Nordic immunohistochemical Quality Control

NordiQC

# Workshop in Diagnostic Immunohistochemistry
# Aalborg University Hospital, 2-4 October 2019

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


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# Technical aspects of immunohistochemistry & pitfalls - Analytical phase

**The total test paradigm:**
**Key elements in the IHC procedure**

## The Analytic phase :

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

**Unlike the pre-analytic factors, analytic 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

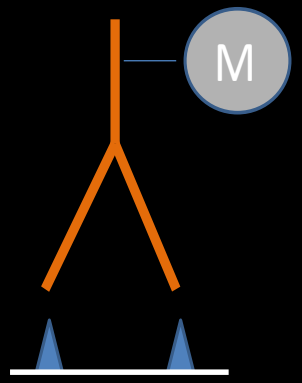
Platform (manual/ Automated)
Epitope retrieval
Blocking
Primary Antibody
Detection system
Chromogen
Counterstain
Mounting

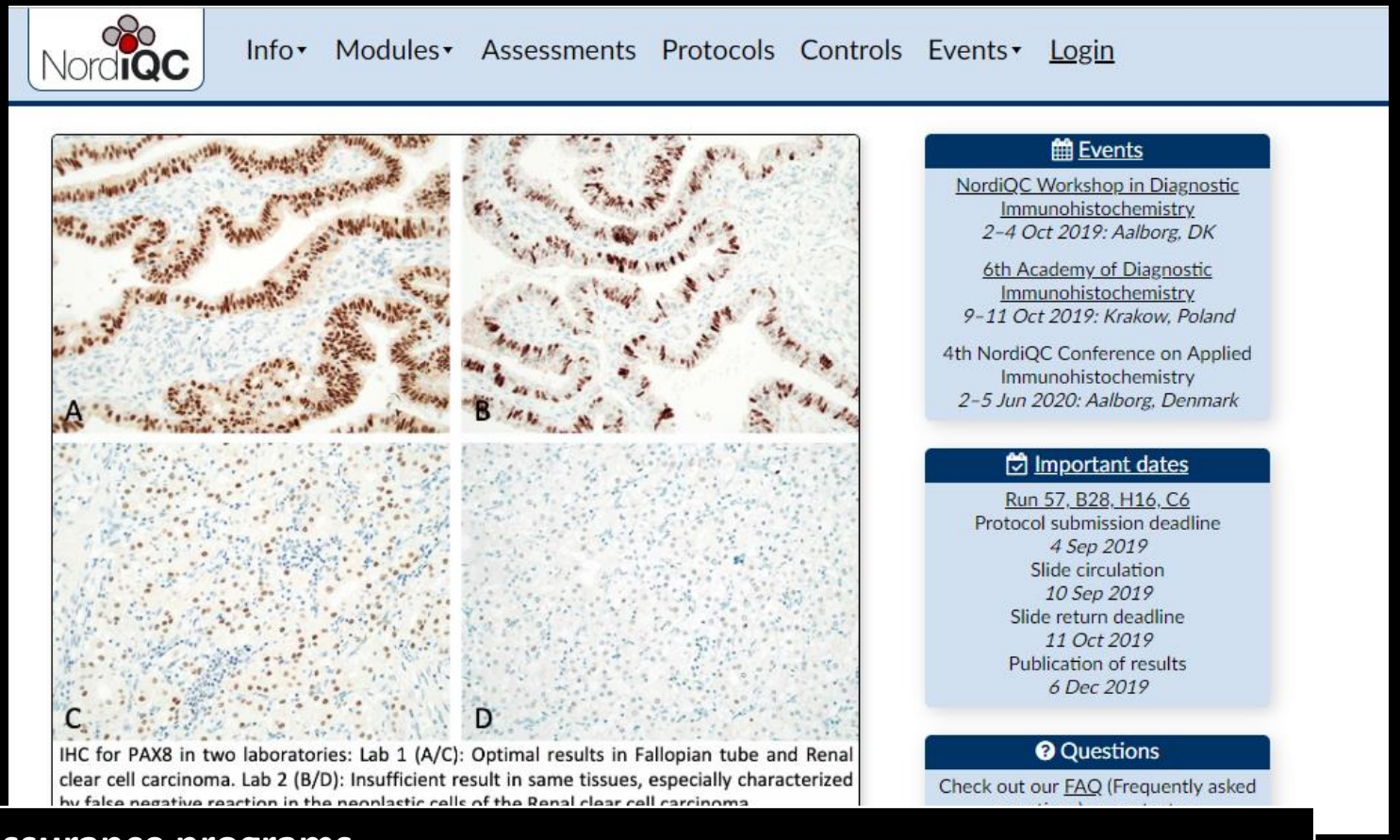
### Post-analytic phase

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

# Technical aspects of immunohistochemistry & pitfalls - 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**

# Technical aspects of immunohistochemistry & pitfalls - Analytical phase

## Optimization of the IHC assay - topics to be addressed

- Purpose and intended use of an 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)

# Technical aspects of immunohistochemistry & pitfalls - 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 …….)**

# Technical aspects of immunohistochemistry & pitfalls - Analytical phase

## Purpose and Intended use

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

**Malignant melanoma** → **Optimal results (NQC run 56)**

mAb A103
mAb BS52
mAb M2-7C10
rmAb EP43

**Steroid producing tumors (e.g. Granulosa cell tumor of the ovary)** → **Optimal results (NQC run 56)**

mAb A103

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

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

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

# Purpose and Intended use

## Melan A/MART-1 (melanoma assessment)/Run 56

**MLA, A103 (melanoma assessment): Suff. 60%**

**- Opt. results - Efficient HIER (Alkaline buffer), High conc. of the primary Ab (app. 1:50) and a sensitive detection system (3-step)**

**MLA, A103 (melanoma + steroid hormone assessment): Suff. 29%**

**- Autostainer (Dako)** ☺

**- Benchmark (Ventana), Omnis (Dako) and Bond (Leica)** ☹

**Melan A (MLA) / MART-1:**

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

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

# Technical aspects of immunohistochemistry & pitfalls - Analytical phase

**MLA, A103**

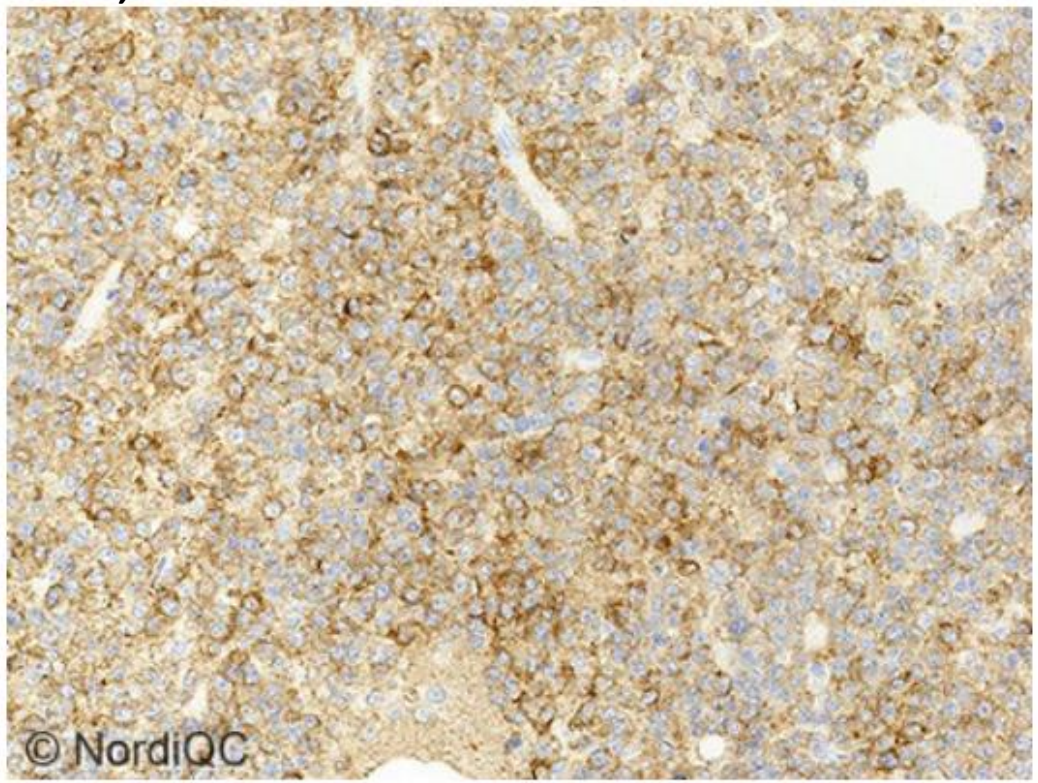



Fig. 8a
Optimal MLA staining of the malignant melanoma tissue core no. 4 (low-level expressor) using same protocol as in Figs. 7a and 7b. The majority of neoplastic cells show a weak to moderate cytoplasmic staining reaction. Compare with Figs. 7a and 8b, same protocol. Also compare with Figs. 5a and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

**MLA, EP43**

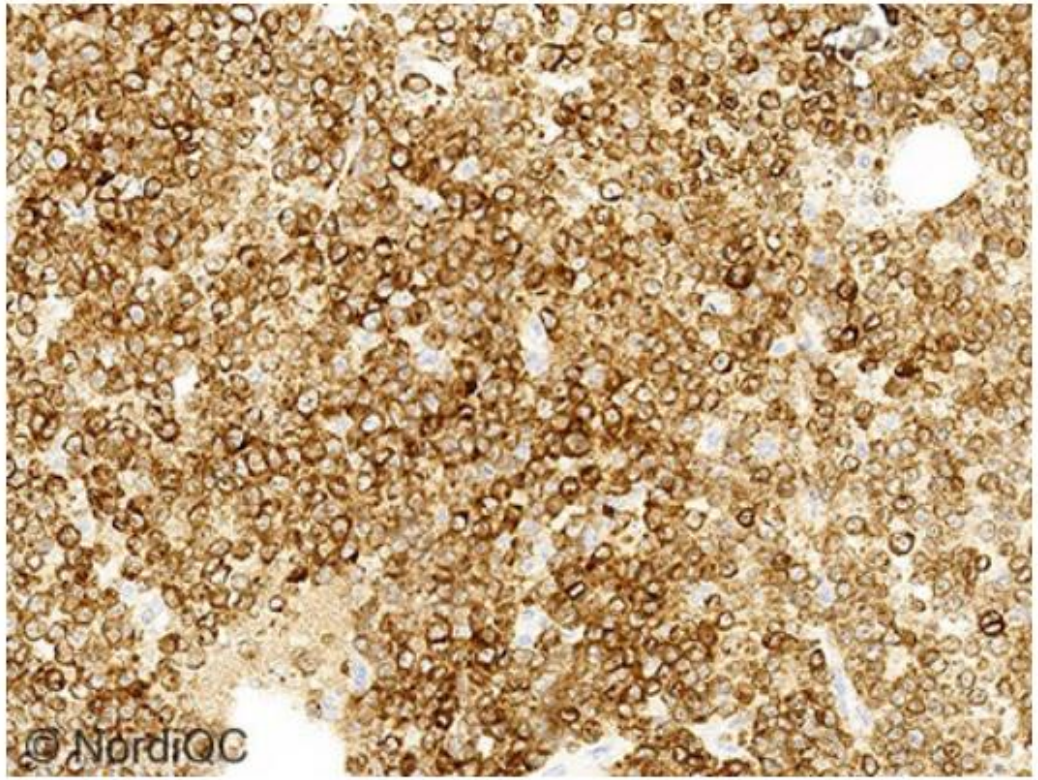
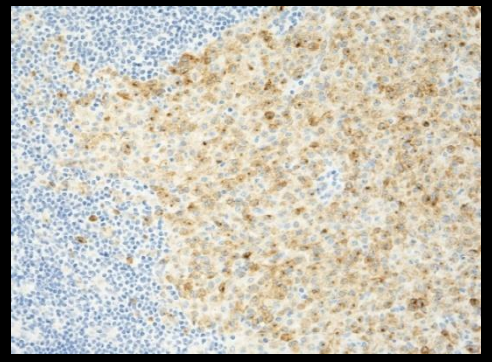
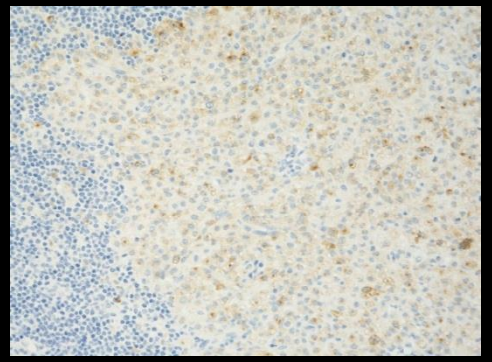
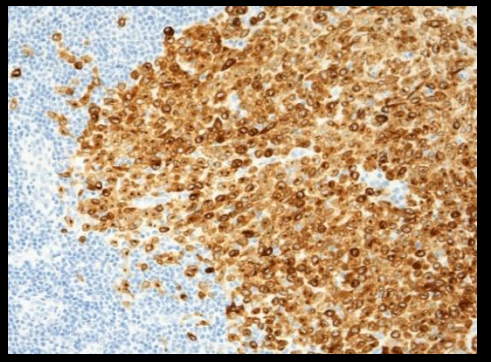
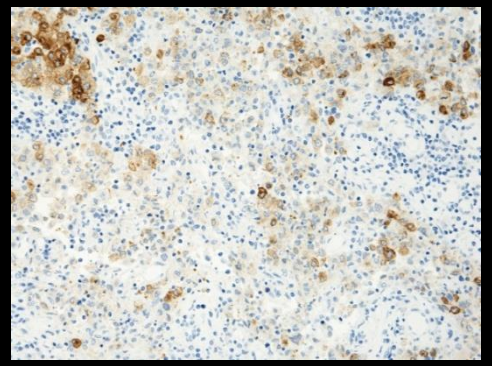
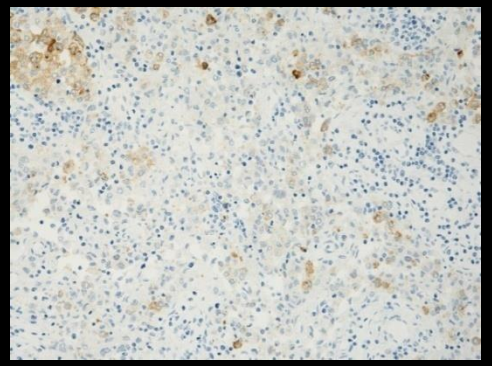
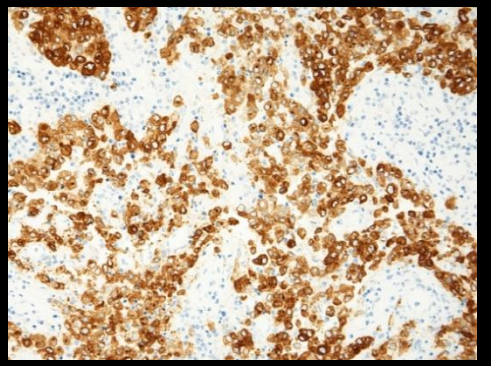
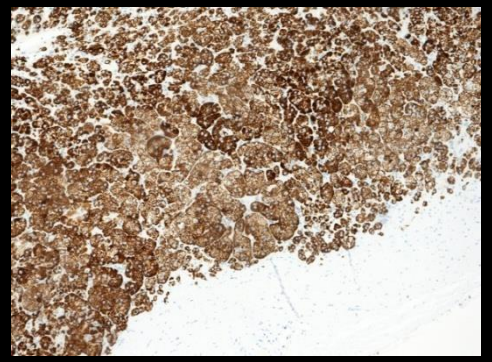
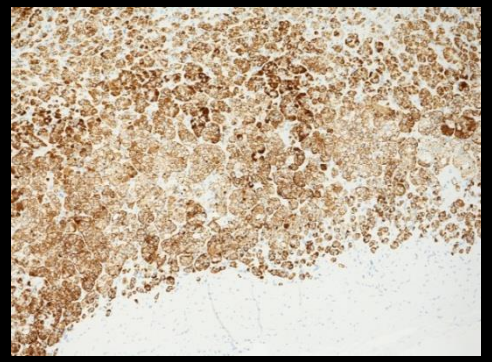
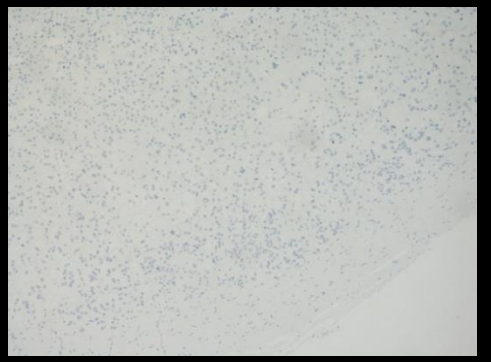



Fig. 8b
Optimal MLA staining of the malignant melanoma tissue core no. 4 (low-level expressor) using **rmAb EP43** in similar protocol settings as in Fig. 8a (and 7a and 7b) All neoplastic cells show a moderate to strong cytoplasmic staining reaction. In melanomas, rmAb EP43 produce very strong reactions.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

# Technical aspects of immunohistochemistry & pitfalls - Analytical phase

**Melan A /MART1**

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

**TRS High pH/Flex+ protocols**

# Technical aspects of immunohistochemistry & pitfalls - 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 robust controls providing information of each test performance in daily practice.

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

# Technical aspects of immunohistochemistry & pitfalls - 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 an antigen of interest before implementation in the routine

Test with robust, specific & sensitive detection system

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

Compare results with external quality assurance programs, literature or colleagues

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

Hadi Yaziji and Todd Barry – Adv Anat Pathol • Vol13, Number 5, September 2006

# Technical aspects of immunohistochemistry & 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 |

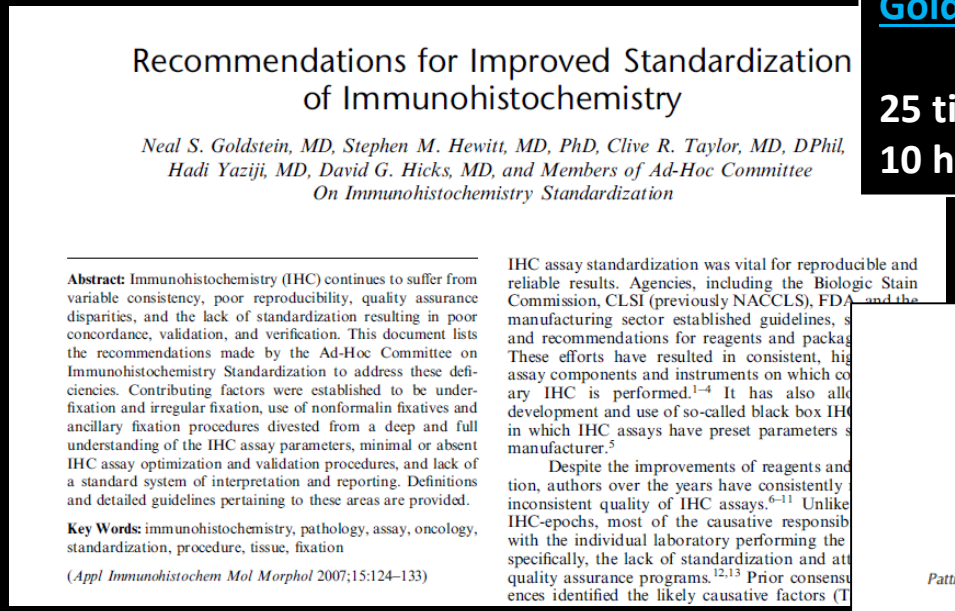
| | |
|---|---|
| Protocol A: | 0.5 % |
| Protocol B: | 2.0 % |
| Protocol C: | 10.0 % |
| Protocol D: | 83.5 % |
| Protocol E: | 1.0 % |
| Protocol F: | 3.0 % |
| Protocol G: | 0 % |
| Protocol H: | 0 % |

* 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 immunohistochemistry & 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

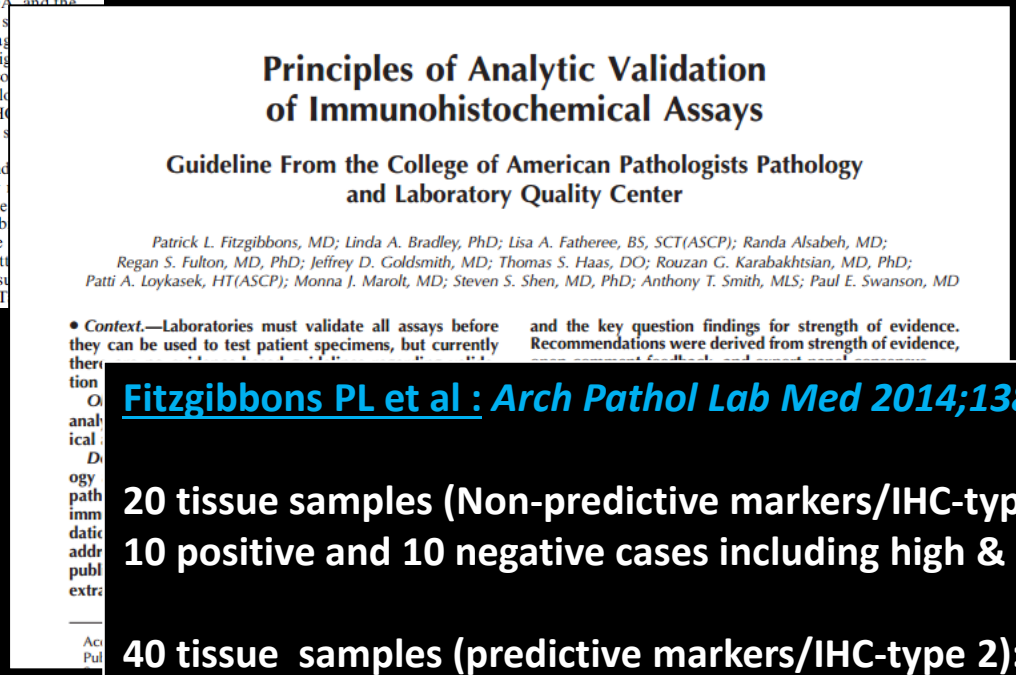
(*Appl Immunohistochem Mol Morphol* 2007;15:124–133)

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

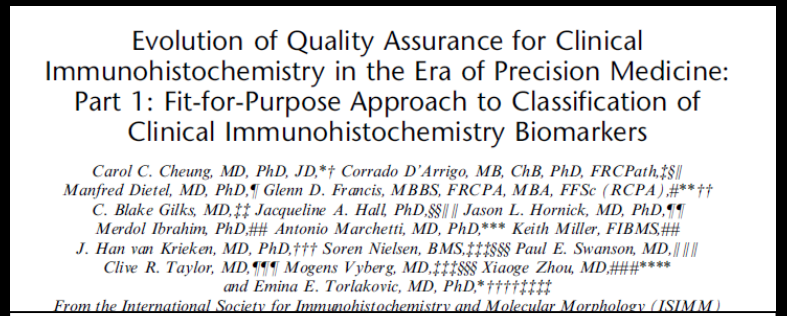
and the key question findings for strength of evidence. Recommendations were derived from strength of evidence,

**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 ?**



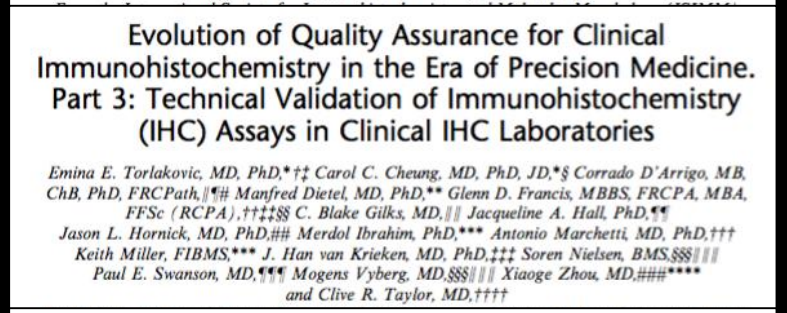
Evolution of Quality Assurance for Clinical Immunohistochemistry in the Era of Precision Medicine: Part 1: Fit-for-Purpose Approach to Classification of Clinical Immunohistochemistry Biomarkers

*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)*

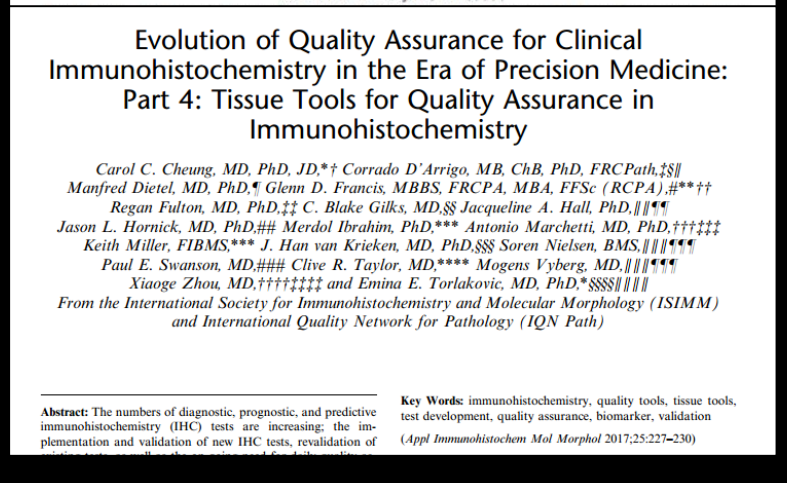
Evolution of Quality Assurance for Clinical Immunohistochemistry in the Era of Precision Medicine – Part 2: Immunohistochemistry Test Performance Characteristics

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



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

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



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

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

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

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

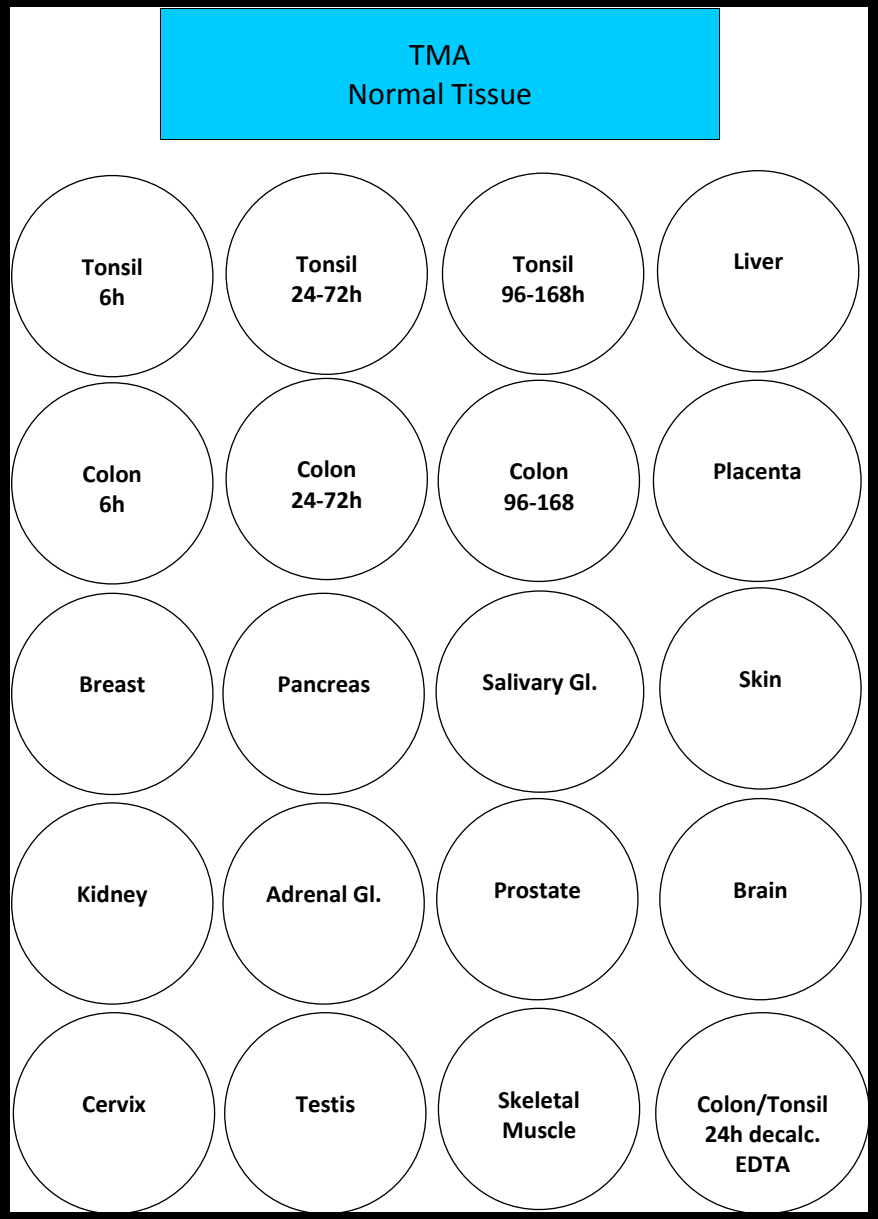
**Key Words:** immunohistochemistry, quality tools, tissue tools, test development, quality assurance, biomarker, validation

(*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 - what is required for a full technical validation and implementation of a new test.**

**Full technical validation**

# Technical aspects of immunohistochemistry & pitfalls - Analytical phase




**Protocol set-up: Evaluate analytic sensitivity and specificity**

**Normal tissue including fixation and decalcification controls**

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

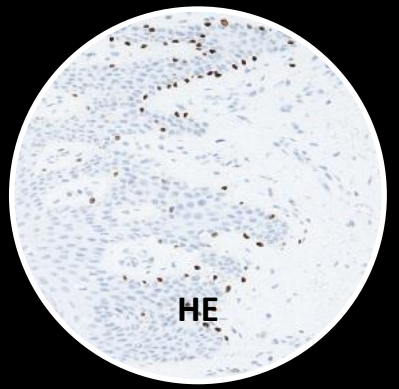
**SOX10, BS7; HIER High pH 24`; 1:350 RR; Flex+Mouse linker**

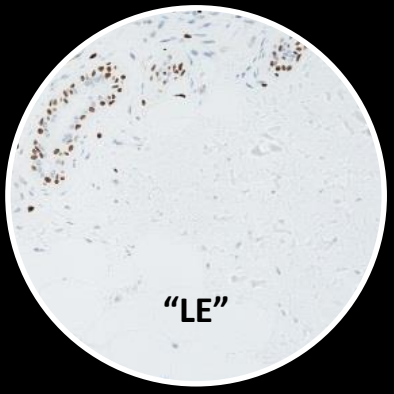
**Establishing robustness of the IHC assay / pre-analytic parameter`s ?**

**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

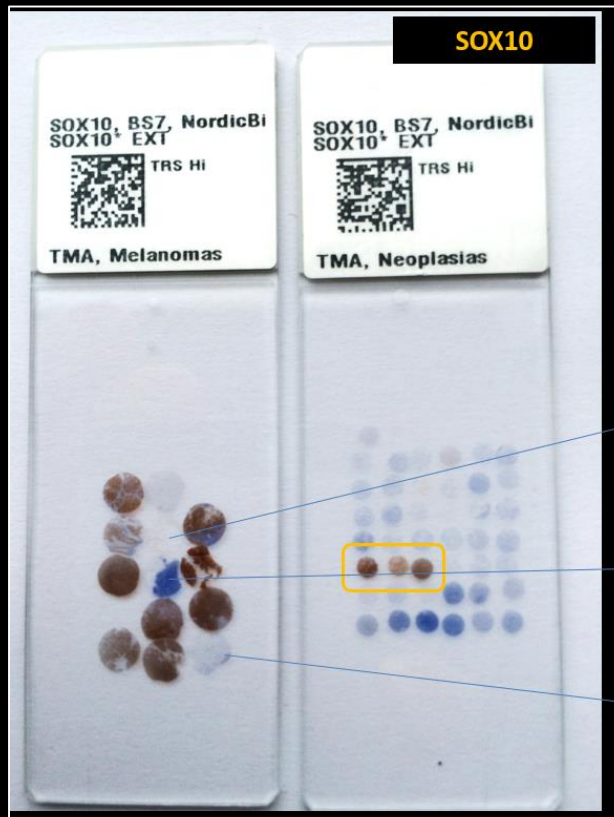
TMA
Malignant Melanomas

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

TMA
Mixed tumors

SOX10

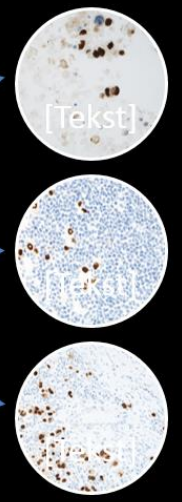
| | | | | | |
|---|---|---|---|---|---|
| Liver | | | | | |
| Mamma Ductal carcinoma | Mamma Ductal carcinoma | Mamma Lobular carcinoma | Lung Adeno carcinoma | Lung Adeno carcinoma | Lung Squam. carcinoma |
| Colon Adeno carcinoma | Colon Adeno carcinoma | Kidney Clear cell carcinoma | Kidney Clear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




**SOX10, BS7:**

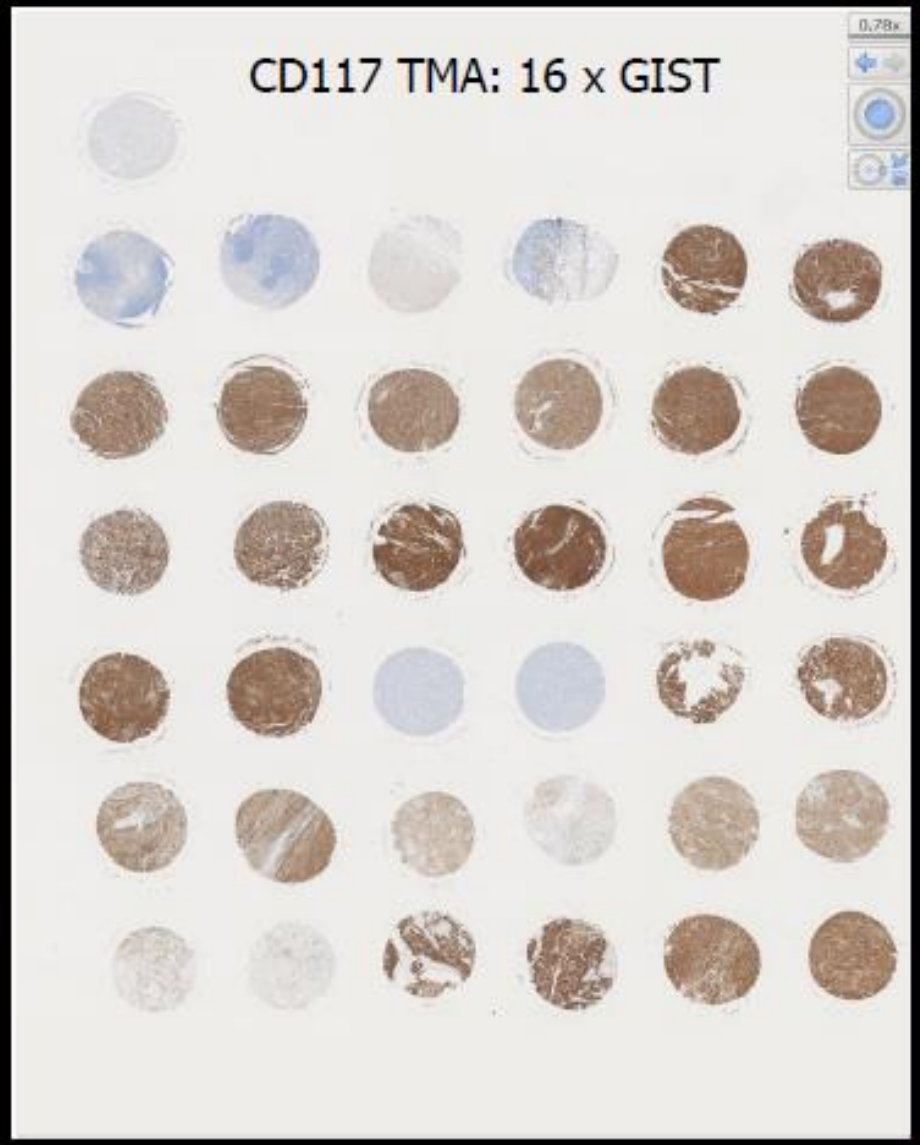
15/15 Melanomas were positive

37/37 other neoplasm`s were negative




**Other SOX10+ tumours: Schwannoma´s, neurofibroma`s, myoepithelial carcinomas……..?**

# 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]

**Main causes of insufficient staining reactions are related to:**

- The choice of antigen retrieval method
- The choice of primary antibody (Concentrate or RTU)
  a) Calibration of the 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
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]

## 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**



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
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   a. Heat-induced impaired morphology
   b. Proteolysis induced impaired morphology
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   e. Excessive counterstaining impairing interpretation

**27% insuff.**

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

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

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

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

Virchows Arch (2011) 458:665–670
DOI 10.1007/s00428-011-1076-z

ORIGINAL ARTICLE

# Detection of smoothelin expression in the urinary bladder is strongly dependent on pretreatment conditions: a critical analysis with possible consequences for cancer staging

Claes Lindh · Robert Nilsson · Marie Louise Lindstrom · Lilian Lundin · Goran Elmberger

**Distinguishing urinary bladder muscularis propria (MP) from muscularis mucosae (MM) is crucial in bladder cancer staging.**

**Table 1** Intensity of smoothelin IHC staining depending on pretreatment conditions

| Intensity | | Muscularis mucosae (%) | | Muscularis propria (%) |
|---|---|---|---|---|
| Enzymatic pretreatment | | | | |
| Negative | 0 | 17/18 (94) | Negative | 14/18 (78) |
| Weak | 2+ | 1/18 (6) | | 4/18 (22) |
| | 3+ | 0/18 (0) | | 0/18 (0) |
| Strong | 4+ | 0/18 (0) | | 0/18 (0) |
| HIER in acidic buffer | | | | |
| Negative | 0 | 10/18 (56) | Intermediate | 1/18 (5.5) |
| Weak | 1+ | 7/18 (39) | | 10/18 (56) |
| | 2+ | 1/18 (5) | | 6/18 (33) |
| Strong | 3+ | 0/18 (0) | | 1/18 (5.5) |
| HIER in alkaline buffer | | | | |
| Negative | 0 | 1/18 (6) | Positive | 0/18 (0) |
| Weak | 1+ | 6/18 (33) | | 0/18 (0) |
| | 2+ | 7/18 (39) | | 1/18 (6) |
| Strong | 3+ | 4/18 (22) | | 17/18 (94) |



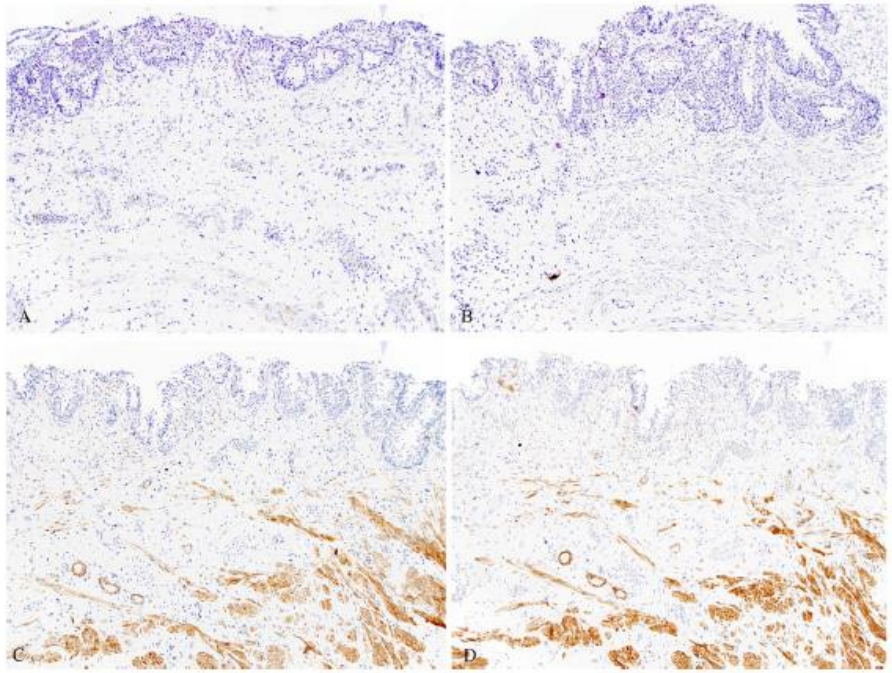



**Fig. 1** Staining intensity of the muscularis mucosae, muscularis propria and smooth muscle in blood vessels depended strongly on pretreatment conditions. Staining without pretreatment resulted in weak staining of smooth muscle in the blood vessels (**a**) while staining was virtually absent when using enzymatic pretreatment (**b**). HIER in acidic buffer resulted in weak–moderate staining (**c**), but the strongest staining was achieved using HIER in alkaline buffer as pretreatment (**d**) (a–d lens magnification ×10)

**The discrepancy between different studies using the same primary antibody for smoothelin in the bladder is properly caused by different technical aspects**

**Table 2** Summary of IHC protocols used by different groups

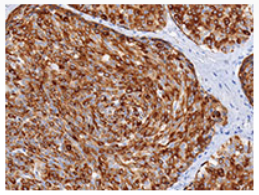
| | Antibody | Dilution | HIER | Platform |
|---|---|---|---|---|
| Paner et al. | R4A (Abcam Inc.) | 1:150 | Citrate buffer (acidic, pH 6.0) | Ventana Benchmark System |
| Council et al. | R4A (Chemicon International) | 1:400 | Citrate buffer (acidic) | Ventana Benchmark System |
| Miyamoto et al. | R4A (Abcam Inc.) | 1:200 | Mild CC1 buffer (high pH) | Ventana Benchmark System |
| Lindh et al. | R4A (Biocare Medical) | 1:100 | EDTA (alkaline, pH 9.0) | Bond Max |

**In conclusion, smoothelin IHC is strongly dependent on the chosen epitope retrieval method, and smoothelin staining did not discriminate reliably between MP and MM with any of the tested pretreatment protocols.**

# 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<br>BenchMark XT instrument: 16 minutes, 37°C<br>BenchMark GX instrument: 16 minutes, 37°C |
| | |
| | inutes |
| | |

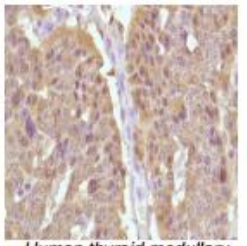
**Optimizing an assay can be confusing**

**(Vendor recommendations)**

SPRING BIOSCIENCE

## Rabbit Anti-Human Calcitonin Monoclonal Antibody (Clone SP17)

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



*Human thyroid medullary carcinoma stained with anti-calcitonin antibody*

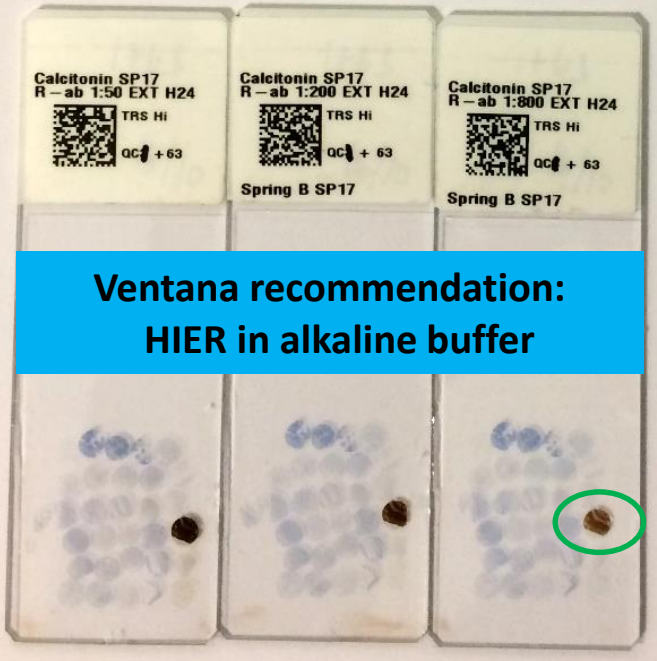
| | |
|---|---|
| **INTENDED USE:** | For Research Use Only. Not for use in diagnostic procedures. |
| **CLONE:** | SP17 |
| **IMMUNOGEN:** | Synthetic human calcitonin 1-32 amino acid peptide. |
| **IG ISOTYPE:** | Rabbbit IgG |
| **EPITOPE:** | Not determined |
| **MOLECULAR WEIGHT** | 15kDa |
| **SPECIES REACTIVITY:** | Human (tested). (See www.springbio.com for information on species reactivity predicted by sequence homology.) |
| **DESCRIPTION:** | Calcitonin is a 32 amino acid peptide which can be demonstrated in C cells of the normal and hyperplastic thyroid. Staining for calcitonin may be used for the identification of a spectrum of C cell proliferative abnormalities ranging from C cell hyperplasia to invasive tumors. Staining for calcitonin in medullary carcinoma of the thyroid produces a fine granular pattern in the cytoplasm. Amyloid deposits within the tumor may also exhibit varying degrees of calcitonin activity. |
| **APPLICATIONS:** | Immunohistochemistry (IHC) |
| **IHC PROCEDURE:** | **Specimen Preparation:** Formalin-fixed, paraffin-embedded tissues are suitable for use with this primary antibody.<br>**Deparaffinization:** Deparaffinize slides using xylene or xylene alternative and graded alcohols.<br>**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.<br>**Antigen Retrieval:** None<br>**Primary Antibody Incubation:** Incubate for 30 minutes at room temperature.<br>**Slide Washing:** Slides must be washed in between steps. Rinse slides with PBS/0.05% Tween.<br>**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?

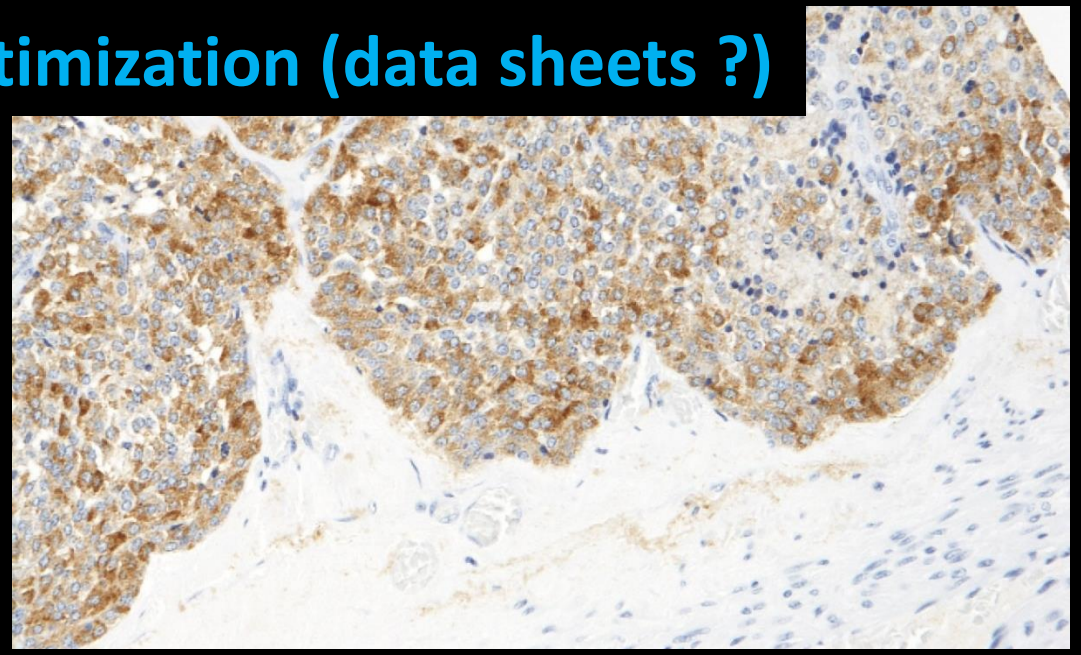
# Calcitonin optimization (data sheets ?)

Omnis, Flex+

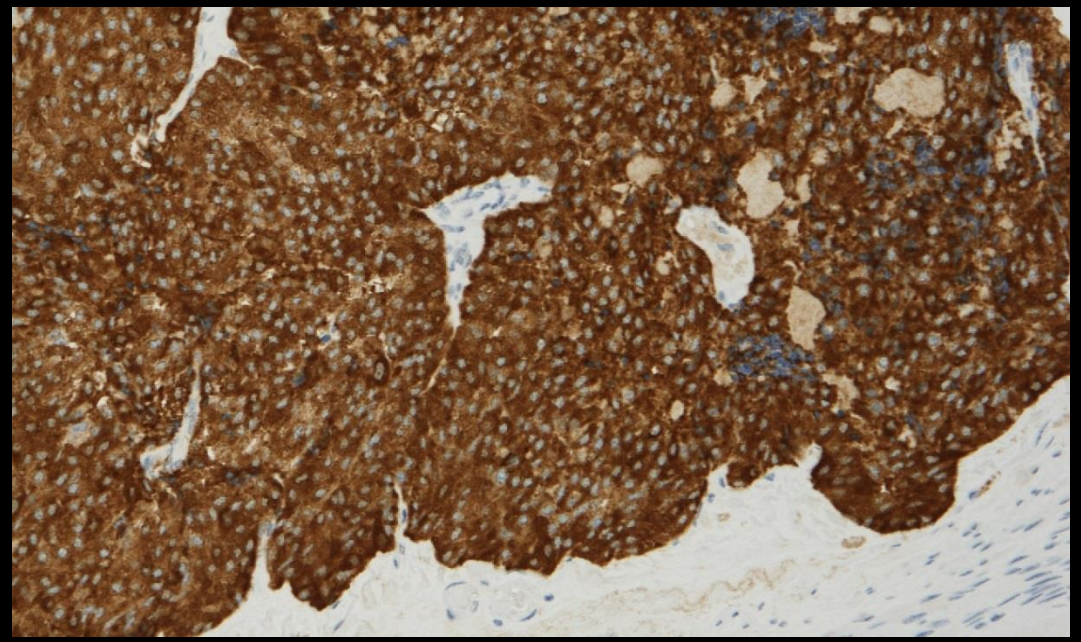

**Spring B recommendation :**
**No pretreatment**




**Ventana recommendation:**
**HIER in alkaline buffer**



**Thyroid medullary carcinoma**



# Technical aspects of immunohistochemistry & pitfalls - Analytical phase

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

**Antigen retrieval procedures for formalin fixed tissue:**

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

# Technical aspects of immunohistochemistry & pitfalls - Analytical phase

0022-1554/91/$3.30
The Journal of Histochemistry and Cytochemistry
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 Laborat*

Received for publi

We describe a ne
formalin-fixed, p

**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.**

# Technical aspects of immunohistochemistry & pitfalls - Analytical phase

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

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

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

ORIGINAL PAPER

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

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

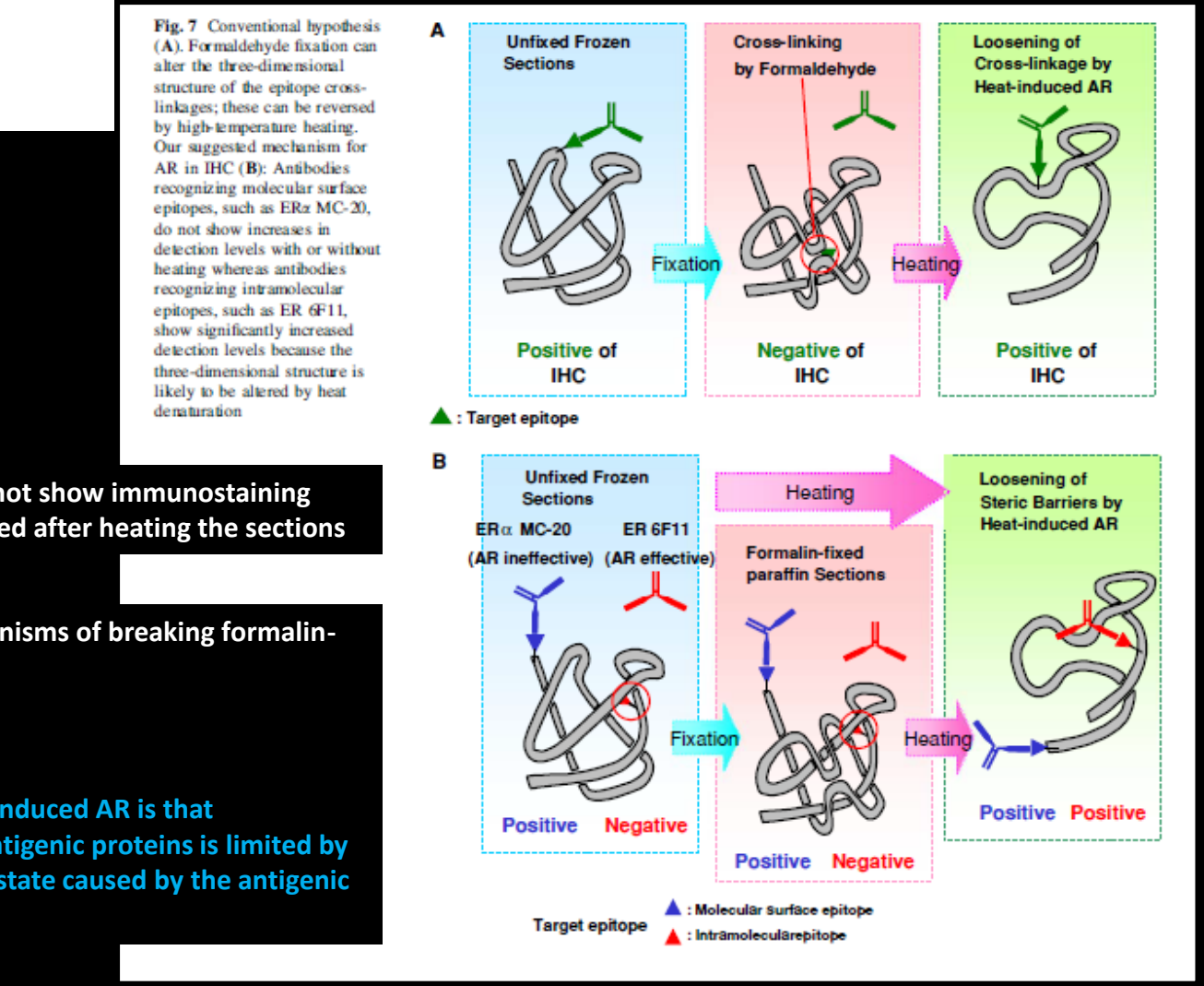



Fig. 7 Conventional hypothesis (A). Formaldehyde fixation can alter the three-dimensional structure of the epitope cross-linkages; these can be reversed by high-temperature heating. Our suggested mechanism for AR in IHC (B): Antibodies recognizing molecular surface epitopes, such as ERα MC-20, do not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

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

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

**The authors proposed that :**

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

# Technical aspects of immunohistochemistry & pitfalls - Analytical phase

## Efficient HIER depends on:

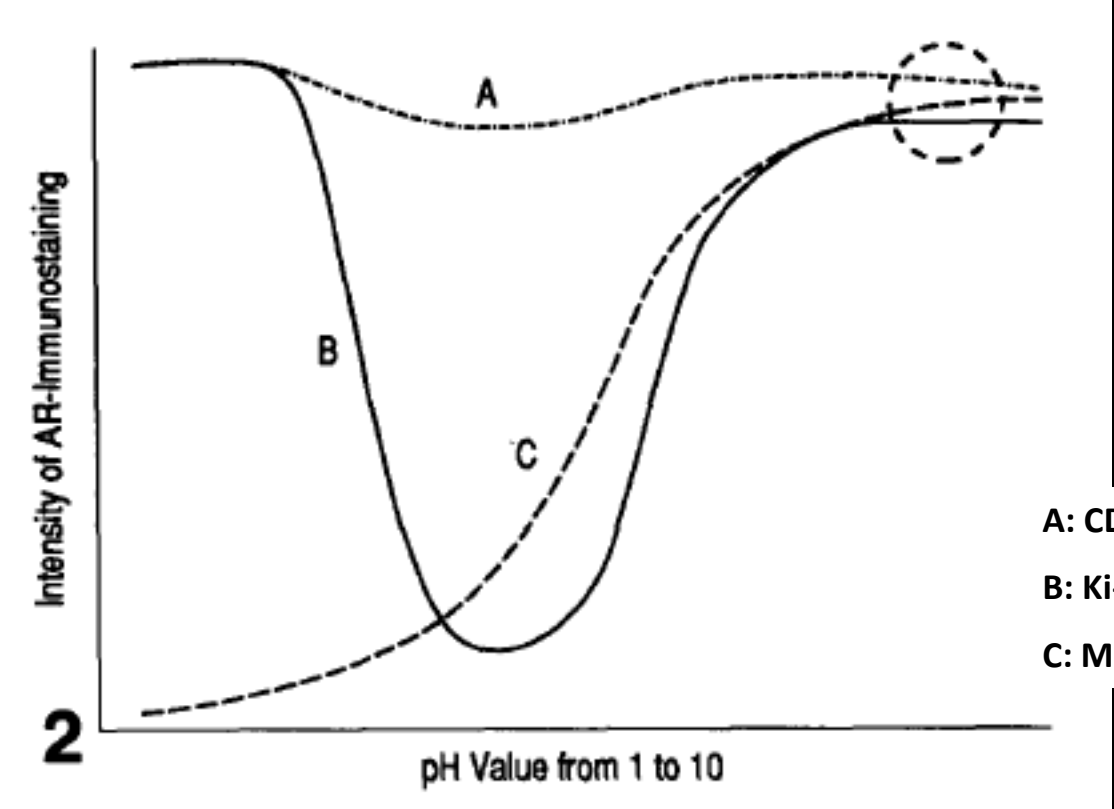
- ❑ pH of the HIER buffer
- ❑ Temperature
- ❑ Time
- ❑ Elementary nature of the HIER buffer (e.g. Citrate; TRIS; EDTA; TE)
- ❑ "Fixation time in formalin"

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

> 95% of all commonly used antibodies require HIER

# Technical aspects of immunohistochemistry & pitfalls - Analytical phase

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




## Efficient HIER - Influence of pH

**A: CD20 (clone L26)**

**B: Ki-67 (clone MIB1)**

**C: MSA (clone HMB45)**

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

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

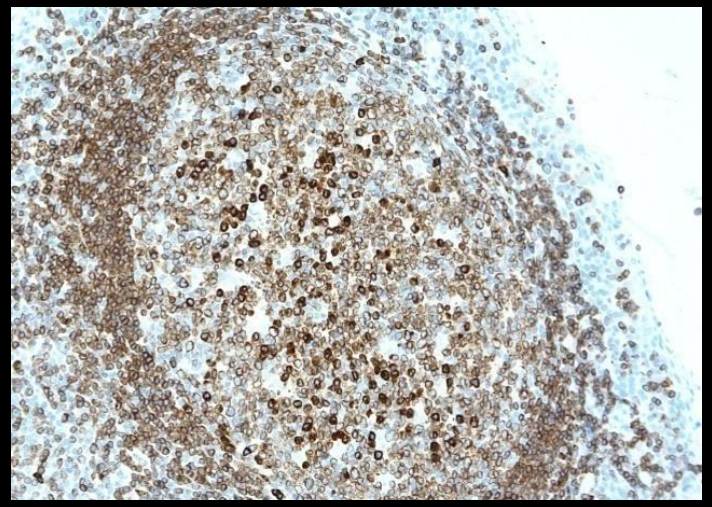
# Technical aspects of immunohistochemistry & pitfalls - Analytical phase

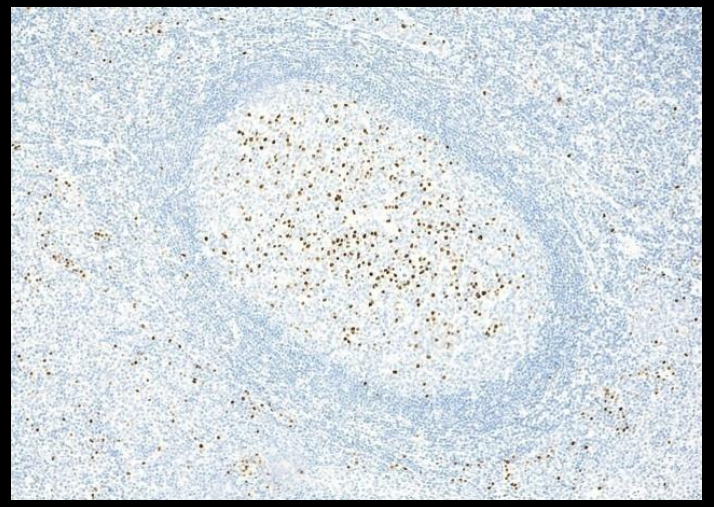
## Efficient HIER - Influence of pH

CD79, JCB117 (1:300)

MUM-1, MUM1p (1:400)

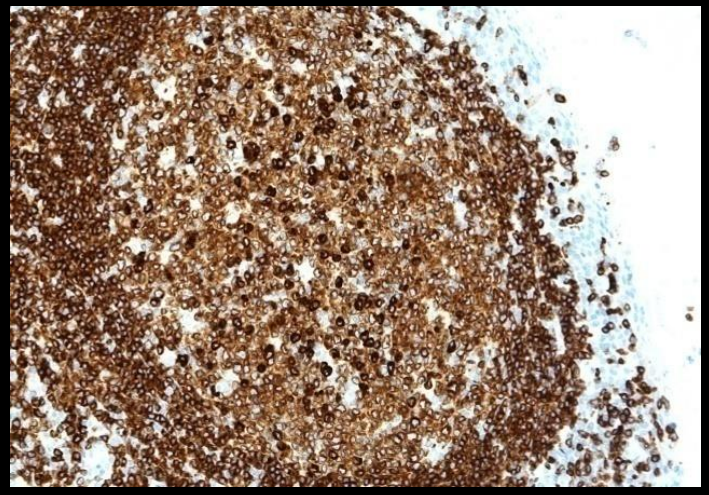
HIER in TRS pH 6.1
(20 min at 97°C)

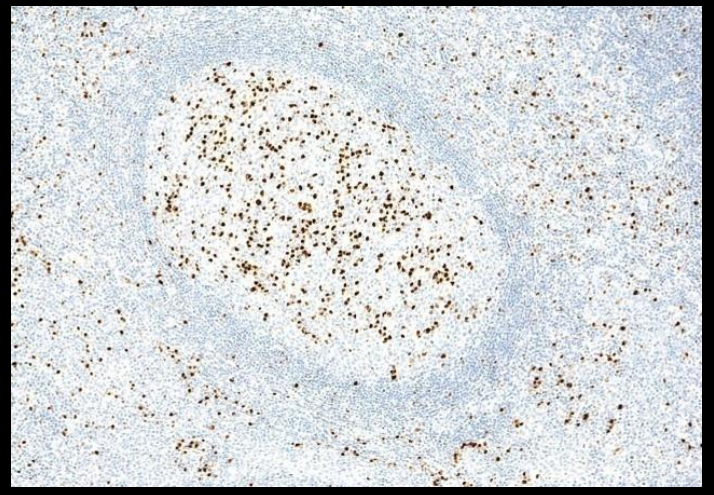




Autostainer: Flex+ as the detection system

HIER in TRS pH 9
(20 min at 97°C)





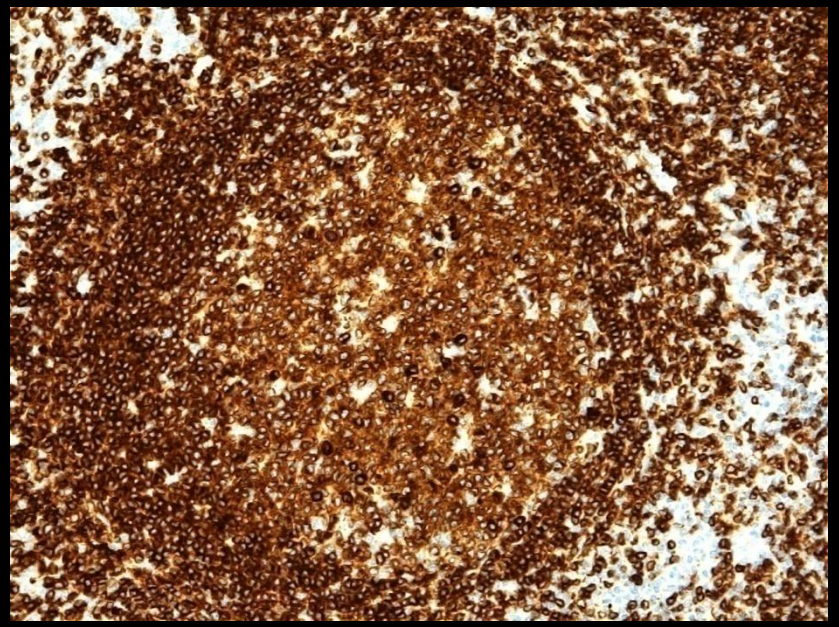
Tonsillar tissue fixed in 10% NBF (48h).

# Technical aspects of immunohistochemistry & pitfalls - Analytical phase

## Efficient HIER - Influence of pH

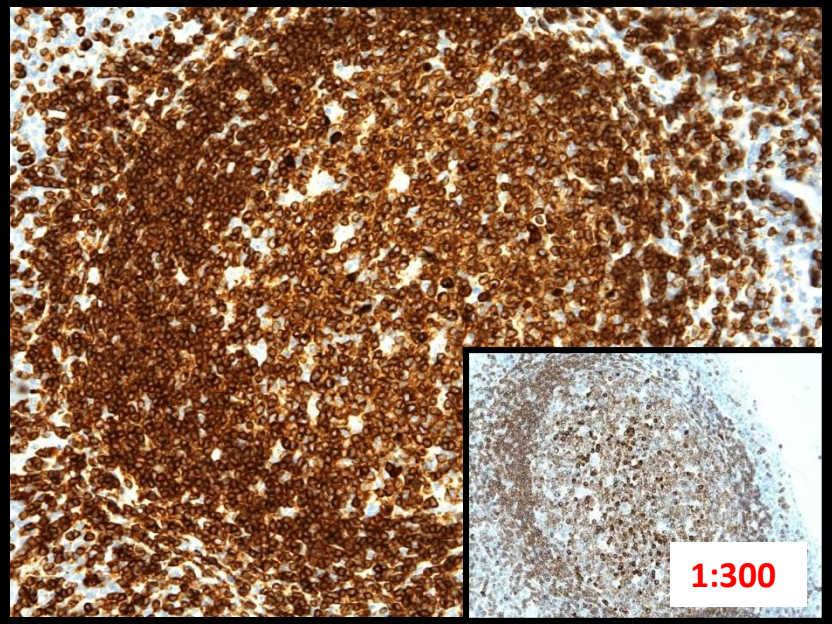
HIER in TRS pH 9

CD79, JCB117 (1:300)



HIER in TRS pH 6.1

CD79, JCB117 (1:50)




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

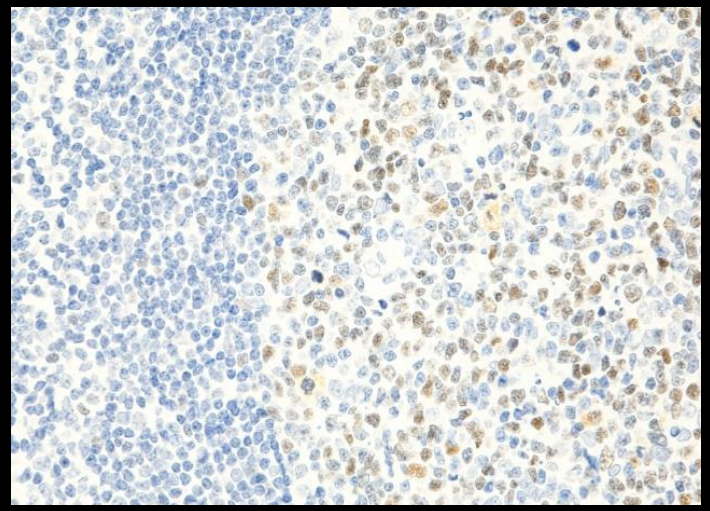
# Technical aspects of immunohistochemistry & pitfalls - Analytical phase

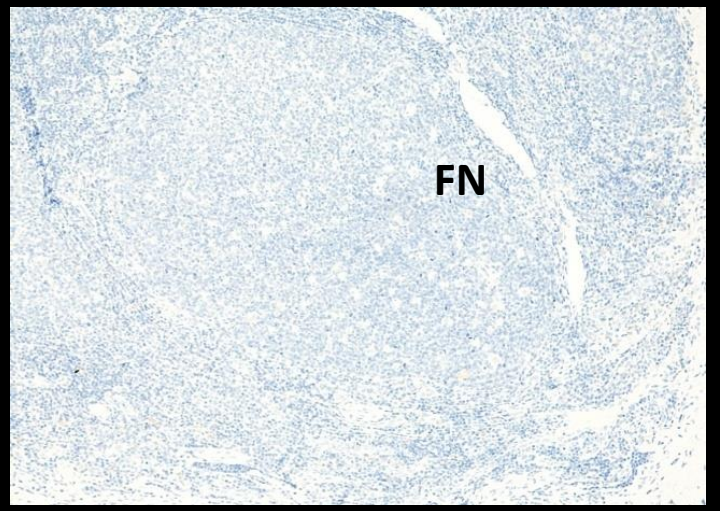
## Efficient HIER - Influence of pH

BCL-6, LN22 (1:100)

CD163, MRQ-26 (1:200)

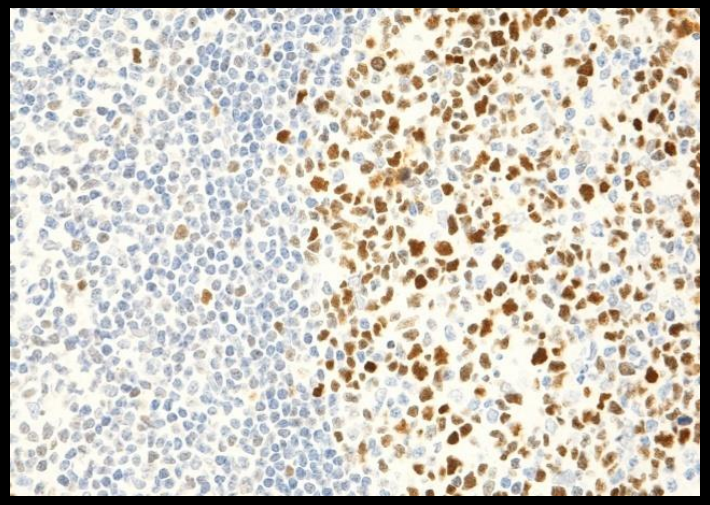
HIER in TRS pH 6.1
(20 min at 97°C)

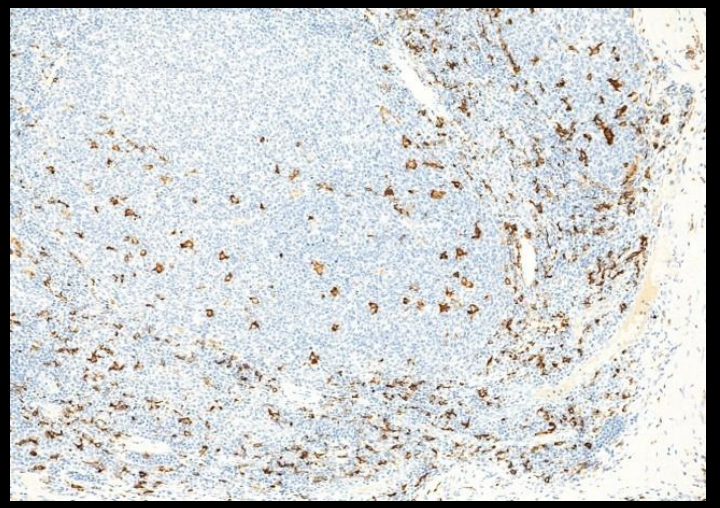





Autostainer: Flex+ as the detection system

HIER in TRS pH 9
(20 min at 97°C)





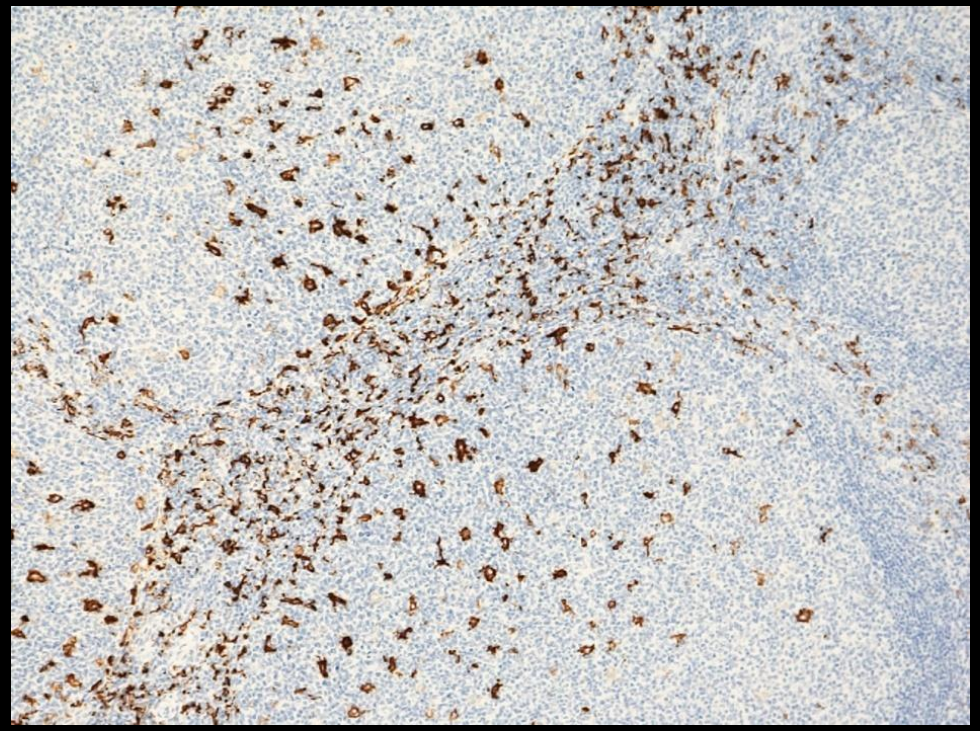
Tonsillar tissue fixed in 10% NBF (48h).

# Technical aspects of immunohistochemistry & pitfalls - Analytical phase

## Efficient HIER - Influence of pH

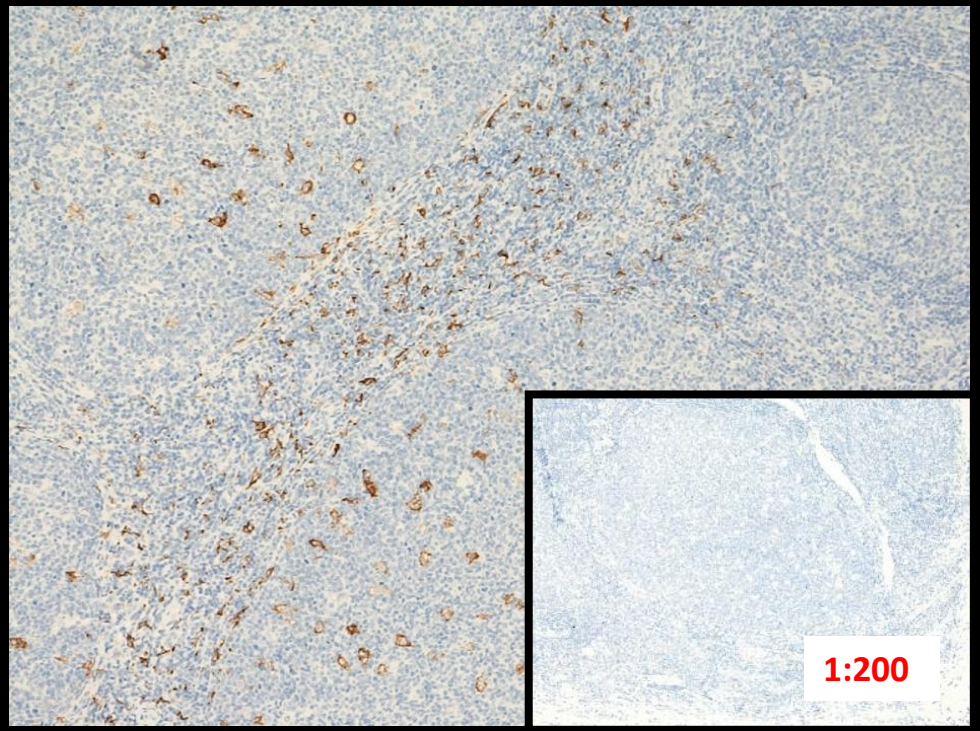
**HIER in TRS pH 9**

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



**HIER in TRS pH 6.1**

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




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

# Technical aspects of immunohistochemistry & pitfalls - Analytical phase

## Efficient HIER - Influence of time and temperature

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

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

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

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

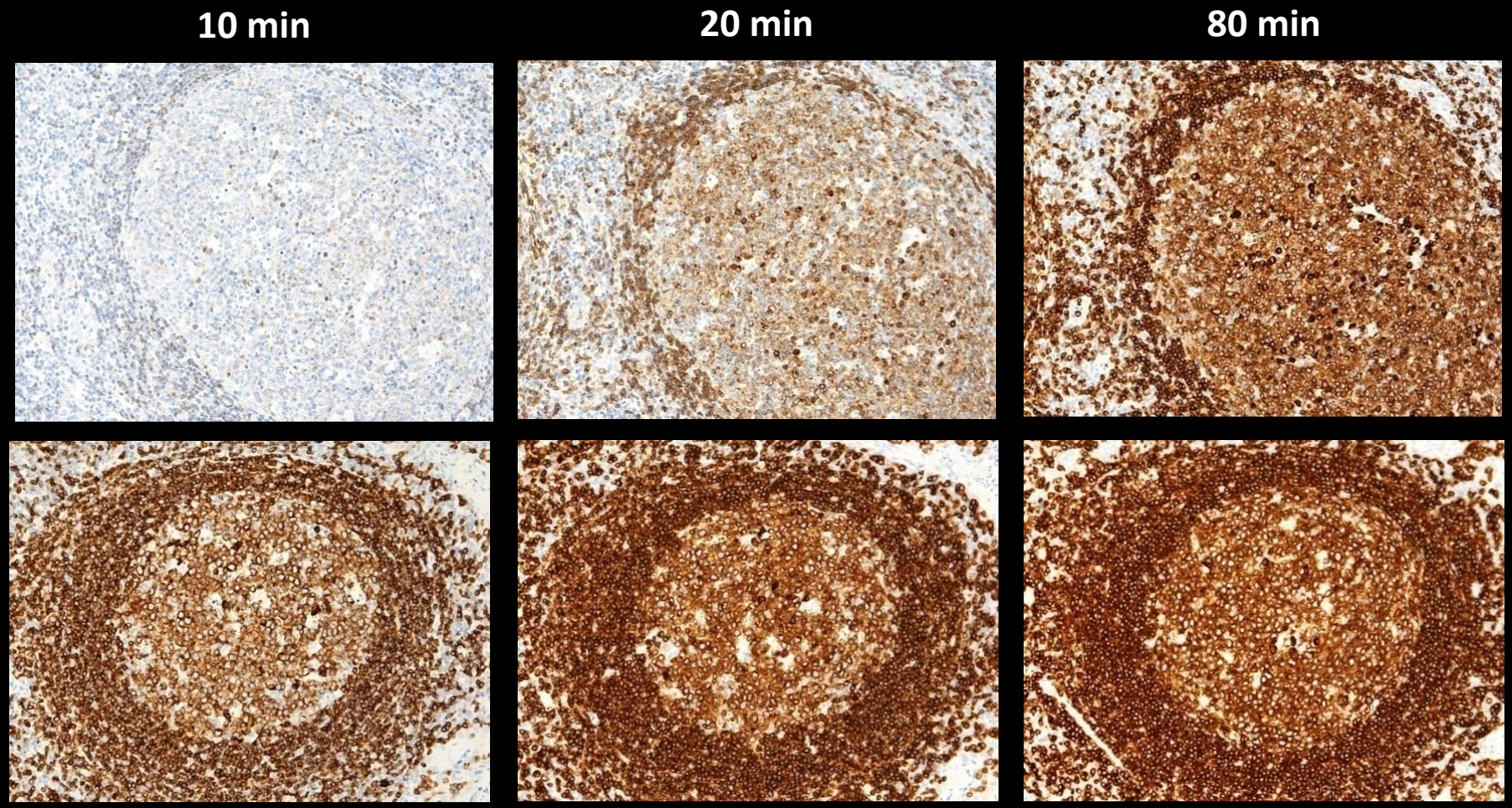
**Optimal staining intensity could be generated by the following heating conditions:**

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

# Technical aspects of immunohistochemistry & pitfalls - Analytical phase

## Efficient HIER - Influence of time and temperature

CD79, JCB117 (1:300)

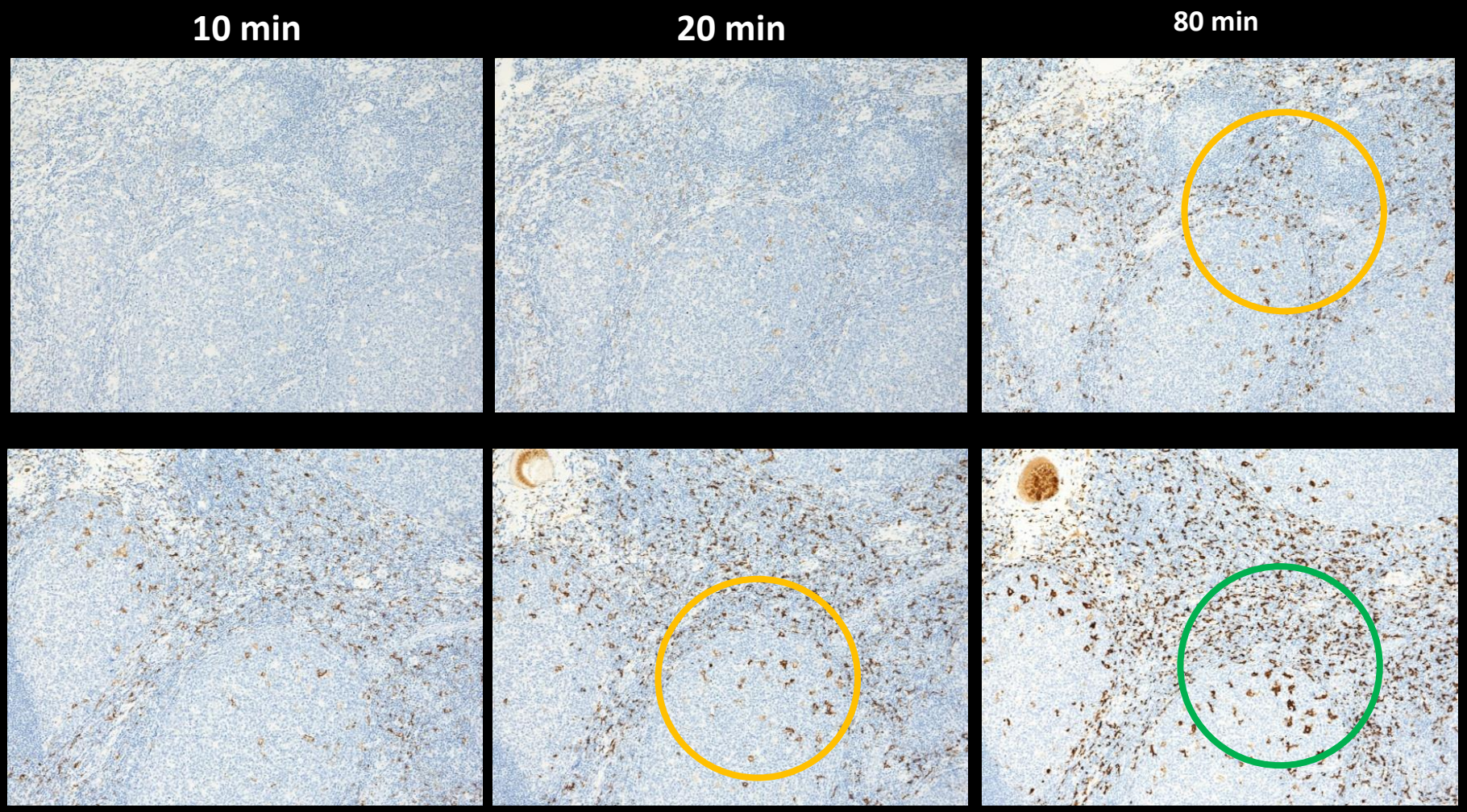



Tonsillar tissue fixed in 10% formalin (48h).

# Technical aspects of immunohistochemistry & pitfalls - Analytical phase

## HIER buffer - Influence of time and temperature

CD163, MRQ-26 (1:200)



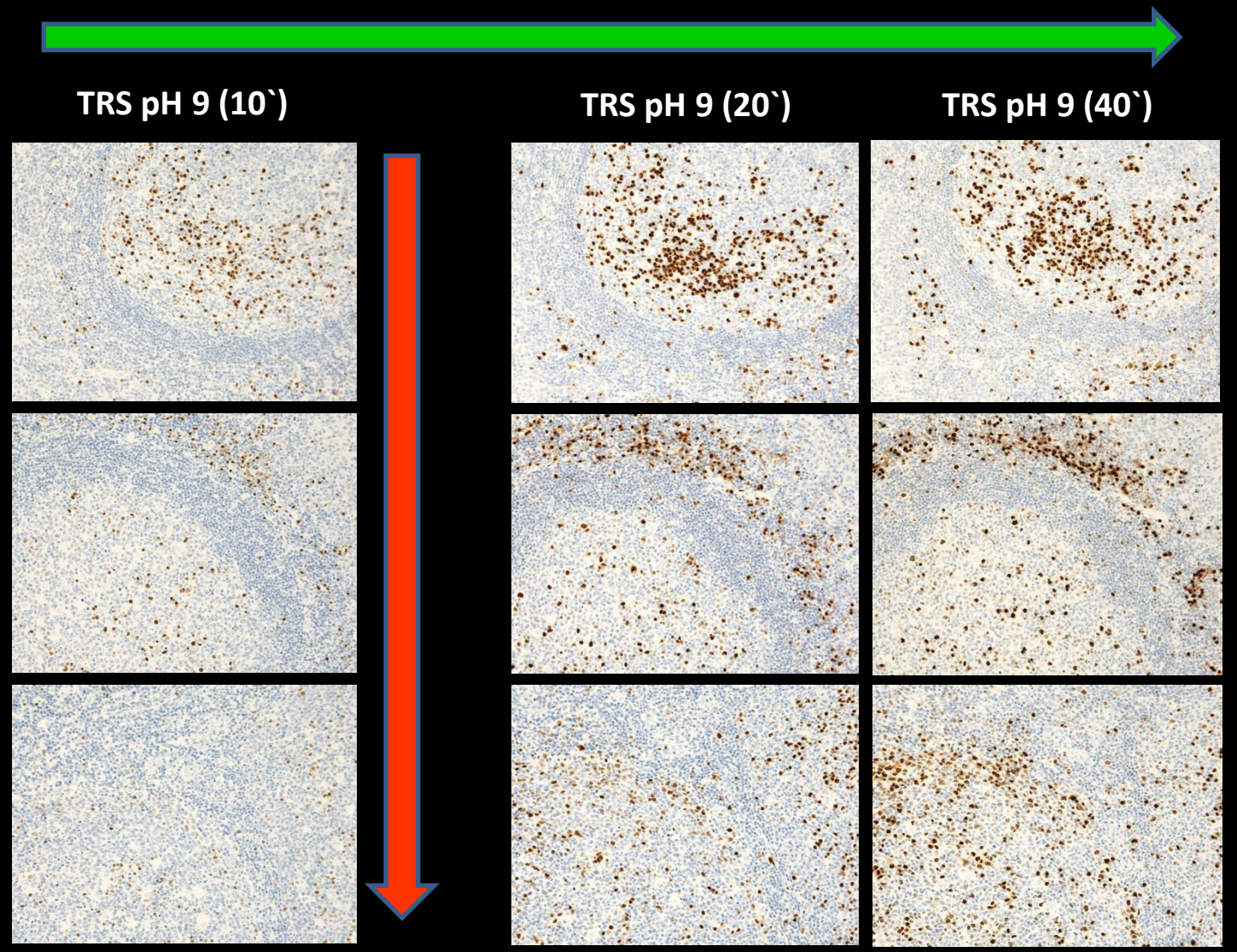

Tonsillar tissue fixed in 10% formalin (48h).

# Technical aspects of immunohistochemistry & pitfalls - Analytical phase

**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

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

# ANTIGEN RETRIEVAL TECHNIQUES IN IMMUNOHISTOCHEMISTRY: COMPARISON OF DIFFERENT METHODS

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

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## Chemical composition of the HIER buffer`s

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

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

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

## Overall best performance:

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



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

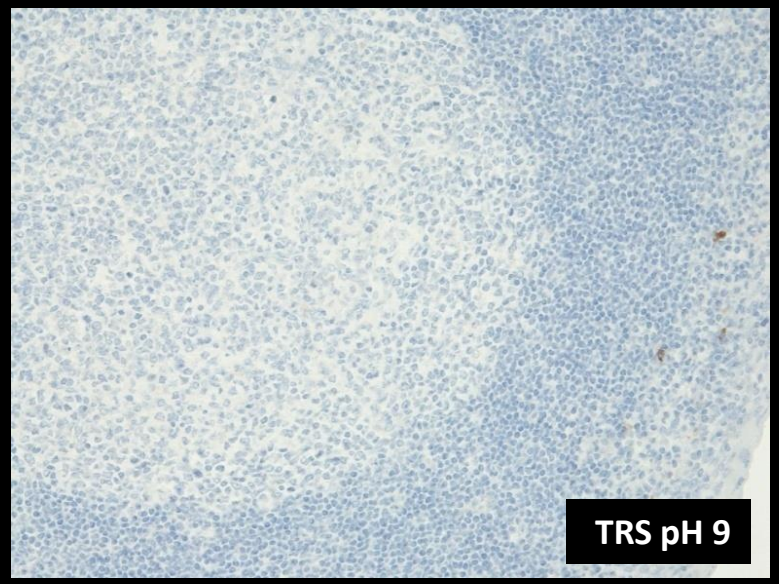
| Clone | Specificity | Source | Dilution | No AgR | PT | HBAR+ citrate | HBAR+ Tris-HCl pH8 | HBAR+ EDTA pH8 |
|---|---|---|---|---|---|---|---|---|
| O10 | CD1a | Immunotech | 1:40 | – | – | +– – – | ++– – | ++++ |
| Poly | CD3 | DAKO | 1:300 | – | ++– – | +– – – | ++– – | ++++ |
| C8/144B | CD8 |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

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

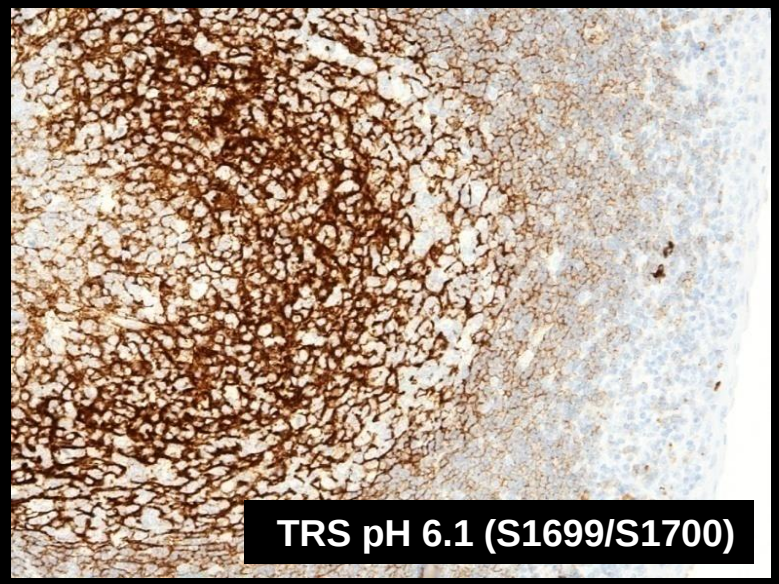
# Technical aspects of immunohistochemistry & pitfalls - Analytical phase

## Modified low pH buffers

CD21, clone 1F8




CD21, clone 1F8




CD21, clone 2G9


HIER time 20`/ Flex+

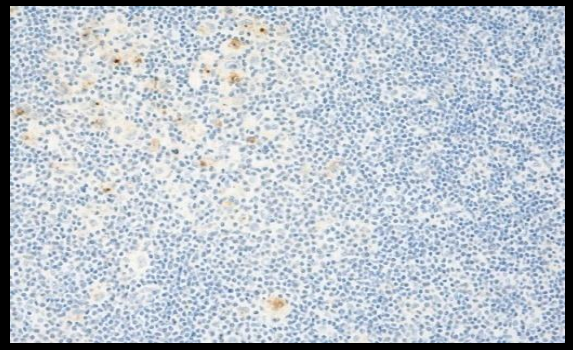
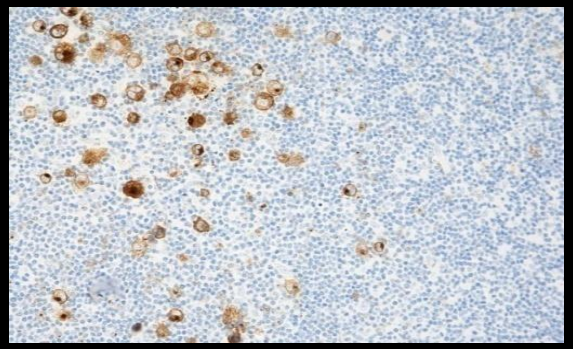
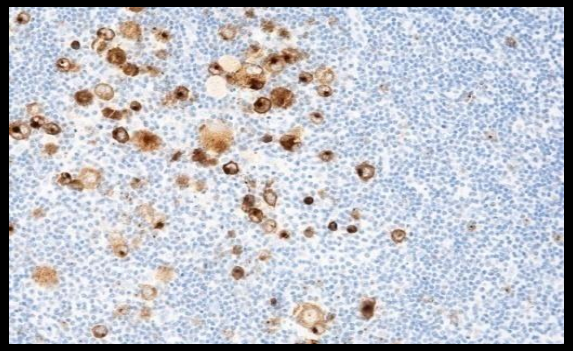
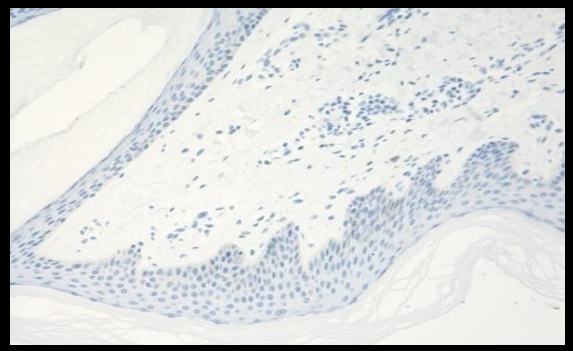
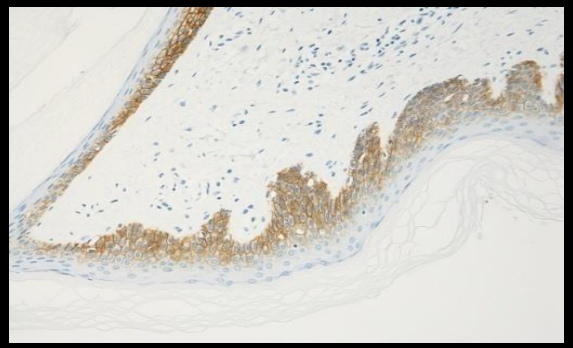
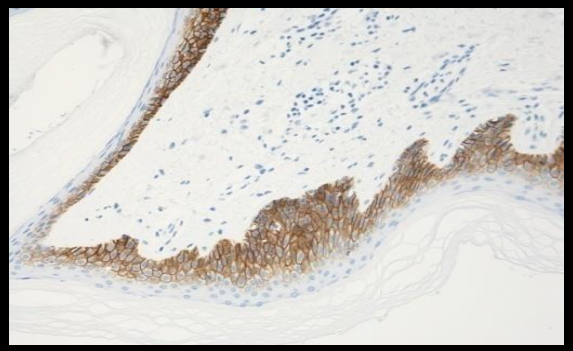
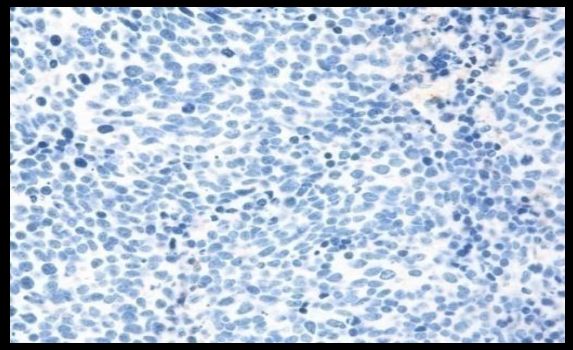
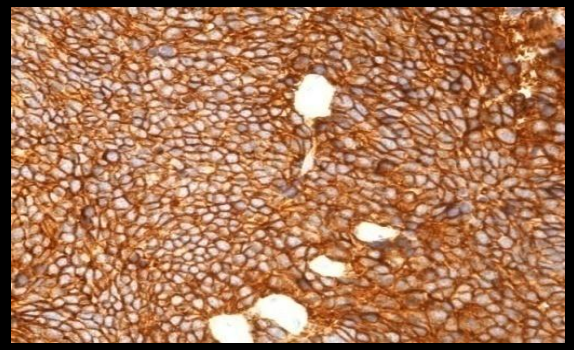
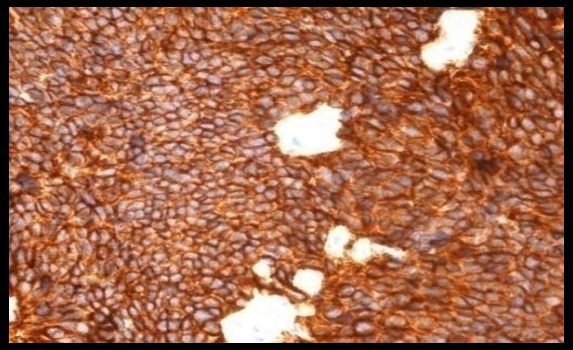
**Markers requiring the TRS Low pH 6.1 (Dako, S1699/S1700) or Diva Decloaker pH 6.2 (Biocare, DV2004) :**

**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**

# Technical aspects of immunohistochemistry & pitfalls - Analytical phase

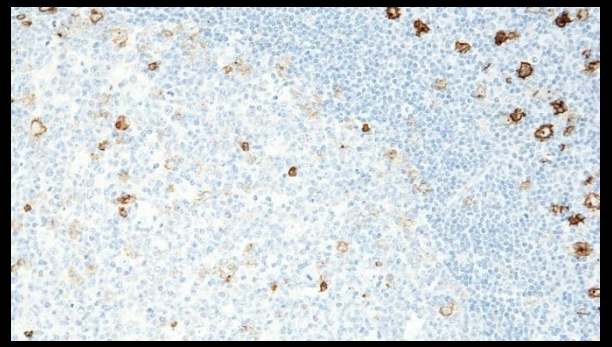
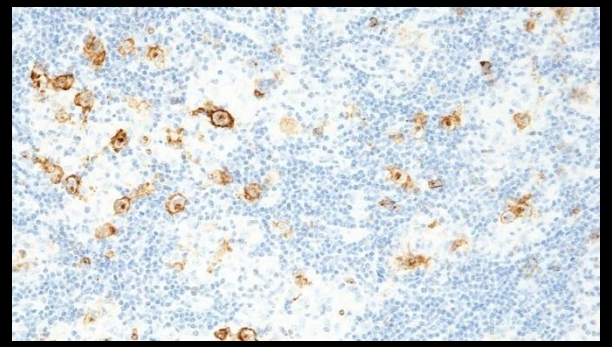
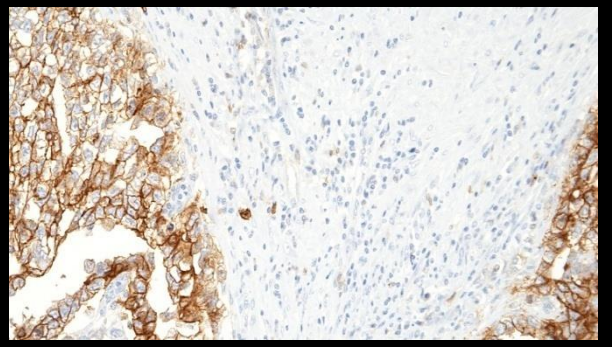
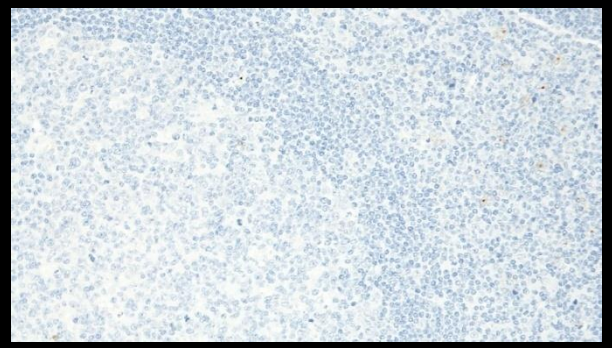
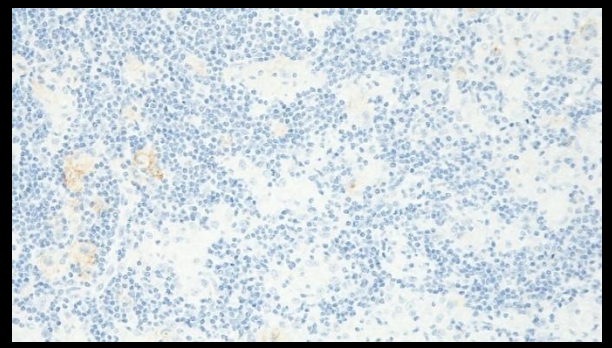
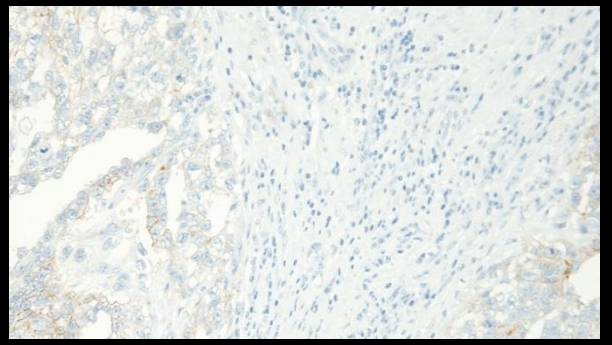
**Modified low pH buffers**

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

# Technical aspects of immunohistochemistry & pitfalls - Analytical phase

## Modified low pH buffers

### CD30 clone ConD6/B5

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

# Technical aspects of immunohistochemistry & pitfalls - Analytical phase

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

**Antigen retrieval procedures for formalin fixed tissue:**

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

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

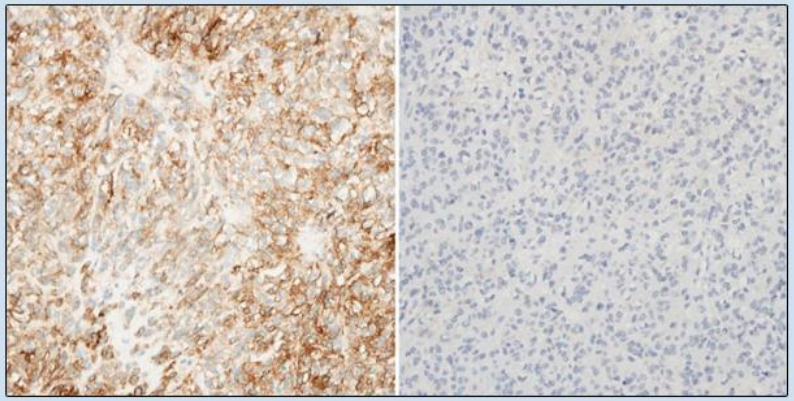
NordiQC Info Modules Assessments Protocols Controls Events Login

**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**

# Technical aspects of immunohistochemistry & pitfalls - Analytical phase

## ”Optimal” enzymatic digestion depends on:

Enzyme type

Concentration

Time

Temperature

Fixation type & time

Tissue type

**Most common Enzymes**

Proteinase K
Pronase XIV
Pronase XXIV
Pepsin
Trypsin

**Difficult to control and to standardizes within routine LAB**

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

**Markers requiring enzymatic pretreatment :**

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

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

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

# Technical aspects of immunohistochemistry & pitfalls - Analytical phase

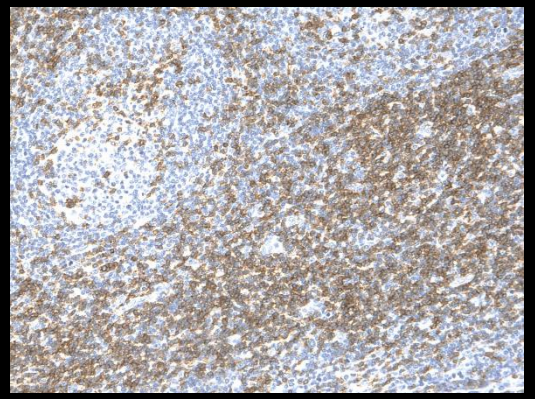
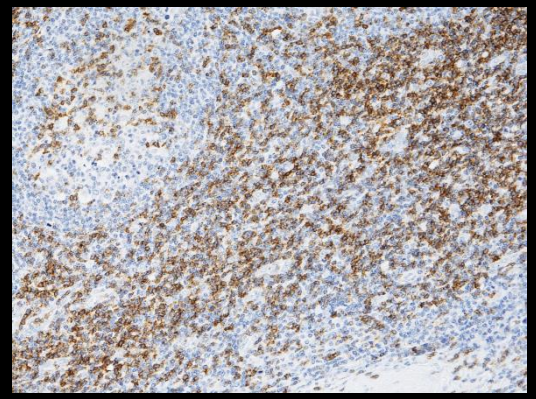
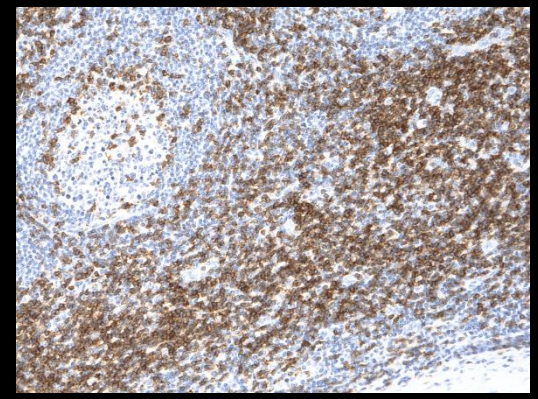
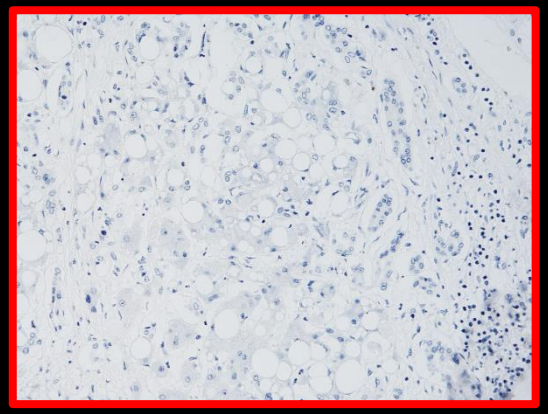
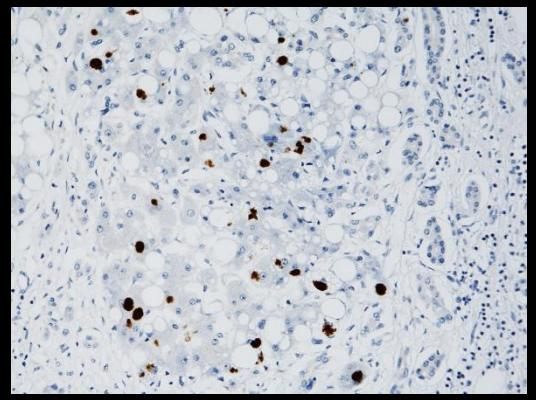
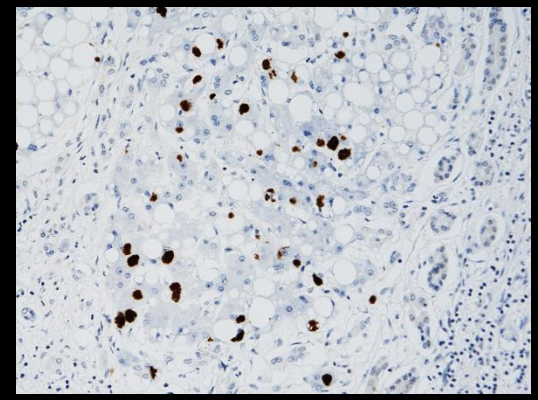
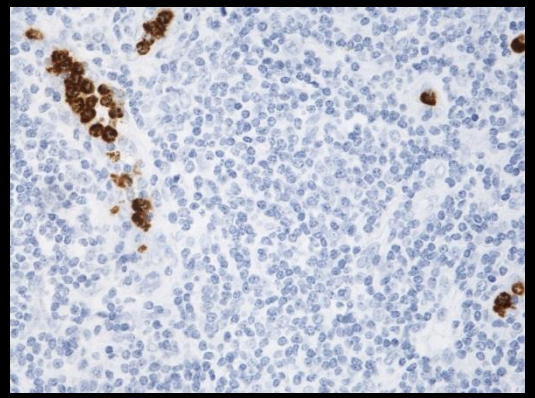
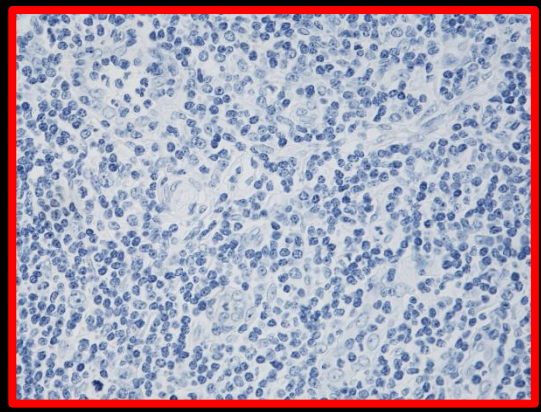
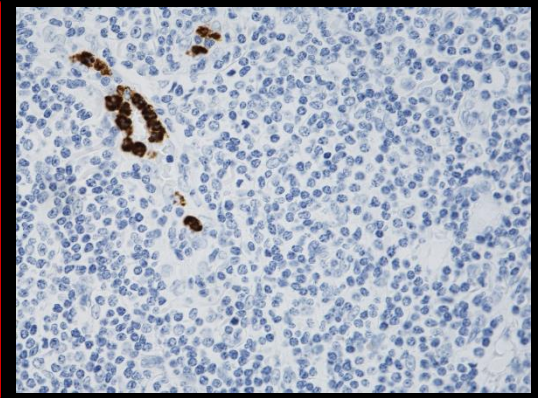
| Enzyme | Typical working conc. | Activation Temperature | Typical Incubation time | Cleavage nature |
|---|---|---|---|---|
| Proteinase K | 0.1%, pH 8.0 | 25-37 °C | 5-10 min. | Broad, all amino acids |
| Trypsin | 0.1-0.25%, pH 7.6 | 37 °C | 10 min. | Arginin / Lysin |
| Pepsin | 0.2-0.4%, pH 2.0 | 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 |

**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) ……..

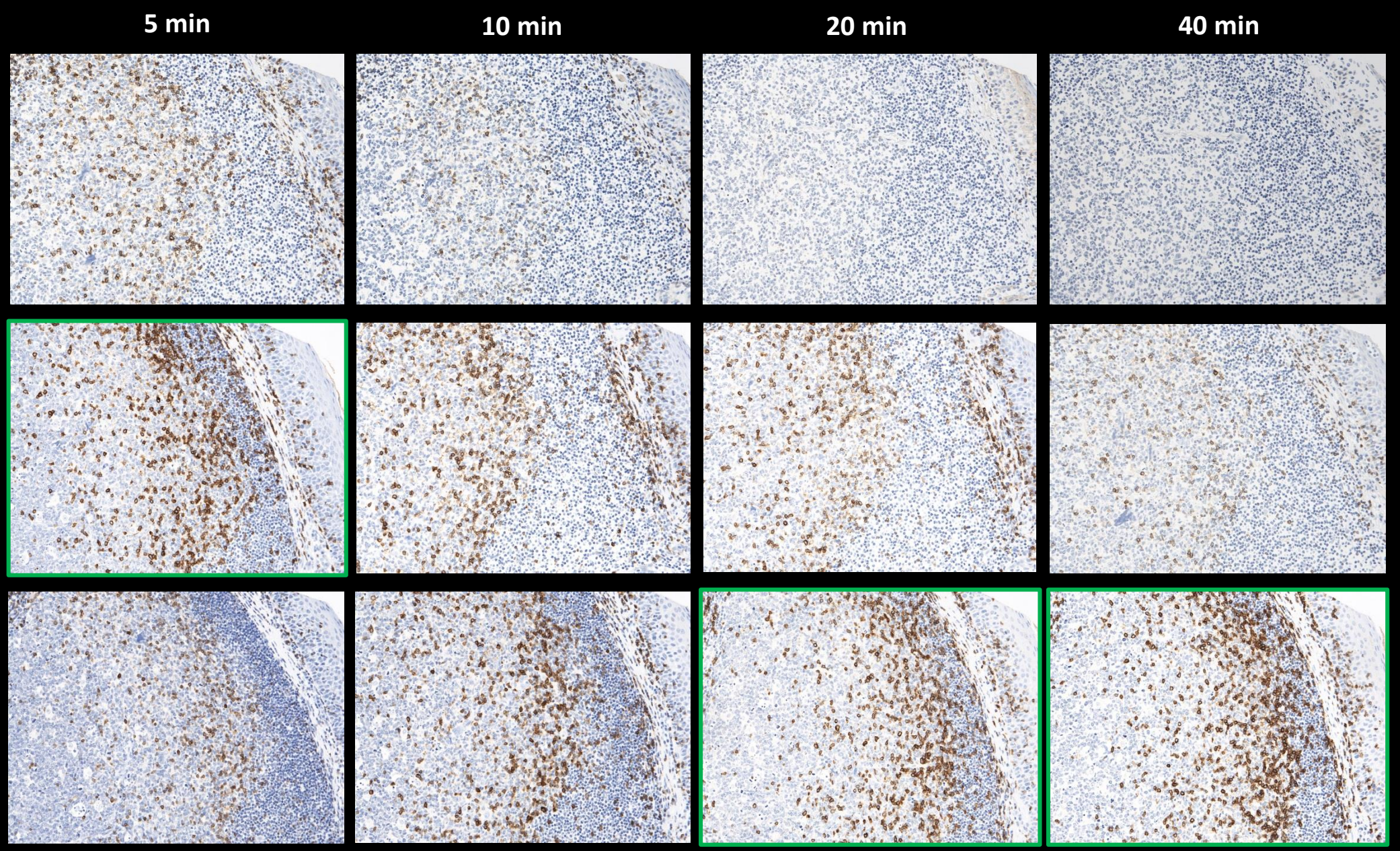
# Choice of proteolytic enzyme

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

# Proteolytic enzyme & digestion time ?

Tonsil NBF 48h

Digestion temp. 32°C



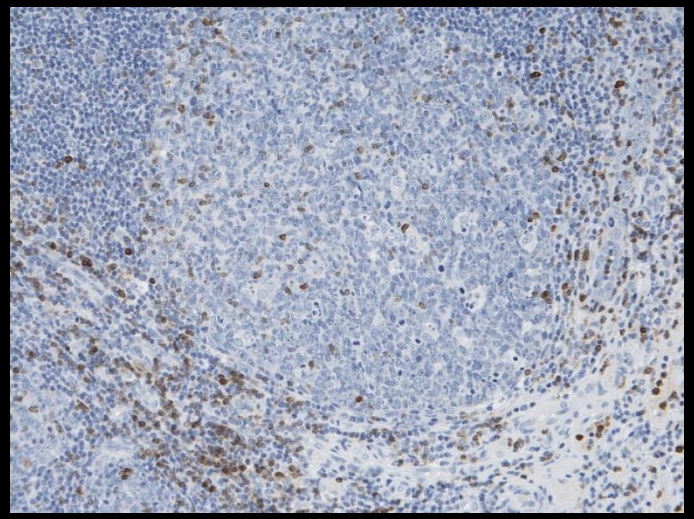

TCR β clone 8A3 (1:200 RR) / Flex+ (Omnis)

# Technical aspects of immunohistochemistry & pitfalls - Analytical phase

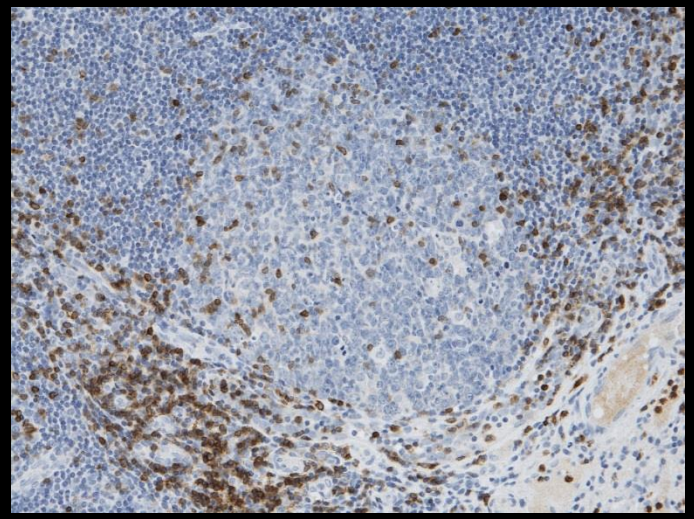
## Proteolytic enzyme & digestion temperature ?

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

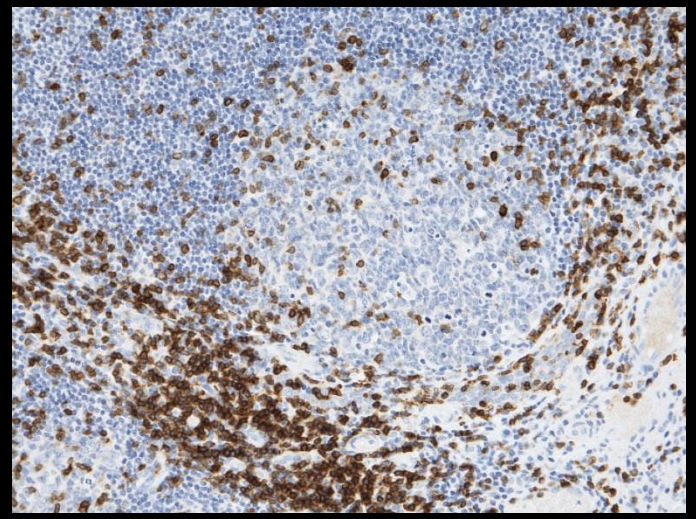
Trypsin Digestion temp. 4°C (10 `)



Trypsin Digestion temp. 24°C (10`)



Trypsin Digestion temp. 37°C (10`)



Increased intensity of TCR β positive T-cells

# Technical aspects of immunohistochemistry & pitfalls - Analytical phase

**Enzymatic digestion (Influence of fixation time)**

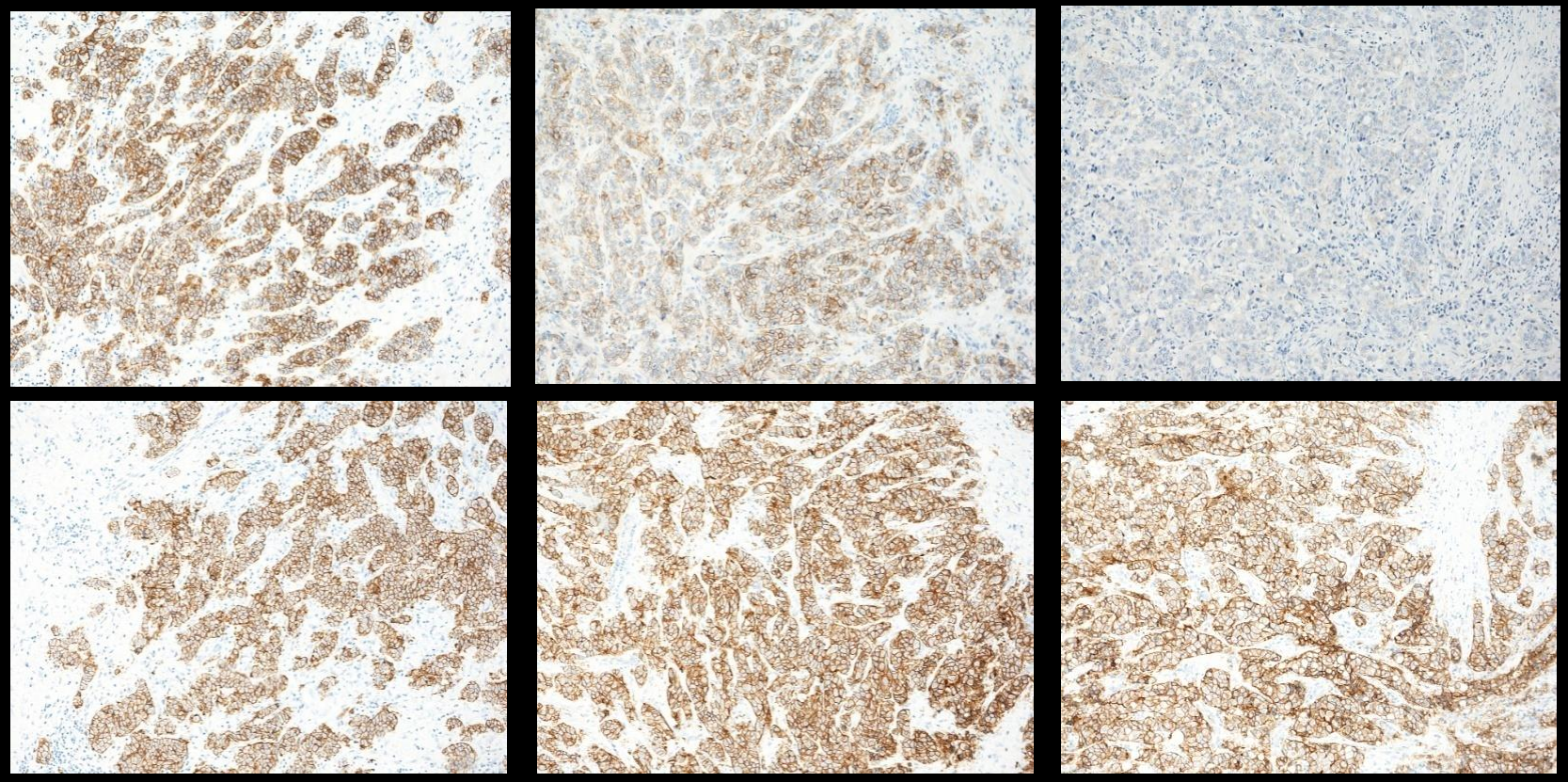
**EP-CAM, clone MOC-31, dilution 1:20**

**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
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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

[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**

# Technical aspects of immunohistochemistry & pitfalls - Analytical phase

**Proteolytic pretreatment (excessive retrieval procedures)**

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

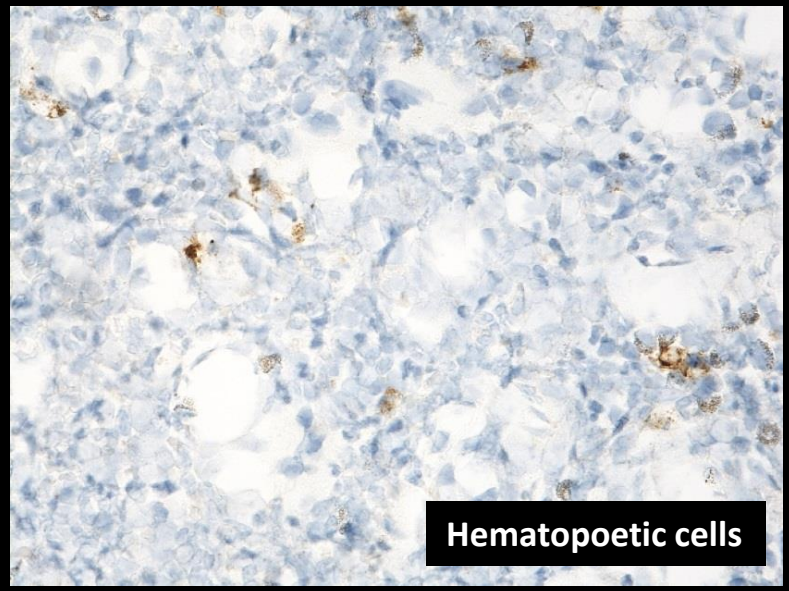
**HIER (excessive retrieval procedures)**

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

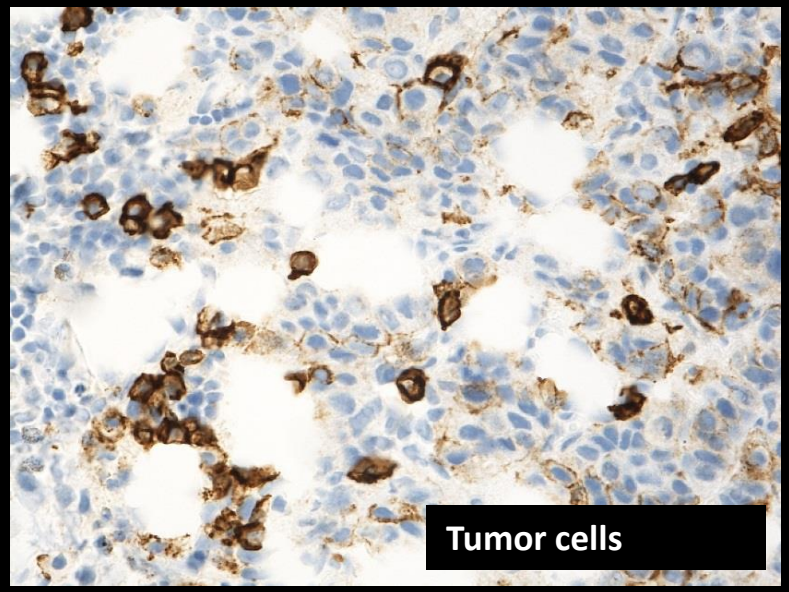
**HIER applying standard procedures (excessive retrieval procedures)**

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

Bone marrow Trephine: Breast Carcinoma



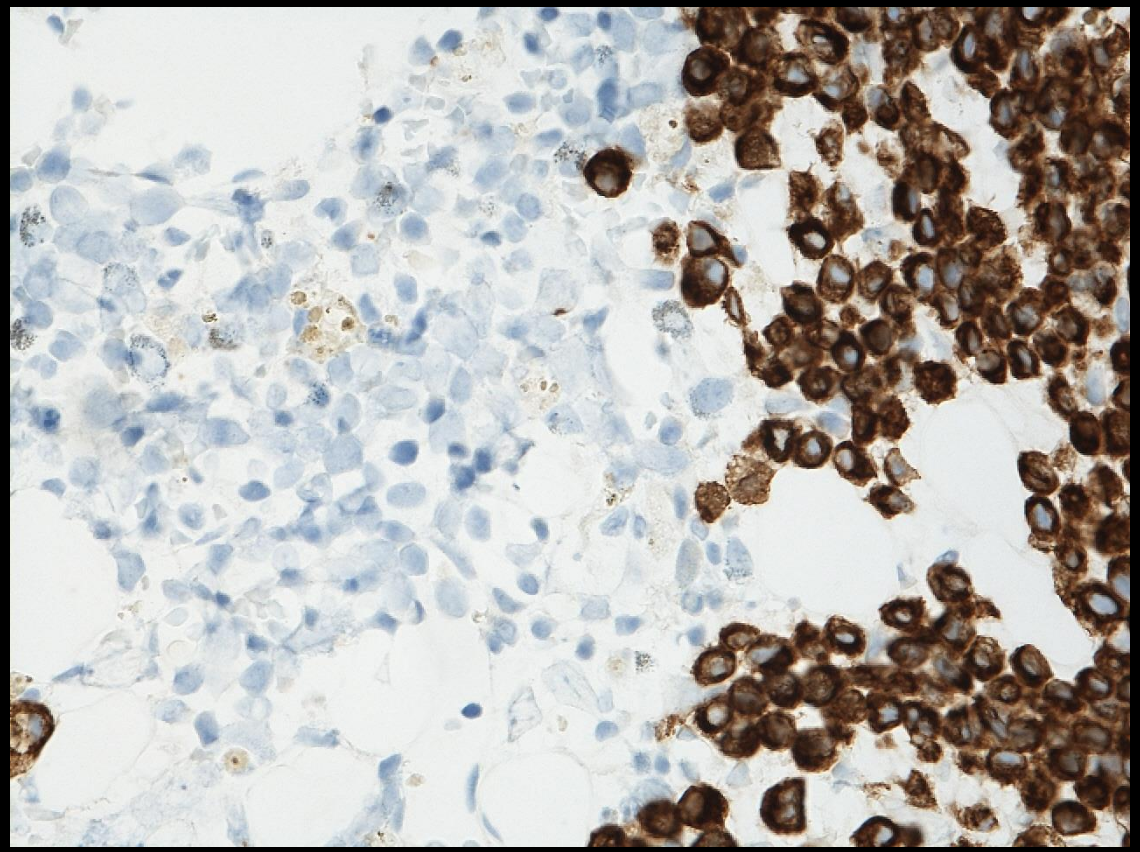
Hematopoetic cells



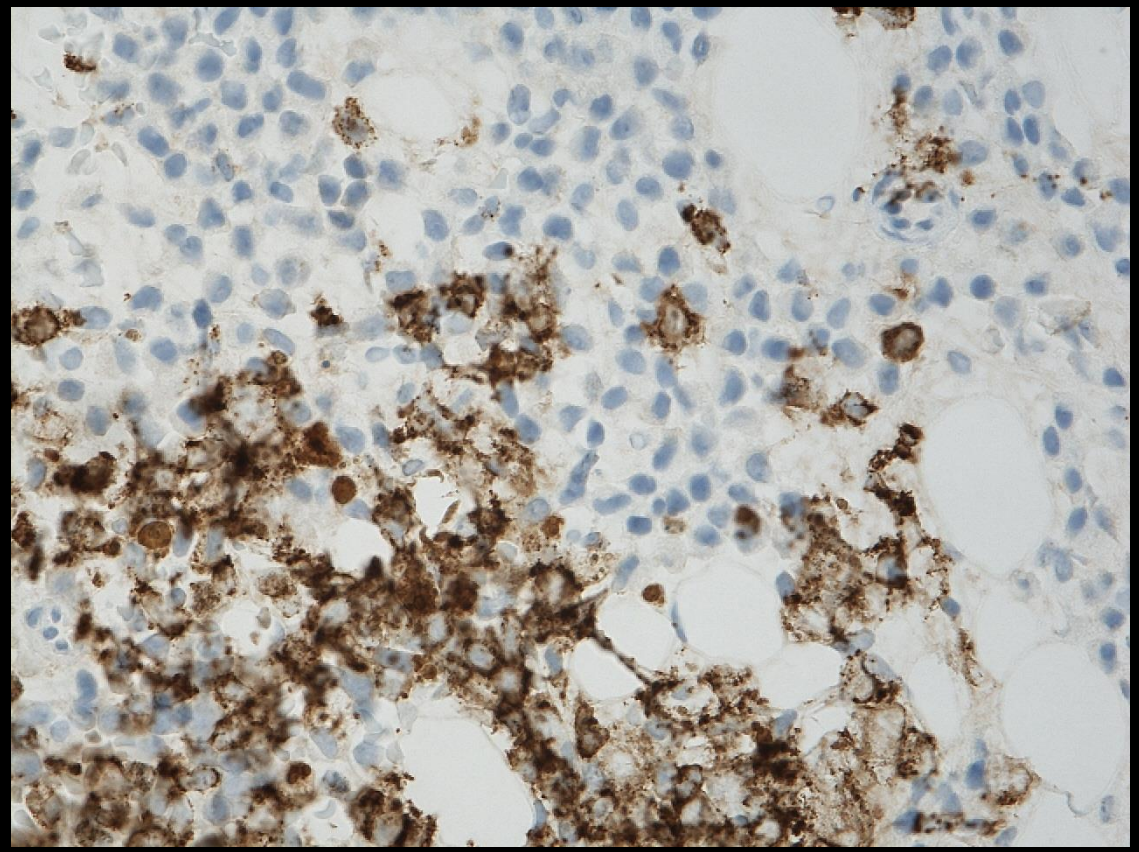
Tumor cells

Morphology ?

## CKPAN



## MPO

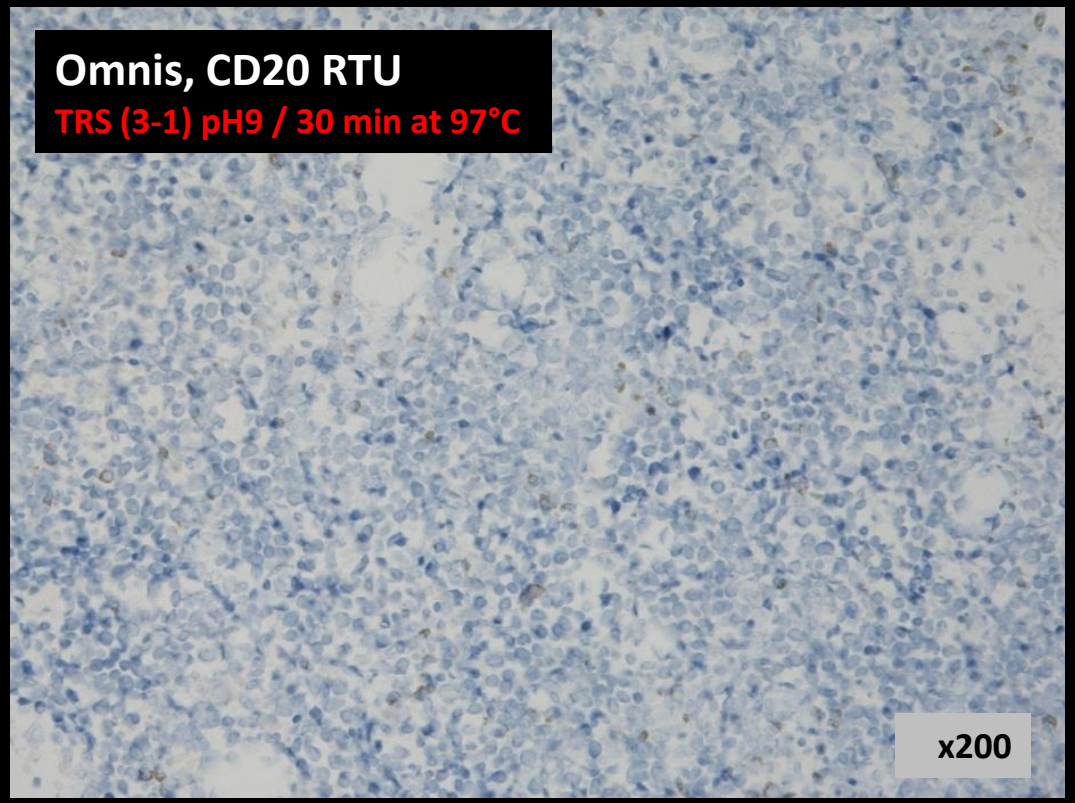


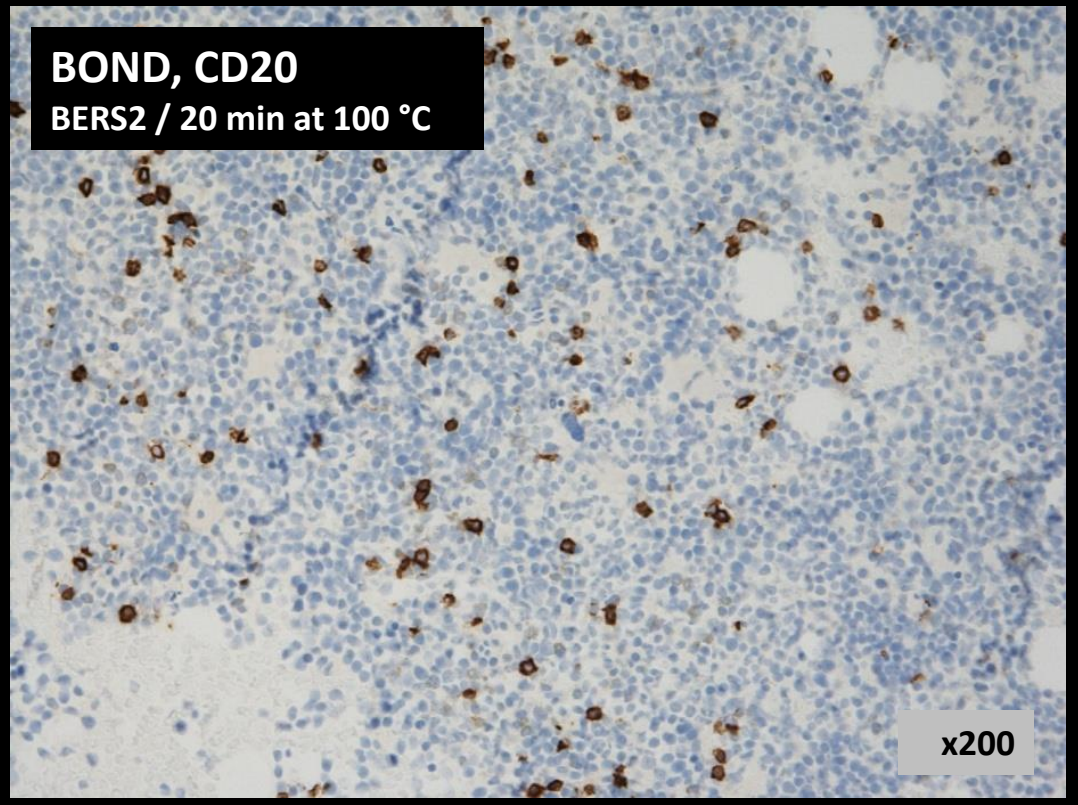
Bone marrow Trephine: Breast Carcinoma

# Technical aspects of immunohistochemistry & pitfalls - Analytical phase

## CD20 clone L26

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




**HIER settings: Recommendations given by the manufacturer`s**

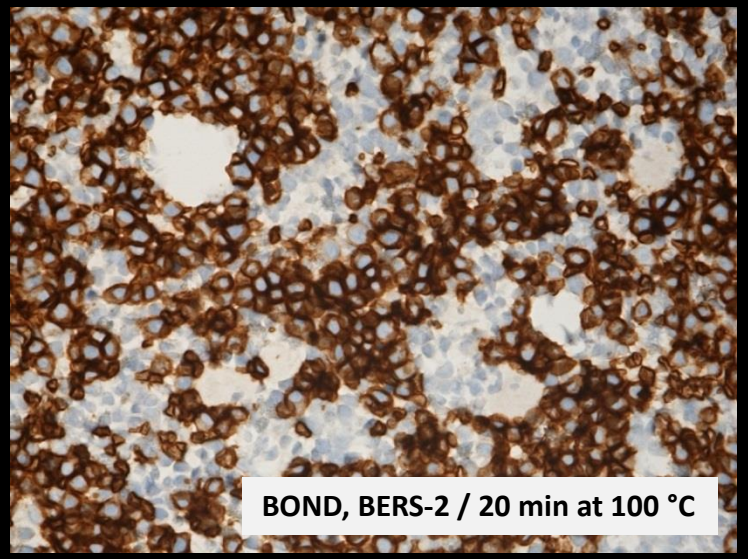
# Technical aspects of immunohistochemistry & pitfalls - Analytical phase

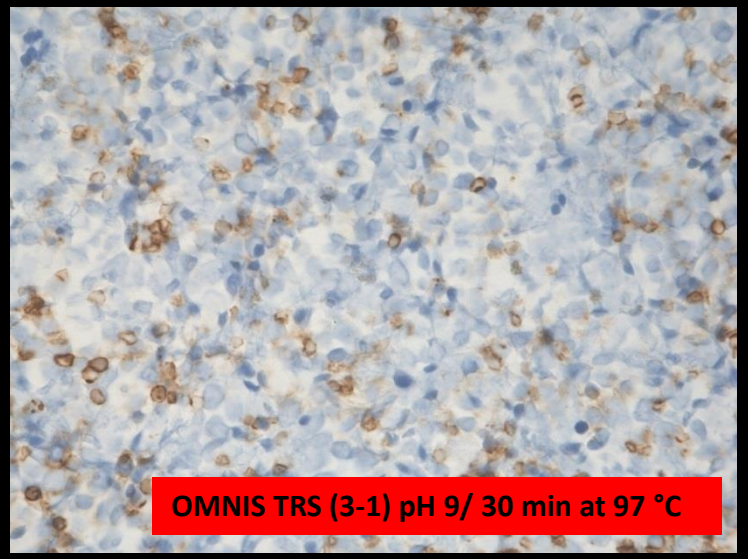
**Bone marrow clot (NBF 24h)**

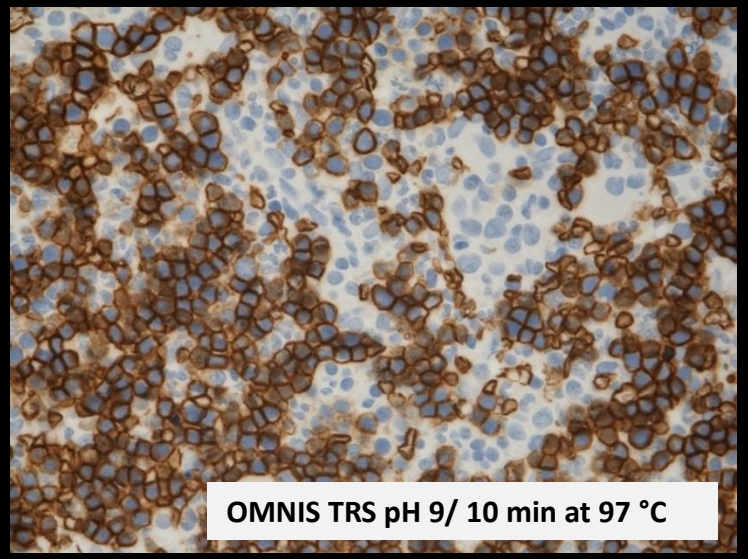
**Morphology ?**

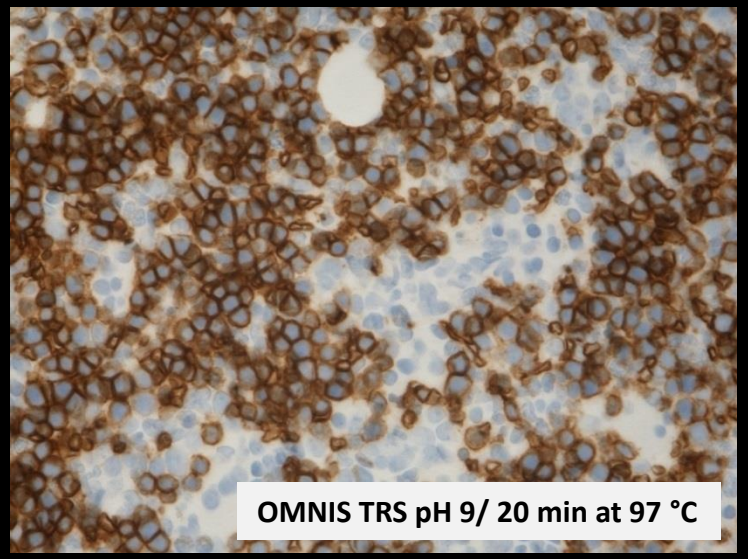
**Glycophorin A clone JC159 (1:500)**

**Flex+**



# Technical aspects of immunohistochemistry & pitfalls - Analytical phase

Bone Marrow cloth (NBF 24 h)

Morphology ?

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

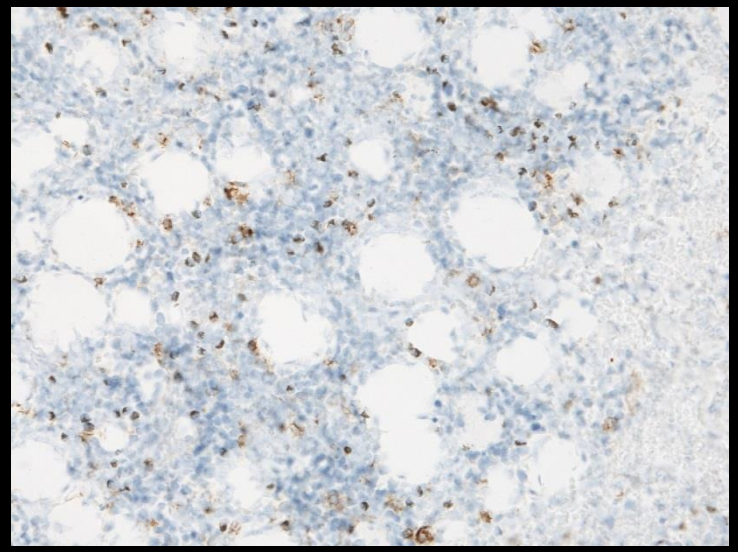
Agilent/Dako (Omnis, Flex+)

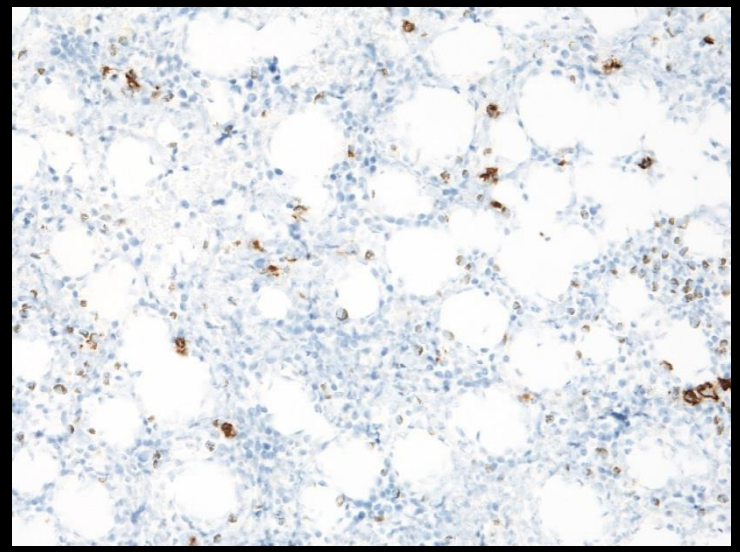
HIER buffer H, 24` at 97C

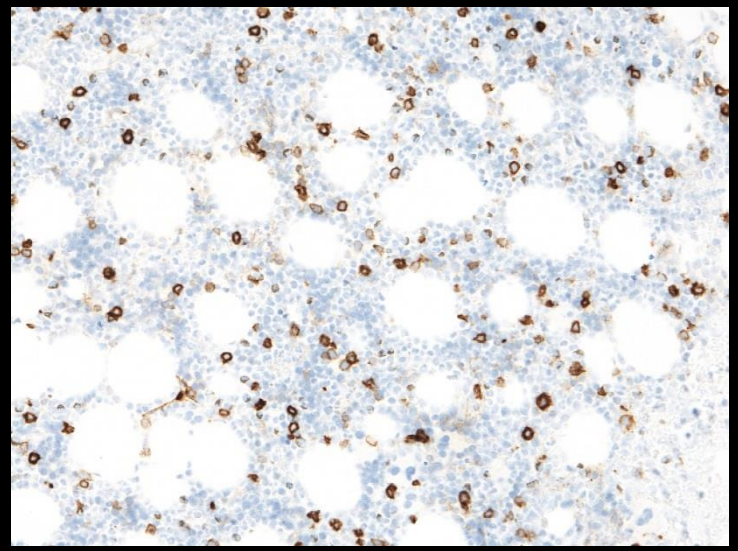
Thermo S./ LabVision (Omnis, Flex+)

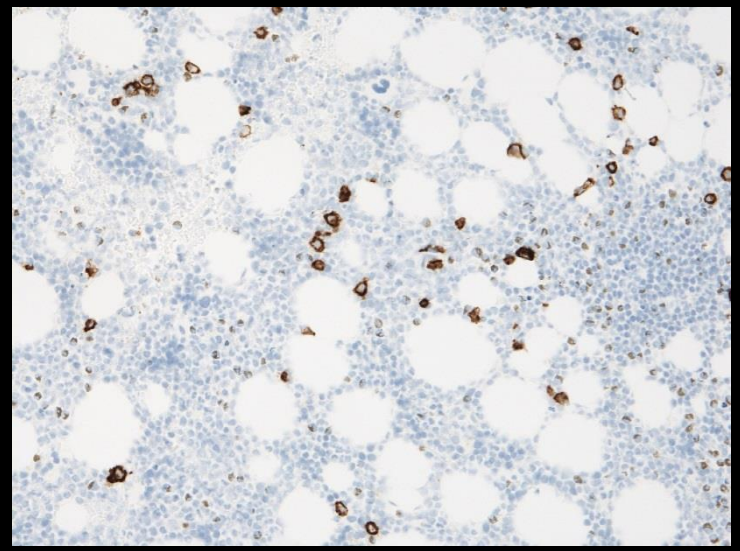
CD117, EP10 (1:25 RR)

CD138, B-A38 (1:1000)









# BoneSTATION
# Advanced system for fixation and decalcification of bone tissues

A new and innovative workstation for complete control of the all important pre-analytical step.
Provides accurate, reliable diagnostic results for morphology and molecular studies.
The BoneSTATION consists of two work platforms complete with glass modules for fixation/ decalcification.

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

Two user-friendly work platforms, for easy handling of bone specimens, complete the unit. The BoneSTATION station can be used with ANY type of fixative/decalcifier, even with strong mineral acids (HCl - HNO3), as all contact surfaces are either glass or PTFE polymer. The magnetic stirrer assures homogeneity of temperature throughout the solution.
The PTFE cover condenses vapors, generating a reflux of the reagent for consistent and safe protocols at constant pH.
A touch-screen terminal allows the user to optimize, standardize and fully document all the processes.


**At arrival in our department - New fixative is added and post fixation for 24h of the bone marrow material has improved morphology.**

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

# Pause

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
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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% insuff.**

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

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

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

## Problems related to:

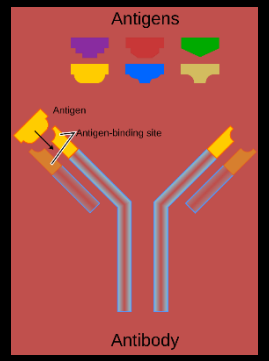
- ❑ **The choice and use of the primary antibody (Concentrate or RTU)**
  - **Inappropriate primary antibody**
    - **Provide low sensitivity/specificity**
  - **Appropriate primary antibody**
    - **Inapp. titre (too low or too high concentration)**
  - **Stainer platform dependent antibodies**
    - **Provide low sensitivity / specificity**

**False positive or false negative results**

# Technical aspects of immunohistochemistry & pitfalls - 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**



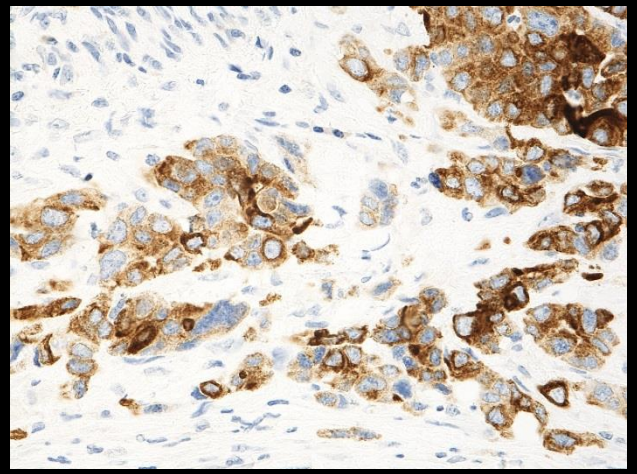
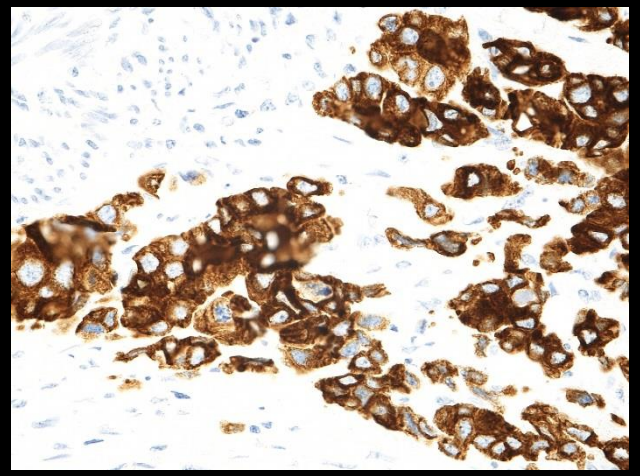
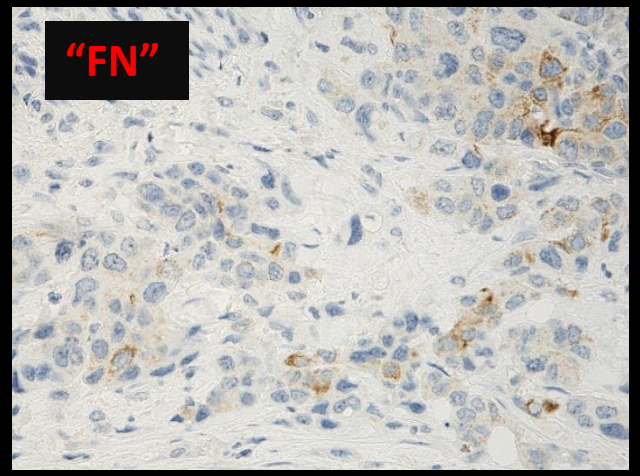
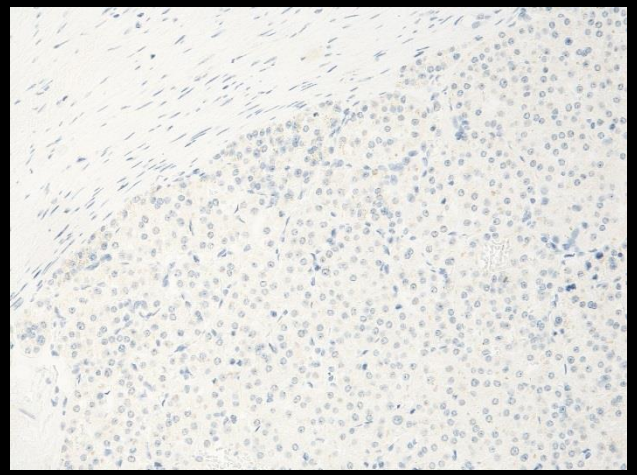
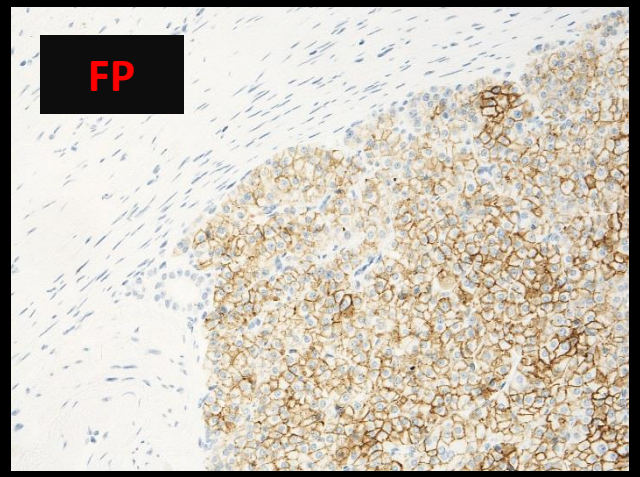
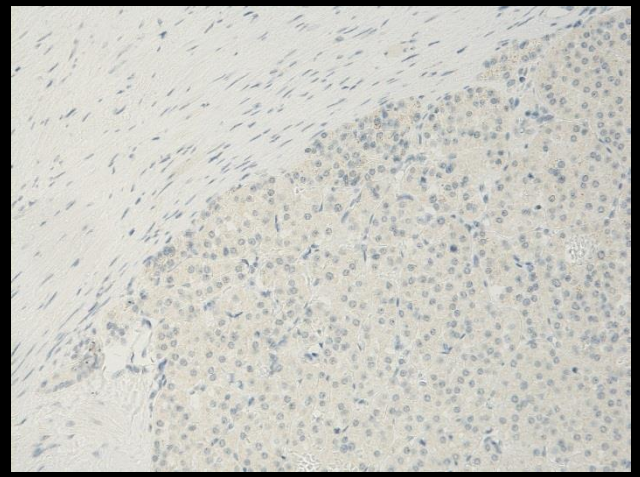

**Incubation time**
**Incubation temperature**
**Sensitive to endogenous peroxidase blocking**

**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**

# Technical aspects of immunohistochemistry & pitfalls - Analytical phase

**Problem: Sensitivity & Specificity**

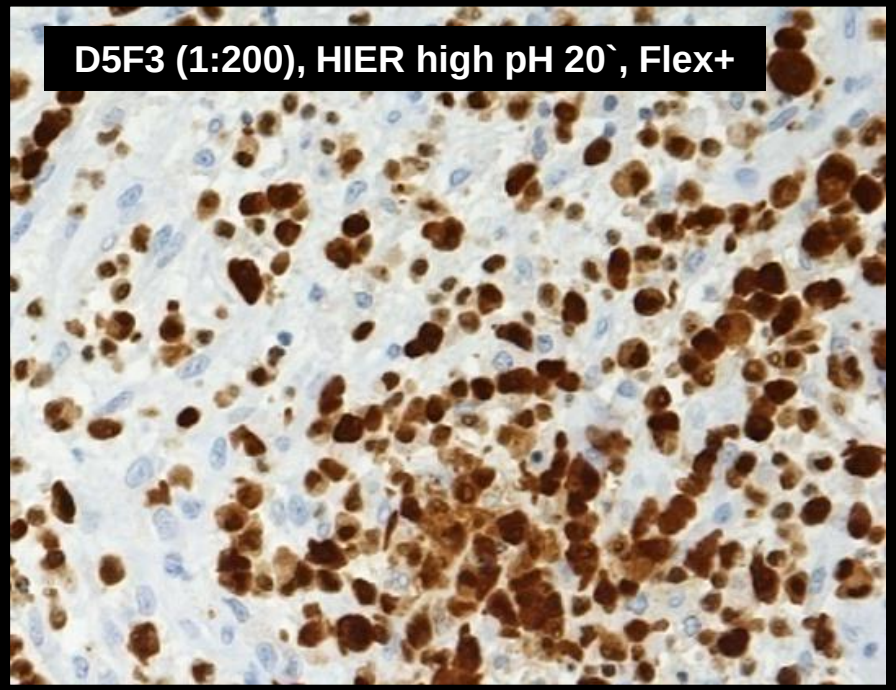
| | CK20 clone Ks20.8 (RTU) | CK20 clone EP23 (1:100) | CK20 clone BS101 (1:100) |
|---|---|---|---|
| Bladder cancer (Urothelial carcinoma) |  |  |   |
| Kidney cancer (Clear Cell Carcinoma) |  |   |  |

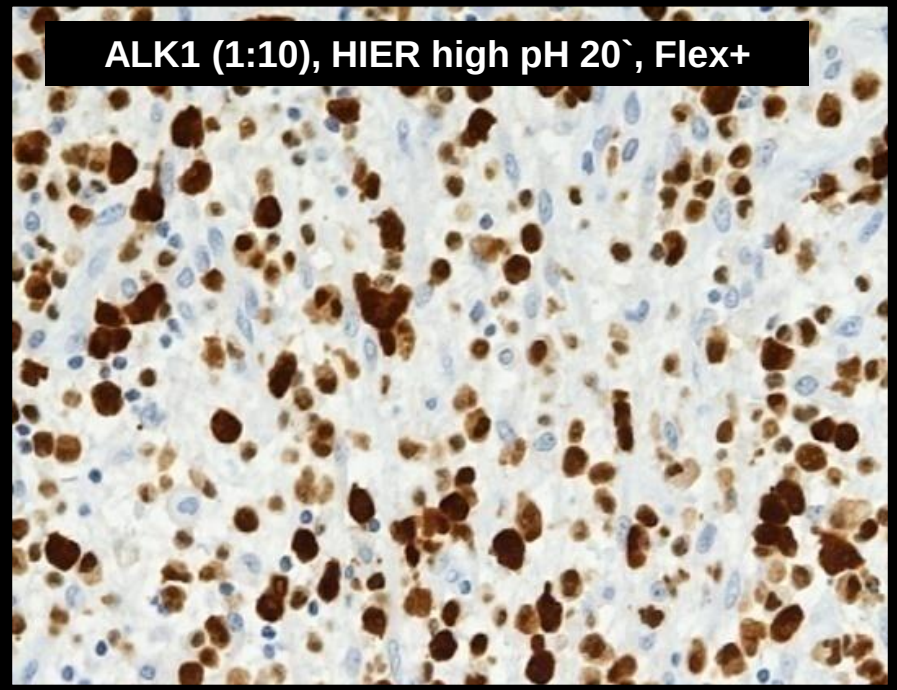
Omnis: HIER/HIGH pH 24`, Flex+ (10+20`)

# Technical aspects of immunohistochemistry & pitfalls - Analytical phase

**Anaplastic lymphoma kinase (ALK)**

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

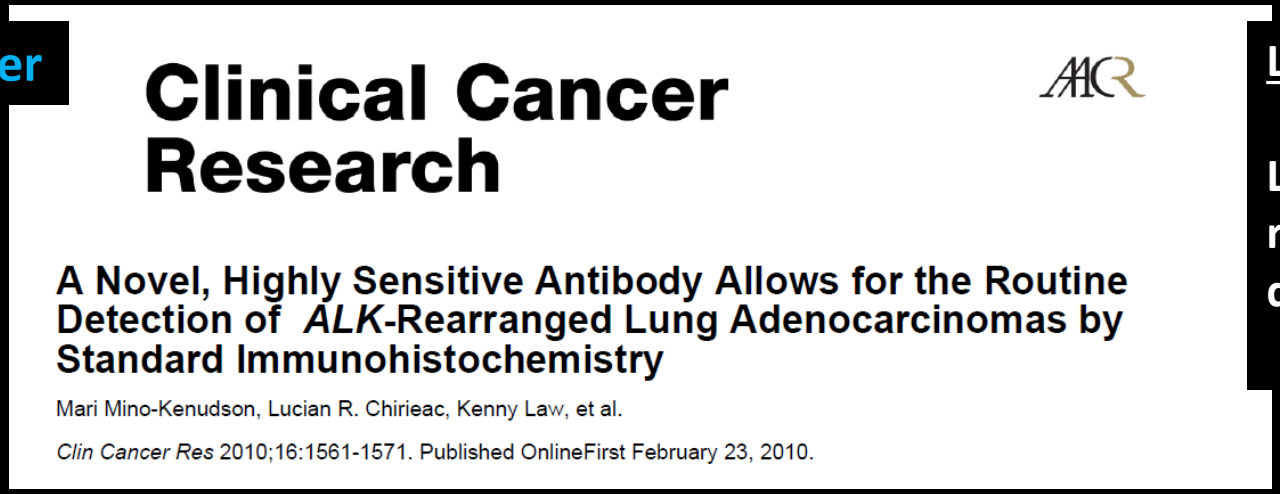



**Anything wrong ?**

# Technical aspects of immunohistochemistry & pitfalls - Analytical phase

IHC-Type 2 marker



Clinical Cancer Research

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**

Histopathology

*Histopathology* 2014, **65**, 398–407. DOI: 10.1111/his.12399

# Immunohistochemistry as a screening tool for *ALK* rearrangement in NSCLC: evaluation of five different ALK antibody clones and *ALK* FISH

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**Table 2.** ALK antibody clones and immunohistochemical staining results

| ALK antibody clone Working dilution | Detection system | No. (%) of cases stained, ($n$ = 303) | Staining intensity of all stained cases | No. (%) of rearranged cases stained ($n$ = 14) | Staining intensity of rearranged cases |
|---|---|---|---|---|---|
| 5A4 (Novocastra) 1:10 | Envision Flex | 23 (7.59) | 4 × 3+<br>9 × 2+<br>10 × 1+ | 14 (100) | 4 × 3+<br>4 × 2+<br>6 × 1+ |
| D5F3 (Cell Signaling) 1:250 | Envision Flex | 25 (8.25) | 3 × 3+<br>12 × 2+<br>10 × 1+ | 14 (100) | 3 × 3+<br>7 × 2+<br>4 × 1+ |
| D5F3 (Ventana) Ready to use | OptiView Benchmark XT + AMP | 128 (42.2) | 14 × 3+<br>7 × 2+<br>107 × 1+ | 14 (100) | 14 × 3+ |
| 5A4 (Novocastra) 1:100 | Envision Flex | 15 (4.95) | 1 × 3+<br>9 × 2+<br>5 × 1+ | 12 (86.5) | 1 × 3+<br>6 × 2+<br>5 × 1+ |
| SP8 (Abcam) 1:50 | Envision Flex | 41 (13.5) | 2 × 3+<br>9 × 2+<br>30 × 1+ | 9 (64) | 9 × 1+ |
| ALK1 (Dako) Ready to use | Envision Flex | 10 (3.30) | 0 × 3+<br>0 × 2+<br>10 × 1+ | 7 (50) | 7 × 1+ |
| ALK01 (Ventana) Ready to use | Benchmark XT | 18 (5.94) | 0 × 3+<br>1 × 2+<br>17 × 3+ | 7 (50) | 1 × 2+<br>6 × 1+ |

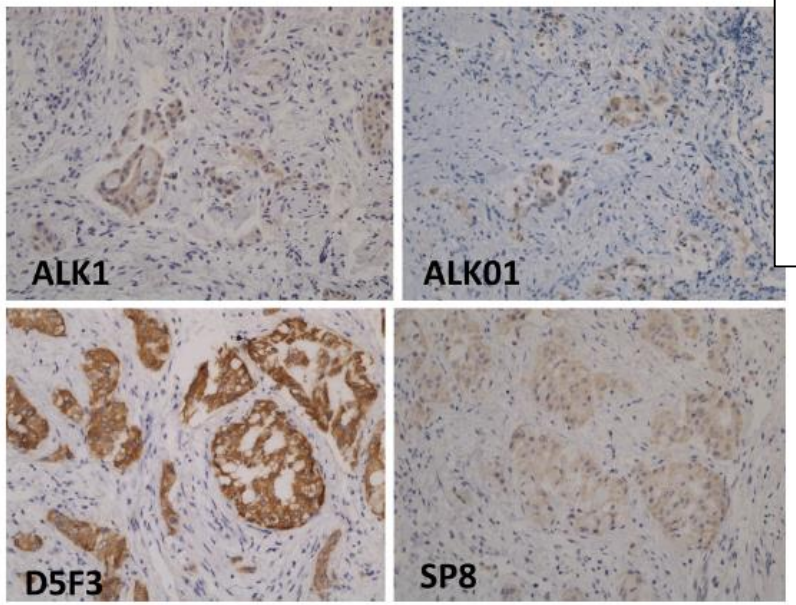



**Figure 4.** Conventional immunohistochemical staining without amplification. ALK1, ALK01 and SP8 staining of score 1+; D5F3 staining of score 3+.

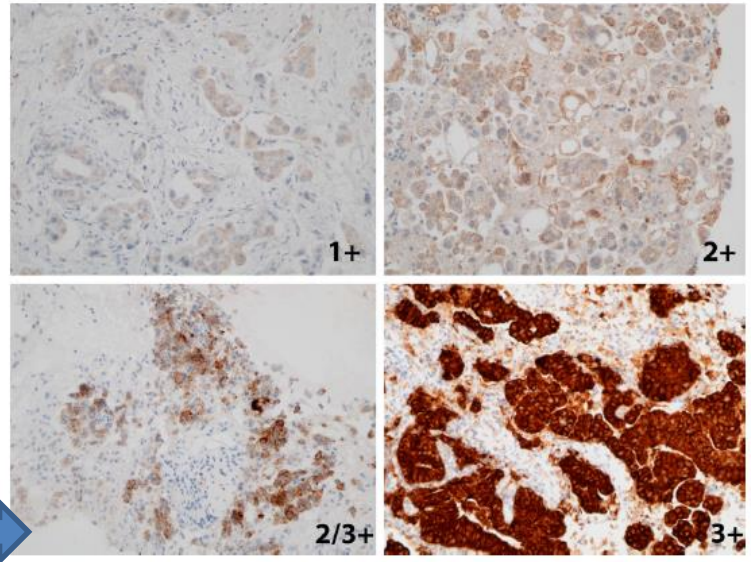



**Figure 5.** Staining using D5F3 (Ventana) and OptiView. The numbers represent scores using the four-tiered system; using the binary system both upper images are negative, and both lower images are positive. The image on the lower left shows a few strongly stained tumour cells (3+), and this case was also proved to be rearranged in *ALK* FISH analysis.

**Cases visualized with OptiView were evaluated using both the four-tiered system and also a binary system from Ventana which classifies strong granular cytoplasmic staining in any percentage of tumour cells as a positive result, and the absence of strong granular cytoplasmic staining as a negative result.**

# Technical aspects of immunohistochemistry & pitfalls - Analytical phase

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

| Concentrated antibodies | n | Vendor | Optimal | Good | Borderline | Poor | Suff.[1] | Suff. OPS[2] |
|---|---|---|---|---|---|---|---|---|
| mAb clone **5A4** | 46 | Leica/Novocastra | | | | | | |
| | 3 | Thermo/NeoMarkers | | | | | | |
| | 2 | Monosan | | | | | | |
| mAb clone **5A4 API3041** | 1 | Biocare | 1 | 0 | 0 | 0 | - | - |
| mAb clone **5A4** | 1 | Maixin | 1 | 0 | 0 | 0 | - | - |
| **790-4794** | | | | | | | | |
| 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.

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

| Concentrated antibodies | n | Vendor | Optimal | Good | Borderline | Poor | Suff.[1] | Suff. OPS[2] |
|---|---|---|---|---|---|---|---|---|
| mAb clone **5A4** | 43 | Leica/Novocastra | | | | | | |
| | 1 | Abcam | | | | | | |
| | 1 | Biocare | 1 | 15 | 24 | 7 | 34% | 22% |
| mAb **5A4** **MAD-001720QD** | 1 | Master Diagnostica | 0 | 0 | 1 | 0 | - | - |
| mAb clone **5A4** | 1 | ThermoFisher | 0 | 1 | 0 | 0 | - | - |
| **790-4796**[3] | | Ventana | | | | | | |
| mAb clone **OTI1A4** **8344-C010** | 1 | Sakura Finetek | 1 | 0 | 0 | 0 | - | - |
| Total | 189 | | 72 | 43 | 43 | 31 | - | |
| Proportion | | | 38% | 23% | 23% | 16% | 61% | |

1) Proportion of sufficient stains (optimal or good).
2) Proportion of sufficient stains with optimal protocol settings only, see below. . 3) RTU system developed for the Ventana BenchMark systems (Ultra/XT) but used by laboratories on different platforms (e.g Dako Autostainer)

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

**It does not ”fit-for-purpose”**

**D5F3, OTI1A4, 5A4**

**RUN 45 & 51**

**58 protocols were based on ALK1:**

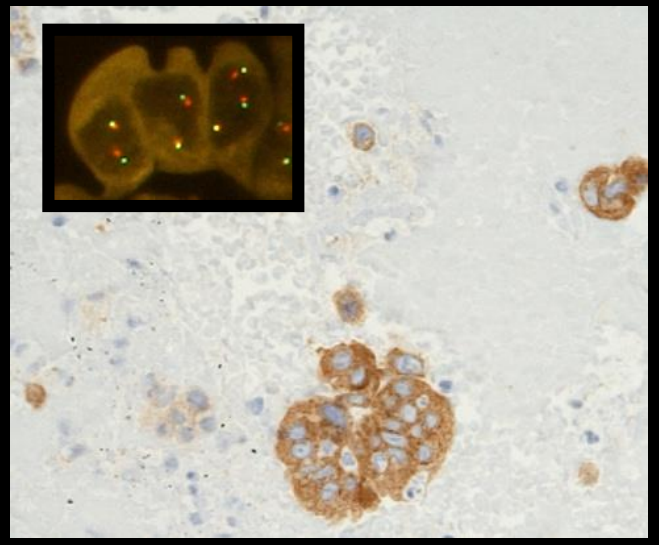
**Only one protocol (2%) were assessed as sufficient, none were optimal**

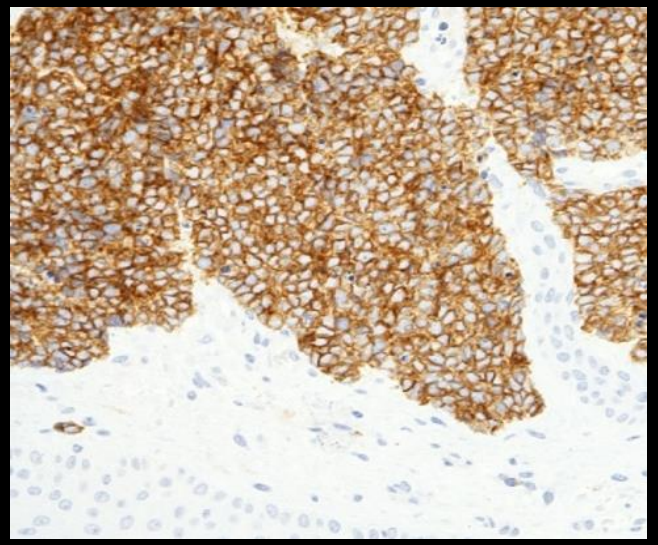
Lung AC (ALK-EML4)

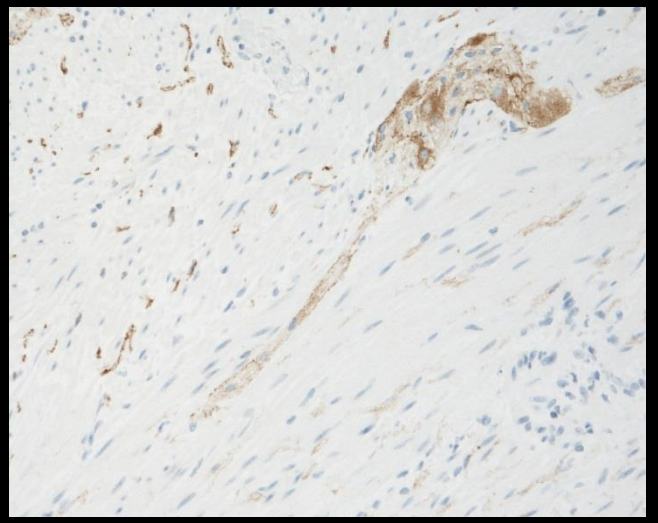
MCC (Skin)

Appendix

ALK, D5F3 (1:200)

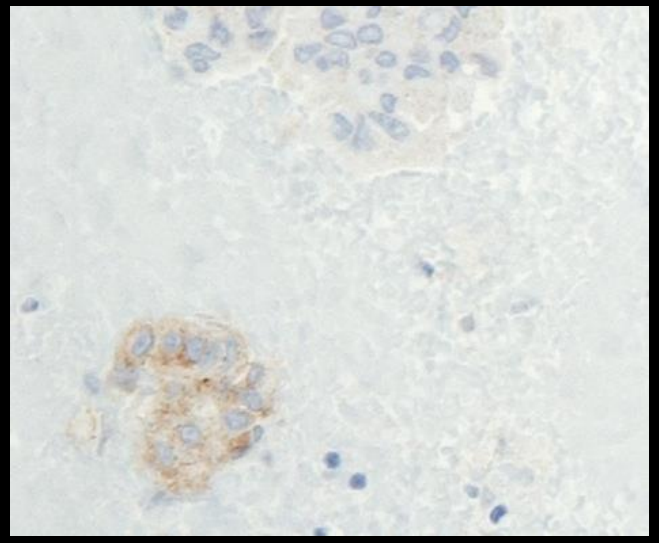


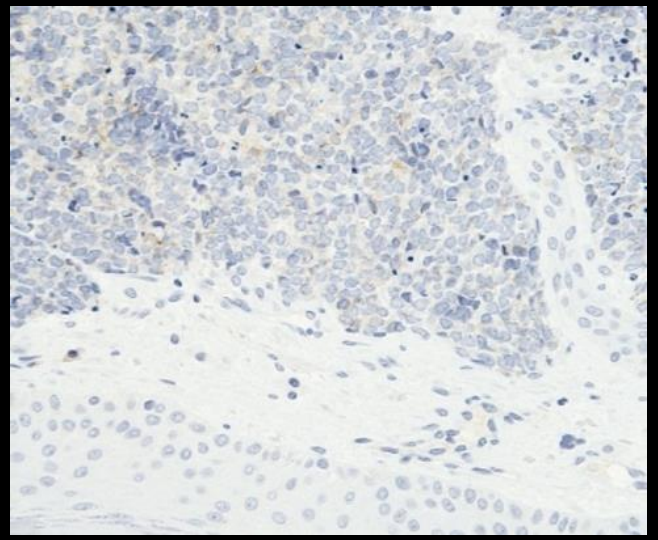


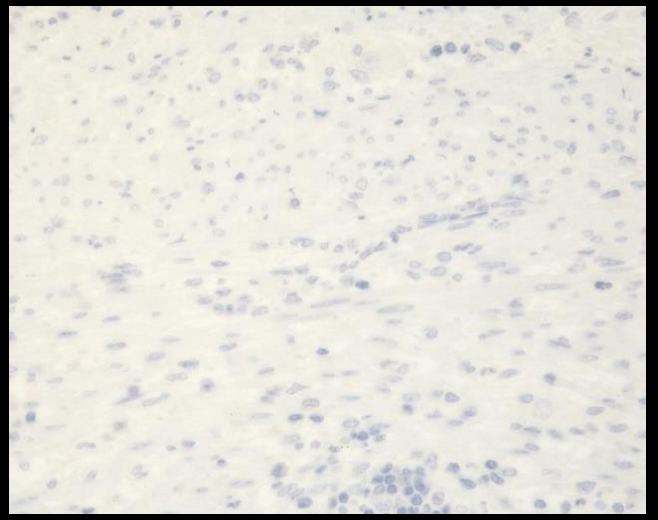


Ganglion and peripheral nerve cells

ALK, ALK1 (1:10)







HIER in high pH buffer 20`, Flex+

Clone ALK1 provides low sensitivity

# Technical aspects of immunohistochemistry & pitfalls - Analytical phase

**Problem: Primary antibody provides  low sensitivity**

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<br>1<br>1 | Biocare<br>Thermo/Neomarkers<br>Zytomed | 0 | 0 | 2 | 8 | - | - |
| rmAb clone **EP111** | 20<br>8<br>1 | Agilent/Dako<br>Cell Marque<br>BioSB | 7 | 7 | 11 | 4 | 48% | 53% |
| rmAb clone **EPR3864** | 8<br>2 | Abcam/Epitomics<br>Zeta Corporation | 6 | 2 | 1 | 1 | 80% | 100% |

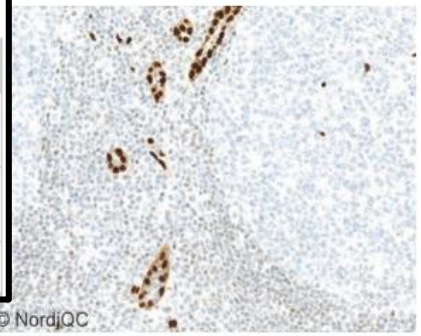



Fig. 1a
Optimal staining for ERG of tonsil using the rmAb clone EP111 within a laboratory developed assay optimally calibrated, using HIER in an alkaline buffer and a 3-step multimer based detection system, OptiView Ventana. Virtually all endothelial cells show a strong nuclear staining reaction, while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

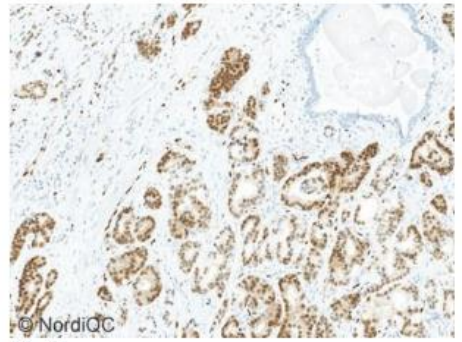



Fig. 3a
Optimal ERG staining of the prostate adenocarcinoma, tissue core no. 5, using same protocol as in Figs. 1a and 2a.
A moderate to strong and distinct nuclear staining reaction is seen in virtually all neoplastic cells. A weak cytoplasmic staining reaction is seen, but in general, a high signal-to-noise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

**ERG, 9FY, prostate adenocarcinoma TMPRSS2-ERG gene fusion ?**

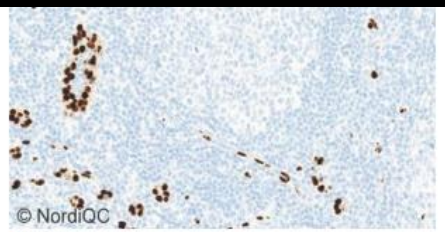



Fig. 5a
Staining for ERG of tonsil using the mAb clone 9FY within a laboratory developed test using HIER in an alkaline buffer and a 3-step polymer based detection system.
This antibody does not react with lymphocytes, whereas an intense nuclear staining reaction in endothelial cells is seen.
However 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.

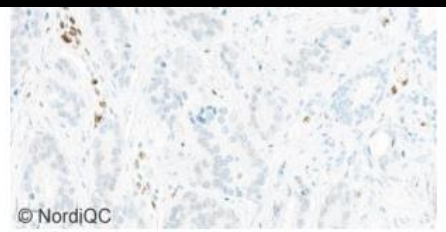



Fig. 5b
Insufficient ERG staining of prostate adenocarcinoma, tissue core no. 5 with high level ERG expression  using same protocol as in Fig. 5a.
Only the endothelial cells show a distinct nuclear staining reaction, while virtually all the neoplastic cells are negative.
Compare with Fig. 3a showing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

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

**ERG (Ets-Related-Gene) clone 9FY**
**ALK clone ALK1**
**GATA3 clone HG3-31**
**CEA clone II-7**
**CGA clone DAK-A3**
**P63 clone 7JUL**
**……**

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

Mol Cell. 2015 Sep 17;59(6):904-16. doi: 10.1016/j.molcel.2015.07.025. Epub 2015 Sep 3.

## Truncated ERG Oncoproteins from TMPRSS2-ERG Fusions Are Resistant to SPOP-Mediated Proteasome Degradation.

An J[1], Ren S[2], Murphy SJ[3], Dalangood S[4], Chang C[4], Pang X[5], Cui Y[4], Wang L[6], Pan Y[7], Zhang X[7], Zhu Y[2], Wang C[8], Halling GC[3], Cheng L[9], Sukov WR[10], Karnes RJ[11], Vasmatzis G[12], Zhang Q[4], Zhang J[10], Cheville JC[13], Yan J[4], Sun Y[14], Huang H[15].

⊕ Author information

**Abstract**

SPOP mutations and TMPRSS2-ERG rearrangements occur collectively in up to 65% of human prostate cancers. Although the two events are mutually exclusive, it is unclear whether they are functionally interrelated. Here, we demonstrate that SPOP, functioning as an E3 ubiquitin ligase substrate-binding protein, promotes ubiquitination and proteasome degradation of wild-type ERG by recognizing a degron motif at the N terminus of ERG. Prostate cancer-associated SPOP mutations abrogate the SPOP-mediated degradation function on the ERG oncoprotein. Conversely, the majority of TMPRSS2-ERG fusions encode N-terminal-truncated ERG proteins that are resistant to the SPOP-mediated degradation because of degron impairment. Our findings reveal degradation r uncharacterized mechanism that contributes to elevation of truncated ERG proteins in p suggest that overcoming ERG resistance to SPOP-mediated degradation represents a v prostate cancers expressing either mutated SPOP or truncated ERG.

**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**

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.



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

# Technical aspects of immunohistochemistry & pitfalls - 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<br>1<br>1 | Agilent/Dako<br>Diagnostic Biosystem<br>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<br>1<br>1 | Cell Marque<br>Menarini<br>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**
**ECAD clone EP700Y**
**PAX5 clone SP34**
**Many pAbs (e.g. P40 and SOX10)**
**……**

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

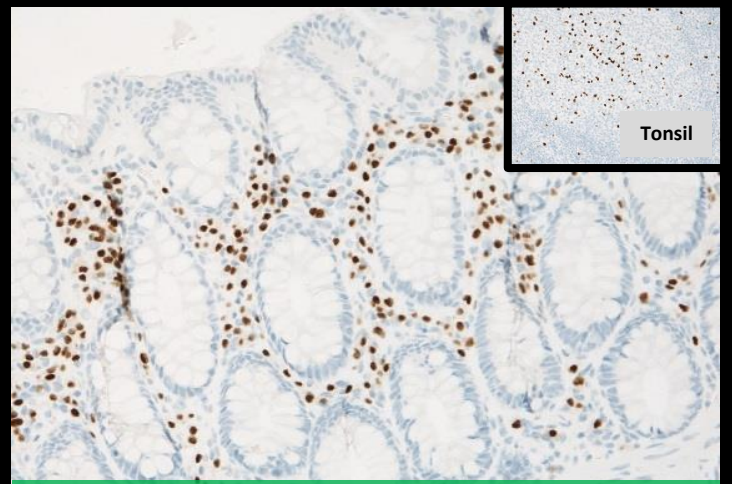
**MUMp1, EAU32 & EP190**

# Technical aspects of immunohistochemistry & pitfalls - Analytical phase

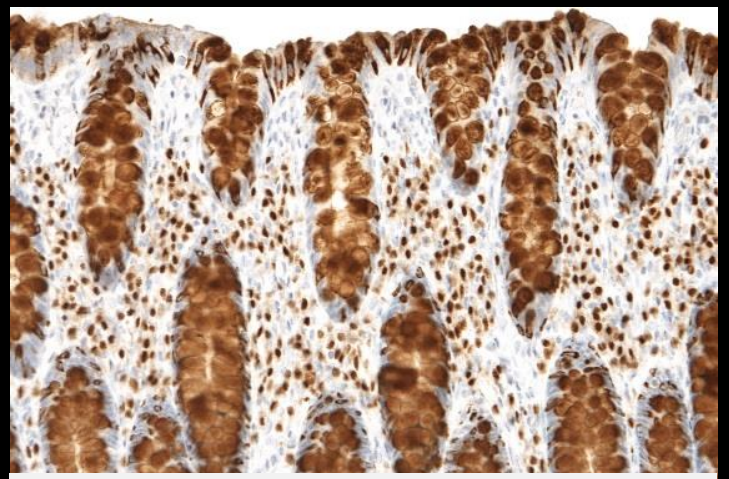
**Problem: Primary antibody provides  low specificity and/or poor signal-to-noise ration**

**Clones providing optimal results:**

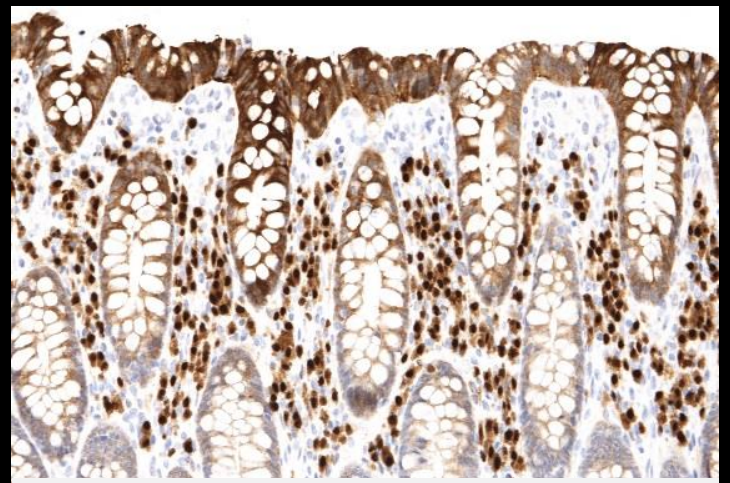
**MUMp1, EAU32 & EP190**



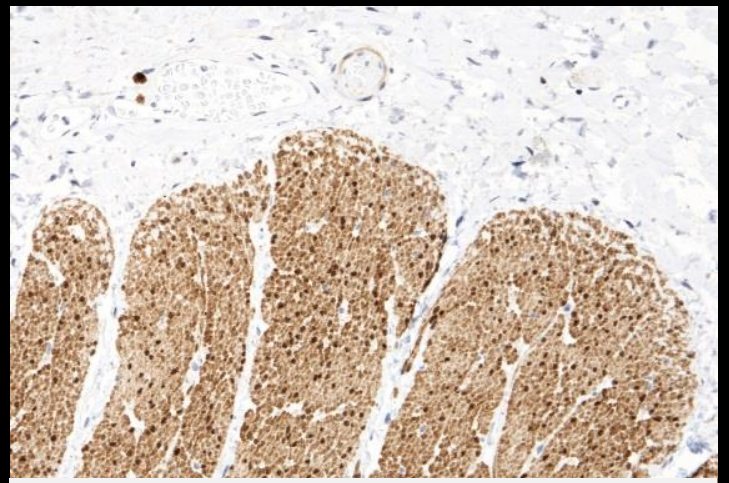

MUMp1, optimal



BC5, aberrant cytoplasmic staining result



MRQ-43, aberrant cytoplasmic staining result



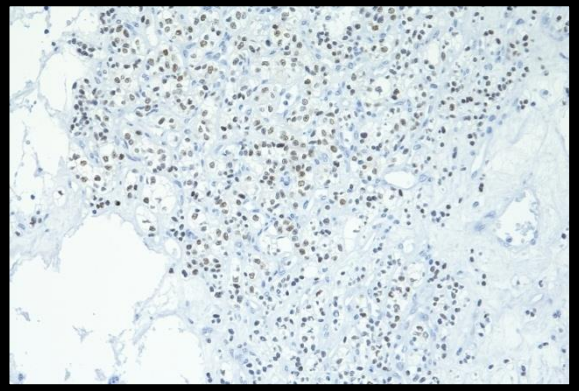
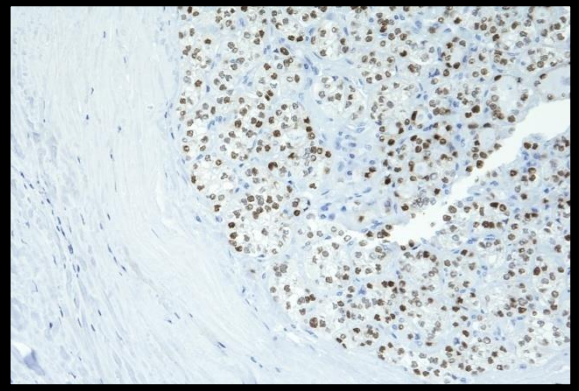
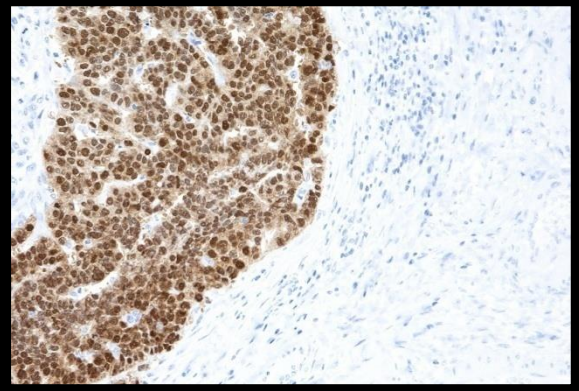
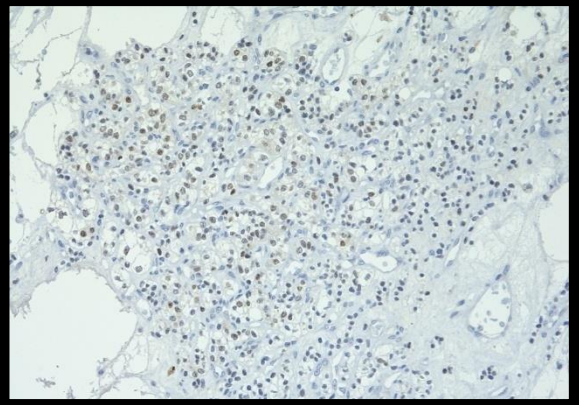
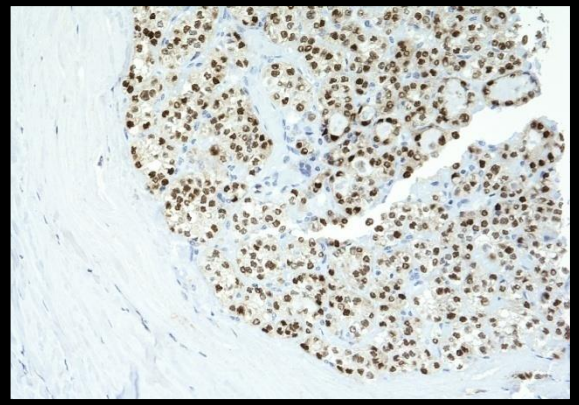
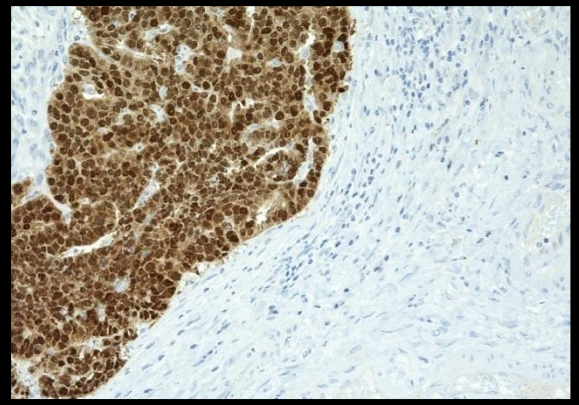
MRQ-43, false positive

Colon

# Technical aspects of immunohistochemistry & pitfalls - Analytical phase

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

**Which antibody ?**

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

# Technical aspects of immunohistochemistry & pitfalls - Analytical phase

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

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

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

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

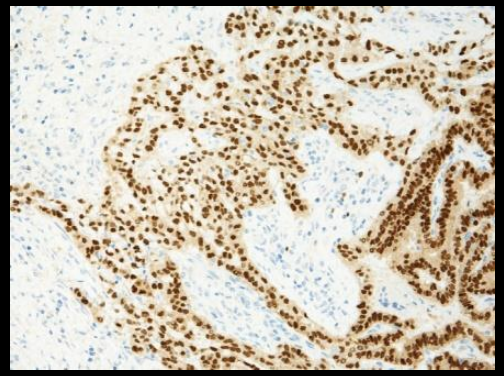
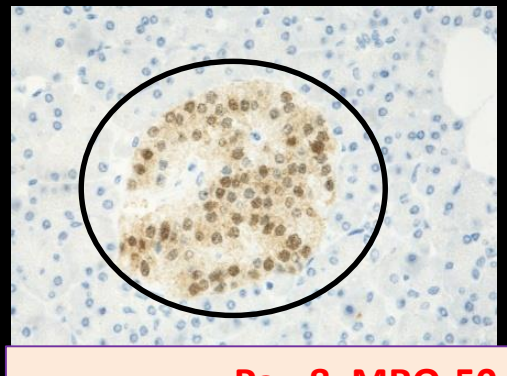
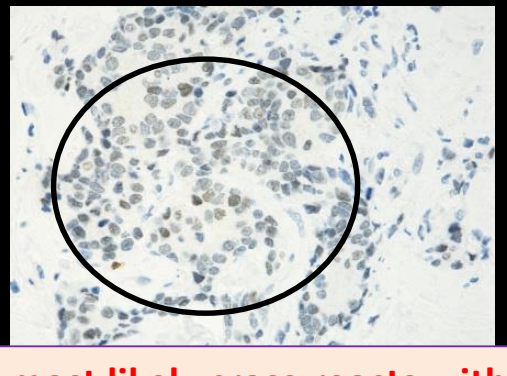
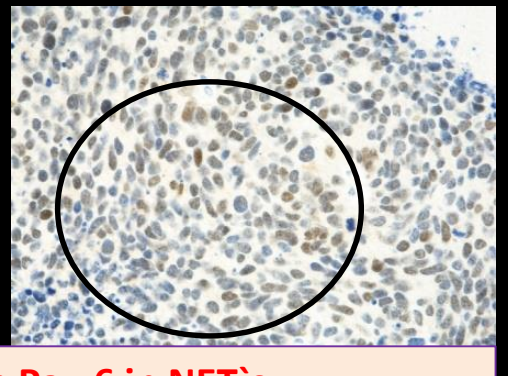
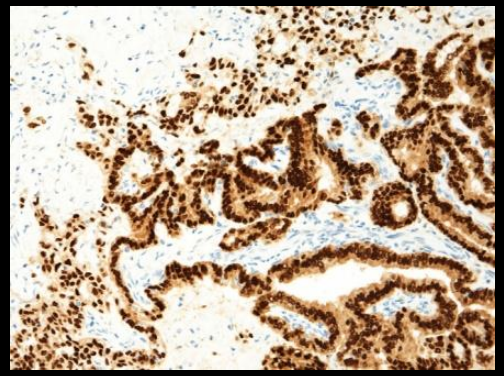
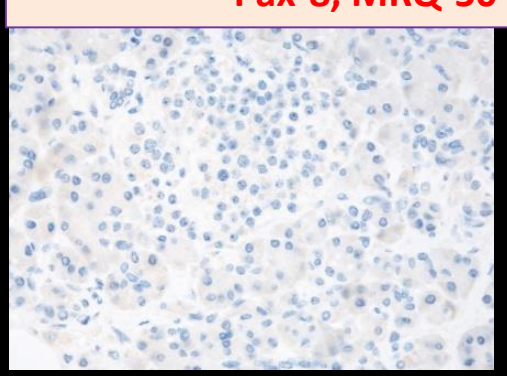
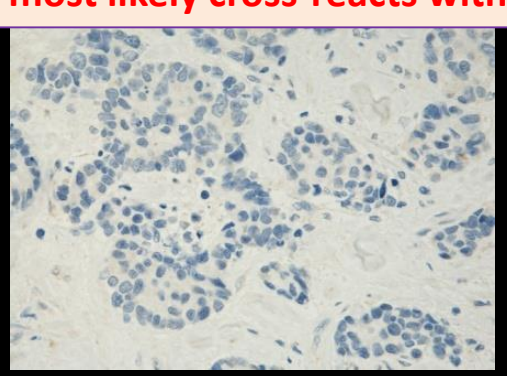
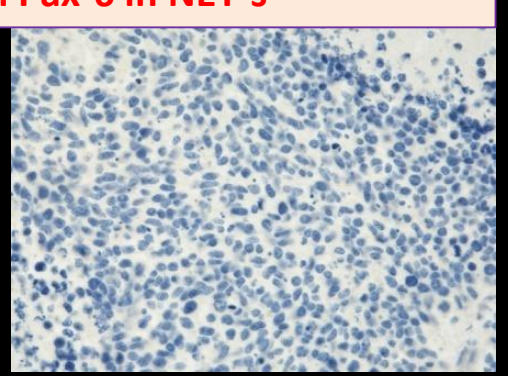
**Moretti L et al. : *Mod Pathol. 2012; 25 : 231-236***

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

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

# Technical aspects of immunohistochemistry & pitfalls - Analytical phase

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

| | Papillary carcinoma (Thyroid) | Pancreas | Carcinoid (Appendix) | SCLC (Lung) |
|---|---|---|---|---|
| PAX-8, MRQ-50 |  |  |  |  |
| PAX-8, ZR1 (BC12) |  |  |  |  |

**Pax-8, MRQ-50 most likely cross-reacts with Pax-6 in NET`s**

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

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

# Technical aspects of immunohistochemistry & pitfalls - Analytical phase

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

Table 1. **Antibodies and assessment marks for PAX8, run 56**

| Concentrated antibodies | n | Vendor | Optimal | Good | Borderline | Poor | Suff.[1] | Suff. OPS[2] |
|---|---|---|---|---|---|---|---|---|
| mAb clone **BC12*** | 13<br>1 | Biocare<br>Zytomed Systems | - | 4 | 4 | 6 | 29% | - |
| mAb clone **DBM15.48** | 1 | Diagnostic Biosystems | - | - | 1 | - | - | - |
| mAb clone **MRQ-50** | 59<br>2 | Cell Marque<br>Master Diagnostica | 8 | 19 | 22 | 12 | 44% | 38% |
| mAb clone **MX062*** | 1 | Maixin | - | 1 | - | - | - | - |
| mAb clone **PAX8R1** | 1 | abcam | - | - | - | 1 | - | - |
| rmAb clone **EP298*** | 7<br>5<br>1<br>2 | Cell Marque<br>Epitomics<br>BIO SB<br>Nordic Biosite | 3 | 6 | 3 | 3 | 60% | 100% |
| rmAb clone **EP331** | 1 | Path n situ | - | - | 1 | - | - | - |
| rmAb clone **SP348*** | 2<br>2 | Spring Biosciences<br>abcam | 3 | - | 1 | - | 75% | 100% |
| rmAb clone **ZR-1*** | 1<br>1<br>1 | Zeta Corporation<br>Abcam<br>Gene tech | 1 | 2 | - | - | - | - |
| pAb, **10336-1-AP** | 26 | Protein Tech | 9 | 6 | 11 | - | 58% | 60% |
| pAb, **363A-15** | 5 | Cell Marque | - | 2 | 1 | 2 | - | - |
| pAb, **AP10903** | 2 | Gennova | - | 1 | - | 1 | - | - |
| pAb, **CP379** | 5 | Biocare | - | 4 | 1 | - | - | - |
| pAb, **Pax8** | 1 | Menapath | - | 1 | - | - | - | - |
| pAb, **RBG047** | 1 | Zytomed | - | - | - | 1 | - | - |
| pAb, **RBK047** | 1 | Diagomic | - | - | - | 1 | - | - |
| Ready-To-Use antibodies | | | | | | | | |
| mAb clone **MRQ-50 760-4618** | 91 | Ventana/Cell Marque | 3 | 16 | 41 | 31 | 21% | 20% |
| mAb clone **MRQ-50 363M** | 28 | Cell Marque | 3 | 4 | 12 | 9 | 25% | - |
| pAb **363A-17/363A-18** | 3 | Cell Marque | 1 | - | 1 | 1 | - | - |
| Total | 264 | | 31 | 66 | 99 | 68 | - | |
| Proportion | | | 12% | 25% | 37% | 26% | 37% | |

1) Proportion of sufficient stains (optir
2) Proportion of sufficient stains with c
*Clones that do not show cross reactiv

**Conclusion**
Optimal staining results could be obtained with the mAb clone **MRQ-50**, the rmAb clones **EP298**, **SP348** and **ZR-1** and the pAbs **10336-1-AP** and **363A-17/18**. Irrespective of the clone applied, efficient HIER, use of a sensitive 3-step polymer/multimer based detection system and careful calibration of the primary antibody were the most important prerequisites for an optimal staining result.
The rmAbs clones **EP298**, **SP348** and **ZR-1** gave encouraging results and a high proportion of sufficient results on the main fully automated platforms where no cross-reaction with e.g. PAX5 was observed. In contrast, the mAb clone **MRQ-50** provided a poor performance especially on the Ventana BenchMark and Dako Omnis platforms and at the same time also labelled PAX5 in B-cells. In coming assessments cross-reaction with other PAX epitopes will be downgraded.

**BC12 (platform dependent)**

**"ZR1" (lot variations/antibody diluent dependent)**

**EP298**

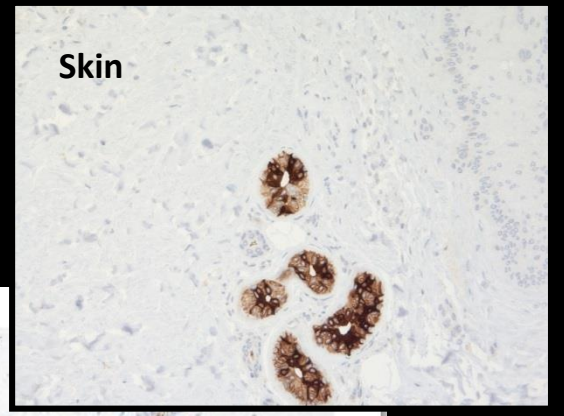
**SP348**

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

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

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

# Technical aspects of immunohistochemistry & pitfalls - Analytical phase




## Problem: Primary antibody poorly calibrated providing low sensitivity

The right primary antibody

The right protocol (AR procedure and detection system)

Poorly calibrated primary Ab ?

### Tissue controls are the key element

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

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

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

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

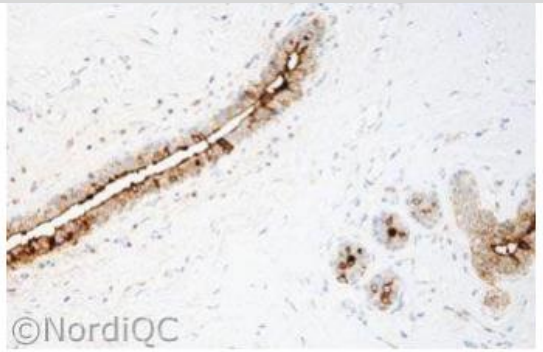



Fig. 1a
Optimal staining for GCDFP-15 of the breast hyperplasia using the mAb clone 23A3 optimally calibrated as a concentrate, HIER in an alkaline buffer and a polymer based detection system. The majority of the ductal epithelia cells show a distinct moderate to strong cytoplasmic staining reaction.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

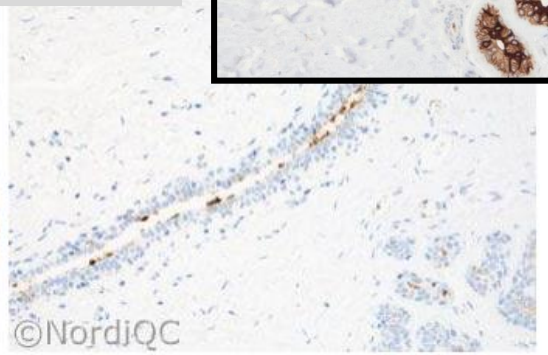



Fig. 1b
Insufficient staining for GCDFP-15 of the breast hyperplasia applying the mAb clone 23A3 as a concentrate using exactly the same protocol settings as used in Fig 1a, except for a 20 fold dilution of the primary antibody. The proportion and the intensity of the cells demonstrated are significantly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

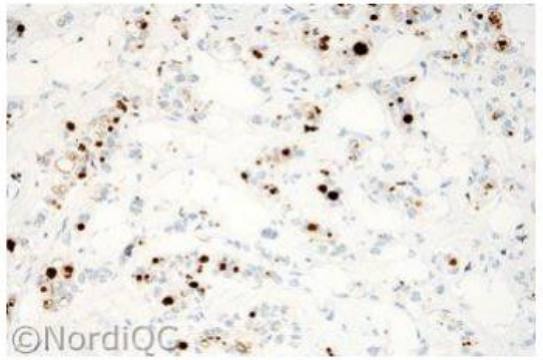



Fig. 2a
Optimal staining for GCDFP-15 of the breast carcinoma no. 5 using same protocol as in Fig. 1a.
The majority of the neoplastic cells show a moderate to strong dot-like cytoplasmic staining reaction.

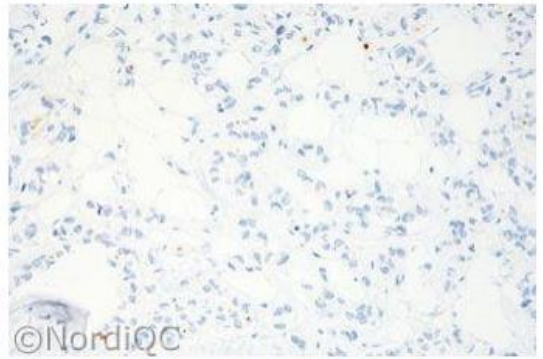
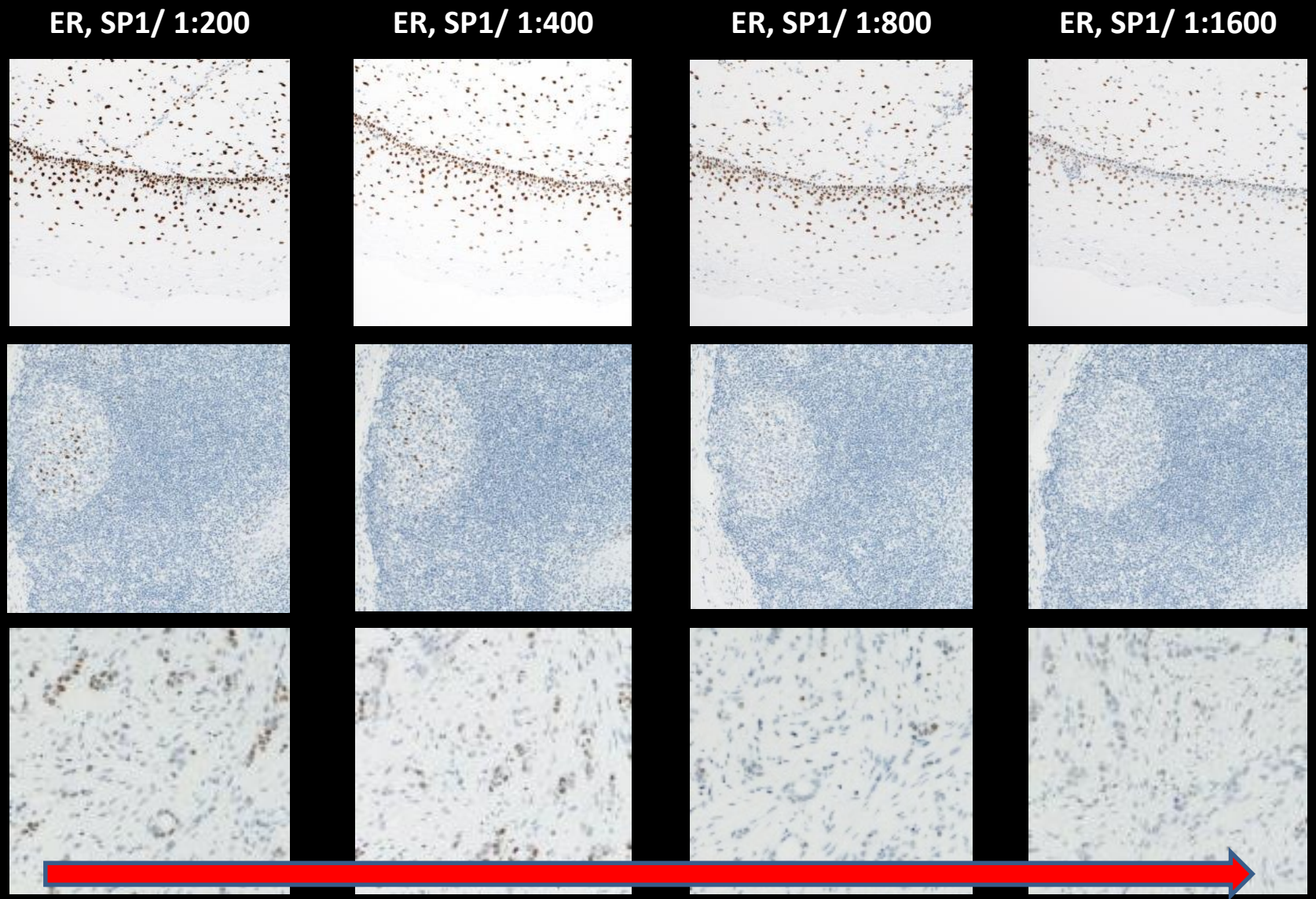



Fig. 2b
Insufficient staining GCDFP-15 of the breast carcinoma no. 5 using same protocol as in Fig. 1b. - same field as in Fig. 2a.
Only scattered neoplastic cells show a faint dot-like reaction.

# Technical aspects of immunohistochemistry & pitfalls - Analytical phase

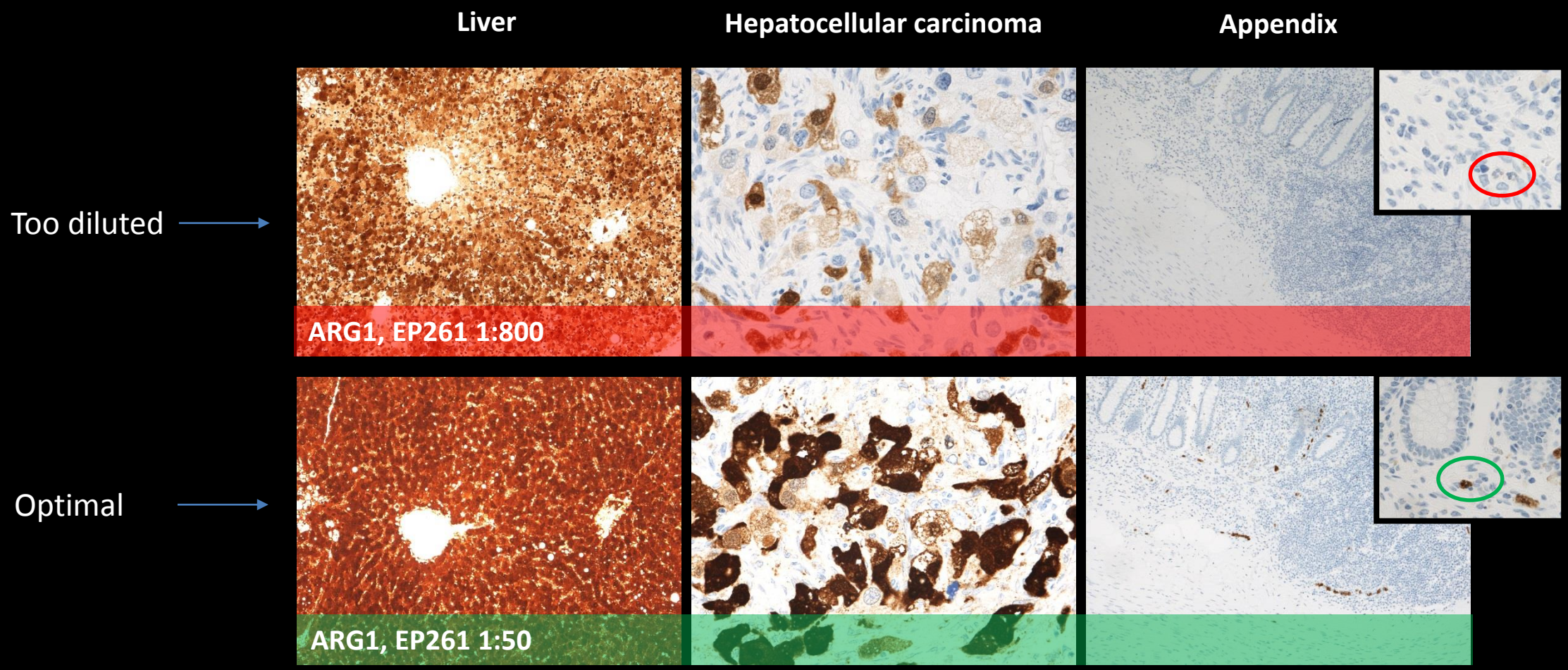
## Problem: Primary antibody poorly calibrated providing low sensitivity




High pH 24`, Flex+Rabbit

# Technical aspects of immunohistochemistry & pitfalls - 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 immunohistochemistry & pitfalls - Analytical phase

**HIER buffers used by NordiQC laboratories**

**App. 80-90 % of all pretreatment protocols**

| In house | Agilent 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 | | | | | |

## Challenges:

**The platform often dictates the choice of HIER buffers**

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

**Assessment Run 56 2019**

# Epithelial cell-cell adhesion molecule (Ep-CAM)

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

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

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

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

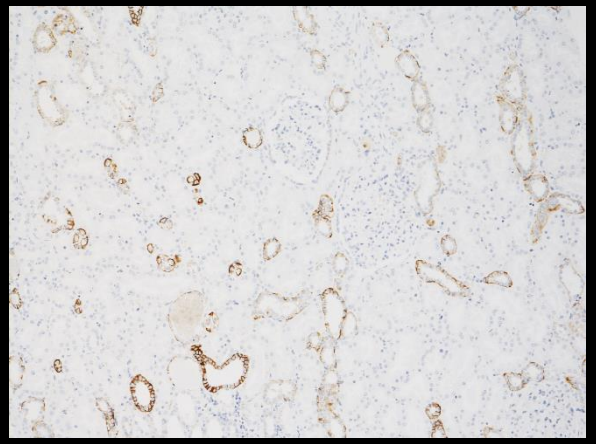
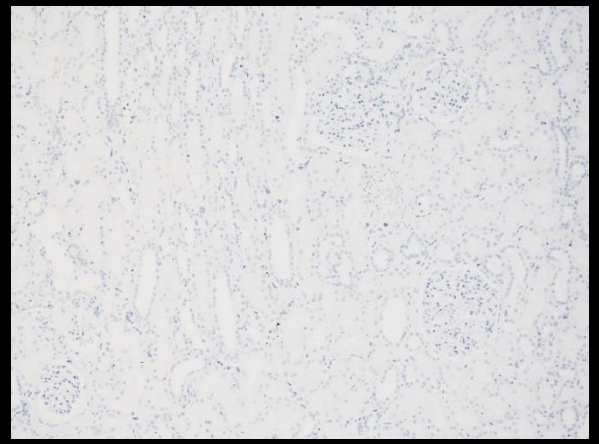
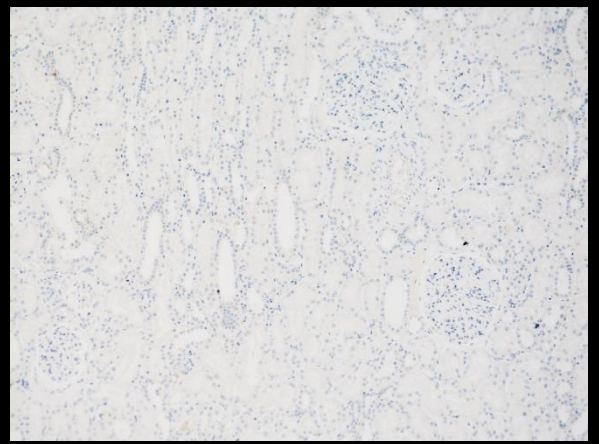
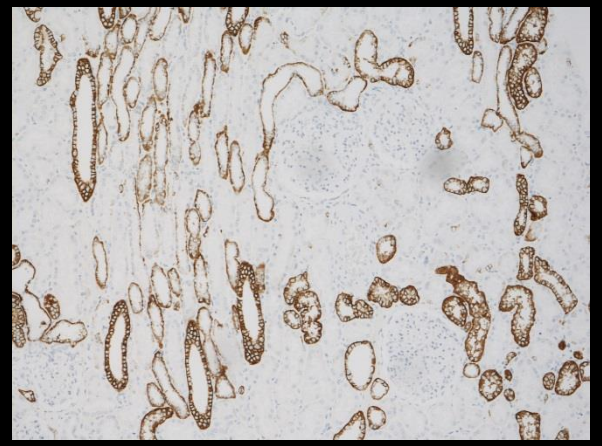
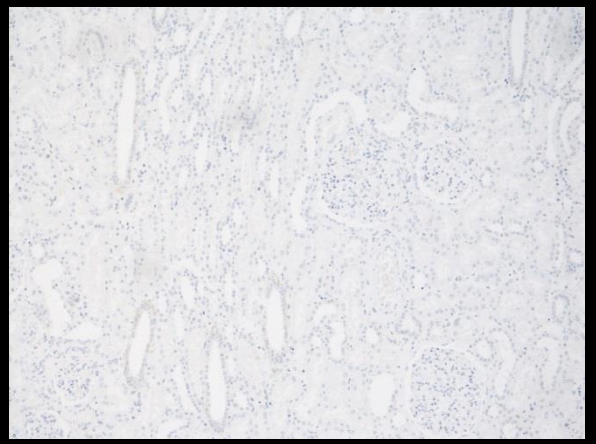
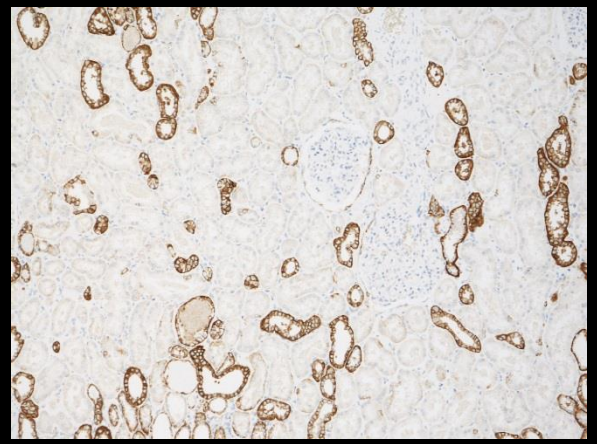
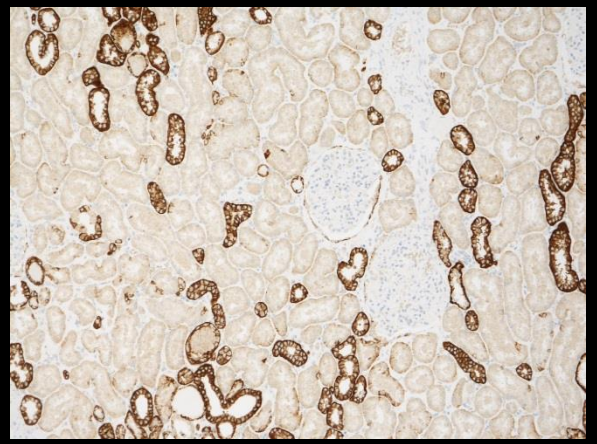
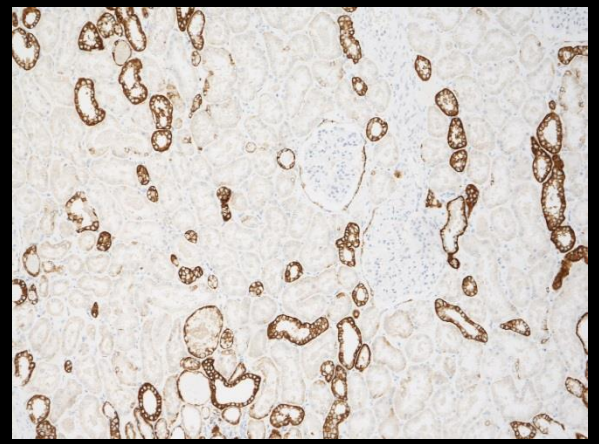
**OPS based on HIER in mod. Low pH buffers (Dako)**

**Omitted from HIER in mod. Low pH buffers (Dako)**

**HIER in mod. Low pH buffers (Dako)**

# Technical aspects of immunohistochemistry & pitfalls - Analytical phase

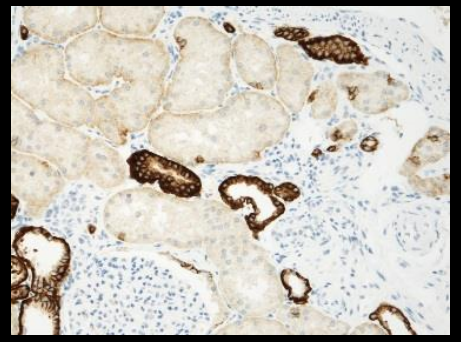
## EPCAM clone EP4 or BS14

| | 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 |  |  |  |  |

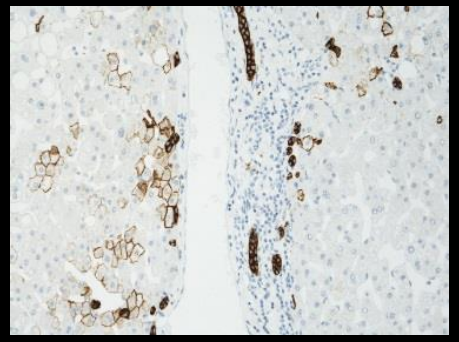
Kidney

# Omnis

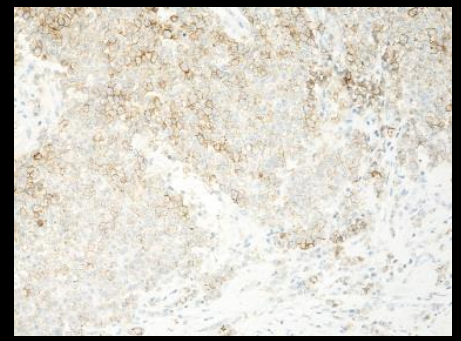

Kidney



Hepar

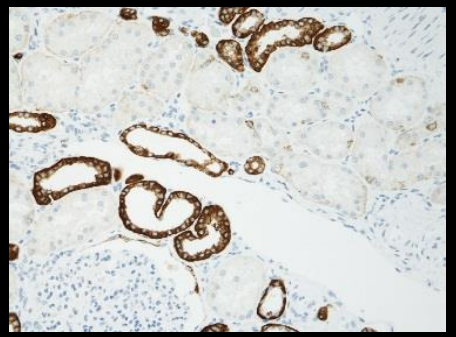


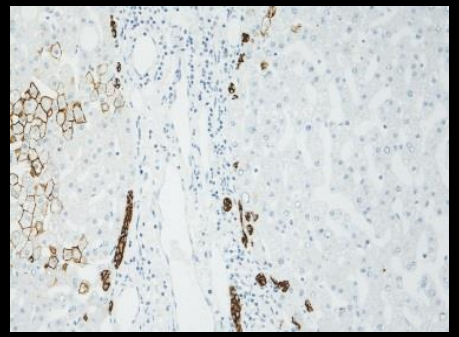
Breast tumor

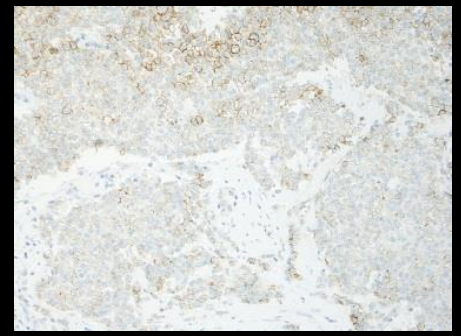


**EPCAM, BS14 (1:500) / TRS pH 9.0**

**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.**



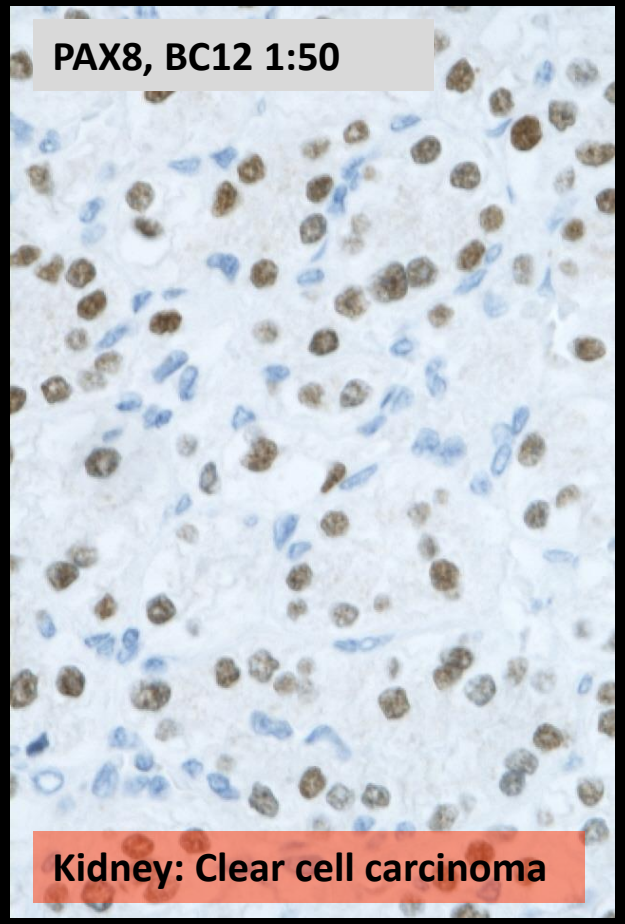
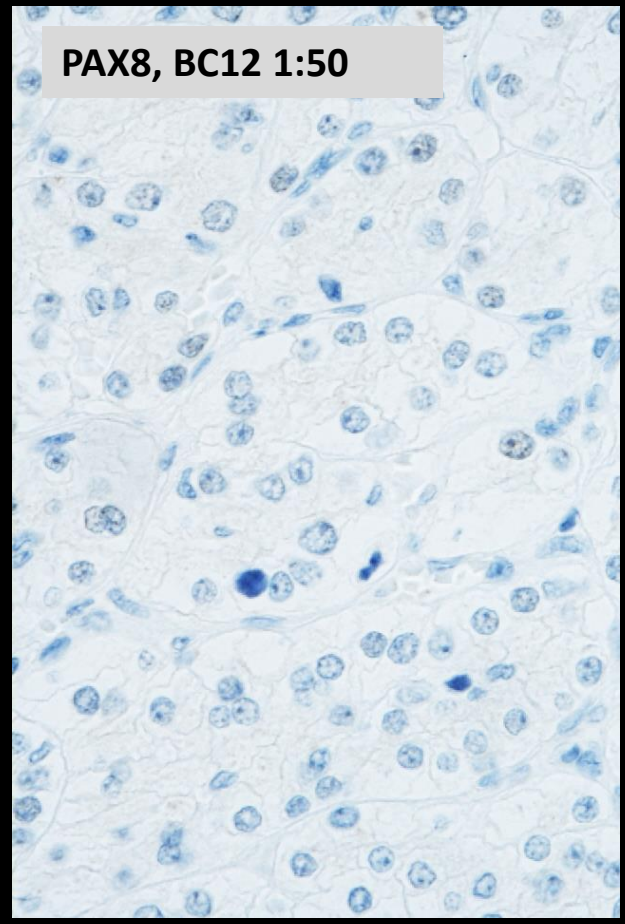
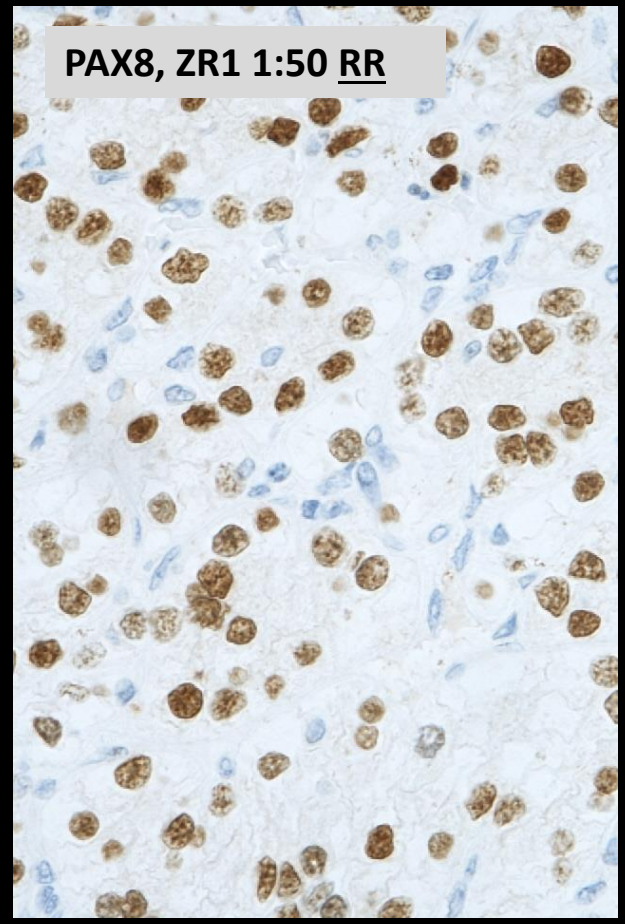




**EPCAM, MOC31 (1:25) / TRS pH 6.1**

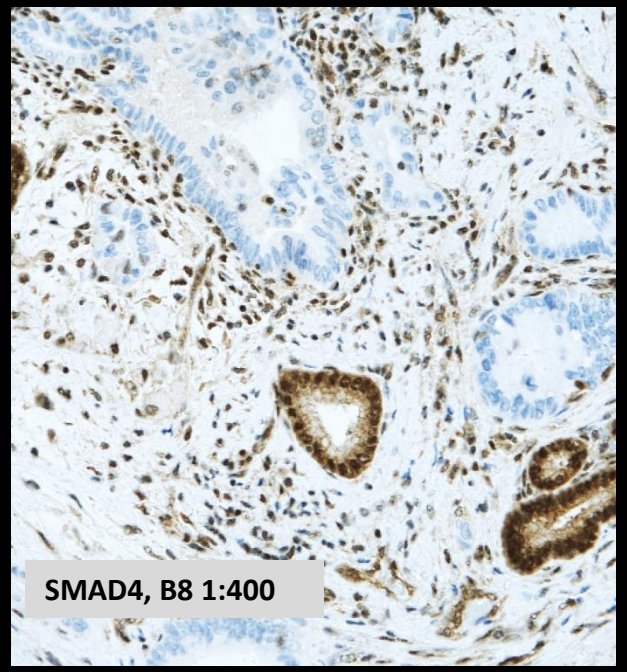
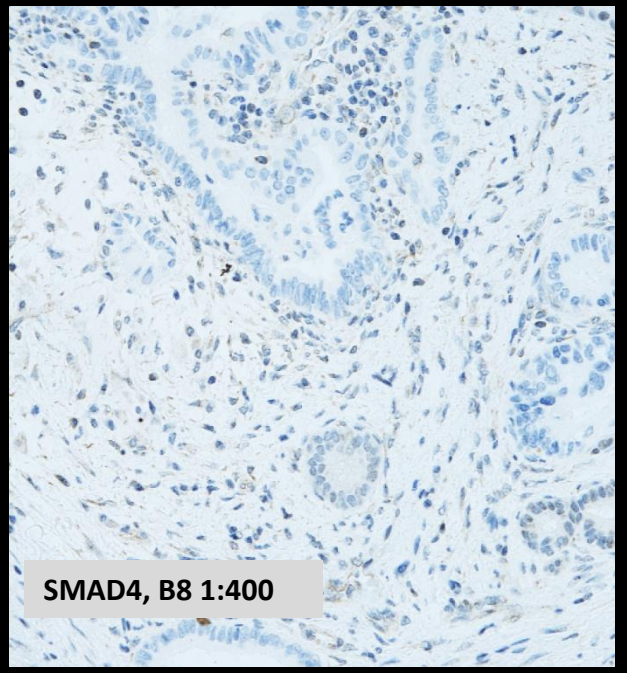
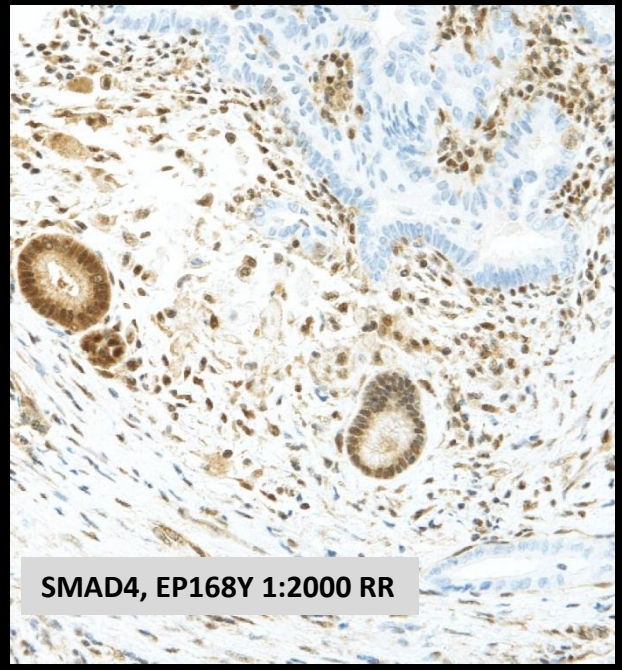
# Technical aspects of immunohistochemistry & pitfalls - Analytical phase

**Primary antibodies sensitive to the chosen platform**

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

# Technical aspects of immunohistochemistry & pitfalls - Analytical phase

**Primary antibodies sensitive to the chosen platform**

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

**Pancreatic Adenocarcinoma**

# Challenging and platform dependent antibodies (NQC results)

| Marker | Clone |
|---|---|
| ASMA | 1A4/BS66 |
| BCL2 | 124 /E17 |
| 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 | BER-EP4/BS14 |
| Melan A | A103/EP43 (melanomas) |
| OCT 3/4 | C-10/MRQ-10 or N1NK |
| PAX8 | MRQ-50/SP348 or EP298 |
| Podop | D2-40 |
| WT1 | 6F-H2/D817F or EP122 |
| …… | (e.g. PAN-CK, AE1/AE3 on the BOND) |

Alternative antibodies: Antibody clones applied on the Omnis (Dept. of surgical Pathology, Region Zealand, Denmark) - These clones might also work on other platforms (e.g. Benchmark Ulta or Bond MAX/III).

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

# Technical aspects of immunohistochemistry & pitfalls - Analytical phase

**Primary antibodies sensitive to the chosen platform**


**Implementing a new platform has been a challenge**

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

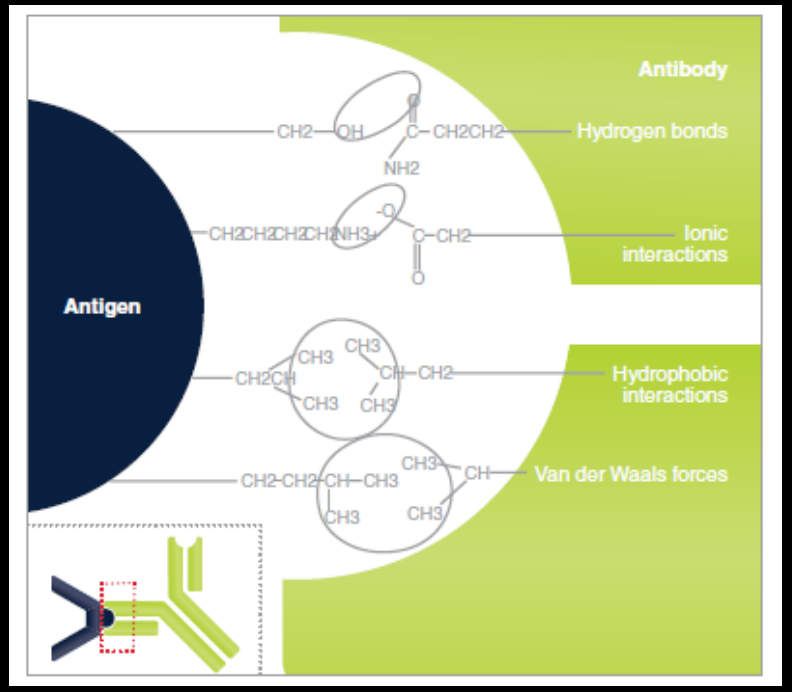
**Changing the primary Ab**

**Changing Ab-Ag reaction microenvironment (Diluent)**

**Low affinity primary antibodies**

# Technical aspects of immunohistochemistry & pitfalls - Analytical phase

## Antibody-Antigen reaction – Antibody Diluents




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

- Hydrogen bonds
- Hydrophobic interactions
- Van der Waals forces

### Antibody diluents

Commercial antibody diluents are buffered solutions

- often based on TRIS-HCL buffers at neutral pH (7.0-7.6)
- often contains detergent, NaCl and stabilizers
- may contain protein-based background reducing agents
  - BSA
  - Serum proteins
  - Caseins

Protein blockers act by occupying the non-specific tissue binding sites (protein adsorbance ) minimizing unwanted non-specific reaction with the primary antibody of interest.

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

# Technical aspects of immunohistochemistry & pitfalls - Analytical phase

## Antibody diluents

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


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

Thomas Boenisch, M.S.

**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)*

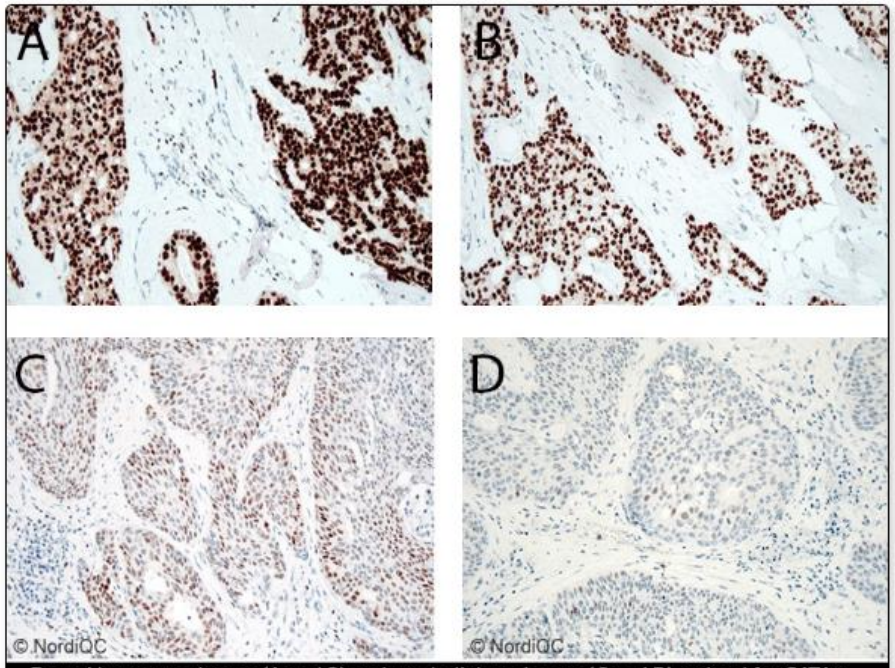
| | | TB | | TBS | | PBS |
|---|---|---|---|---|---|---|
| Clone | pH | 6.0 | 8.6 | 6.0 | 8.6 | 7.3 |
| BLA.36 | | 2 | 4 | 1 | 2 | 1 |
| UCHL1 | | 4 | 3 | 2 | 1 | 1 |
| L26 | | 4 | 3 | 3 | 3 | 2 |
| PC10 | | 4 | 3 | 4 | 4 | 3 |
| N10/2 | | 3 | 2 | 1 | 2 | 1 |
| V9 | | 4 | 3 | 4 | 4 | 2 |
| TAL1B5 | | 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.

# Technical aspects of immunohistochemistry & pitfalls - Analytical phase

NordiQC

Info Modules Assessments Protocols Controls Events Login




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

Results - Run 54, B26, H14, C4

14-Dec-2018

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

All news

**Events**

NordiQC Workshop in Diagnostic Immunohistochemistry
*2–4 Oct 2019: Aalborg, DK*

6th Annual Course of Academy of Immunohistochemistry "Diagnostic Immunohistochemistry and Molecular
*9–11 Oct 2019: Krakow, Poland*

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

**Important dates**

Run 55, B27, H15
Protocol submission deadline
*3 Jan 2019*
Slide circulation
*9 Jan 2019*
Slide return deadline
*13 Feb 2019*
Publication of results
*20 Apr 2019*

**Questions**

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

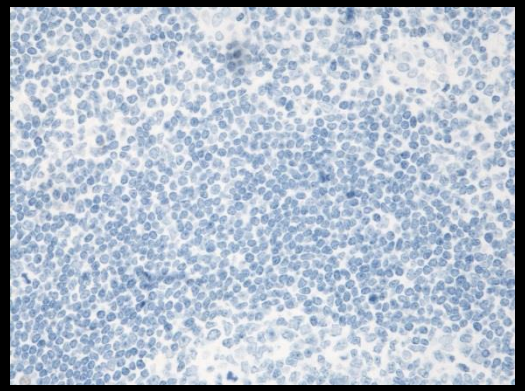
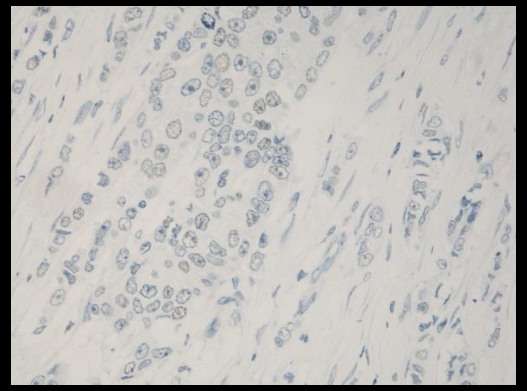
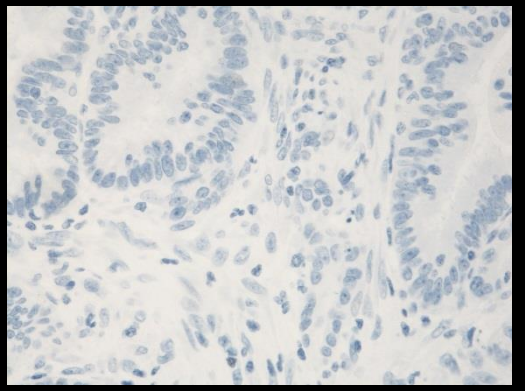
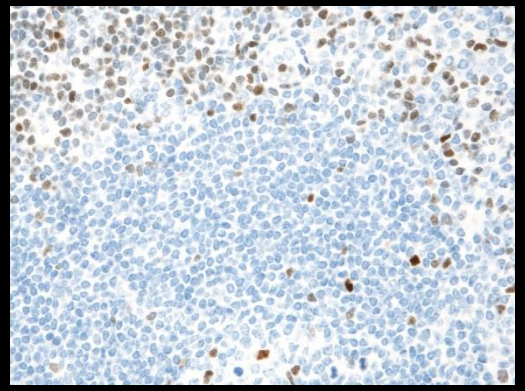
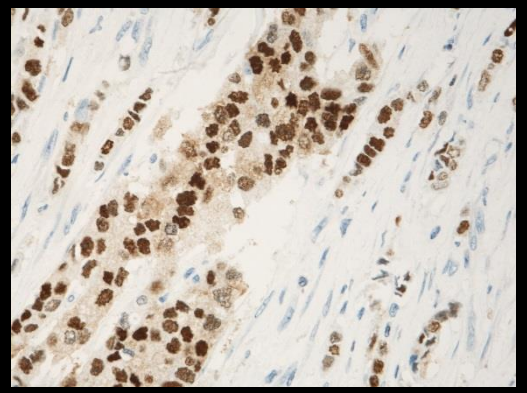
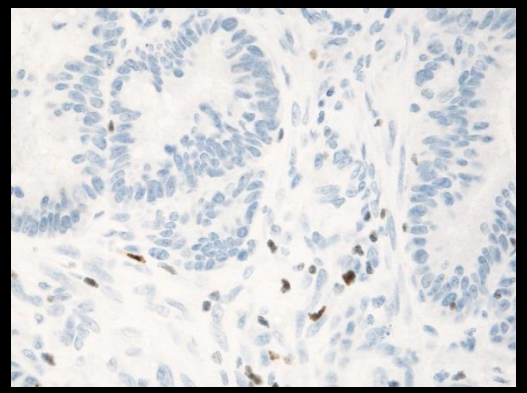
## GATA3

## Antibody diluents

# Technical aspects of immunohistochemistry & pitfalls - Analytical phase

**Antibody diluents**

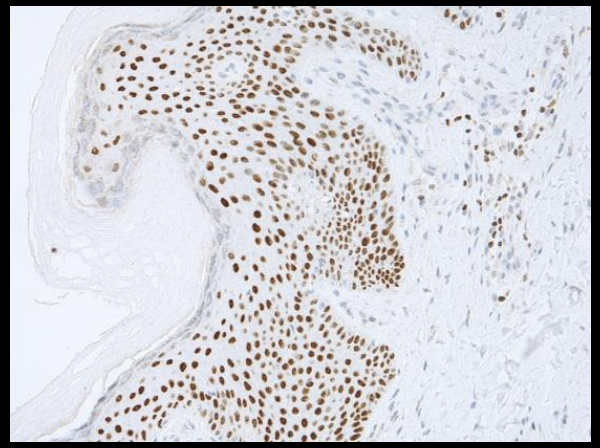
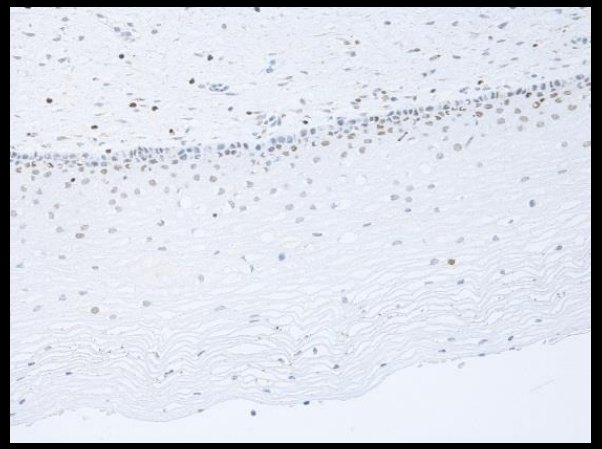
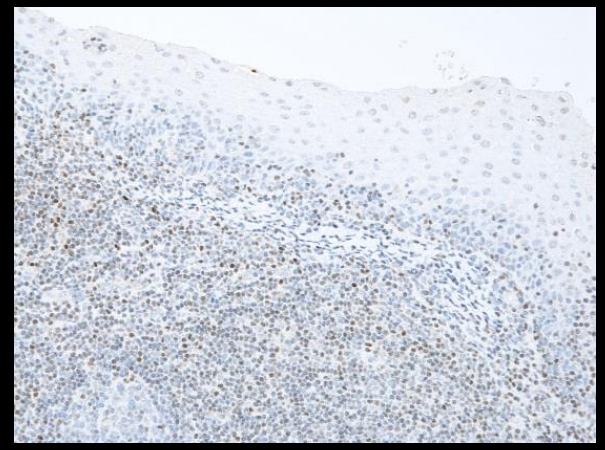
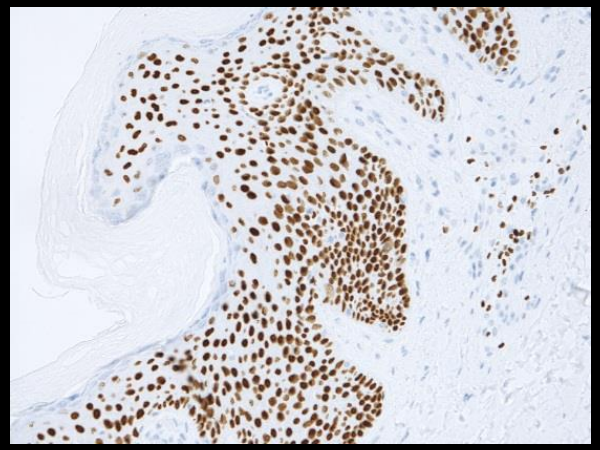
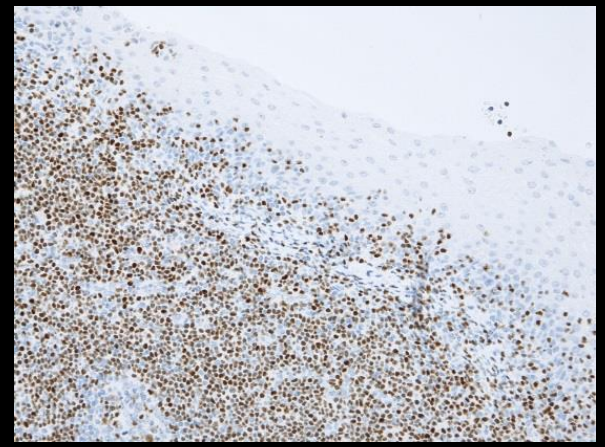
## GATA3, L50-823 (Biocare) 1:800

| | Tonsil | Breast adenocarc. | Colon adenocarc. |
|---|---|---|---|
| Dako Dil. pH7.3 |  |  |  |
| Renoir Red pH 6.2 |  |  |  |

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

# Technical aspects of immunohistochemistry & pitfalls - Analytical phase

## Tissue controls and critical stain indicators: Squamous epithelium ?

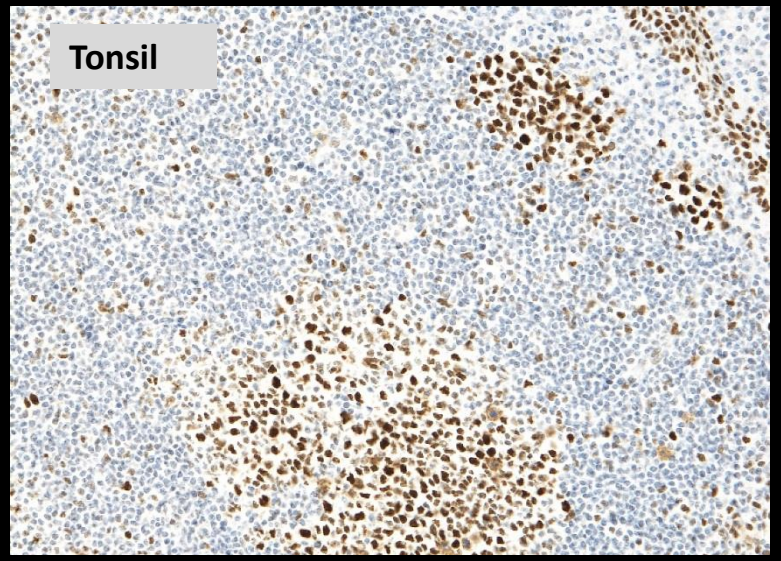
| | Skin (HE) | Cervix (LE) | Tonsil (NE) |
|---|---|---|---|
| GATA3, L50-823<br>mAb 1:50<br>Dako Dil. pH7.3 |  |  |  |
| GATA3, EPR16651<br>rmAb 1:300<br>Renoir R pH 6.2 |  |  |  |

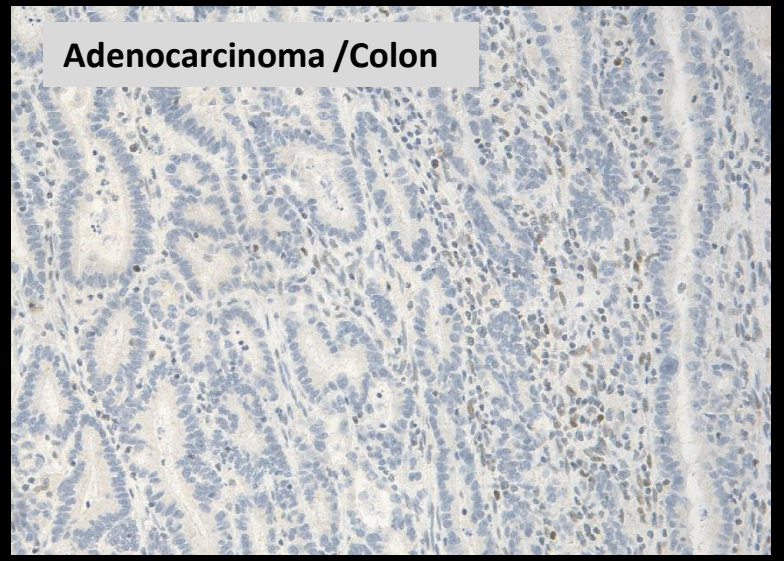
Omnis: HIER/HIGH pH 24` at 97°C, Flex+ Mouse or Flex+ Rabbit (10+20`)

# Technical aspects of immunohistochemistry & pitfalls - Analytical phase

**Antibody diluents**

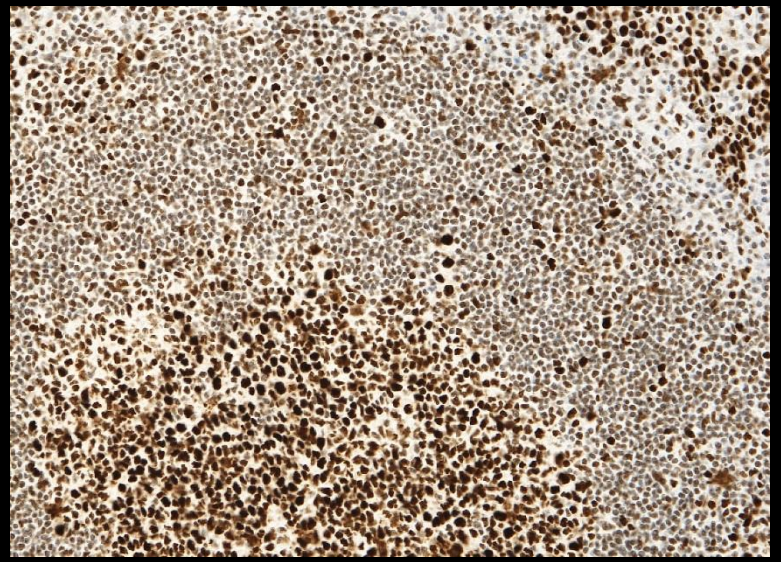
**MSH2, FE11 1:50**
**Dako dil. pH 7.3**

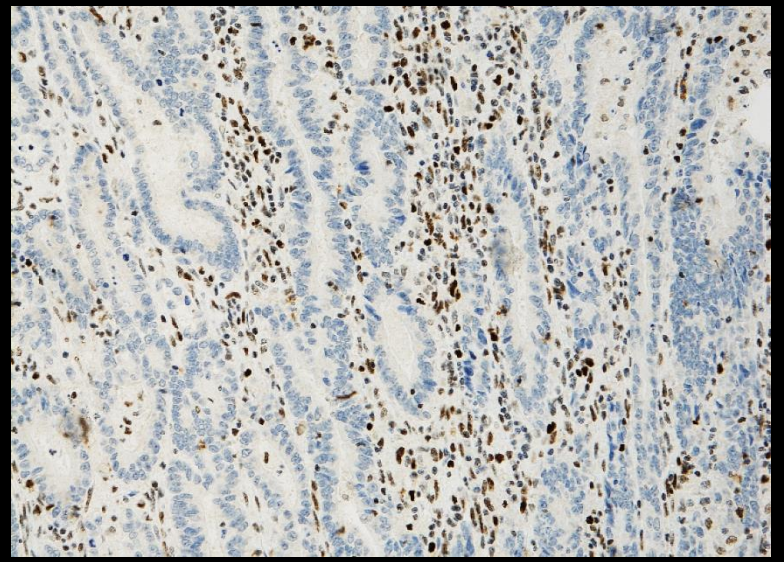



**Omnis: HIER/HIGH pH 24`, Flex+ Mouse (10+20`)**

**MSH2, FE11 1:50**
**Renoir Red pH 6.2**

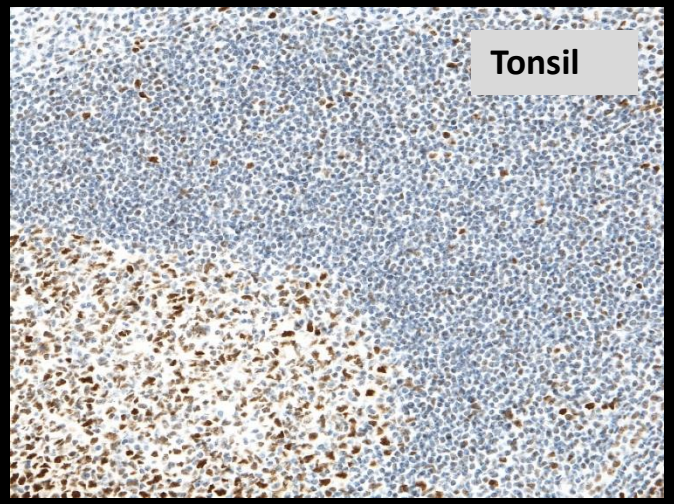


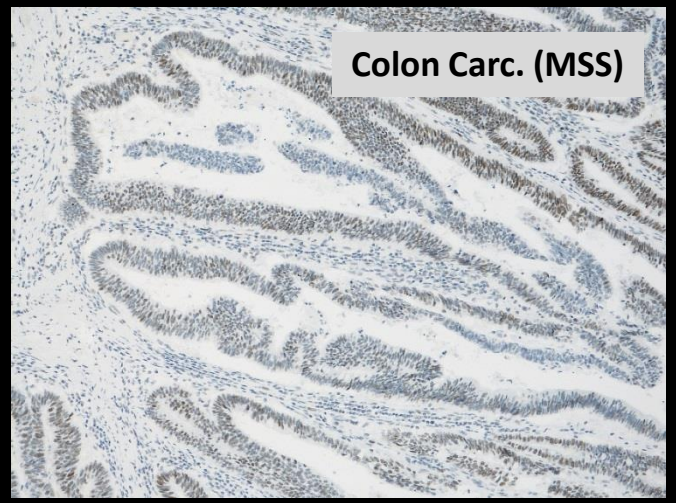


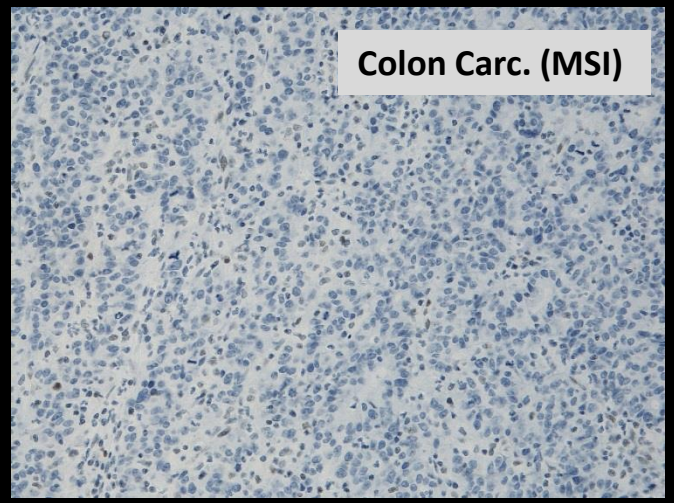
# Technical aspects of immunohistochemistry & pitfalls - Analytical phase

## Antibody diluents

MLH1, BC23 1:40
Dako dil. pH 7.3

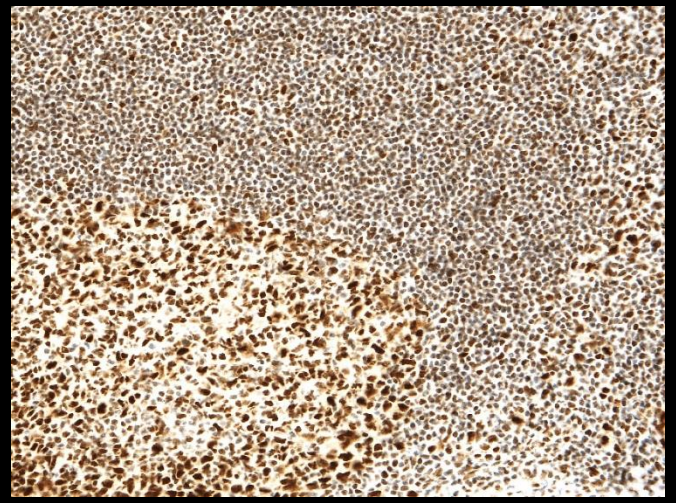


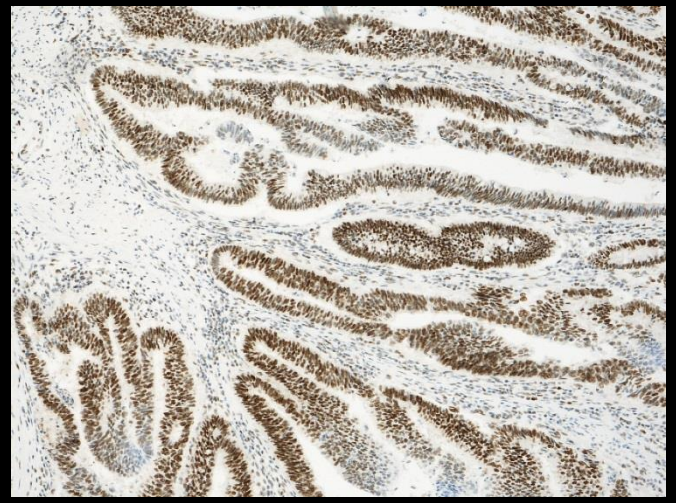



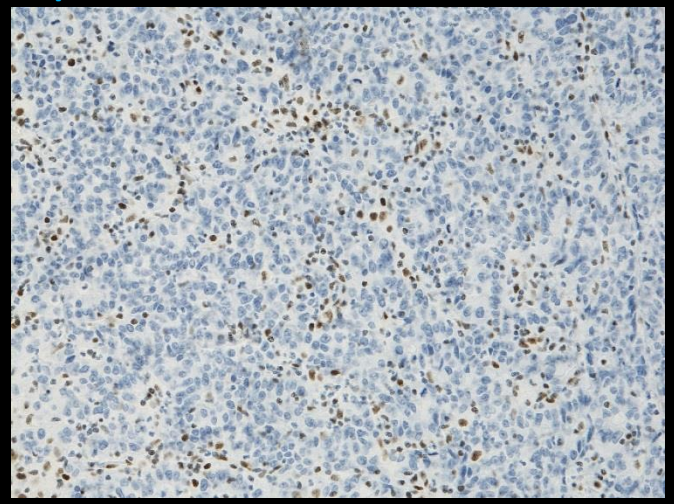




Omnis: HIER/HIGH pH 24`, Flex+ Mouse (10+20`)

MLH1, BC23 1:40
Renoir Red pH 6.2



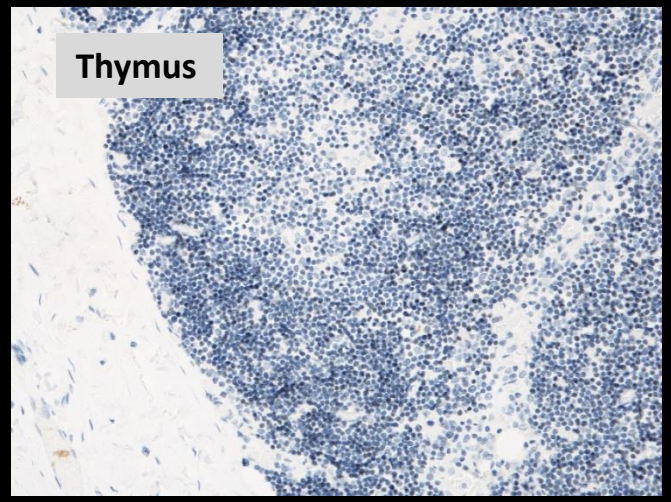


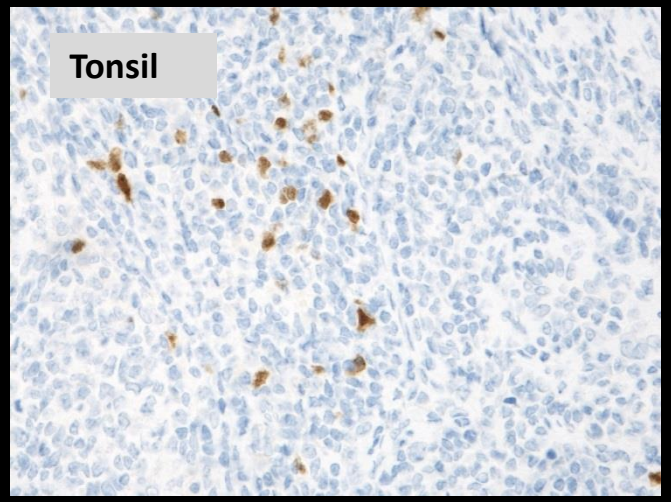


# Technical aspects of immunohistochemistry & pitfalls - Analytical phase

## Antibody diluents

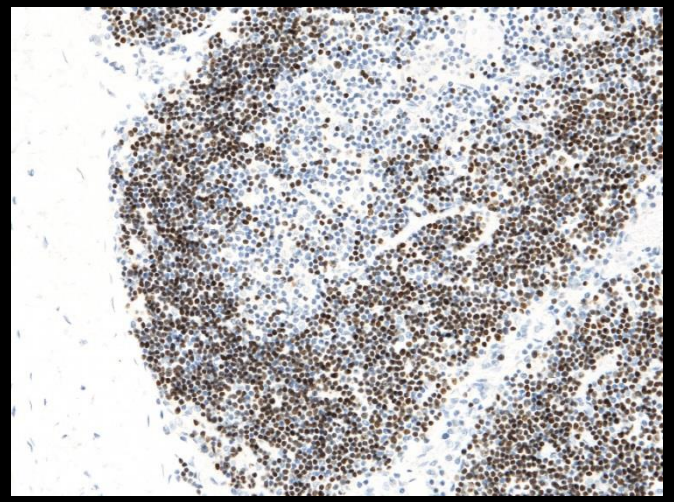
TdT, SEN28 1:50
Dako dil. pH 7.3

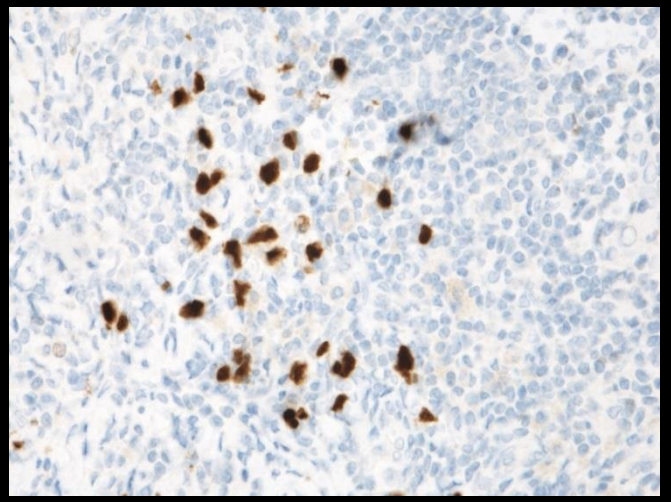



Omnis: HIER/HIGH pH 24`, Flex+ Mouse (10+20`)

TdT, SEN28 1:50
Renoir Red pH 6.2

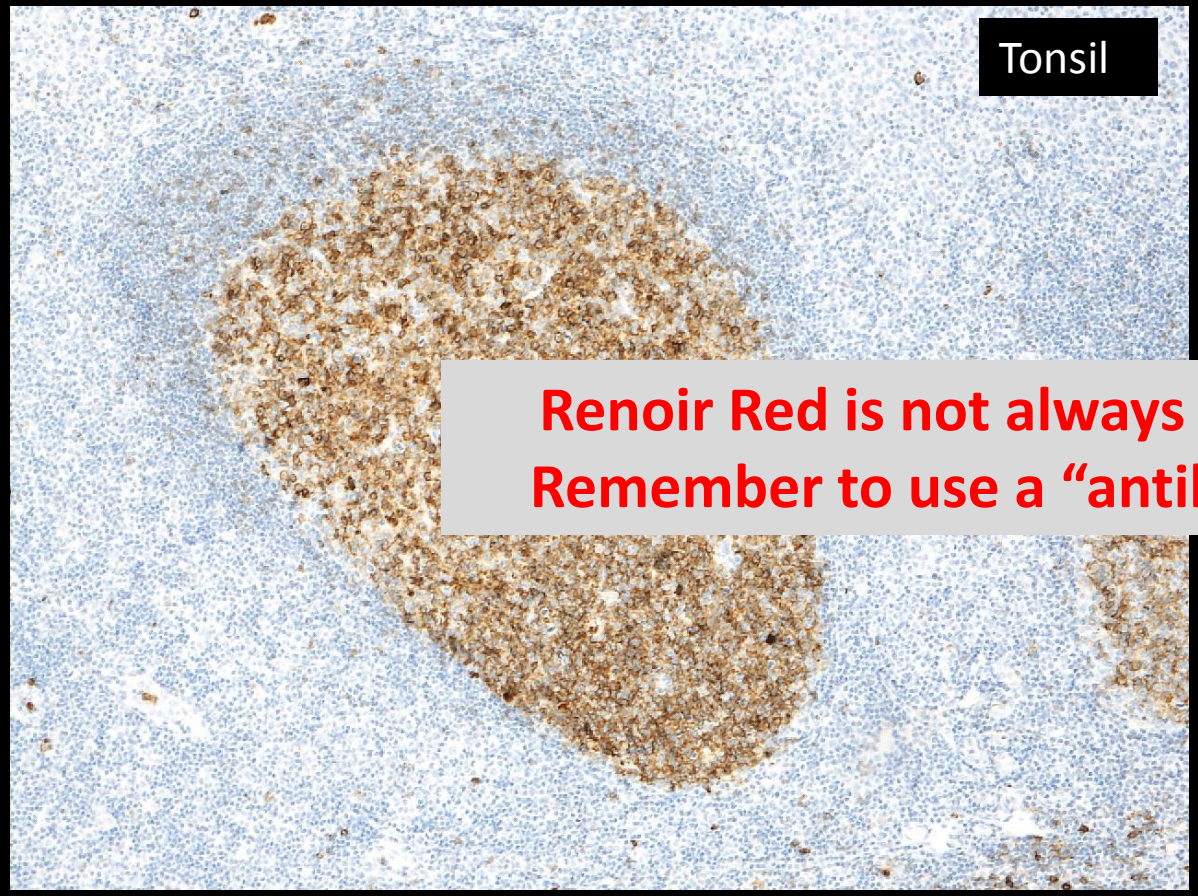




# Technical aspects of immunohistochemistry & pitfalls - Analytical phase

**Problem: Antibody diluent**

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




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

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

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

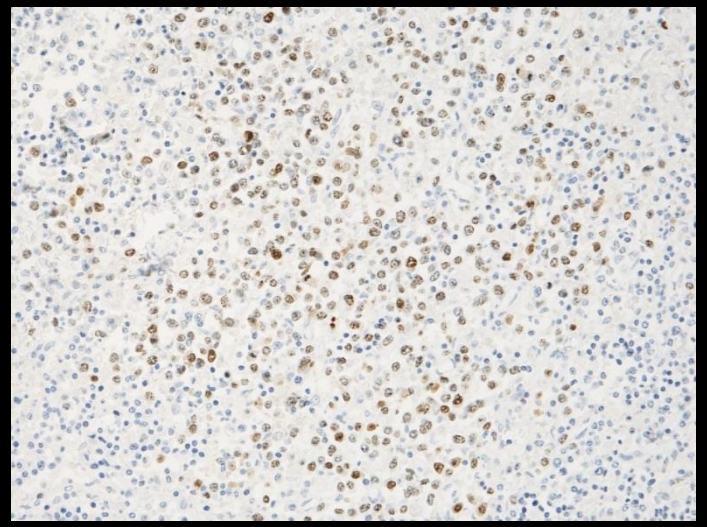
# Technical aspects of immunohistochemistry & pitfalls - Analytical phase

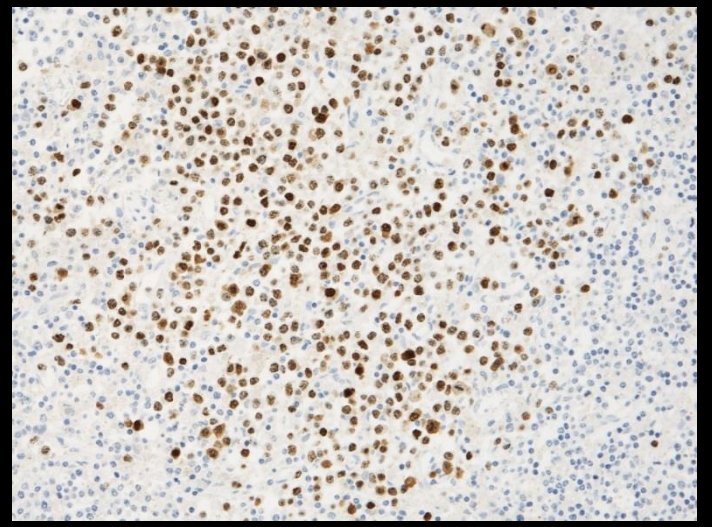
**Antibody diluents**

**HHV8, 13B10**
**1:100 Renoir Red pH 6.2**

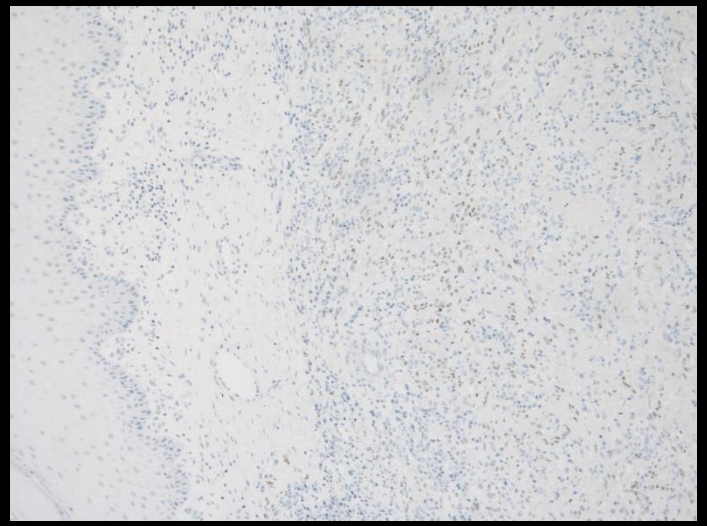
**HHV8, 13B10**
**1:100 Dako dil pH 7.3**

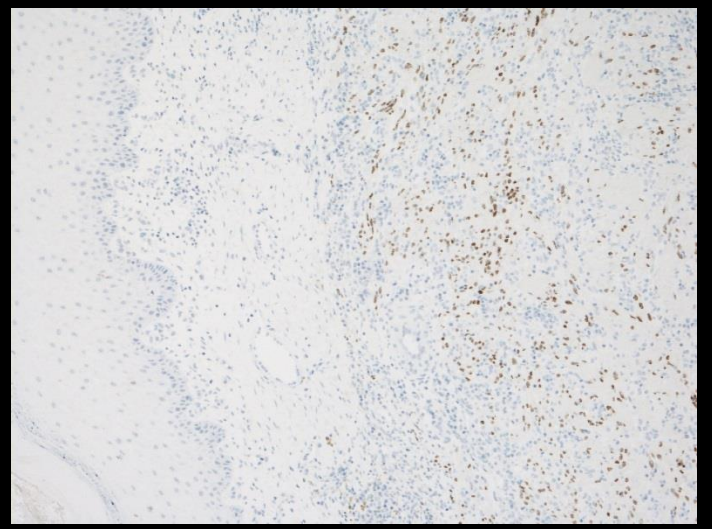
**Case 1**





**Case 2**





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

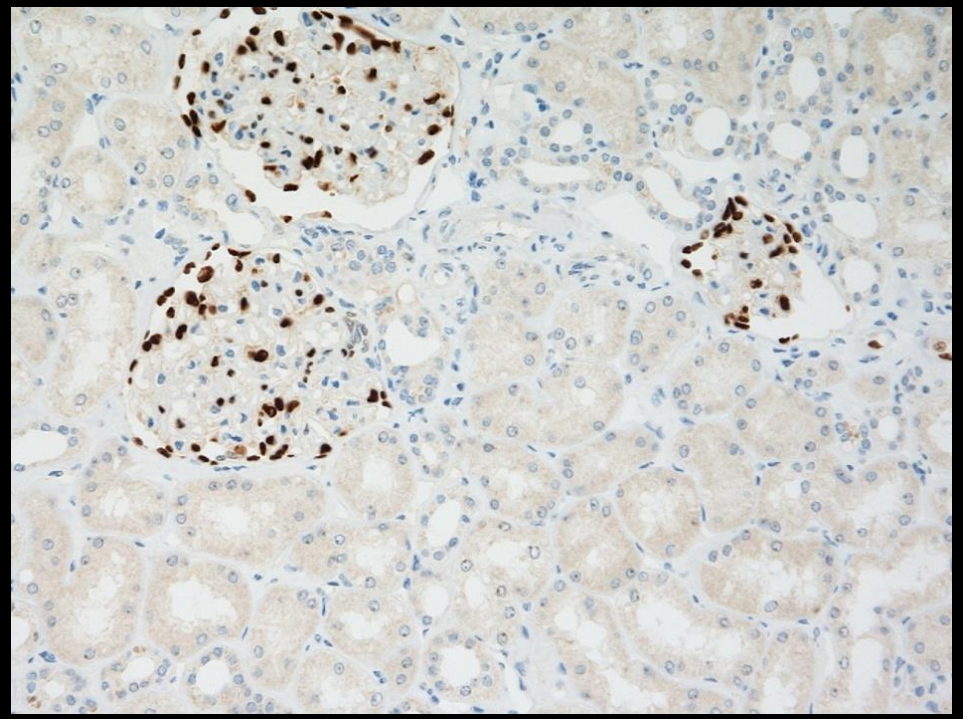
**Immunodeficient patients Kaposi's sarcoma, Castleman's disease, Primary effusion lymphoma ……**

# Technical aspects of immunohistochemistry & pitfalls - Analytical phase

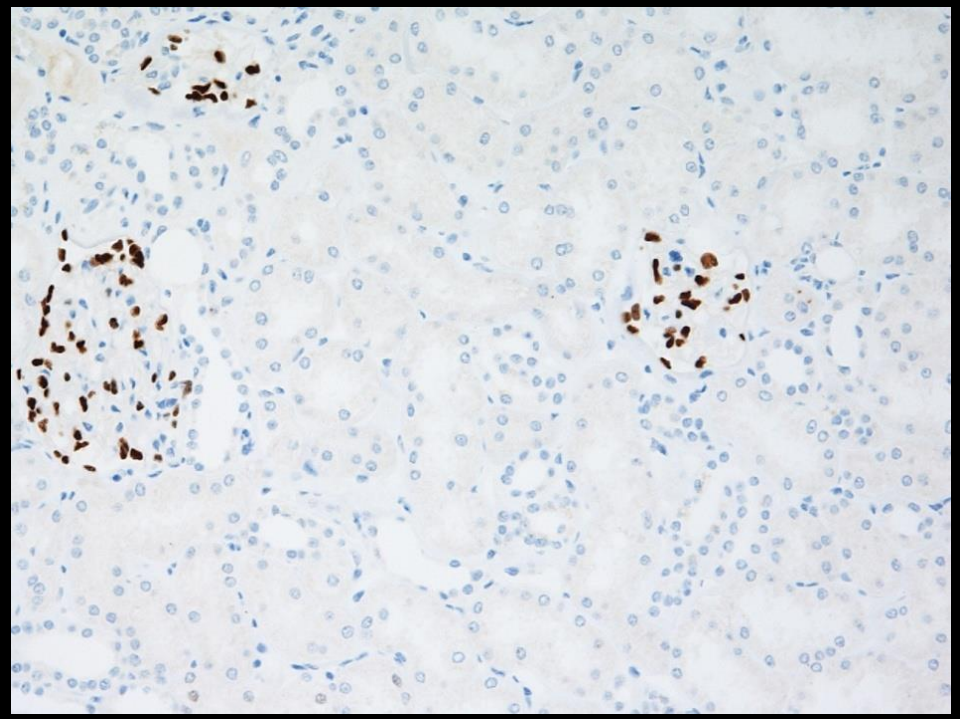
**Antibody diluents**

**Kidney**

**WT1,EP122 1:25**
**Renoir Red (Biocare)**



**WT1,EP122 1:25**
**Background Sniper (Biocare)**



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

**HIER TRS pH9 (24` /97°C) + Pep © (3`)**

# Technical aspects of immunohistochemistry & pitfalls - Analytical phase

## Antibody diluents

**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 (BC23, ES05 & GM011), MSH2 (G219-1129), MSH2 (FE11), MSH6 (EP49), NKX 3.1 (poly), SALL4 (6E3), PAX8 (ZR1), PMS2 (EP51), SOX10 (EP268), SOX11 (C1 & MRQ58), TdT (SEN28 & EP266), UP-II (BC21), WT1 (WT49) and …….

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

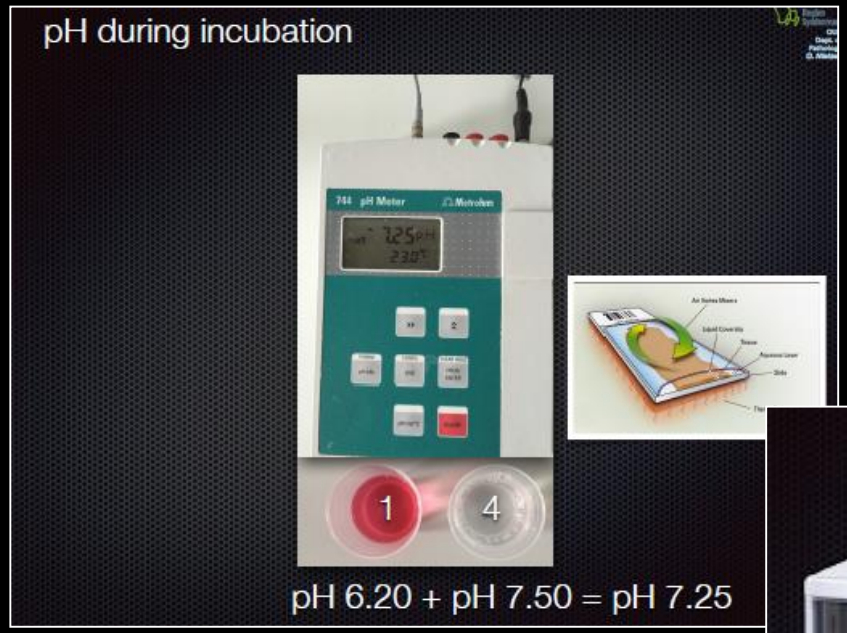
BCL2 (124), BCL6 (LN22 & PG-B6p & GI191E/A3), CR (DAK-Calret1), CD10 (56C6), CD163 (MRQ26), CD21 (2G9), CD5 (4C7), ER (SP1), HHV8 (13B10), Mammaglobin (304-1A5), MUC5AC (CLH2), MUC6 (CLH5), and …….

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

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

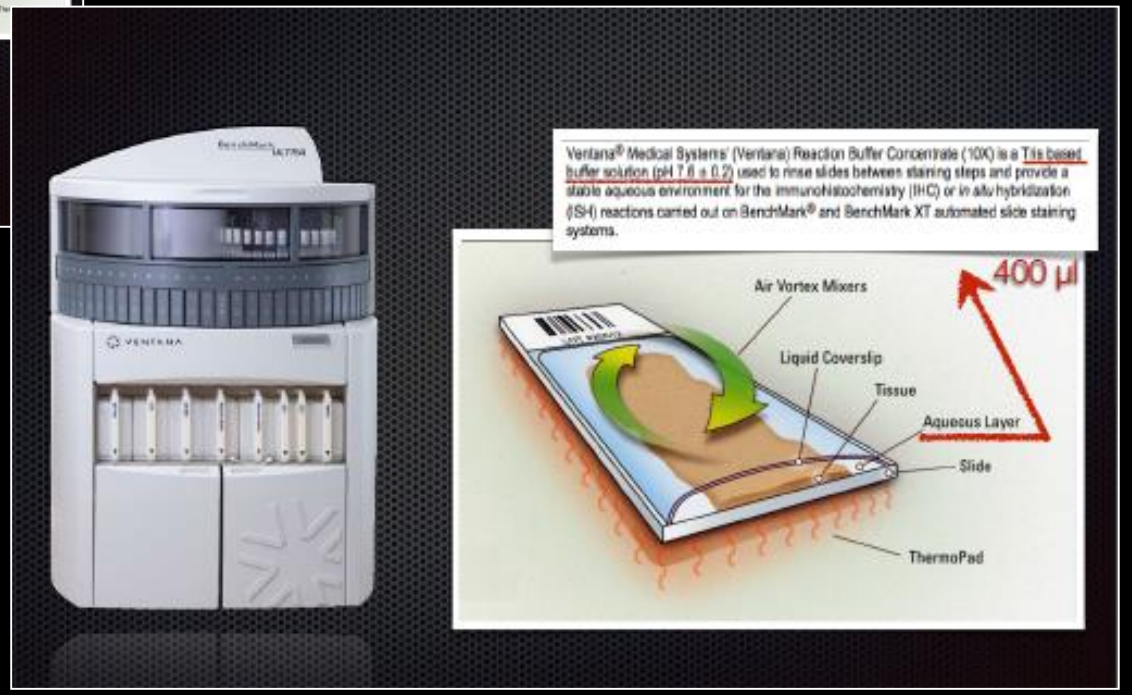
# Technical aspects of immunohistochemistry & pitfalls - Analytical phase

## 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]

## Problems related to the choice of the detection system:

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

**False positive or false negative results**



**Table 3** Major causes of insufficient staining reactions

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

**19% insuff.**

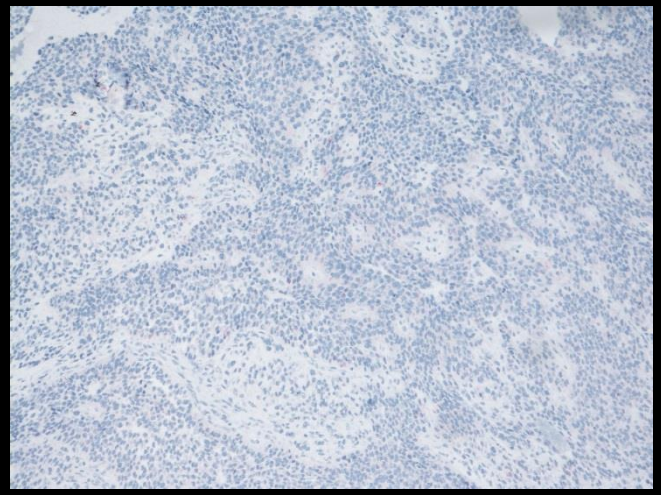
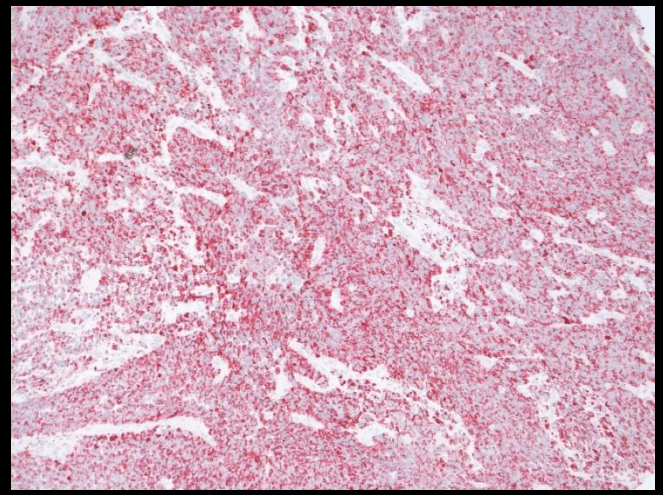
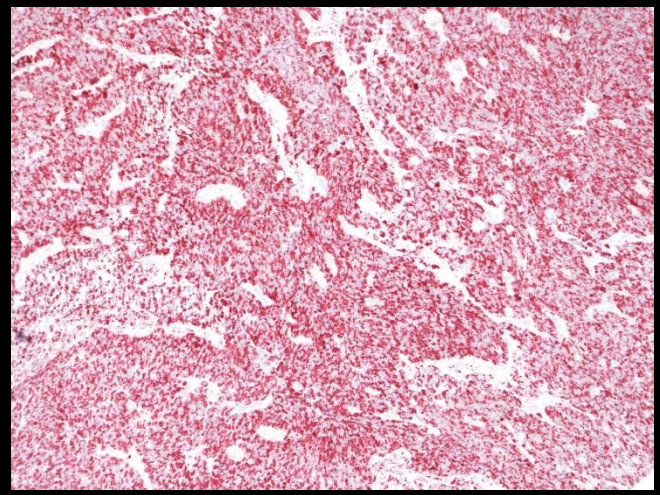
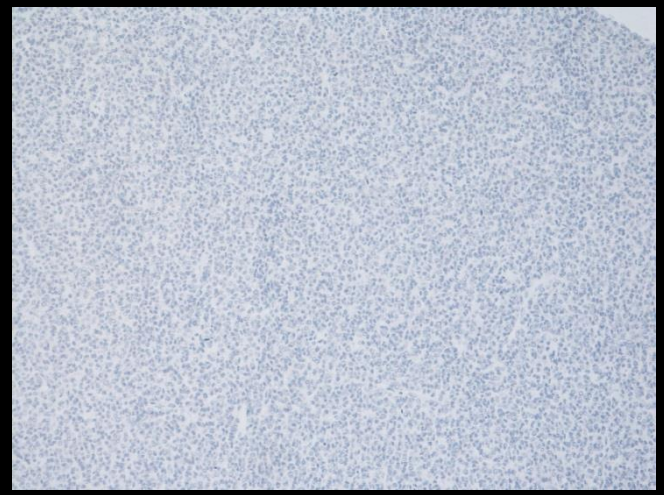
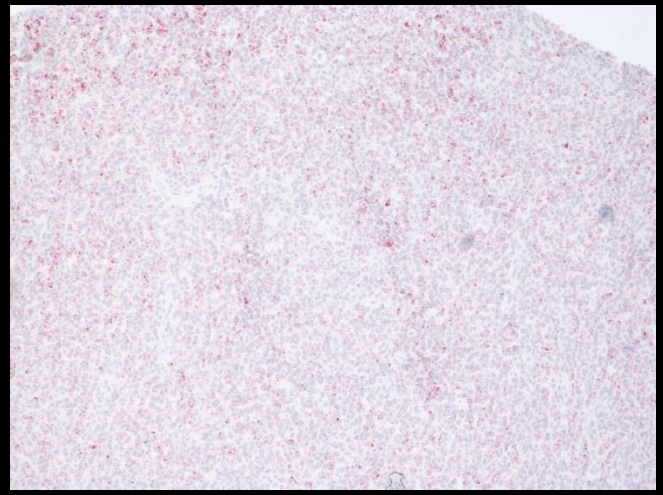
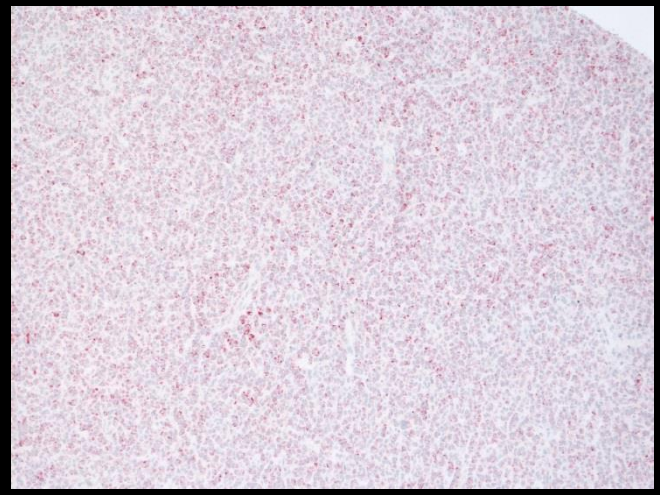
[a] Consistently gives false negative or false positive staining or a poor signal-to-noise ratio in one or more assessment runs

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

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

# Technical aspects of immunohistochemistry & pitfalls - Analytical phase

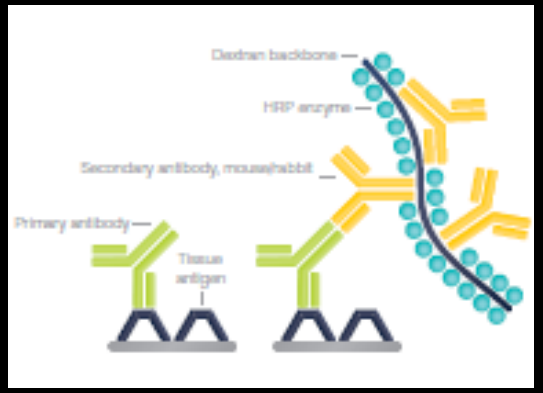
**Omnis**
**MLA, A103 (1:50)**
**HIER High pH 24`**

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

# Technical aspects of immunohistochemistry & pitfalls - Analytical phase

## Detection systems (polymer/multimer) used by NordiQC participants

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

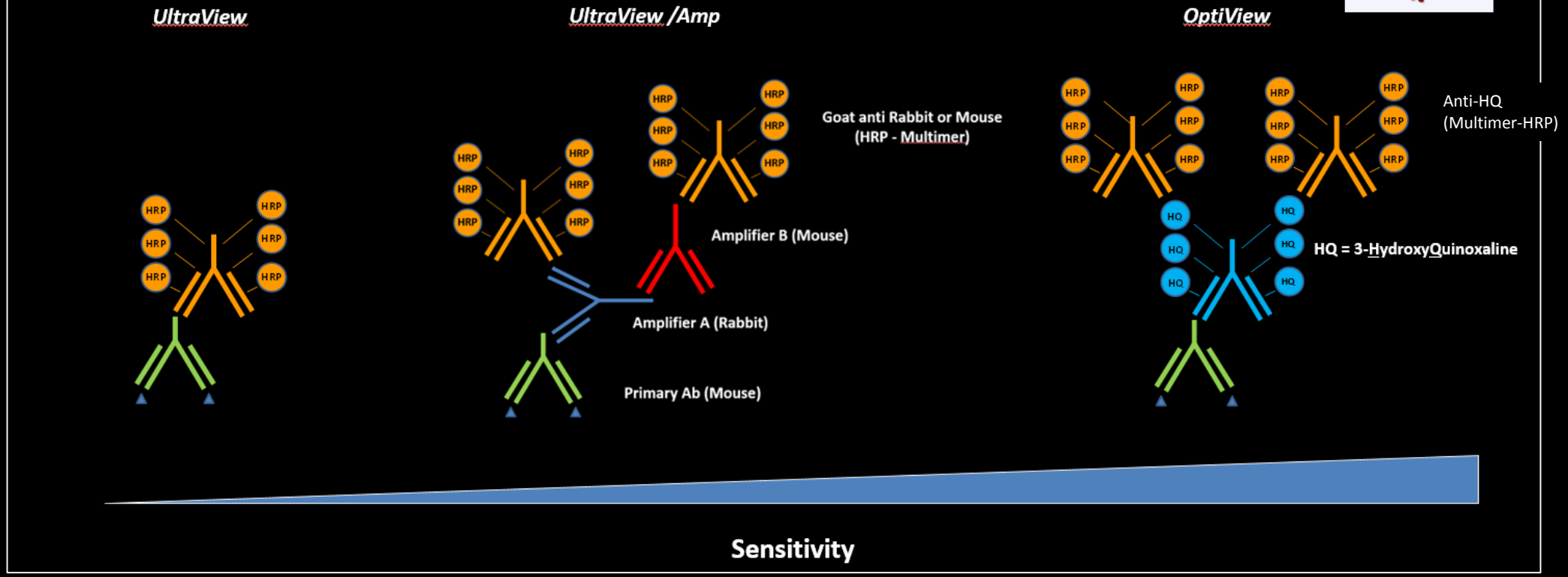



**App. 98% of all NordiQC participants use a polymer/multimer based detection systems**

### Considerations related to the choice of detection system:

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

## Multimer detection systems (Ventana/Roche)

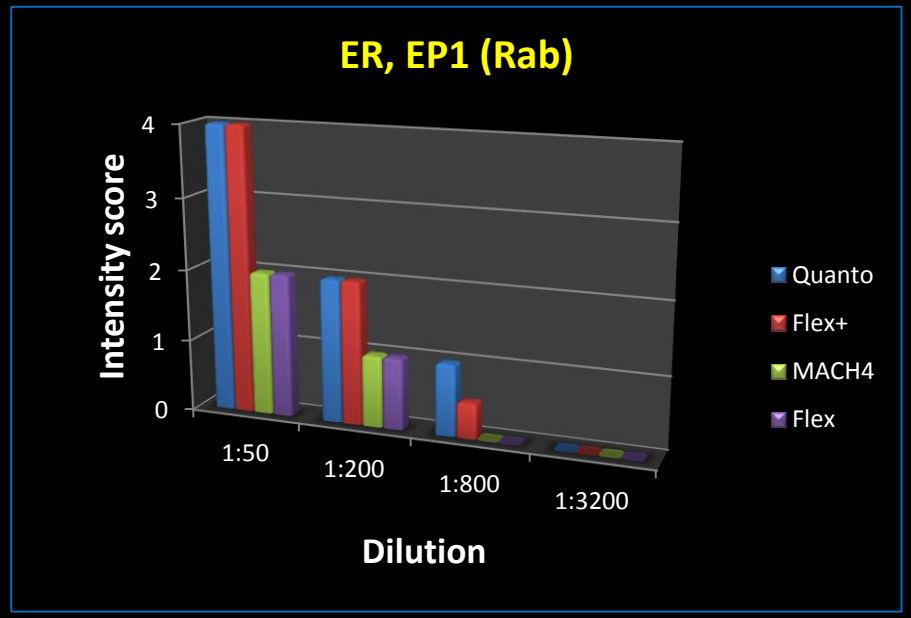

**HQ-Linker**
**Amplifier (A/B)**

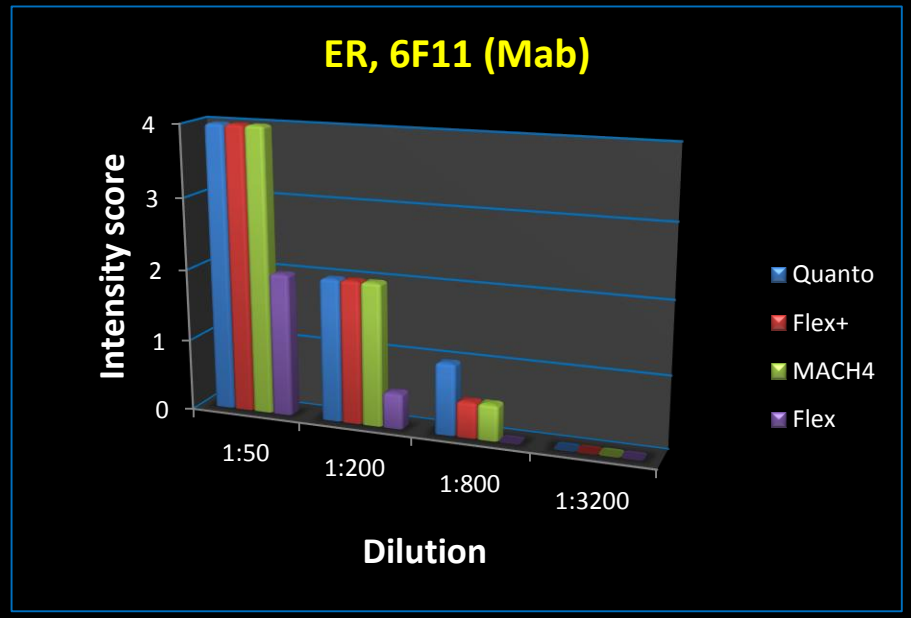
**Linker (Mouse/Rabbit)**
**Enhancer**
**Universal Linker**
**Post Blocking**
**…….**

**Increases sensitivity**

# Technical aspects of immunohistochemistry & pitfalls - Analytical phase

Detection systems - Performance Testing




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

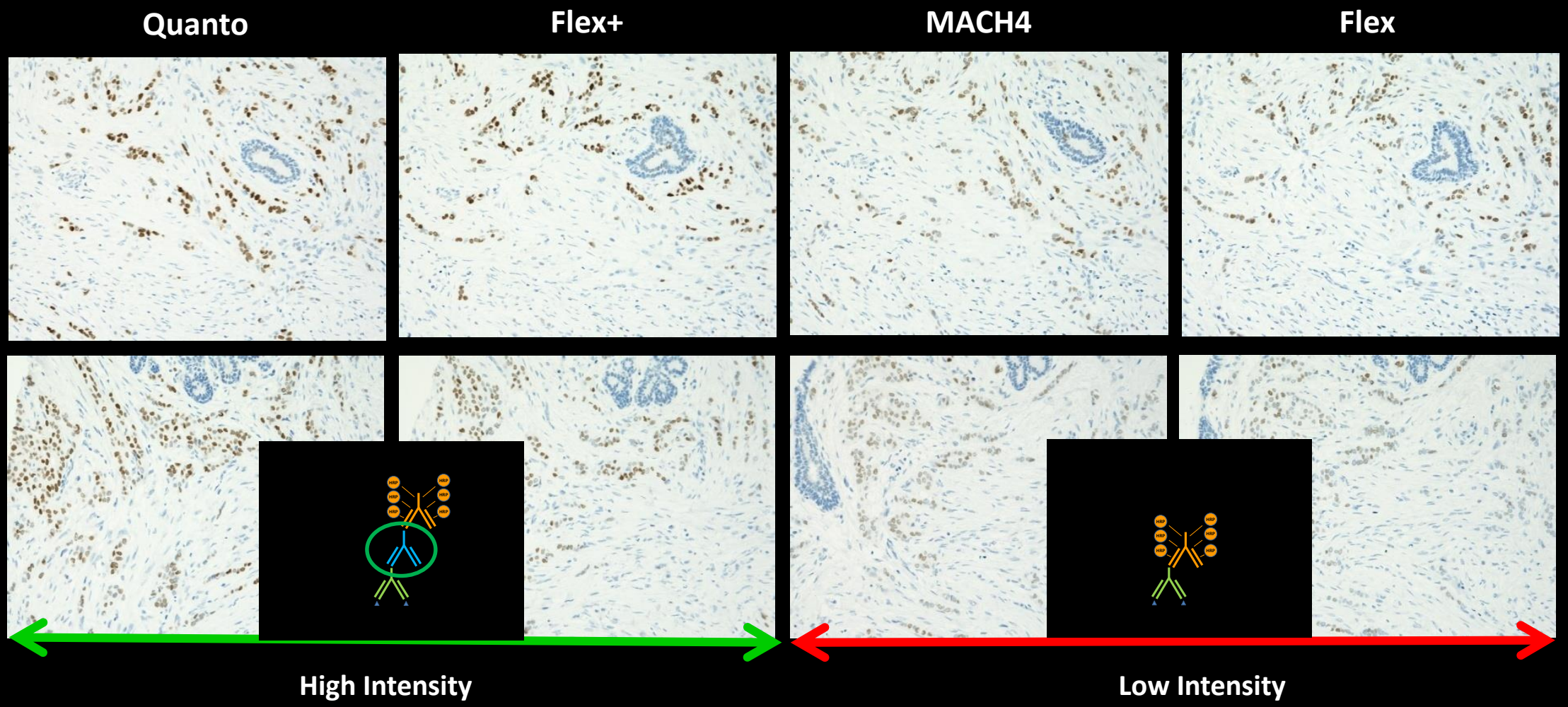
- **The 3-step polymer detection systems Quanto and Flex+ - produced the overall highest intensity.**
- **The 3-step polymer detection system MACH4 only enhances reactions with mouse monoclonal Abs (ER,6F11).**
- **"Optimal staining" was highly influenced by the concentration of the primary Abs and the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

# Technical aspects of immunohistochemistry & pitfalls - Analytical phase

**Performance testing of detection systems (Vendor recommended protocol settings)**

**Breast tumor**

## ER, EP1 (Rab)

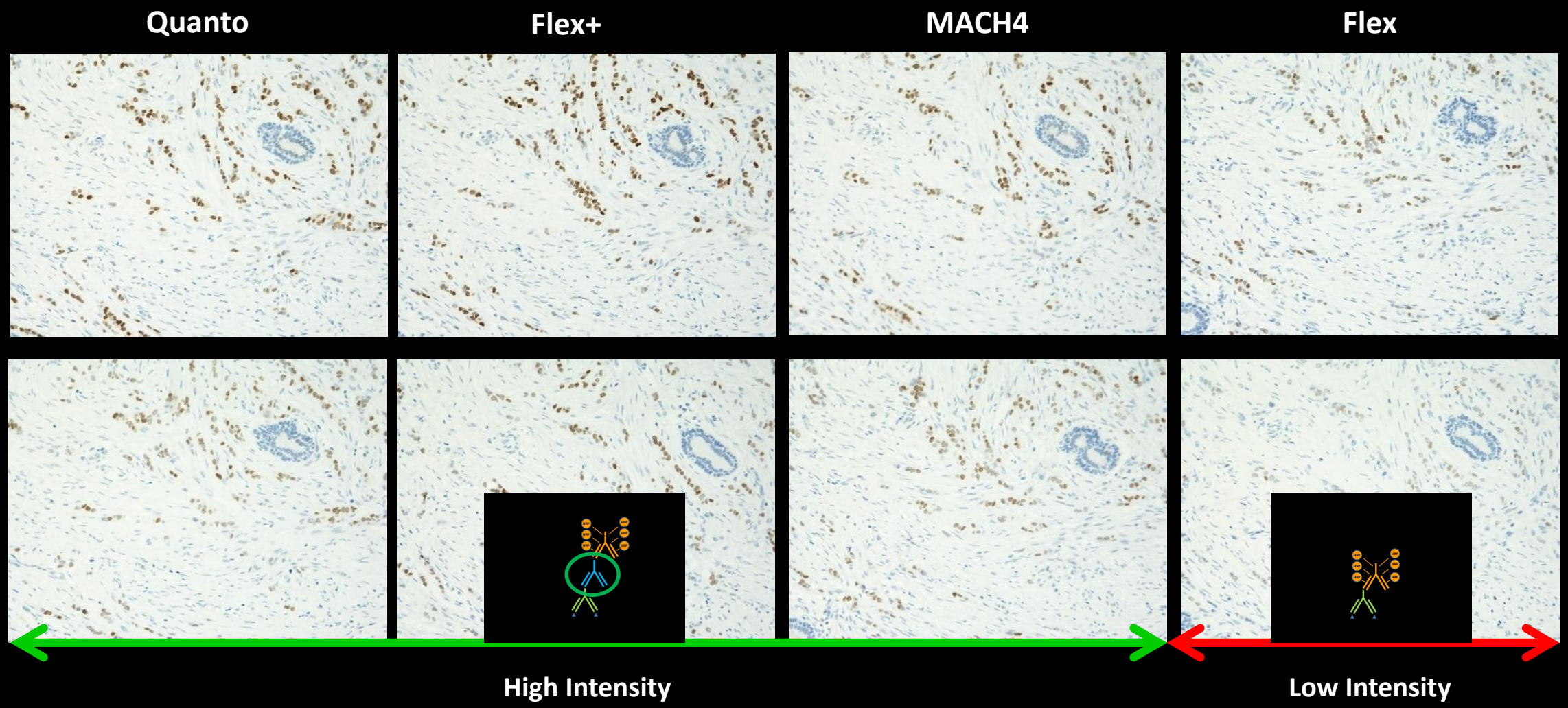


# The technical test approach – Analytical phase

**Performance testing of detection systems (Vendor recommended protocol settings)**

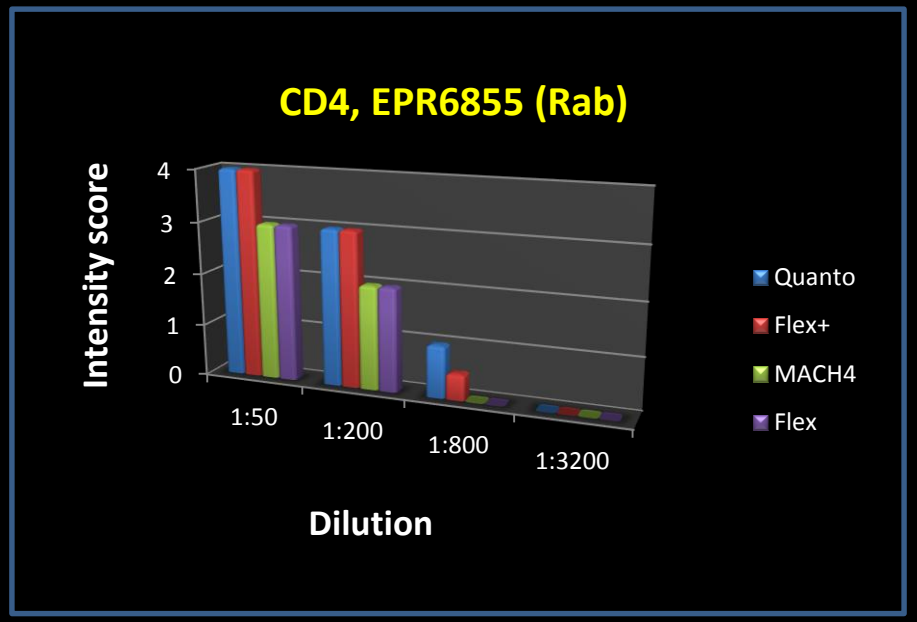
**Breast tumor**

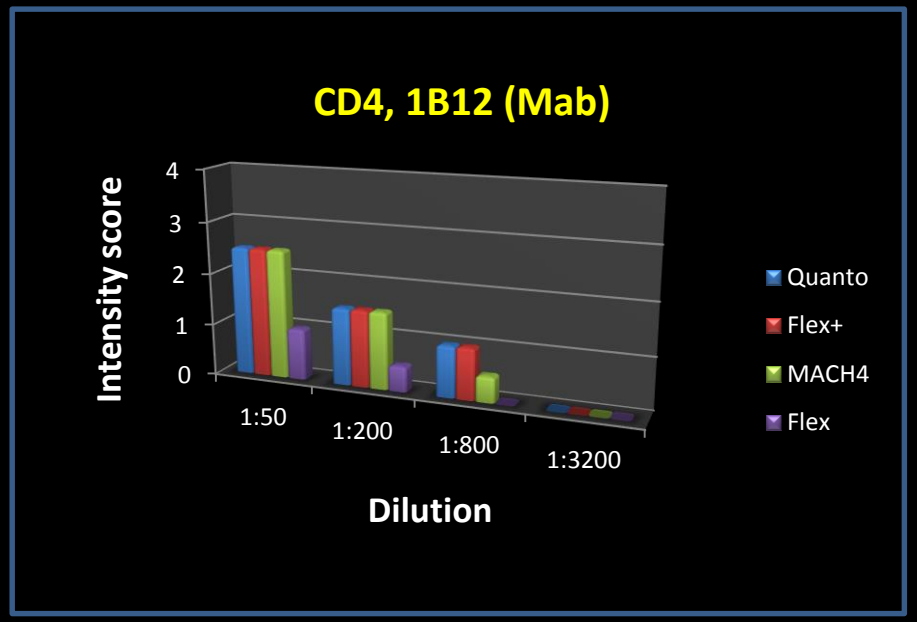
## ER, 6F11 (Mab)



# Technical aspects of immunohistochemistry & pitfalls - Analytical phase

## Detection systems - Performance Testing




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

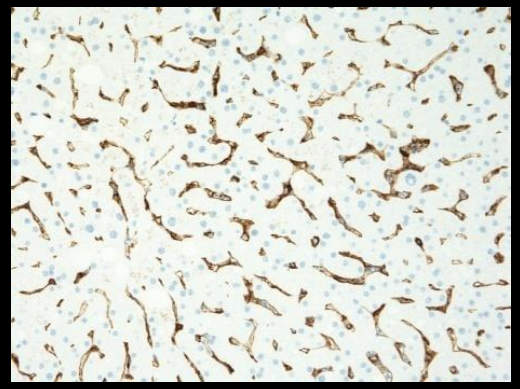
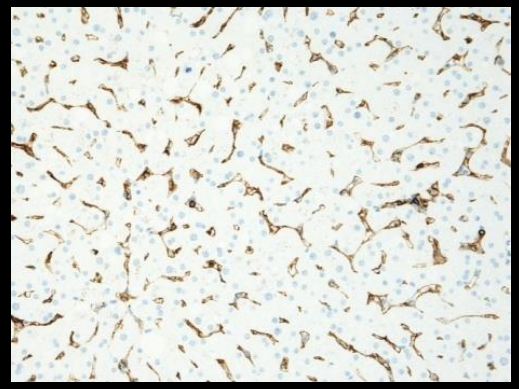
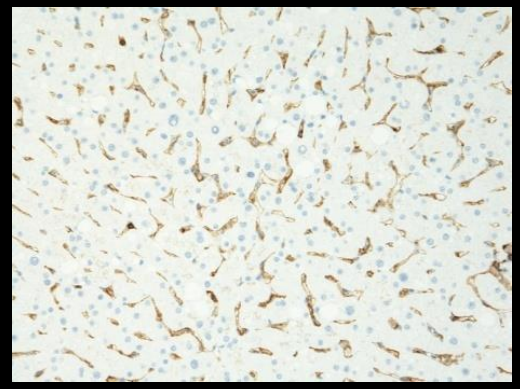
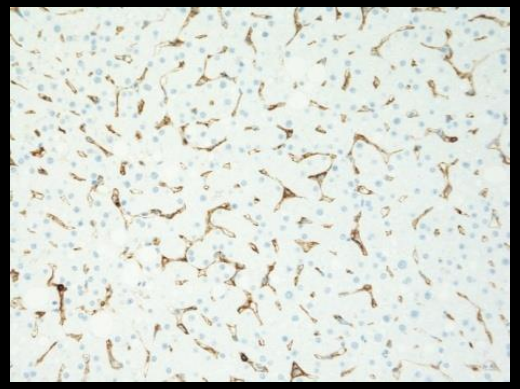
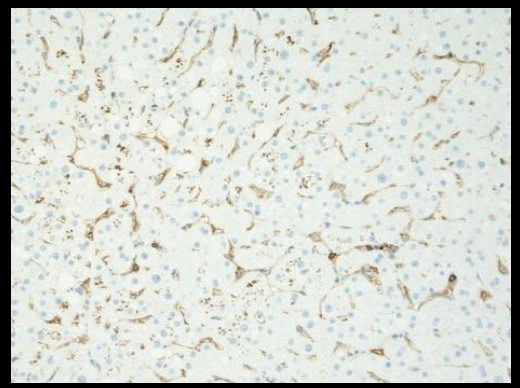
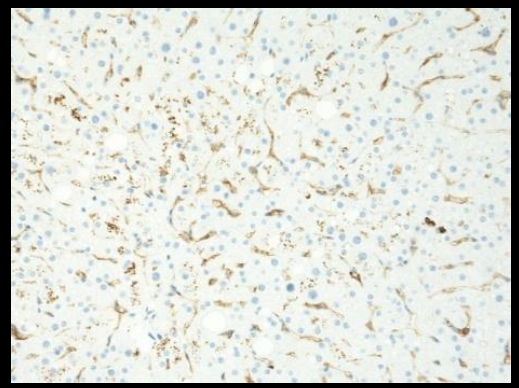
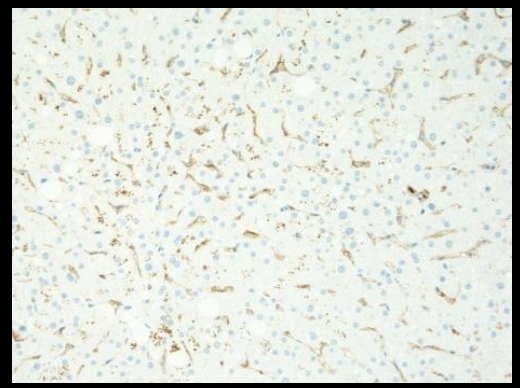
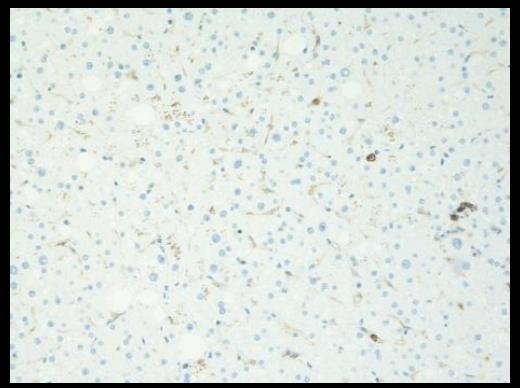
- The 3-step polymer detection systems Quanto and Flex+ - produced the overall highest intensity.
- Intensity was highly influenced by the nature of primary Ab and "optimal" staining could only be obtained with the Rab (CD4, EPR6855) used in combination with the 3- step polymer detection systems Quanto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

# Technical aspects of immunohistochemistry & pitfalls - Analytical phase

**Performance testing of detection systems (Vendor recommended protocol settings)**

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

**Liver**

| | Quanto | Flex+ | MACH4 | Flex |
|---|---|---|---|---|
| EPR6855 |  |  |  |  |
| 1B12 |  |  |  |  |

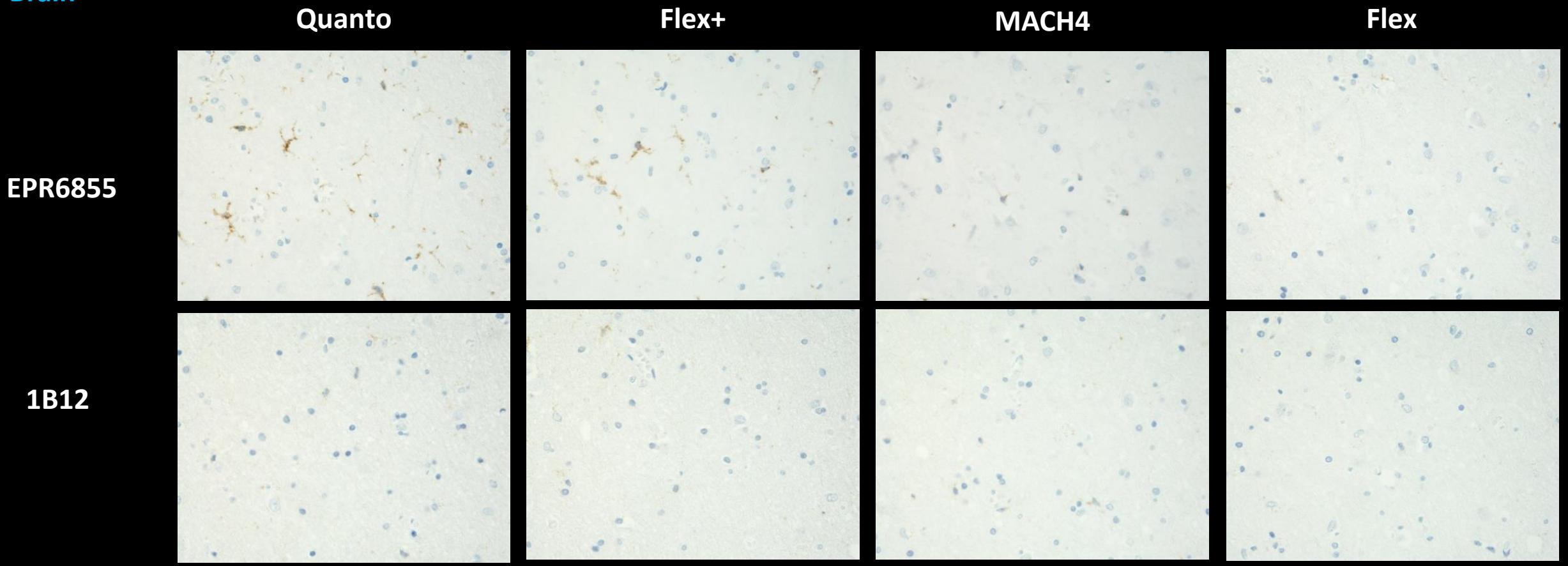
**Strong staining of hepatic sinusoidal endothelial cells and kupffer cells using the Rab (CD4, EPR6855) with all the detection system tested (2-step or 3-step polymer systems) . Intensity is significantly reduced using the Mab (CD4, 1B12).**

# Technical aspects of immunohistochemistry & pitfalls - Analytical phase

**Performance testing of detection systems (Vendor recommended protocol settings)**

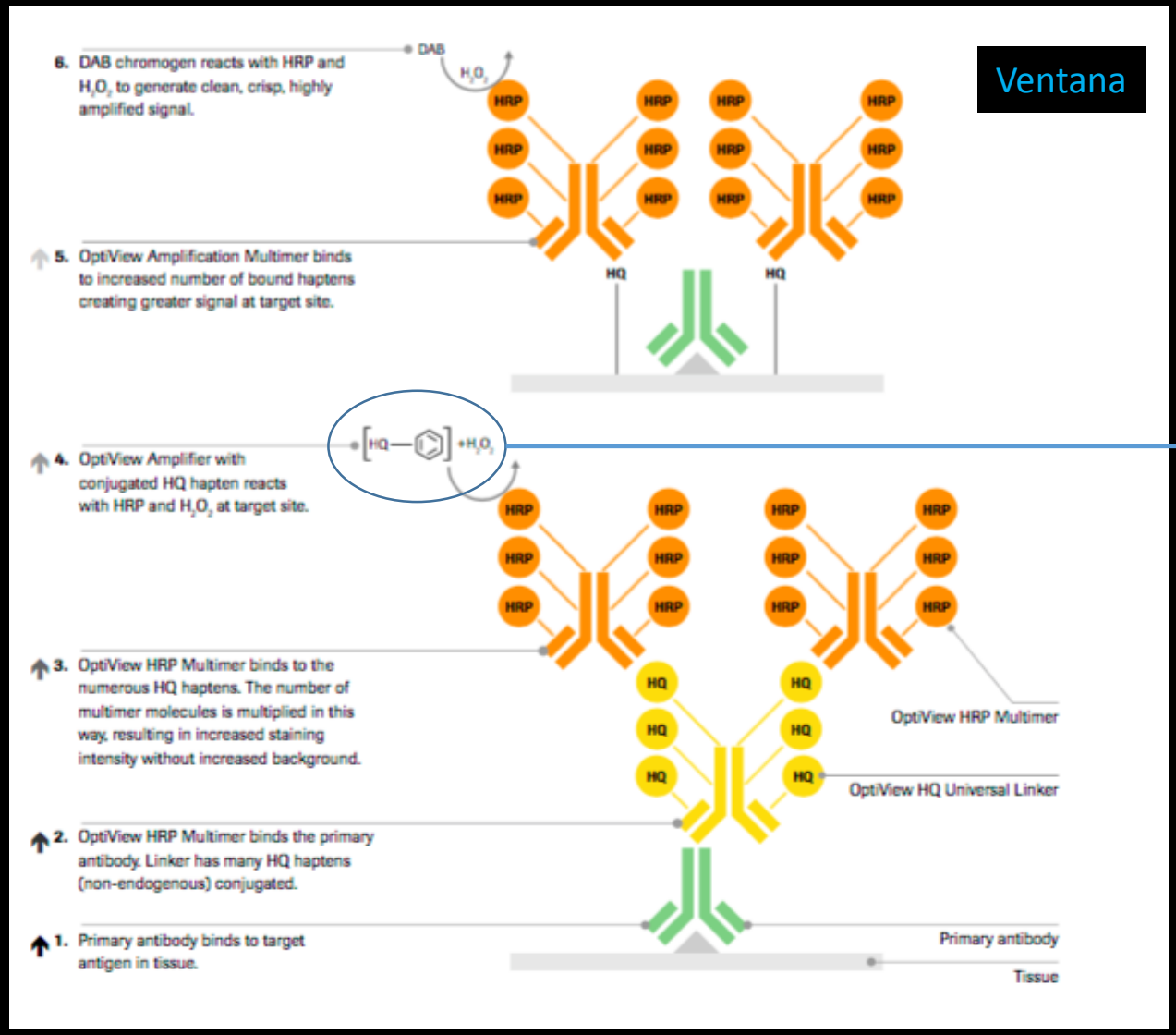
**CD4, EPR6855 (Rab, 1:50) and 1B12 (Mab, 1:50)**

**Brain**




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

# Technical aspects of immunohistochemistry & pitfalls - Analytical phase




## Tyramide Signal Amplification

**Mechanism of Tyramide amplification:**

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

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

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

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

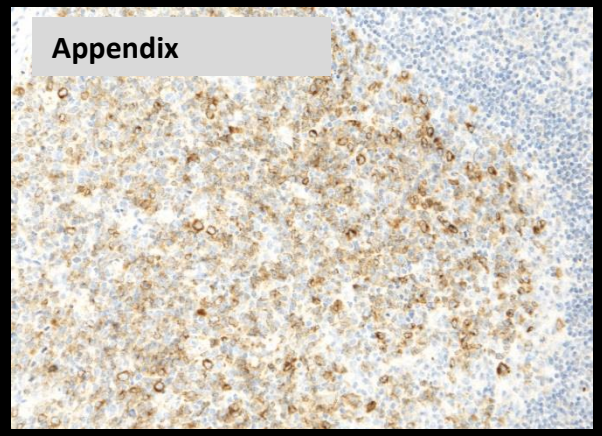
- Detection of HQ with anti-HQ / HRP Multimer
- Visualization with DAB

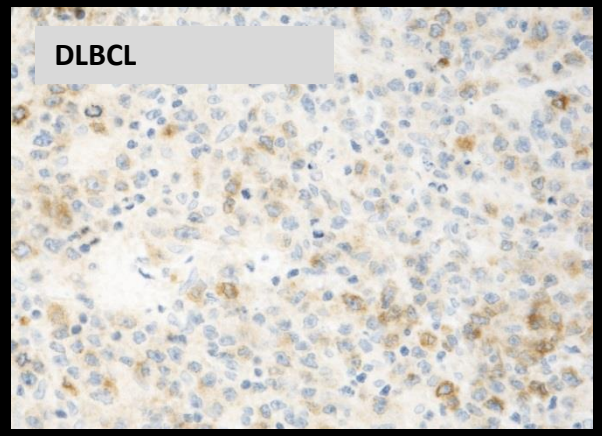
# Technical aspects of immunohistochemistry & pitfalls - Analytical phase

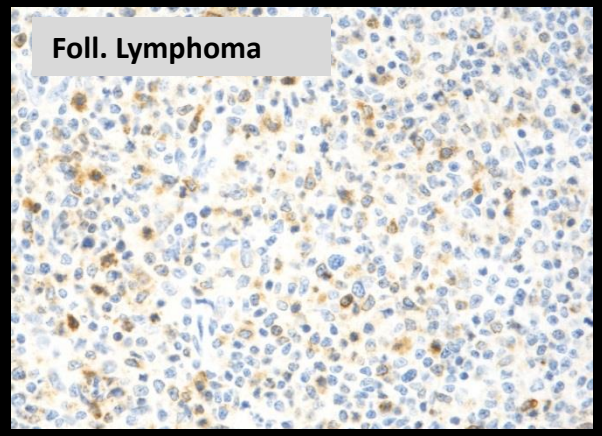
**Primary antibodies sensitive to the chosen platform**

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

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

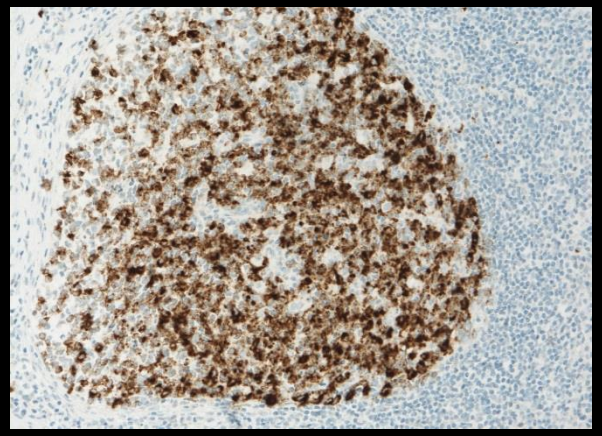


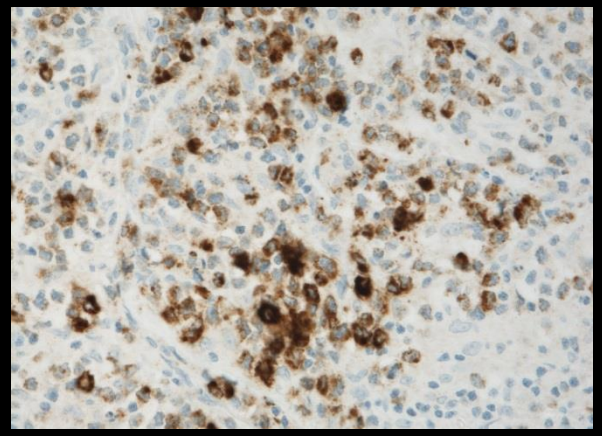



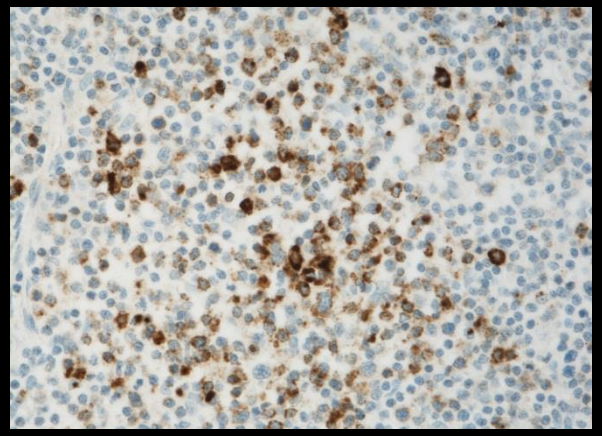




**Appropriate protocol library and immunoreagents (Omnis) ?**

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





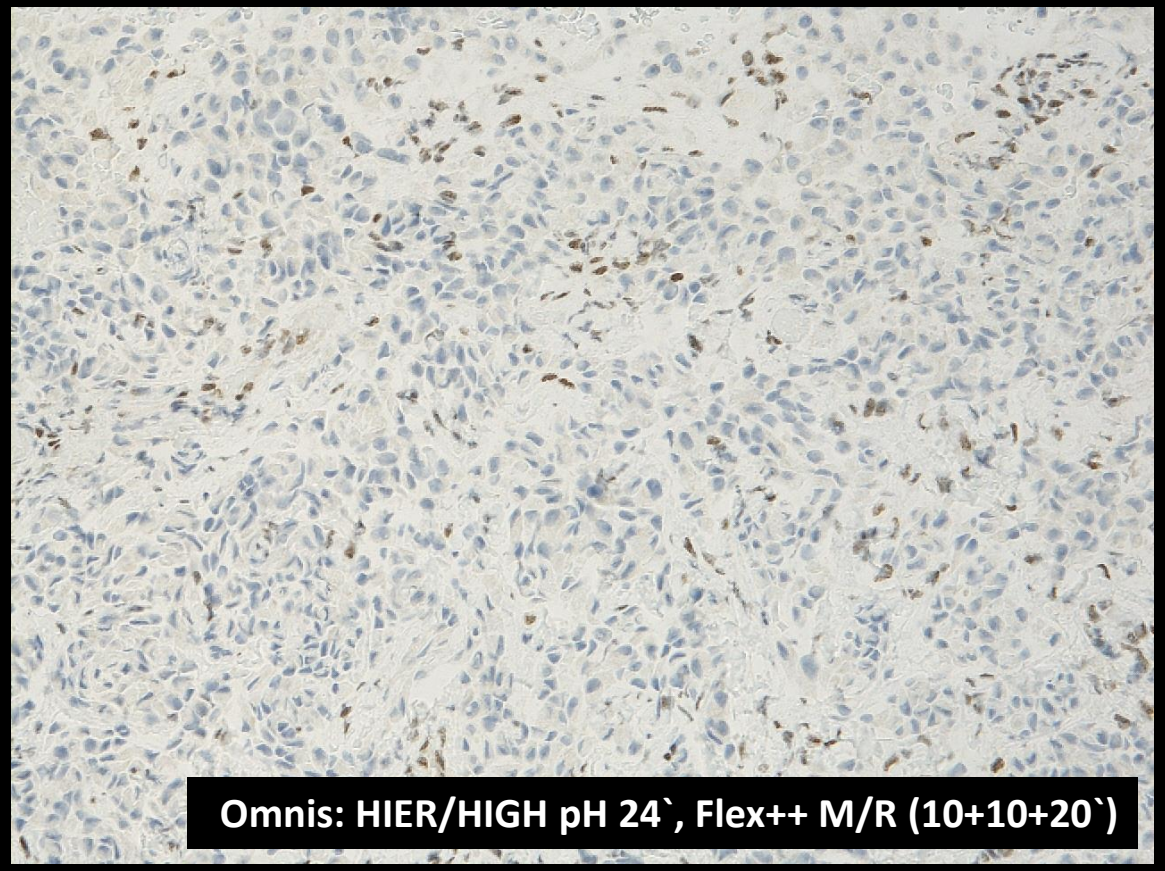


Data & method kindly provided by Ole Nielsen, Dept. of Pathology, Odense, Denmark

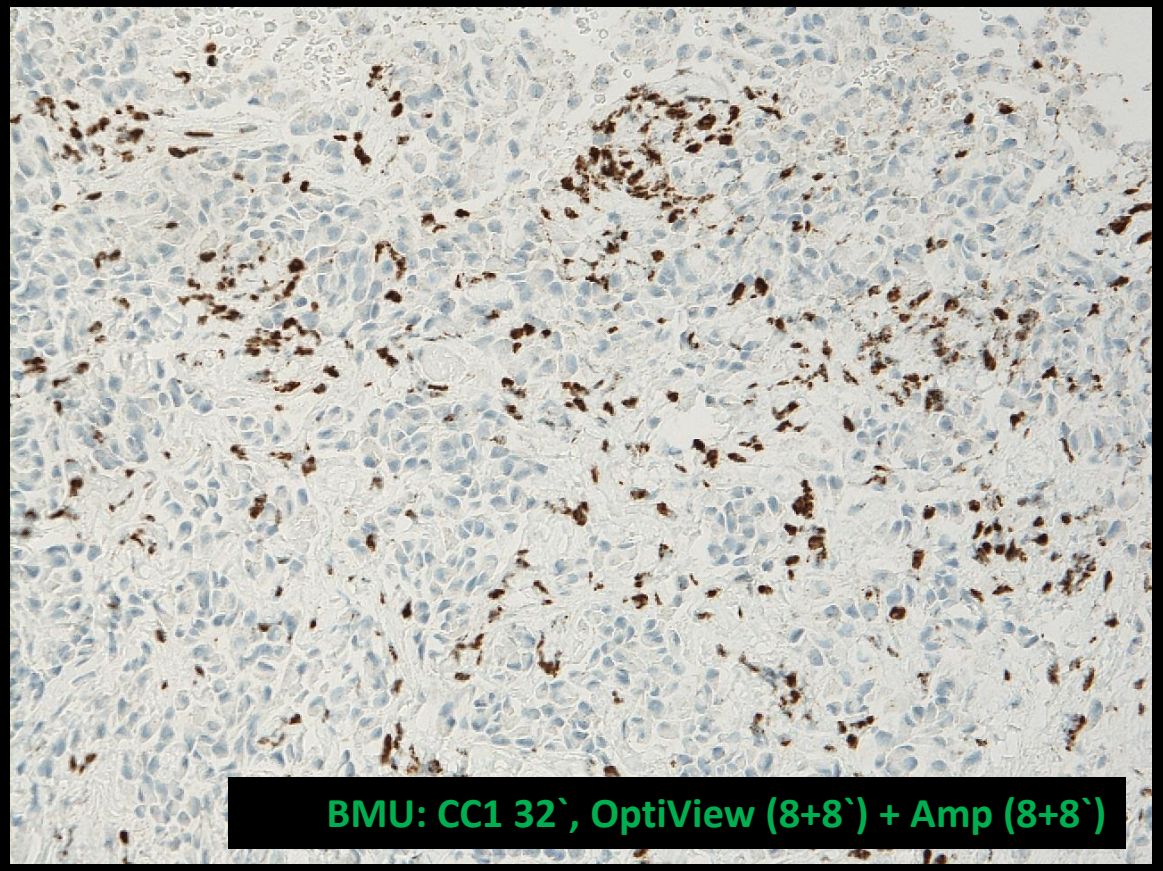
# Technical aspects of immunohistochemistry & pitfalls - Analytical phase

**Problem: Detection System**

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



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



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

# Technical aspects of immunohistochemistry & pitfalls - Analytical phase

## 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<br>3<br>2<br>1<br>1<br>1 | Leica/Novocastra<br>Thermo/NeoMarkers<br>Monosan<br>Abcam<br>Biocare<br>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<br>1 | Cell Signaling<br>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 provides optimal results but……..**

**Ready-To-Use antibodies and corresponding systems**

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

mAb clone **5A4**, product no. **MAB-0281**, Maixin, manual staining:
One protocol with an optimal result was based on HIER using Citrate buffer pH 6 (efficient heating time 2 min. at 120°C), 60 min. incubation of the primary Ab. and KIT-5230 (Maixin) as detection system.

rmAb clone **D5F3** product no. **790-4794**, Ventana, BenchMark GX, XT and Ultra:
Protocols with optimal results were typically based on HIER using Cell Conditioning 1 (efficient heating time 32-92 min.), 16-44 min. incubation of the primary Ab. and OptiView (760-700) + amplification kit (760-099) as detection system. Using these protocol settings, 45 of 47 (96%) laboratories produced a sufficient staining result.

rmAb clone **D5F3** product no. **790-4843**, CDx Assay, Ventana, BenchMark XT and Ultra:
Protocols with optimal results were typically based on HIER using Cell Conditioning 1 (efficient heating time 72-92min.), 16-36 min. incubation of the primary Ab. and OptiView (760-700) + amplification kit (760-099) as detection system. Using these protocol settings, 3 of 3 (100%) laboratories produced a sufficient staining result.

**TSA**

# Technical aspects of immunohistochemistry & pitfalls - Analytical phase

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


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

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**Author for correspondence*

Received 8 September 1998 and in revised form 1 December 1998

**Summary**

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

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

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

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

## TSA 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

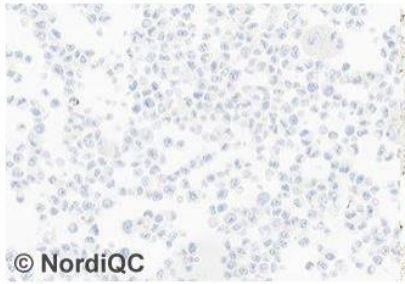



Fig. 6a
Optimal ALK staining of the cell line without ALK rearrangement using same protocol as in Figs. 1a – 5a. All cells are unstained.

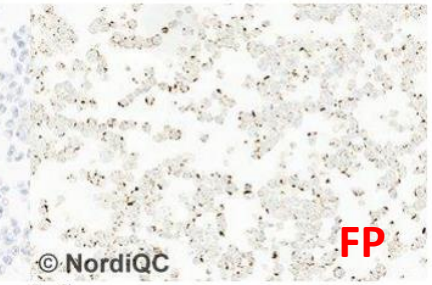



Fig. 6b
Aberrant ALK staining of the cell line without ALK rearrangement using the Ventana Ready-To-Use system based on the rmAb clone D5F3, prod. No. 790-4794. The vast majority of cells show an intracytoplasmic dot-like staining reaction.
This aberrant result was seen in a high number of protocols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

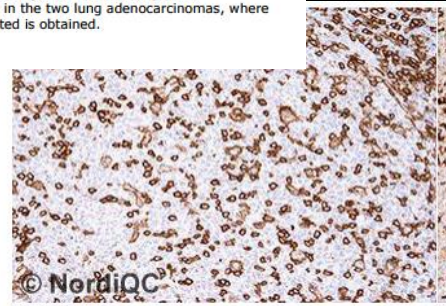



Fig. 1a (x200)
Optimal CD4 staining of the tonsil using the rmAb clone SP35 as a concentrate, HIER in an alkaline buffer (CC1 pH 8.5) and a 3-step multimer based detection system (OptiView, Ventana) - same protocol used in Figs. 2a - 4a. The inducer/helper T-cells show a strong staining reaction, while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

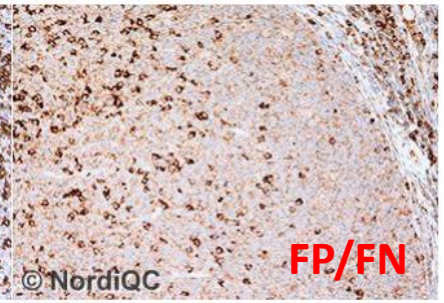



Fig. 1b (x200)
Insufficient CD4 staining of the tonsil using the mAb 4B12 as a concentrate, HIER in an alkaline buffer (CC1 pH 8.5) and a multimer based detection system (OptiView with Tyramide amplification, Ventana) -same protocol used in Figs. 2b-4b. The protocol provided a too low sensitivity, but 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**

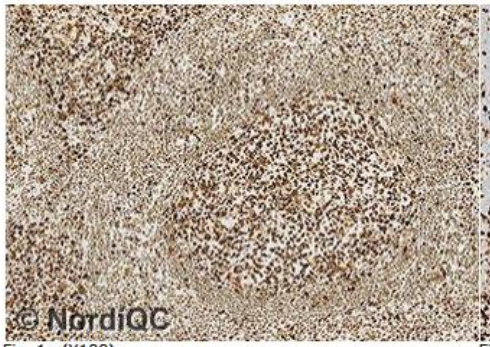



Fig. 1a (X100)
Optimal MSH6 staining reaction of the tonsil using the rmAb clone EP49, optimally calibrated, HIER in an alkaline buffer and a 3-step multimer based detection system (OptiView, Ventana). Virtually all mantle zone B-cells show a moderate and distinct nuclear staining reaction, while the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

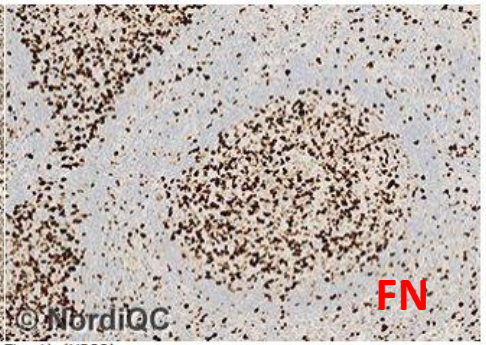



Fig. 1b (X200)
Insufficient MSH6 staining reaction of the tonsil using the rmAb clone EP49 with a protocol providing a too low sensitivity- same field as in Fig. 1a.
Only the germinal centre B-cells are distinctively demonstrated, while mantle zone B-cells expressing low level MSH6 virtually are unstained.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

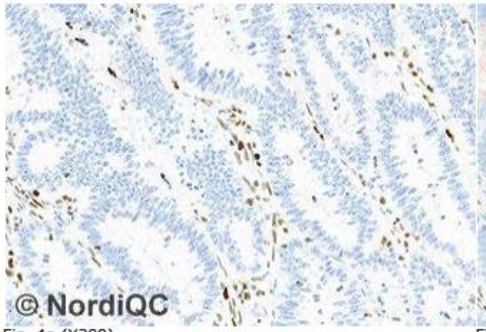



Fig. 4a (X200)
Optimal MSH6 staining reaction of the colon adenocarcinoma no. 4 with loss of MSH6 expression using same protocol as in Figs. 1a - 3a. The neoplastic cells are negative, while stromal cells show a distinct nuclear staining reaction serving as internal positive tissue control.

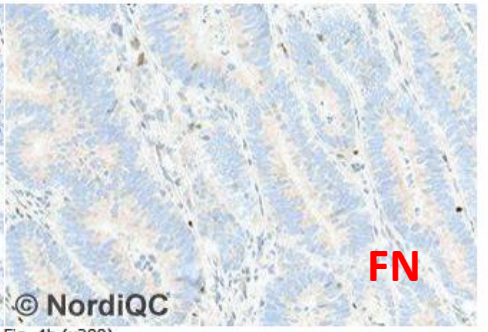



Fig. 4b (x200)
Insufficient MSH6 staining reaction of the colon adenocarcinoma no. 4 with loss of MSH6 expression using same protocol as in Figs. 1b - 3b – same field as in Fig. 4a. No nuclear staining reaction in the neoplastic cells is seen, but as only an equivocal nuclear staining reaction in the 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 key to “low sensitive” detection system ?

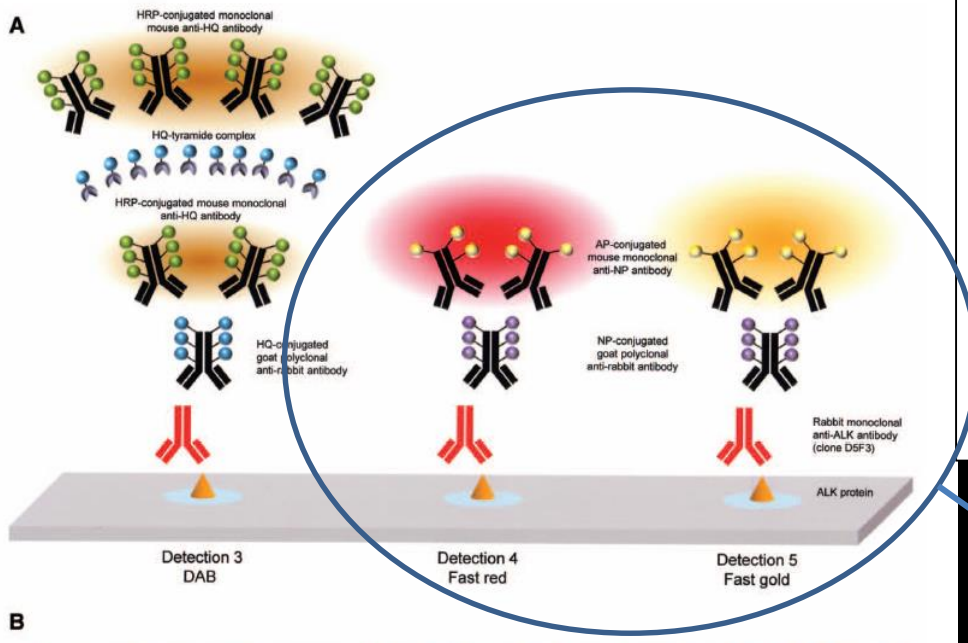



FIGURE 4. Comparison of a tyramide-amplified DAB IHC ALK-detection method (detection 3) with two AP-based IHC ALK-detection methods (detections 4 and 5). *A*, Schematic comparison of the IHC methods. Detection 3, which uses HQ-conjugated, tyramide-amplified IHC detection with HRP-catalyzed deposition 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*

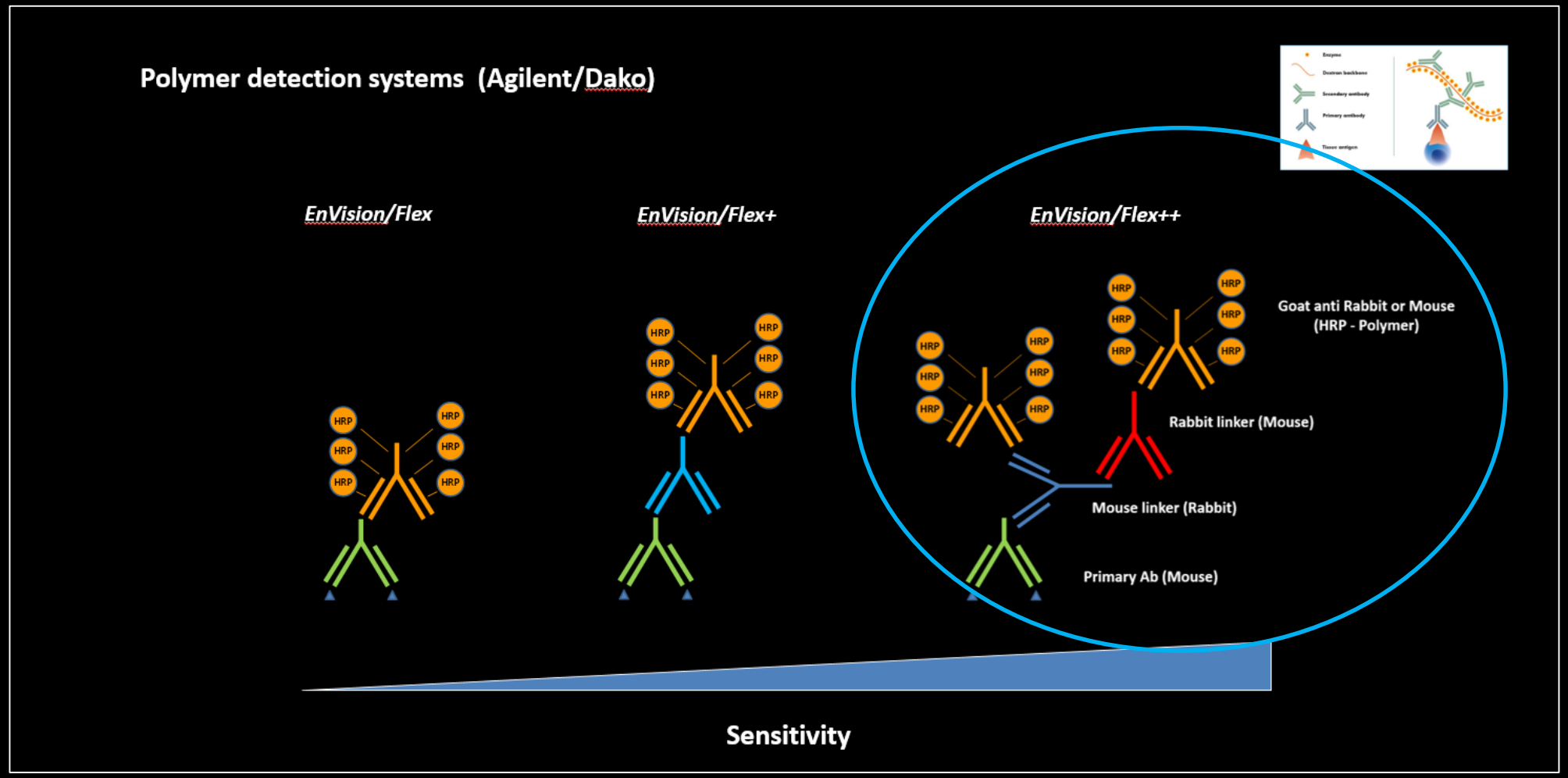
*Hiroaki Nitta, PhD, MBA,* Koji Tsuta, MD, PhD,† Akihiko Yoshida, MD, PhD,† Steffan N. Ho, MD, PhD,‡ Brian D. Kelly, PhD,* Lauren B. Murata, PhD,* Jerry Kosmeder, PhD,* Katie White, PhD,* Sandra Ehser, PhD,§ Penny Towne, MBA,* Crystal Schemp, MPH,* Abigail McElhinny, PhD,* Jim Ranger-Moore, PhD,* Chris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

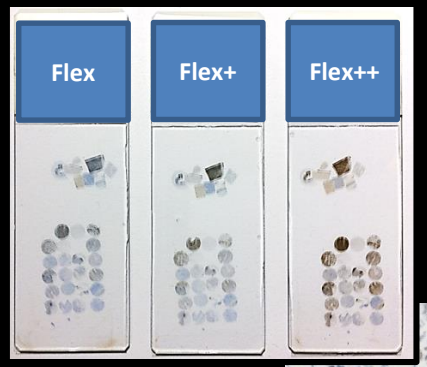
# Technical aspects of immunohistochemistry & pitfalls - Analytical phase




## New option on the Omnis

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

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




FLEX

FLEX+

FLEX++

Colon tumor

MSI

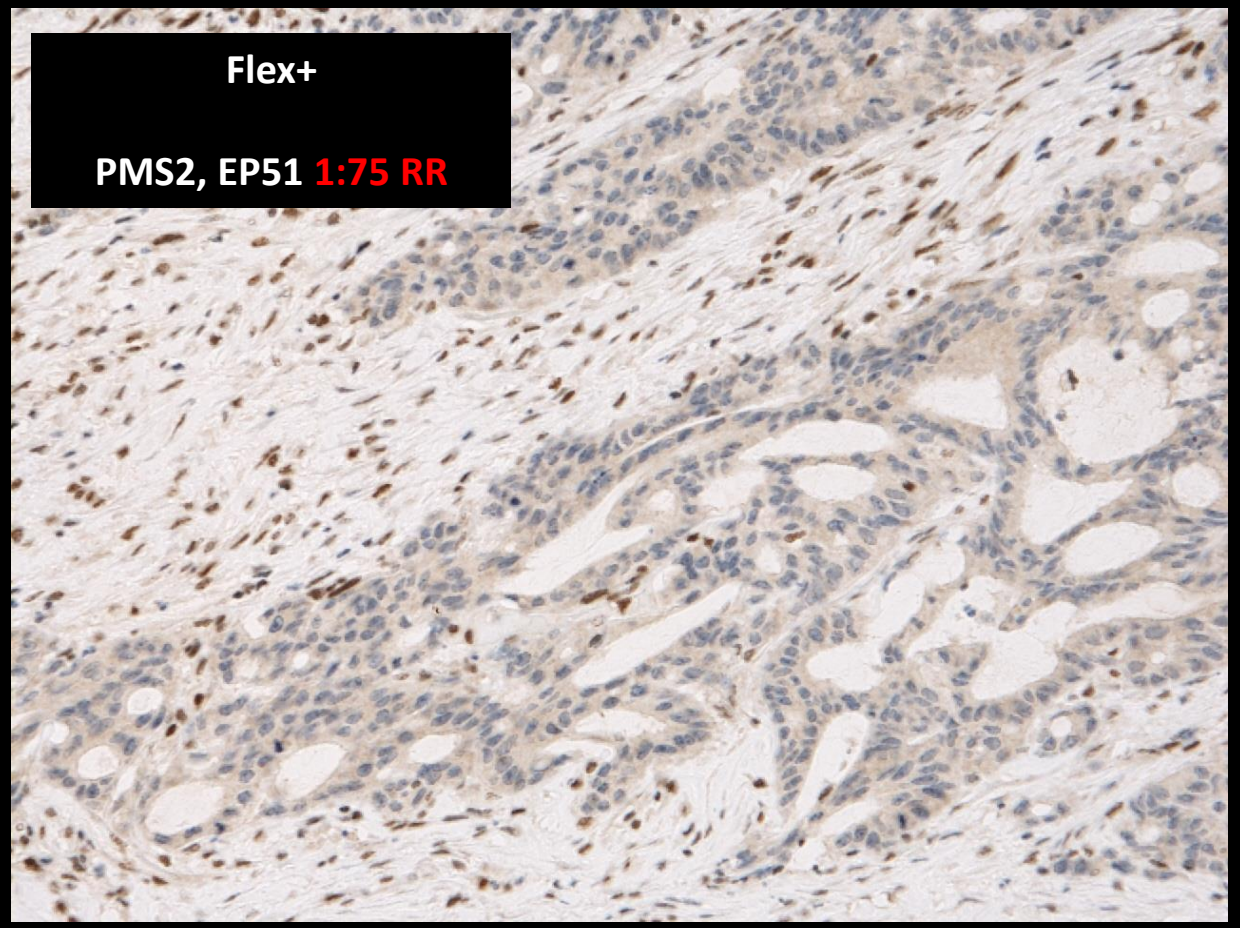
Sensitivity

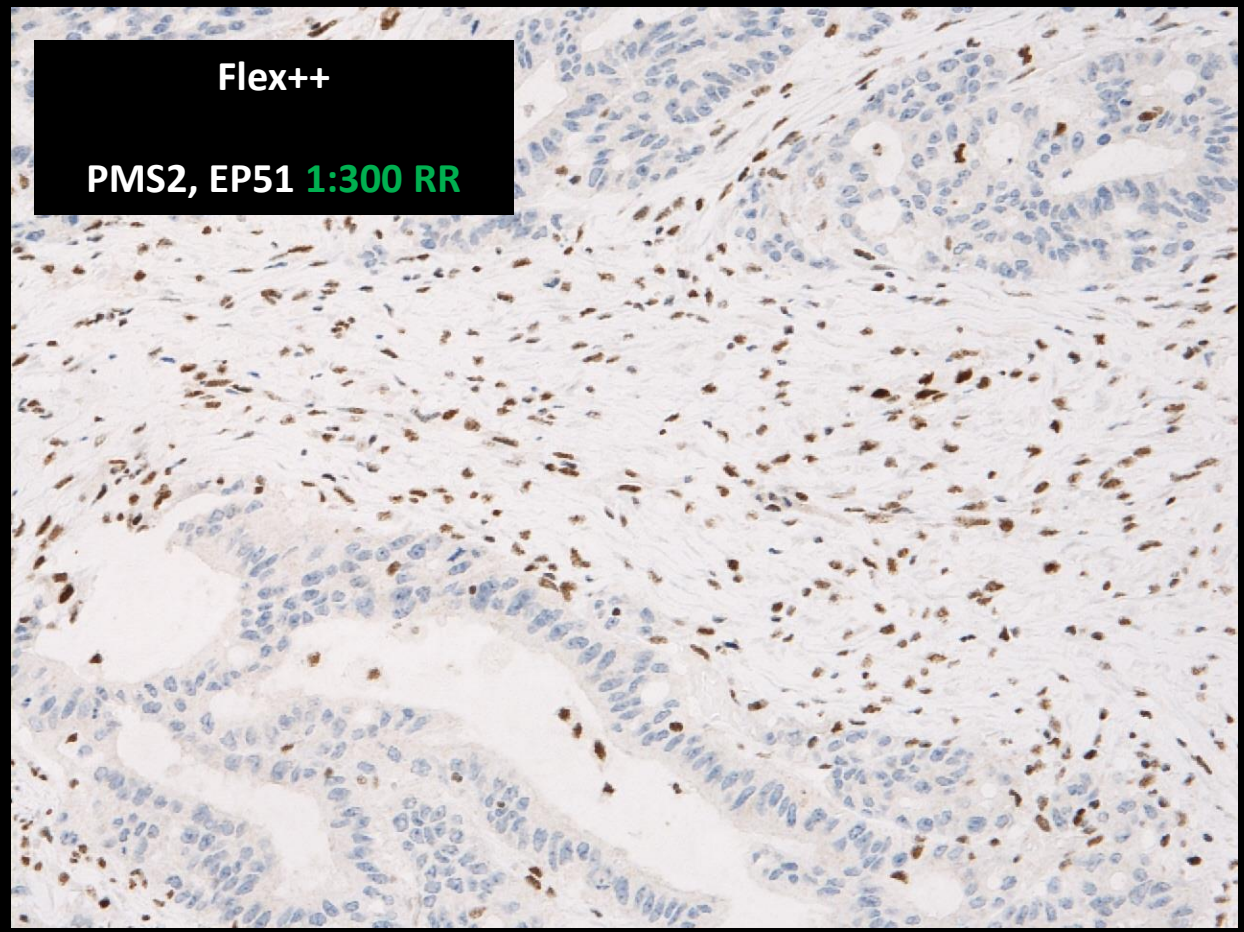
Colon tumor

MSS

# Technical aspects of immunohistochemistry & pitfalls - Analytical phase

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



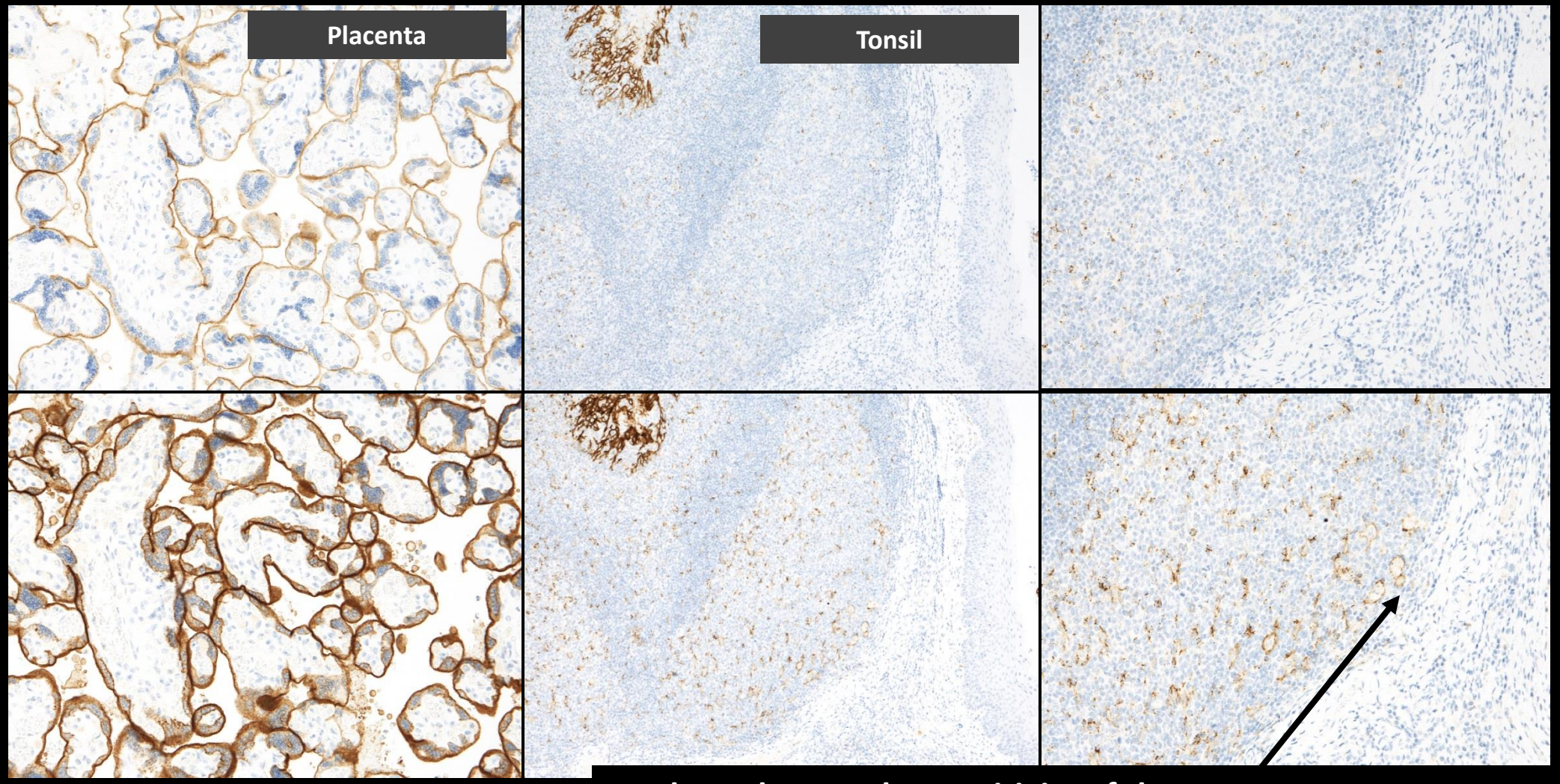

**Omnis: HIER High pH 48`**

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

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

Flex+

Flex++



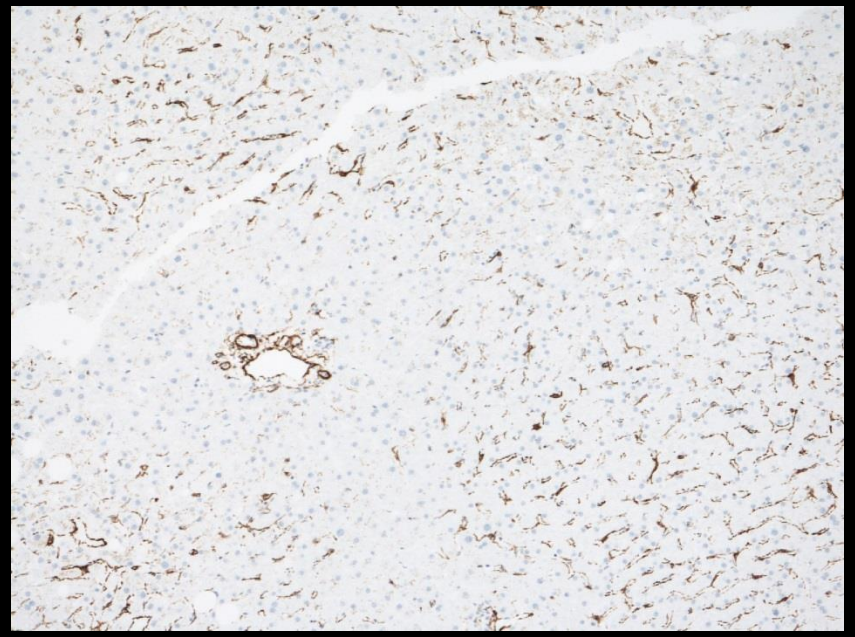

rmAbs: Enhances the sensitivity of the assay

# Technical aspects of immunohistochemistry & pitfalls - Analytical phase

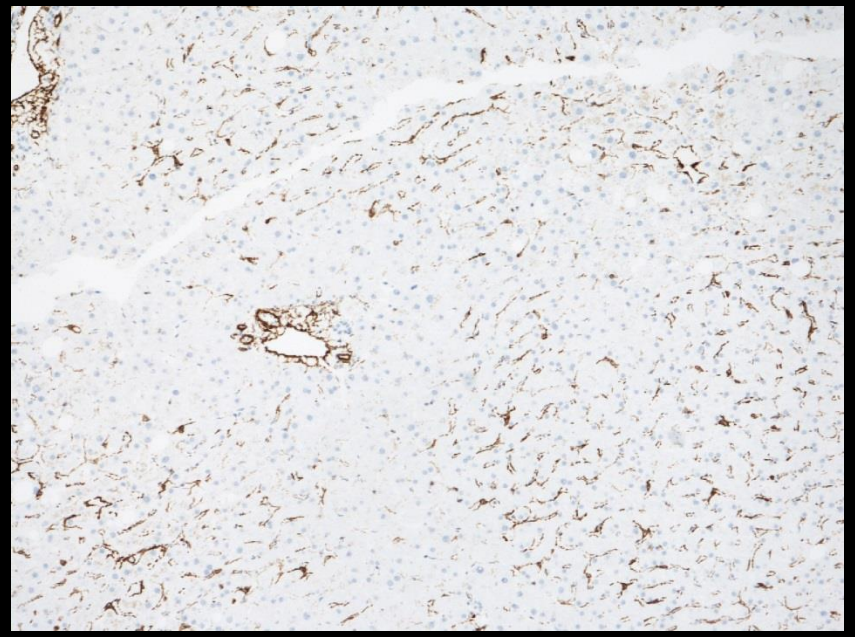
## Flex++ : 4-step polymer detection system (Omnis)

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

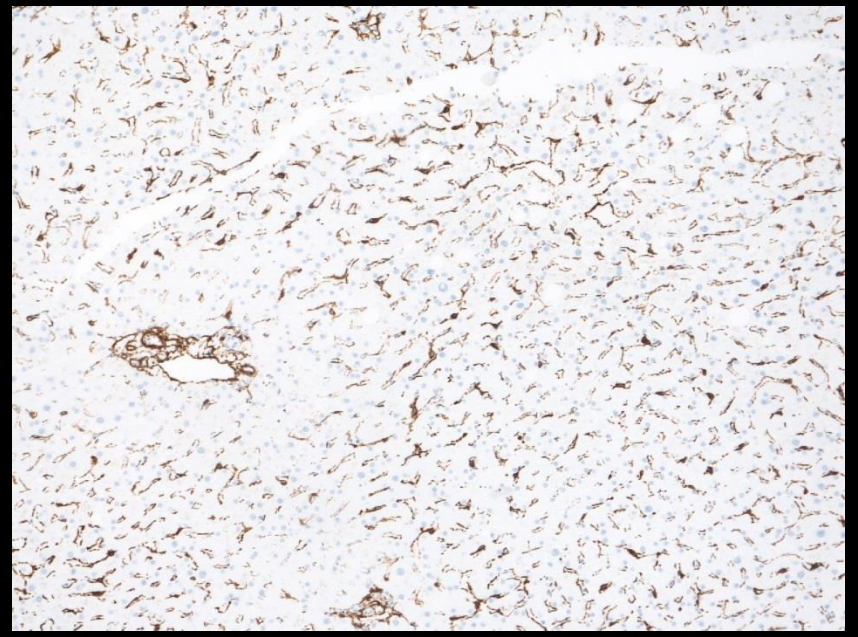
ASMA, 1A4 1:400 / Flex+



ASMA, 1A4 1:400 / Flex++



ASMA, BS66 1:500 / Flex+



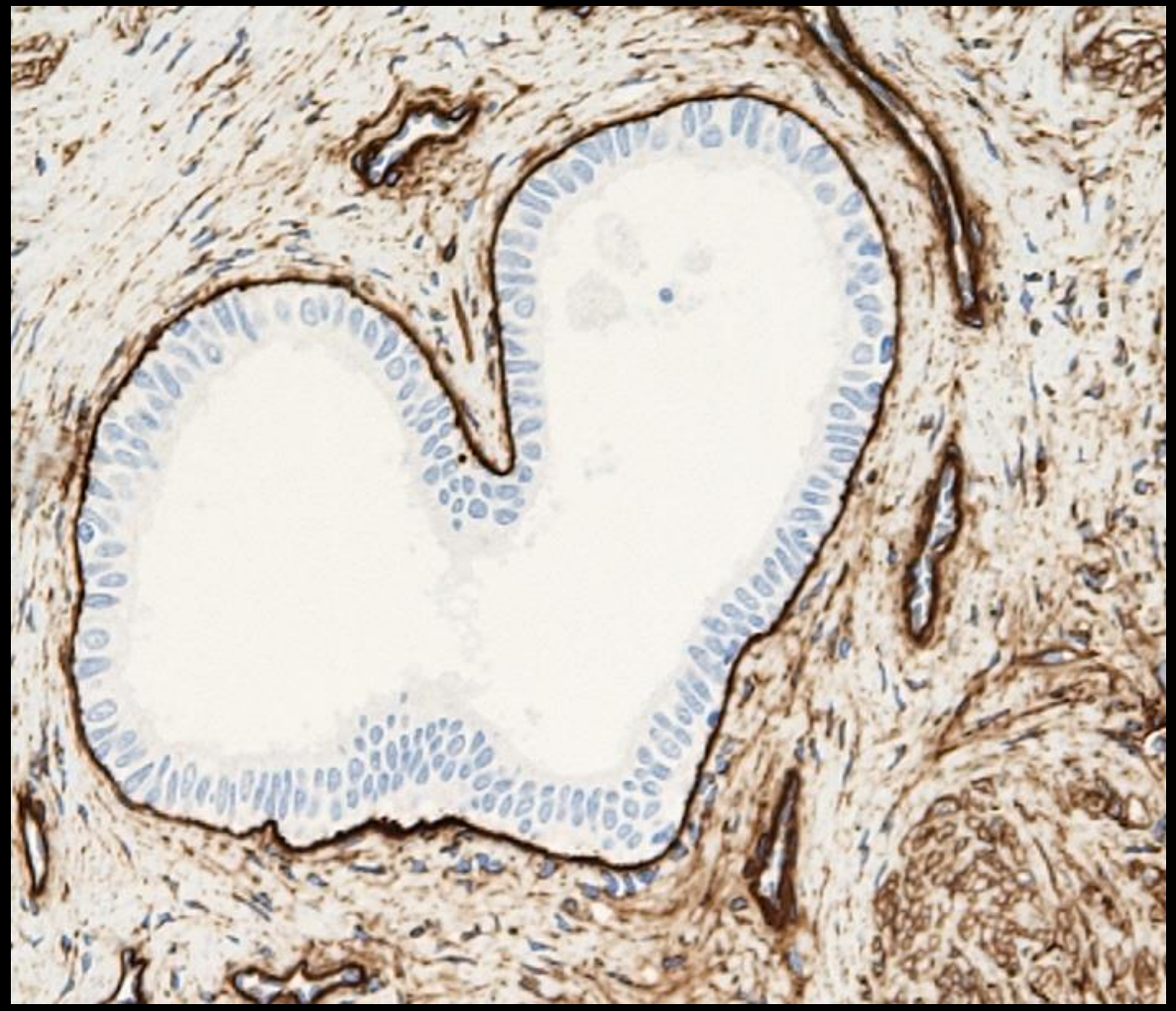
**Mouse monoclonal antibodies**: No improvement in signal intensity using Flex++ compared to Flex+

Liver

# The basal fundament for a technical optimal performance is :

- **Appropriate tissue fixation and processing**
- **Appropriate and efficient epitope retrieval**
  - 95% of the Abs require HIER and app. 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
- **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**
  - Focus on Immunohistochemistry Critical Assay Performance Controls (iCAPCs)

# Thank you for your attention



Volume 53(1): 13–21, 2005
Journal of Histochemistry & Cytochemistry
http://www.jhc.org

RAPID COMMUNICATION

# Mechanisms of Heat-induced Antigen Retrieval: Analyses In Vitro Employing SDS-PAGE and Immunohistochemistry

Shuji Yamashita and Yasunori Okada

Electron Microscope Laboratory (SY) and Department of Pathology (YO), School of Medicine, Keio University, Tokyo, Japan

**SUMMARY** In this study, we examined the mechanism of heat-induced antigen retrieval using analytical procedures involving SDS-PAGE, Western blotting, and immunohistochemistry. Five proteins were treated with 4% formaldehyde in the presence or absence of 25 mM $CaCl_2$, then heated under various conditions after removal of formaldehyde and analyzed on SDS-PAGE. Formaldehyde produced inter- and intramolecular cross-links in the proteins. Heating at high temperatures cleaved these cross-links at all pH ranges examined (pH 3.0, 6.0, 7.5, 9.0) and produced almost the same electrophoregrams as the native proteins. Proteins treated with formaldehyde containing $CaCl_2$ showed similar electrophoretic patterns, observed without heating or after heating at pH 6.0 and pH 9.0 in the presence or absence of 10 mM EDTA. Western blot analyses demonstrated that the soluble forms of β-actin (monomer and oligomers) and fibronectin were present in extracts from deparaffinized mouse uterine sections autoclaved for 15 min but not in extracts from unheated specimens. Nine of ten antigens, independent of their isoelectric points, exhibited much stronger immunoreaction in the sections heated at pH 9.0 than in those heated at pH 6.0. The second heating at pH 6.0 significantly decreased the immunostaining of the antigens that had been boiled at pH 9.0, but the immunostaining was recovered after a third heating at pH 9.0. These results suggest that the main mechanism of heat-induced antigen retrieval is disruption of the cross-links and that pH is an essential factor for a proper refolding of epitopes. (J Histochem Cytochem 53:13–21, 2005)

**KEY WORDS**
antigen retrieval
SDS-PAGE
Western blot
immunohistochemistry
epitope conformations

**Table 1** pH-dependent antigen retrieval in mouse tissues[a]

| | | Heating procedures | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Antigens | pI | No heating | PH 6 | pH 6–9 | pH 6–9–6 | pH 9 | pH 9–6 | pH 9–6–9 |
| ERα | 8.3 | ± | − | + | ± | +++ | + | +++ |
| ERβ | 8.8 | − | − | + | ± | ++ | − | ++ |
| AR | 6.0 | − | − | ++ | + | +++ | + | +++ |
| GR | 6.0 | − | − | + | + | +++ | ++ | +++ |
| P300 | 8.8 | ± | + | ++ | + | +++ | ++ | +++ |
| SRC-1 | 5.7 | − | ± | ++ | + | +++ | + | +++ |
| α-Amylase | 6.5 | +++ | ++ | ++ | ++ | ++ | ++ | ++ |
| β-Actin | 5.2 | + | + | ++ | ++ | +++ | ++ | +++ |
| Fibronectin | 5.9 | + | − | ++ | − | ++ | ± | +++ |
| Laminin | 5.4 | ++ | − | + | − | + | − | + |

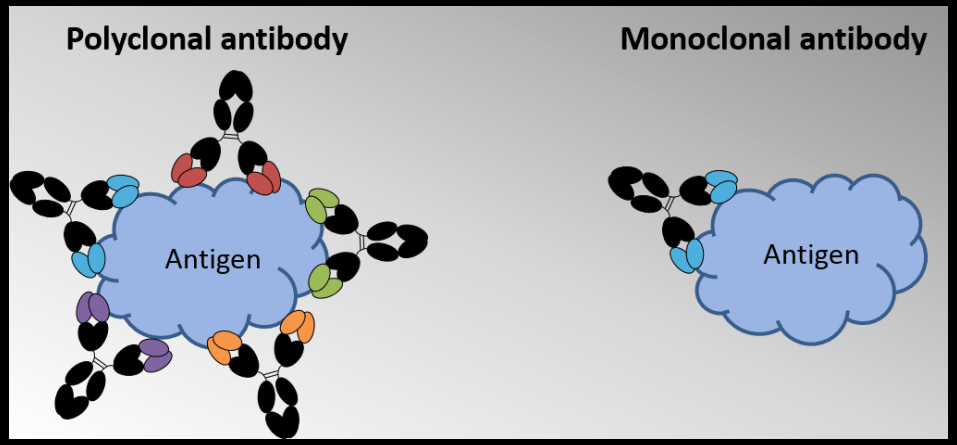
[a]Immunostaining was scored as follows: +++, strong; ++, moderate; +, weak; ±, faint; −, negative.

**Results from this study suggested that:**

**The main mechanism of heat-induced antigen retrieval is disruption of the cross-links formed by formalin fixation (confirming earlier hypothesis to this subject)**

**pH of the antigen retrieval buffer is an essential factor for a proper refolding of epitopes favoring better reactions with respective antibodies**

**High pH antigen retrieval buffers seems to be more efficient (Table 1)**




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

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

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

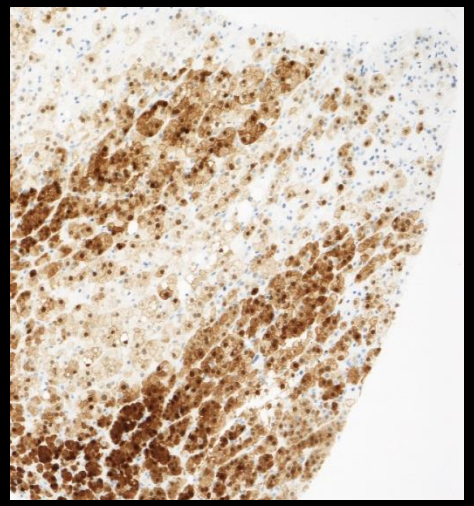
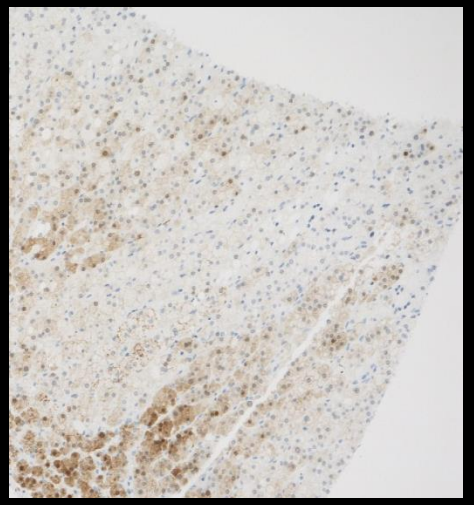
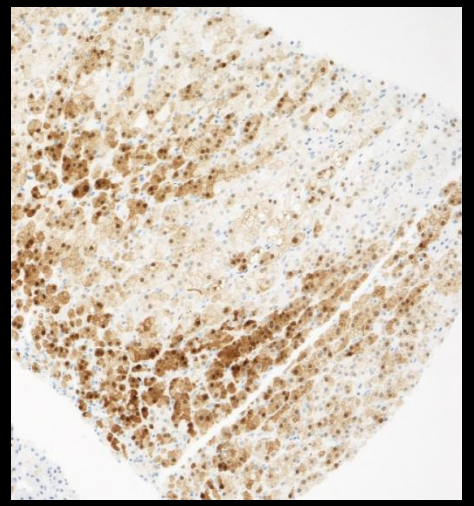
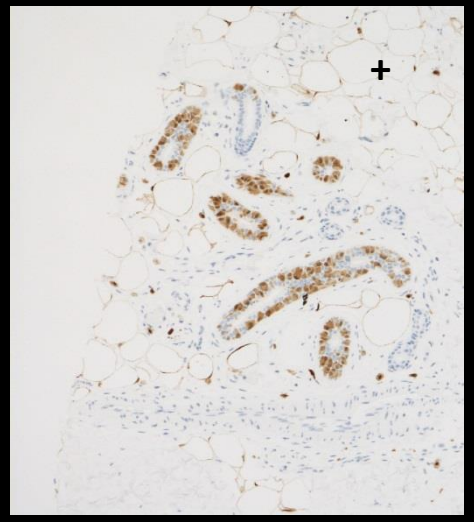
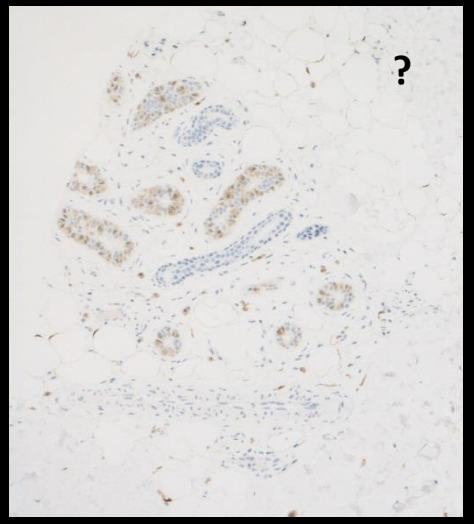
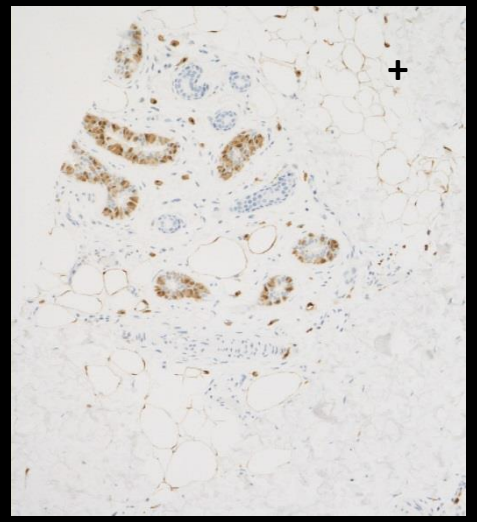
## Immunohistochemistry : Key differences between Polyclonal and Monoclonal Antibodies

| Polyclonal | Monoclonal |
|---|---|
| Heterogeneous population of antibodies reacting with different epitopes of an antigen | Homogenous population of a specific antibody reacting with one epitope of an antigen |
| Not Epitope Specific | Epitope Specific |
| Increased likelihood for cross-reactivity with similar antigens | Low cross-reactivity |
| Increased likelihood for background noise | Low background noise |
| Lot Variability | Identical lots |
| Many host species options<br>- Normally Rabbit antibodies | Few host species options<br>- Normally Mouse or Rabbit |

## Is monoclonal primary Abs always better to use ?

# Technical aspects of immunohistochemistry & pitfalls - Analytical phase

## Poly versus monoclonal antibody ?

| | CR, poly (18-0211) 1:200 | CR, DAK-Calret1 1:15 | CR, CAL6 1:15 RR |
|---|---|---|---|
| Adrenal Gl. |  |  |  |
| | **HIER High pH, Flex+ Rabbit** | **HIER High pH, Flex+ Mouse** | **HIER High pH, Flex+ Mouse** |
| Sweat Gl. |   |   |   |

**Omnis**

Fat and eccrine epithelia cells ?

# Technical aspects of immunohistochemistry & pitfalls - Analytical phase

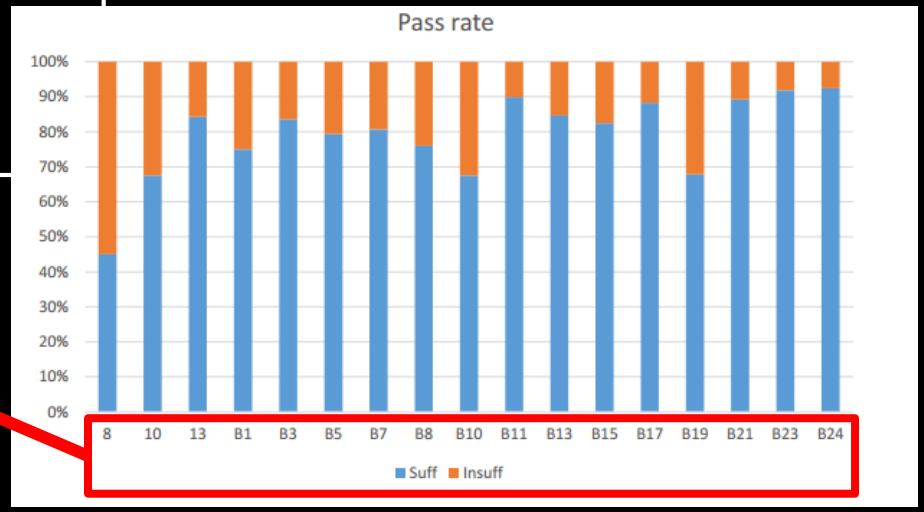
## Problem: Primary antibody poorly calibrated providing low sensitivity

| 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)

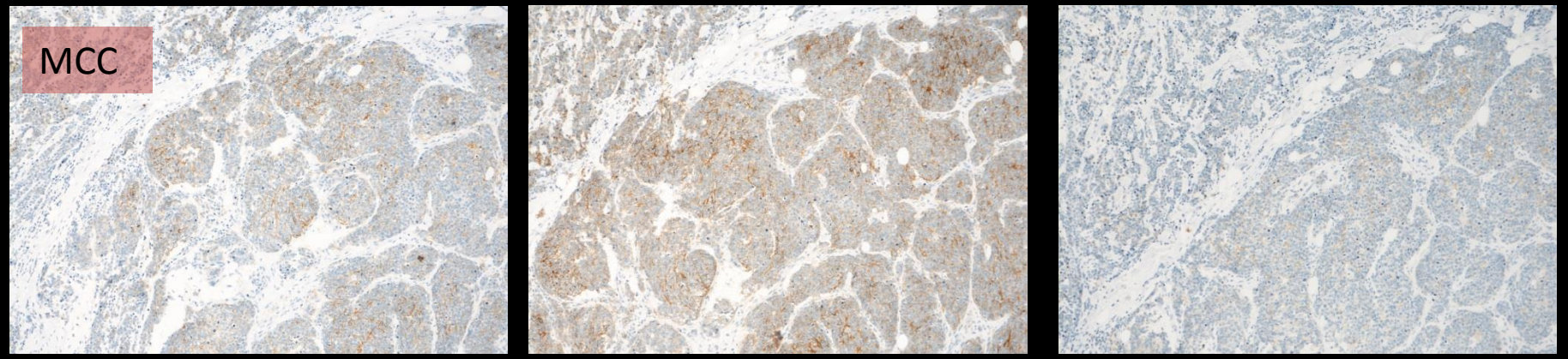
# Technical aspects of immunohistochemistry & pitfalls - Analytical phase

## Antibody diluents

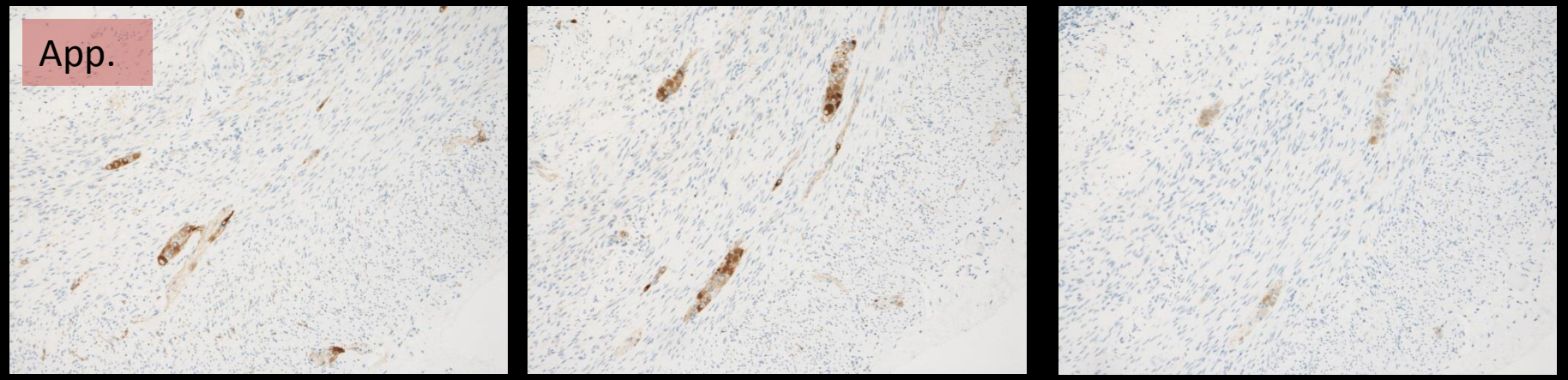
ALK, 1A4 1:300
Dako Dil. pH 7.3

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`)



# Technical aspects of immunohistochemistry & pitfalls - 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**

# 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

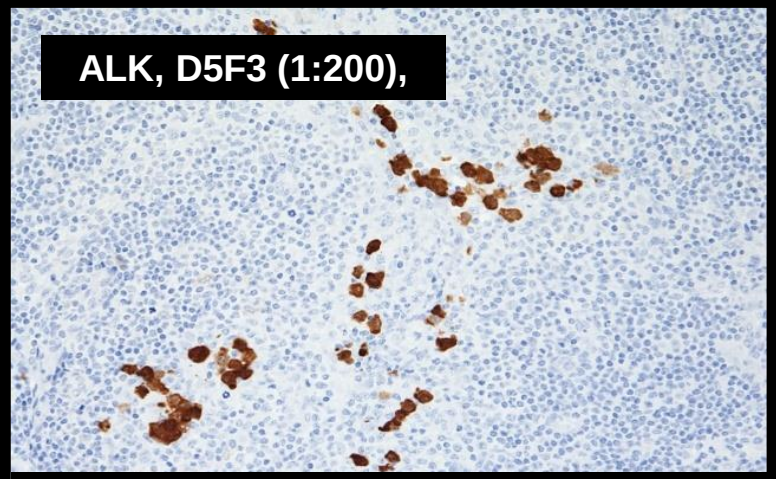
- Appropriate protocol library

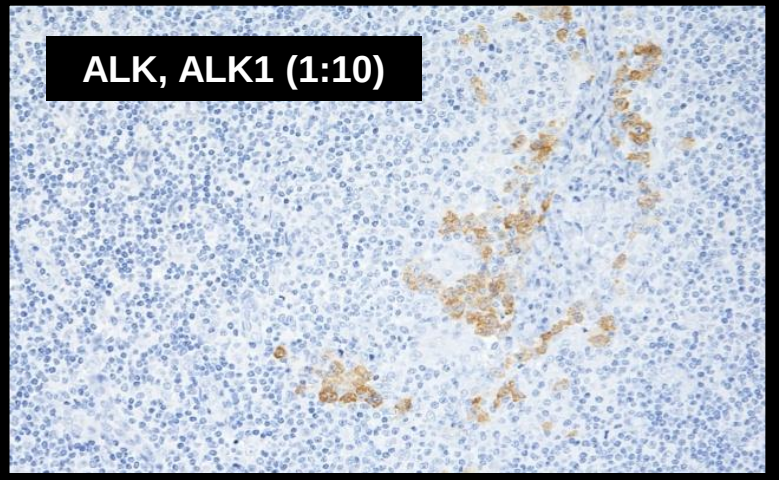
Do we have access to appropriate tissue, reflecting the range of different antigen expression levels,  both for the optimization process but also for the laboratory`s internal quality assurance program (control tissue) – monitoring specificity and sensitivity of the assays

# The technical test approach – Analytical phase

HIER in high pH buffer, Flex+

ALCL

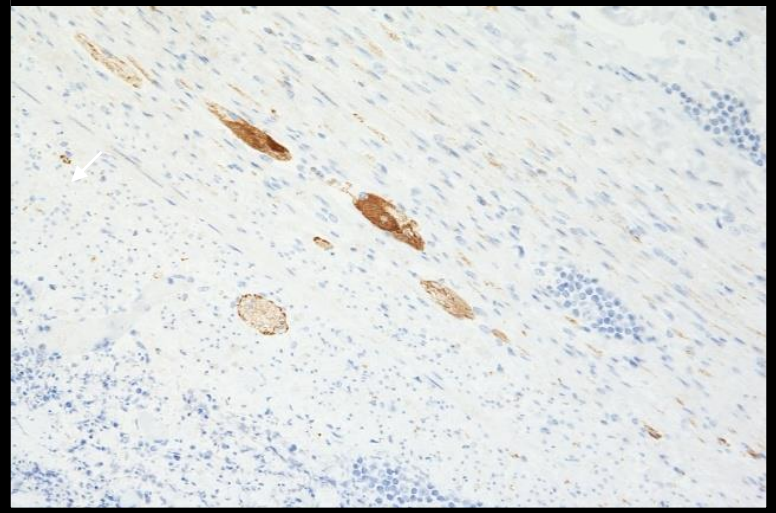


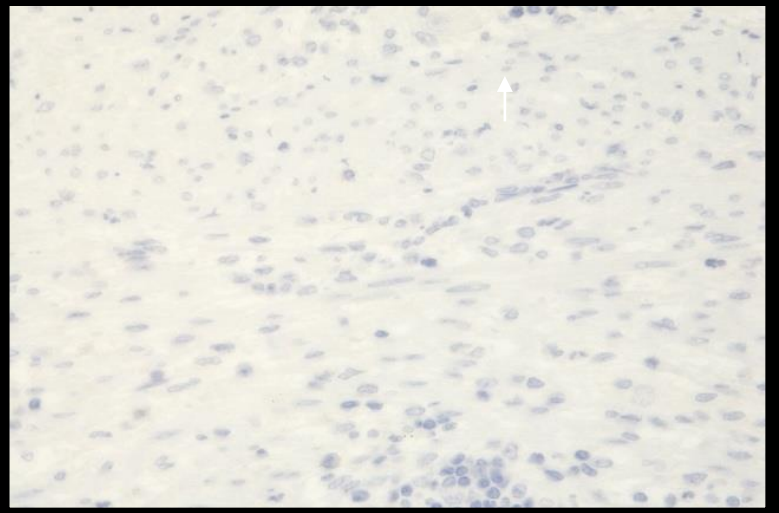




**Clone ALK1 provides low sensitivity**

”iCAPS” : Ganglion and peripheral nerve cells ?

Appendix





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

***Effect of Heat-Induced Antigen Retrieval Following Inconsistent Formalin Fixation***

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(1) | 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.

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

5`

| Antigen | Antibody Clone | AR 121°C | AR 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.

**Demonstrated that:**

**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

**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<br>6<br>2<br>2<br>1 | Biocare<br>Zytomed<br>Menarini<br>abcam<br>Nordic Biosite | 52 | 24 | 10 | 2 | 86% | 89% |
| rmAb clone **ZR8** | 12<br>1<br>1 | Immunologic<br>Zeta Corporation<br>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

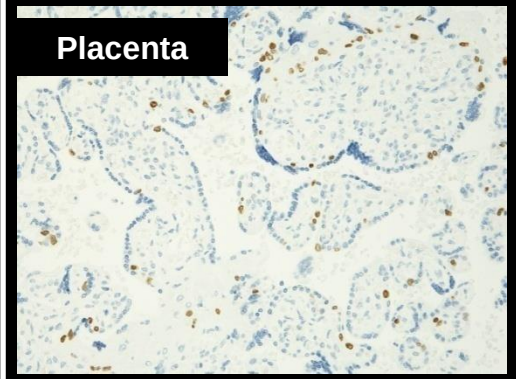
**No optimal results / pass rate of 23% (5 of 22)**

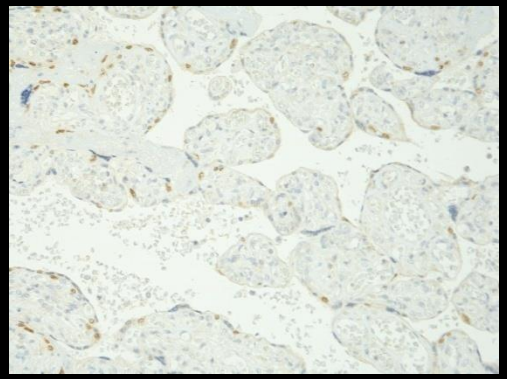
**The insufficient results were typically characterized by a poor signal-to-noise ratio and aberrant staining reaction compromising the interpretation.**

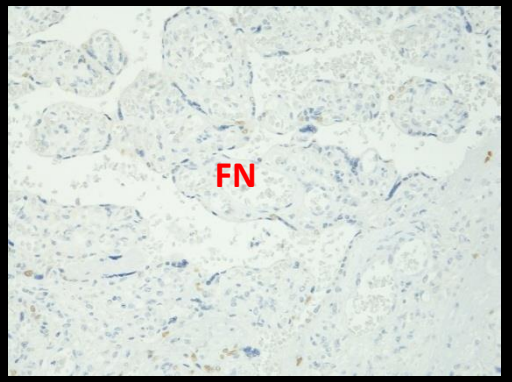
p40 dil. 1:25 clone BC28

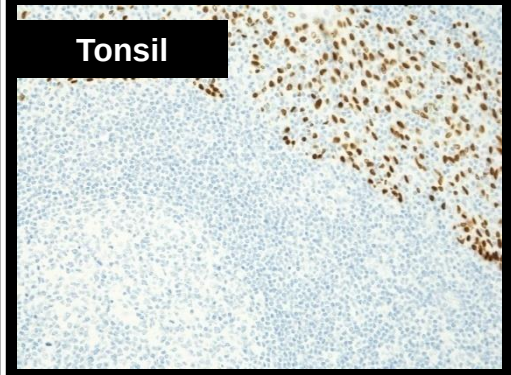
p40 dil. 1:50 polyclonal

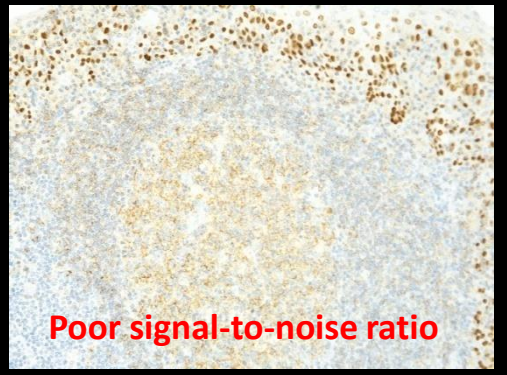
P40 dil. 1:200 polyclonal

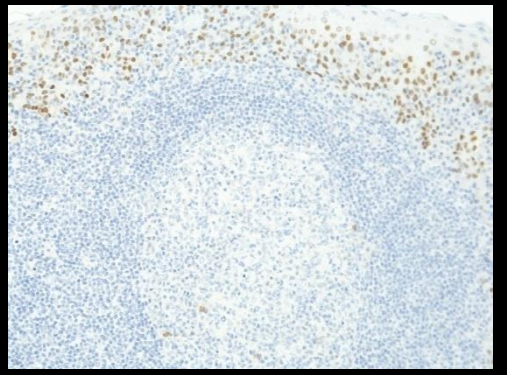




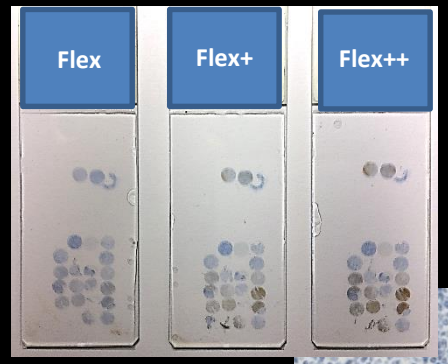



**Both primary antibodies are from BIOCARE**

# Omnis: BRAF clone VE1 (1:1000 RR)

## High pH 48`




FLEX

FLEX+

FLEX++

Colon tumor

BRAFV600E mut

Sensitivity

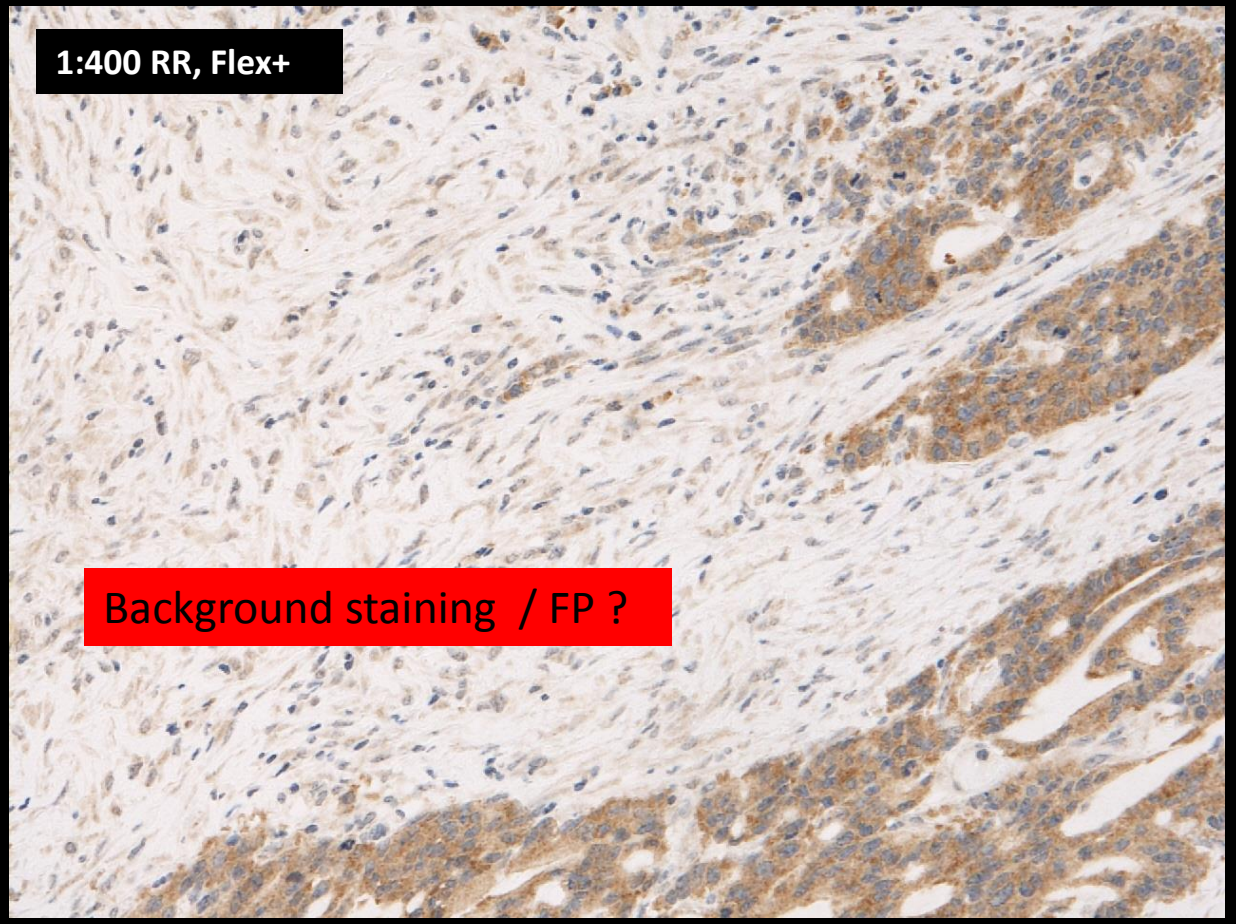
Colon tumor

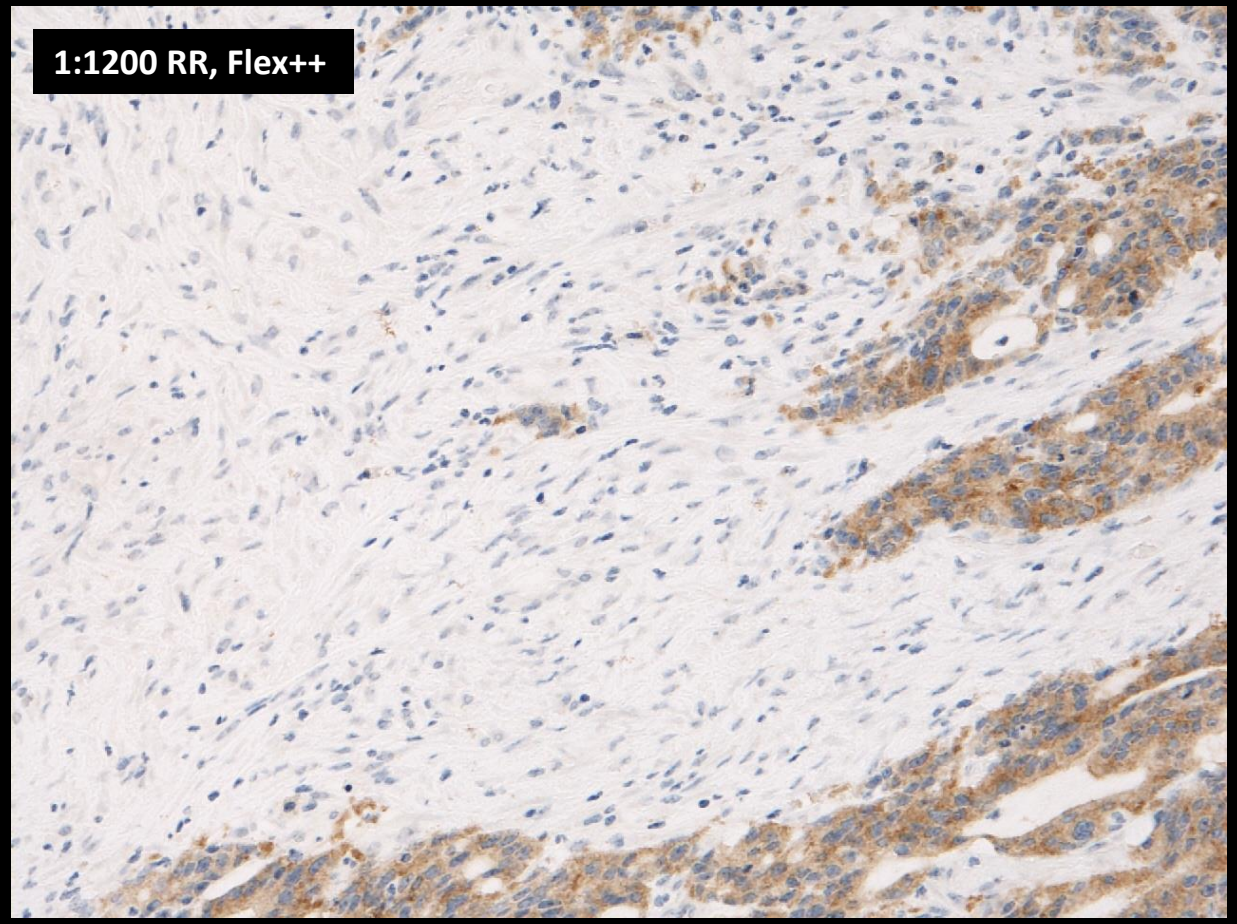
KRAS mut

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

mAb BRAF, VE1 (mutation specific)

Colon tumor BRAF V600E mutated

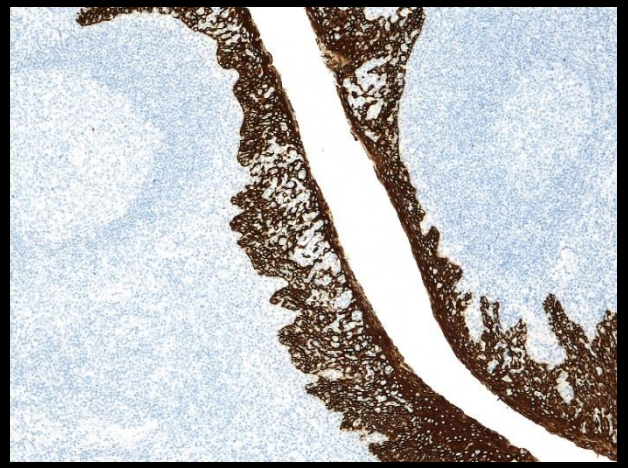
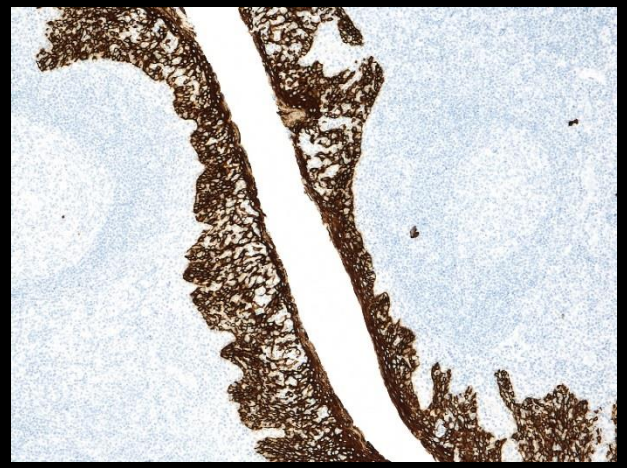
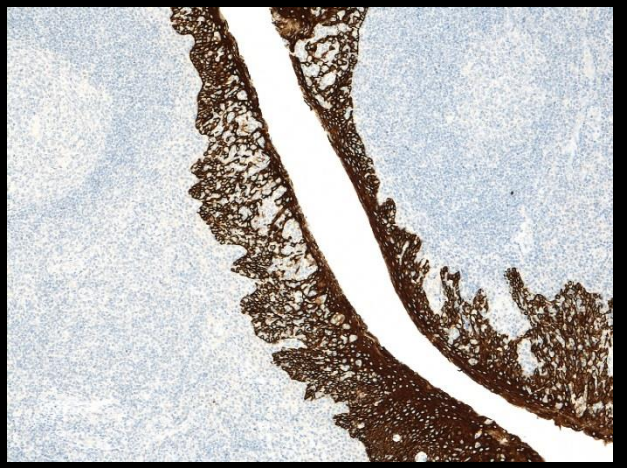
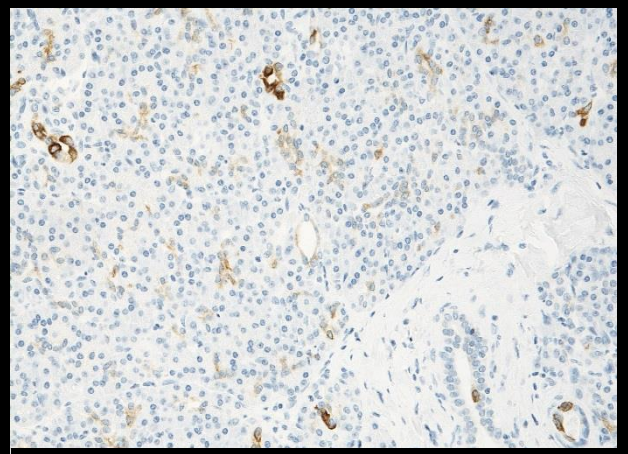
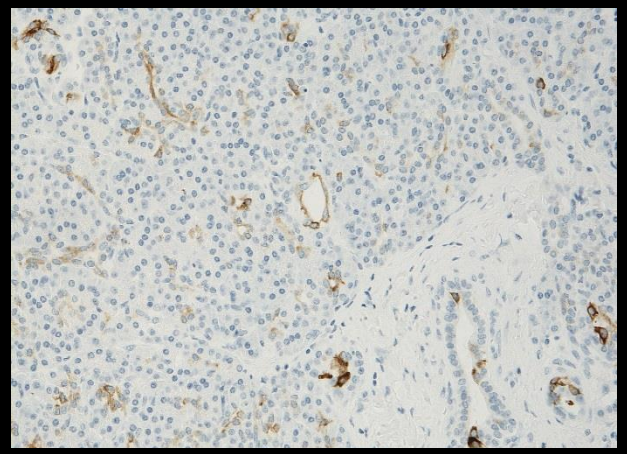
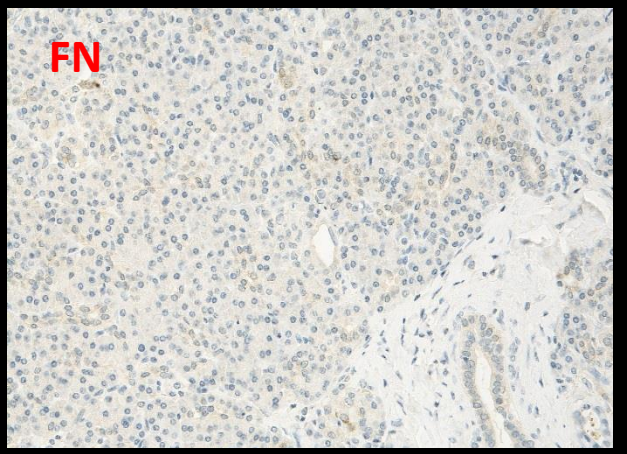



HIER in High pH 48`

# Technical aspects of immunohistochemistry & pitfalls - Analytical phase

**Problem: Primary antibody provides  low sensitivity**

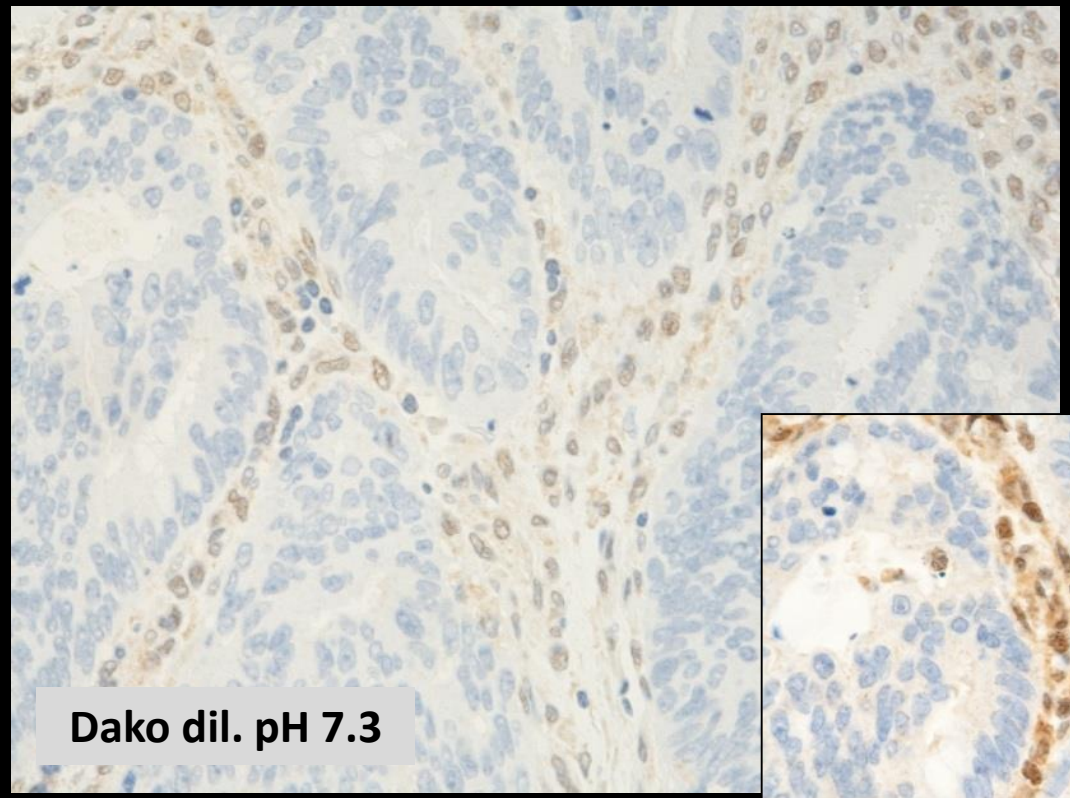
| | CK5 clone BSR55 (1:200) | CK5 clone XM26 (1:200) | CK5 clone BS42 (1:200) |
|---|---|---|---|
| Tonsil |  |  |  |
| Pancreas |  |  |   |

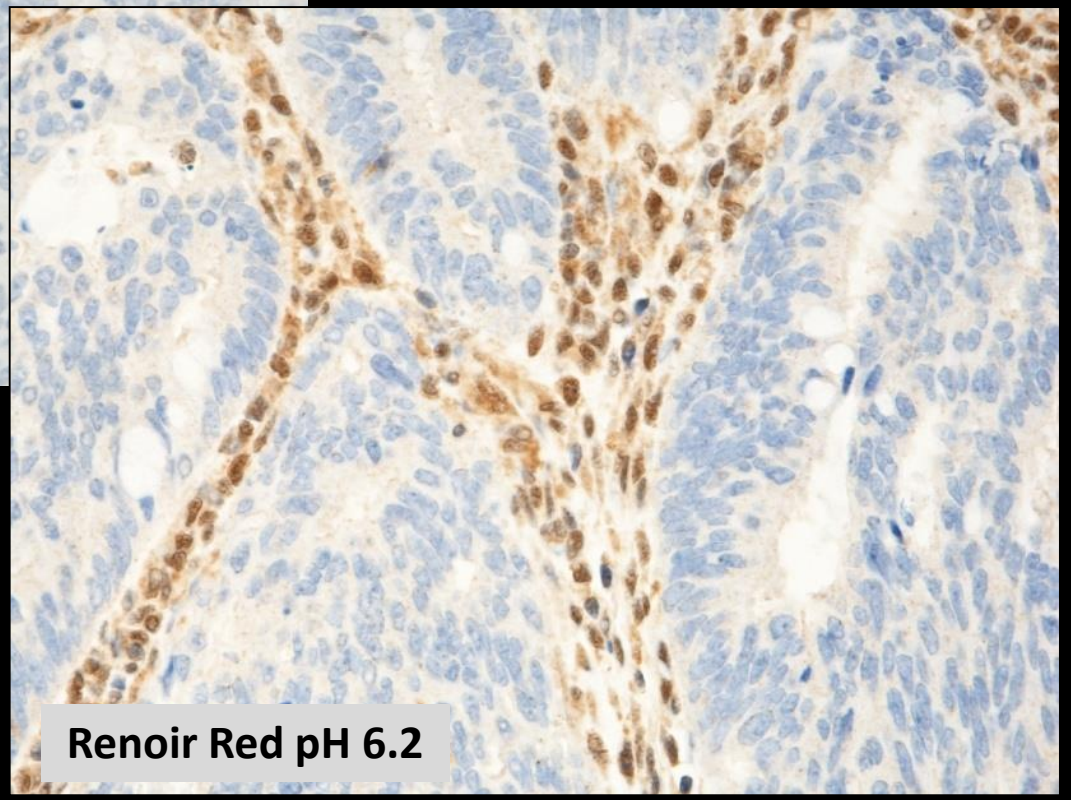
Omnis: HIER/HIGH pH 24`, Flex+ (10+20`)

# Antibody diluents

**SMAD4, Clone EP168Y 1:1500**

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

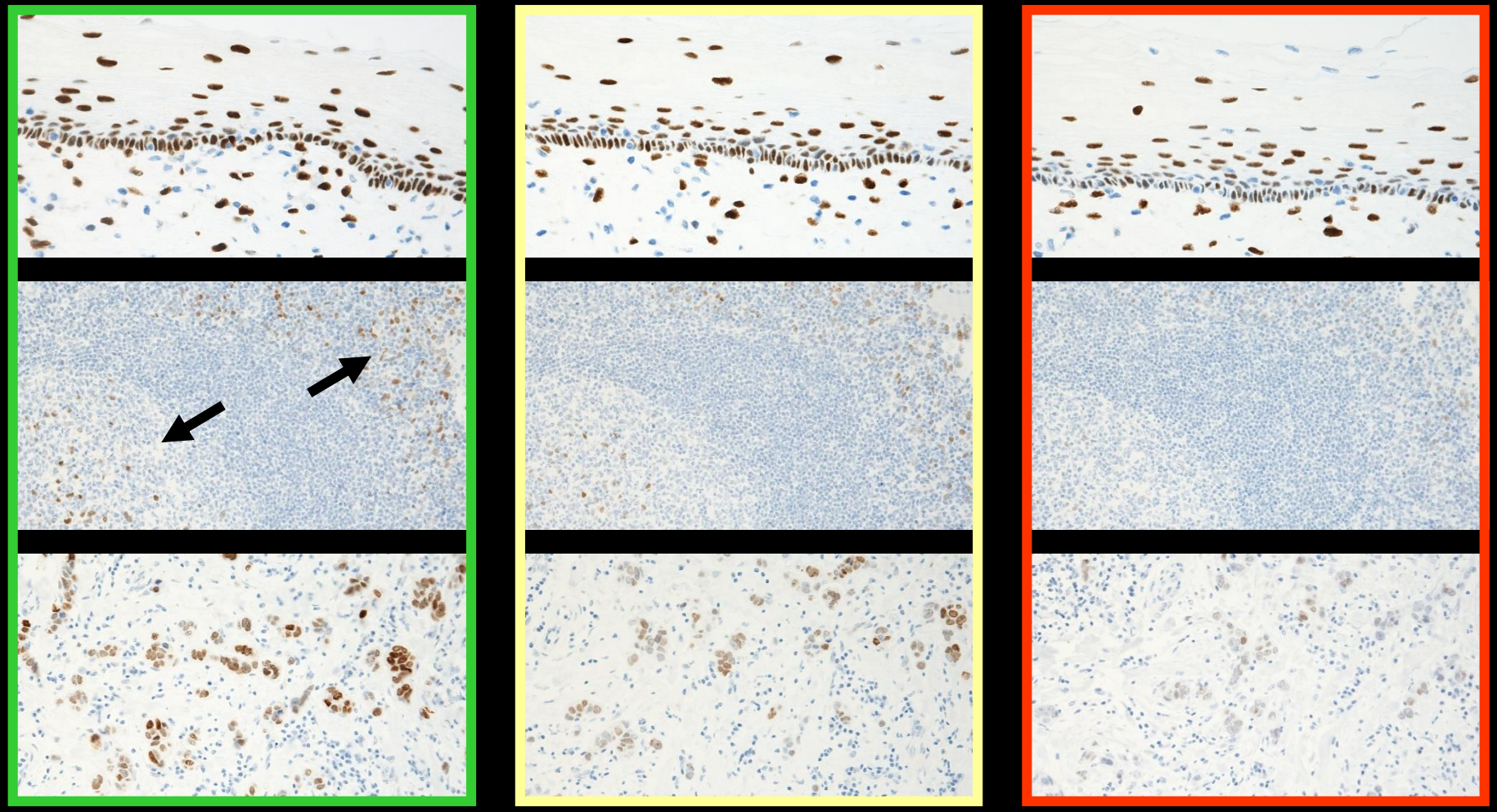



Colon Adenocarcinoma

# Technical aspects of immunohistochemistry & pitfalls - Analytical phase

## Problem: Primary antibody poorly calibrated providing low sensitivity




High pH 20`, Flex+Mouse

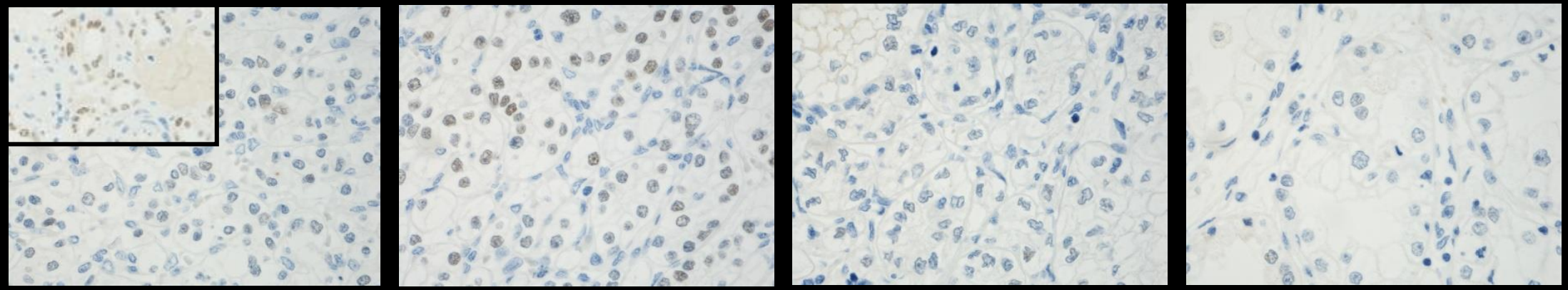
Staining indicators are extremely important - helping us to calibrate the IHC assay correctly

# Technical aspects of immunohistochemistry & pitfalls - Analytical phase

## Antibody diluents

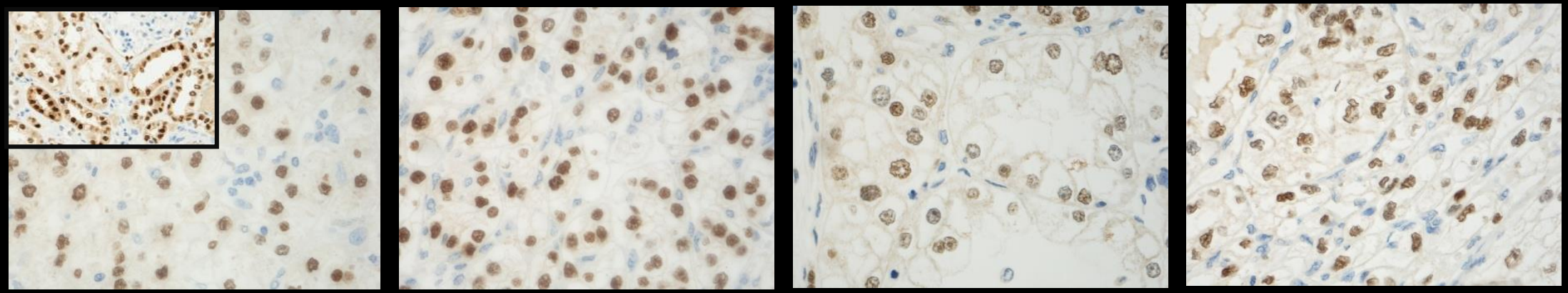
PAX8, ZR1 1:50

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`)

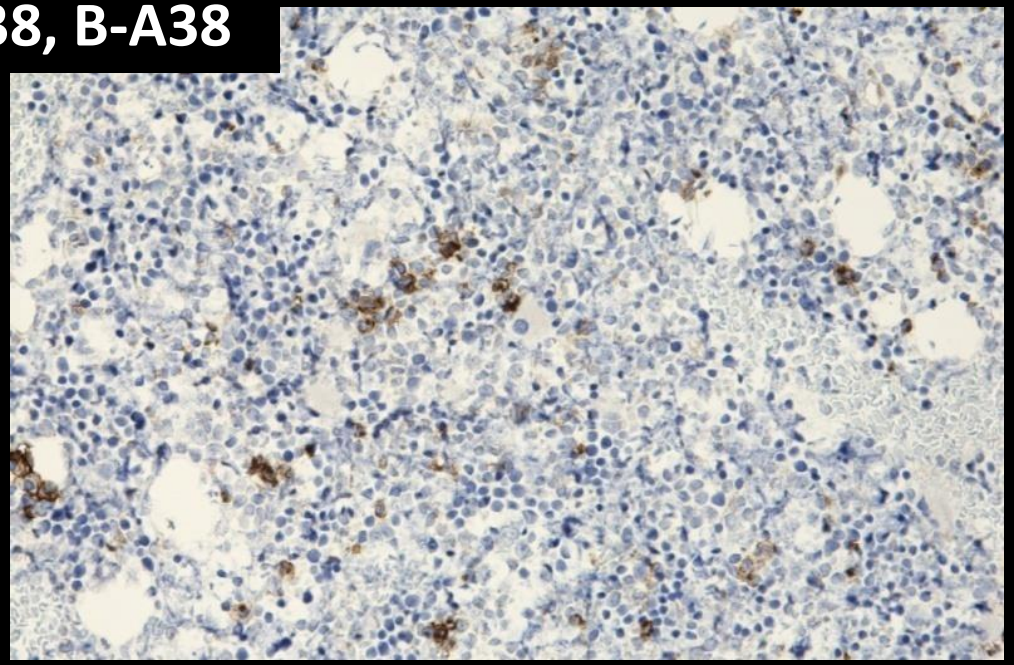
# Excessive antigen retrieval related to the PT-module (Dako)

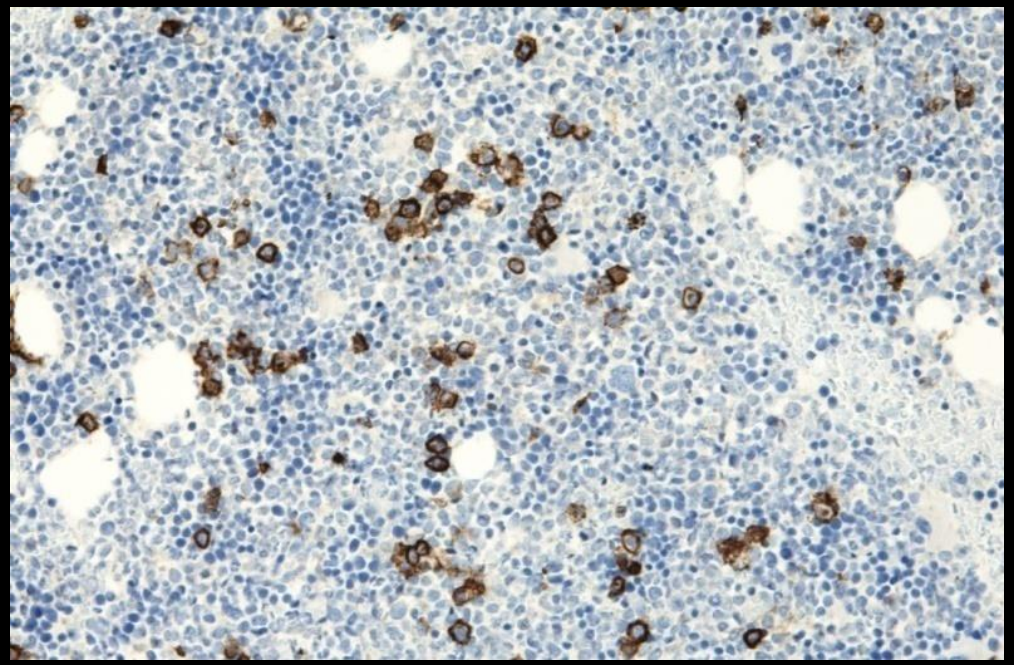
## Influence of pre-heat temperature (65°C versus 85°C)

**P/E 65°C**

**P/E 85°C**

**CD138, B-A38**





**Bone marrow clot, NBF 96h**

**PT, High pH (3-1) 95ºC, / 20 min**

# Technical aspects of immunohistochemistry & pitfalls - Analytical phase

**Bone marrow clot**

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

**Morphology ?**

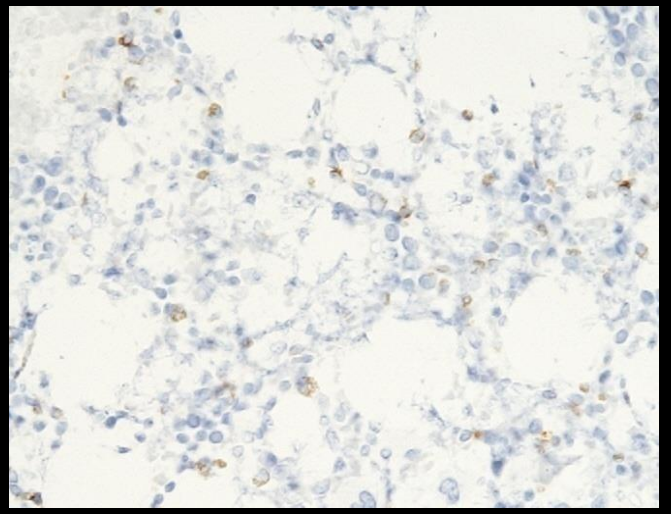
**CD5 clone SP19**

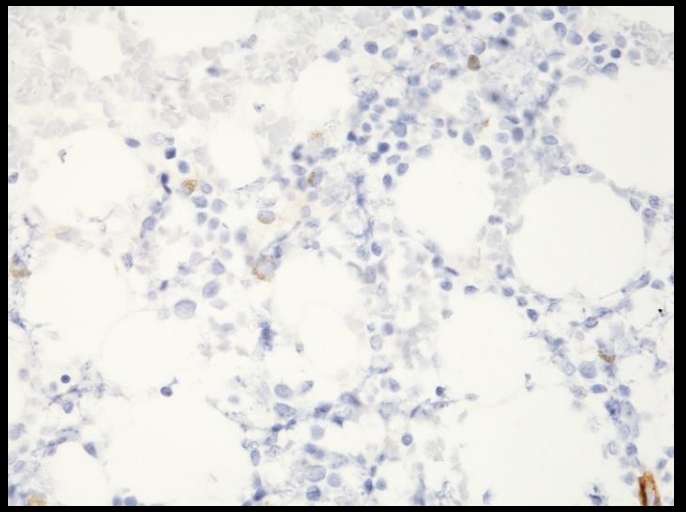
**CD34 clone QBEND-10**

**High pH (3-1) (Dako)**

**Recommended settings:**

**65℃**





**HIER buffer H (LabVision)**

**Recommended settings:**

**85℃**