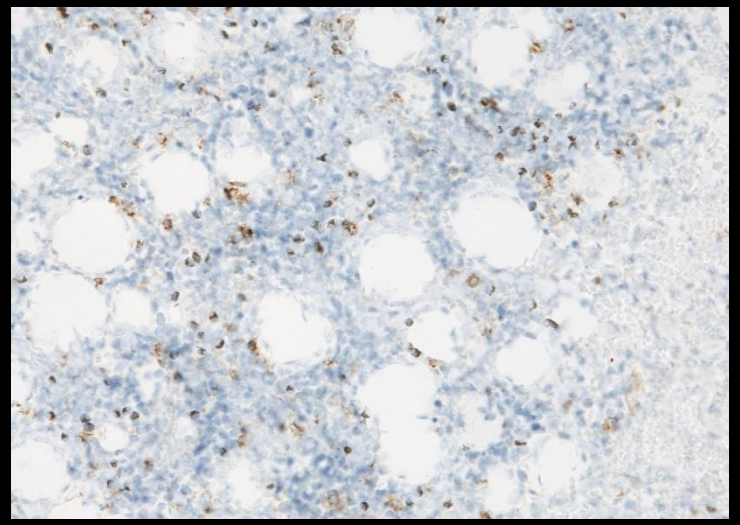
Agilent/Dako

HIER buffer H, 24` at 97C

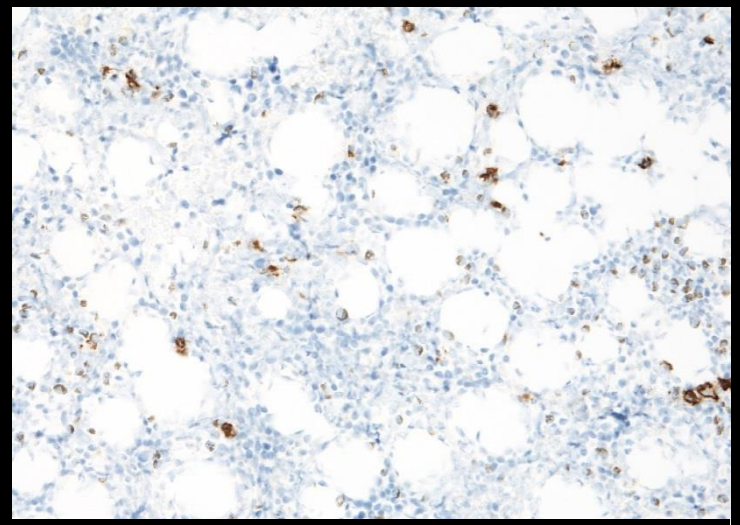
Thermo S./ LabVision

Chemical composition of the HIER buffer

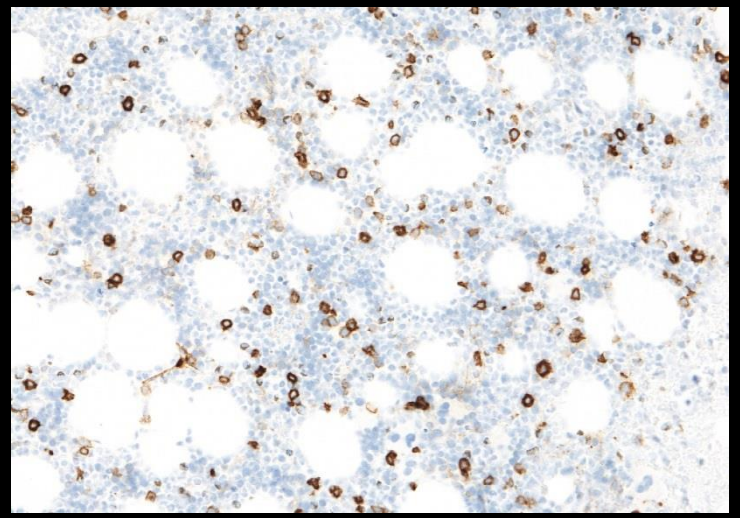
CD117, EP10 (1:25 RR)

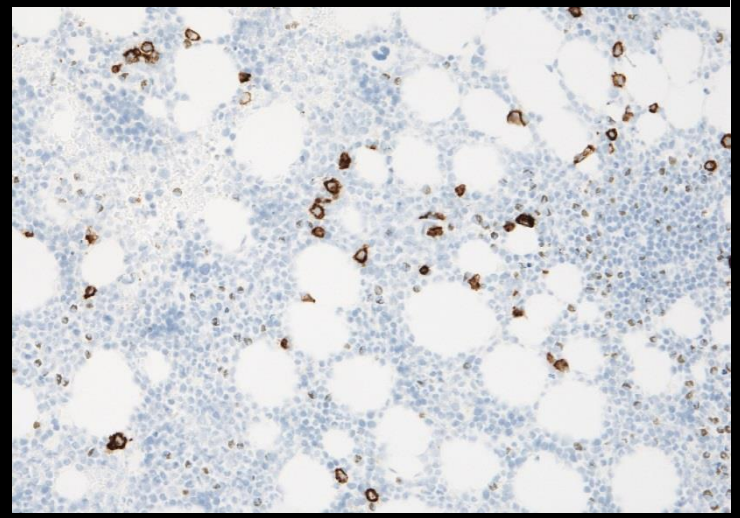


CD138, B-A38 (1:1000)



Omnis: Flex+

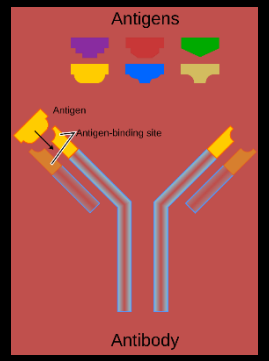




Pause

The technical test approach – Analytical phase

Parameters related to the primary Ab 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

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

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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%

[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)
  - Stainer platform dependent antibodies
    - Provide low sensitivity / specificity

False positive or false negative results

The technical test approach – Analytical phase

Problem: Primary antibody provides low sensitivity

Table 1. **Antibodies and assessment marks for ERG, run 50**

Concentrated antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	Suff. OPS[2]
mAb clone **9FY**	8 1 1	Biocare Thermo/Neomarkers Zytomed	0	0	2	8	-	-
rmAb clone **EP111**	20 8 1	Agilent/Dako Cell Marque BioSB	7	7	11	4	48%	53%
rmAb clone **EPR3864**	8 2	Abcam/Epitomics Zeta Corporation	6	2	1	1	80%	100%

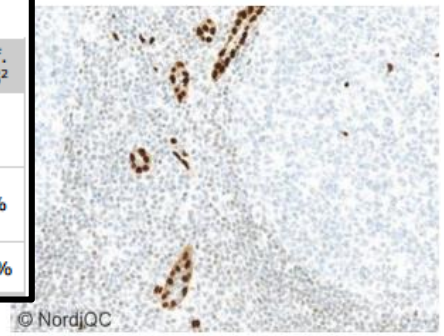

Fig. 1a
Optimal staining for ERG of tonsil using the rmAb clone EP111 within a laboratory developed assay optimally calibrated, using HIER in an alkaline buffer and a 3-step multimer based detection system, OptiView Ventana. Virtually all endothelial cells show a strong nuclear staining reaction, while mantle zone B-cells and interfollicular lymphocytes show a weak but distinct nuclear staining reaction. .
Also compare with Figs. 2a – 4a, same protocol.

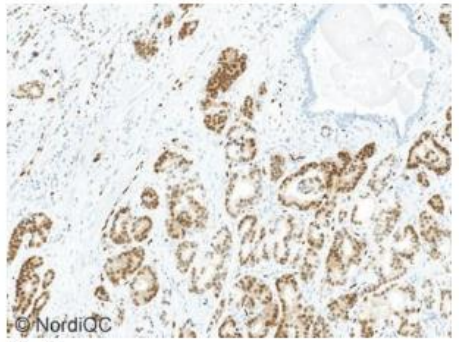

Fig. 3a
Optimal ERG staining of the prostate adenocarcinoma, tissue core no. 5, using same protocol as in Figs. 1a and 2a.
A moderate to strong and distinct nuclear staining reaction is seen in virtually all neoplastic cells. A weak cytoplasmic staining reaction is seen, but in general, a high signal-to-noise ratio is observed.

Primary antibodies providing low sensitivity (NordiQC results/Latest run)

ERG (Ets-Related-Gene) clone 9FY
ALK-Lu clone ALK1
GATA3 clone HG3-31
“CEA clone II-7”
CGA clone DAK-A3
……

Focus on clones giving optimal results and use app. tissue control material

ERG, 9FY – prostate adenocarcinoma / TMPRSS2-ERG gene fusion ?

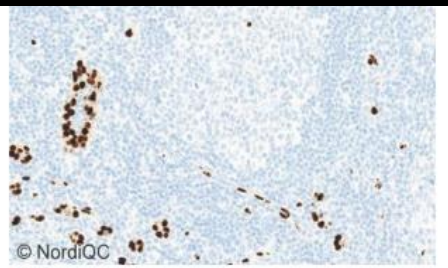

Fig. 5a
Staining for ERG of tonsil using the mAb clone 9FY within a laboratory developed test using HIER in an alkaline buffer and a 3-step polymer based detection system.
This antibody does not react with lymphocytes, whereas an intense nuclear staining reaction in endothelial cells is seen.
However despite this intense staining reaction an insufficient result in the prostate adenocarcinomas was seen – see also Fig. 5b, same protocol.
14 of 15 protocols based on mAb clone 9FY provided an insufficient result and only one sufficient result assessed as good.

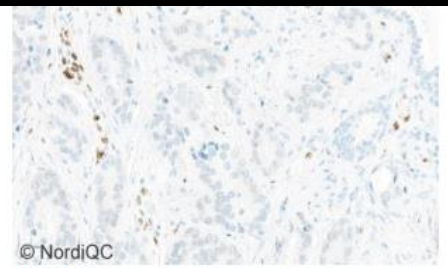

Fig. 5b
Insufficient ERG staining of prostate adenocarcinoma, tissue core no. 5 with high level ERG expression using same protocol as in Fig. 5a.
Only the endothelial cells show a distinct nuclear staining reaction, while virtually all the neoplastic cells are negative.
Compare with Fig. 3a showing the level expected.

The technical test approach – Analytical phase

Pathol Res Pract. 2014 Aug;210(8):508-13. doi: 10.1016/j.prp.2014.04.005. Epub 2014 Apr 18.

Immunoreactivity using anti-ERG monoclonal antibodies in sarcomas is influenced by clone selection.

Machado I[1], Mayordomo-Aranda E[2], Scotlandi K[3], Picci P[3], Llombart-Bosch A[4].

Author information

Abstract

The aim of the present study was to explore ERG immunoreactivity in a series of sarcomas, GIST and malignant rhabdoid tumor (MRT), considering the not fully elucidated specificity and sensitivity of this antibody. Paraffin-embedded tissue microarrays from those tumors were stained with anti-ERG against the C-terminus [(EPR3864(2)] and N-terminus (Clone 9FY). EPR3864(2) was positive in almost all angiosarcomas, and MRT.GIST were positive in a large proportion of cases (38.4%), and more than half the synovial sarcomas (52.7%) revealed EPR3864(2) staining. Several chondrosarcomas, osteosarcomas, rhabdomyosarcoma and Ewing's sarcoma family of tumors (ESFT) presented EPR3864(2) expression in a lower number of cases. 9FY was positive in most of the angiosarcomas; however, only sporadic ESFT and synovial sarcoma were positive and the other tumors tested were negative. Fourteen ESFT with EWSR1/Fli-1 gene fusion presented positive nuclear staining for EPR3864(2). Similarly, 5 ESFT with EWSR1/Fli-1 gene fusion presented positive staining for 9FY. We must stress that the difference between the present and previous studies may be due to the source of the anti-ERG employed, anti-ERG against C or N-terminus, protein cross-reactivity and dilution. In conclusion, specificity for ERG staining in sarcomas should be considered with caution and the immunoexpression is undoubtedly influenced by clone and antibody selection.

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KEYWORDS: ERG; Ewing's sarcoma; Immunohistochemistry; Sarcomas

Detection of ERG using clone 9FY in prostate adenocarcinomas - antibody raised against the N-terminal part of the ERG (wt) protein ?

TMPRSS2-ERG rearrangements often encodes N-terminal truncated ERG proteins

The technical test approach – Analytical phase

Problem: Primary antibody providing low sensitivity

Table 1. **Abs and assessment marks for CGA, run 31**

Concentrated Abs	N	Vendor	Optimal	Good	Borderl.	Poor	Suff.[1]	Suff. OPS[2]
mAb clone **LK2H10**	13 5 2 2 1 1	NeoMarkers BioGenex Chemicon/Millipore Leica/Novocastra EuroProxima Zytomed	5	13	6	0	75 %	91 %
mAb clones **LK2H10 + PHE5**	8 3	NeoMarkers Biocare	3	5	3	0	73 %	80 %
mAb clone **DAK-A3**	16	Dako	0	2	12	2	13 %	-
mAb clone **5H7**	4	Leica/Novocastra	0	2	0	2	-	-
rmAb clone **SP12**	3 1 1 1	Spring Bioscience DSC Master Diagnostica NeoMarkers	0	0	5	1	0%	-
pAb **A0430**	53	Dako	36	15	2	0	96 %	100 %
pAb **18-0054**	2	Zymed	0	1	1	0	-	-
pAb **RB-9003-P**	1	NeoMarkers	0	0	1	0		

Table 1. **Antibodies and assessment marks for CGA, run 46**

Concentrated antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	Suff. OPS[2]
mAb clone **5H7**	4	Leica/Novocastra	0	0	3	1	-	-
mAb clone **DAK-A3**	36	Dako/Agilent	0	2	17	17	6%	-
mAb clone **LK2H10**	22 18 6 3 2 2 1 1 1 1 1	Thermo/Neomarkers Cell Marque Immulologic Biogenex Millipore Zytomed Abcam A.Menarini Diagnostic Biosystems Europroxima Monosan Unknown	24	31	0	4	93%	98%
mAb clone **PHE5**	1	Unknown	0	0	1	0	-	-
mAb clones **LK2H10+PHE5**	6 5	Thermo/Neomarkers Biocare	3	8	0	0	100%	100%
rmAb clone **EP38**	1	Epitomics	0	1	0	0	-	-
rmAb clone **SP12**	1 1	Master Diagnostica Thermo/NeoMarkers	0	0	0	2	-	-

CGA

Substituting Dako`s old polyclonal Ab A430 (discontinued by the manufacturer) with the monoclonal DAK-A3 is not a good decision (Run 31/46)

mAb LK2H10

mAb´s LK2H10 + PHE5

The technical test approach – Analytical phase

Table 1. **Antibodies and assessment marks for MLA, Run 49**

Concentrated antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	Suff. OPS[2]
mAb clone **A103**	73 18 2 7 1 1 4	Dako/Agilent Leica/Novocastra Monosan Cell Marque Immunologic Zeta Corp. Thermo Scientific	26	31	32	17	54%	60%
mAb clone **M2-7C10**	1 1	Cell Marque Zytomed	1	1	0	0	-	-
mAb clone cocktail **M2-7C10+M2-9E3**	2 3	NeoMarkers Biocare	1	2	2	0	-	-
mAb clone cocktail **HMB45+MC-7310 +M2-9E3+T311**	4	Biocare	2	1	0	1	-	-
mAb clone cocktail **A103+M2-7C10+ M2-9E3**	1	Life technologies	0	0	1	0	-	-
mAb clone **BS52**	1	Nordic Biosite	1	0	0	0	-	-
rmAb **EP43**	3 1	Epitomics Cell Marque	4	0	0	0	-	-
Ready-To-Use antibodies								
mAb clone **A103, IR633/IS633**	57	Dako/Agilent						
mAb clone **A103 790-2990**	60	Ventana/Roche						
mAb clone **A103, PA0233**	5	Leica/Novocastra						
mAb clone **A103, 281M-87/281M-88**	4	Cell Marque						
mAb clone **A103, API3114**	1	Biocare						
mAb clone **A103, PDM153**	1	Diagnostic BioSystems						
mAb clone **A103, MAB-0275**	1	maixin						
mAb clone **M2-7C10+M2-9E2 MAD-001767QD**	1	Master Diagnostica						
mAb clone cocktail **HMB45+A103+T311 790-4677**	1	Ventana/Roche						

Melan A /MART1

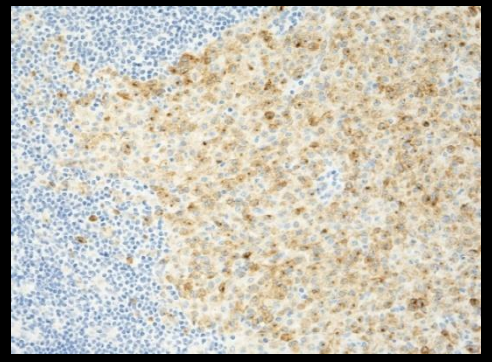
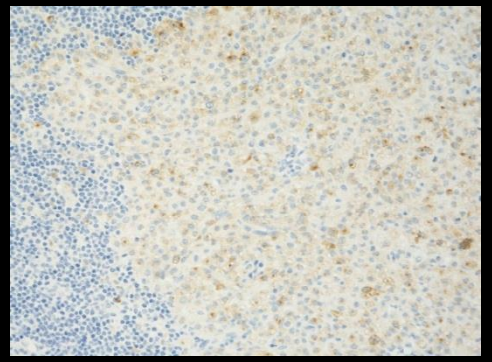
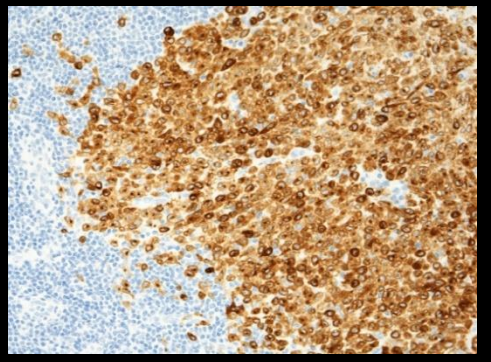
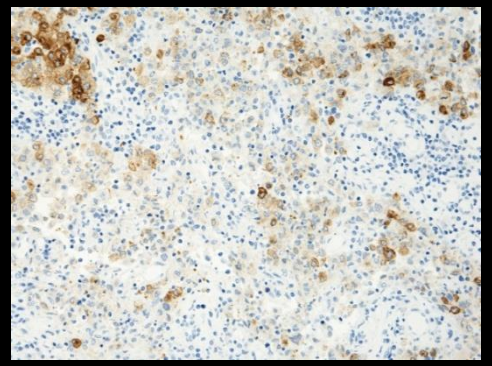
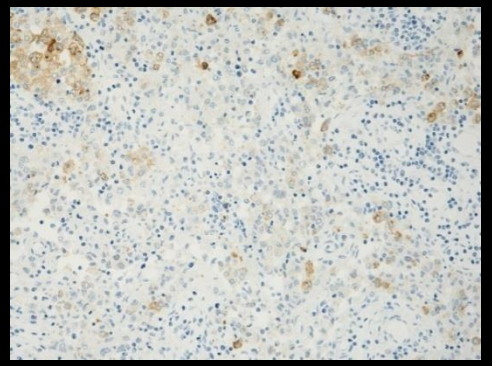
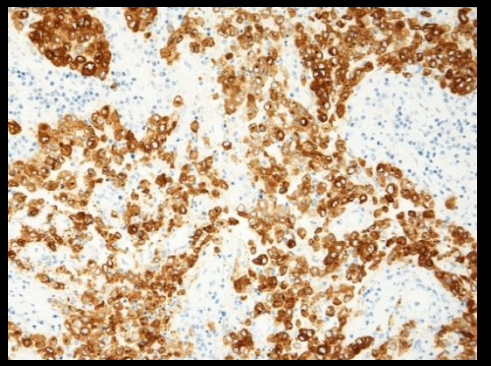
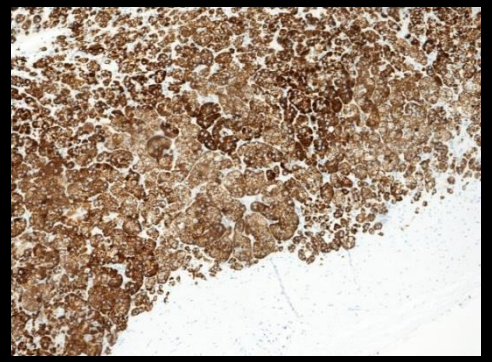
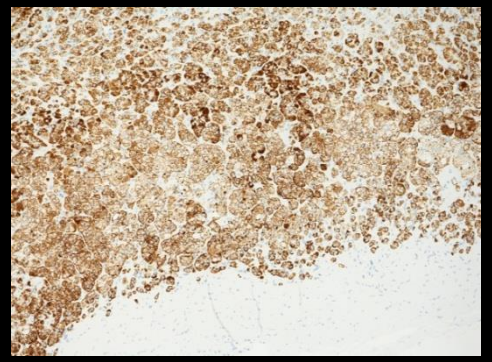
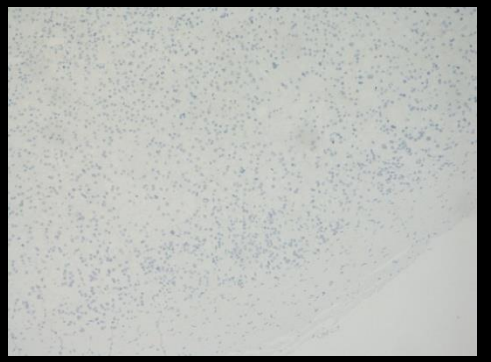
<u>Melan A (MLA) / MART-1:</u>

238 participants ~ 93% used clone A103 (single or in cocktail antibody solutions)

Is MLA , A103 the best primary Ab for detection of melanomas and does it “fit-for-purpose” ?

The technical test approach – Analytical phase

Melan A /MART1

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

The technical test approach – Analytical phase

MUM1

Table 1. **Antibodies and assessment marks for MUM1, run 48**

Concentrated antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	Suff. OPS[2]
mAb clone **MUMp1**	84 1 1	Agilent/Dako Diagnostic Biosystem GeneMed	52	19	11	4	83%	86 %
mAb clone **MRQ-8**	3	Cell Marque	0	0	2	1	-	-
mAb clone **BC5**	3	Biocare Medical	0	0	3	0	-	-
mAb clone **EAU32**	3	Leica/Novocastra	0	2	1	0	-	-
rmAb clone **MRQ-43**	5 1 1	Cell Marque Menarini Zeta	0	0	3	4	-	-
rmAb clone **SP114**	1	Thermo S./ LabVision	0	1	0	0	-	-
Ready-To-Use antibodies								
mAb clone **MUMp1 GA644**	18	Agilent/Dako	8	7	2	1	83%	88 %
mAb clone **MUMp1 IR/IS644**	28	Agilent/Dako	13	12	3	0	89%	88 %
mAb clone **MUMp1 GA644, IR/IS644**[3]	5	Agilent/Dako	3	0	2	0	-	-
mAb clone **MUMp1 MAD-000470QD**	3	Master Diagnostica	1	1	1	0	-	-
mAb clone **MUMp1 MAB-0573**	1	Maixin	1	0	0	0	-	-
mAb clone **EAU32 PA0129**	6	Leica Biosystems	5	1	0	0	100%	100%
rmAb clone **MRQ-43 760-4529**	31	Ventana/Roche	0	0	25	6	0%	0%
rmAb clone **MRQ-43 358R-77/78**	15	Cell Marque	0	0	13	2	0%	0%
rmAb clone **EP190 358R-17/18**	1	Cell Marque	1	0	0	0	-	-
Total	211		84	43	66	18	-	
Proportion			40%	20%	31%	9%	60%	

1) Proportion of sufficient stains (optimal or good).
2) Proportion of sufficient stains with optimal protocol settings only (see below).
3) RTU systems developed for Agilent/Dako`s automatic systems (Omnis/Autostainer) but used by laboratories off-label on the platforms Ventana Benchmark/Ultra or Leica BOND III.

Primary antibodies providing low specificity and/or poor signal-to-noise ration (NordiQC results/Latest run)

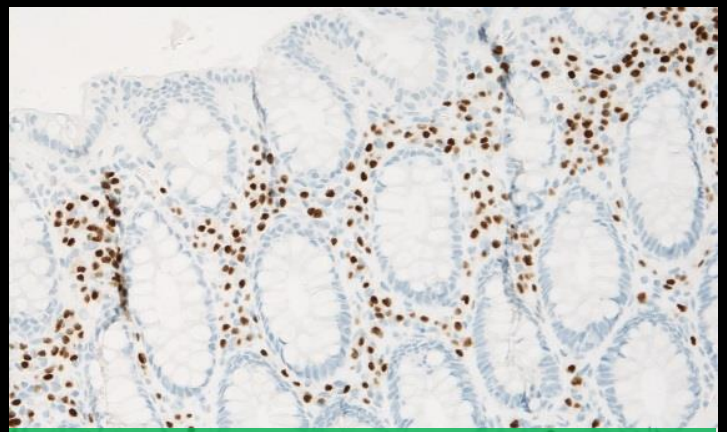
MUM1 clone MRQ-43 & BC5
CK-HMW clone 34βE12
PR clone 1E12
Many pAbs (e.g. P40 and SOX10)
……

Focus on clones giving optimal results and use app. tissue control material (colon and tonsil)

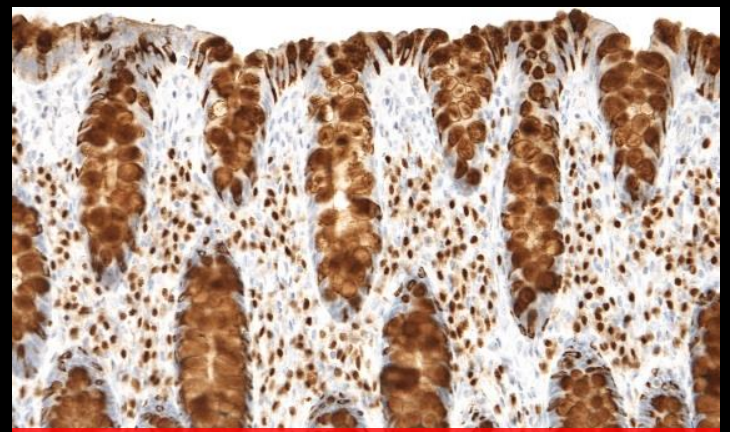
MUMp1, EAU32 & EP190

The technical test approach – Analytical phase

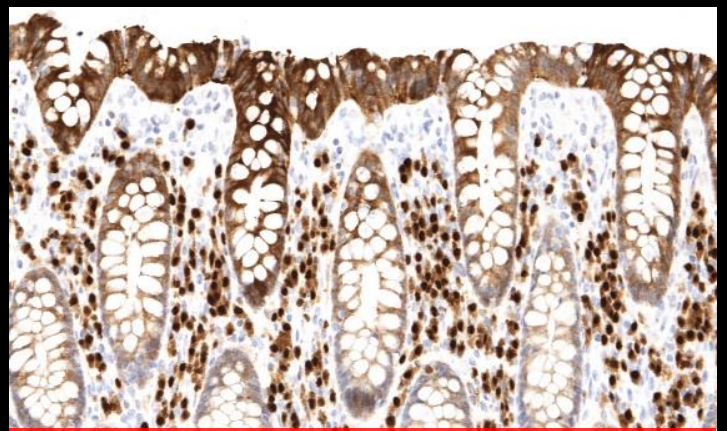
Problem: Primary antibody provides low specificity and/or poor signal-to-noise ration



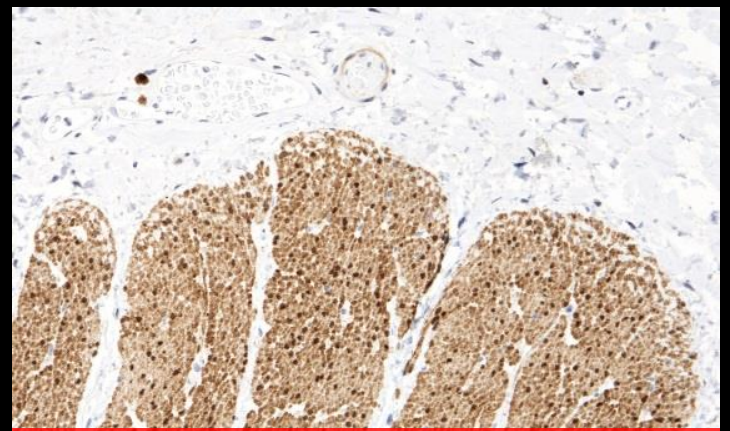
MUMp1, optimal



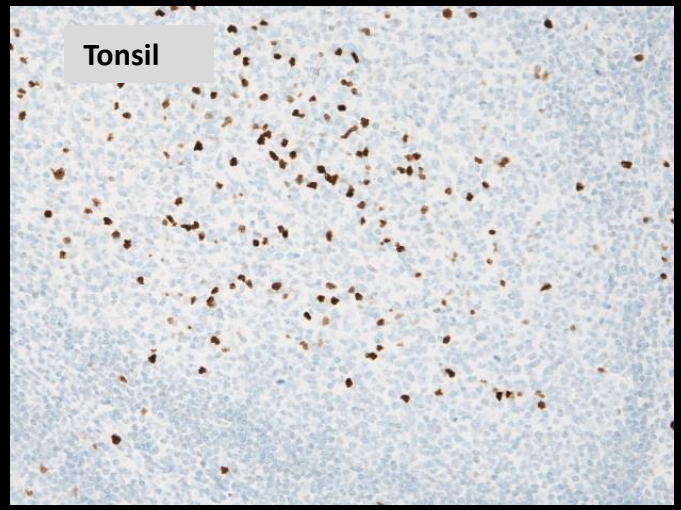
BC5, aberrant cytoplasmic staining result



MRQ-43, aberrant cytoplasmic staining result



MRQ-43, false positive



Clones providing optimal results:

MUMp1, EAU32 & EP190

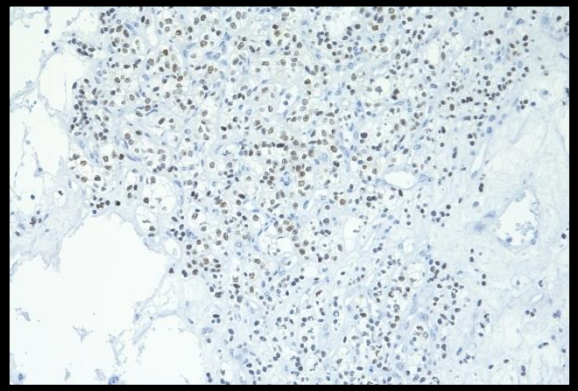
The technical test approach – Analytical phase

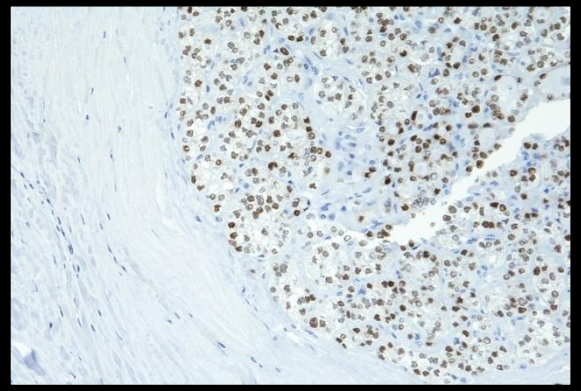
Problem: Primary antibody provides low specificity and/or poor signal-to-noise ration

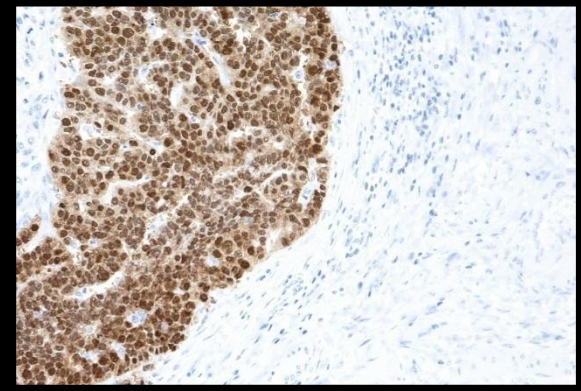
Renal Cell Carcinoma (CC)

Thyroid Carcinoma (Pa)

Ovary Carcinoma (Se)

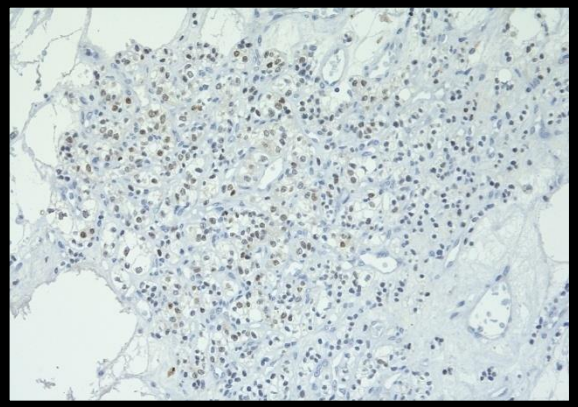


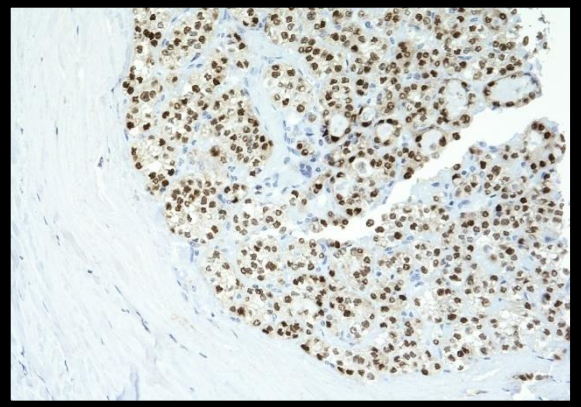


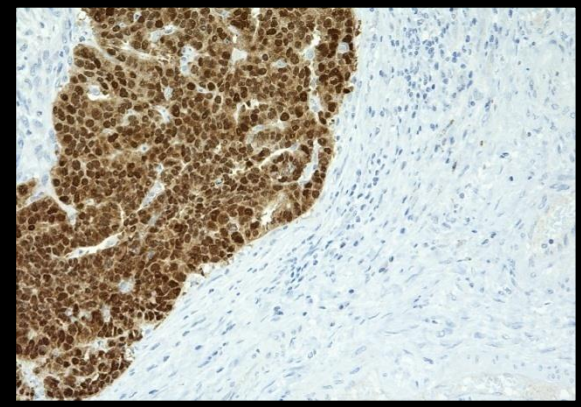


Which antibody ?

Pax-8 / CM / Dil 1:2000 / Clone MRQ-50 - Mab







Pax-8 / BC / Dil 1:150/ Clone BC12 - Mab

The technical test approach – Analytical phase

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

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

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

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

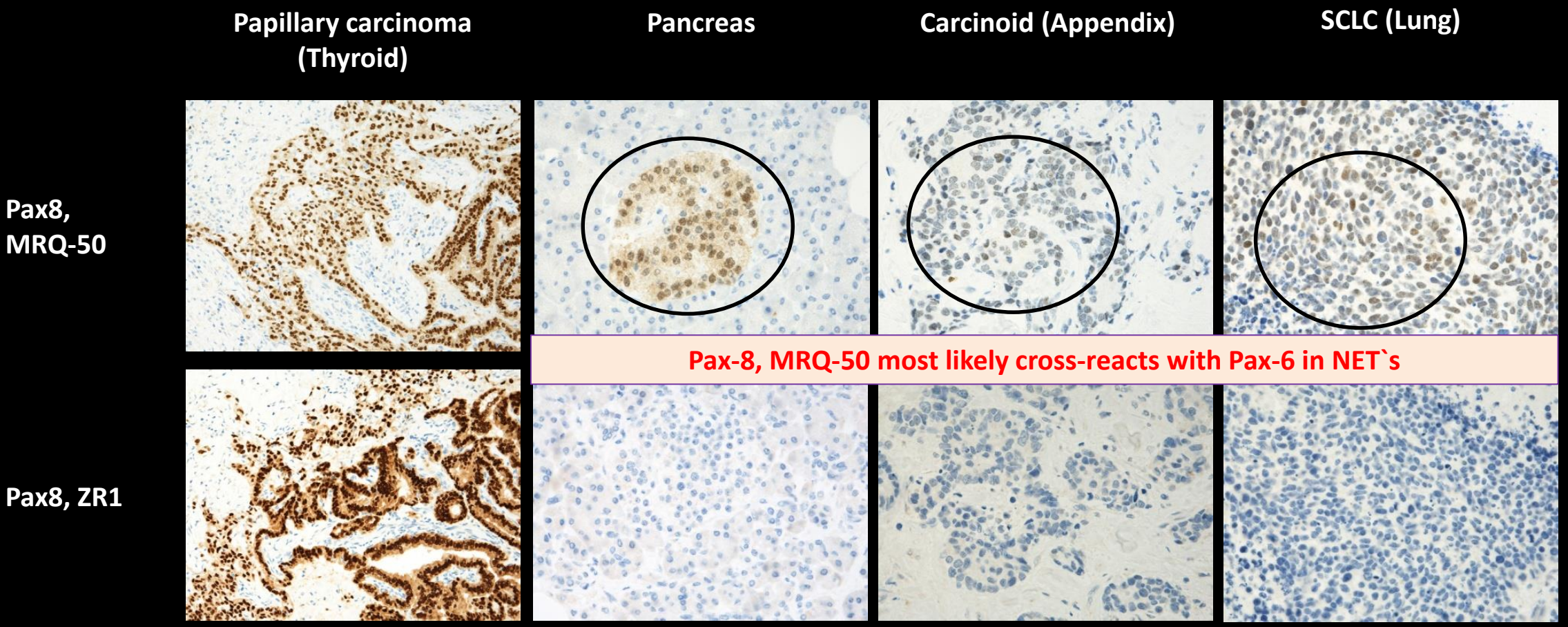
Moretti L et al. : *Mod Pathol. 2012; 25 : 231-236*

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

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

The technical test approach – Analytical phase

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



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

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

The technical test approach – Analytical phase

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

Table 1. Antibodies and assessment marks for PAX8, run 42

Concentrated antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	
mAb clone **MRQ-50**	33	Cell Marque	19	8	6	0	82%	
mAb clone **BC12**	7	BioCare	1	3	1	2	57%	
mAb clone **ILQ-150**	1	Immunologic	1	0	0	0	-	
mAb clone **PAX8R1**	1	Abcam	0	1	0	0	-	
rmAb clone **ZR-1**	1 1 1	Abcam Zeta Zhongshan	2	0	0	1	-	
pAb, **363A**	11	Cell Marque	0	4	7	0	36%	
pAb, **10336-1-AP**	11	Protein Tech	5	5	0	1	91%	
pAb, **CP379**	4	Biocare	1	2	1	0	-	-
pAb, **RBK047**	2	Zytomed Systems	0	1	1	0	-	-
pAb, **HP**								-
pAb, **ILP**								-
pAb, **ABE671**	1	Millipore	0	0	1	0	-	-
pAb, **NBP1-32440**	1	Novus	1	0	0	0	-	-

?

Cross react with other Pax proteins in the family (e.g. PAX5)

BC12 (sensitive to the certain platforms)

"ZR1" (lot variations/antibody diluent dependent)

EP298

SP348

Question`s:

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 ?

The technical test approach – Analytical phase

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

Table 1. **Antibodies and assessment marks for p40, run 48**

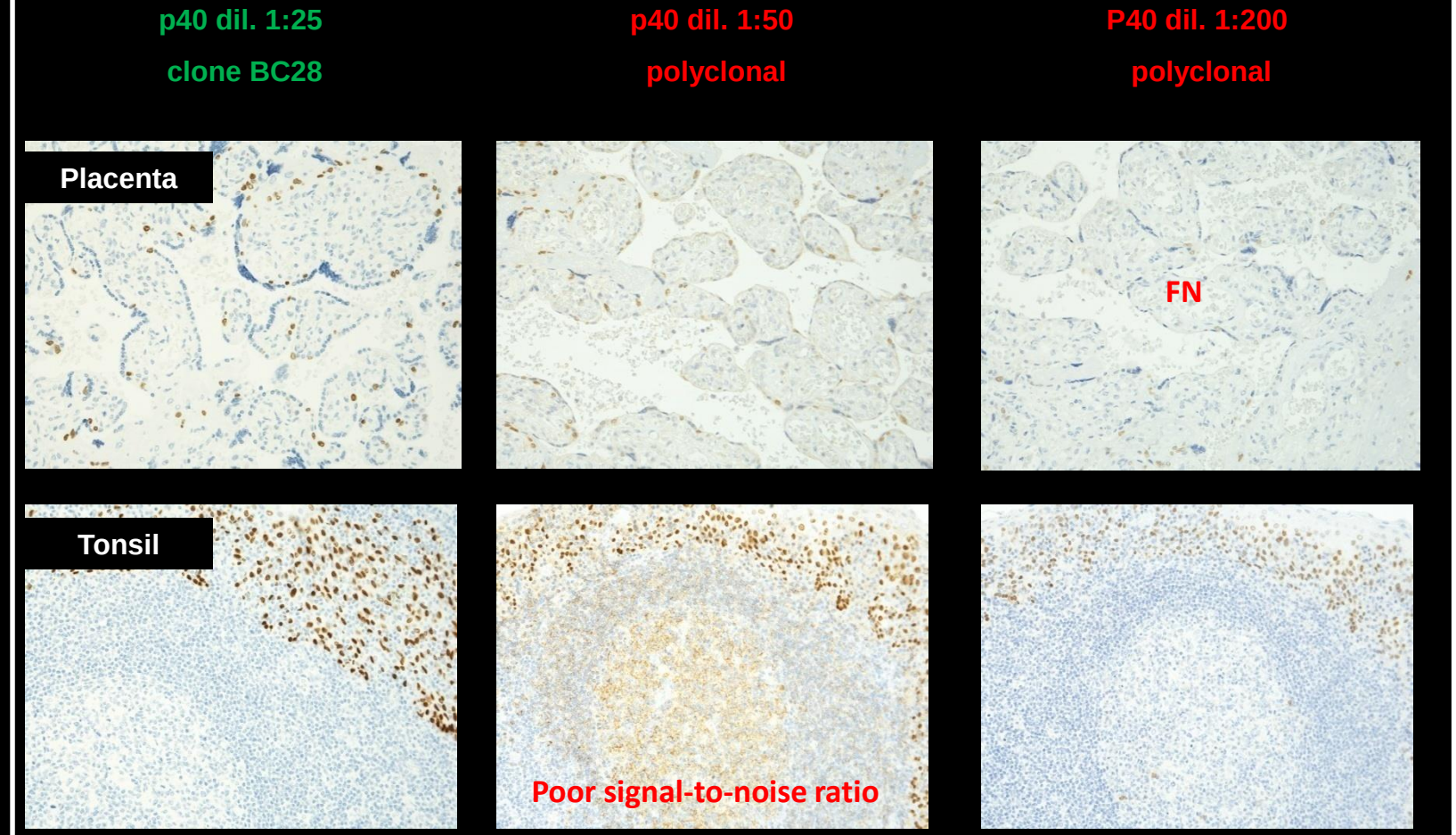
Concentrated antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	Suff. OPS[2]
mAb clone **BC28**	77 6 2 2 1	Biocare Zytomed Menarini abcam Nordic Biosite	52	24	10	2	86%	89%
rmAb clone **ZR8**	12 1 1	Immunologic Zeta Corporation BioSB	1	6	2	5	50%	67%
pAb **AC13030**	8	Biocare	0	2	6	0	-	-
pAb **RP163**	5	Diagnostic Biosystems	0	1	1	3	-	-
pAb **PC373**	4	Calbiochem, Merck	0	1	0	3	-	-
pAb **RBK054**	3	Zytomed	0	0	1	2	-	-
pAb **PI049**	1	DCS	0	1	0	0	-	-
pAb **PP123**	1	Pathnsitu	0	0	1	0	-	-
Ready-To-Use antibodies								
mAb clone **BC28 API/IPI/AVI 3066**	13	Biocare	5	8	0	0	100%	100%
mAb clone **BC28 790-4950**	39	Ventana	19	15	5	0	87%	94%
mAb clone **BC28 MSG097**	1	Zytomed	1	0	0	0	-	-
mAb clone **ZR8 MAD-000686QD**	3	Master Diagnostica	0	2	1	0	-	-
pAb **API 3030**	6	Biocare	0	0	4	2	-	-
pAb **RAB-066**	1	Maixin	0	1	0	0	-	-
pAb **A00112**	1	Loxo GmbH	0	0	1	0	-	-
Total	188		78	61	32	17	-	
Proportion			42%	32%	17%	9%	74%	

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

pAbs

No optimal results / pass rate of 23% (5 of 22)

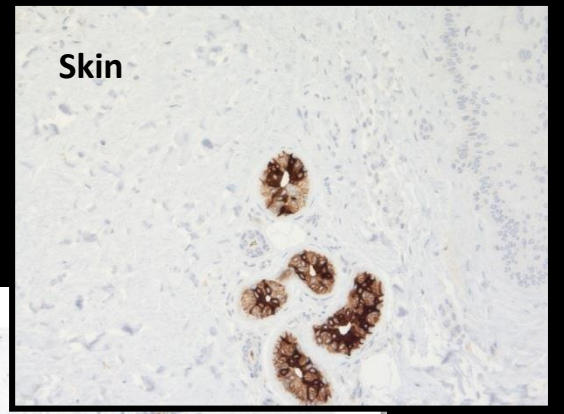
The insufficient results were typically characterized by a poor signal-to-noise ratio and aberrant staining reaction compromising the interpretation.



Both primary antibodies are from BIOCARE

The technical test approach – 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)

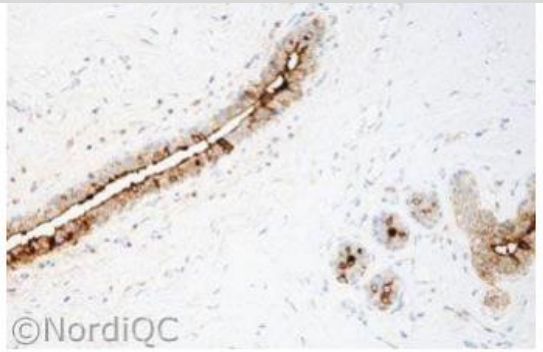


Fig. 1a
Optimal staining for GCDFP-15 of the breast hyperplasia using the mAb clone 23A3 optimally calibrated as a concentrate, HIER in an alkaline buffer and a polymer based detection system. The majority of the ductal epithelia cells show a distinct moderate to strong cytoplasmic staining reaction.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

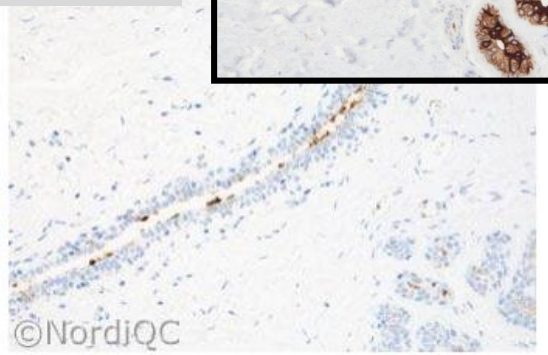


Fig. 1b
Insufficient staining for GCDFP-15 of the breast hyperplasia applying the mAb clone 23A3 as a concentrate using exactly the same protocol settings as used in Fig 1a, except for a 20 fold dilution of the primary antibody. The proportion and the intensity of the cells demonstrated are significantly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

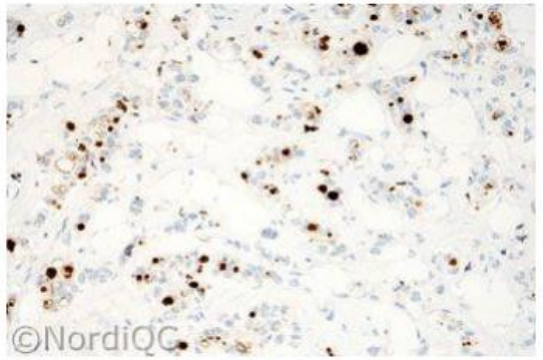


Fig. 2a
Optimal staining for GCDFP-15 of the breast carcinoma no. 5 using same protocol as in Fig. 1a.
The majority of the neoplastic cells show a moderate to strong dot-like cytoplasmic staining reaction.

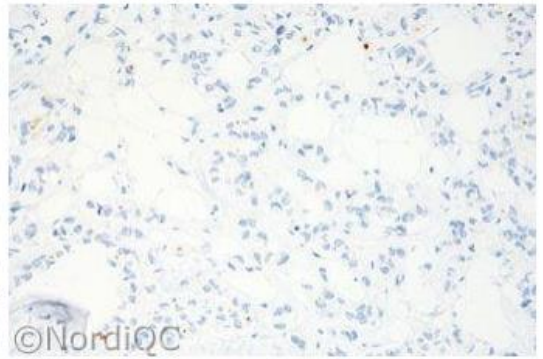


Fig. 2b
Insufficient staining GCDFP-15 of the breast carcinoma no. 5 using same protocol as in Fig. 1b. - same field as in Fig. 2a.
Only scattered neoplastic cells show a faint dot-like reaction.

The technical test approach – Analytical phase

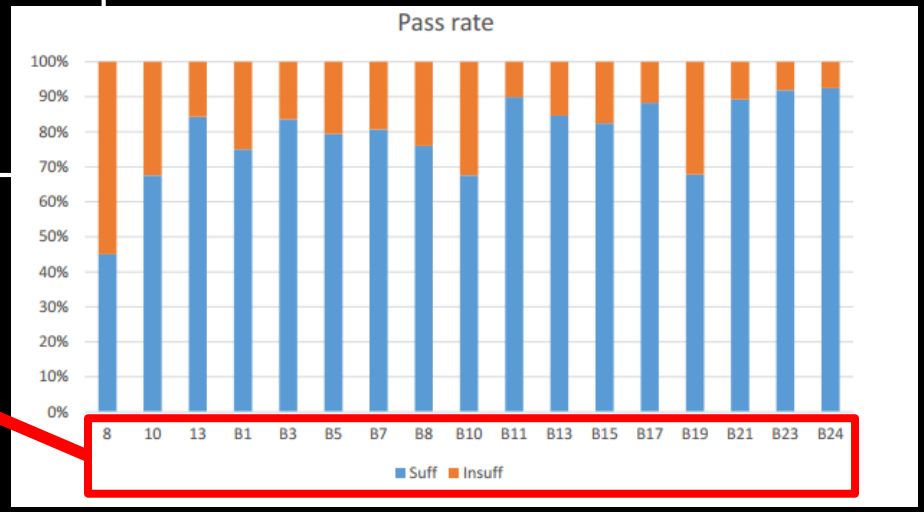
Problem: Primary antibody poorly calibrated providing low sensitivity

Estrogen Receptor (ER), NQC Run B24		Optimal	Good	Borderl.	Poor	Suff
Total protocols assessed	386	276	81	22	7	-
Proportion		71%	36%	6%	2%	92%

* All Ab clones and protocol settings

The most frequent causes of insufficient staining reactions were:

- Less successful primary Ab.
- Insufficient HIER - too short efficient HIER time and/or use of a non-alkaline buffer
- Too low concentration of the primary Ab.

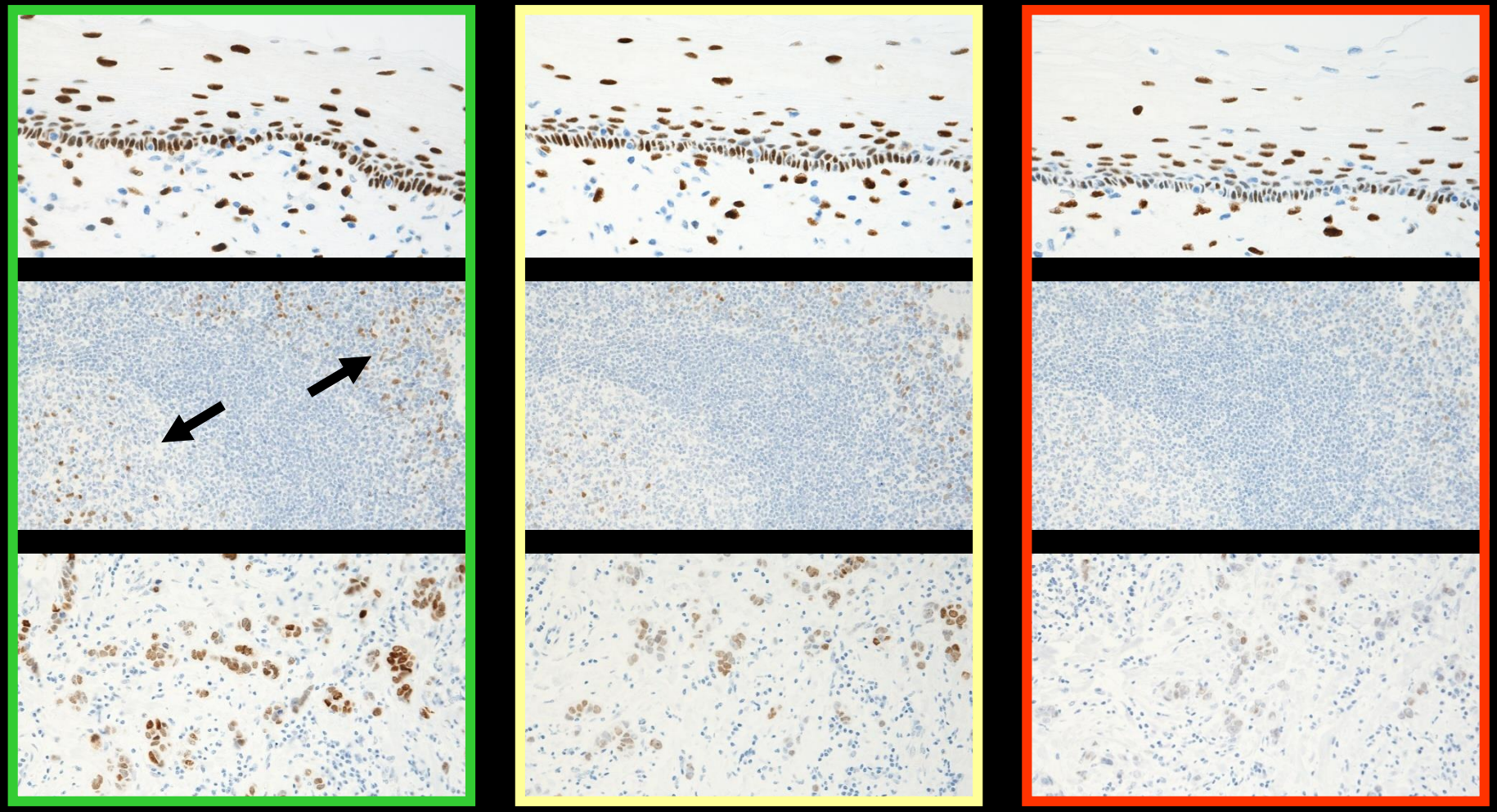


Estrogen receptor - Control tissue

- Normal cervix (high and non-expressors)
- Breast tumor´s x 3 (non, low and high-expressors)
- Tonsil (Normal tissue – low and non-expressors)

The technical test approach – Analytical phase

Problem: Primary antibody poorly calibrated providing low sensitivity

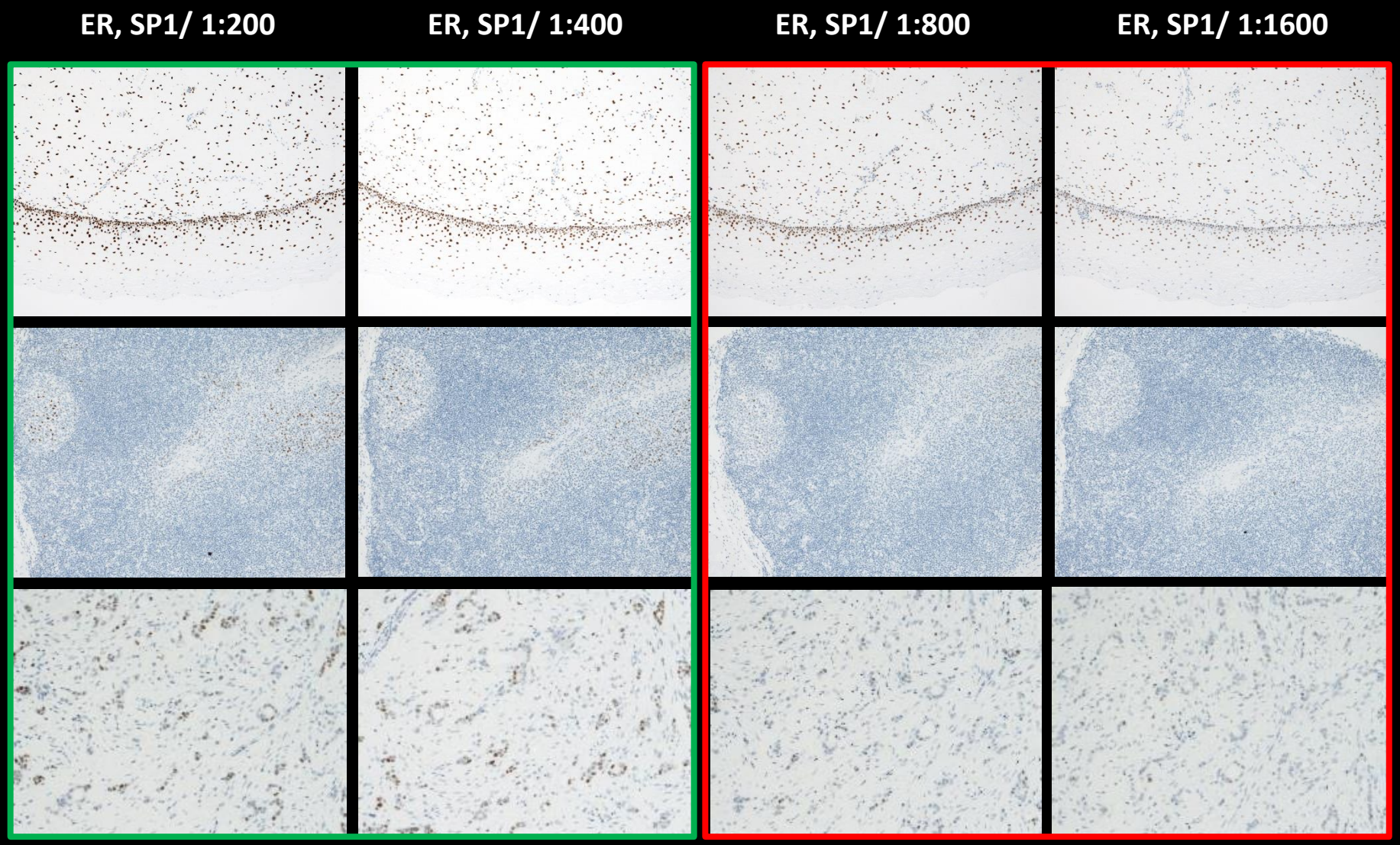


High pH 20`, Flex+Mouse

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

The technical test approach – Analytical phase

Problem: Primary antibody poorly calibrated providing low sensitivity

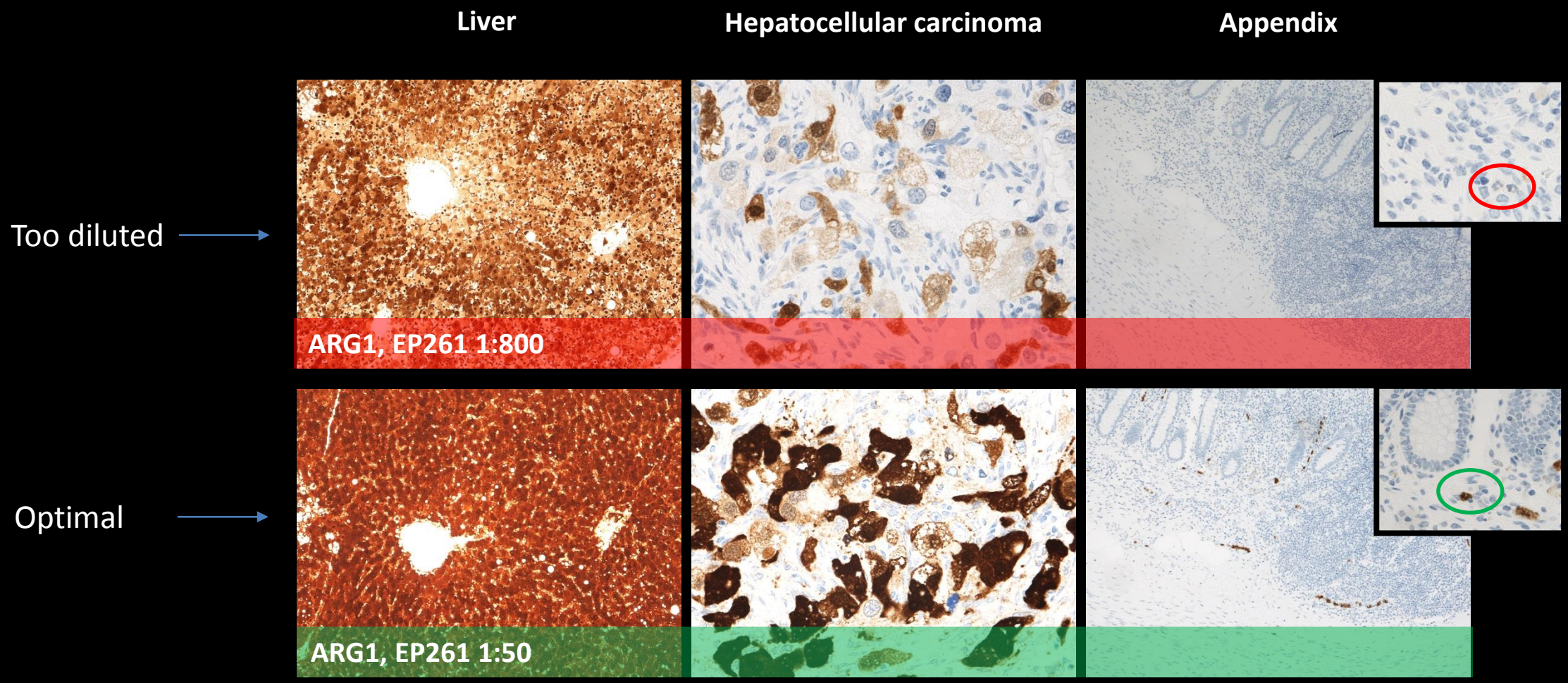


High pH 24`, Flex+Rabbit

Reduced intensity and proportion of cells expected to be stained

The technical test approach – Analytical phase

Problem: Primary antibody poorly calibrated providing low sensitivity



HIER High pH 24`; Flex+ Rabbit linker

In collaboration with Ole Nielsen, Department of Pathology, Odense

IHC: Technical considerations to intended use and “fit-for-purpose” approach

Do we have the right antibody (IHC type markers 1 & 2) – can it provide appropriate sensitivity and specificity

Does the antibody work on the chosen automatic platform(s)

Does the automatic platform come with appropriate reagents fulfilling purpose and intended use of the IHC assay

- Appropriate Antigen Retrieval solutions (enzymes and HIER buffers)
- Appropriate antibody diluents and wash buffers
- Appropriate detection and visualization products
- Appropriate protocol library

Do we have access to appropriate tissue, reflecting the range of different antigen expression levels, both for the optimization process but also for the laboratory`s internal quality assurance program (control tissue) – monitoring specificity and sensitivity of the assays

Technical aspects of IHC and pitfalls– Analytical phase

HIER buffers used by NordiQC laboratories

In house	Dako	Roche Ventana	Leica Microsystems	Biocare	Thermo S LAB Vision
Low pH buffers					
Citrate buffer pH 6 / pH6.7	TRS Low pH 6.1	CC2 pH 6	BERS-1 pH 6	Diva Decloaker pH 6.2	
High pH buffer					
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	App. 80-90 % of all pretreatment protocols				

TRS ~ Target Retrieval Solution ~ Autostainer (Link/Classic) / Omnis

CC ~ Cell Conditioning ~ Benchmark (XT/Ultra)

BERS ~ Bond Epitope Retrieval Solution ~ Bond (Max/III)

Decloaker` s ~ IntelliPATH

HIER High H ~ Autostainer (480S-2D/720-2D)

Restrictions:

The instrumentation / platforms dictates the choice of HIER buffers

For some antigens, the HIER buffers dictate`s the choice of primary Ab

NordiQC

Assessment Run 45 2015

Epithelial cell-cell adhesion molecule (Ep-CAM)

Recommended Ep-CAM protocols Recommended Ep-CAM control tissue

Table 1. **Antibodies and assessment marks for Ep-CAM, run 45**

Concentrated antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	Suff. OPS[2]
mAb clone **9C4**	1	BioLegend	0	0	0	1	-	-
mAb clone **BS14**	2	Nordic Biosite	2	0	0	0	-	-
mAb clone **C-10**	1	Santa Cruz Biotech	0	0	1	0	-	-
mAb clone **Ber-Ep4**	77 2 2	Dako Diagnostic BioSystems Thermo/NeoMarkers	9	16	38	18	31%	89%
mAb clone **MOC-31**	19 3 1 1	Dako Leica/Novocastra Cell Marque Monosan	9	6	6	3	63%	100%
mAb clone **VU-1D9**	3 3 1 1	Novocastra Thermo/LabVision Merck Millipore Thermo/Pierce	3	3	2	0	75%	75%
rmAb clone **E144**	1	Abcam	0	0	0	1	-	-
Ready-To-Use antibodies								
mAb clone **Ber-Ep4 760-4383**	36	Ventana/Cell Marque	0	6	21	9	17%	-
mAb clone **Ber-Ep4 IR/IS637**	19	Dako	4	12	1	2	84%	100%
mAb clone **Ber-Ep4 GA637**	9	Dako	7	1	1	0	89%	100%
mAb **Ber-Ep4 PM107**	1	Biocare	0	0	0	1	-	-
mAb **Ber-Ep4 MAD-001709QD**	1	Master Diagnostica	0	0	1	0	-	-
mAb clone **Ber-Ep4 MON-RTU1096**	1	Monosan	0	0	1	0	-	-
mAb clone **MOC-31 790-4561**	3	Ventana	0	1	2	0	-	-
mAb clone **MOC-31 248M-18**	1	Cell Marque	0	0	1	0	-	-
mAb clone **MOC-31 PA0797**	1	Leica/Novocastra	0	1	0	0		
mAb clone **MOC-31 MAB-0280**	1	Maixin	0	1	0	0	-	-
mAb clone **VU-1D9**	1	Unknown	0	0	1	0		
Total	192		34	47	76	35	-	
Proportion			18%	25%	39%	18%	43%	

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

Optimal results with HIER in High pH buffers e.g. CC1 (Ventana) (with or without gentle enzymatic digestion performed after HIER)

No optimal results with HIER in High pH buffer CC1 (Ventana) or proteolytic pretreatment

Optimal results with HIER in mod. Low pH buffers (Dako)

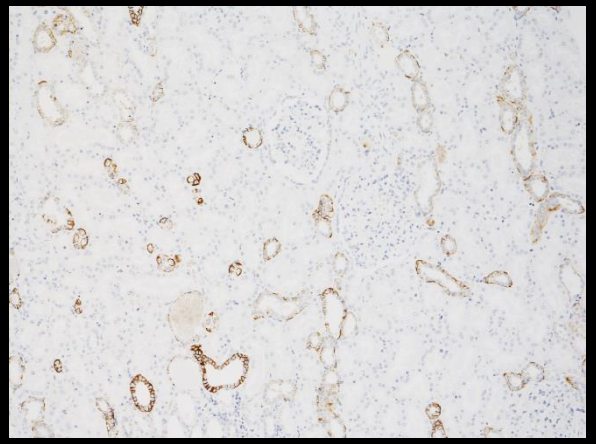
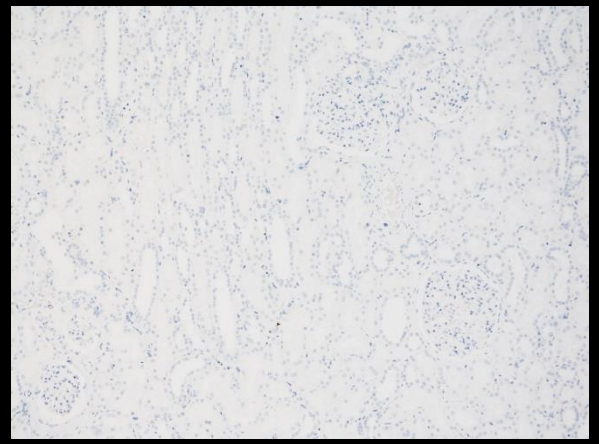
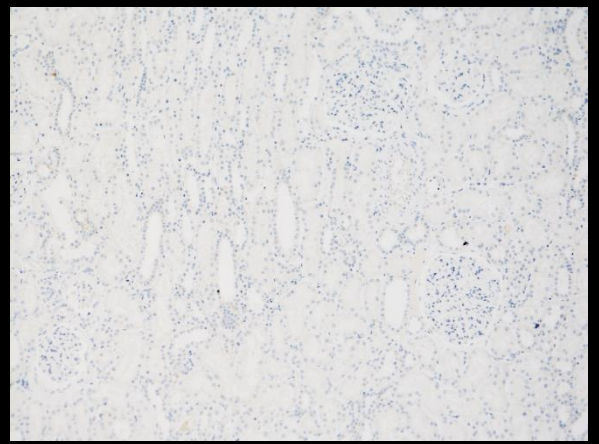
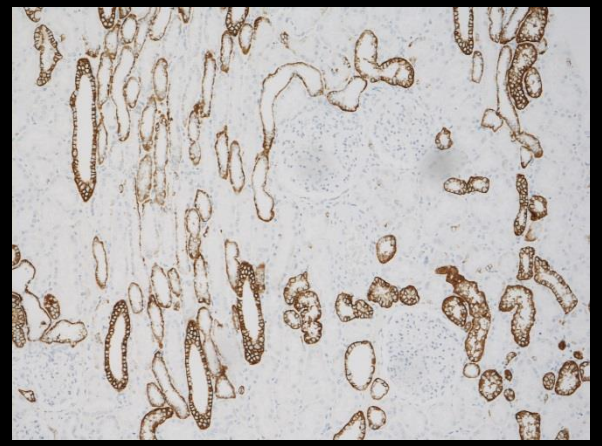
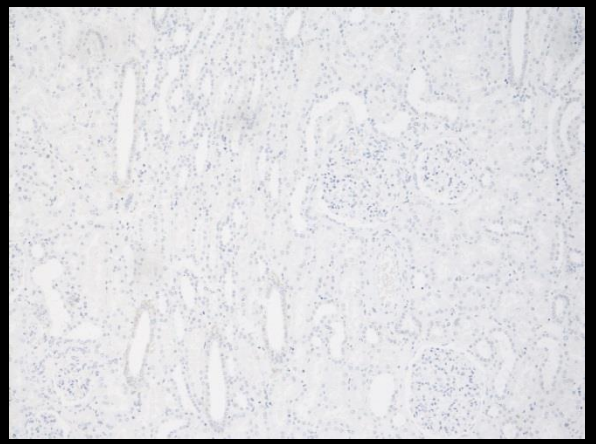
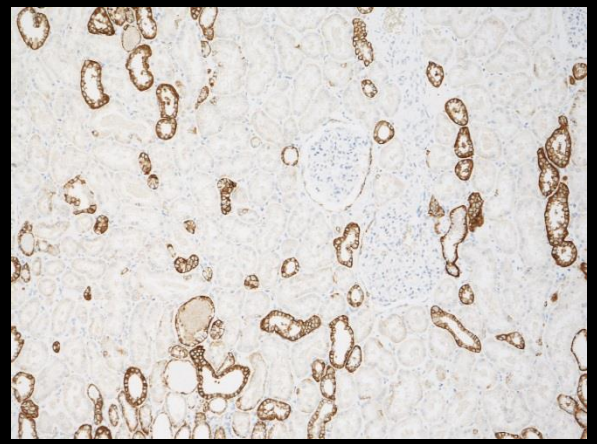
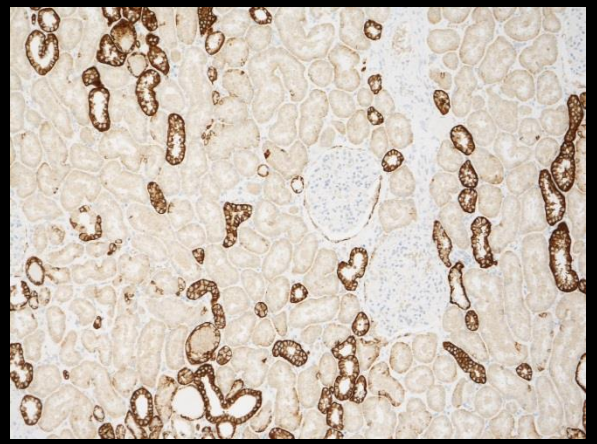
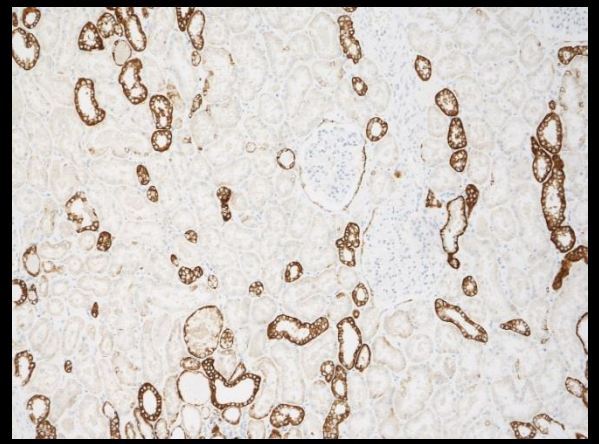
BS14 could be an alternative to Ber-EP4 on platforms excluded from the use of modified low pH buffers e.g. Diva pH 6.2 (Biocare) or TRS pH6.1 (Dako)

The most frequent causes of insufficient staining reactions were:

- Less successful performance of mAb clone Ber-EP4 on BenchMark and BOND IHC platforms.
- Proteolytic pre-treatment
- Too low concentration of the primary Ab
- Use of low sensitive detection systems

The technical test approach – Analytical phase

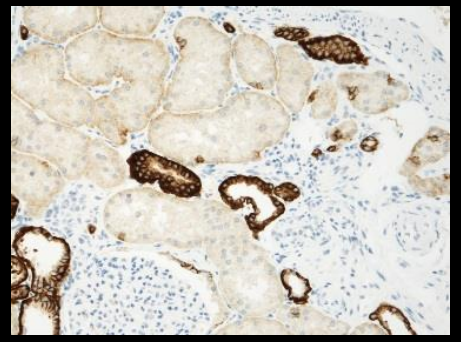
EPCAM clone EP4 or BS14

	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				
BS 14				

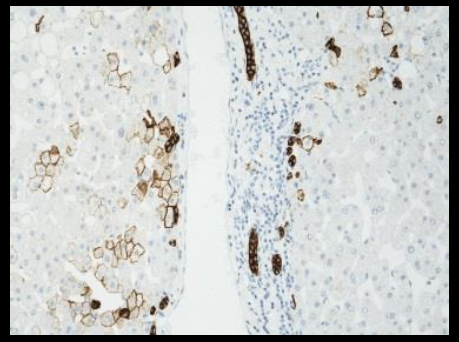
Kidney

Omnis

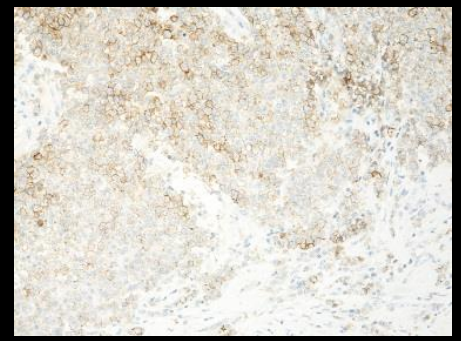
Kidney



Hepar

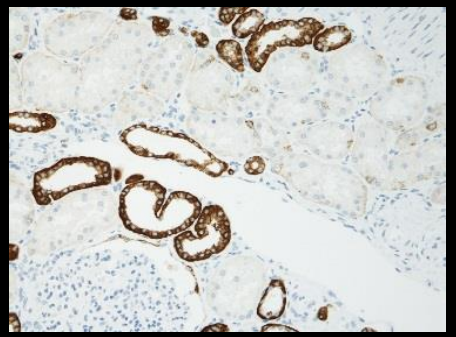


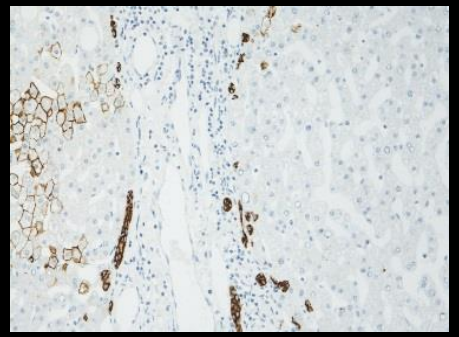
Breast tumor

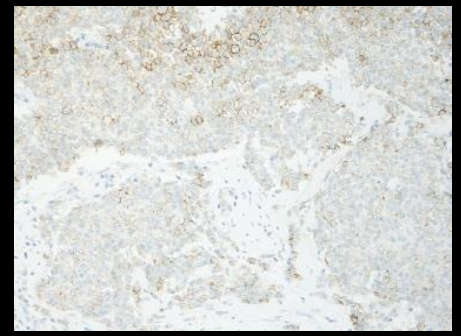


EPCAM, BS14 (1:500) / TRS pH 9.0

EPCAM, BS14 (Nordic Biosite) is a better alternative than EPCAM MOC31 or Ber-EP4 for automated platforms not offering the possibility to use mod. low pH buffers.



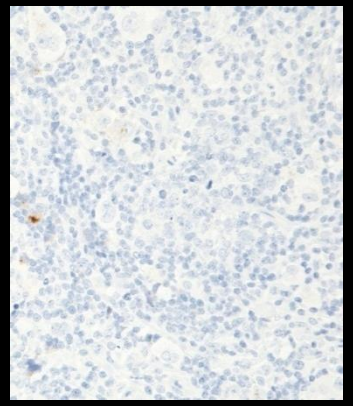
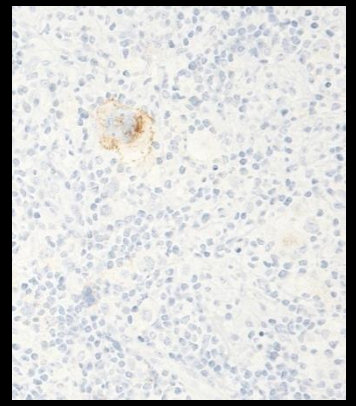
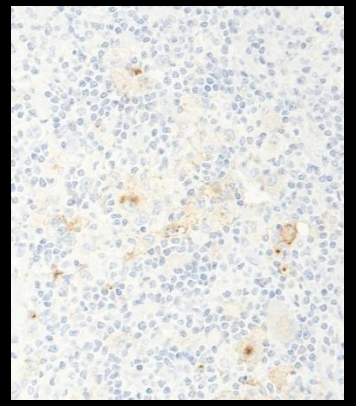
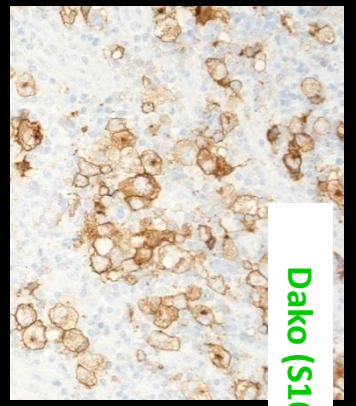
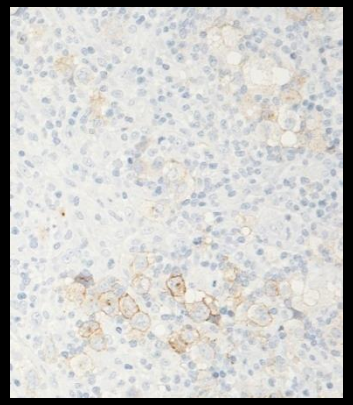
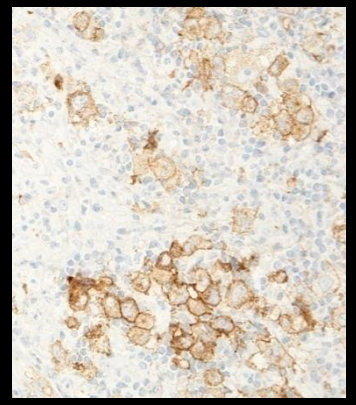
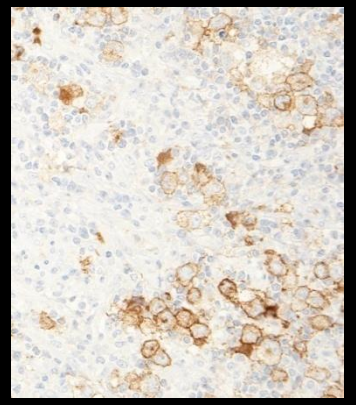
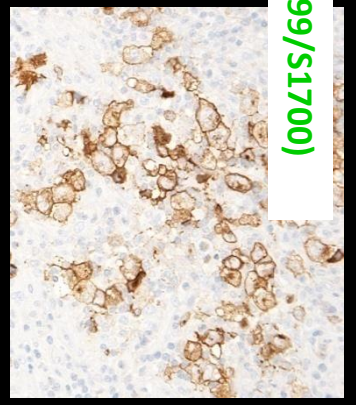




EPCAM, MOC31 (1:25) / TRS pH 6.1

The technical test approach – Analytical phase

Important questions: Which platform - Which antibody - Which antigen retrieval procedure

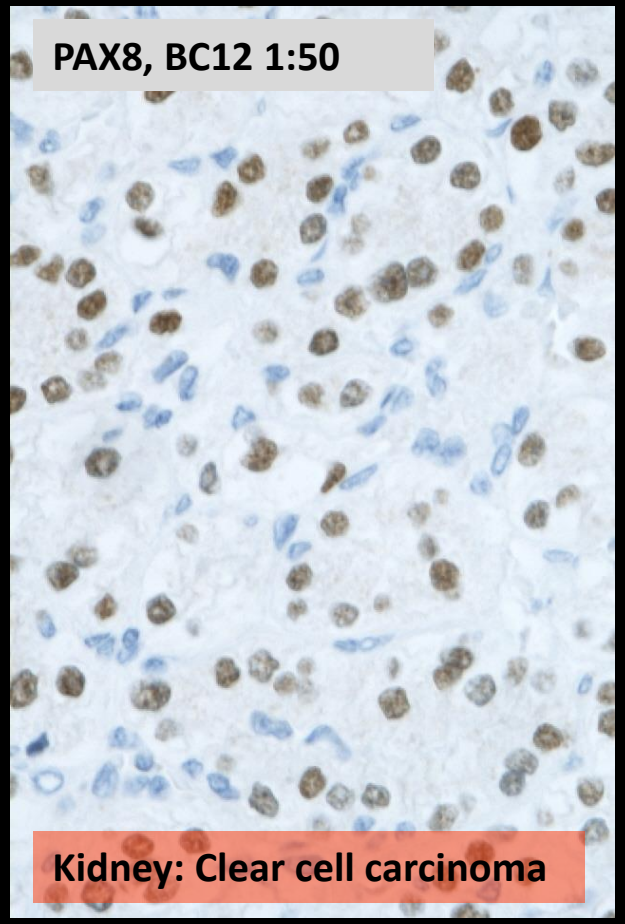
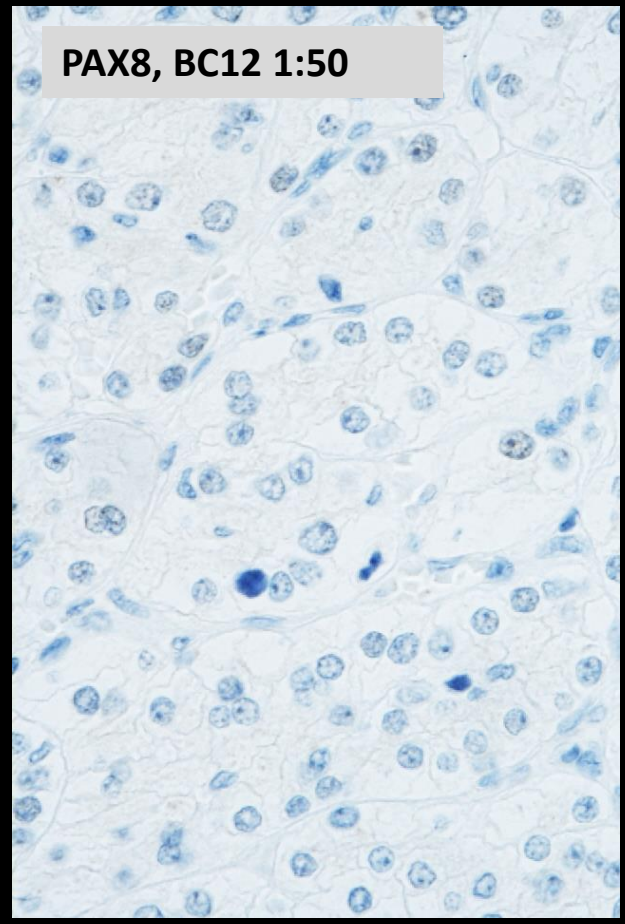
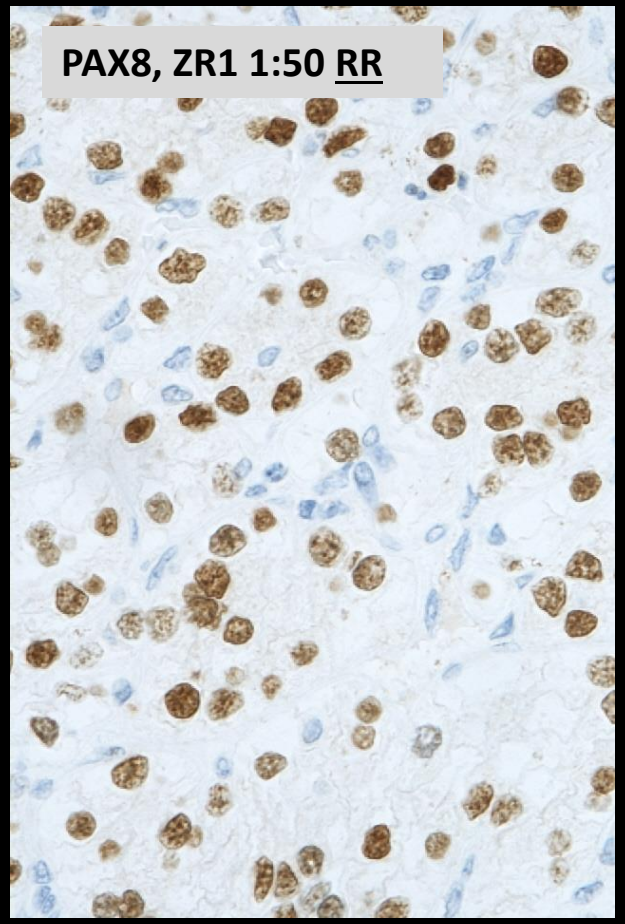
	Ci pH 6 MWO / 20 min	EDTA pH 8 MWO / 20 min	TE pH 9 MWO / 20 min	TRS pH 6.1 MWO / 20 min
CD30 Clone ConD6/B5				
CD30 Clone Ber-H2				

Dako (S1699/S1700)

Hodgkin Lymphoma

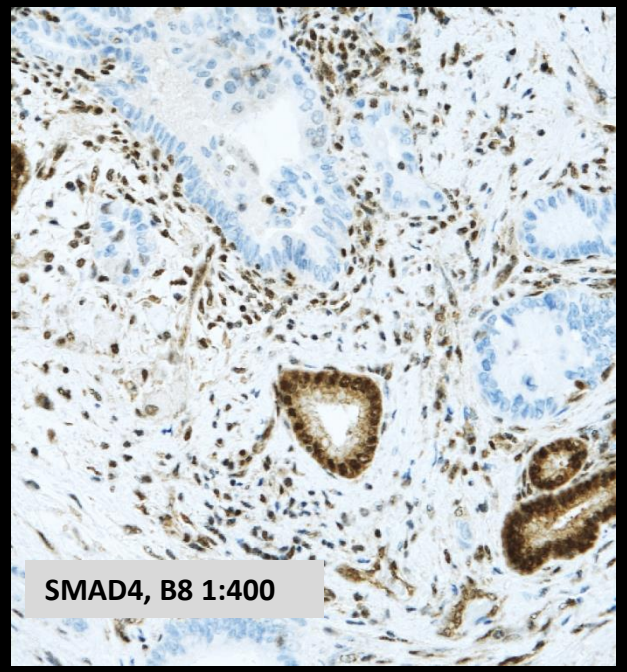
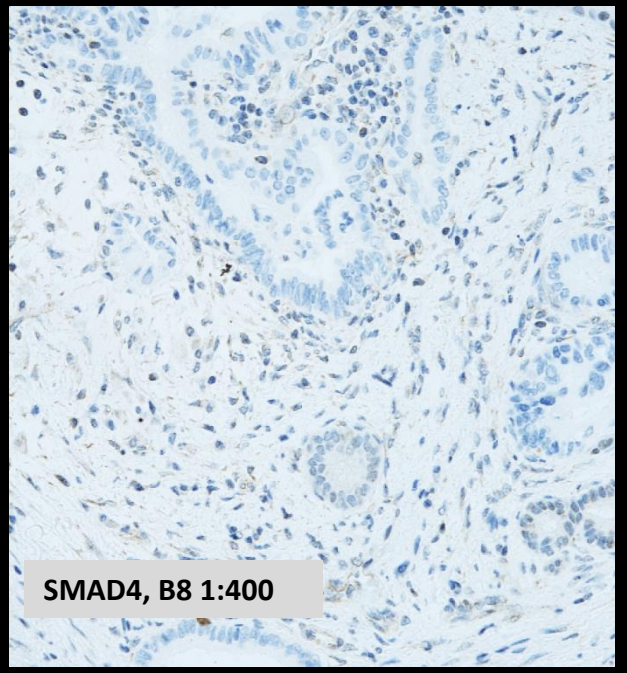
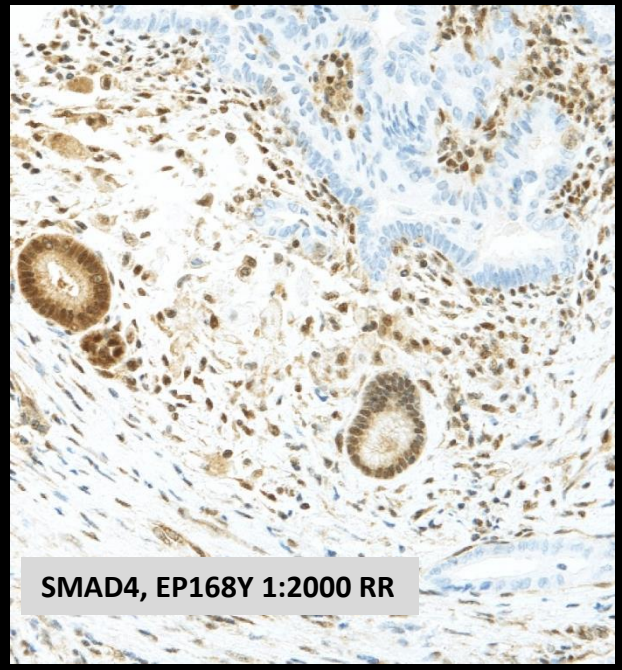
The technical test approach – Analytical phase

Primary antibodies sensitive to the chosen platform

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

The technical test approach – Analytical phase

Primary antibodies sensitive to the chosen platform

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

Pancreatic Adenocarcinoma

Platform dependent antibodies (NordiQC results/Latest run):

Marker	Clone
ASMA	1A4/BS66
BCL2	124
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

Marker	Clone
CR	DAK-Calret1/CAL6
Desmin	D33/BS21
EPCAM	EP4/BS14
Melan A	A103/EP43
OCT 3/4	C-10/MRQ-10
PAX8	MRQ-50/SP348 or EP298
Podop	D2-40
WT1	6F-H2/D817F or EP122

Antibody clones applied on the Omnis (Dept. of surgical Pathology, Region Zealand, Denmark

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

The technical test approach – 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

The technical test approach – Analytical phase

Antibody diluents

Applied Immunohistochemistry & Molecular Morphology 9(2): 176–179, 2001
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Formalin-Fixed and Heat-Retrieved Tissue Antigens: A Comparison of Their Immunoreactivity in Experimental Antibody Diluents

Thomas Boenisch, M.S.

Demonstrated that:

pH of the Ab-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).

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

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

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

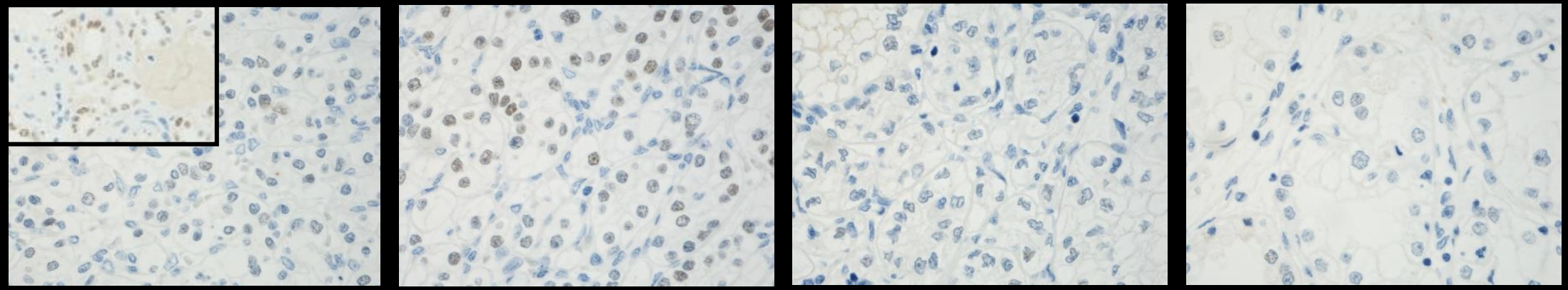
ND, not done.

The technical test approach – Analytical phase

Antibody diluents

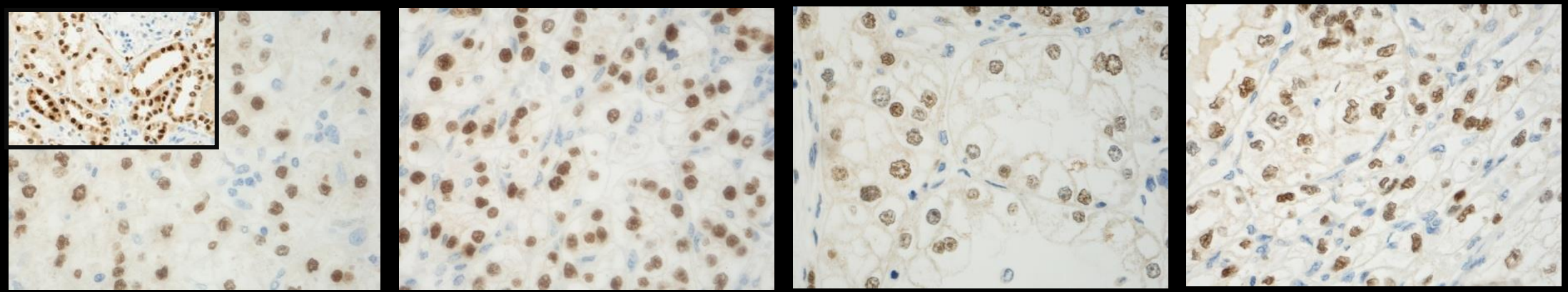
PAX8, ZR1 1:50

Dako Dil. pH7.3



PAX8, ZR1 1:50

Renoir R pH 6.2



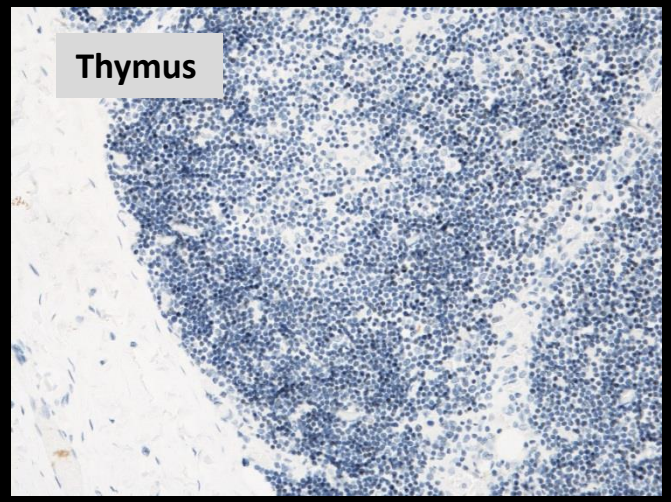
4x Clear Cell Carcinomas (Kidney)

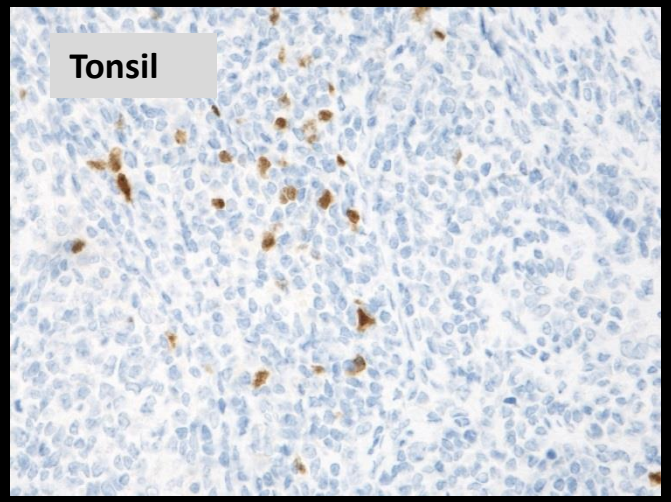
PK (2` at RT/ off-board) + HIER (Dako, S2367 pH9) (30` at 97°C) / PK ~ Proteinase K Solution RTU (Dako cat.no.S3020) diluted 1:10 in TBS pH7.6 /Flex+ Rabbit (10+20`)

The technical test approach – Analytical phase

Antibody diluents

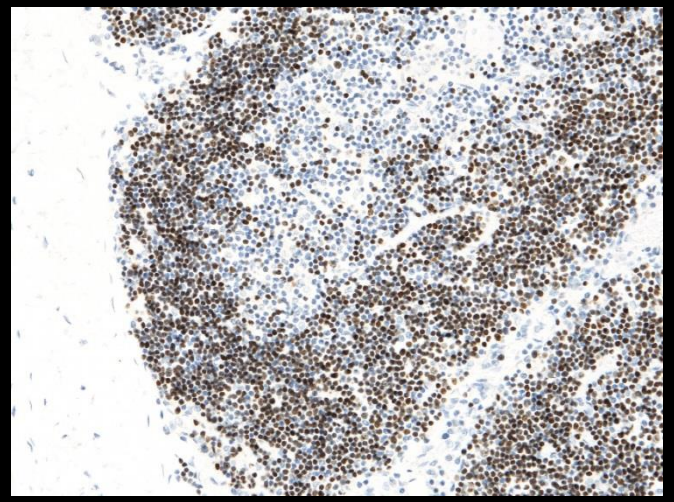
TdT, SEN28 1:50
Dako dil. pH 7.3

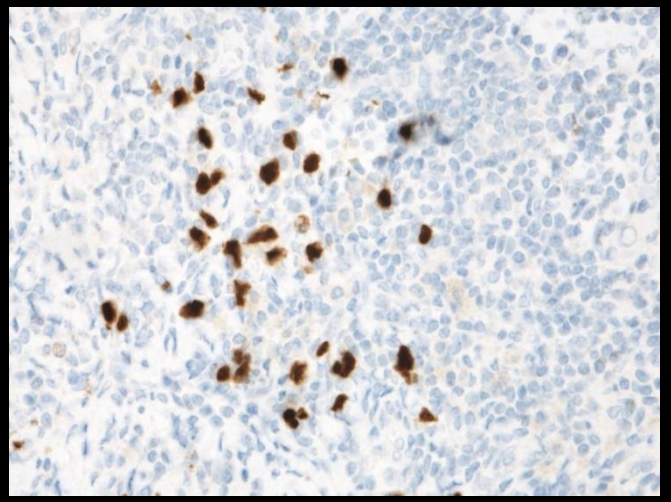




Omnis: HIER/HIGH pH 24`, Flex+ Mouse (10+20`)

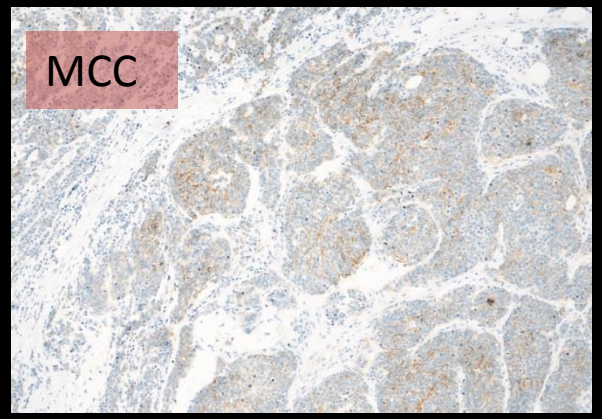
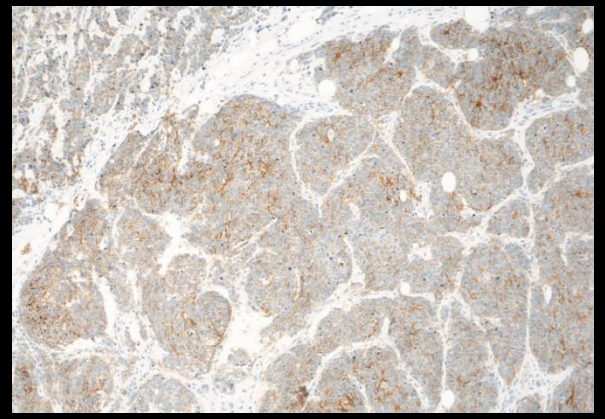
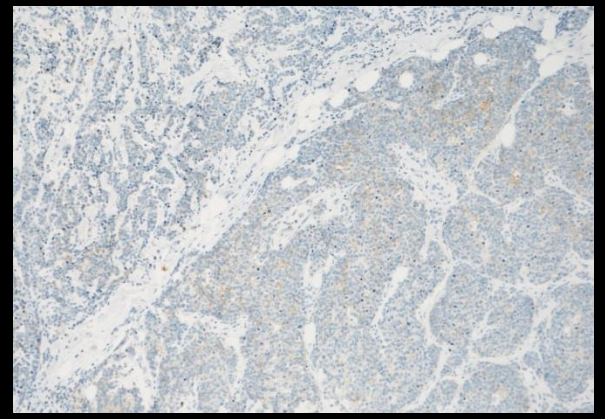
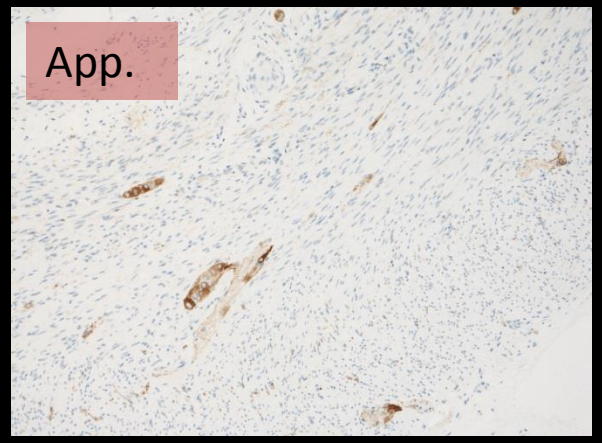
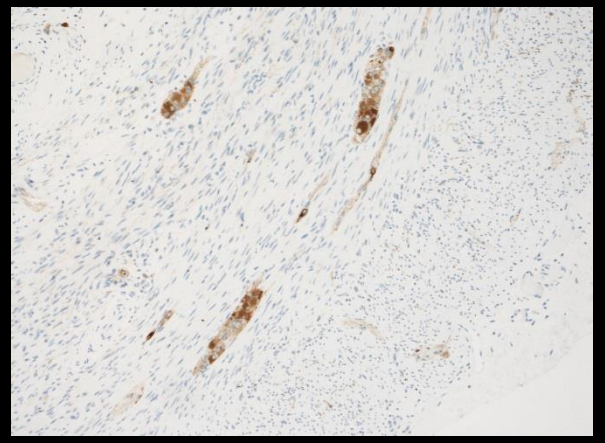
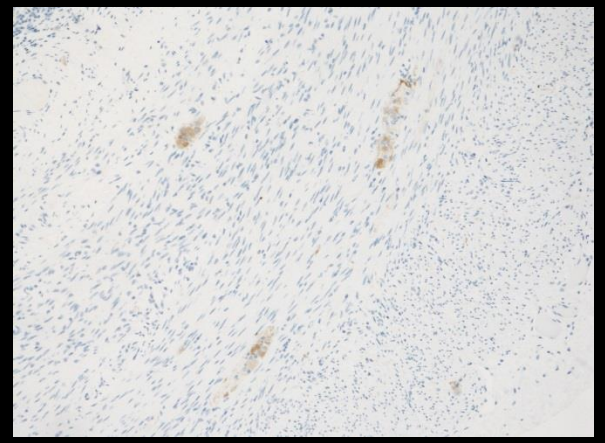
TdT, SEN28 1:50
Renoir Red pH 6.2





The technical test approach – Analytical phase

Antibody diluents

ALK, 1A4 1:300 Dako Dil. pH 7.3	ALK, 1A4 1:1200 Renoir R pH 6.2	ALK, 1A4 1:1200 Dako Dil. pH7.3
		
		

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

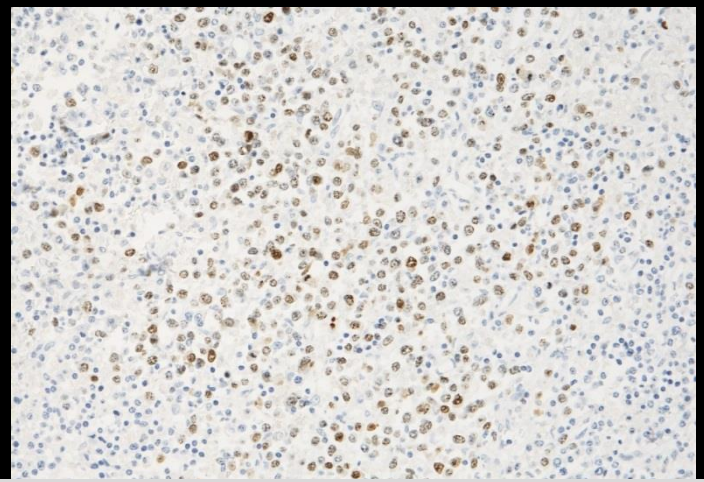
The technical test approach – Analytical phase

Antibody diluents

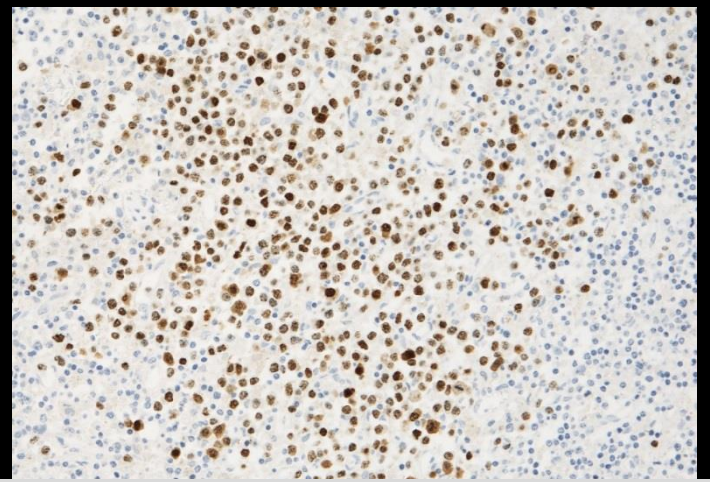
HHV8, 13B10
1:100 Renoir Red pH 6.2

HHV8, 13B10
1:100 Dako dil pH 7.3

Case 1

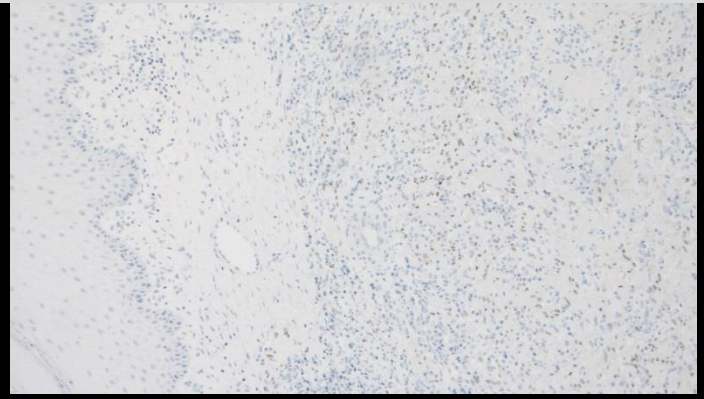


Flex+ Rabbit (10+20')

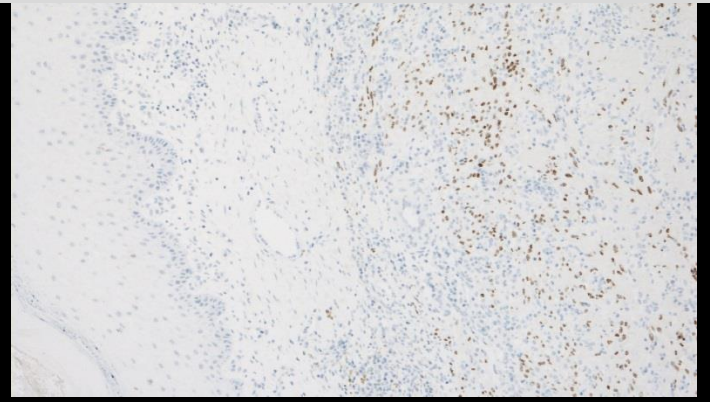


Renoir Red is not always the best antibody diluent
Remember to use a "antibody diluent test battery"

Case 2



Omnis: HIER/HIG



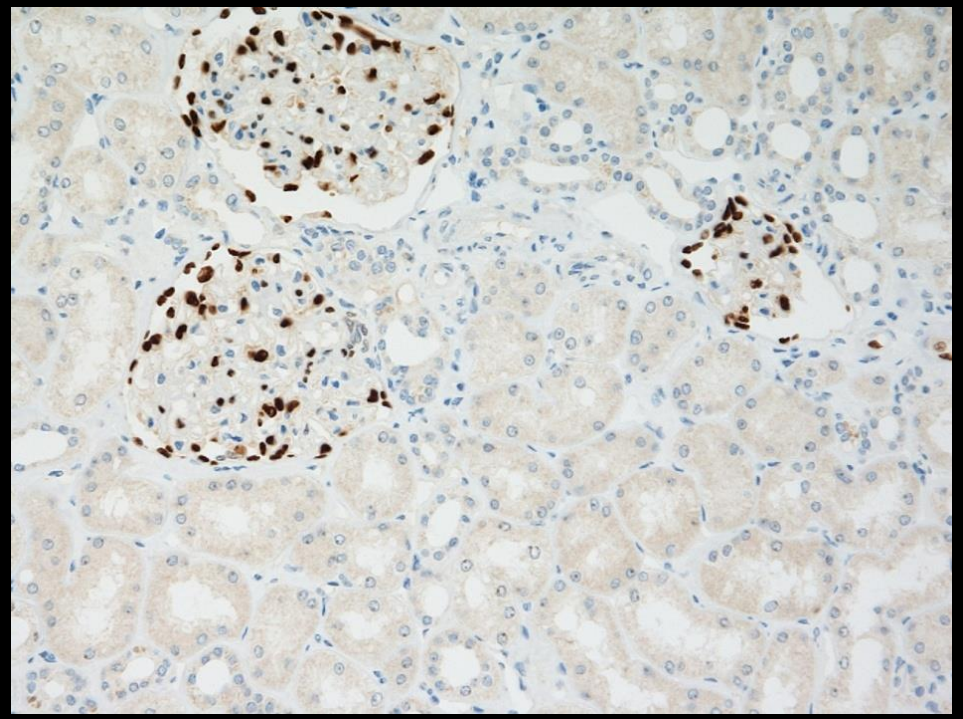
Immunodeficient patients Kaposi's sarcoma, Castleman's disease, Primary effusion lymphoma ……

The technical test approach – Analytical phase

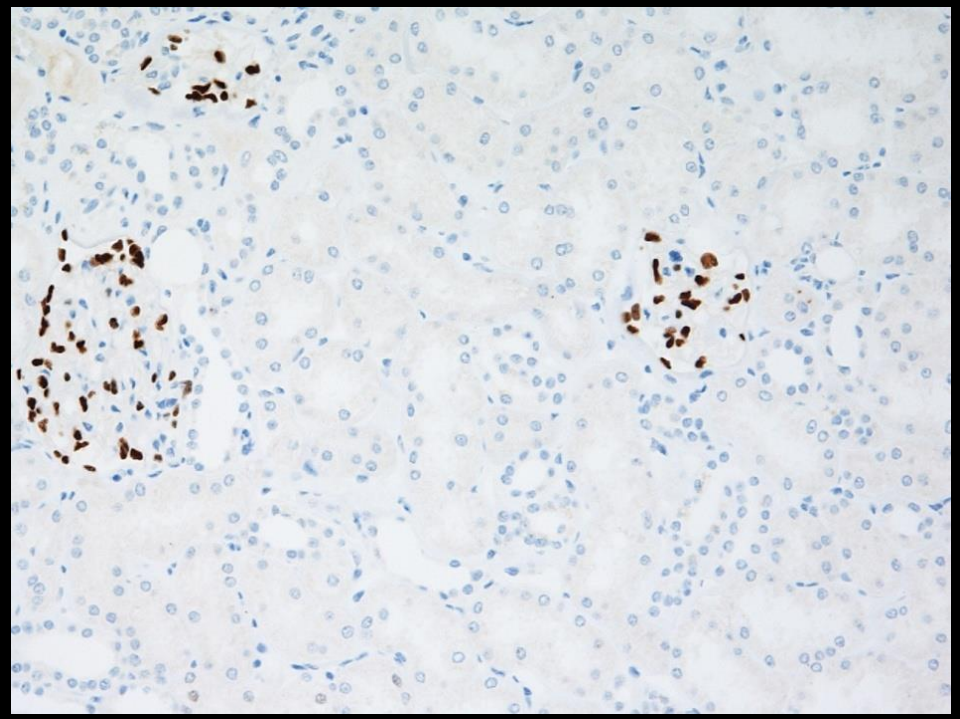
Antibody diluents

Kidney

WT1,EP122 1:25
Renoir Red (Biocare)



WT1,EP122 1:25
Background Sniper (Biocare)



The choice of antibody diluent has a high impact on unwanted / unspecific background staining

HIER TRS pH9 (24` /97°C) + Pep © (3`)

The technical test approach – Analytical phase

Antibody diluents

Omnis (Department of Surgical Pathology, Region Zealand, Denmark)

Markers 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 (ES05 & GM011), MSH2 (G219-1129), 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 …….

Markers that don`t benefit from dilution in Renoir Red pH 6.2:

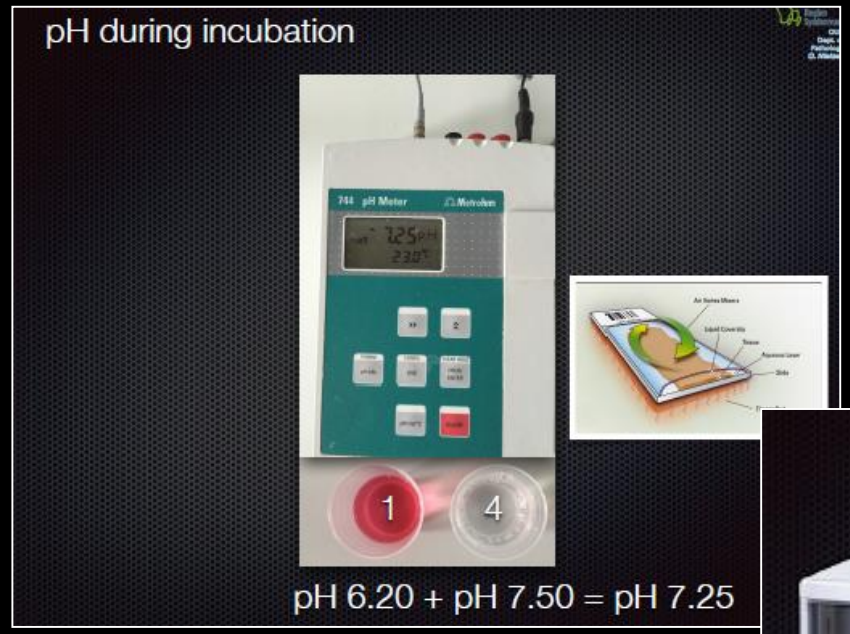
BCL2 (124), BCL6 (LN22 & PG-B6p & GI191E/A3), CR (DAK-Calret1), CD163 (MRQ26), CD21 (2G9), CD5 (4C7), ER (SP1), HHV8 (13B10), Mammaglobin (304-1A5), MUC5AC (CLH2), MUC6 (CLH5), and …….

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

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

The technical test approach – Analytical phase

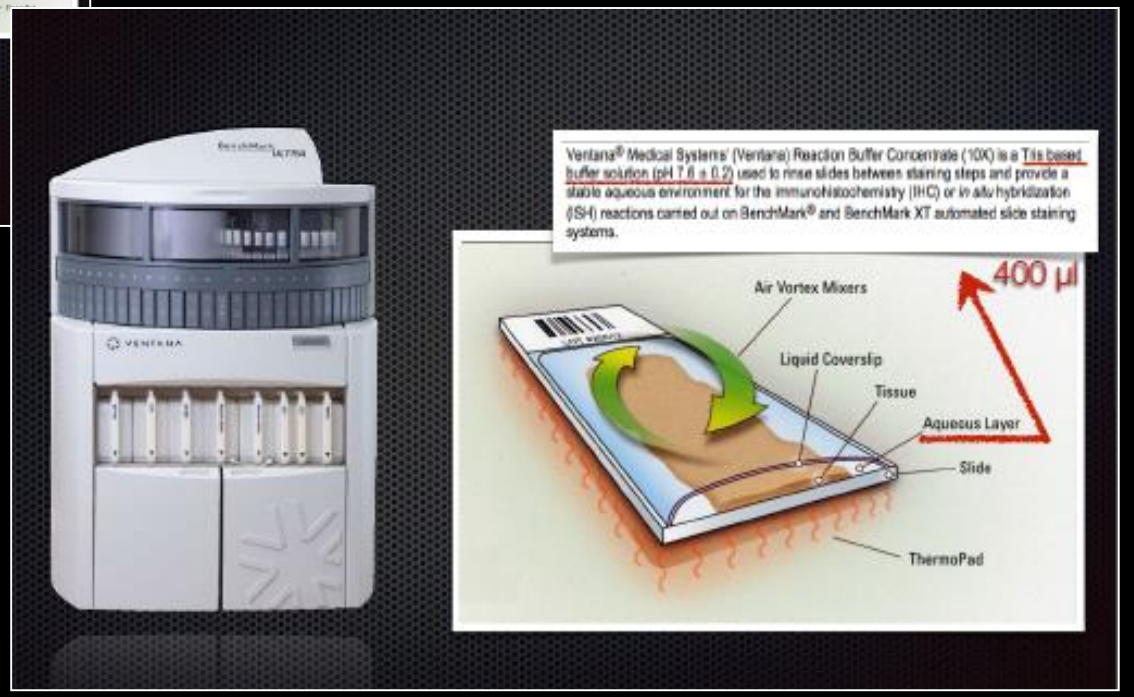
Antibody diluents



[Ventana Benchmark Ultra]

The "full effect" of the antibody diluents may depend on the chosen platform

Courtesy Ole Nielsen, Dept. of Pathology, OUH, Denmark



Virchows Arch (2016) 468:19–29
DOI 10.1007/s00428-015-1829-1

ANNUAL REVIEW ISSUE

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

Mogens Vyberg[1,2] · Søren Nielsen[1]

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%

[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

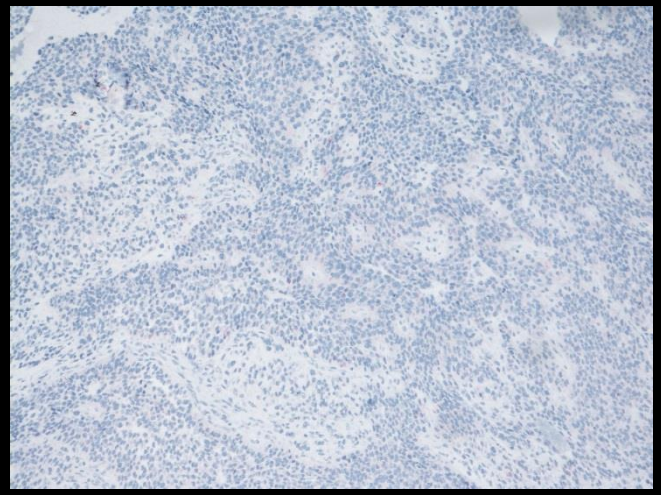
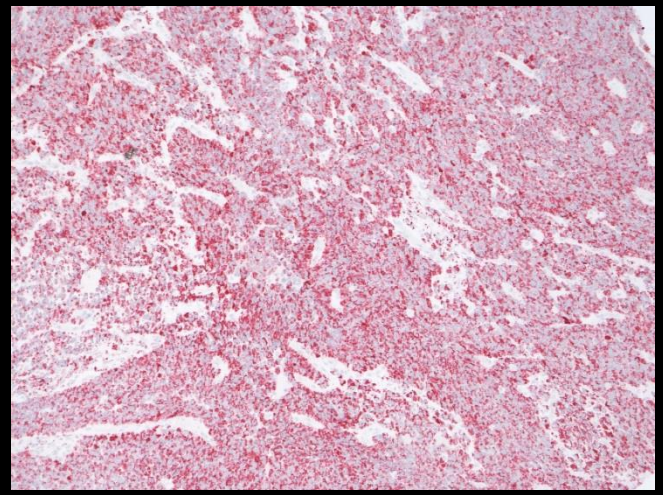
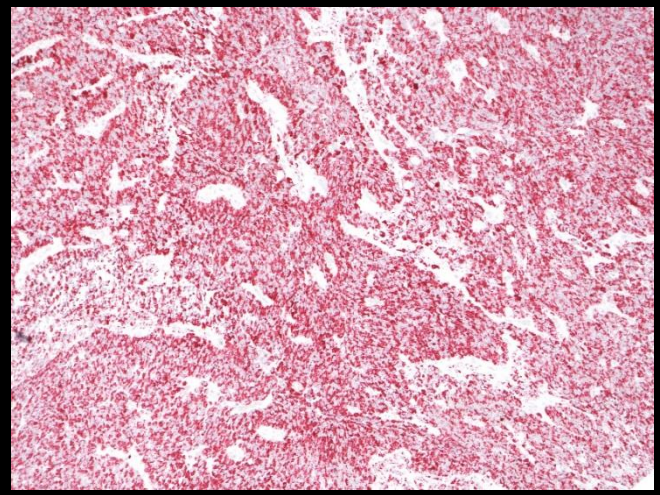
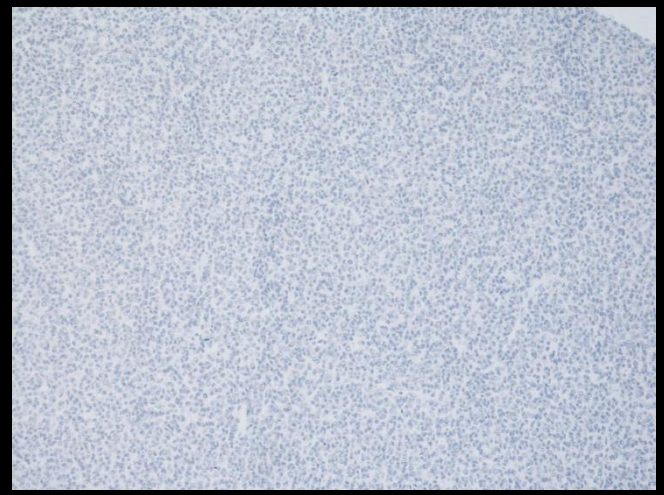
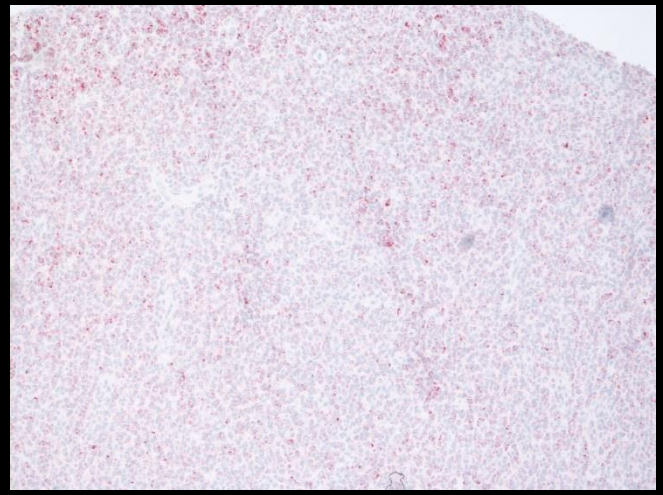
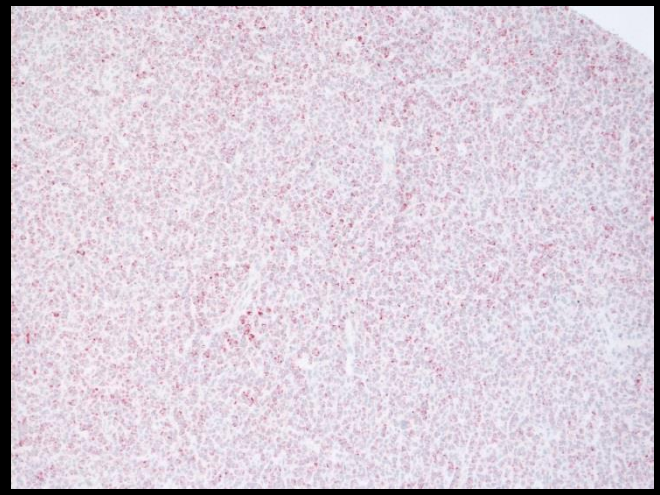
Problems related to the choice of the detection system:

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

False positive or false negative results

The technical test approach – Analytical phase

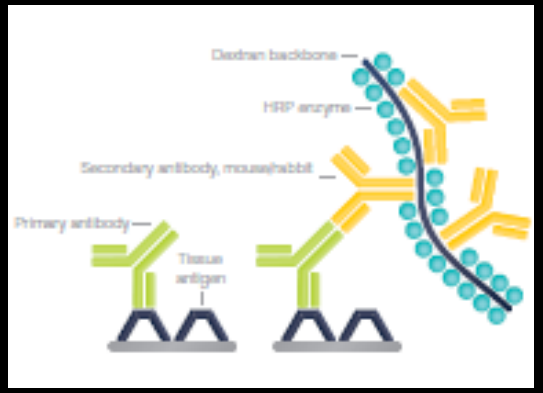
Omnis
MLA, A103 (1:50)
HIER High pH 24`

	Envision G2-AP (Dako) 30/10/20/PR10	PoLink-2 plus /AP (GBI Labs) 30/15/15/PR10	Modified Histo-AP (Nordic Biosite) 30/10/20/PR10
Granulosa cell tumor High expressor			
Granulosa cell tumor Low expressor			

The technical test approach – Analytical phase

Detection systems (polymer/multimer) used by NordiQC participants

Vendor	Detection System 2- Step	Detection System 3-step	Amplifier	Cat.no
Dako	EnVision EnVision +/Flex	Envision Flex+	Anti -Ms/Rb	K4001 K8000 /10 (K5007) K8002/12
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	UltaVision 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……..)				

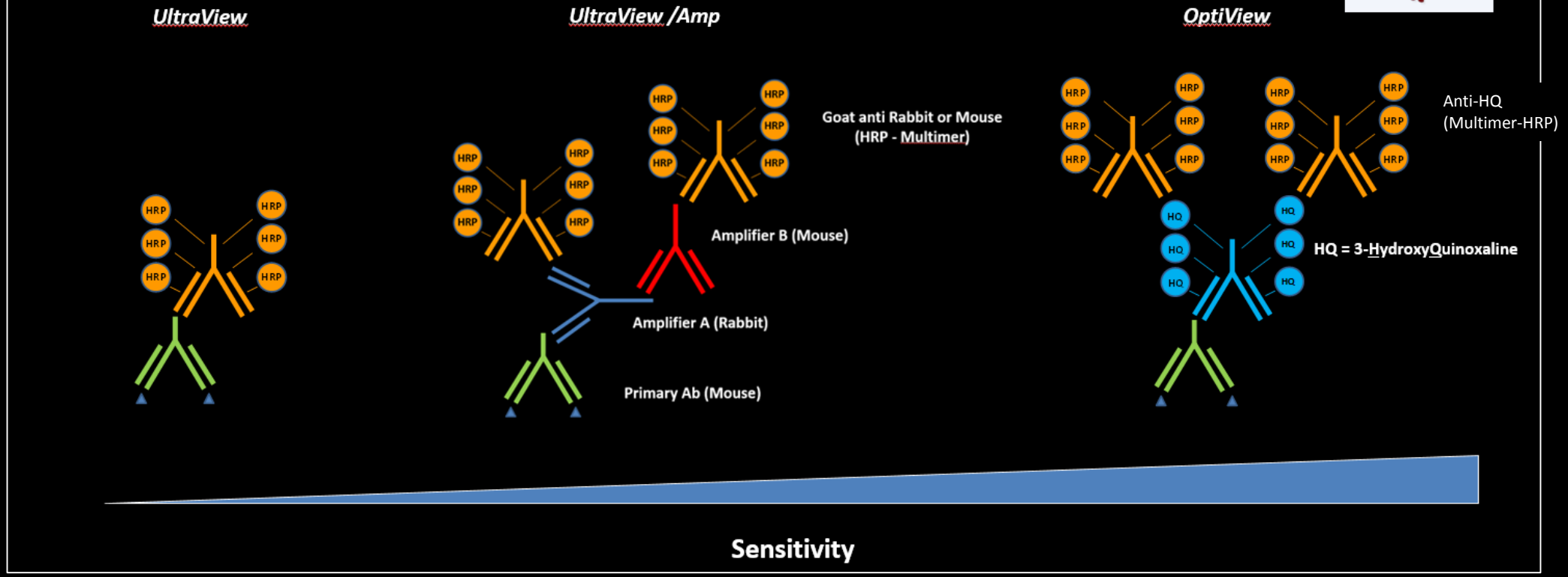


App. 95% of all NordiQC participants use a polymer/multimer based detection systems

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)



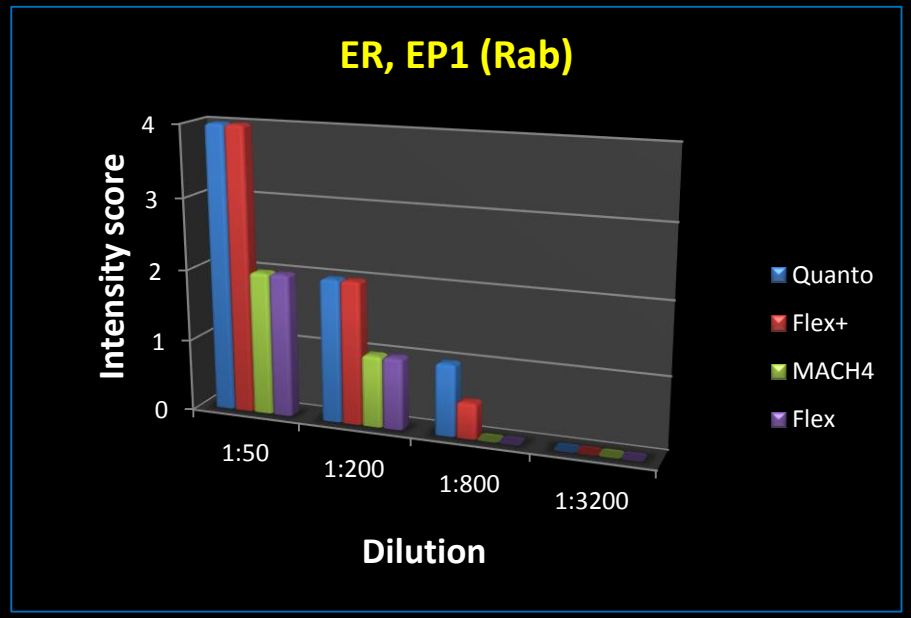
HQ-Linker
Amplifier (A/B)

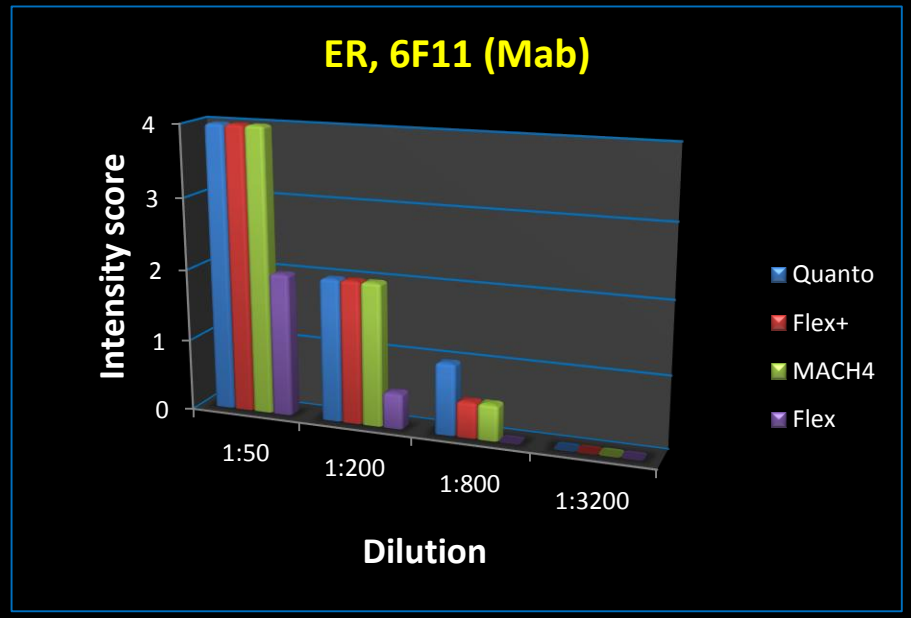
Linker (Mouse/Rabbit)
Enhancer
Universal Linker
Post Blocking
…….

Increases sensitivity

The technical test approach – Analytical phase

Detection systems - Performance Testing





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

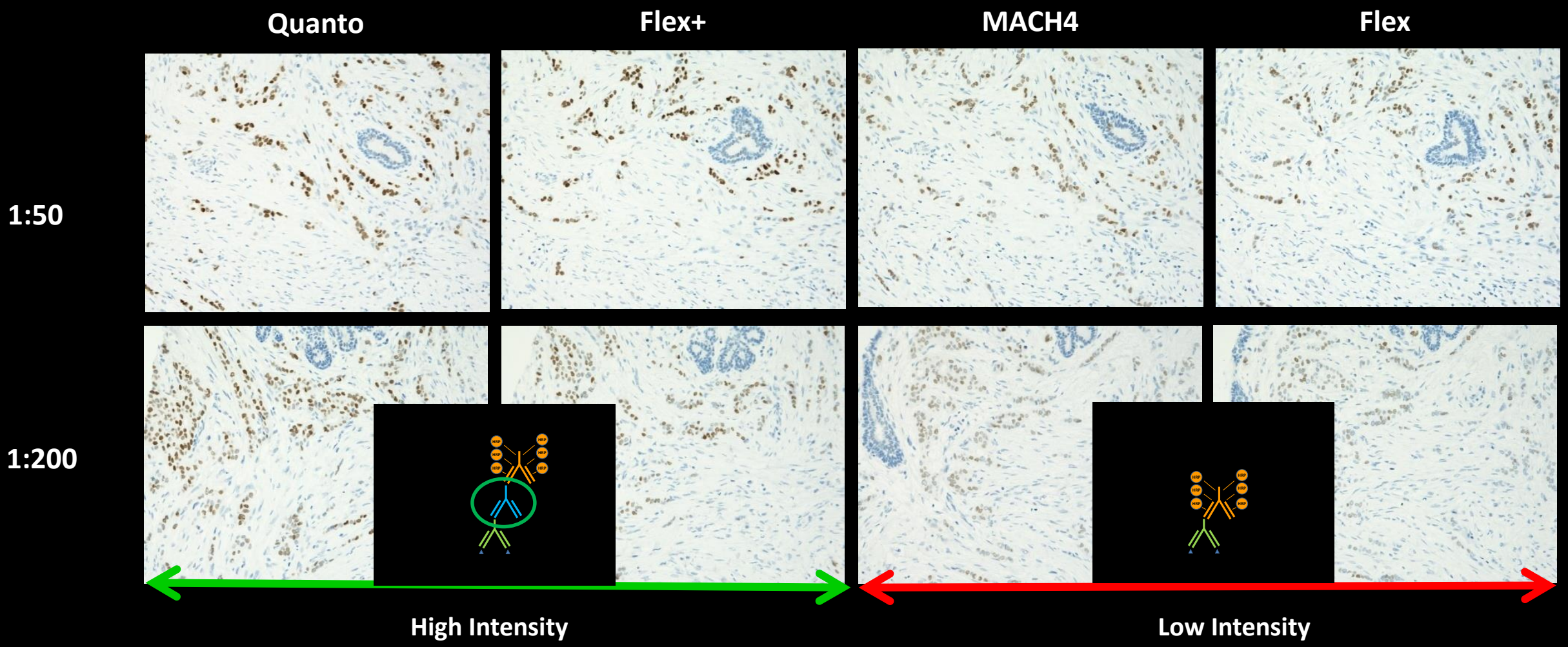
- **The 3-step polymer detection systems Quanto and Flex+ - produced the overall highest intensity.**
- **The 3-step polymer detection system MACH4 only enhances reactions with mouse monoclonal Abs (ER,6F11).**
- **"Optimal staining" was highly influenced by the concentration of the primary Abs and the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

The technical test approach – Analytical phase

Detection systems - Performance Testing

Breast tumor

ER, EP1 (Rab)

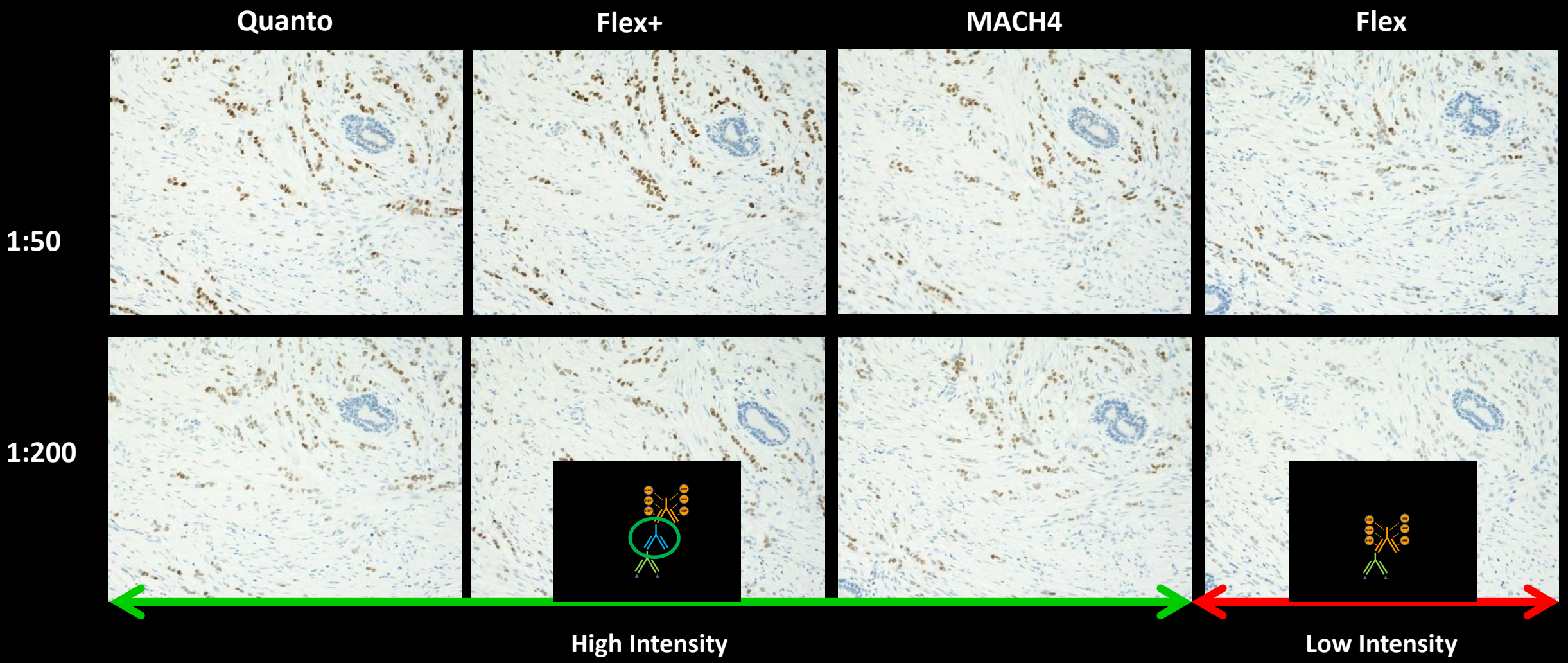


The technical test approach – Analytical phase

Detection systems - Performance Testing

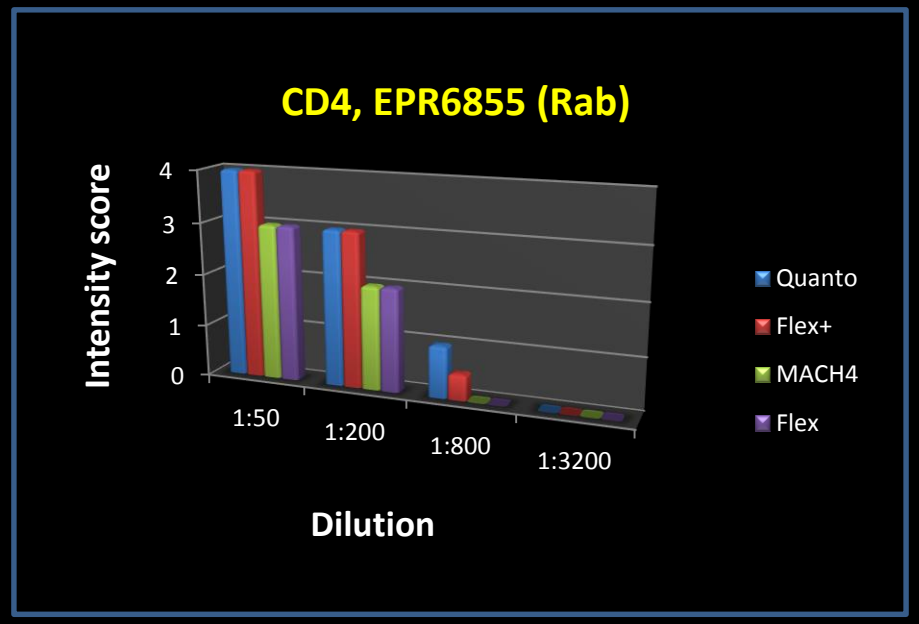
Breast tumor

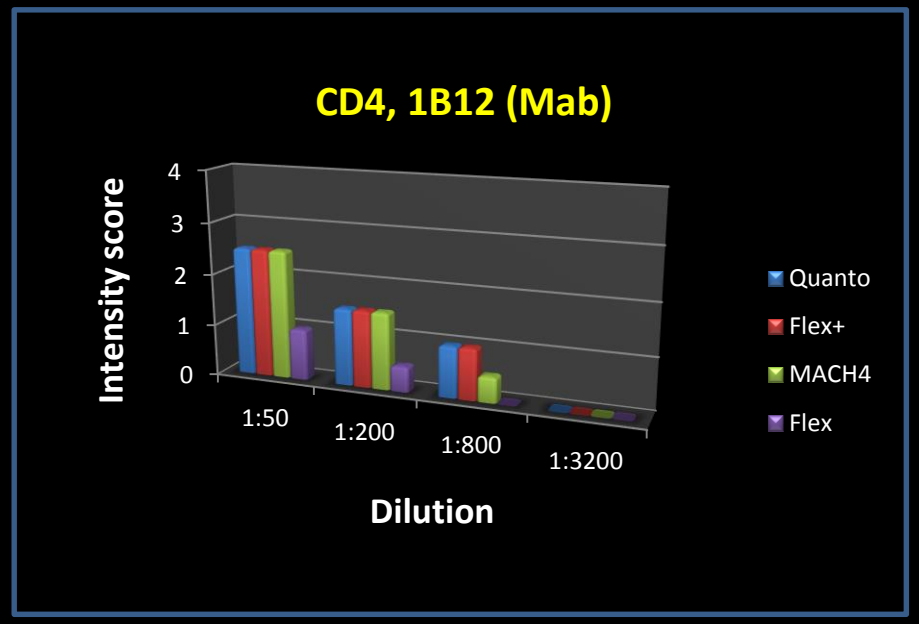
ER, 6F11 (Mab)



The technical test approach – Analytical phase

Detection systems - Performance Testing





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

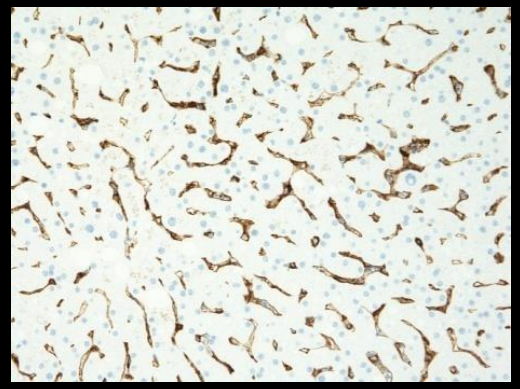
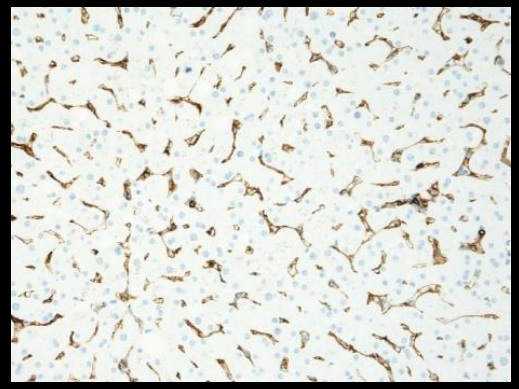
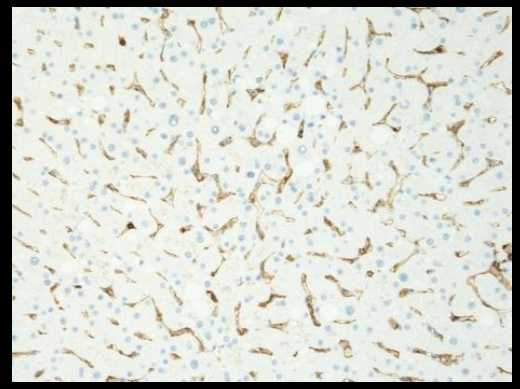
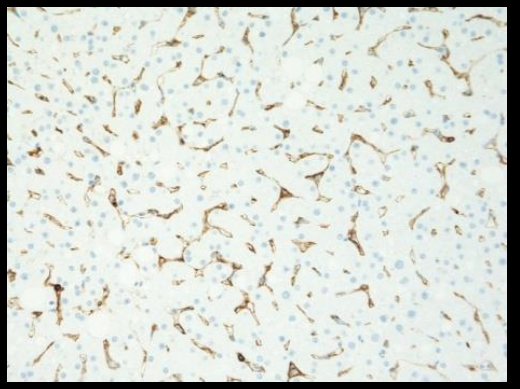
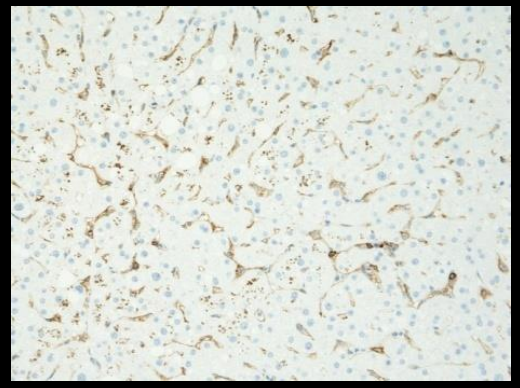
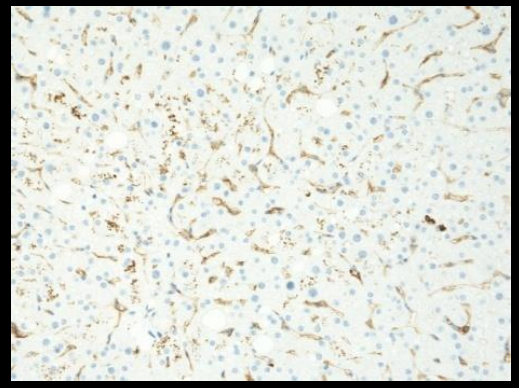
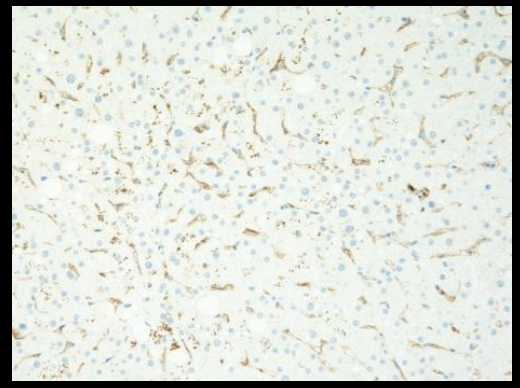
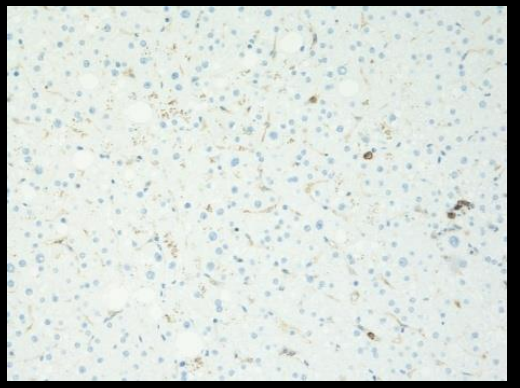
- **The 3-step polymer detection systems Quanto and Flex+ - produced the overall highest intensity.**
- **Intensity was highly influenced by the nature of primary Ab and "optimal" staining could only be obtained with the Rab (CD4, EPR6855) used in combination with the 3 step – polymer detection systems 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) in combination with the use of a 3 step polymer system (Quanto, Flex+ or MACH4)**

The technical test approach – Analytical phase

Performance Testing using incubation times recommended by the vendors

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

Liver

	Quanto	Flex+	MACH4	Flex
EPR6855				
1B12				

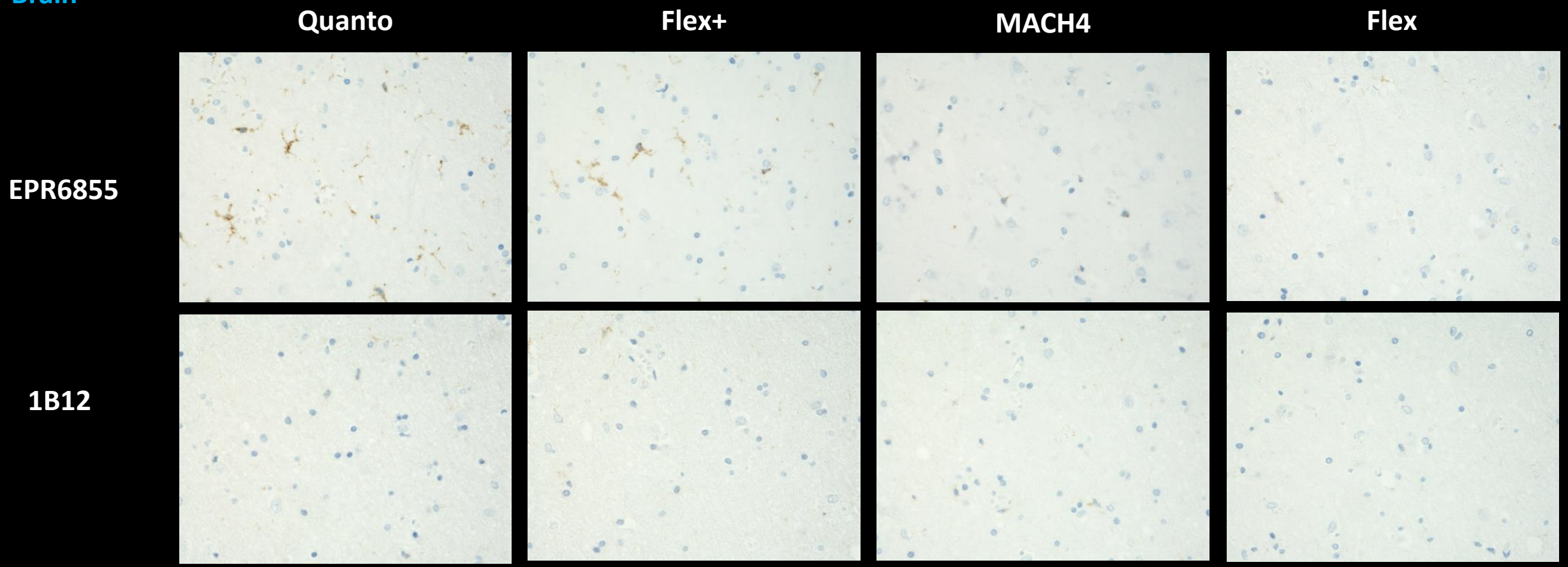
Note: Strong staining of hepatic sinusoidal endothelial cells and kupffer cells using the Rab (CD4, EPR6855) in combination with all the detection system tested (2-step or 3-step polymer systems) . Intensity is significantly reduced using the Mab (CD4, 1B12).

The technical test approach – Analytical phase

Detection systems - Performance Testing

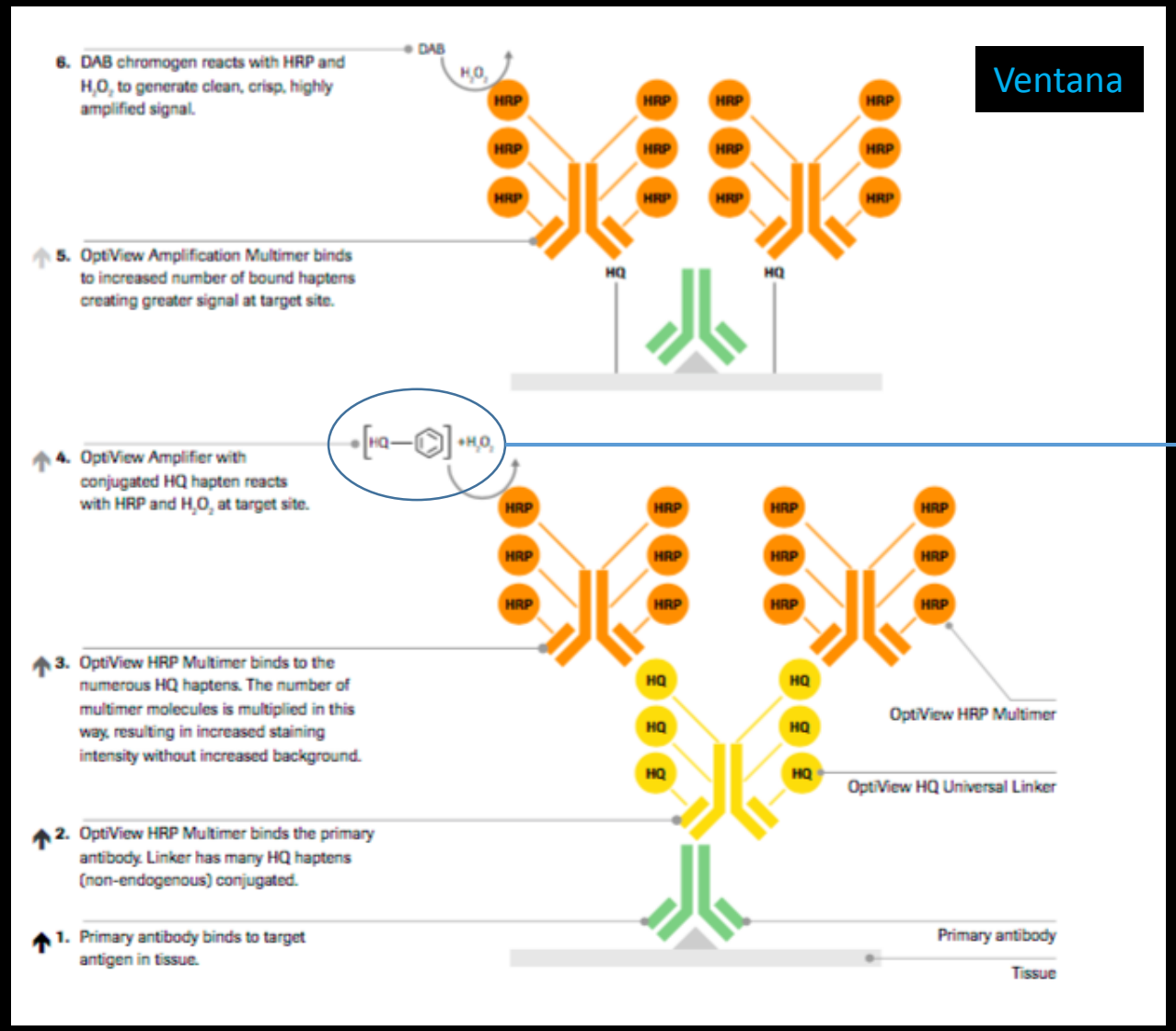
CD4, EPR6855 (Rab, 1:50) and 1B12 (Mab, 1:50)

Brain



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

The technical test approach – Analytical phase



Tyramide Signal Amplification

<u>Mechanism of Tyramide amplification:</u>

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

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

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

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

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

The technical test approach – 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 3 2 1 1 1	Leica/Novocastra Thermo/NeoMarkers Monosan Abcam Biocare Zytomed	24	16	13	1	74%	81%
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 1	Cell Signaling PrimeBioMed	18	2	1	1	91%	95%
rmAb clone **SP8**	2	Thermo/NeoMarkers	0	0	1	1	-	-
Ready-To-Use antibodies								
mAb clone **5A4 PA0306**	3	Leica/Novocatra	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 can provide 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

The technical test approach – Analytical phase

The Histochemical Journal **31:** 195–200, 1999.
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Concentration dependent and adverse effects in immunohistochemistry using the tyramine amplification technique

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Received 8 September 1998 and in revised form 1 December 1998

Summary

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

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

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

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

TSA 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

OptiView + TSA

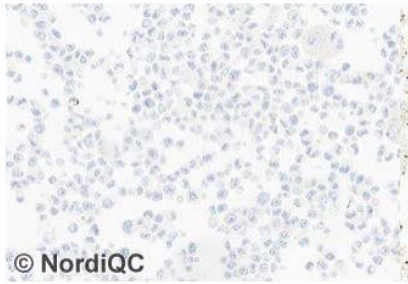


Fig. 6a
Optimal ALK staining of the cell line without ALK rearrangement using same protocol as in Figs. 1a – 5a. All cells are unstained.

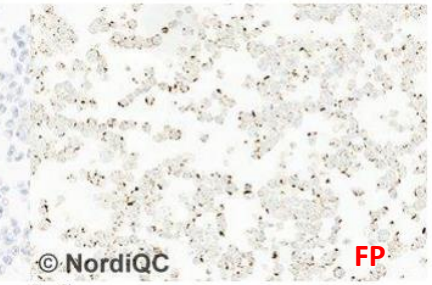


Fig. 6b
Aberrant ALK staining of the cell line without ALK rearrangement using the Ventana Ready-To-Use system based on the rmAb clone D5F3, prod. No. 790-4794. The vast majority of cells show an intracytoplasmic dot-like staining reaction.
This aberrant result was seen in a high number of protocols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

Lu-ALK

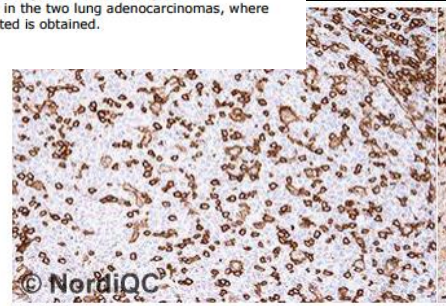


Fig. 1a (x200)
Optimal CD4 staining of the tonsil using the rmAb clone SP35 as a concentrate, HIER in an alkaline buffer (CC1 pH 8.5) and a 3-step multimer based detection system (OptiView, Ventana) - same protocol used in Figs. 2a - 4a. The inducer/helper T-cells show a strong staining reaction, while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

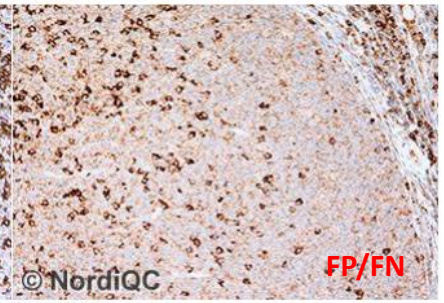


Fig. 1b (x200)
Insufficient CD4 staining of the tonsil using the mAb 4B12 as a concentrate, HIER in an alkaline buffer (CC1 pH 8.5) and a multimer based detection system (OptiView with Tyramide amplification, Ventana) -same protocol used in Figs. 2b-4b. The protocol provided a too low sensitivity, but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

CD4

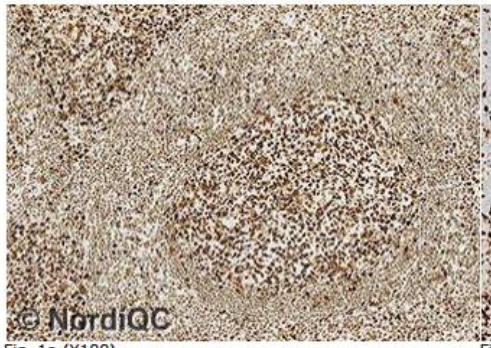


Fig. 1a (X100)
Optimal MSH6 staining reaction of the tonsil using the rmAb clone EP49, optimally calibrated, HIER in an alkaline buffer and a 3-step multimer based detection system (OptiView, Ventana). Virtually all mantle zone B-cells show a moderate and distinct nuclear staining reaction, while the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

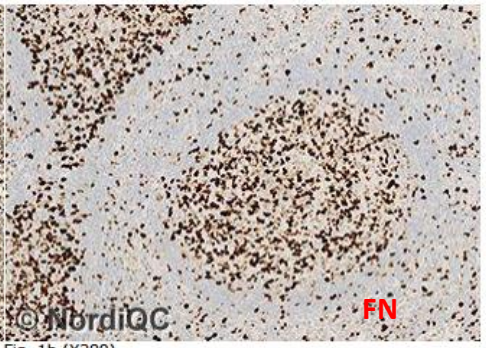


Fig. 1b (X200)
Insufficient MSH6 staining reaction of the tonsil using the rmAb clone EP49 with a protocol providing a too low sensitivity- same field as in Fig. 1a.
Only the germinal centre B-cells are distinctively demonstrated, while mantle zone B-cells expressing low level MSH6 virtually are unstained.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

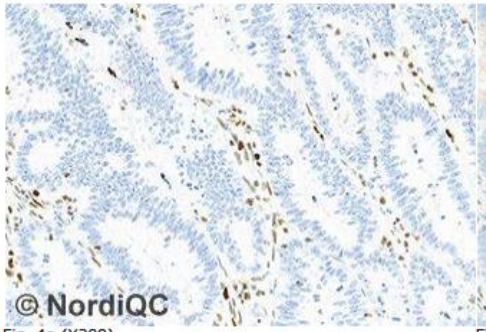


Fig. 4a (X200)
Optimal MSH6 staining reaction of the colon adenocarcinoma no. 4 with loss of MSH6 expression using same protocol as in Figs. 1a - 3a. The neoplastic cells are negative, while stromal cells show a distinct nuclear staining reaction serving as internal positive tissue control.

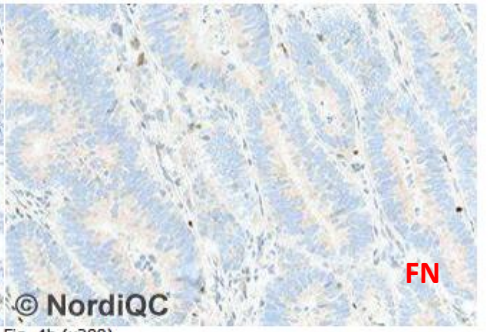


Fig. 4b (x200)
Insufficient MSH6 staining reaction of the colon adenocarcinoma no. 4 with loss of MSH6 expression using same protocol as in Figs. 1b - 3b – same field as in Fig. 4a. No nuclear staining reaction in the neoplastic cells is seen, but as only an equivocal nuclear staining reaction in the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

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 ?

The future - The solution to “low sensitive” detection system ?

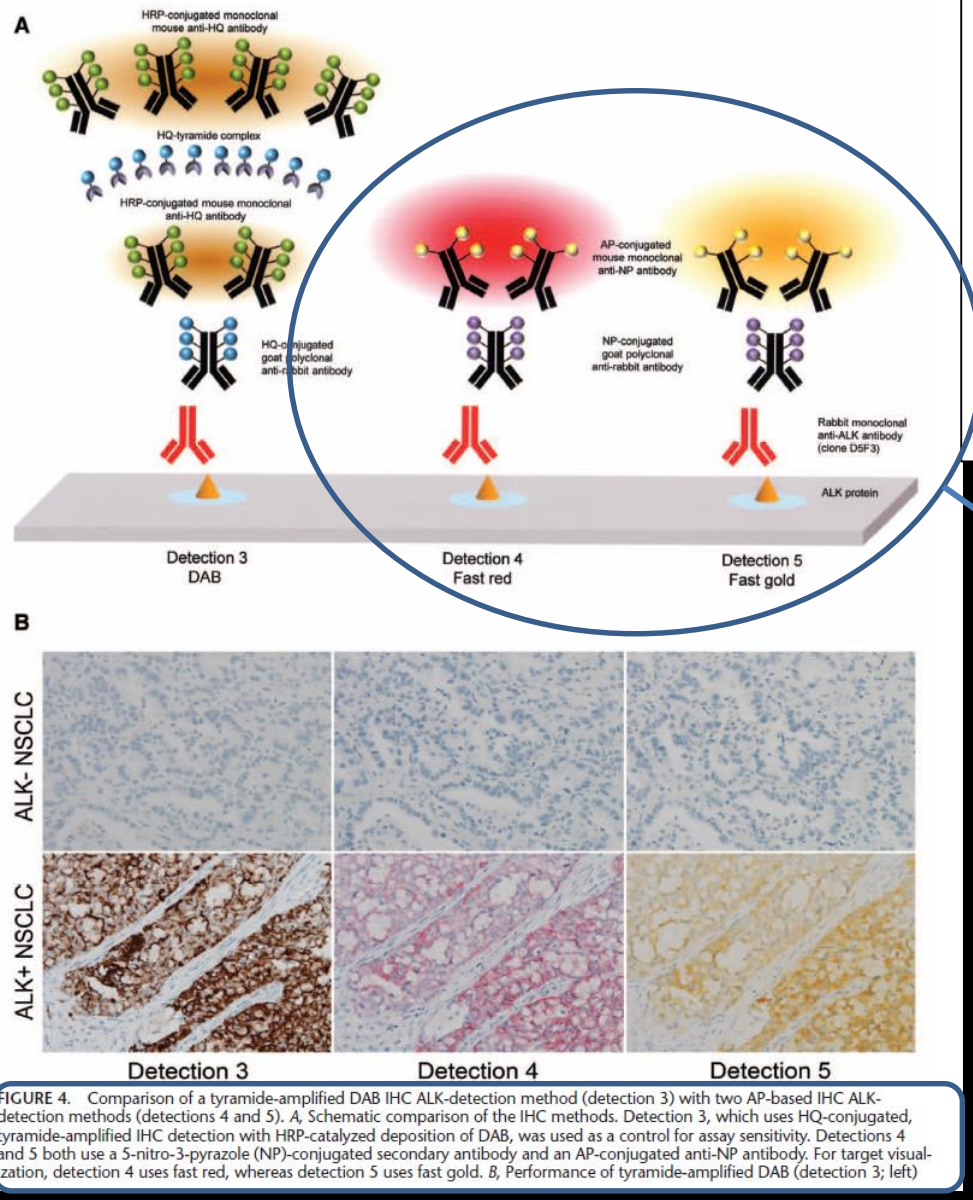


FIGURE 4. Comparison of a tyramide-amplified DAB IHC ALK-detection method (detection 3) with two AP-based IHC ALK-detection methods (detections 4 and 5). *A*, Schematic comparison of the IHC methods. Detection 3, which uses HQ-conjugated, tyramide-amplified IHC detection with HRP-catalyzed deposition of DAB, was used as a control for assay sensitivity. Detections 4 and 5 both use a 5-nitro-3-pyrazole (NP)-conjugated secondary antibody and an AP-conjugated anti-NP antibody. For target visualization, detection 4 uses fast red, whereas detection 5 uses fast gold. *B*, Performance of tyramide-amplified DAB (detection 3; left)

New Methods for *ALK* Status Diagnosis in Non–Small-Cell Lung Cancer

An Improved ALK Immunohistochemical Assay and a New, Brightfield, Dual ALK IHC–In Situ Hybridization Assay

Hiroaki Nitta, PhD, MBA, Koji Tsuta, MD, PhD,† Akihiko Yoshida, MD, PhD,† Steffan N. Ho, MD, PhD,‡ Brian D. Kelly, PhD,* Lauren B. Murata, PhD,* Jerry Kosmeder, PhD,* Katie White, PhD,* Sandra Ehser, PhD,§ Penny Towne, MBA,* Crystal Schemp, MPH,* Abigail McElhinny, PhD,* Jim Ranger-Moore, PhD,* Chris Bieniarz, PhD,* Shalini Singh, MD,* Hitoshi Tsuda, MD, PhD,† and Thomas M. Grogan, MD**

(*J Thorac Oncol.* 2013;8: 1019-1031)

5-nitro-3-pyrazole (NP)-conjugated AP-based systems:

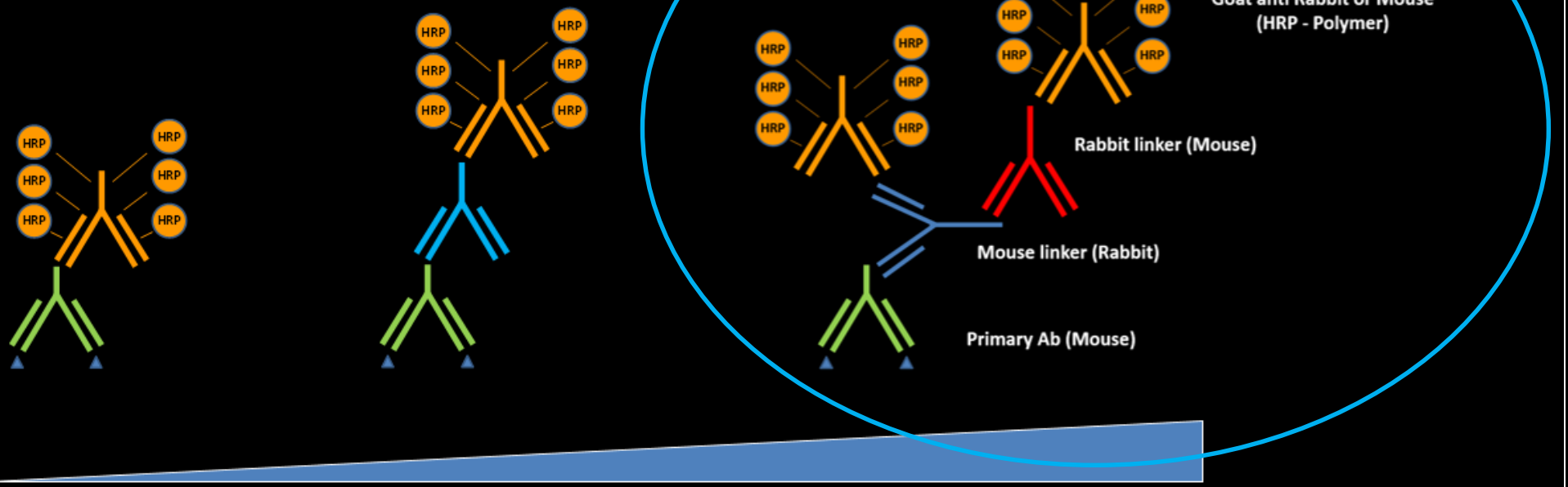
Both of the NP systems demonstrated outstanding sensitivity similar to that observed for the tyramide-based DAB IHC system and superior staining resolution and dynamic range on *ALK* FISH–positive TMA slides (Fig. 4*B*).

Polymer detection systems (Agilent/Dako)

EnVision/Flex

EnVision/Flex+

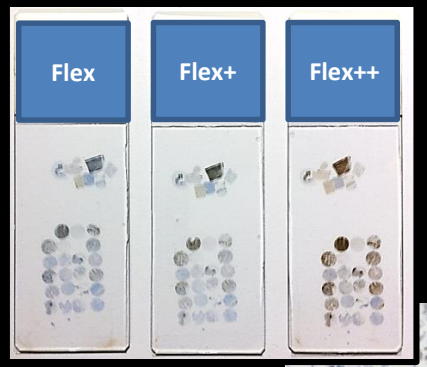
EnVision/Flex++



Sensitivity

New option on the Omnis

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



FLEX

FLEX+

FLEX++

Colon tumor

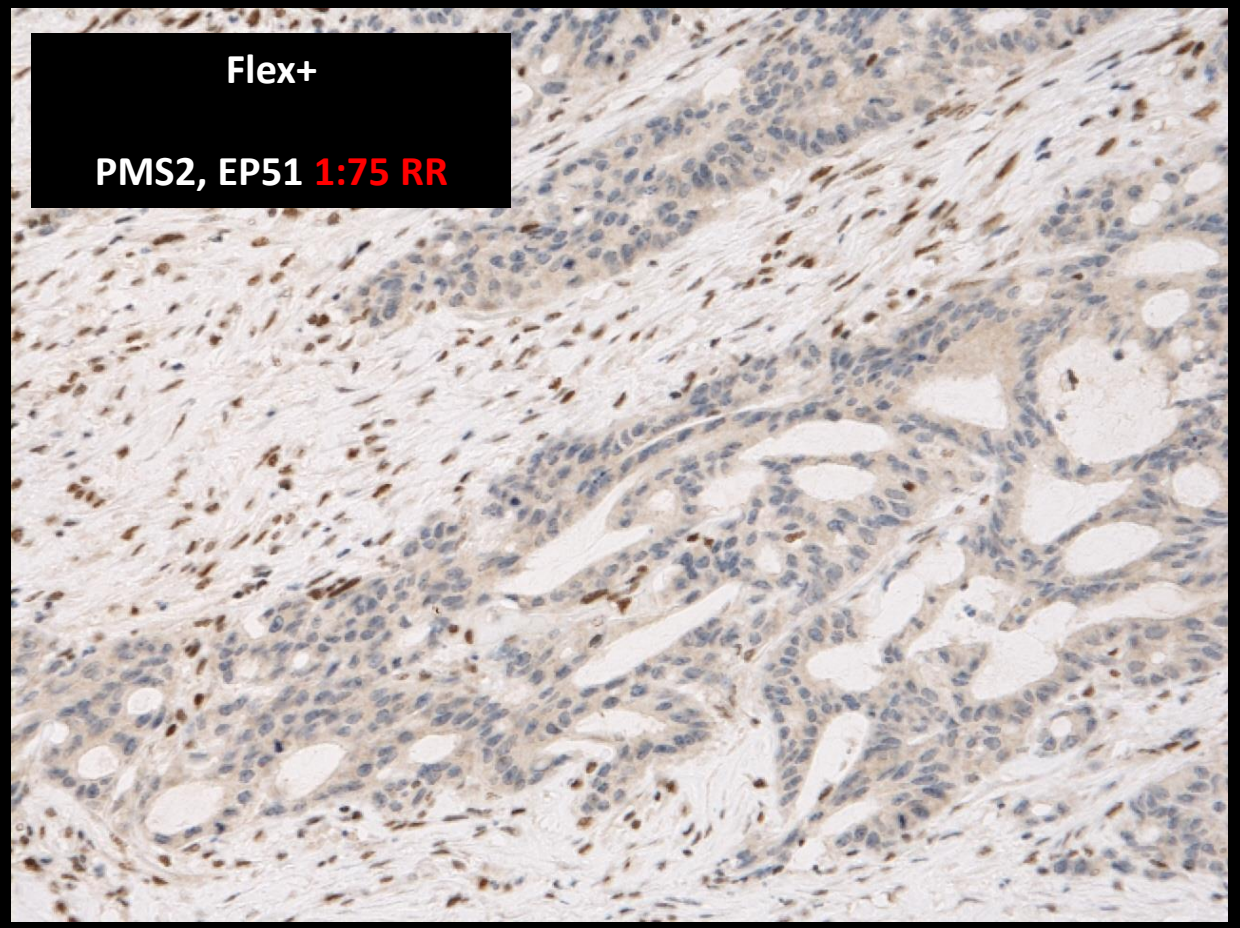
MSI

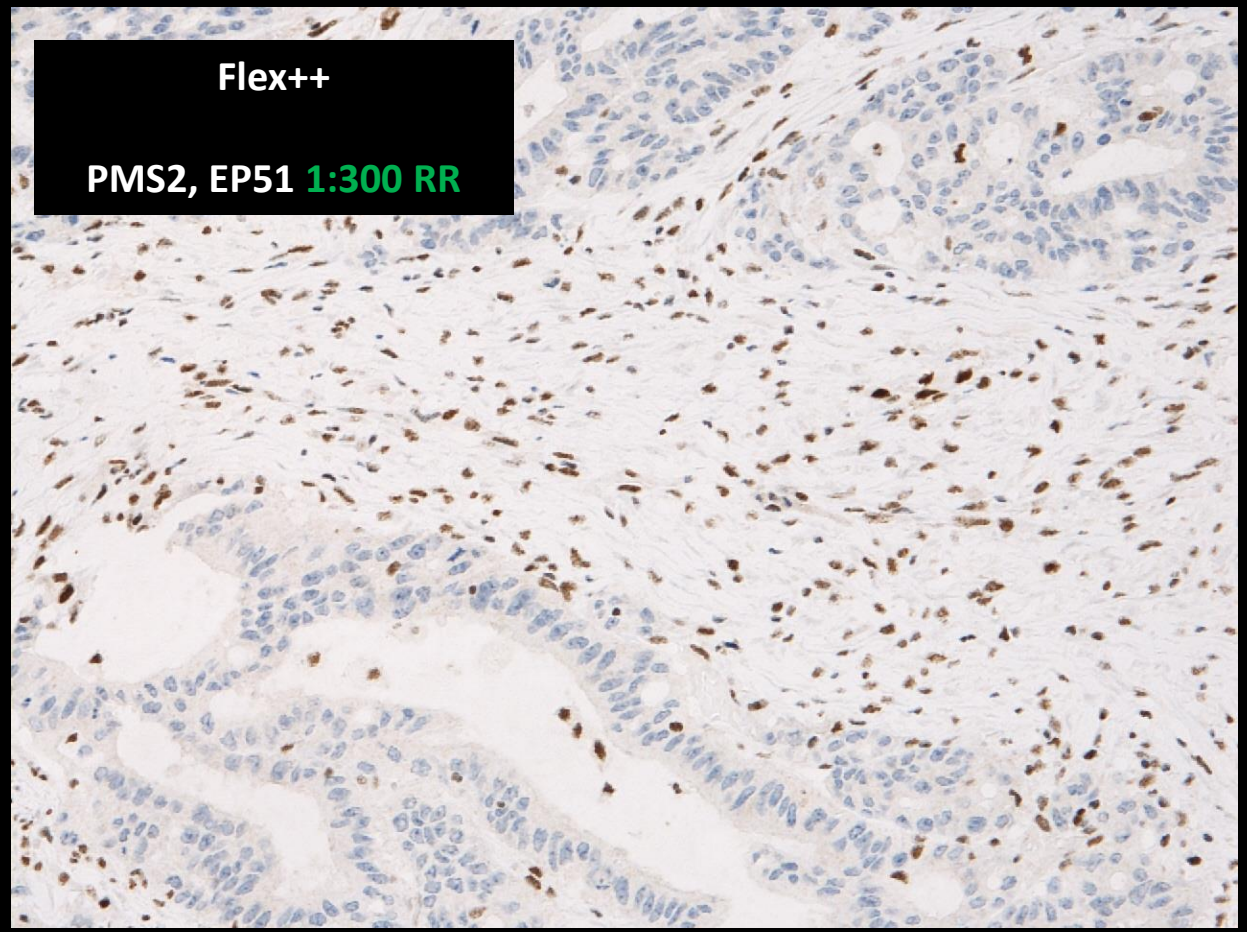
Sensitivity

Colon tumor

MSS

Colon tumor with loss of PMS2





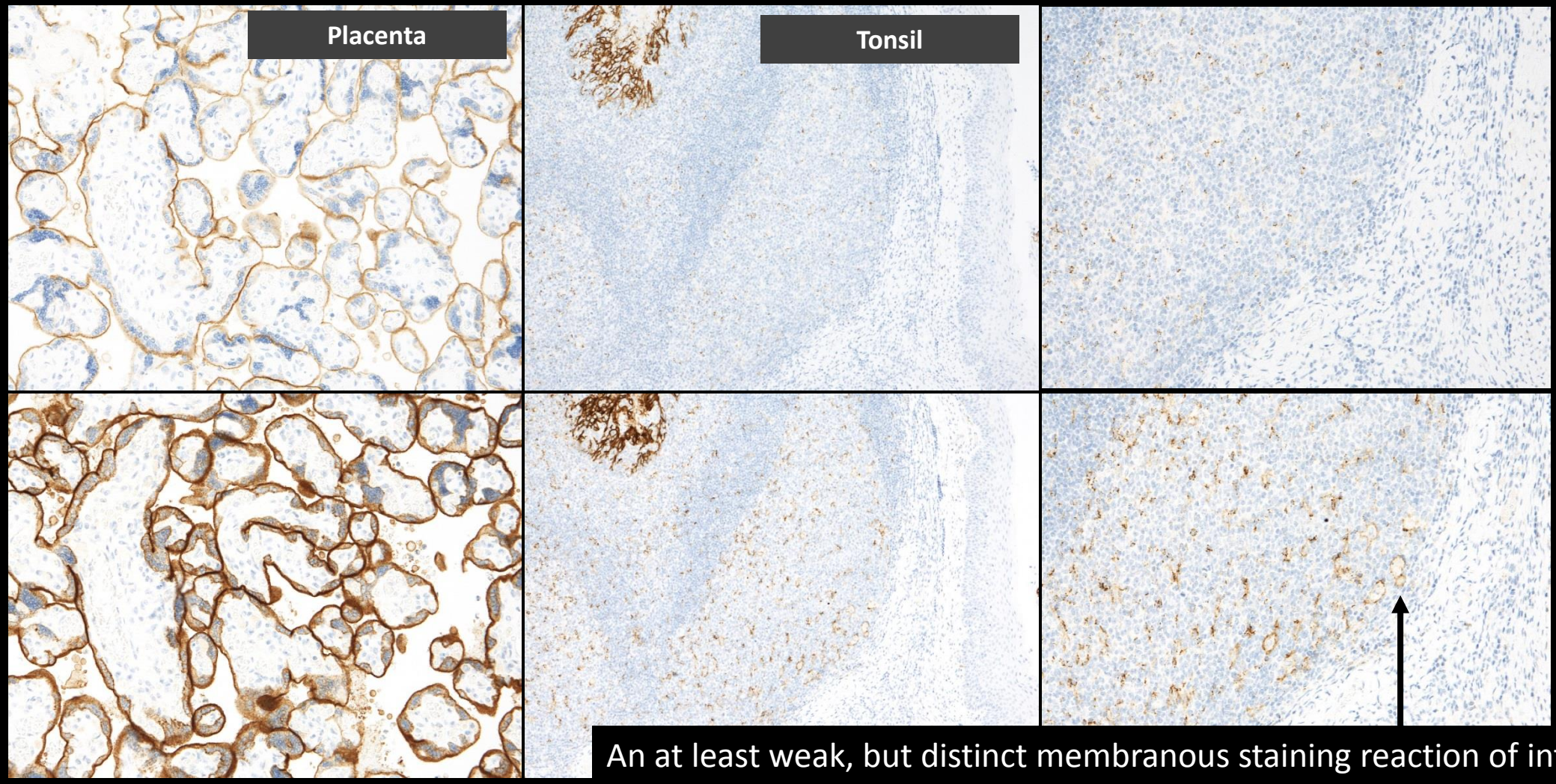
Omnis: HIER High pH 48`

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++



An at least weak, but distinct membranous staining reaction of intra germinal centre macrophages in the tonsil

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

rmAb CMYC, EP121

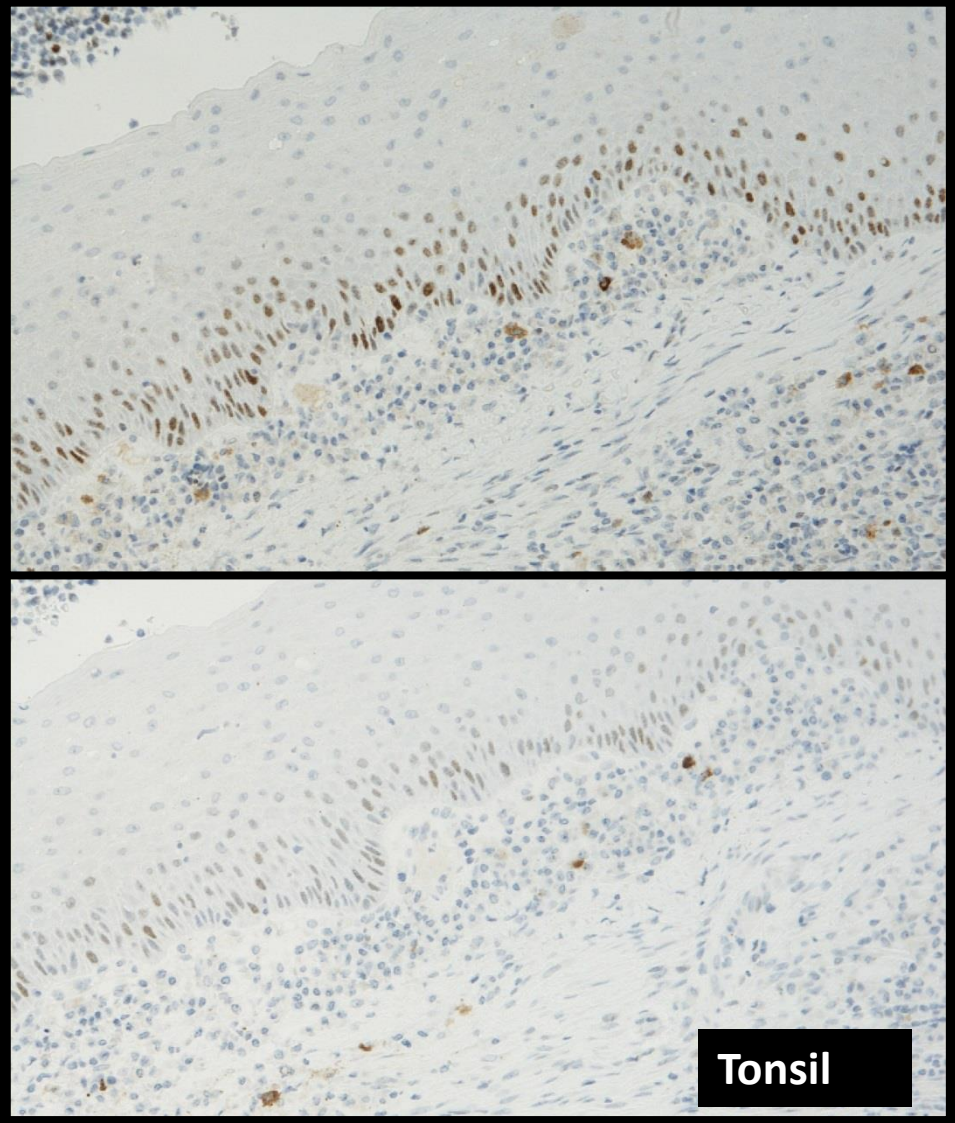
Flex++

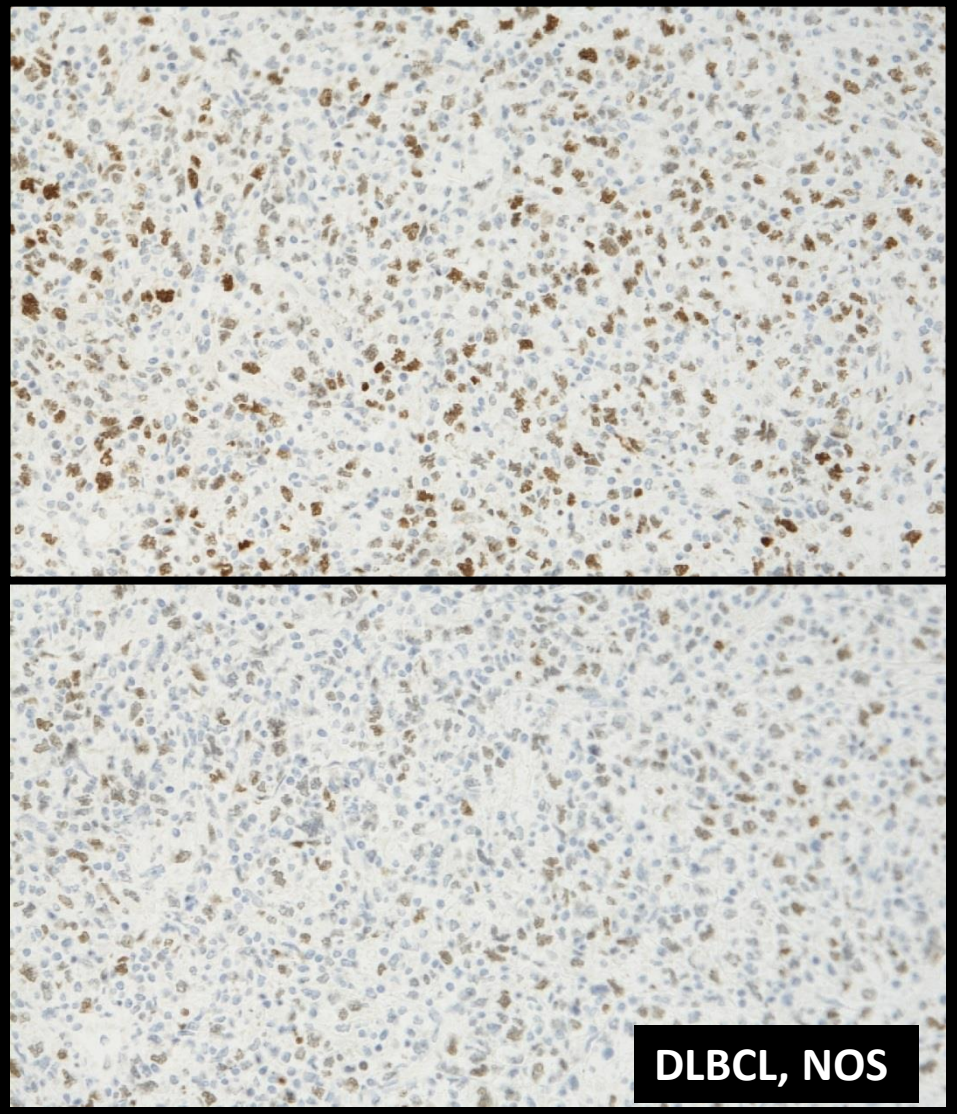
CMYC, EP121
(1:150 RR)

Flex+

CMYC, EP121
(1:75 RR)

HIER in High pH 24`

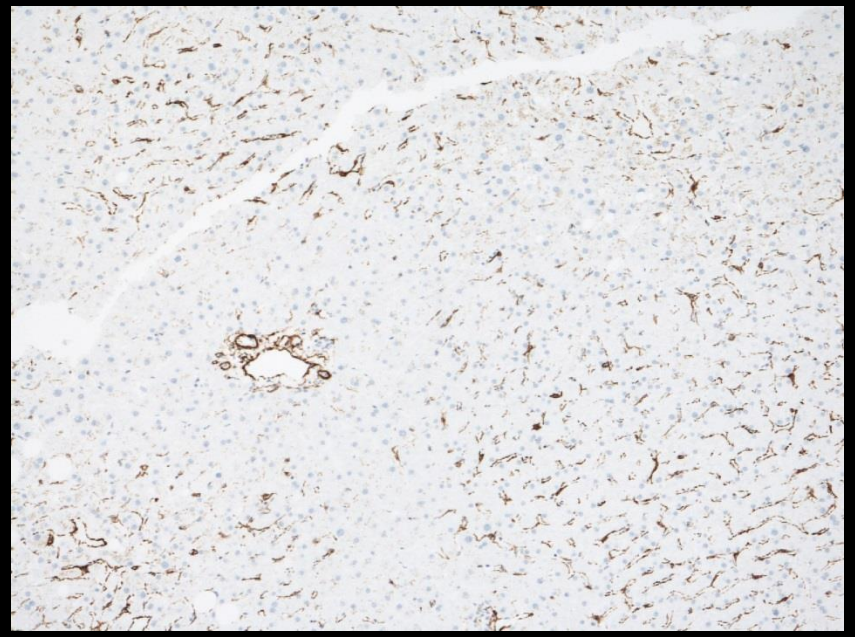




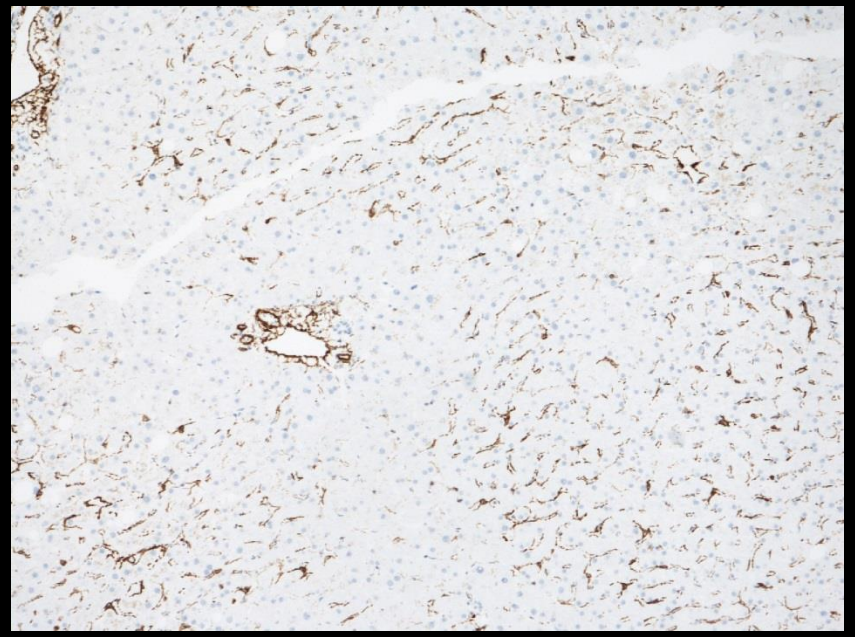
Flex++ : 4-step polymer detection system (Omnis)

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

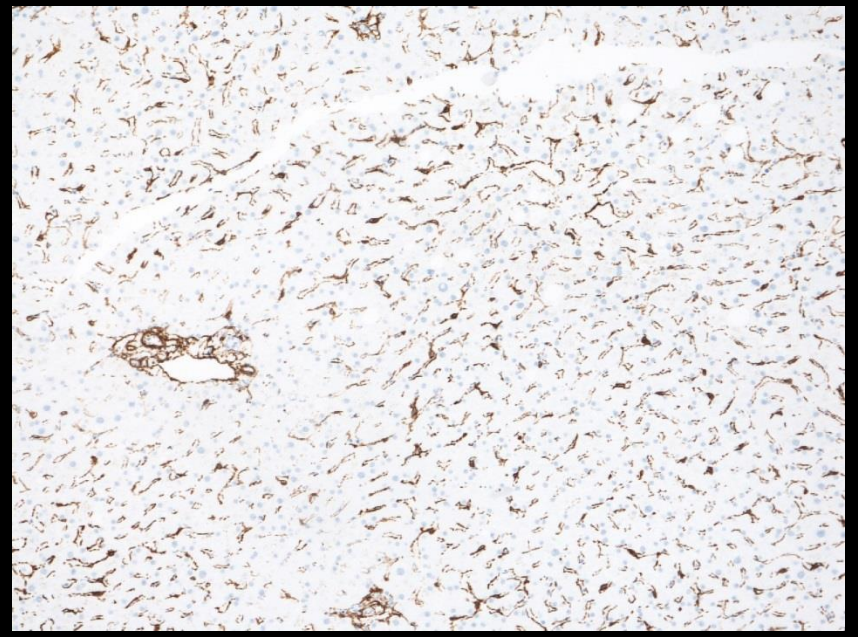
ASMA, 1A4 1:400 / Flex+



ASMA, 1A4 1:400 / Flex++



ASMA, BS66 1:500 / Flex+

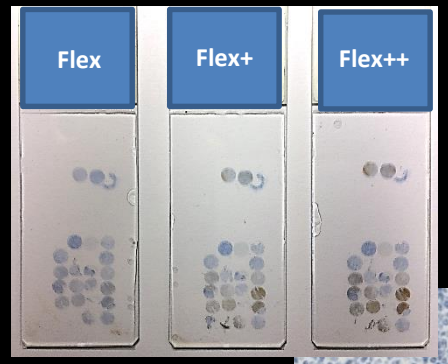


Mouse monoclonal antibodies: No improvement in signal intensity using Flex++ compared to Flex+

Liver

Omnis: BRAF clone VE1 (1:1000 RR)

High pH 48`



FLEX

FLEX+

FLEX++

Colon tumor

BRAFV600E mut

Sensitivity

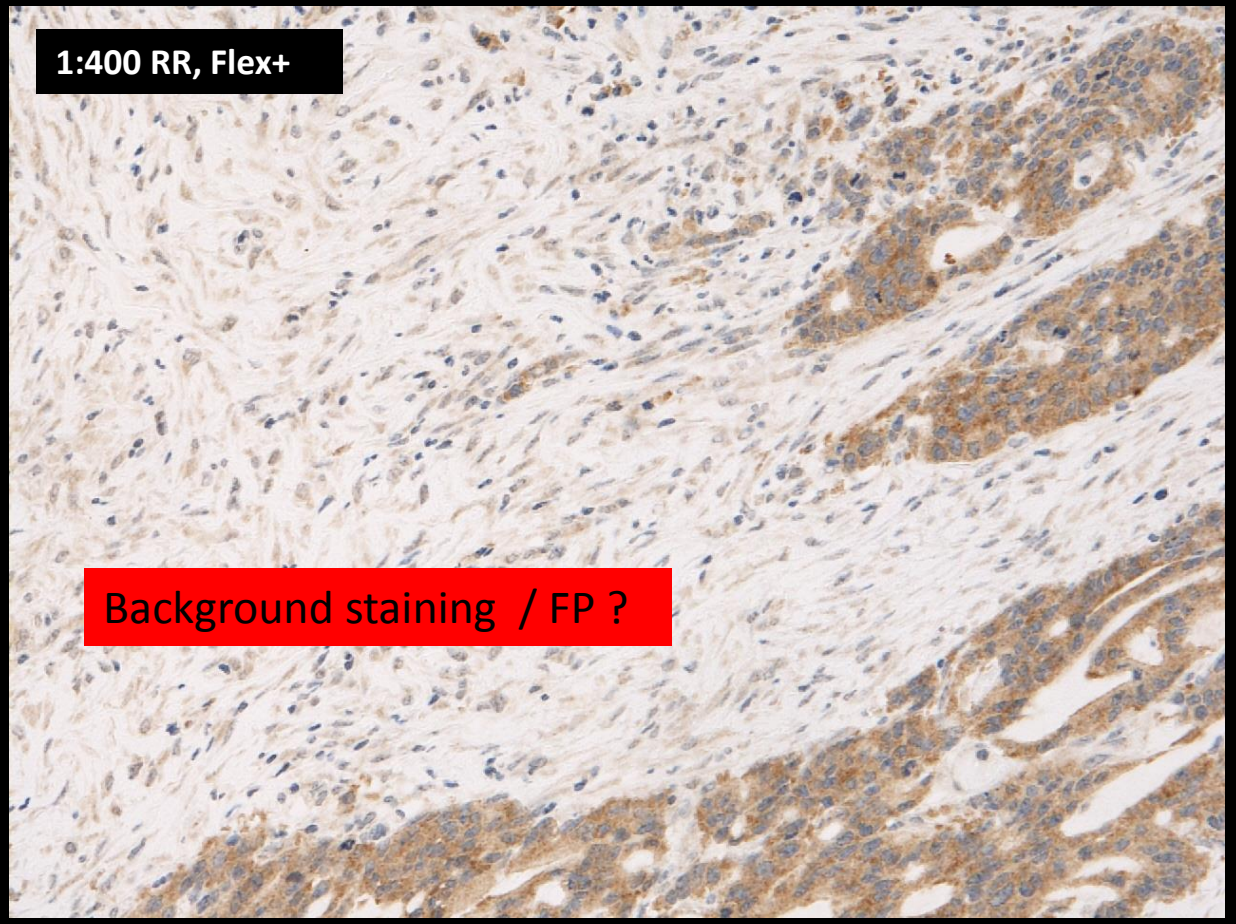
Colon tumor

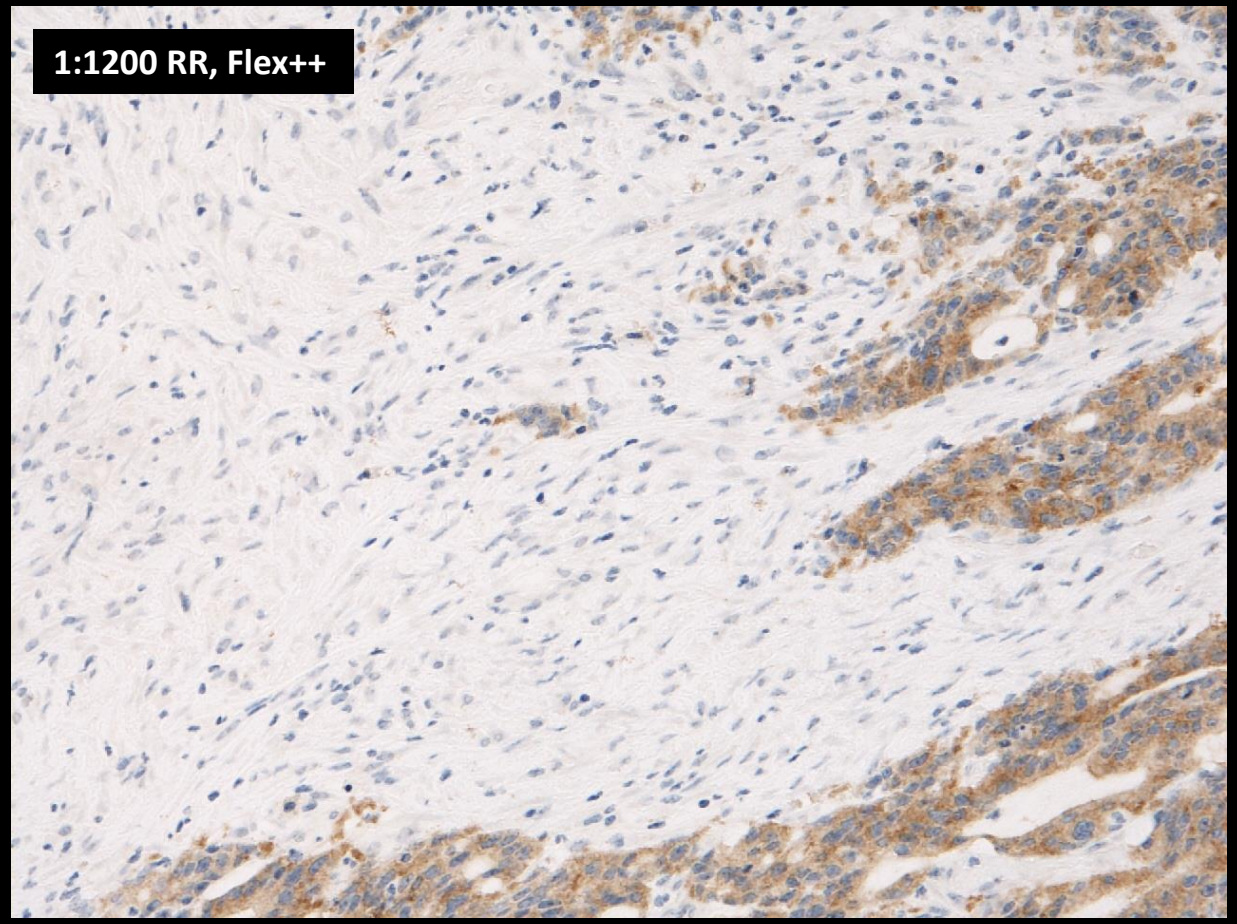
KRAS mut

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

mAb BRAF, VE1 (mutation specific)

Colon tumor BRAF V600E mutated





HIER in High pH 48`

The basal fundament for a technical optimal performance is :

- **Appropriate tissue fixation and processing**
- **Appropriate and efficient epitop retrieval**
  - 95% of the Abs require HIER and app. 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 desired antigen before implementation
  - Calibrate the Ab concentration carefully in relation to Critical Staining Quality Indicators
- **Robust, 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**
  - Include tissue material with low expressors, but also high and non-expressors

Thank you for your attention