Nordic immunohistochemical Quality Control

NordiQC

Workshop in Diagnostic Immunohistochemistry

Aalborg Hospital, 19th – 21th September 2016

The technical test approach

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

Michael Bzorek

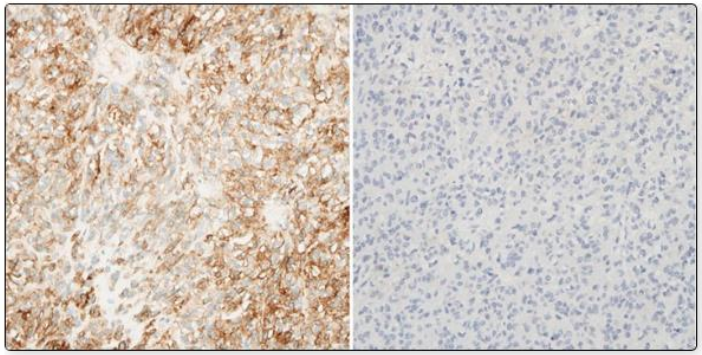
Histotechnologist

Department of Pathology

Næstved Hospital, Denmark

Immunohistochemistry – A simple technique ?

NordiQC Info Modules Assessments Protocols Controls Events Login



Results general module - run 47

11-Jul-2016

The results from general module - run 47 are now available. Individual scores will be mailed to participants. Click to see a summary.

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

CD117 / GIST

All news

Events

NordiQC Workshop in Diagnostic Immunohistochemistry
19–21 Sep 2016: Aalborg, Denmark

NordiQC Academy of Immunohistochemistry
12–14 Oct 2016: Krakow, Poland

Important dates

Run 48, B22, H10
Protocol submission deadline
10 Sep 2016
Slide circulation
18 Sep 2016
Slide return deadline
11 Oct 2016
Publication of results
9 Dec 2016

Questions

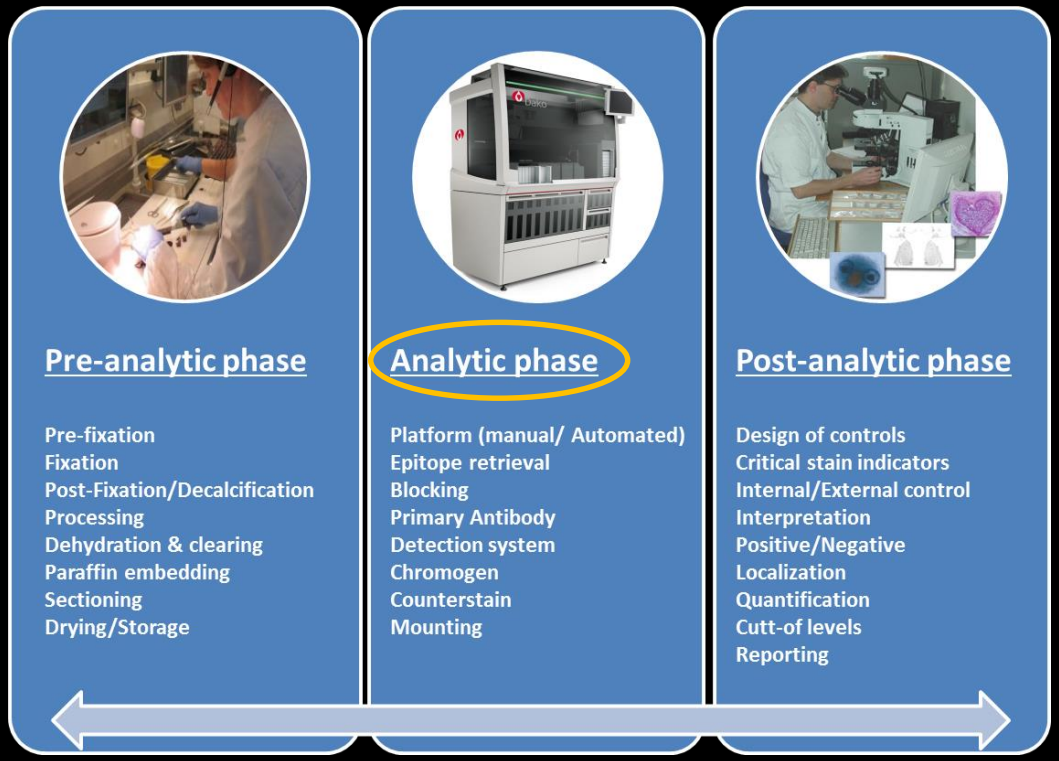
Check out our FAQ (Frequently asked questions) or contact us

Google™ Custom Search Search

External Quality Assurance program

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

The total test paradigm
Key elements in the immunohistochemical procedure



The Analytic phase - definition:

Begins with dewax of the cut slides of the 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.

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

ANNUAL REVIEW ISSUE

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

Mogens Vyberg[1,2] · Søren Nielsen[1]

Major problems 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
- 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 (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)

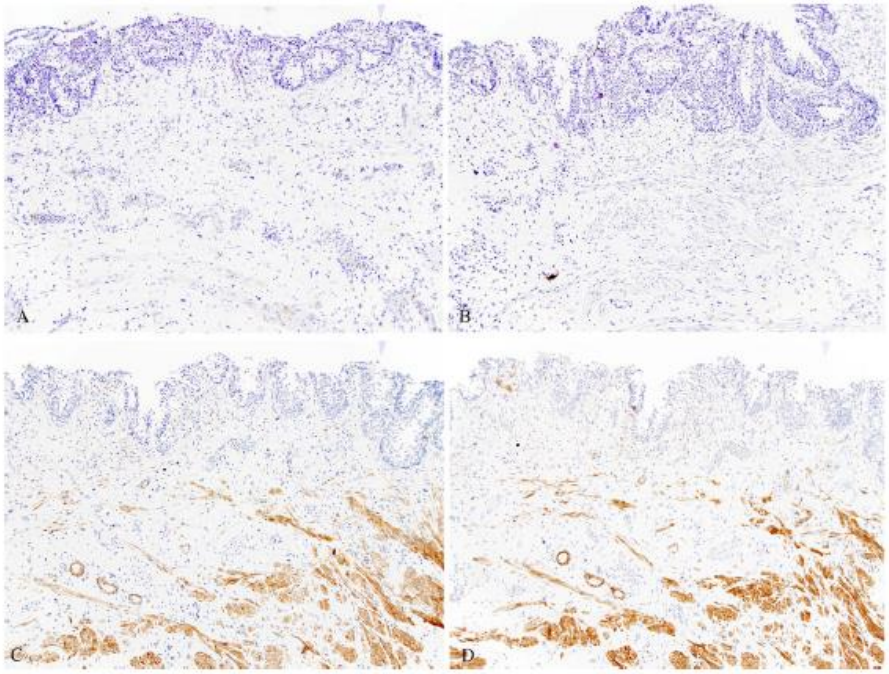


Fig. 1 Staining intensity of the muscularis mucosae, muscularis propria and smooth muscle in blood vessels depended strongly on pretreatment conditions. Staining without pretreatment resulted in weak staining of smooth muscle in the blood vessels (**a**) while staining was virtually absent when using 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

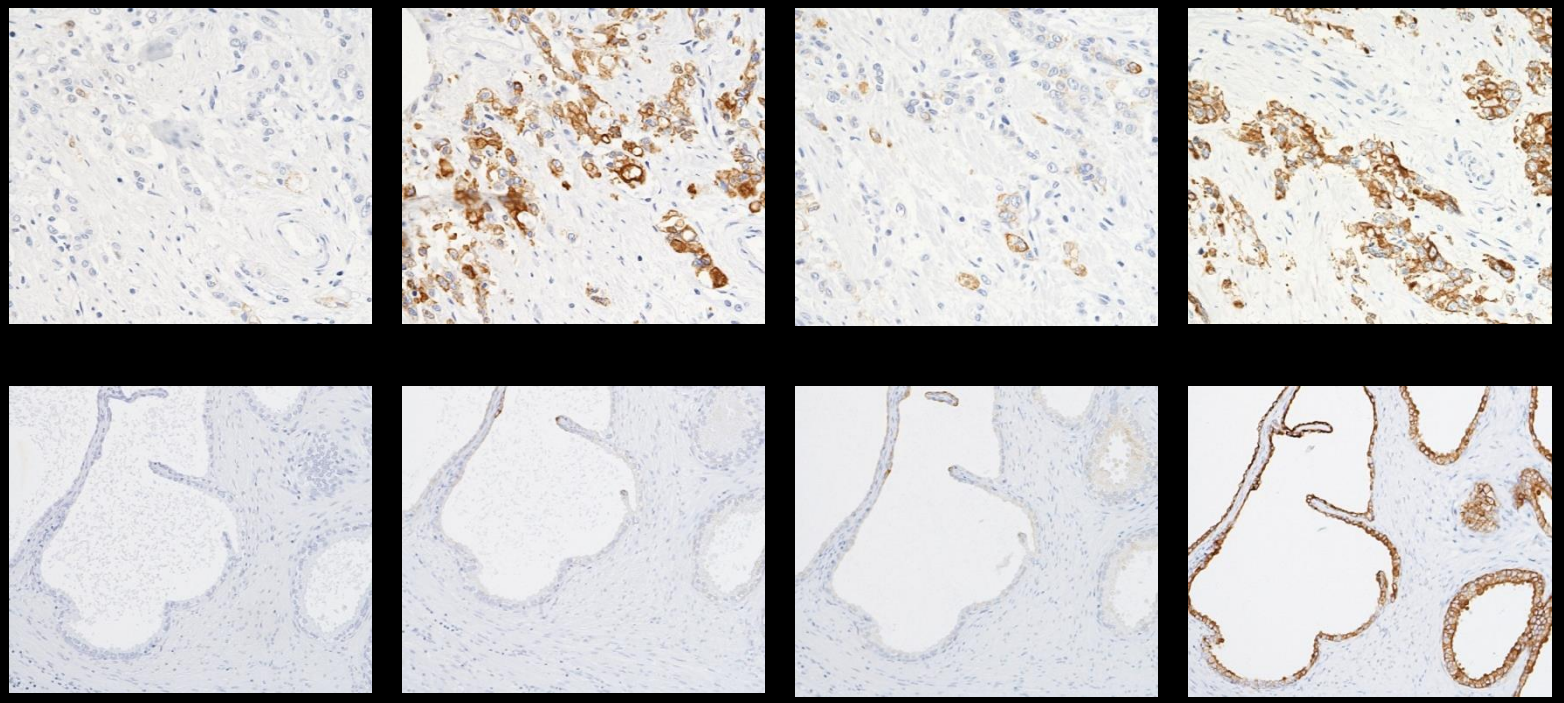
Table 2 Summary of IHC protocols used by different groups

	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.

Effect of different antigen retrieval procedures

CK19 clone b170



Epitope Retrieval

Antigen retrieval procedures for formalin fixed tissue:

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

The purpose of antigen retrieval is to unmask antigen epitopes and recover immuno-reactivity

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 Laboratories, San Ramon, California 94583.

Received for publication January 15, 1991; accepted March 12, 1991 (IC2212).

We describe a new approach for retrieval of antigens from formalin-fixed, paraffin-embedded tissues and their subse-

tions after pre-treatment of the slides with this method. These results showed that after antigen retrieval: (a) enzyme pre-

Shi et al. demonstrated that :

A) Enzyme pre-digestion of tissue could be omitted.

B) Incubation time with primary antibodies could be reduced, or dilutions of primary antibodies could be increased.

C) Staining could be achieved on long-term formalin fixed that failed to stain with conventional methods.

D) Certain antibodies which where typically unreactive with formalin-fixed tissue gave excellent staining.

The mechanism of HIER ?

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

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

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

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

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)

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

Table 2 Effectiveness of antigen retrieval by microwave heating

Antibody and clone/code	IHC				Dot-blot	
	Unfixed[a]		Fixed[b]		Unfixed	
	UH[c]	H[d]	UH	H	UH	H
ER 6F11	−/+[e]	+++	+	+++	1–2[f]	>0.02
ER 88	−	+++	−	+++	2–5	>0.02
ERα 1D5	−	+++	−	+++	0.5	>0.01
ERα MC-20	+	+	+	+	0.2–0.5	0.2–0.5
ERβ 06-629	−	+	−	+	2–5	>0.02
ERβ Y-19	−	+	−	+	2–5	>0.02
PR	−	+++	−	+++	2–5	>0.02
Ki-67 MM1	−	+++	−	+++	0.5–1	>0.02
Ki-67 MIB-5	−	−	−	+++	0.5–1	>0.02
Topo IIα SWT3D1	−	+++	−	+++	0.5–1	>0.02
COX-2	−	+	−	++	1–2	0.1–0.2

[a] Fresh frozen sections; [b] Formalin-fixed paraffin sections; [c] Unheated; [d] Tissue sections were heated by microwave oven at 98°C for 15 min in 10 mM citrate buffer (pH 6.0) before immunostaining; [e] Intensity of positive immunostaining is graded as +++, +++, ++, + and − for strong, moderate, weak and negative, respectively; [f] Last detectable dilution (μg/dot)

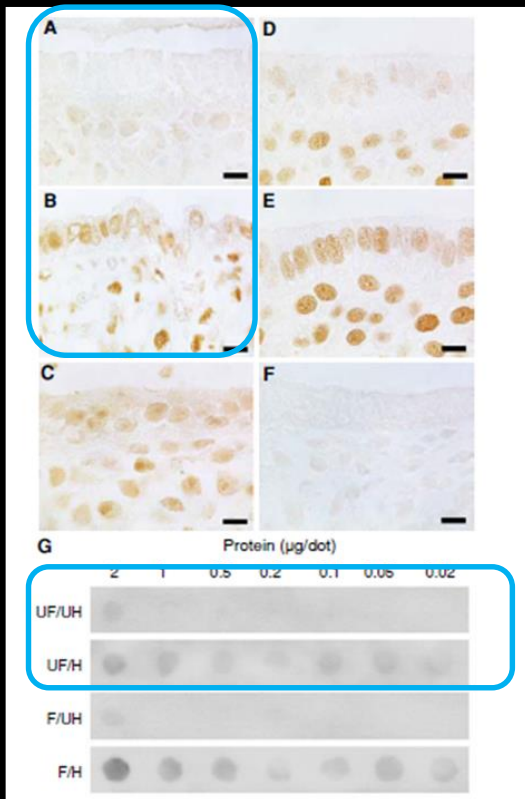


Fig. 1 IHC staining results (**A**–**F**) and dot-blot analysis (**G**) for ER 6F11. Both unfixed frozen sections (**A**) and formalin-fixed paraffin sections (**D**) of rat uterus showed weak or absent immunostaining without heating, the strongest intensity of staining was found with heating (**B**: unfixed frozen sections; **E**: formalin-fixed paraffin sections). Likewise, the sensitivity of detection in dot-blot analyses of ER 6F11 in protein extracts of the rat uterus was increased strongly by heating not only for fixed blots also for unfixed blots (**G**). In addition, the unfixed frozen sections, which showed weak or absent immunostaining without heating, showed positive immunostaining on retrial with a second IHC staining of the same tissues after heating of the sections (**C**). Negative control in an unfixed frozen section with heating (**F**). DAB was used as the chromogen. UF: Unfixed; F: Fixed; UH: Unheated; H: Heated. Bar = 10 μm

Demonstrated that:

9 of 11 markers tested, showed increased staining intensity in unfixed frozen tissue (rat uterus) after HIER treatment

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

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 propose 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.

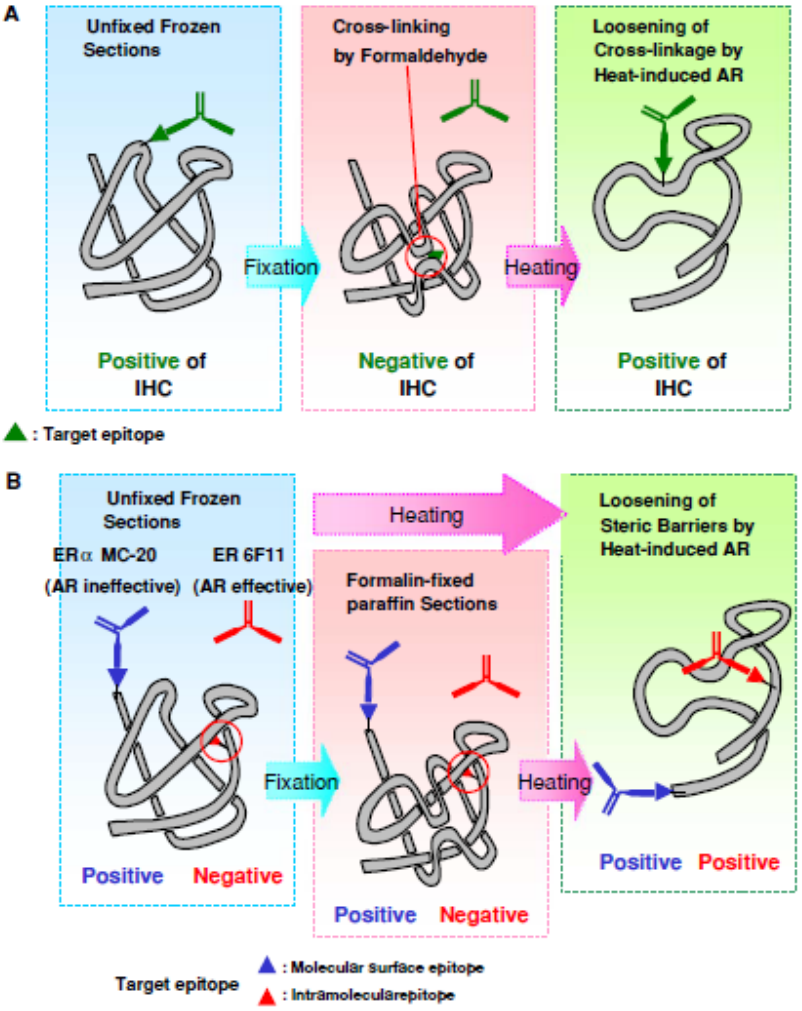


Fig. 7 Conventional hypothesis (**A**). Formaldehyde fixation can alter the three-dimensional structure of the epitope cross-linkages; these can be reversed by high-temperature heating. Our suggested mechanism for AR in IHC (**B**): Antibodies recognizing molecular surface epitopes, such as ERα MC-20, 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

HIER buffers used by NordiQC participants

In house	Dako	Roche Ventana	Leica Microsystems	Biocare	Thermo S LAB Vision
Low pH buffers					
Citrate buffer pH 6 / pH6.7	TRS Low pH 6.1	CC2 pH 6	BERS-1 pH 6	Diva Decloaker pH 6.2	
High pH buffer					
EDTA/EGTA pH 8	TRS High pH 9	CC1 pH 8.5	BERS-2 pH 9	Borg Decloaker pH 9.5	HIER buffer H pH 9
Tris-EDTA/EGTA pH 9	TRS High (3-in-1) pH 9				
Tris-HCL pH 9	App. 80-90 % of all pretreatment protocols				

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

CC ~ Cell Conditioning ~ Benchmark (XT/Ultra)

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

Decloaker` s ~ IntelliPATH

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

Restrictions:

The instrumentation / platforms dictates the choice of HIER buffers

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

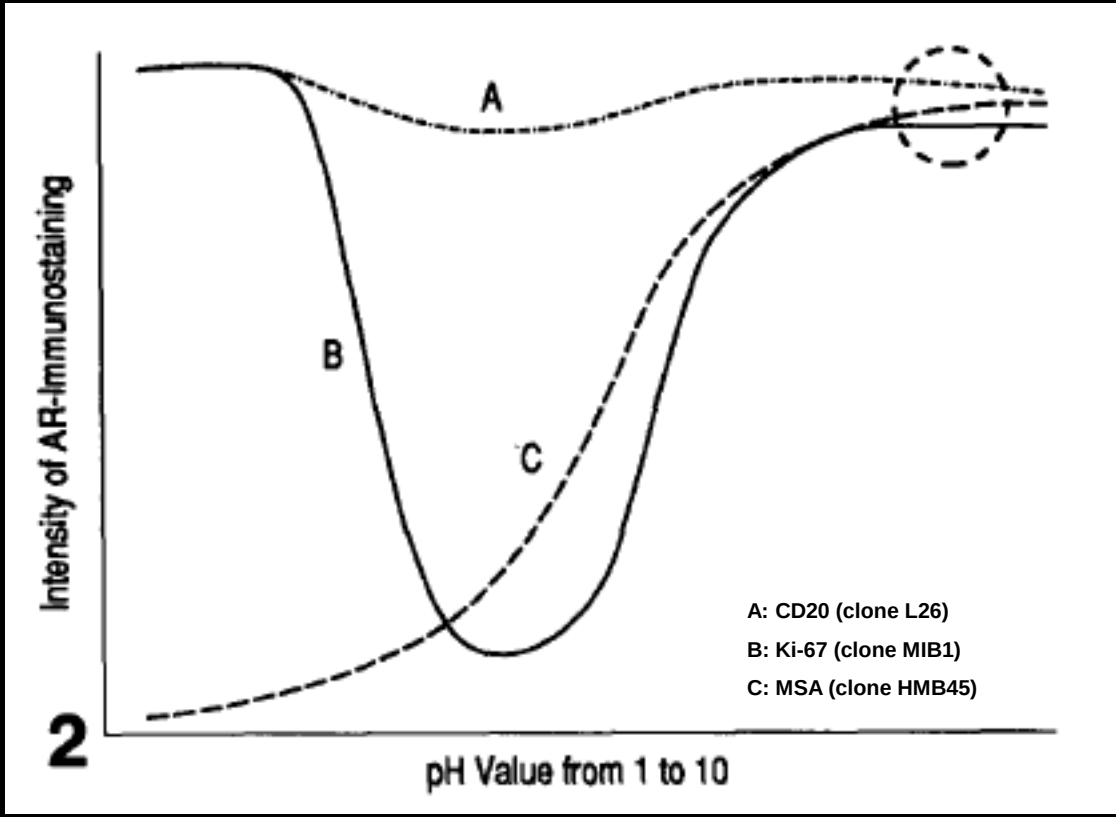
Efficient HIER depends on:

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

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

> 95% of all commonly used antibodies require HIER

HIER buffer - Influence of pH



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

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).

HIER buffer - Influence of pH

Alkaline buffer (TRS pH 9) versus Acidic based buffer (TRS pH 6.1) / HIER (20 min at 97°C)

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

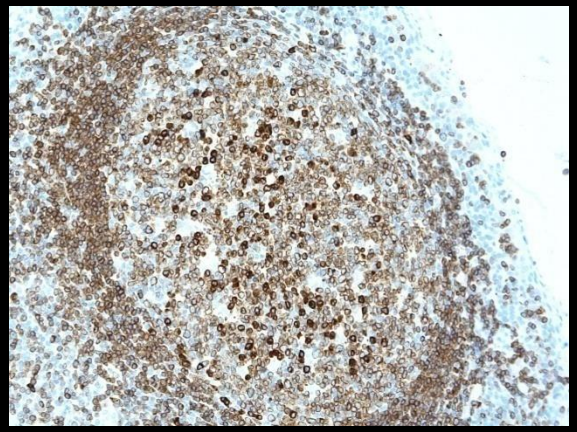
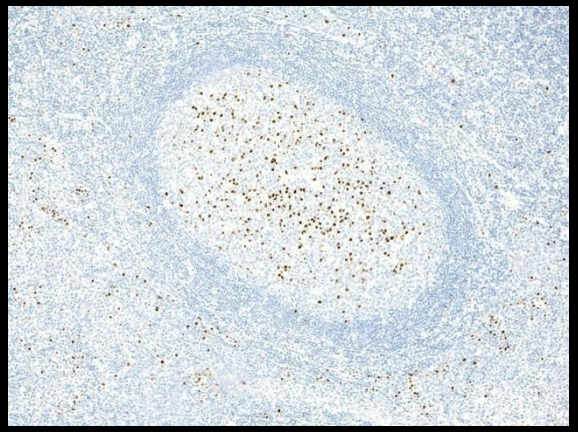
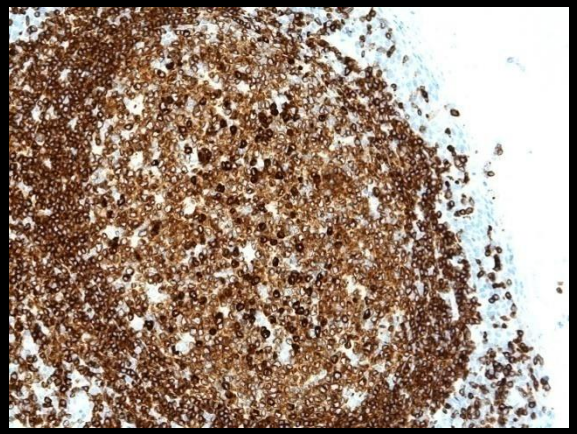
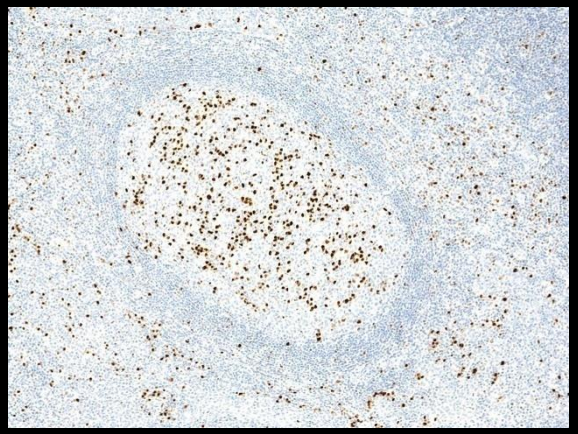
HIER	CD79 (1:300) (JCB117)	BCL-6 (1:100) (LN22)	CD163 (1:200) (MRQ-26)	MUM-1 (1:400) (MUM1p)	CD23 (1:50) (1B12)
TRS / High pH 9	+++(+)	+++	+++	++++	+++(+)
TRS / Low pH 6.1	++	(+)	-	++(+)	++(+)

HIER	CD79 (1:50) (JCB117)	BCL-6 (1:25) (LN22)	CD163 (1:25) (MRQ-26)	MUM-1 (1:50) (MUM1p)	CD23 (1:25) (1B12)
TRS / Low pH 6.1	+++(+)	++	+(+)	++++	+++

Staining Intensity graded from no reaction (-) to highest intensity (++++)

HIER buffer - Influence of pH

Alkaline buffer (TRS pH 9) versus Acidic based buffer (TRS pH 6.1) / HIER (20 min at 97°C)

	CD79, JCB117 (1:300)	MUM-1, MUM1p (1:400)
HIER in TRS pH 6.1		
HIER in TRS pH 9		

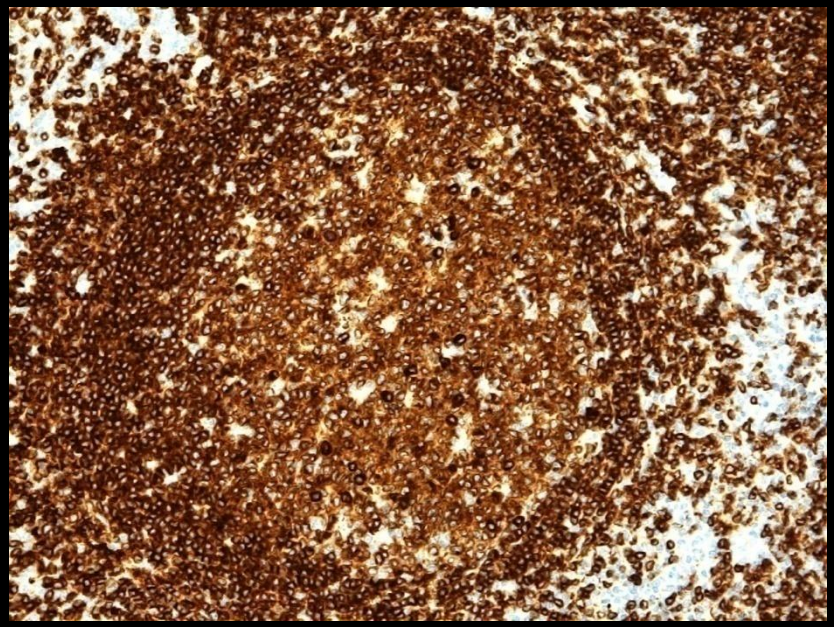
Tonsillar tissue fixed in 10% formalin (48h).

HIER buffer- Influence of pH and concentration of the primary Ab

Alkaline buffer (TRS pH 9) versus Citric based buffer (TRS pH 6.1) / HIER (20 min at 97°C)

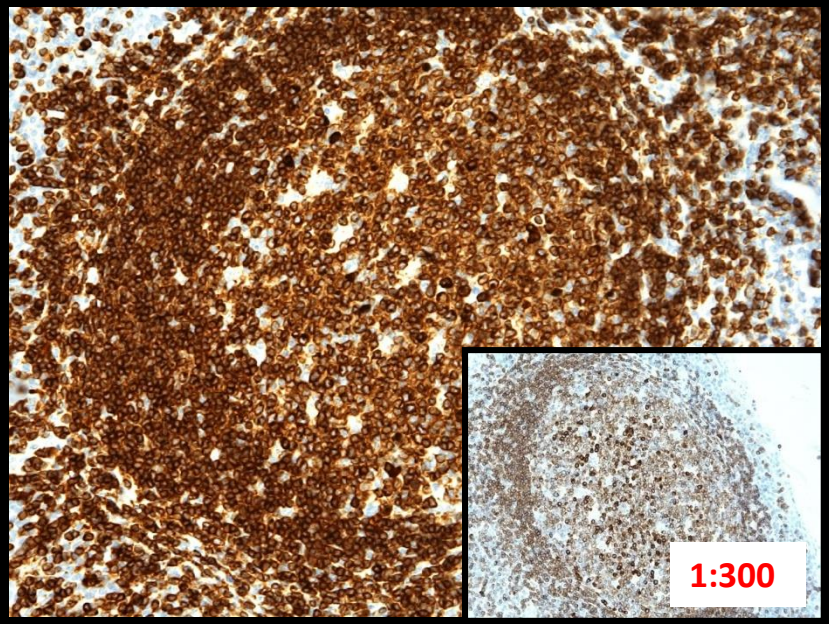
HIER in TRS pH 9

CD79, JCB117 (1:300)



HIER in TRS pH 6.1

CD79, JCB117 (1:50)



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

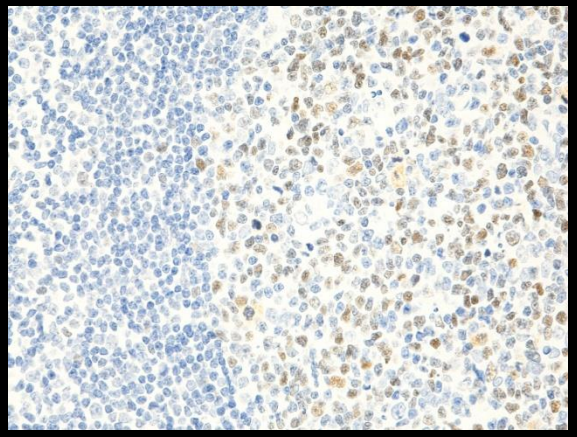
HIER buffer - Influence of pH

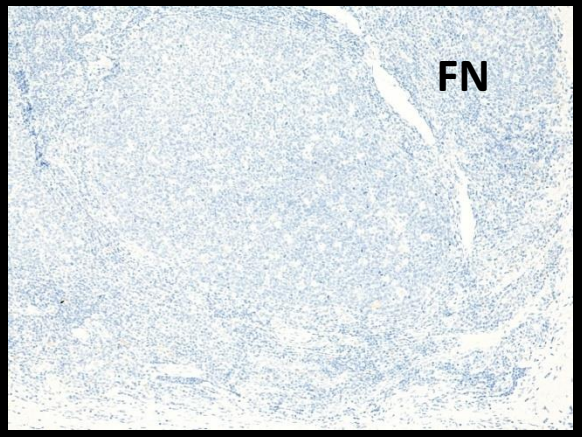
Alkaline buffer (TRS pH 9) versus Acidic based buffer (TRS pH 6.1) / HIER (20 min at 97°C)

BCL-6, LN22 (1:100)

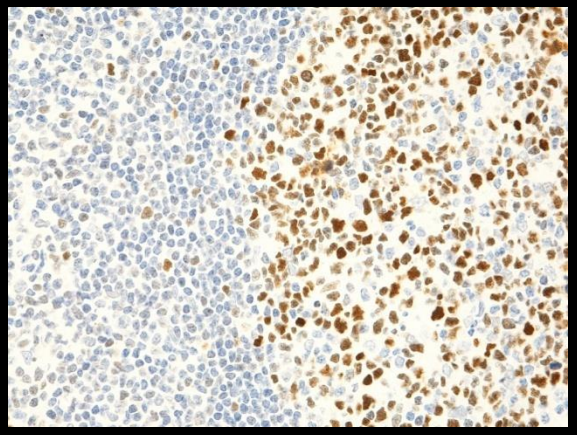
CD163, MRQ-26 (1:200)

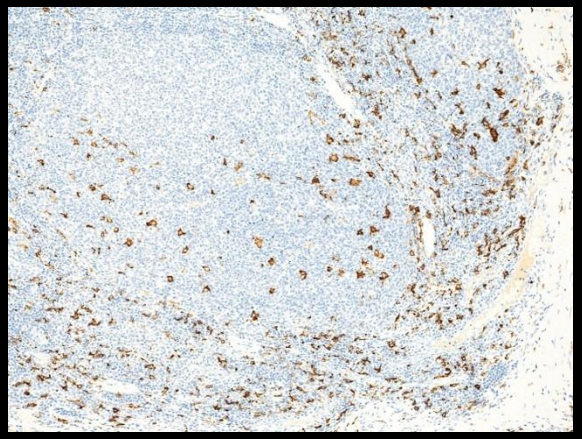
HIER in TRS pH 6.1





HIER in TRS pH 9





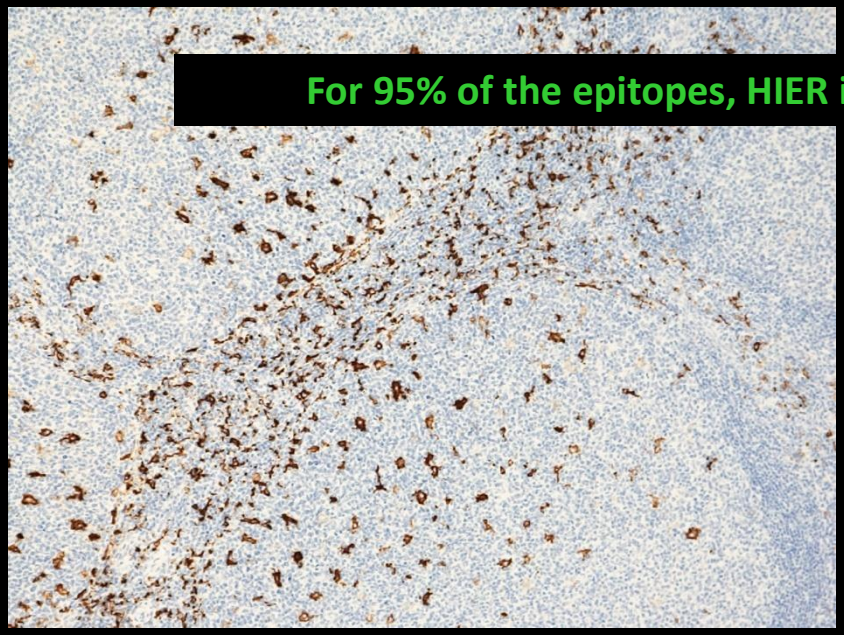
Tonsillar tissue fixed in 10% formalin (48h).

HIER buffer - Influence of pH

Alkaline buffer (TRS pH 9) versus Acidic based buffer (TRS pH 6.1) / HIER (20 min at 97°C)

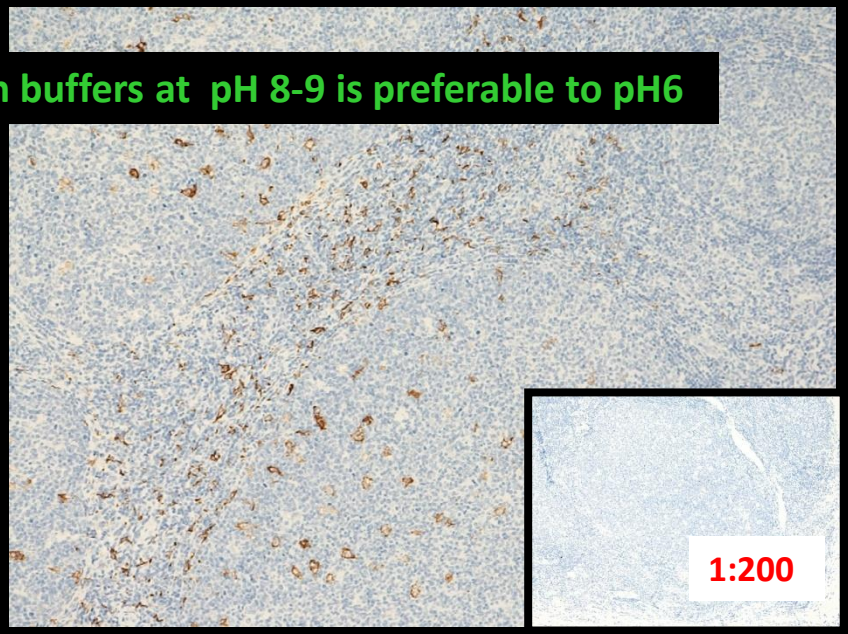
HIER in TRS pH 9

CD163, MRQ-26 (1:200)



HIER in TRS pH 6.1

CD163, MRQ-26 (1:25)



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

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

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

Leong AS-Y et al : *Applied Immunohistochemistry 2002; 10(3) : 263-268*

Demonstrated that superheating at 120°C (MWs under pressure 1.9 bar) produced the best overall results (42 markers) with exception of antibodies to cytokeratin clones Cam 5.2, A1E/3 and 34BE12 (compared to conventional heating in a pressure cooker or MWO at 98°C)

HIER - Influence of time and temperature

Alkaline buffer (TRS / High pH 9) at variable temperature and time

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

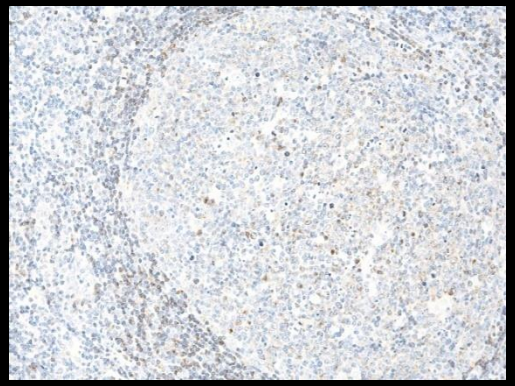
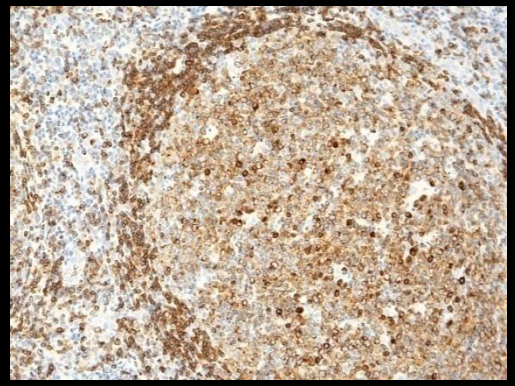
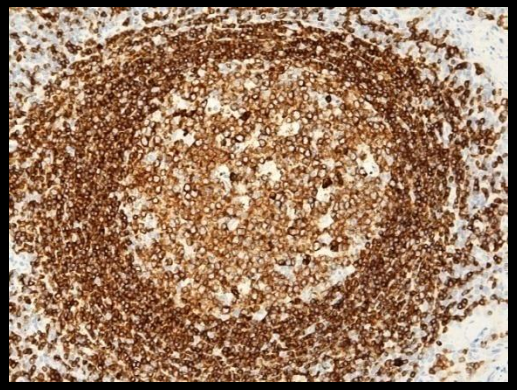
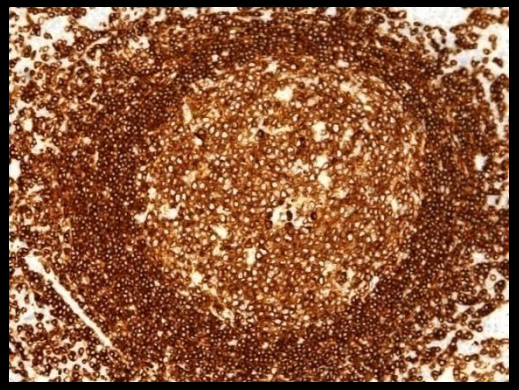
HIER High pH	80°C 5 min	80°C 10 min	80°C 20 min	80°C 40 min	80°C 80 min	97°C 5 min	97°C 10 min	97°C 20 min	97°C 40 min	97°C 80min	80°C 16h
CD79 (JCB117)	+	+	++	+++	+++(+)	+(+)	+++	+++(+)	++++	++++	++++
BCL-6, (LN22)	-	-	-	+	++(+)	-	+	+++	++++	++++	++++
CD163 (MRQ-26)	-	(+)	+	+(+)	++(+)	+	++	+++	++++	++++	+++(+)
MUM-1 (MUM1p)	-	-	(+)	+(+)	+++	(+)	++	++++	++++	++++	++++
CD23 (1B12)	-	-	-	+(+)	+++	(+)	++	+++(+)	++++	++++	+ ?

Staining Intensity graded from no reaction (-) to highest intensity (++++)

HIER - Influence of time and temperature

Alkaline buffer (TRS / High pH 9) at variable temperature and time

CD79, JCB117 (1:300)

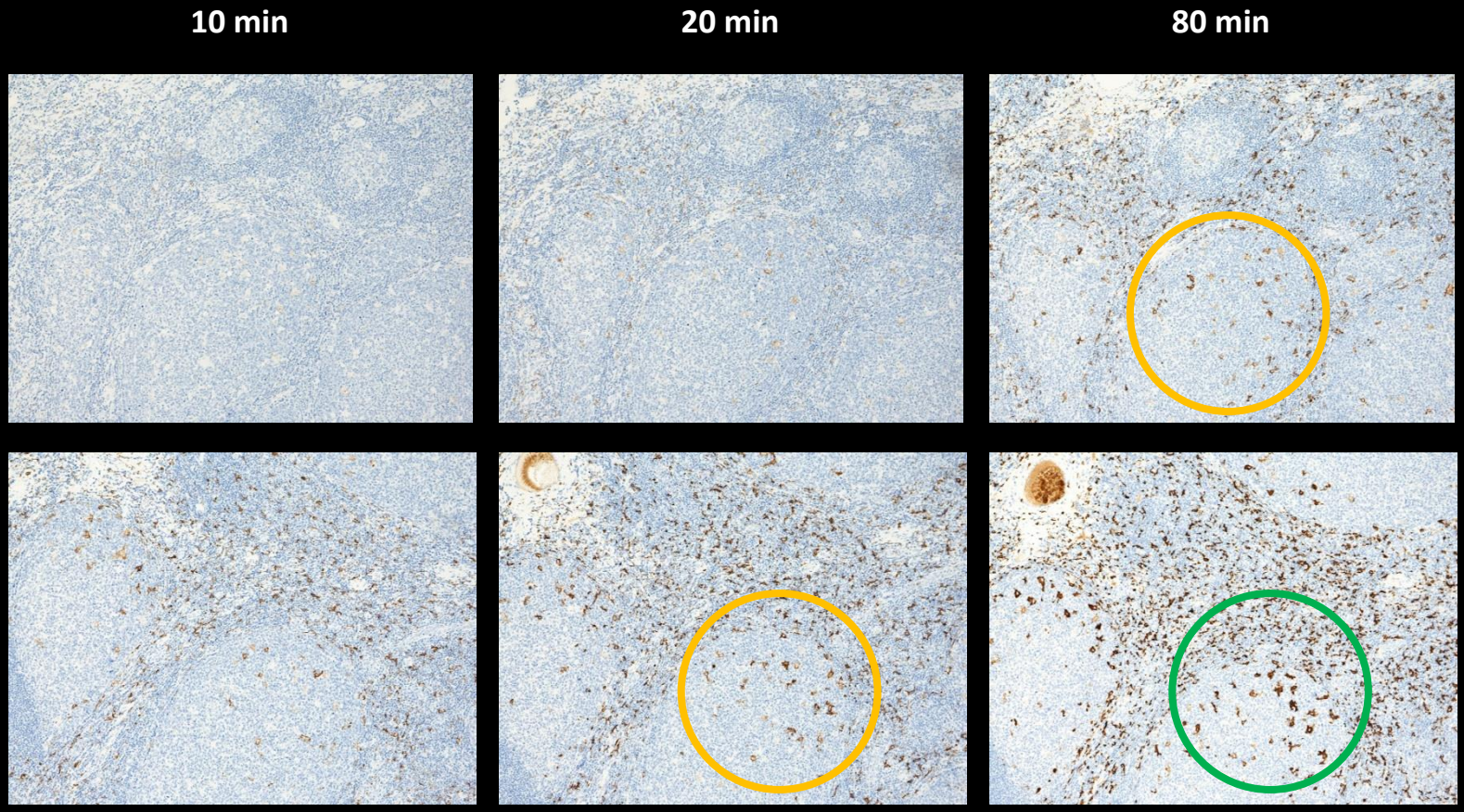
	10 min	20 min	80 min
HIER at 80°C			
HIER at 97°C			

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

HIER - Influence of time and temperature

Alkaline buffer (TRS / High pH 9) at variable temperature and time

CD163, MRQ-26 (1:200)



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

HIER - Influence of time

Alkaline buffer (pH 9) versus standard Citric based buffer (pH 6)

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

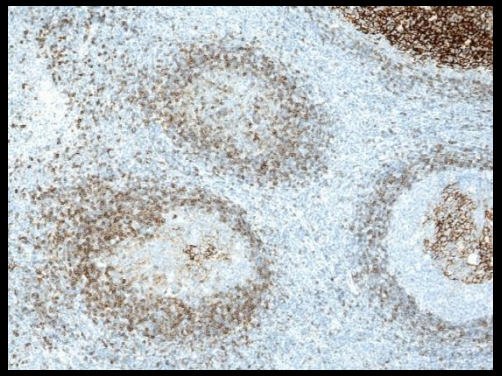
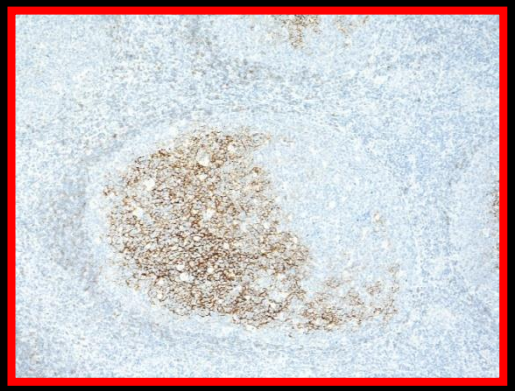
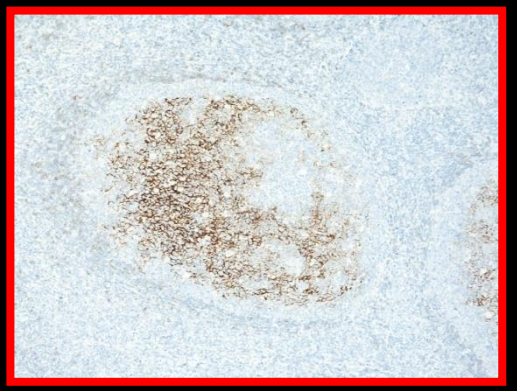
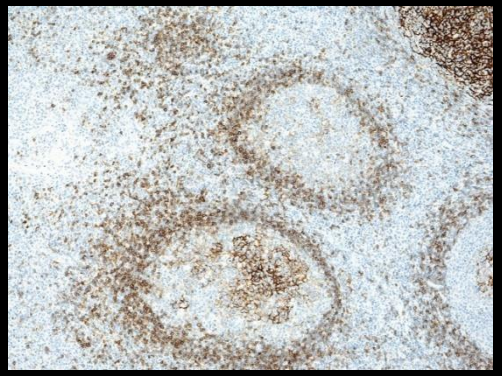
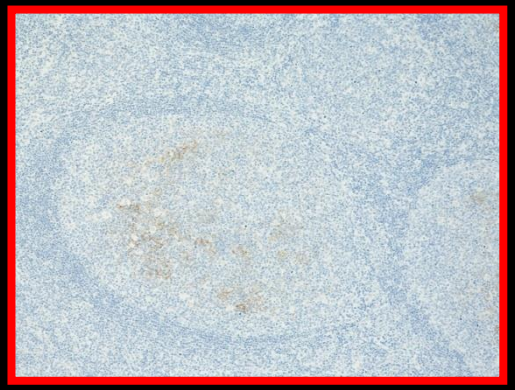
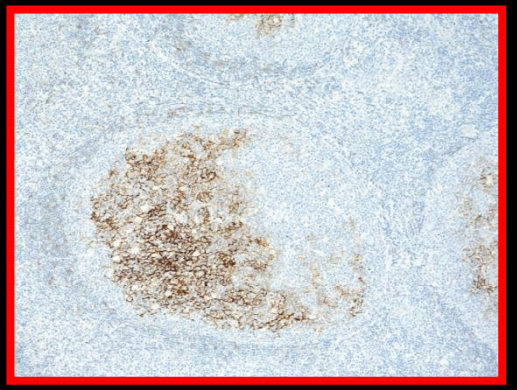
Primary Ab	HIER solution	97°C 5 min	97°C 10 min	97°C 20 min	97°C 40 min	97°C 80min	80°C 16h
CD79 (1:300) (JCB117)	TRS / Low pH pH 6.1	+	+(+)	++	+++	+++	+++
CD79 (1:300) (JCB117)	**TRS /High pH pH 9**	+(+)	+++	+++(+)	++++	++++	++++
MUM-1 (1:400) (MUM1p)	TRS / Low pH pH 6.1	-	+	++(+)	+++	+++	+++
MUM-1 (1:400) (MUM1p)	**TRS /High pH pH 9**	(+)	++	++++	++++	++++	++++
BCL-6 (1:100) (LN22)	TRS / Low pH pH 6.1	-	-	(+)	+	+	+(+)
BCL-6 (1:100) (LN22)	**TRS /High pH pH 9**	-	+	+++	++++	++++	++++
CD163 (1:200) (MRQ-26)	TRS / Low pH pH 6.1	-	-	-	-	(+)	(+)
CD163 (1:200) (MRQ-26)	**TRS /High pH pH 9**	(+)	++	+++	++++	++++	+++(+)
CD23 (1:50) (1B12)	TRS / Low pH pH 6.1	(+)	+(+)	++(+)	++++	+++ (+)	++ ?
CD23 (1:50) (1B12)	**TRS /High pH pH 9**	(+)	++	+++(+)	++++	++++	+ ?

Staining Intensity graded from no reaction (-) to highest intensity (++++)

HIER - Influence of pH and time

Alkaline buffer (TRS pH 9) versus Citric based buffer (TRS pH 6.1) / HIER

CD23, 1B12 (1:50)

	40 min at 97°C	16 h at 80°C	16 h at 70°C
HIER in TRS pH 6.1			
HIER in TRS pH 9			

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

Shi SR et al. : *Applied Immunohistochemistry* 2002 Dec;10(4):368-73.

A modified reduced-temperature antigen retrieval protocol effective for use with a polyclonal antibody to cyclooxygenase-2 (PG 27).

Polyclonal antibody to cyclooxygenase-2 (PG-27) failed to give a positive staining result after orthodox antigen retrieval.

For this particular antibody, a boiling condition yields a negative result

Heating tissue /cell specimens at a reduced temperature (90 degrees C as opposed to 100 degrees C) provided superior immunostaining for cyclooxygenase-2

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

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

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

121°C/ 5`

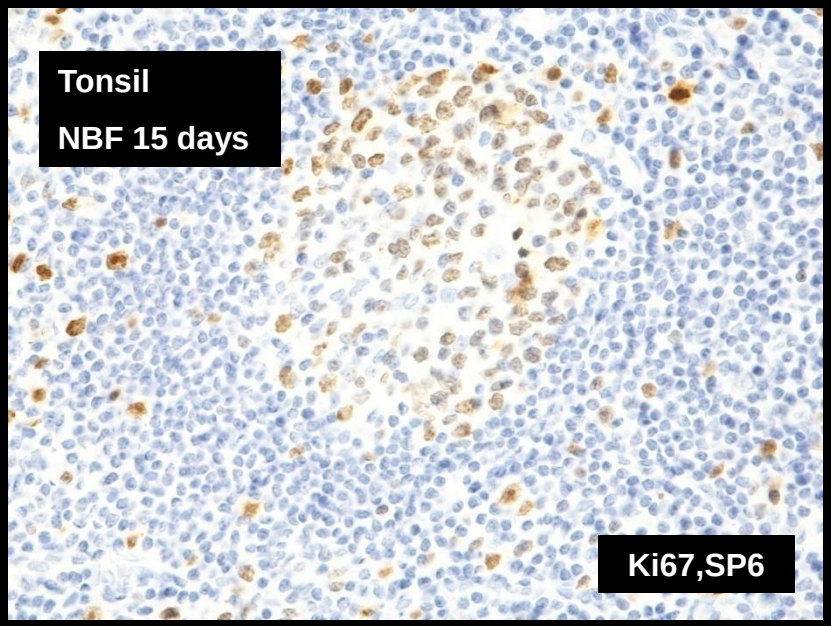
Consistent optimal staining of 26 of the 30 antigens was achieved despite the variable length of fixation (up to 8 days of fixation).

Influence of fixation time (10% formalin)

Prolonging the HIER time ?

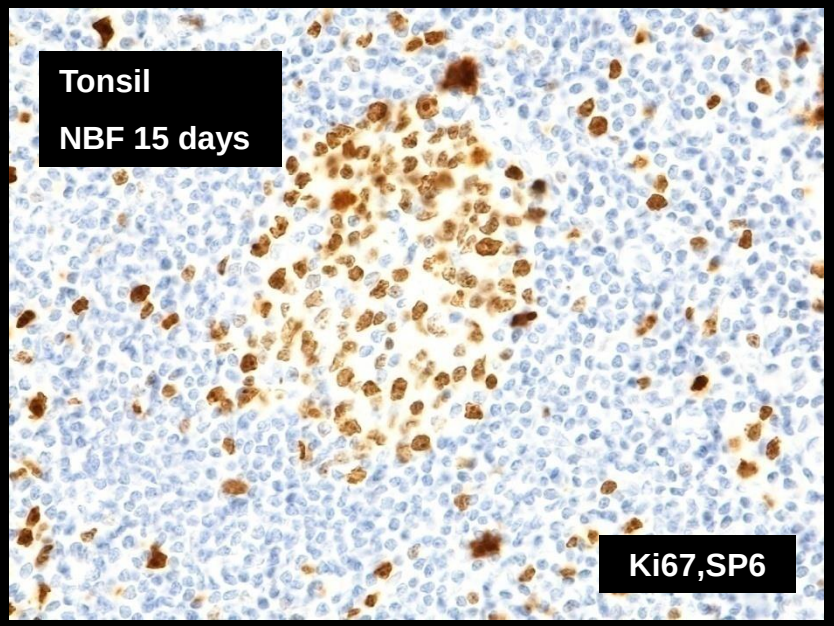
HIER 20`

MWO / TE pH 9 / 100°C



HIER 60`

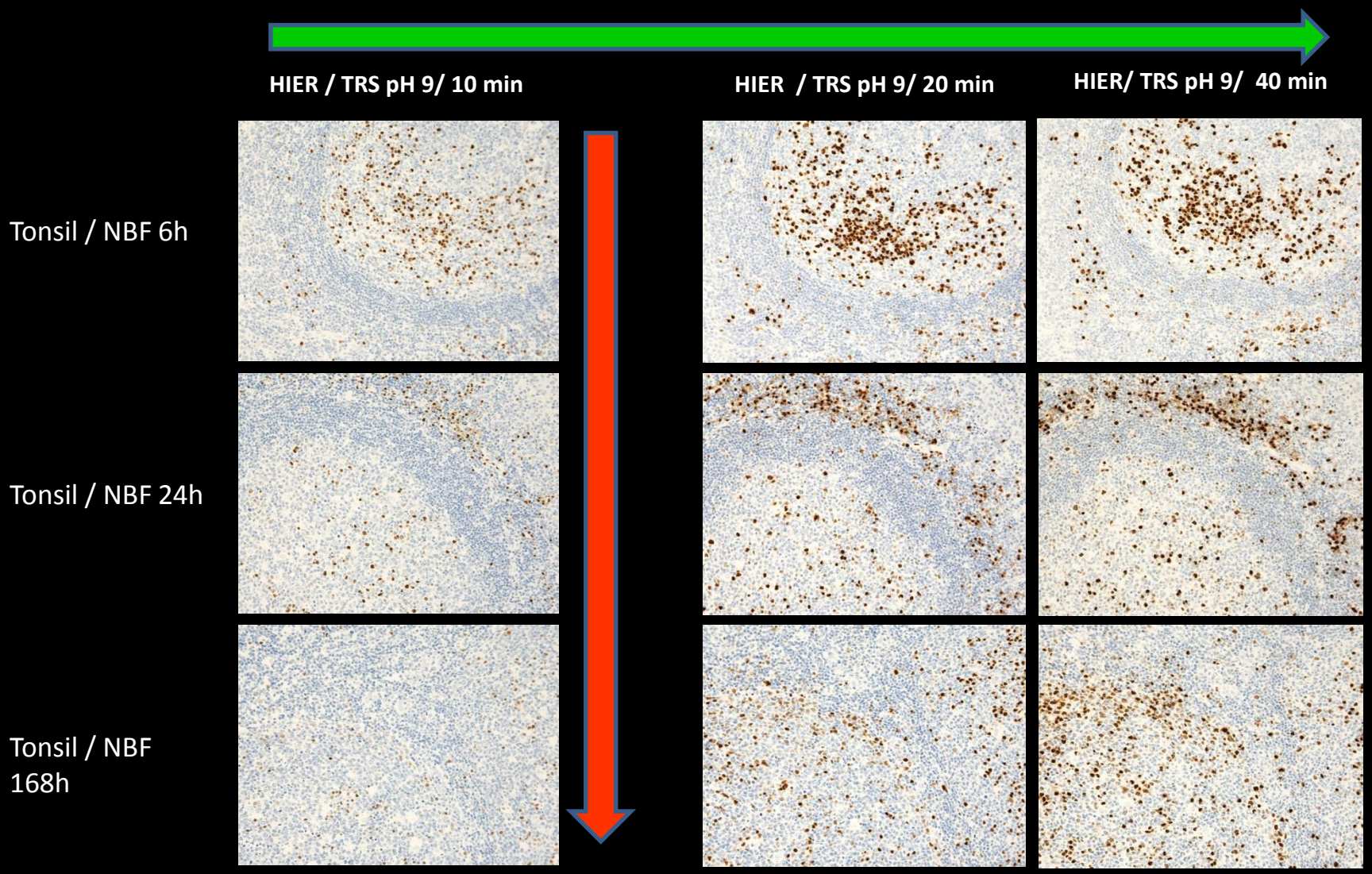
MWO / TE pH 9 / 100°C



An extension of the HIER time may be required to obtain optimal results for tissue section “over fixed” in formalin

Efficient HIER may depend on the chosen source of heat method (e.g. Pressure cooker versus MWO)

MUM-1, MUM1p / HIER in Alkaline buffer (TRS / High pH 9) at 97°C



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

Epitope Retrieval

Epitope retrieval procedures for formalin fixed tissue:

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

The purpose of antigen retrieval is to unmask antigen epitopes and recover immuno-reactivity

Enzymatic digestion

Enzymatic digestion is used to overcome the effects of covalent cross-links that are formed in tissues during formalin fixation.

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

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

Markers requiring enzymatic pretreatment :

FVIII (poly), LMV CK (CAM 5.2), PAN CK (MNF116), EGFR (various), TCR (8A3)……
Extracellulare matrix proteins (COLL-III (poly), Laminin (poly) and COLL-IV (CIV-22) ……

Enzymatic pre-treatment

”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

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

Difficult to control and to standardizes

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

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Original Article

The Influence of Protease Digestion and Duration of Fixation on the Immunostaining of Keratins.

A Comparison of Formalin and Ethanol Fixation[1]

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Division of Anatomic Pathology and the Sylvia Cowan Laboratory of Surgical Pathology, City of Hope National Medical Center Duarte, CA.

Received for publication October 1, 1985 and in revised form January 20, 1986; accepted February 6, 1986 (5A0563).

Table 1. *Immunostaining of several normal epithelial tissues after various periods of formalin fixation and digestion with trypsin*

Tissue	Duration of fixation (min)[a]			
	1 day	1 week	3 weeks	6 weeks
Squamous mucosa	60	180	180	180
Gastric mucosa	10	10	60	120
Skin	60	120	120	180
Pancreas, ducts	30	30	60	120
Kidney, tubules	10	30	30	30
Prostate	30	30	120	120
Thyroid	10	30	120	120

[a] The optimal digestion time for each fixation period is given.

Ethanol fixed tissue:

Even short periods of digestion resulted in disintegration of architectural and cytologic detail and in reduced immunostaining

Formalin fixed tissue:

The increased immunoreactivity of keratins varied with the tissue type and the duration of proteolysis (Trypsin 0.1%, Pronase XIV 0.1% & Pepsin 0.4%), as shown for trypsin in Table 1.

For any given tissue, longer digestion was necessary as the duration of fixation was increased.

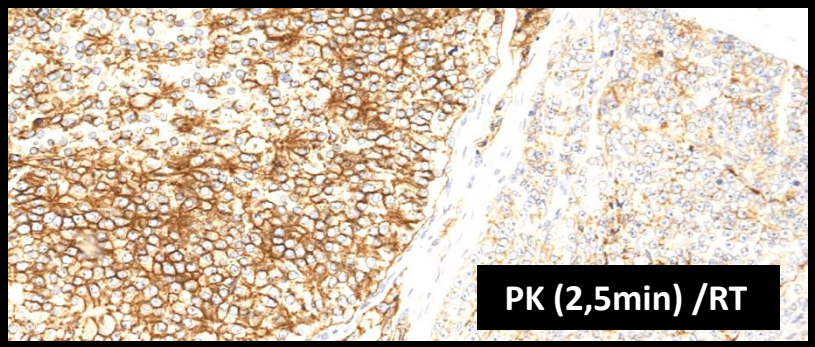
Excessive digestion manifested itself as a reduction rather than an enhancement of the strength of the immunoreaction.

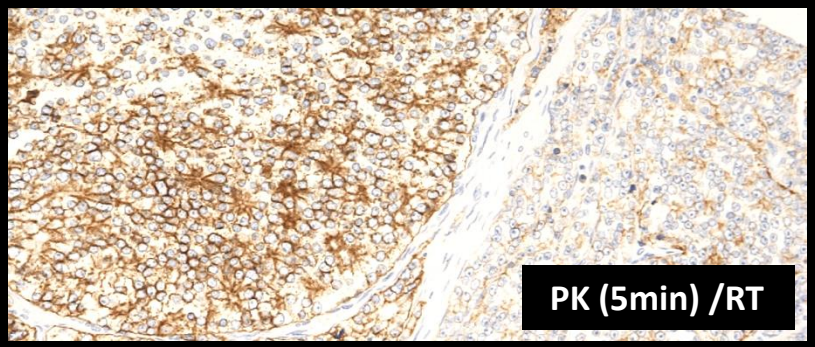
AE1

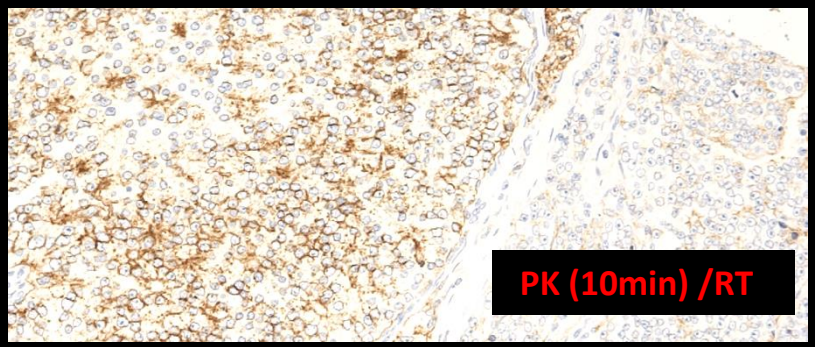
Enzymatic digestion (Influence of digestion time)

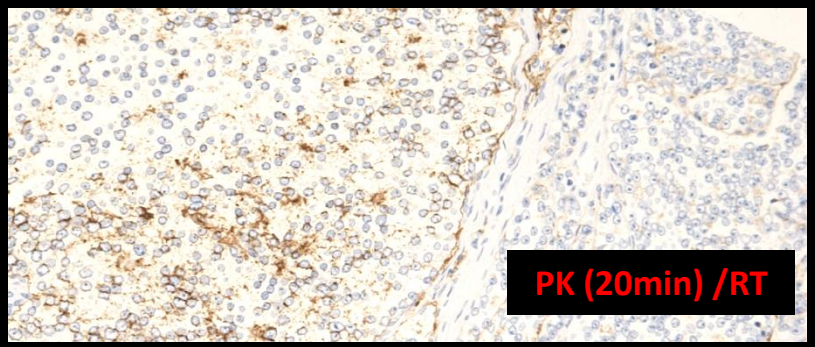
EP-CAM, clone MOC-31, dilution 1:20

Adenocarcinoma (Prostate) fixed in 10% Formalin / 24h









Proteinase K / RTU (Dako, S3020)

Enzymatic digestion (Influence of enzyme type and digestion time)

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

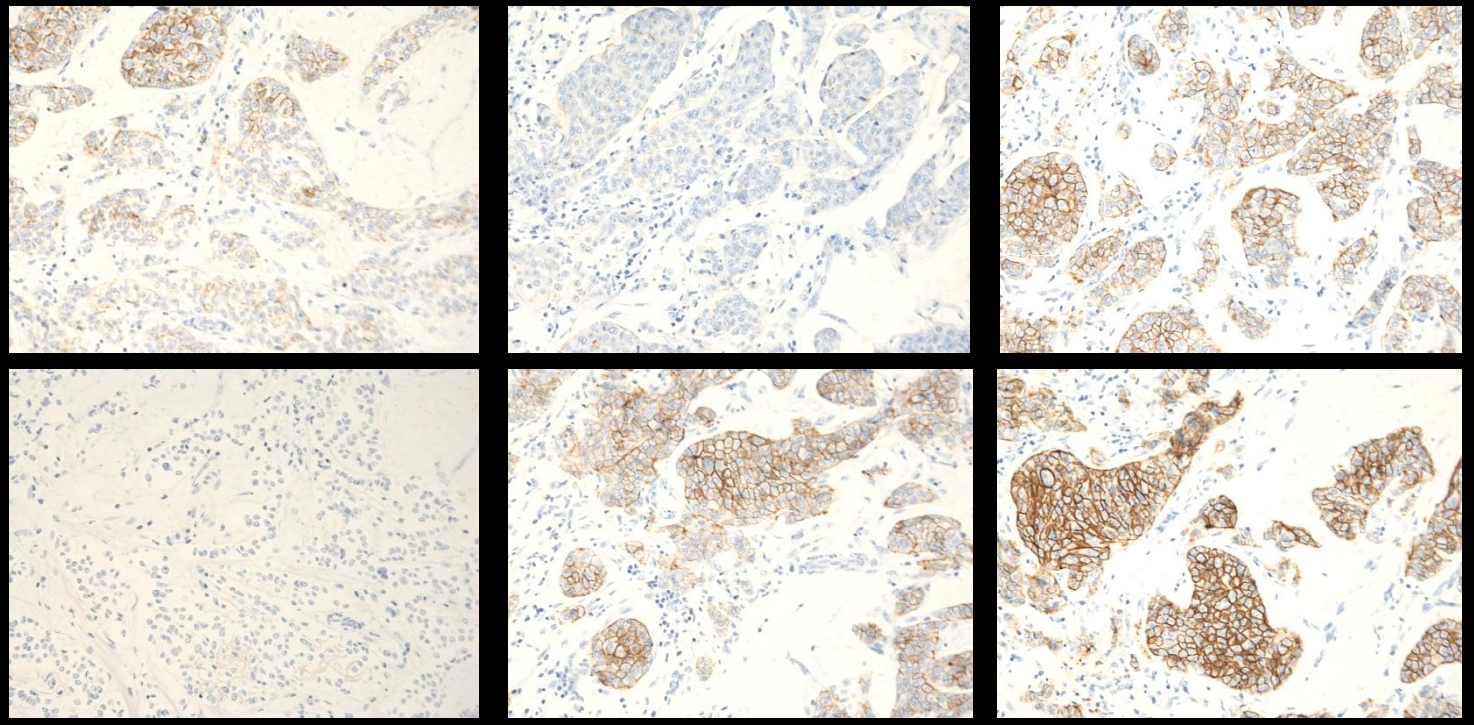
Adenocarcinoma (Breast) fixed in 10% Formalin / 24h

Digestion time 5 min.

37°C

Digestion time 20 min.

37°C



Proteinase K / RTU (Dako, S3020)

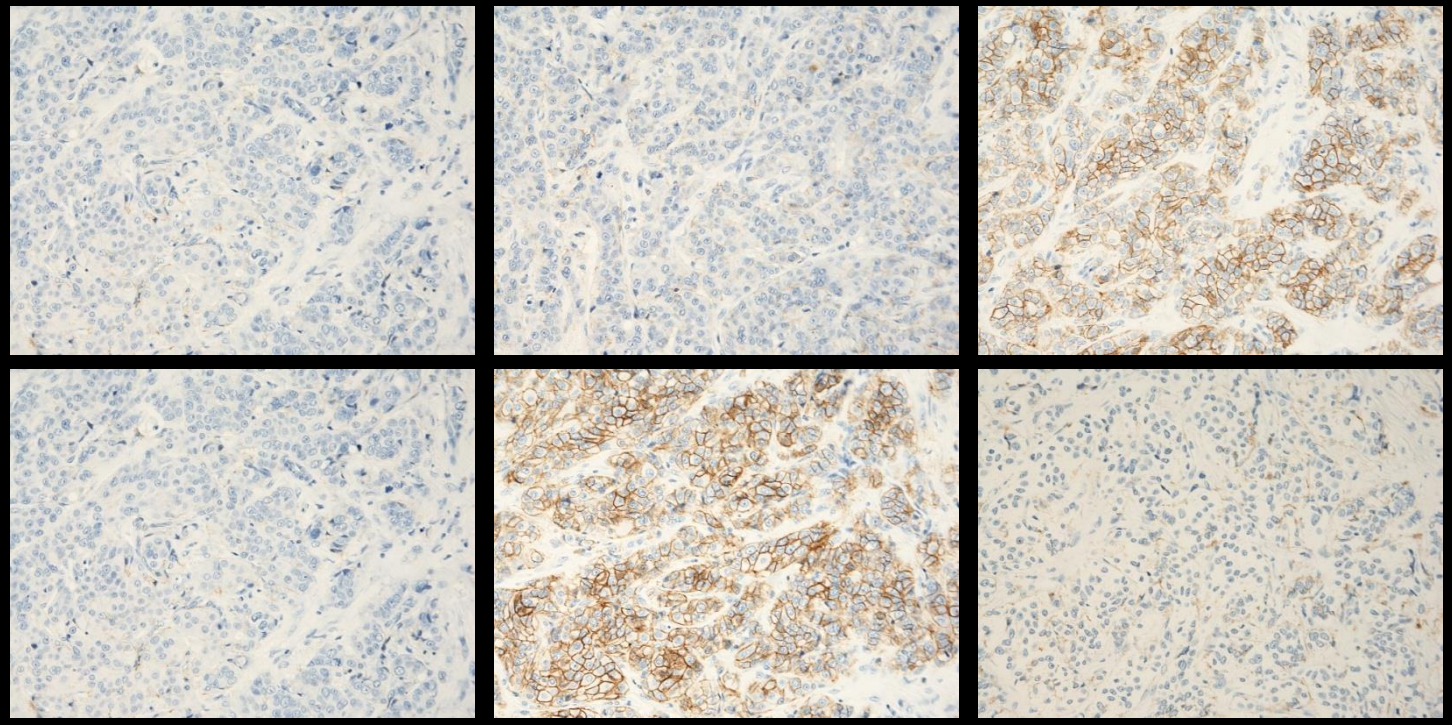
Pronase XIV / 0.05%

Pepsin / (Dako, S3002)

Enzymatic digestion (Influence of temperature and digestion time)

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

Adenocarcinoma (Breast) fixed in 10% Formalin / 48h



Digestion temperature

24°C

Digestion temperature

37°C

Pepsin / (Dako, S3002)

2.5 min

Pepsin / (Dako, S3002)

10 min

Pepsin / (Dako, S3002)

40 min

Enzymatic digestion (Influence of fixation time and “optimal digestion”)

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

Adenocarcinoma (Breast) fixed in 10% Formalin at variable times (24, 48 and 120 h)

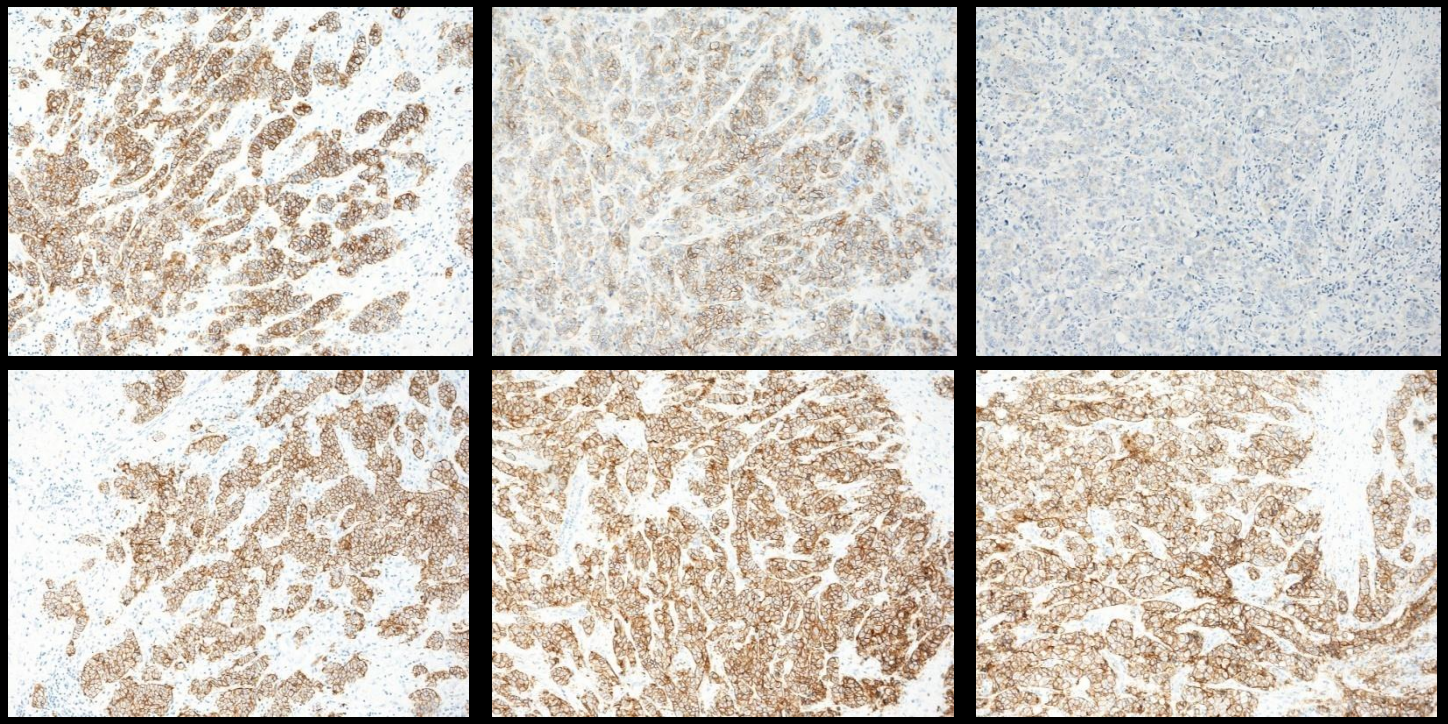
Pepsin / (Dako, S3002)

Digestion time 10 min.

37°C

HIER , Low pH (S1700)

20 min / 97°C



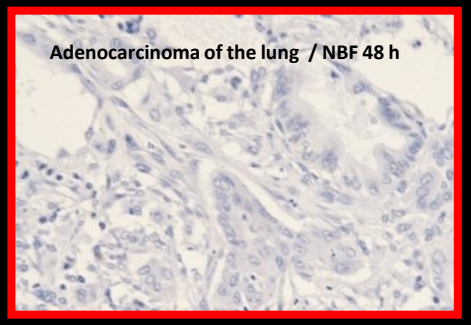
NBF 24 h

NBF 48 h

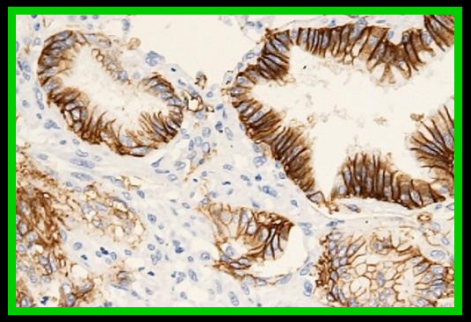
NBF 120h

TRS Low pH 6.1 (Dako, S1699/S1700)

Diva Decloaker pH 6.2 (Biocare, DV2004)



EP-CAM ~ Enzymatic digestion



EP-CAM ~ HIER / TRS low pH 6.1 (S1700)

For certain markers these modified low pH HIER buffers are performing better than:

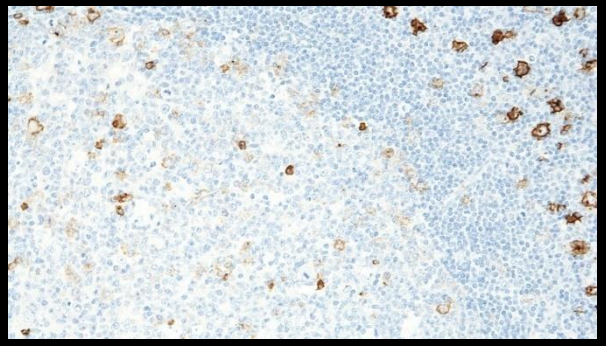
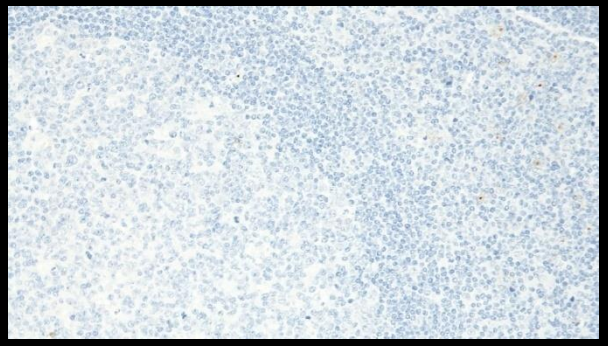
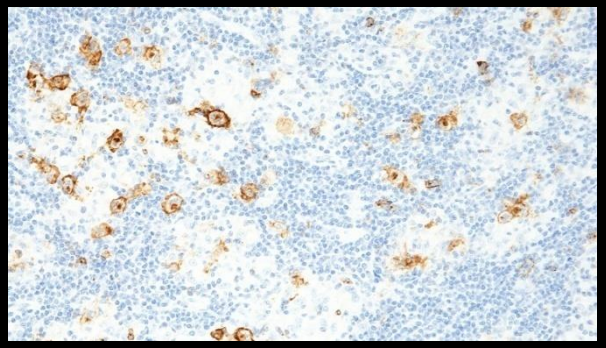
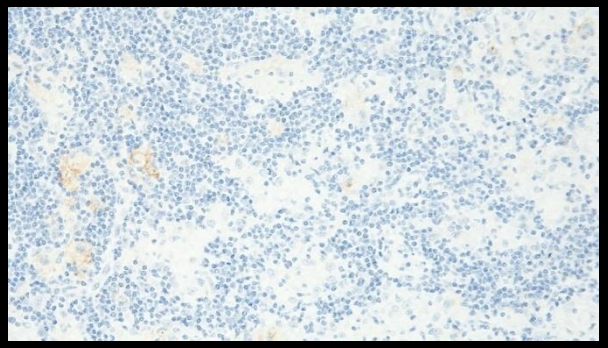
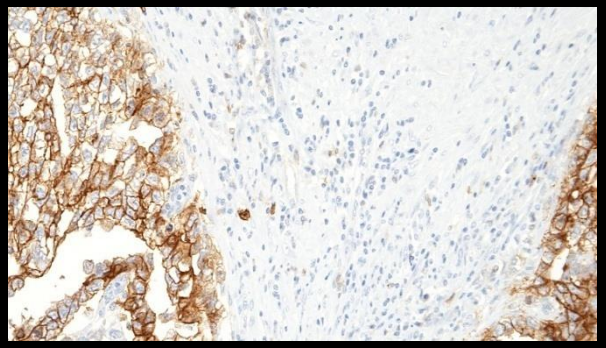
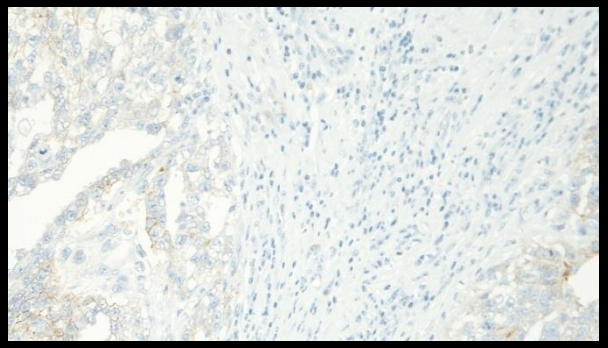
- Enzymatic digestion
- HIER in either an standard low (acidic) or high (alkaline) pH buffer`s

Markers requiring the TRS Low pH 6.1 (Dako) or Diva Decloaker pH 6.2 (Biocare) :

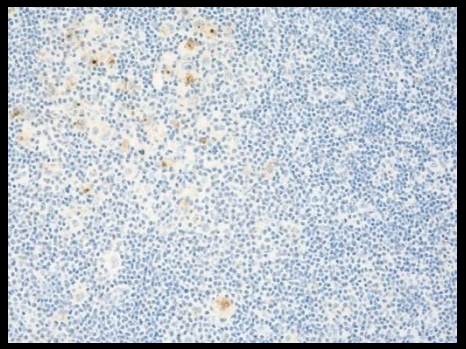
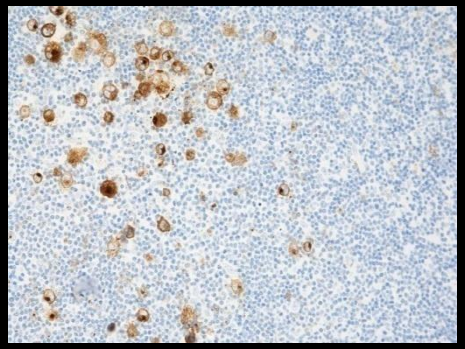
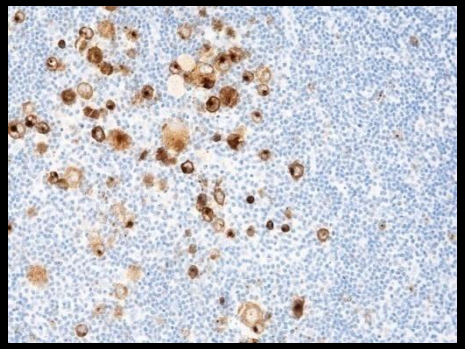
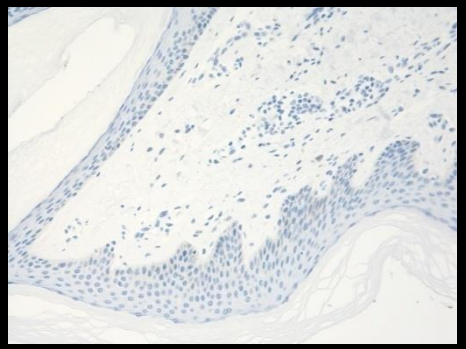
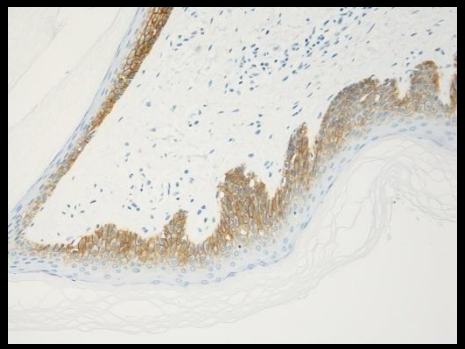
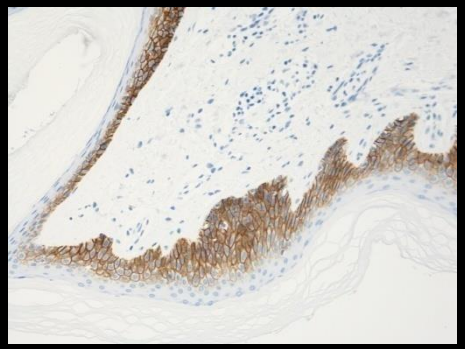
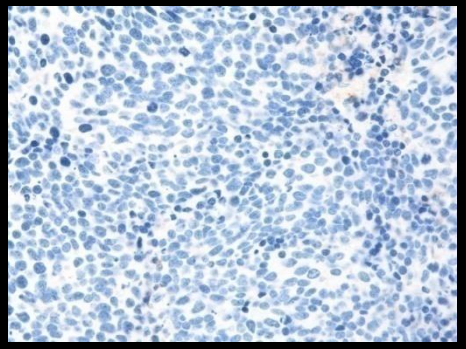
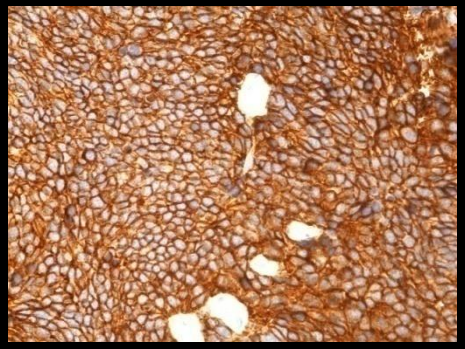
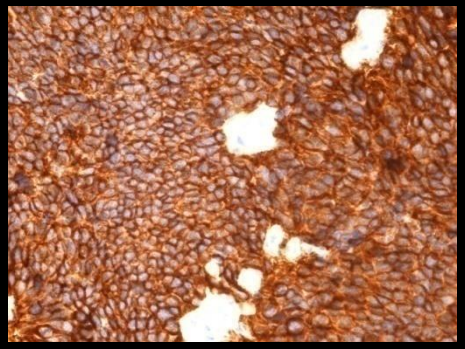
EP-CAM (clone EP-4 or MOC-31 or “VU-1D9”); GP200 (clone SPM 314 or 66.4.C2); CD21 clone 1F8; CD61 clone Y2/51; NGFR clone MRQ-21; Desmoglein-3 clone BC11 and a few more

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

CD30 clone ConD6/B5

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

Modified HIER buffers (low pH) with high impact on the final result

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

BIOCARE MEDICAL

continually researching the most cost-effective and quality-aware diagnostic solutions

Diva Decloaker, 10X

Pretreatment Reagent

Control Number: 901-DV2004X-022912

ISO 9001:13485 CERTIFIED

Catalog Number: DV2004 LX, MX

Description: 100, 500 ml; concentrate

Intended Use:
For In Vitro Diagnostic Use

Summary & Explanation:
Diva Decloaker is a heat retrieval solution that is compatible with virtually all antibodies and eliminates the need for multiple buffers including citrate buffer, EDTA or high pH tris buffers. Diva Decloaker can be used with Biocare's digital electric pressure cooker (Decloaking Chamber), a steamer, a water bath or a microwave oven. Antibody titers are doubled and tripled when compared to citrate buffer, pH 6.0. Diva Decloaker incorporates Assure™ technology, a color-coded high temperatures pH indicator solution. The end-user is assured by visual inspection that the solution is at the correct dilution and pH. This product is specially formulated for superior pH stability at high temperatures and will help prevent the possibility of losing pH sensitive antigens. Diva Decloaker is non-toxic, non-flammable, odorless and sodium azide and thimerosal free.

Known Applications:
Immunohistochemistry (formalin-fixed paraffin-embedded tissues)

4. Retrieve sections under pressure using Biocare's Decloaking Chamber. Follow the recommendations on the antibody data sheet and Table 1 (below).
5. Check solution for appropriate color change. (See Technical Note #1)
6. Gently rinse by gradually adding DI water to the solution, then remove slides and rinse with DI water.

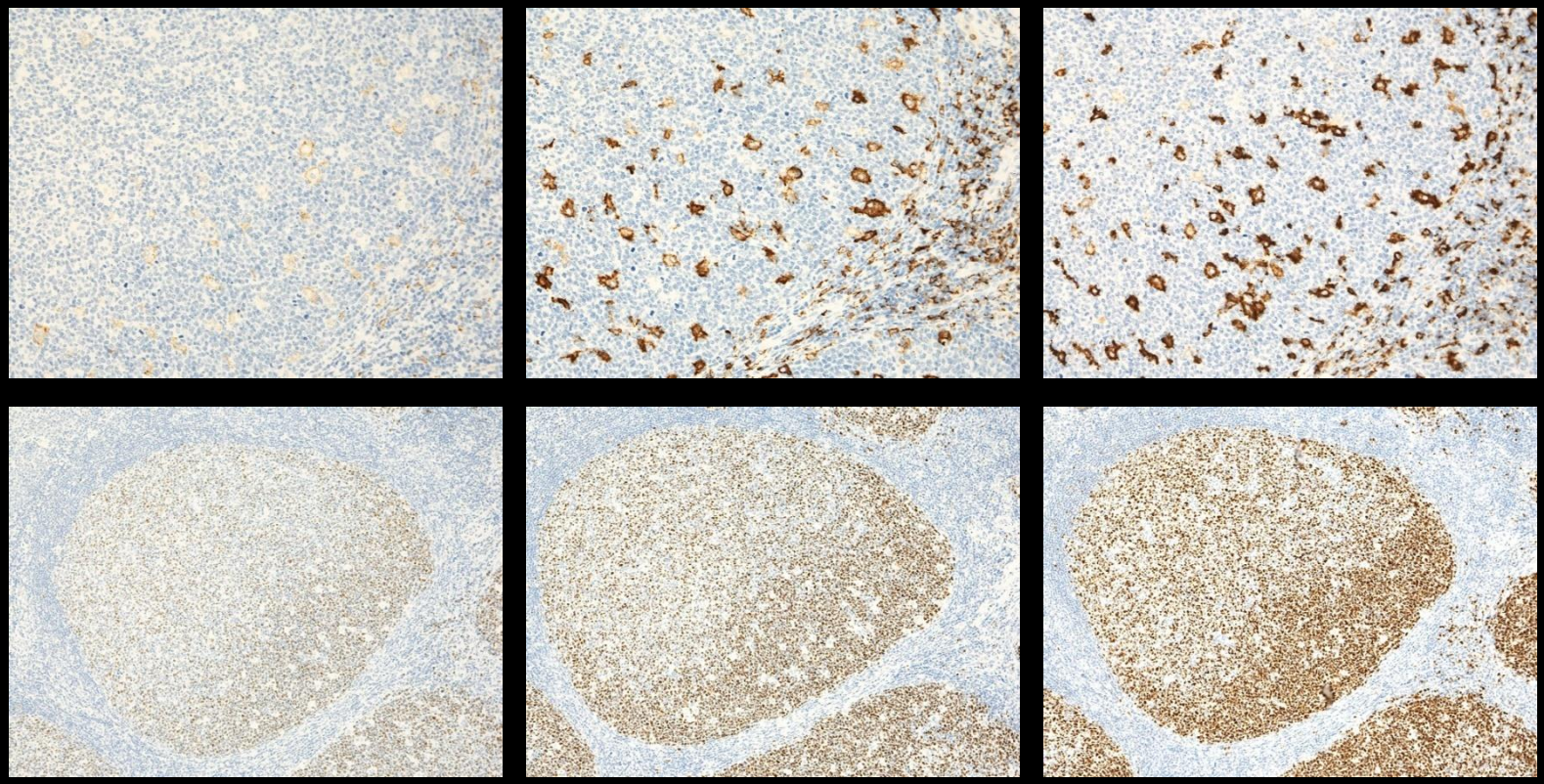
Technical Notes:

1. Concentrated Diva Decloaker is a bright yellow color. RTU or 1X solution is a pale yellow color. When the solution reaches 80-125°C, the solution turns yellow and indicates that the high temperature solution is at correct pH. Should the pH rise above 7.0, the solution turns a fuschia red color. Should the pH drop too low, the solution turns a pink color.
2. Diva may be used with various heat retrieval methods, including a microwave oven, pressure cooker, hot water bath or steamer.
3. If using Biocare's Desert Chamber Pro (a programmable turbo-action drying oven), dry sections at 25°C overnight or at 37°C for 30-60 minutes and then dry slides at 60°C for 30 minutes.
4. Use positive charged slides (use Biocare's Kling-On HIER Slides) and cut tissues at 4-5 microns. Do not use any adhesives in the water bath. Poor fixation and processing of tissues will cause tissue sections to fall off the slides, especially fatty tissues such as breast. Tissues should be fixed a minimum of 6-12 hours.
5. CD5 (CM/PM099) does not work with Diva. Borg Decloaker is recommended.

Be critical about what you read !!!!!

HIER

Diva Decloaker verus Alkaline buffer`s (MWO/ 20 min/99°C)



Tonsillar tissue fixed in 10% formalin (24h).

Automated platforms: The challenge

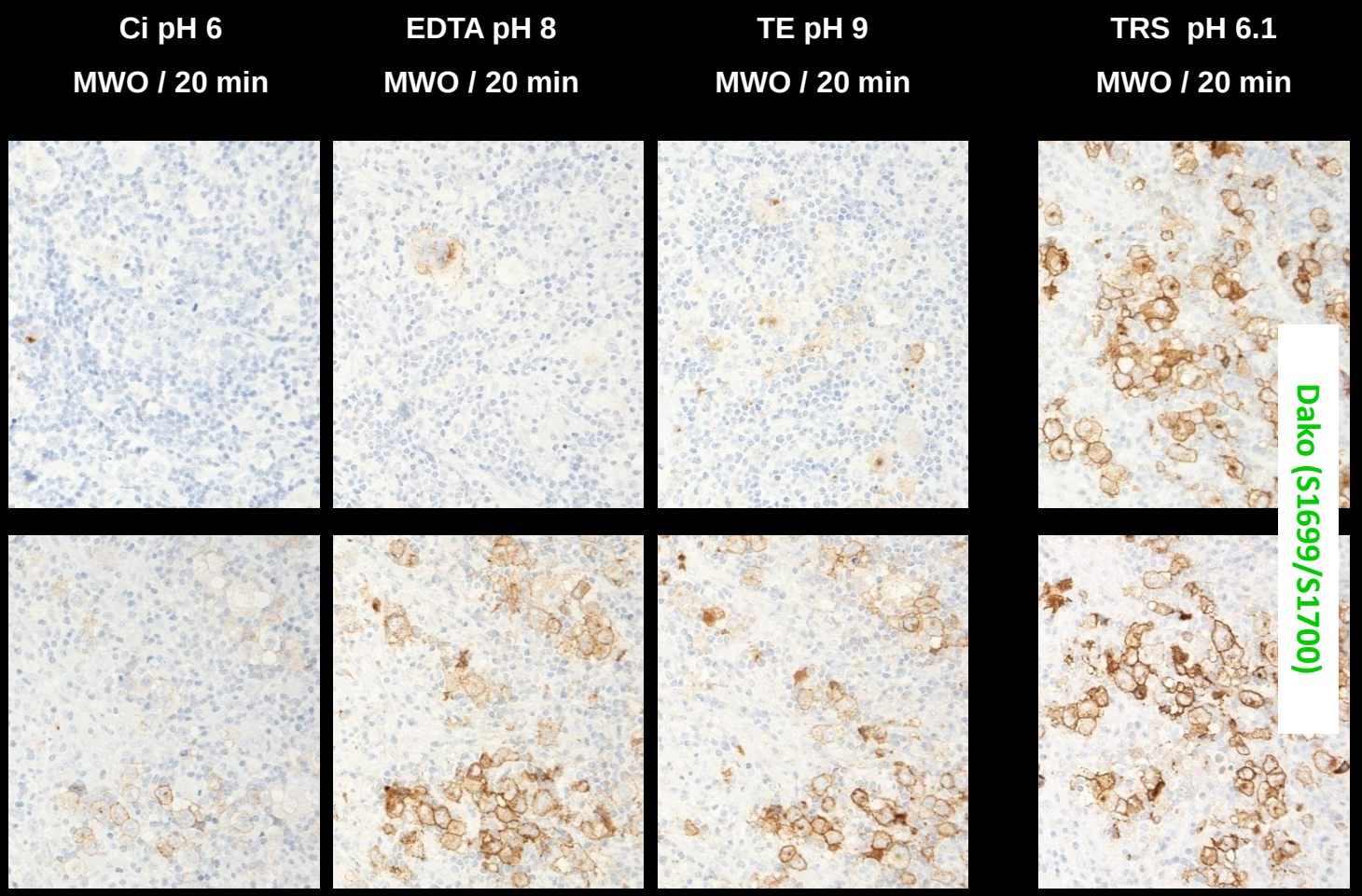
For certain Automated stainer systems (e.g. Ventana Benchmark or BOND) it is advisable to improve immunostainings for makers requiring modified low pH buffers by:

Performing HIER off board (e.g. MWO) with TRS pH 6.1 (S1699/S1700) or Diva Decloaker

Or search for primary antibodies by clones :

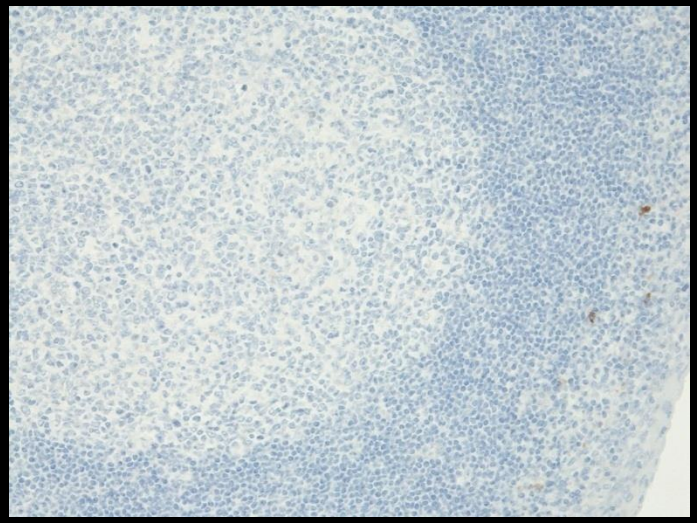
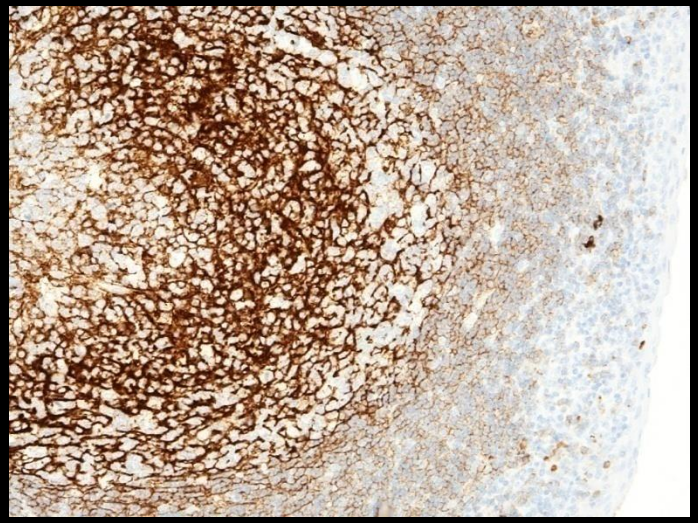
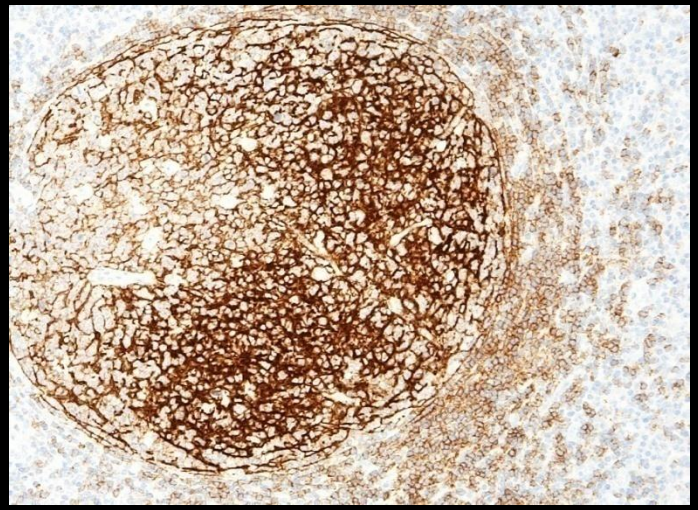
That work with standard HIER retrieval buffers (e.g. TE pH9)

Important questions: Which antibody - Which antigen retrieval procedure – To which platform



Hodgkin Lymphoma

CD21 (substitution)

	TRS, High pH	TRS, Low pH
CD21, Clone 1F8		
CD21, Clone 2G9		

NordiQC

Assessment Run 45 2015

Epithelial cell-cell adhesion molecule (Ep-CAM)

Recommended Ep-CAM protocols Recommended Ep-CAM control tissue

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

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

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

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

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

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

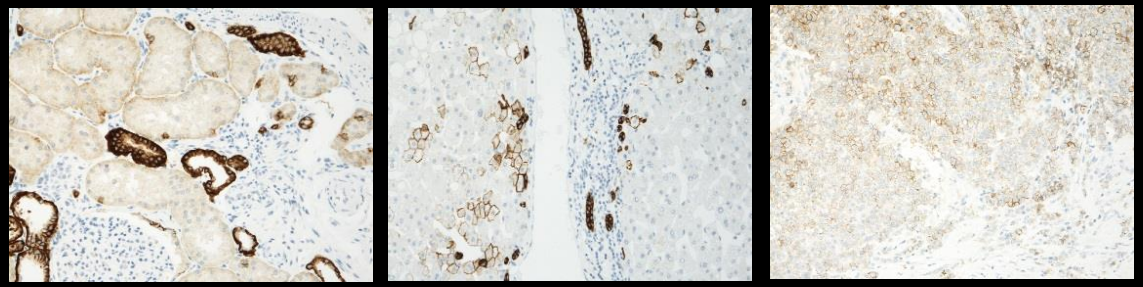
BS14 could be an alternative to Ber-EP4 on platforms excluded from the use of modified low pH buffers e.g. Diva pH 6.2 (Biocare) or TRS pH6.1 (Dako)

The most frequent causes of insufficient staining reactions were:

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

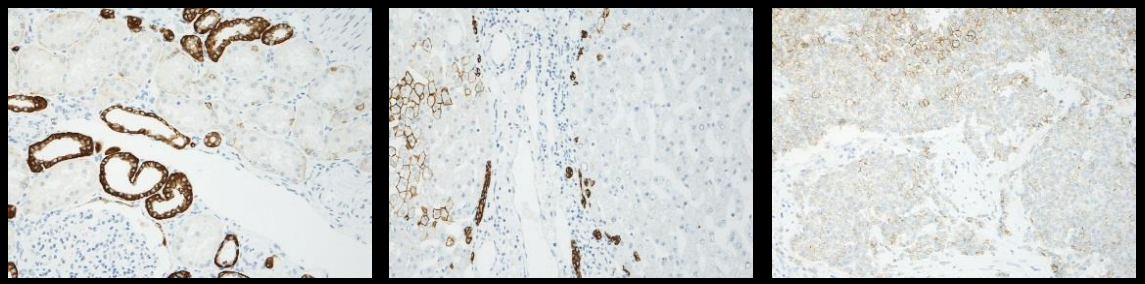
Omnis

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

Epitope Retrieval

Epitope retrieval procedures for formalin fixed tissue:

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

The purpose of antigen retrieval is to unmask antigen epitopes and recover immuno-reactivity

Combined HIER with proteolytic digestion

- Infrequently used today
- Controlled proteolytic digestion can be performed before or after HIER

Merz H et al. J.Pathol. 1993 Jul;170(3):257-64.

Used a combination of protease digestion and microwave treatment (Urea) to improve the staining of surface and cytoplasmic immunoglobulin heavy and light chains

Frost AR et al. Appl Immunohistichem Mol Morphol. 2000 Sep;8(3):236-43.

Demonstrated that better results could be achieved with antibodies to Ki67/MIB 1 and ER in breast carcinomas using a combination of mild microwave heating (10mM citrate buffer at 80°C (2h) following trypsin treatment compared to normal retrieval procedures.

Also, tissue loss (breast samples) was minimized using the combined protocol (HIER at low temperature + Trypsin digestion)

Combined HIER with proteolytic digestion

Applying an combined pre-treatment techniques require careful calibration of all parameters involved both in the HIER and proteolytic digestion process.

These parameters includes as for HIER or enzymatic digestion alone:

- ❑ Type of tissue and fixation conditions
- ❑ Type of HIER Buffer (pH , HIER time and temperature)
- ❑ Type of proteolytic enzyme (specificity, concentration, digestion time and temperature)

In general, an even more gentle approach compared to the "normal" Antigen Retrieval procedures must be performed.

Combined pre-treatment (HIER with Enzymatic digestion) / Naestved LAB

The procedures (Autostainer & Omnis):

A) HIER in Low pH (Dako ~ TRS pH 6.1 /S1700 or S1699), PT module , 97°C /20 min.
Pepsin solution (RTU/Zytovision cat. no. ES-0001-50), RT/5 min.

B) Pepsin solution (RTU/Zytovision cat. no. ES-0001-50), RT/ 8 min.
HIER in High pH (Dako ~ TRS pH 9 (3-1)), PT module , 97°C /20 min.

C) Proteinase K solution (RTU/Dako cat. no. S3020), dil. 1:15/TBS, RT/ 3 min.
HIER in High pH (Dako ~ TRS pH 9 (3-1)), PT module , 97°C /20 min.

D) HIER in Low pH (Dako ~ TRS pH 6.1 /S1700 or S1699), 97°C /20 min.
Cytology Pepsin solution (RTU/Zytovision cat. no. ES-0002-50), 32°/12 min. (Omnis)

E) HIER in pH (Dako ~ TRS pH 9) , 97°C /24 min.
Cytology Pepsin solution (RTU/Zytovision cat. no. ES-0002-50), 32°/3min. (Omnis)

Markers benefitting from combined pre-treatment (Naestved)

Extracellulare matrix proteins such as COLL-3 (polyclonal) , COLL-4 clone CIV-22, Tenascin clone T2H5, LAM5 (γ) clone D4B5, WT1 clone 6H-F5 , WT1 clone EP122, PAX8 clone ZR1, PMS2 clone EP51 and ????

WT1 clone 6H-F2 (RUN43)

HIER in CC1
(Ventana, Benchmark Ultra)

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

NordiQC

Nordic immunohistochemical Quality Control

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NordiQC runs

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

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

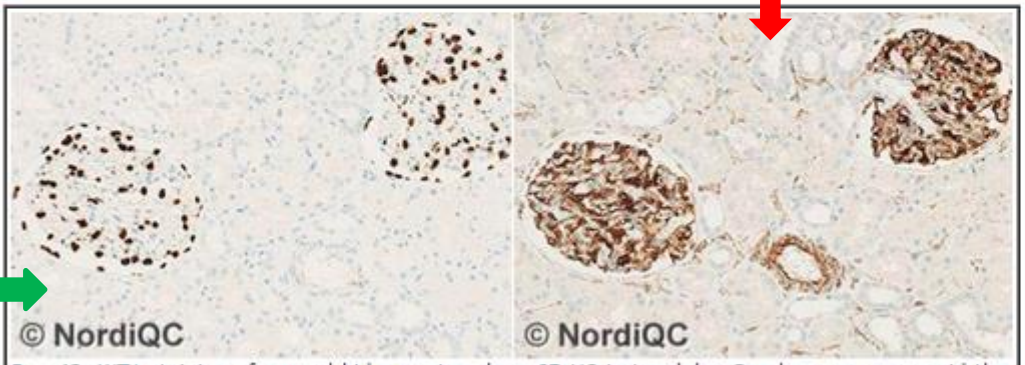
Run 45 (general module), **B20** (breast cancer module) **and H8** (HER-2 ISH module) opens by 12 August. Deadline for protocol submission is 10 September

NordiQC teaching events

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

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

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



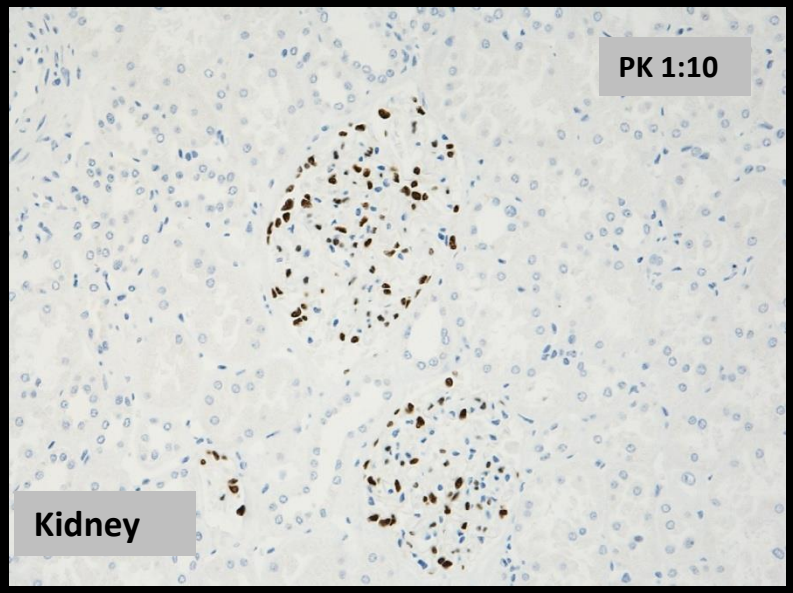
Run 43. WT1 staining of normal kidney using clone 6F-H2 in two labs. See how you can avoid the unwanted cytoplasmic reaction on WT1

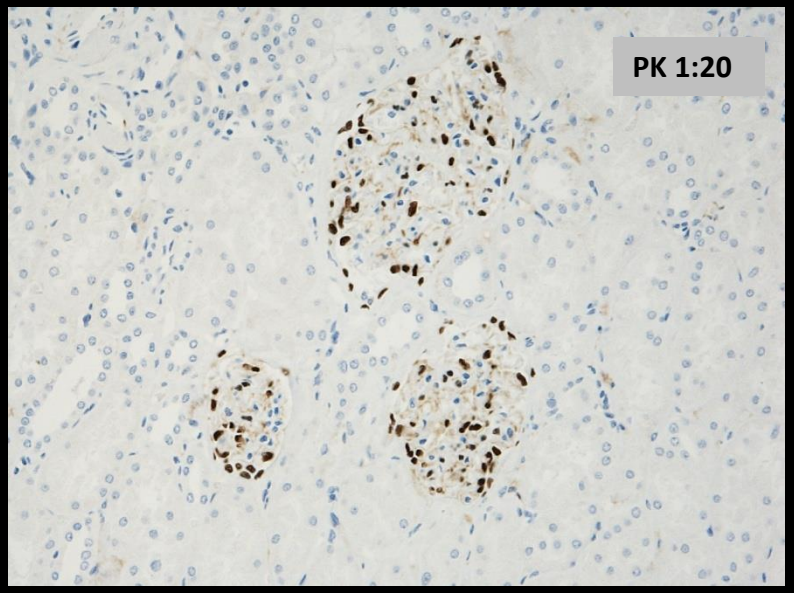
Companies sponsoring NordiQC scientific work have no influence on methods or results

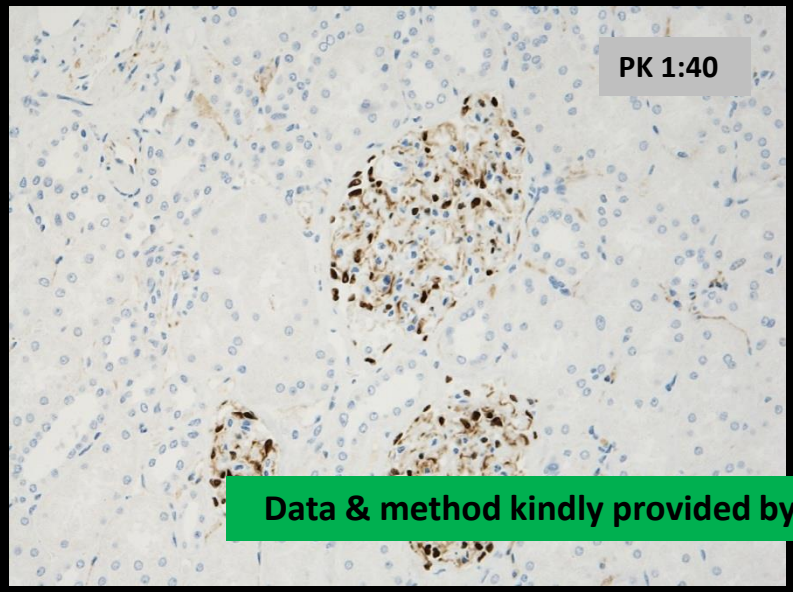
BIOCARE MEDICAL
CELL MARQUE
Dako An Agilent Technologies Company
Diagnostic BioSystems
EPITOMICS
horizon
ImmunoLogic
Leica BIOSYSTEMS
Roche
Thermo SCIENTIFIC
visiopharm

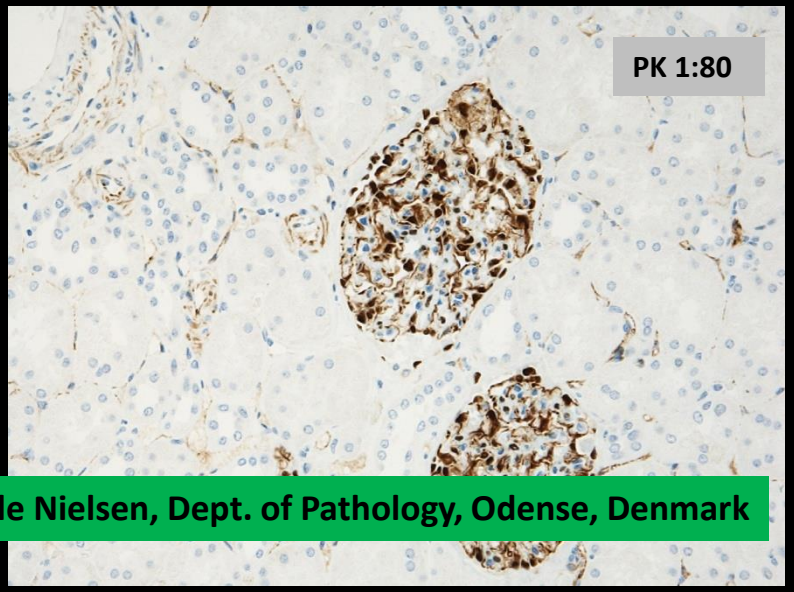
WT1 clone 6H-F2

HIER High pH 20`/97°C followed by diluted RTU Proteinase K (Dako, S3020) 3` RT







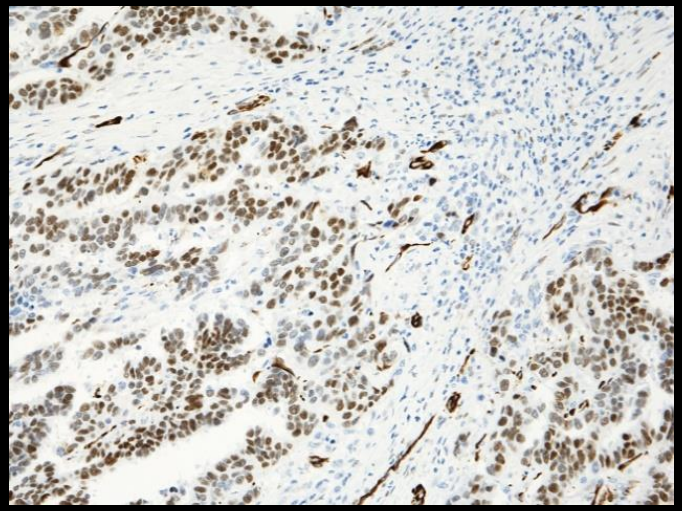


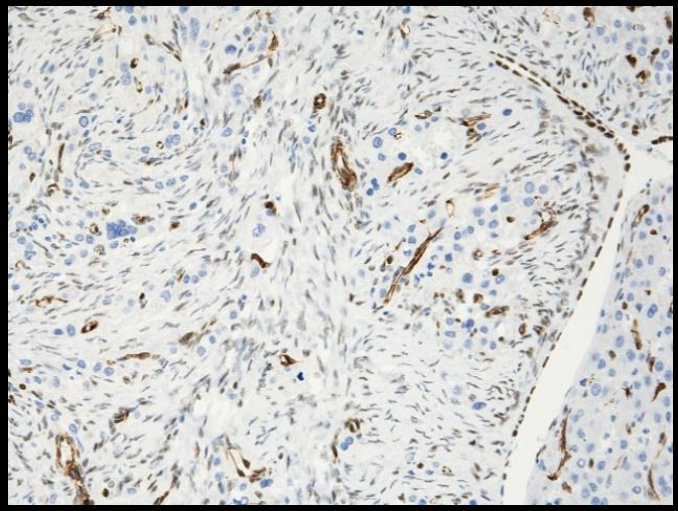
Data & method kindly provided by Ole Nielsen, Dept. of Pathology, Odense, Denmark

Ovarian Serous Carcinoma

Ovarium/Peritoneum
Metastasis (Hepatocellular carc.)

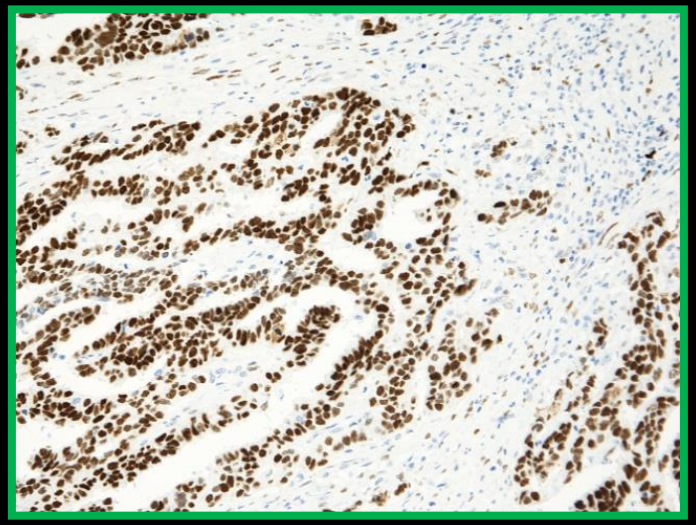
WT-1 clone 6H-F2
HIER High pH 20`/97°C

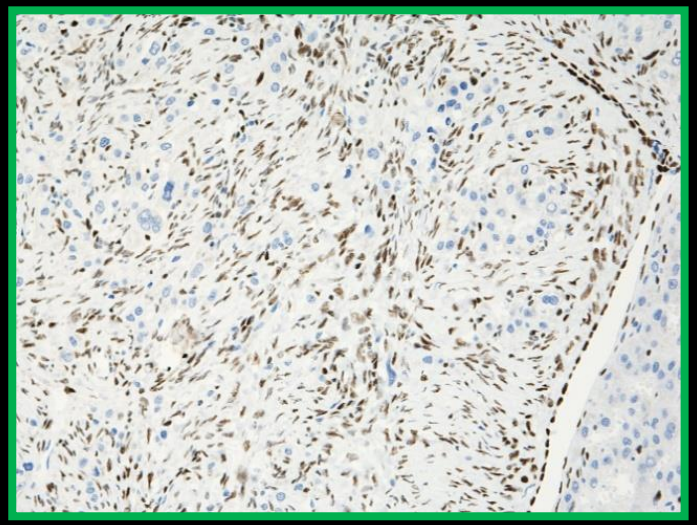




Improves sensitivity & signal to noise ratio

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





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

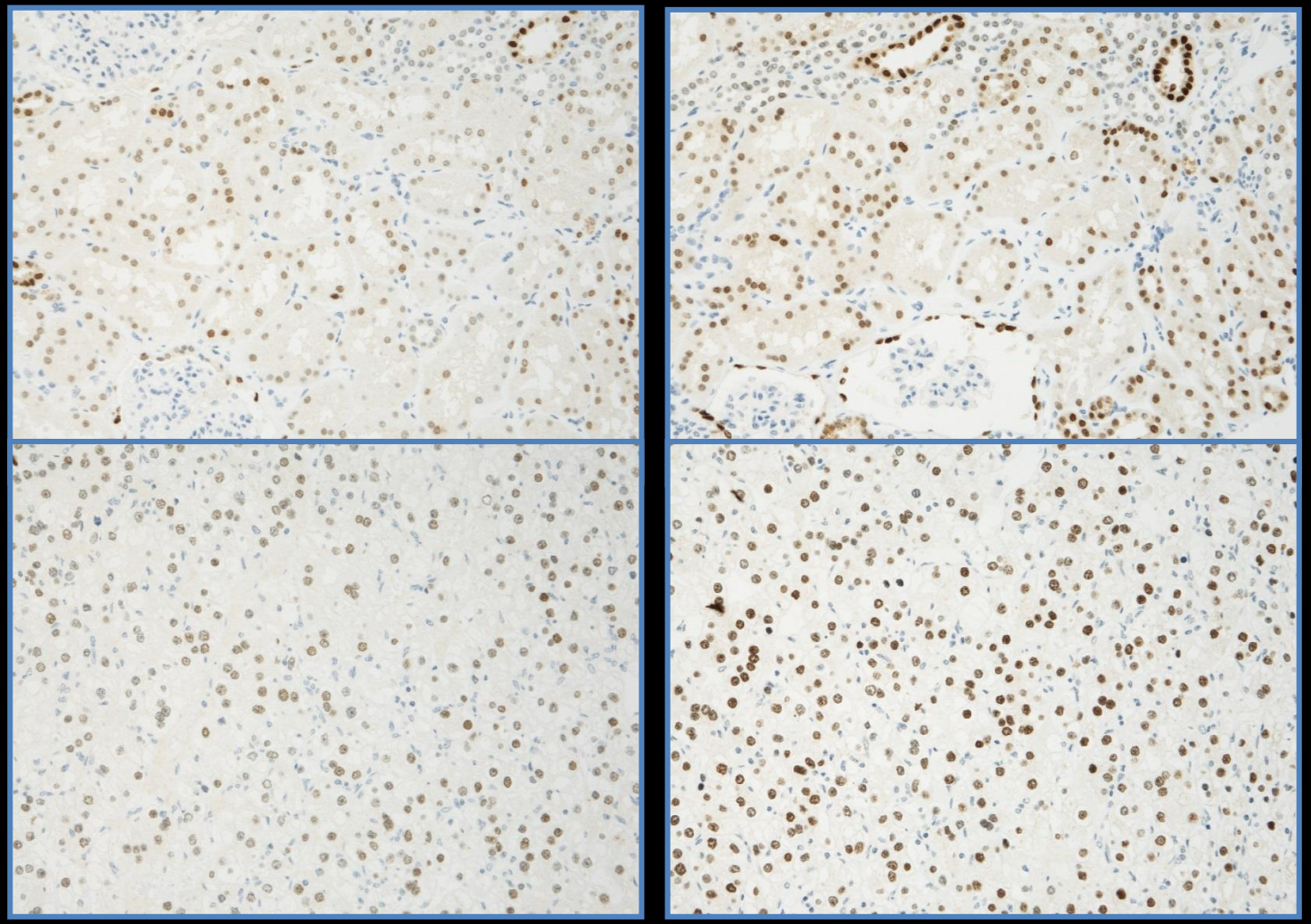
PAX8, ZR1 (1:50 RR /Omnis)

HIER (TRS pH9) / 24 min

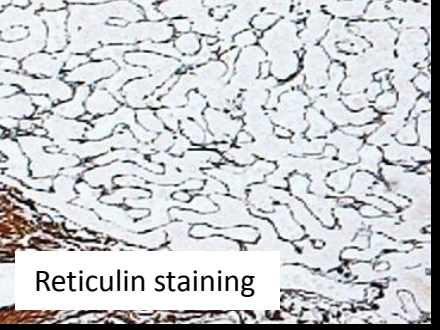
HIER (TRS pH9) / 24min + Pep ©

Kidney

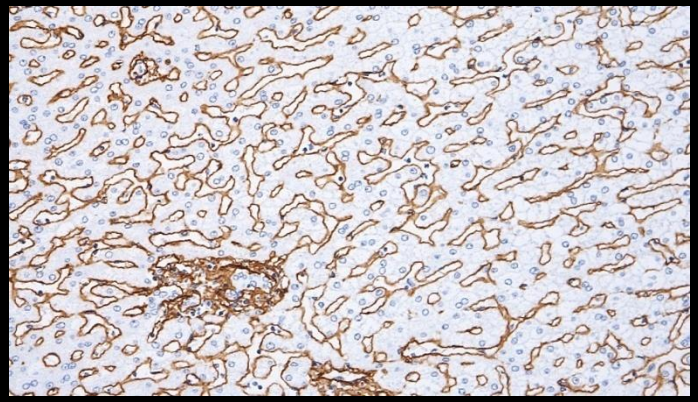
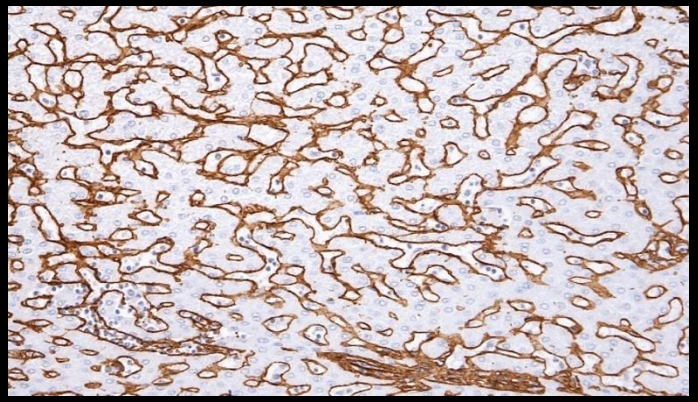
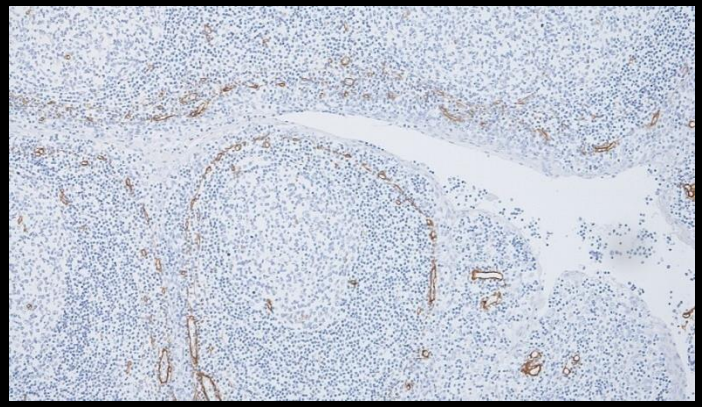
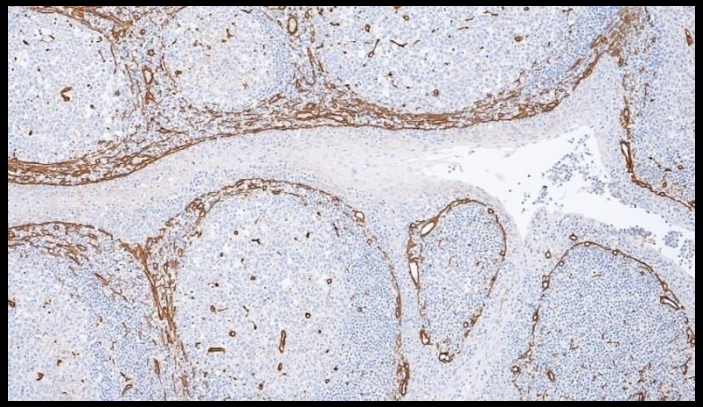
Clear Cell Carcinoma (Kidney)



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

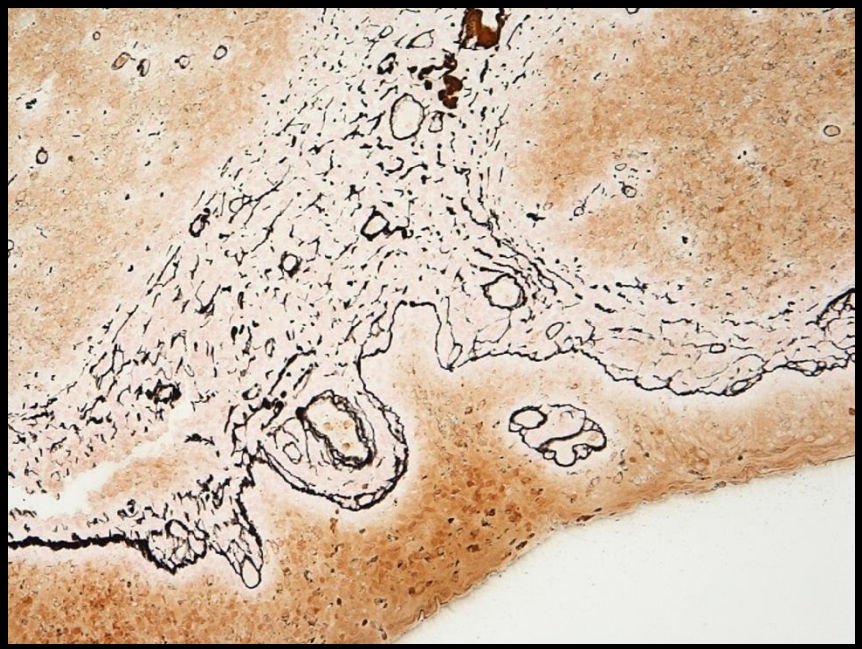

Reticulin staining

Collagen III (pAb 1:1000/ LSBio)

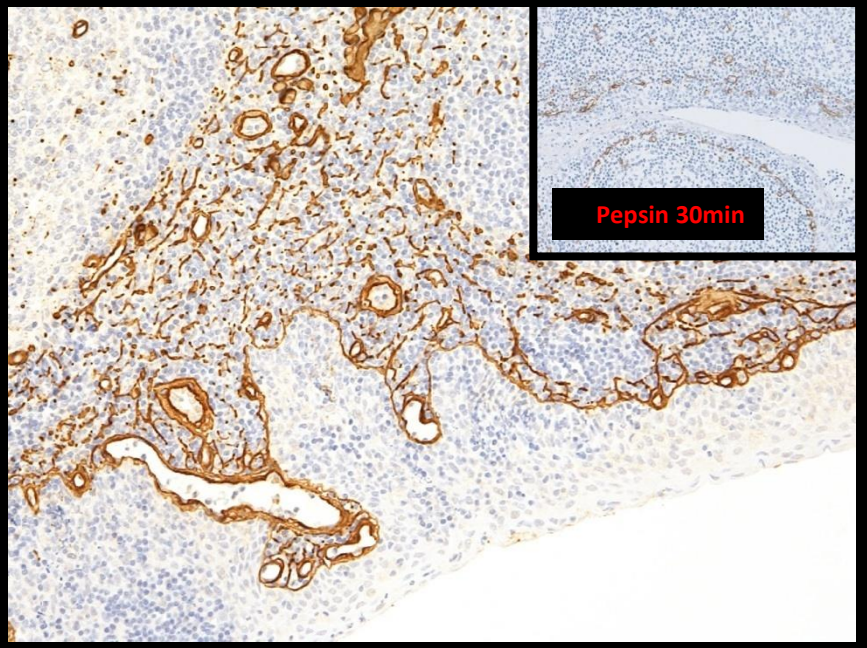
	Pepsin 30min (Dako S3002)	HIER/ Low pH + Pepsin 5 min (RTU/ZytoVision)
Hepar		
Tonsil		

Collagen III (pAb 1:1000)

Immunohistochemical versus Reticulin Staining



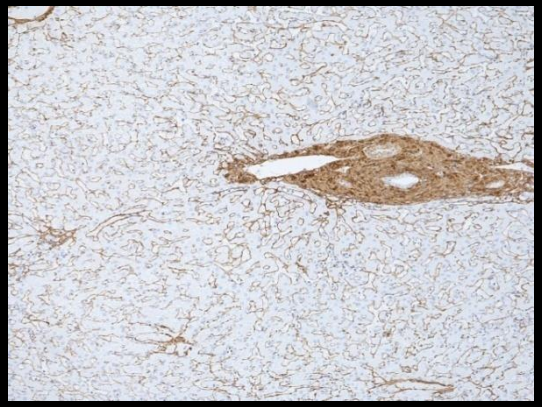
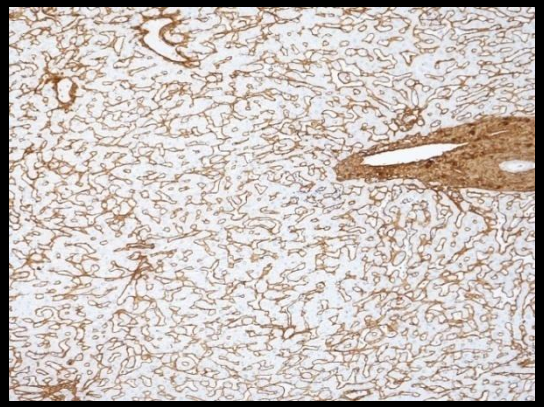
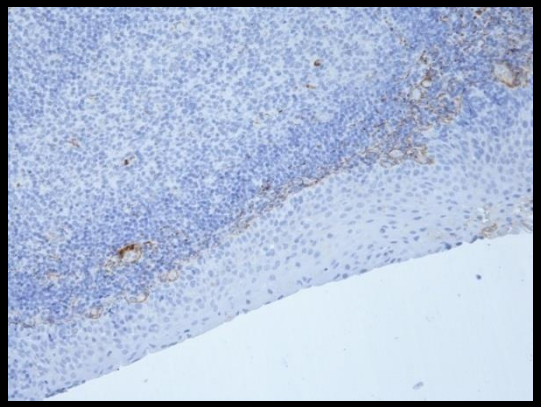
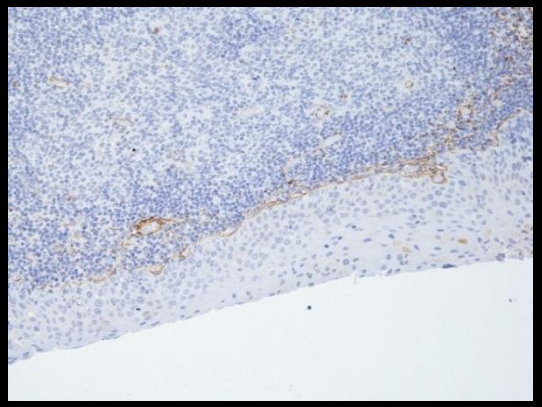
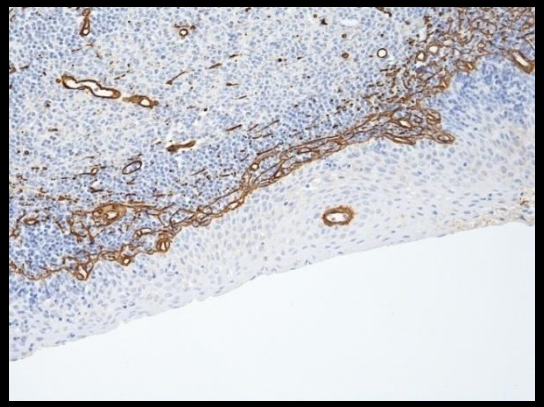
Reticulin – Gordon & Sweet



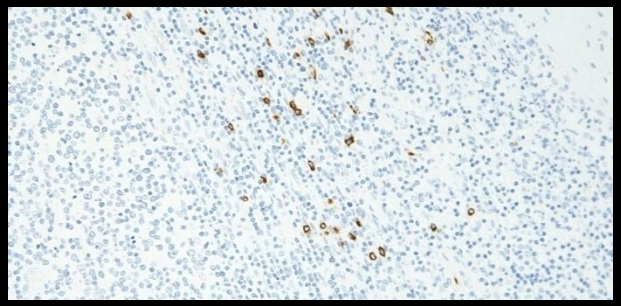
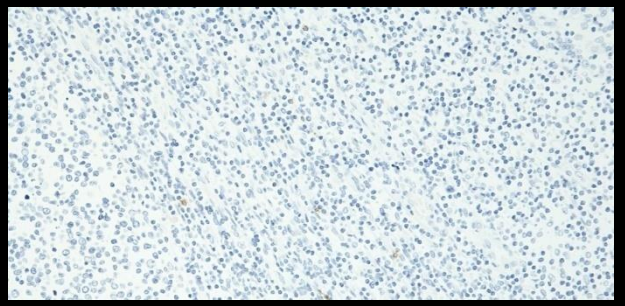
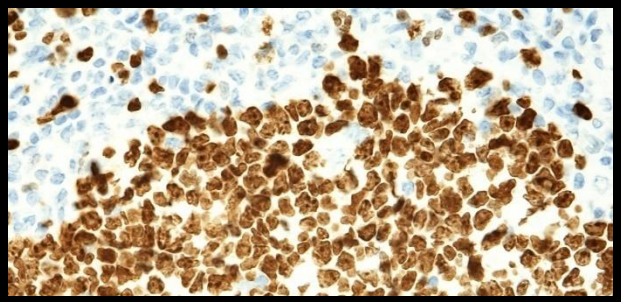
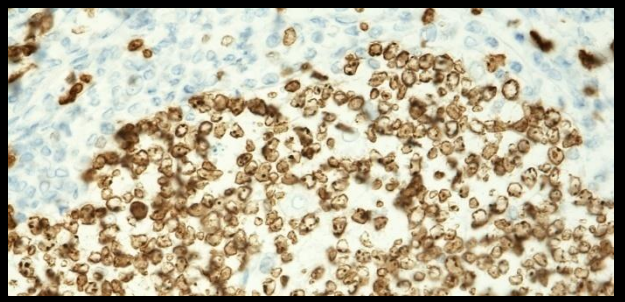
HIER / Low pH + Pepsin 5`

Combined pre-treatment (HIER with Enzymatic digestion)

Collagen III (pAb 1:1000/ LSBio)

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

Excessive retrieval procedures

	Optimal	Excessive
Proteolytic pre-treatment **Mact Cell Tryptase**		
HIER **Ki-67**		

Excessive retrieval:

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

Bone marrow cloth`s and cell cloth´s from cytology

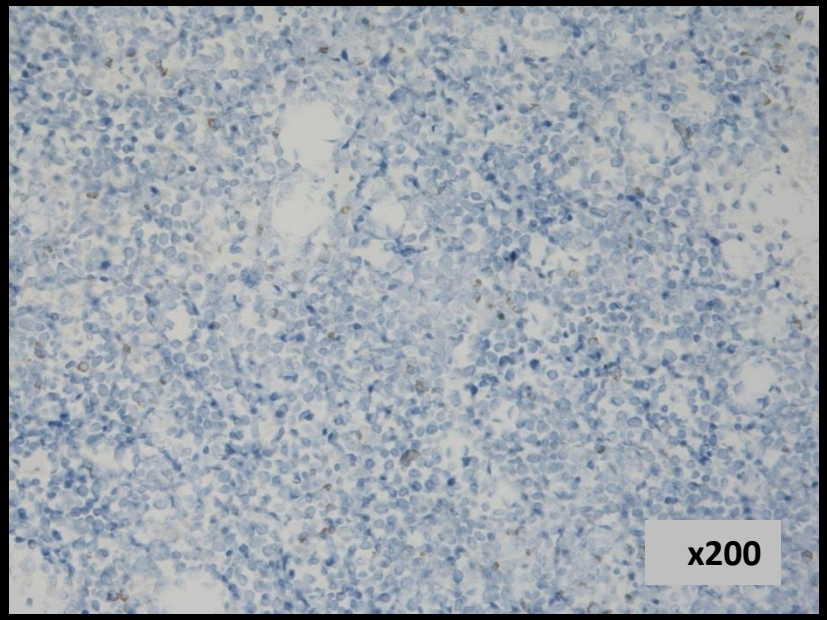
Morphology and IHC reactions dependent on:

- Specimen preparation (bone marrow aspiration technique, anticoagulants, fixation delay……) ?
- Fixation procedure (fixative, concentration, volume, time & temperature) ?
- Sectioning & drying conditions ?
- HIER Buffer (Chemical composition & pH) ?
- HIER temperature ?
- HIER time or time in the buffer ?
- Preheat temperature (PT module/Dako or LabVision) of the HIER buffer ?
- Unknown factors impacting the morphology of the cloth`s ?

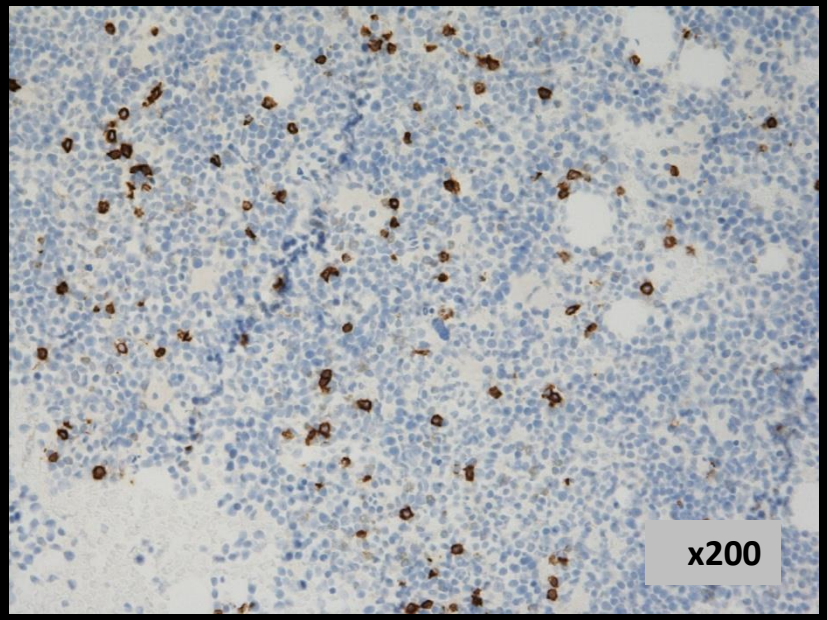
Fragile tissue specimens requiring gentle antigen retrieval procedures ?

CD20 clone L26

Bone Marrow Coagulum (fixed for 24h in 10% formaldehyde)



Omnis, CD20 RTU
TRS (3-1) / 30 min at 97°C

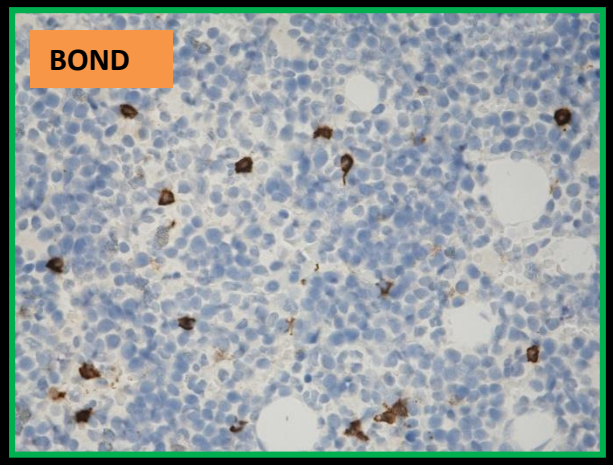


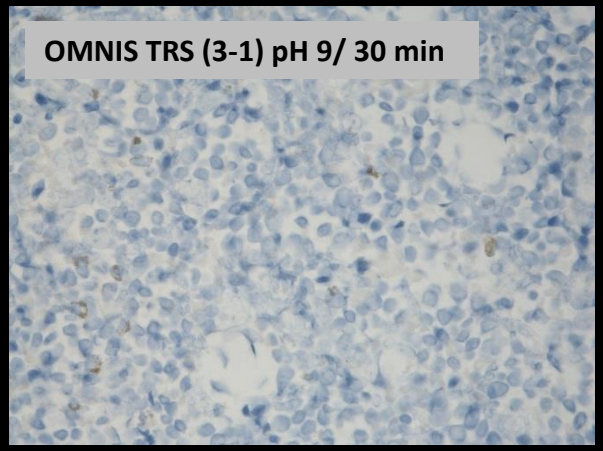
BOND, CD20
BERS-2 / 20 min at 100 °C

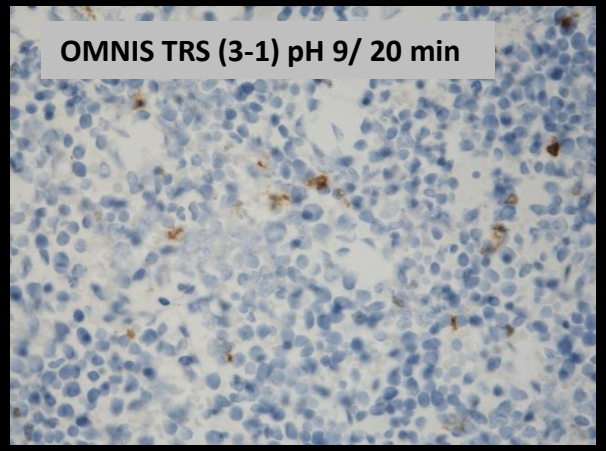
HIER settings: Standard recommendations given by the manufacturer`s

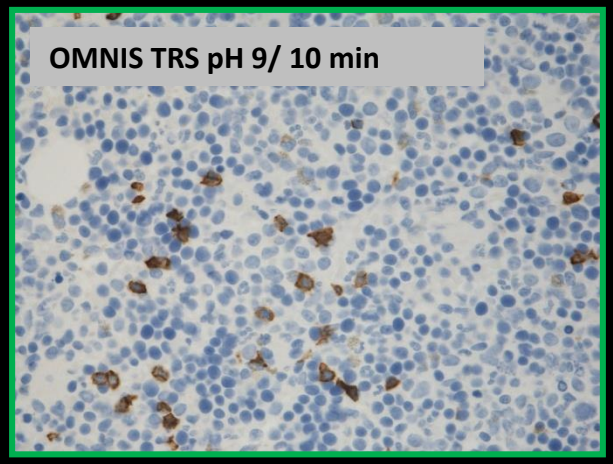
CD20 clone L26

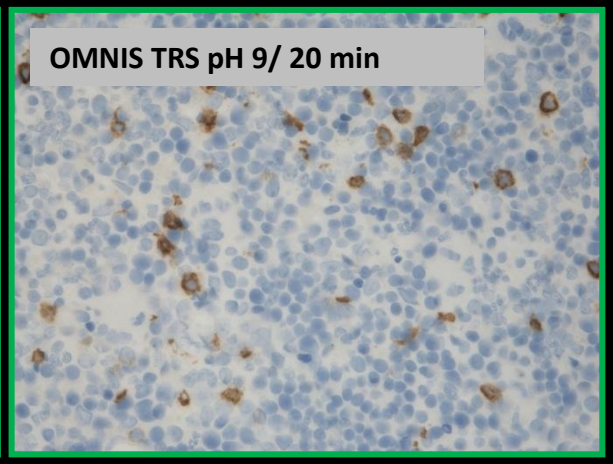
Bone Marrow Coagulum (fixed for 24h in 10% formaldehyde)

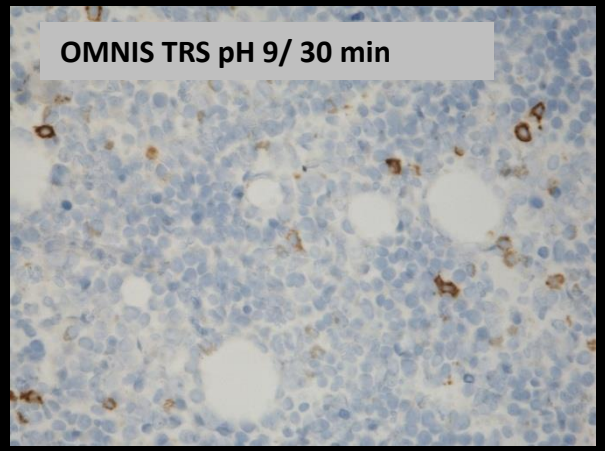








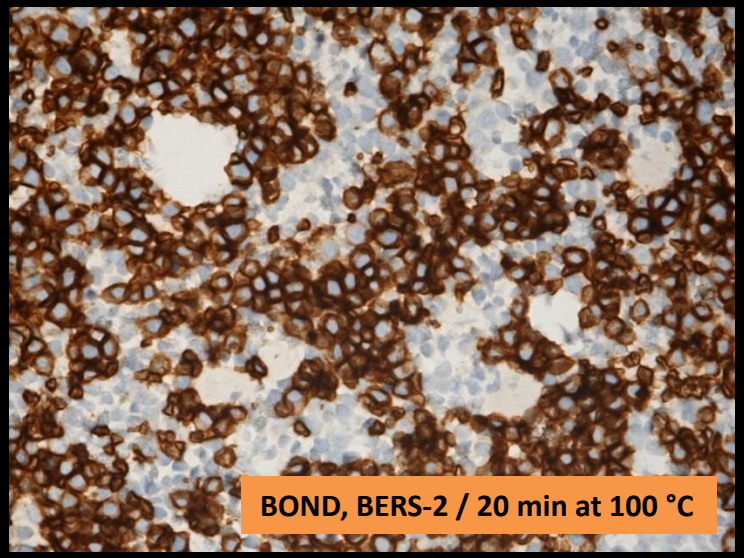


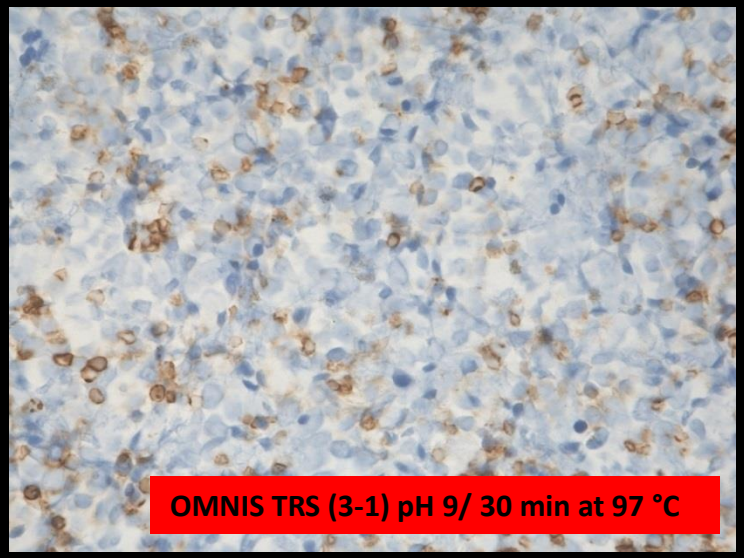


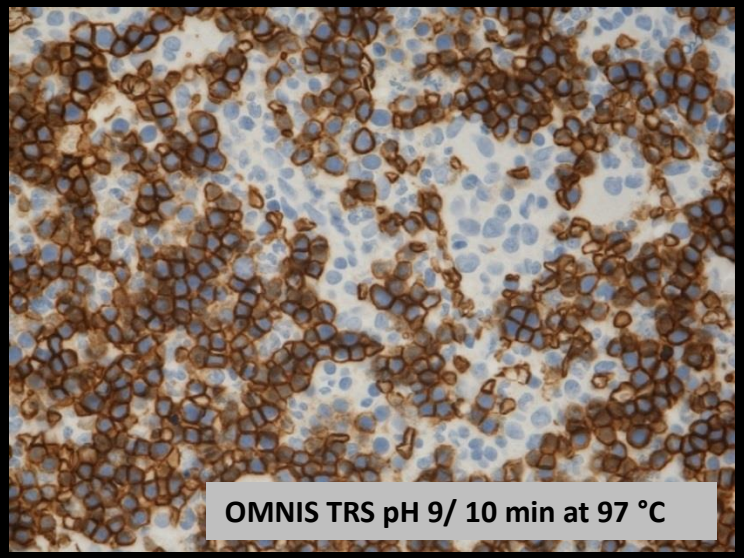
Note: IHC reactivity but also morphological structures of the nuclei`s

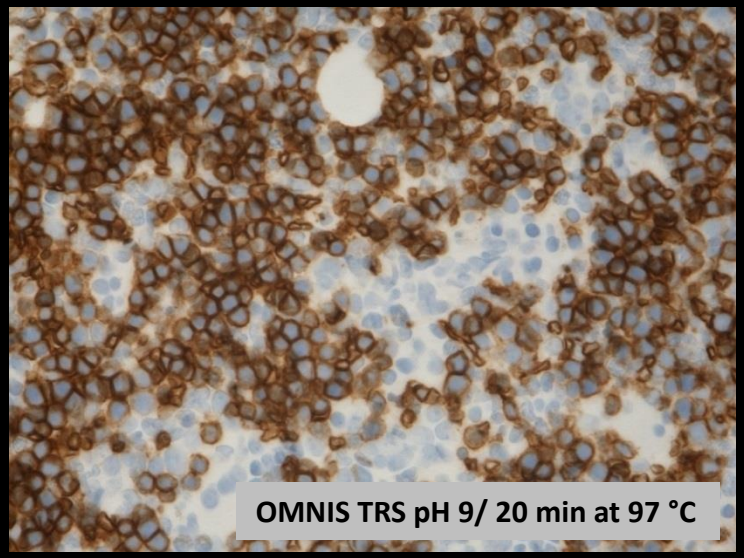
Glycophorin A clone JC159

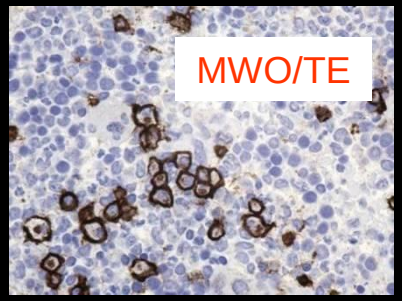
Bone Marrow Coagulum (fixed for 24h in 10% formaldehyde)











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

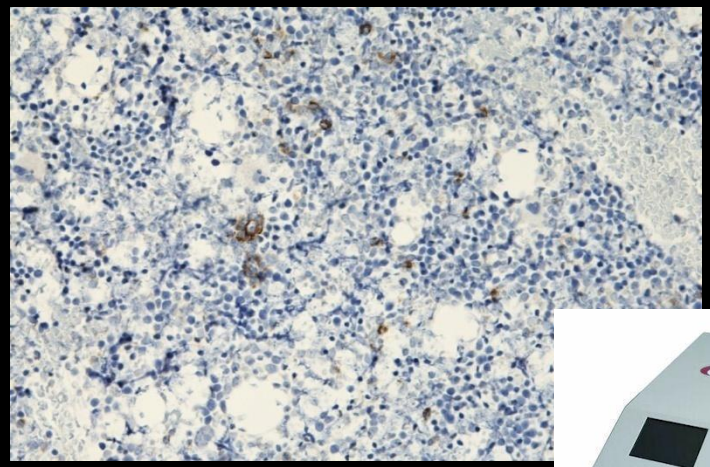
Influence of pre-heat temperature (65°C versus 85°C)

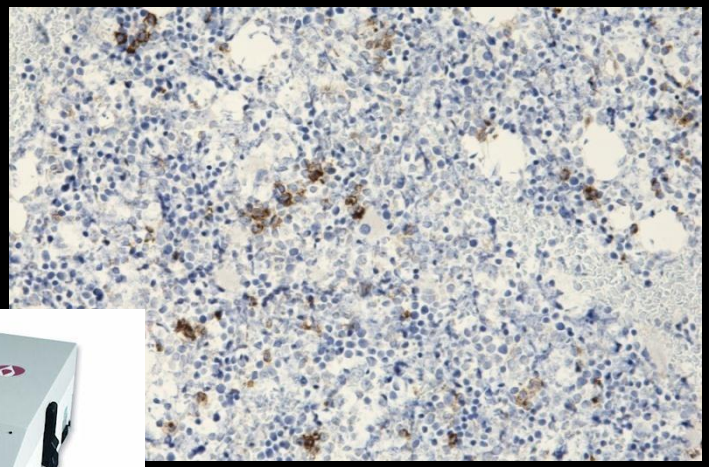
Bone Marrow
NBF 96h
CD138

PT, High pH (3-1), 95ºC / 30 min

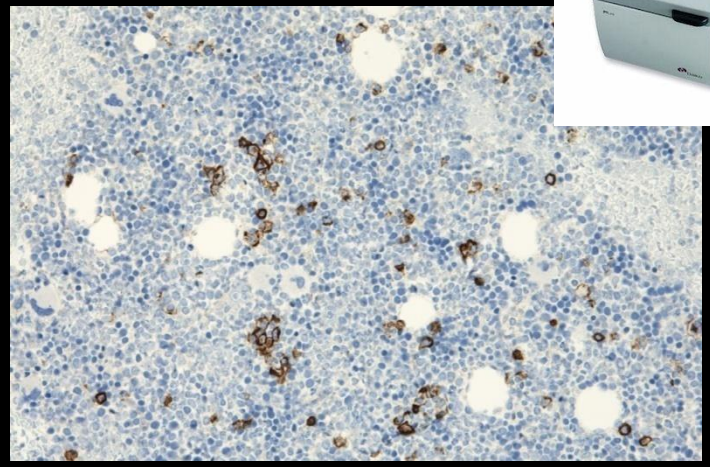
PT, High pH (3-1) 95ºC, / 20 min

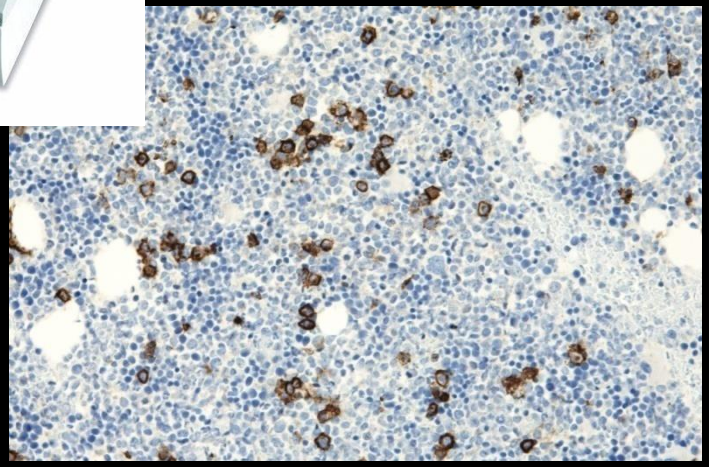
P/E 65ºC





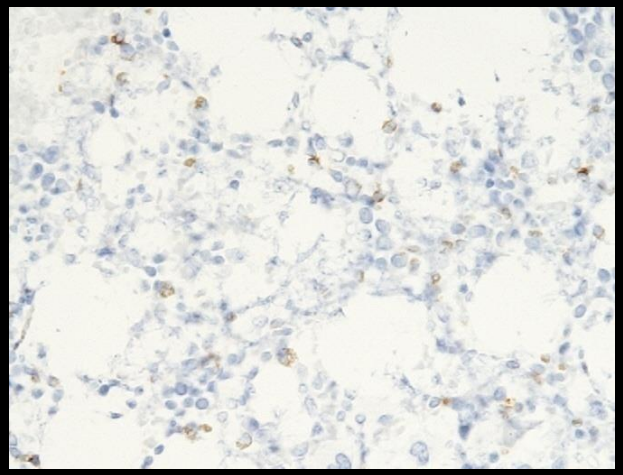
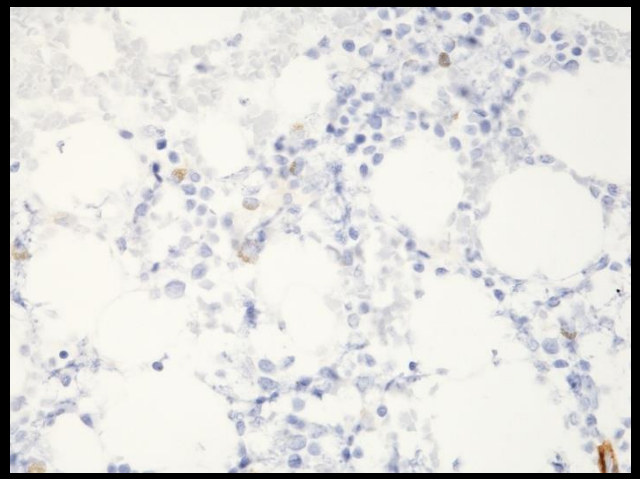
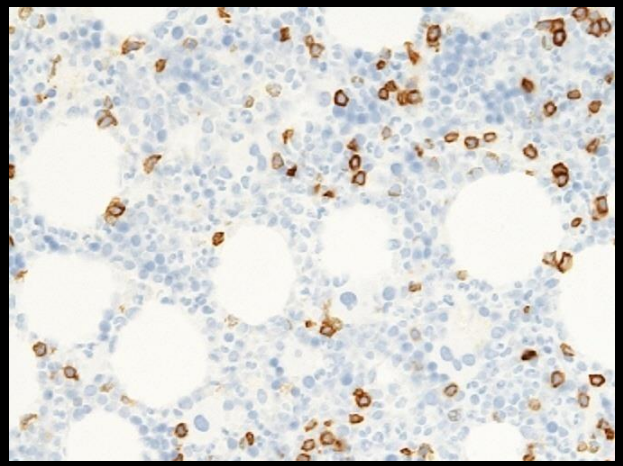
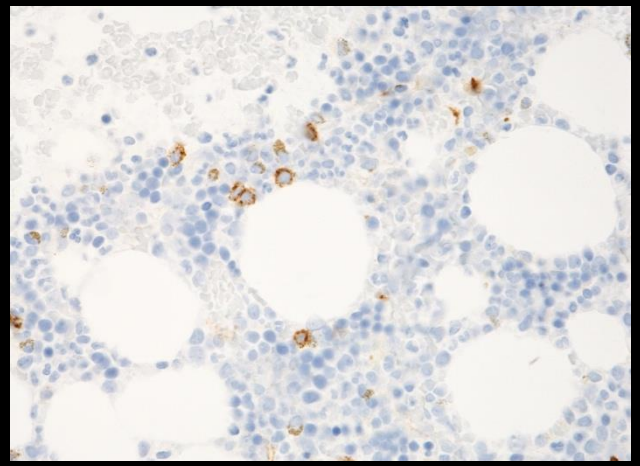
P/E 85ºC



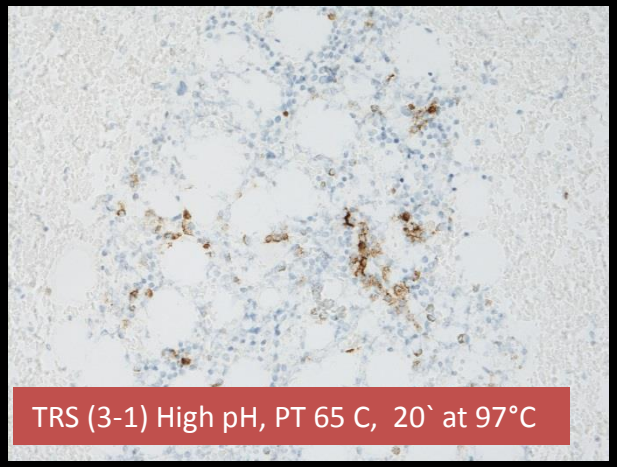


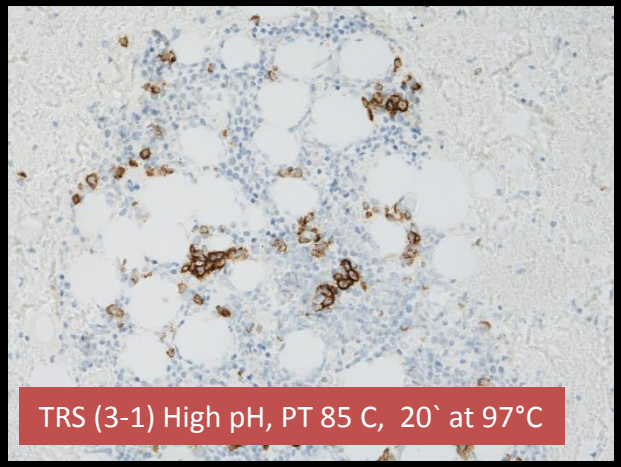
Epitope Retrieval, PT-Link, High pH buffer´s at 97ºC / 20 min.

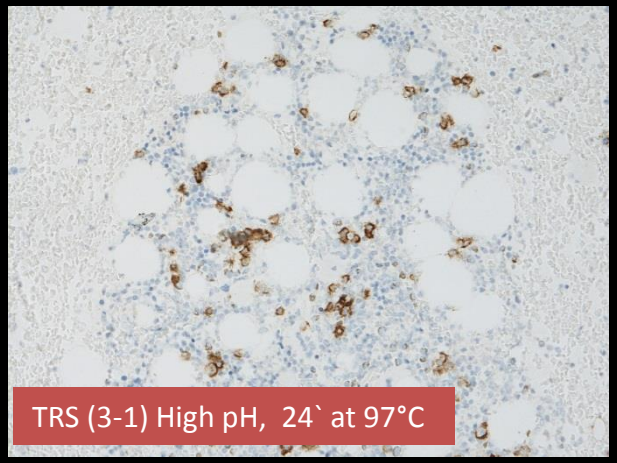
Bone marrow aspirate / Coagulum

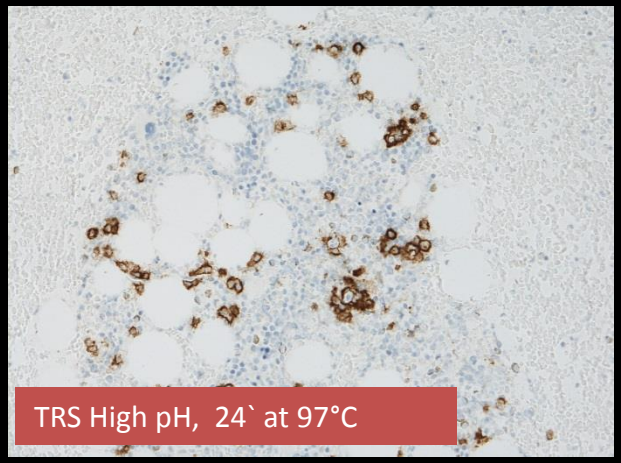
	CD5 clone SP19	CD34 clone QBEND-10
High pH (Dako) Recommended settings: 65ºC		
High pH (LabVision) Recommended settings: 85ºC		

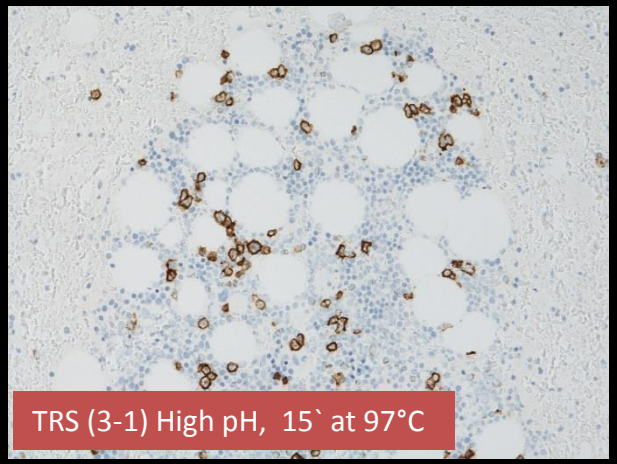
Influence of pre-heat temperature (65ºC versus 85ºC) (same field)

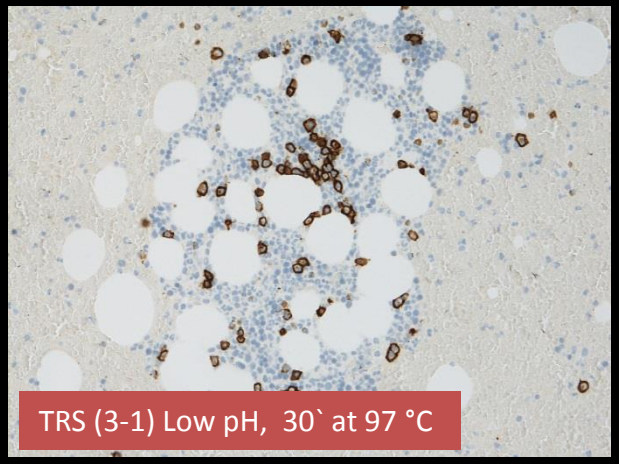












Bone Marrow cloth fixed for 72 h in 10% NBF

CD138, B-A38 1:1000

Autostainer

PT 85C > PT 65 C

Omnis

TRS High pH > TRS (3-1) High pH

Omnis

Reduced HIER time or TRS (3-1) Low pH >

All other parameters tested

Chemical composition of the HIER buffer

Section 3. Composition/information on ingredients 10x

Substance/mixture : Mixture

EDTA based ?

Ingredient name	%	CAS number
Trometamol	1 - 5	77-86-1
Nonylphenol, ethoxylated	0.1 - 1	9016-45-9

Any concentration shown as a range is to protect confidentiality or is due to batch variation.

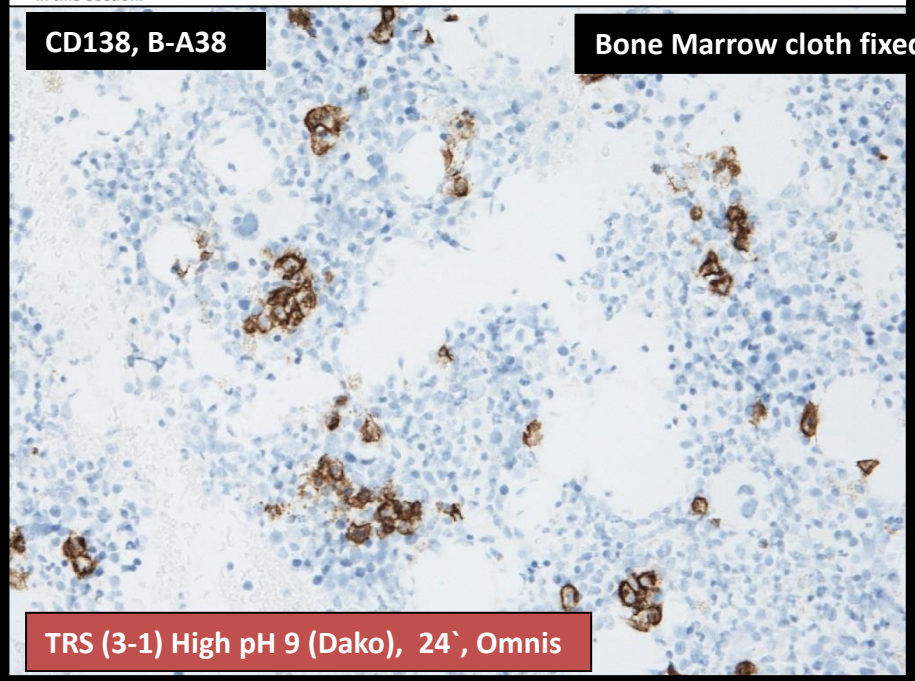
There are no additional ingredients present which, within the current knowledge of the supplier and in the concentrations applicable, are classified as hazardous to health or the environment and hence require reporting in this section.

3. Composition / information on ingredients 100x

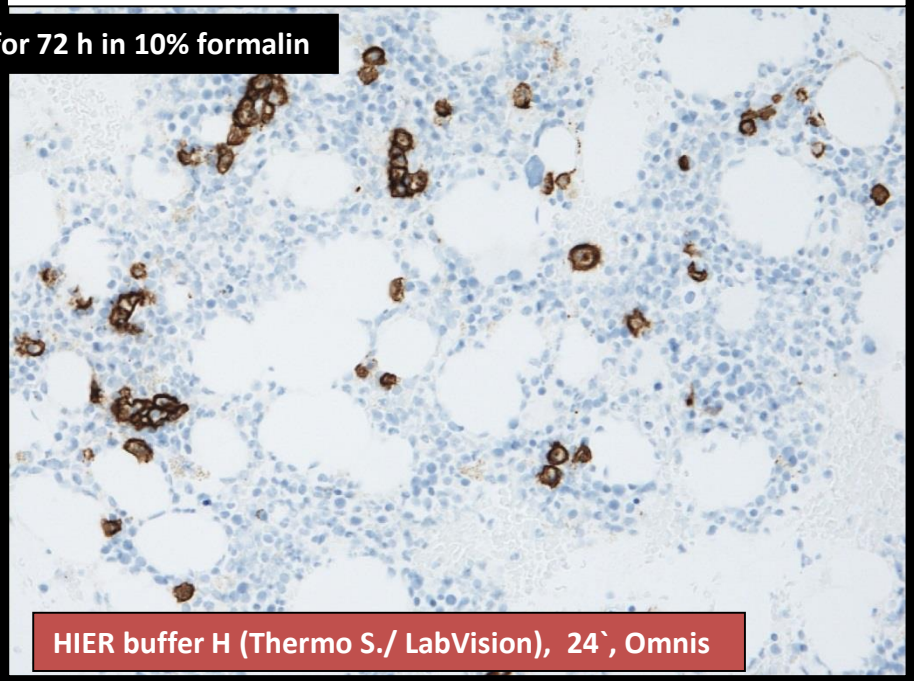
Component	CAS-No	Weight %
Ethylenediamine tetraacetic acid (EDTA)	60-00-4	<1
Tetrasodium EDTA	64-02-8	<1
2-Methyl-3-isothiazolone	2682-20-4	<1
Water	7732-18-5	80-85
Tris (hydroxymethyl) aminomethane	77-86-1	10-12
Triton-X100	9002-93-1	3-5

CD138, B-A38

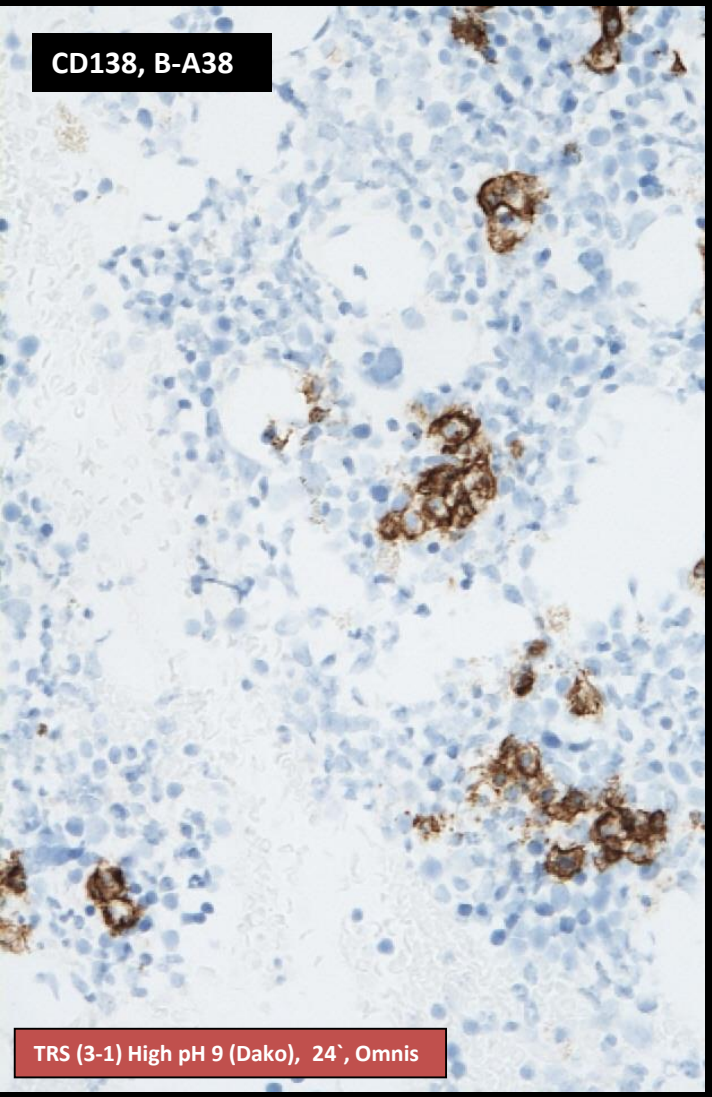
Bone Marrow cloth fixed for 72 h in 10% formalin

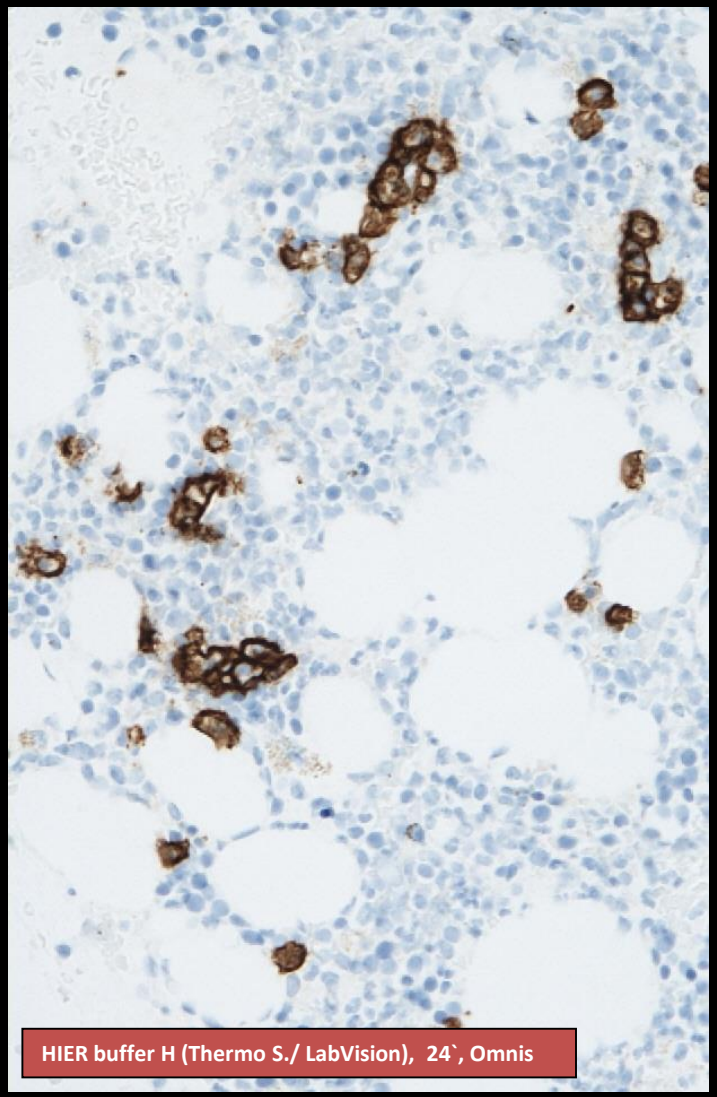


TRS (3-1) High pH 9 (Dako), 24`, Omnis



HIER buffer H (Thermo S./ LabVision), 24`, Omnis





"Morphology of granulocytes"

"Crisp immunoreactivity"

Coffee Break

Questions ?

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

ANNUAL REVIEW ISSUE

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

Mogens Vyberg[1,2] · Søren Nielsen[1]

Major problems 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
- 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

Antibody-Antigen reaction

Analytic specificity:

Ability of a test to detect substance (antigen) without interference from cross-reacting substances

- No false positive results

Analytic sensitivity:

Ability of a test to detect very small amounts of a substance (antigen)

- No false negative results

How to optimize of a new marker ?

A simple approach to implement a new marker

Concentrated primary antibody (Class I - non predictive markers)

Use a "Test battery approach"

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

- Validate on own processed tissue material on a chosen platform (manual/automated).
- Optimal - Include tissue that has been fixed in NBF between 6-168h.
- Optimal - Include tissue that has been decalcified corresponding to your normal procedure

Test > one clone against antigen of interest before implementation

Test with robust, specific & sensitive detection system

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

Antibody Performance Testing

Test Battery (Næstved / Autostainer)

	Dil. 1	Dil.2	Dil.3
A	None	None	None
B	Enzyme 3 & 10 min.	Enzyme 3 & 10 min .	Enzyme 3 & 10 min.
C	HIER TRS Low pH 6.1*	HIER TRS Low pH 6.1	HIER TRS Low pH 6.1
D	HIER TRS High pH 9.0 *	HIER TRS High pH 9.0	HIER TRS High pH 9.0
E	TRS Low * + Pep 4 & 8 min	TRS Low + Pep 4& 8 min	TRS Low +Pep 4 & 8min
F	Pep 6 & 10 min + TRS High *	Pep 6 & 10 min + TRS High	Pep 6 & 10 min + TRS High
G	**HIER** EDTA pH8 *	HIER EDTA pH8	HIER EDTA pH8

HIER time 20 min at 97 °C
Flex+ DAB, Dako Autostainer

Protocol A: 0,5 %
Protocol B: 2,0 %
Protocol C: 4,0 %
Protocol D: 91,5 %

Protocol E-F: 1,5 %
Protocol G: 0,5 %

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

Protocol set-up: Evaluated analytic sensitivity and specificity

Normal tissue including fixation and decalcification controls

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

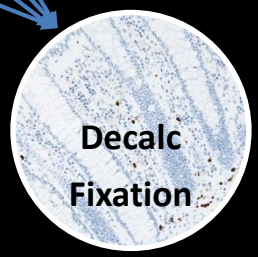
SOX10, BS7; HIER High pH 24`; 1:350 RR; Flex+Mouse linker

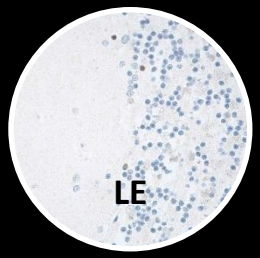
Establishing robustness of the IHC assay / pre-analytic parameter`s ?

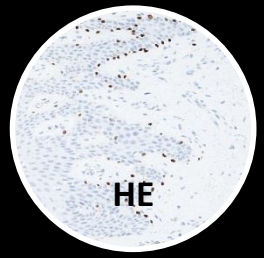
SOX10, BS7; Robust to both fixation time in NBF and de-calcification

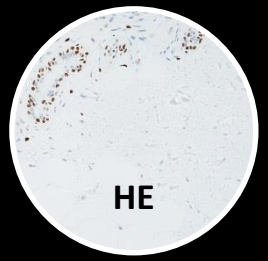
Identification of robust controls

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



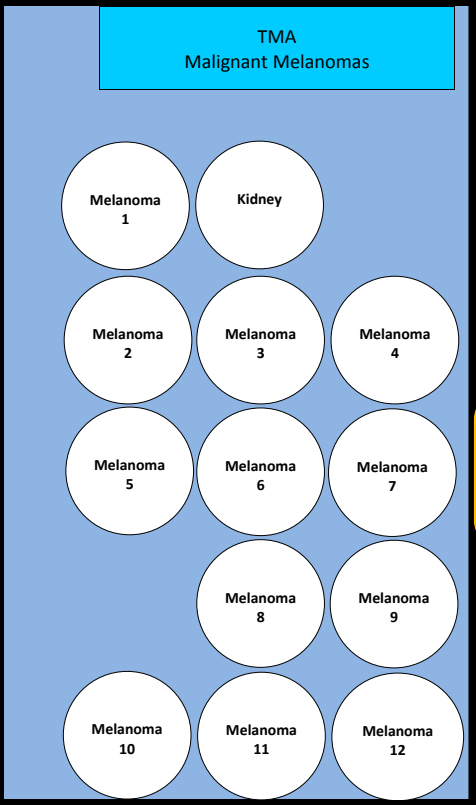


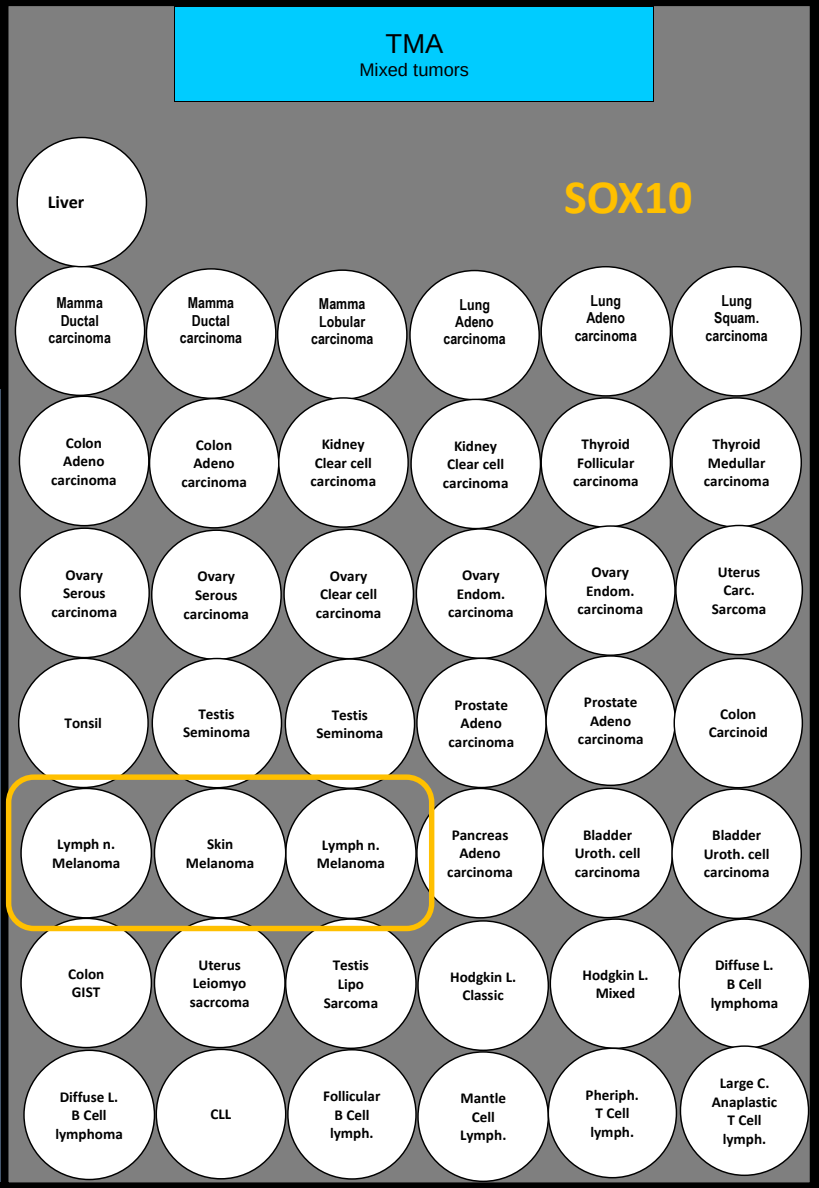




Inspired and TMA modified after the Aalborg procedure, Søren Nielsen, Aalborg, DK

Diagnostic potential





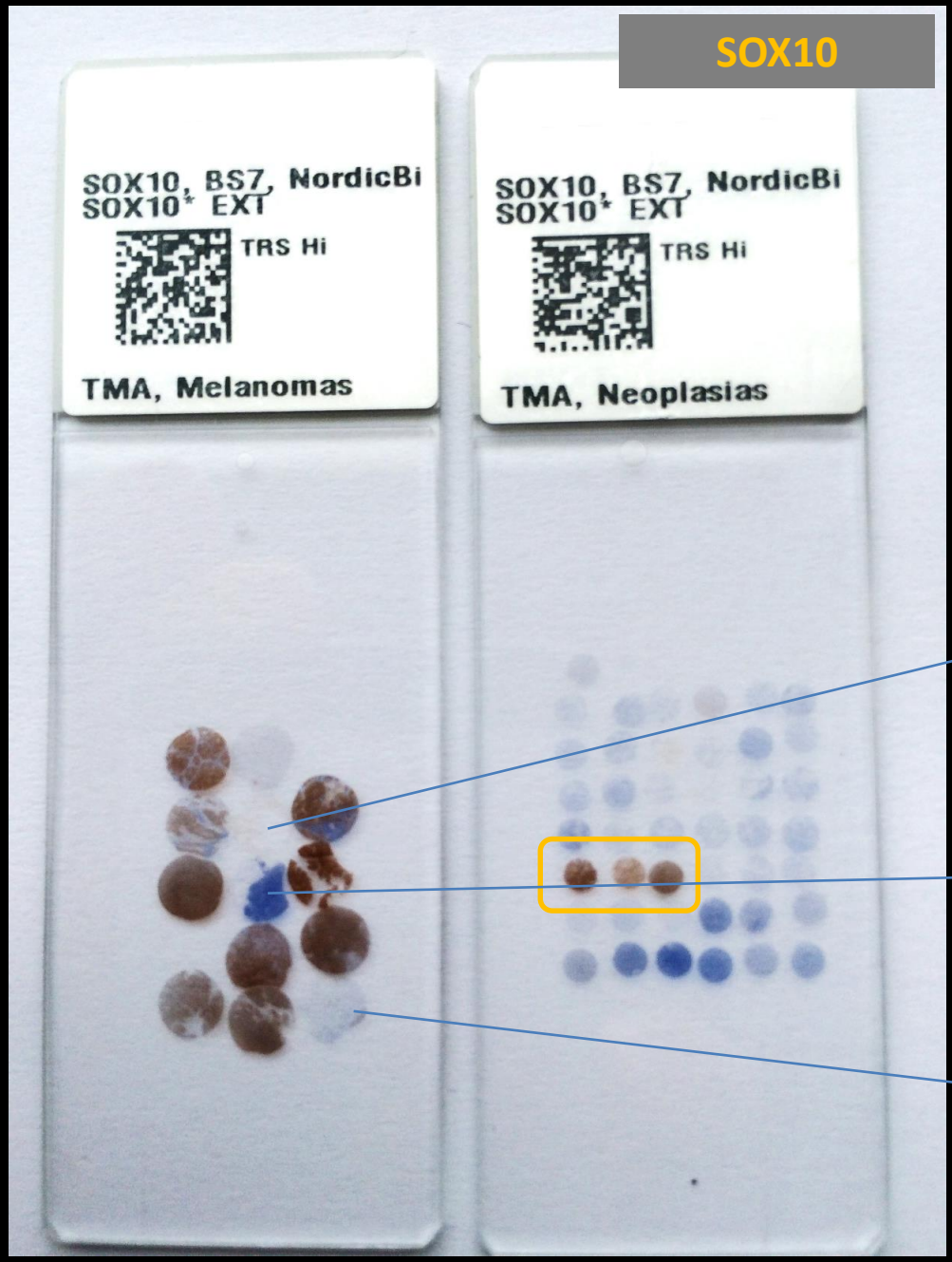
Analytical validation

Recommendations / Guidelines for material included - non-predictive markers (not for markers as ER, HER2,..)

- **CAP: 10 pos & 10 neg (including high & low expressors).**
- **CLSI: 20 cases (pos & neg)**
- **Ad-Hoc: 10 strongly pos, 10 low to moderate pos & 5 negative cases**

Use your common sense ?

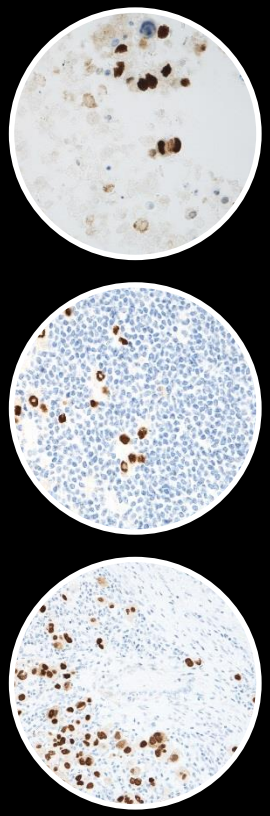
Inspired and TMA modified after the Aalborg procedure, Søren Nielsen, Aalborg, DK

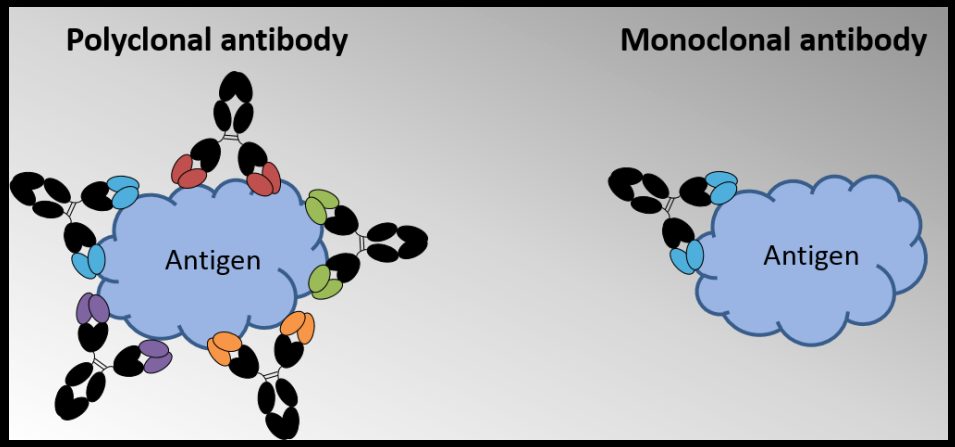


SOX10, BS7:

15/15 Melanomas were positive

37/37 other neoplasm`s were negative





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 - Normally Rabbit antibodies	Few host species options - Normally Mouse or Rabbit

Is monoclonal primary Abs always better to use ?

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

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

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

Concentrated antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	Suff. OPS[2]
mAb clone **5H7**	4	Leica/Novocastra	0	0	3	1	-	-
mAb clone **DAK-A3**	36	Dako/Agilent	0	2	17	17	6%	-
mAb clone **LK2H10**	22 18 6 3 2 2 1 1 1 1 1 1	Thermo/Neomarkers Cell Marque Immulologic Biogenex Millipore Zytomed Abcam A.Menarini Diagnostic Biosystems Europroxima Monosan Unknown	24	31	0	4	93%	98%
mAb clone **PHE5**	1	Unknown	0	0	1	0	-	-
mAb clones **LK2H10+PHE5**	6 5	Thermo/Neomarkers Biocare	3	8	0	0	100%	100%
rmAb clone **EP38**	1	Epitomics	0	1	0	0	-	-
rmAb clone **SP12**	1 1	Master Diagnostica Thermo/NeoMarkers	0	0	0	2	-	-
pAb **A0430***	38	Dako/Agilent	8	17	8	5	66%	-
pAb **NB120-17064**	1	Novus Biologicals	0	1	0	0	-	-
pAb **RB-9003**	1	Thermo/NeoMarkers	0	1	0	0		

pAb A430 not available (discontinued)

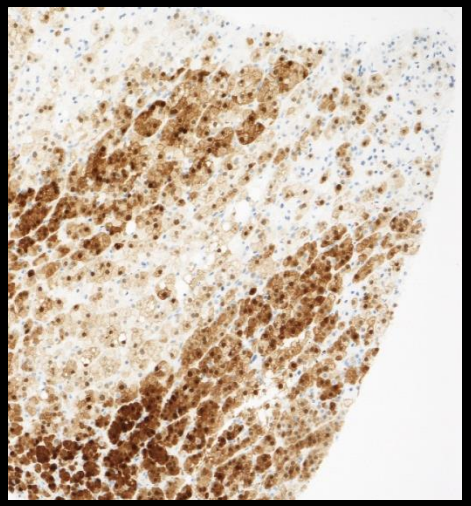
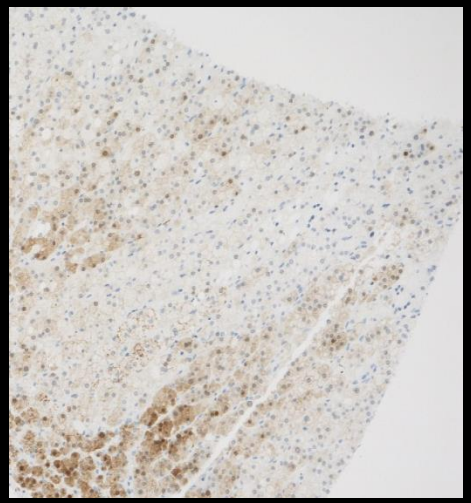
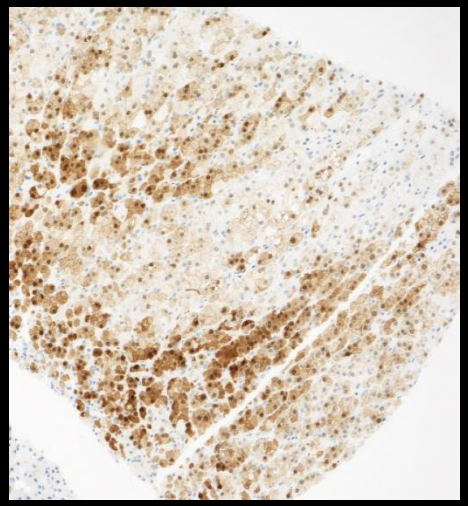
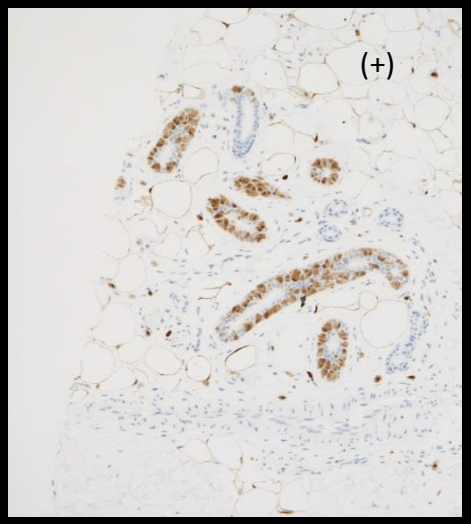
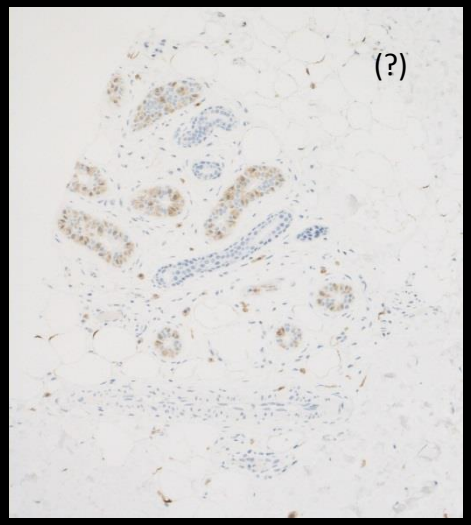
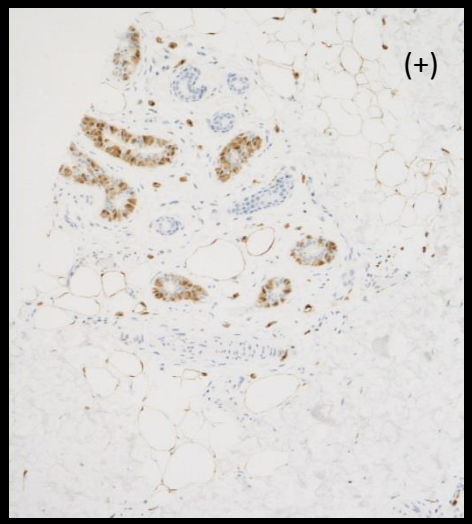
CGA

Shifting from Dako`s old polyclonal Ab A430 (discontinued by the manufacturer) to the monoclonal DAK-A3 is not a good decision

mAb LK2H10

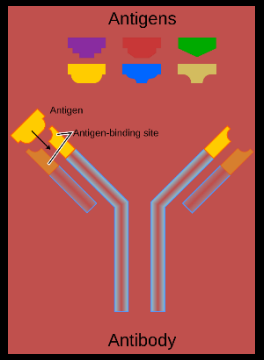
mAb´s LK2H10 + PHE5

Poly versus monoclonal antibody ?

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

Fat and eccrine epithelia cells ?

Antibody-Antigen reaction



Parameters affecting antibody-antigen reactions in tissue:

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

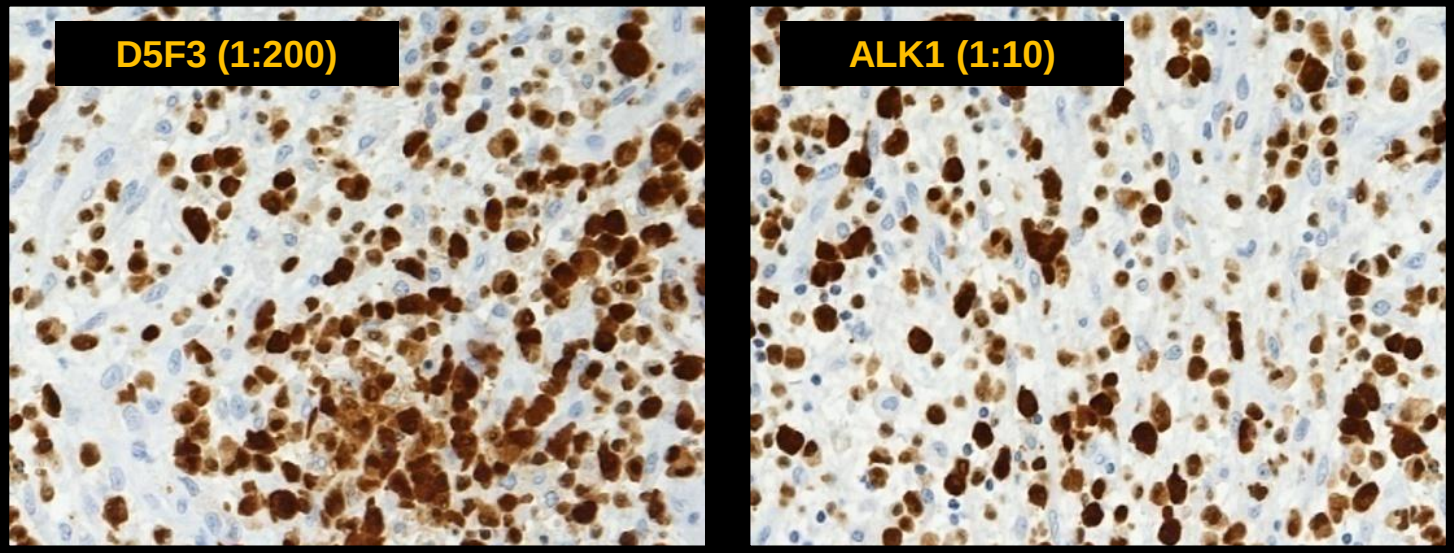
Incubation time
Incubation temperature
Sensitive to endogenous peroxidase blocking

Storage of concentrated primary antibodies
Storage of diluted primary antibodies

Antibody-Antigen reaction – Antibody choice / Sensitivity

Anaplastic lymphoma kinase (ALK)

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



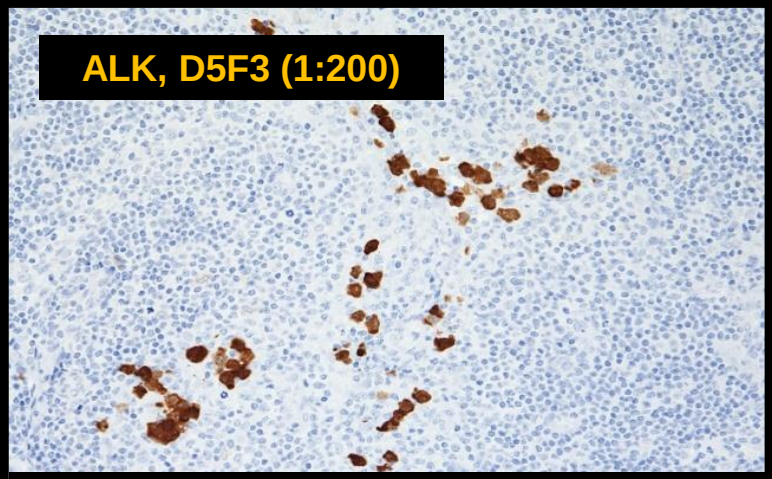
HIER in high pH buffer, Flex+

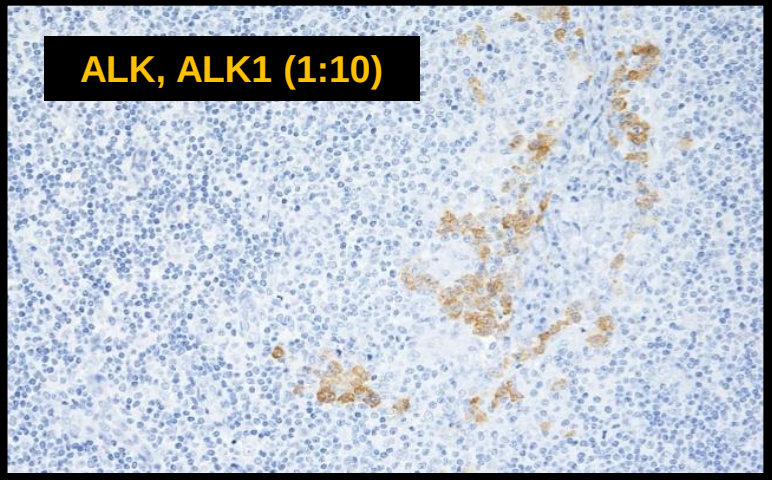
Anything wrong ?

Antibody-Antigen reaction – Antibody choice / Sensitivity

HIER in high pH buffer, Flex+

ALCL

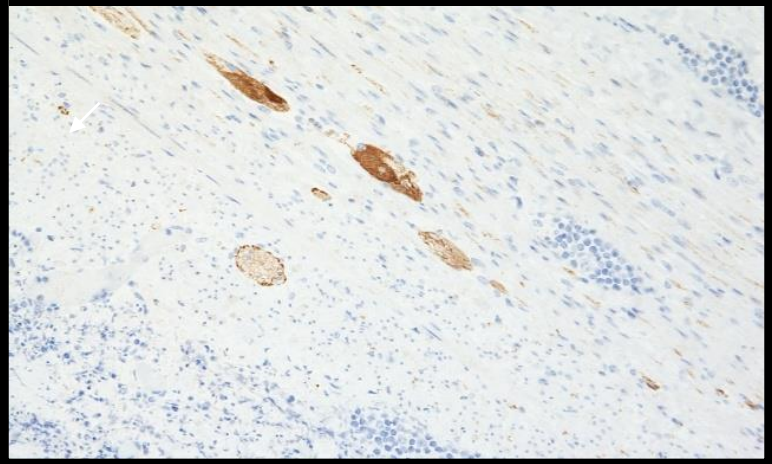


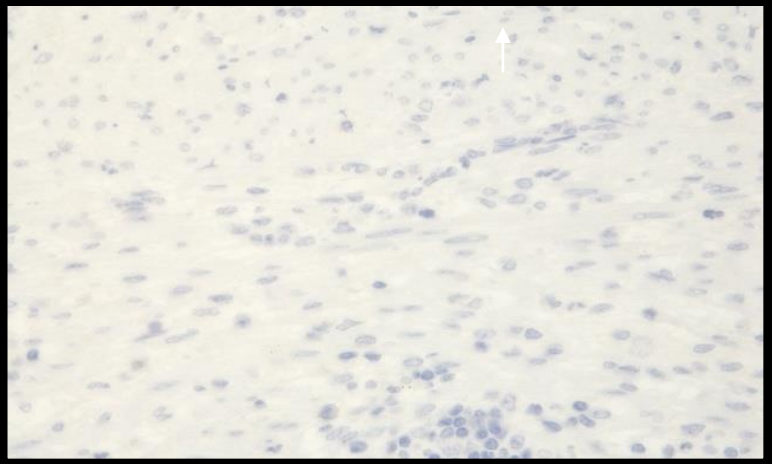


Clone ALK1 provide low sensitivity

iCAPS : Ganglion and peripheral nerve cells ?

Appendix





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

Georg Hutarew, Cornelia Hauser-Kronberger, Felix Strasser, Ida C Llenos & Otto Dietze

Department of Pathology, University Hospital and Paracelsus Medical University Salzburg, Salzburg, Austria

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+ 9 × 2+ 10 × 1+	14 (100)	4 × 3+ 4 × 2+ 6 × 1+
D5F3 (Cell Signaling) 1:250	Envision Flex	25 (8.25)	3 × 3+ 12 × 2+ 10 × 1+	14 (100)	3 × 3+ 7 × 2+ 4 × 1+
D5F3 (Ventana) Ready to use	OptiView Benchmark XT + AMP	128 (42.2)	14 × 3+ 7 × 2+ 107 × 1+	14 (100)	14 × 3+
5A4 (Novocastra) 1:100	Envision Flex	15 (4.95)	1 × 3+ 9 × 2+ 5 × 1+	12 (86.5)	1 × 3+ 6 × 2+ 5 × 1+
SP8 (Abcam) 1:50	Envision Flex	41 (13.5)	2 × 3+ 9 × 2+ 30 × 1+	9 (64)	9 × 1+
ALK1 (Dako) Ready to use	Envision Flex	10 (3.30)	0 × 3+ 0 × 2+ 10 × 1+	7 (50)	7 × 1+
ALK01 (Ventana) Ready to use	Benchmark XT	18 (5.94)	0 × 3+ 1 × 2+ 17 × 3+	7 (50)	1 × 2+ 6 × 1+

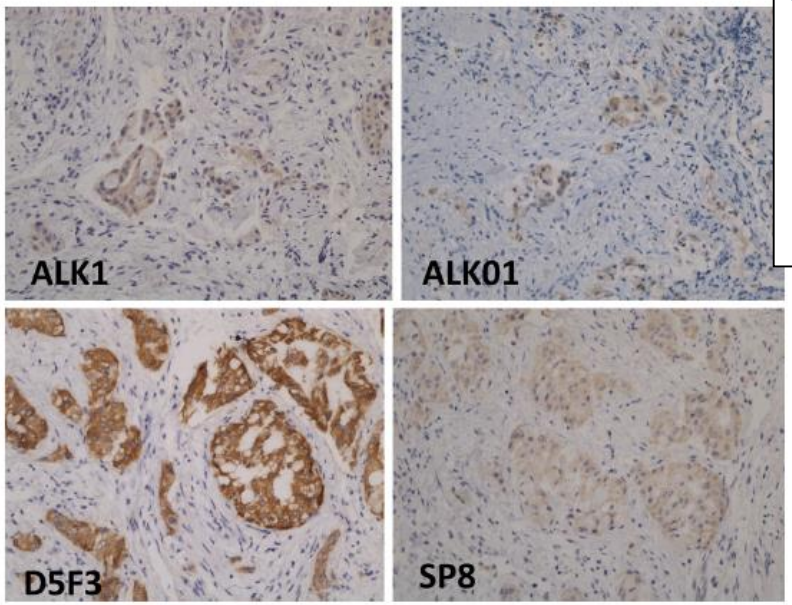


Figure 4. Conventional immunohistochemical staining without amplification. ALK1, ALK01 and SP8 staining of score 1+; D5F3 staining of score 3+.

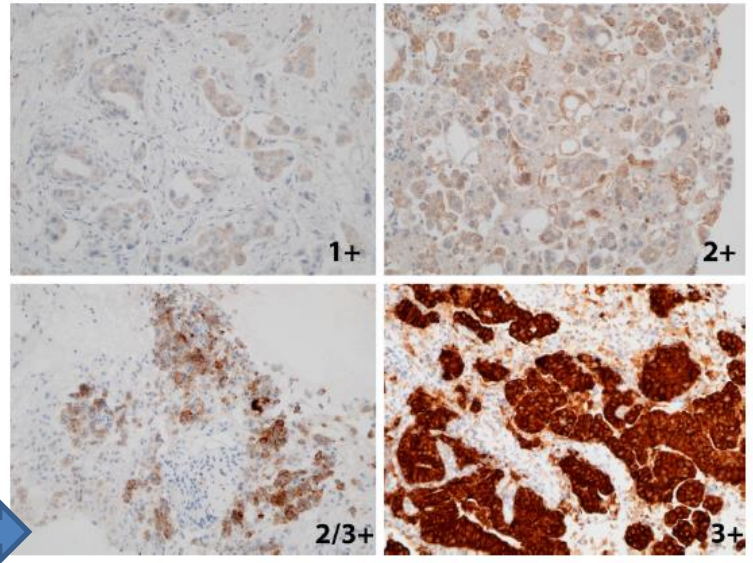


Figure 5. Staining using D5F3 (Ventana) and OptiView. The numbers represent scores using the four-tiered system; using the binary system both upper images are negative, and both lower images are positive. The image on the lower left shows a few strongly stained tumour cells (3+), and this case was also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

Clinical Cancer Research

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

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

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

Lung tumors

Low amount of fused protein = require high sensitive antibody for detection

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

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Human PATHOLOGY

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Original contribution

Expression of anaplastic lymphoma kinase in Merkel cell carcinomas☆

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

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

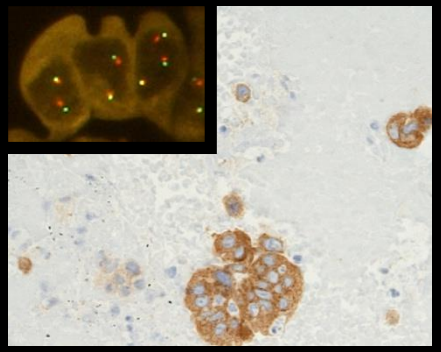
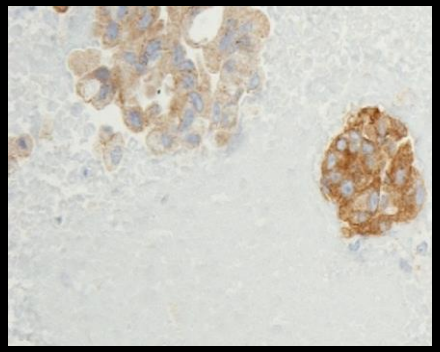
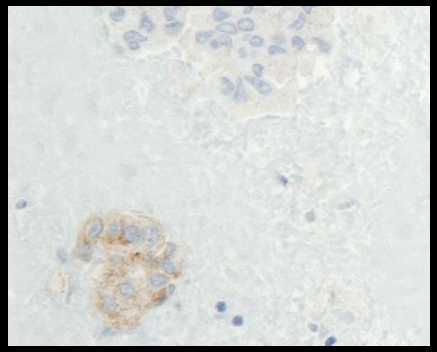
MCC

ALK,D5F3 = 94% pos

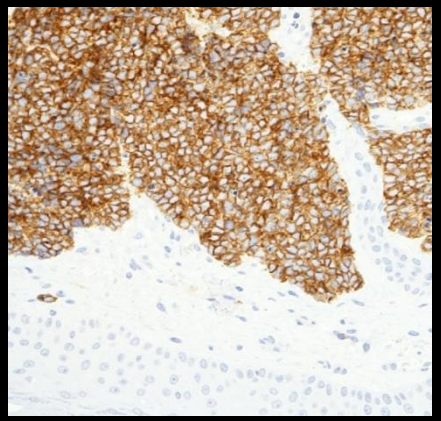
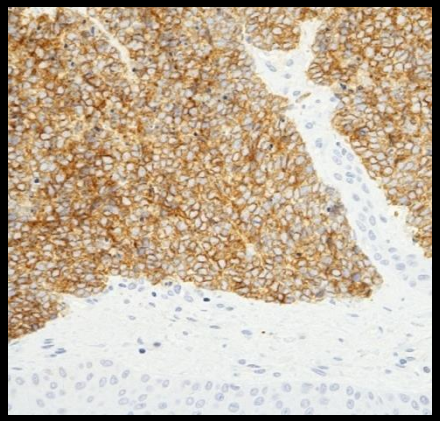
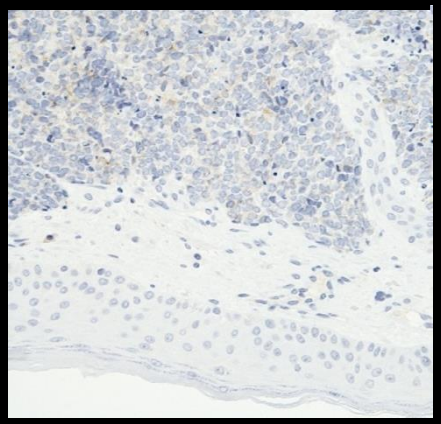
ALK,5A4 = 88% pos

ALK, ALK1 = 13% pos

Antibody-Antigen reaction – Antibody choice / Sensitivity

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

Clone ALK1 provide low sensitivity

Merkel cell carcinoma Skin			

HIER in high pH buffer, Flex+

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

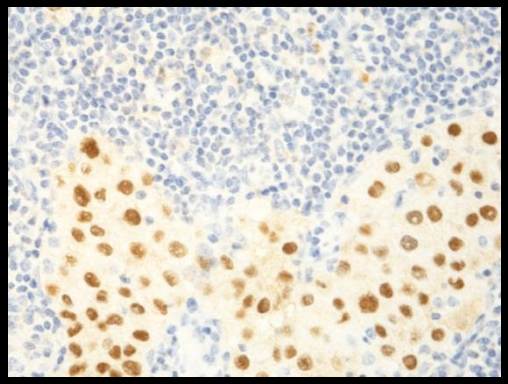
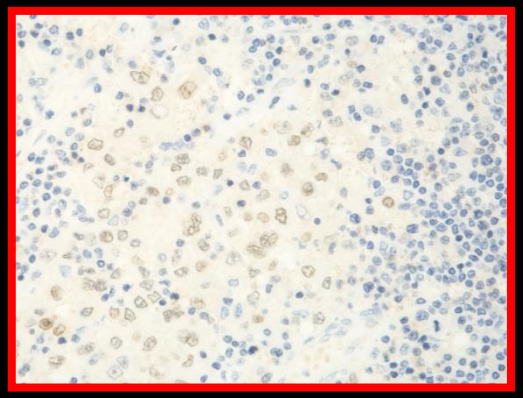
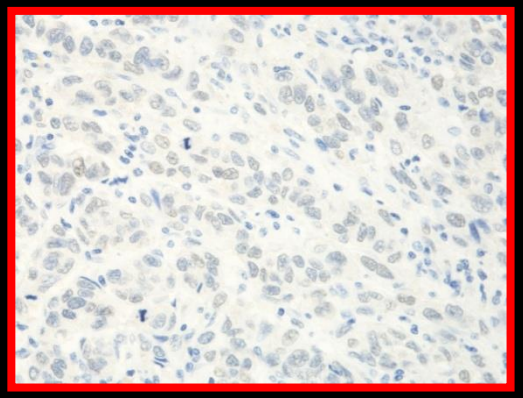
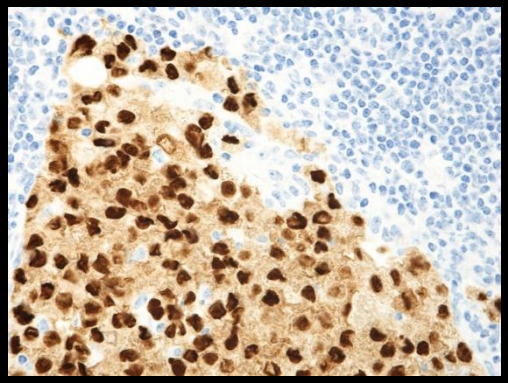
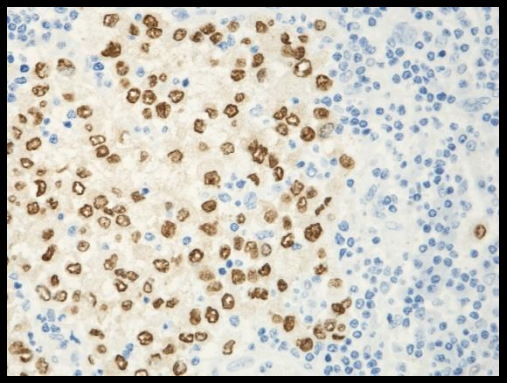
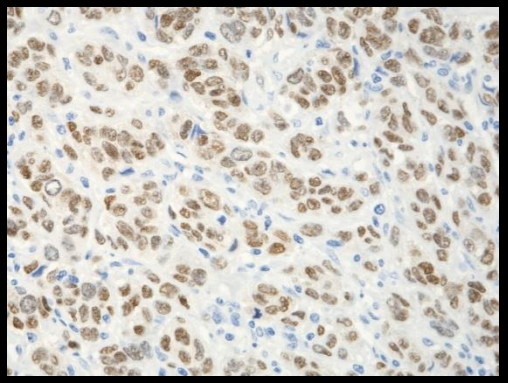
Concentrated antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	Suff. OPS[2]
mAb clone **5A4**	46 3 2 1 1 1	Leica/Novocastra Thermo/NeoMarkers Monosan Abcam Biocare Zytomed	24	16	13	1	74%	81%
mAb clone **ALK1**	8	Dako	0	0	3	5	0%	-
mAb clone **OTI1A4**	5	ORIGENE	4	1	0	0	100%	100%
rmAb clone **D5F3**	21 1	Cell Signaling PrimeBioMed	18	2	1	1	91%	95%
rmAb clone **SP8**	2	Thermo/NeoMarkers	0	0	1	1	-	-
Ready-To-Use antibodies								
mAb clone **5A4 PA0306**	3	Leica/Novocatra	0	1	2	0	-	-
mAb clone **5A4 API3041**	1	Biocare	1	0	0	0	-	-
mAb clone **5A4 MAB-0281**	1	Maixin	1	0	0	0	-	-
mAb **5A4 MAD-001720QD**	1	Master Diagnostica	0	0	0	1	-	-
mAb **ALK1 IR641**	15	Dako	0	0	4	11	0%	-
mAb clone **ALK1 790/800-2918**	10	Ventana	0	1	6	3	10%	-
mAb clone **ALK1 204M-18**	1	Cell Marque	0	0	0	1	-	-
mAb clone **ALK1 GA641**	1	Dako	0	0	0	1	-	-
rmAb clone **D5F3 790-4794**	47	Ventana	41	4	2	0	96%	96%

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

D5F3, 5A4, OTI1A4

35 protocols were based on ALK1: Only one protocol (3%) were assessed as sufficent, none were optimal

Antibody-Antigen reaction – Antibody choice / Sensitivity

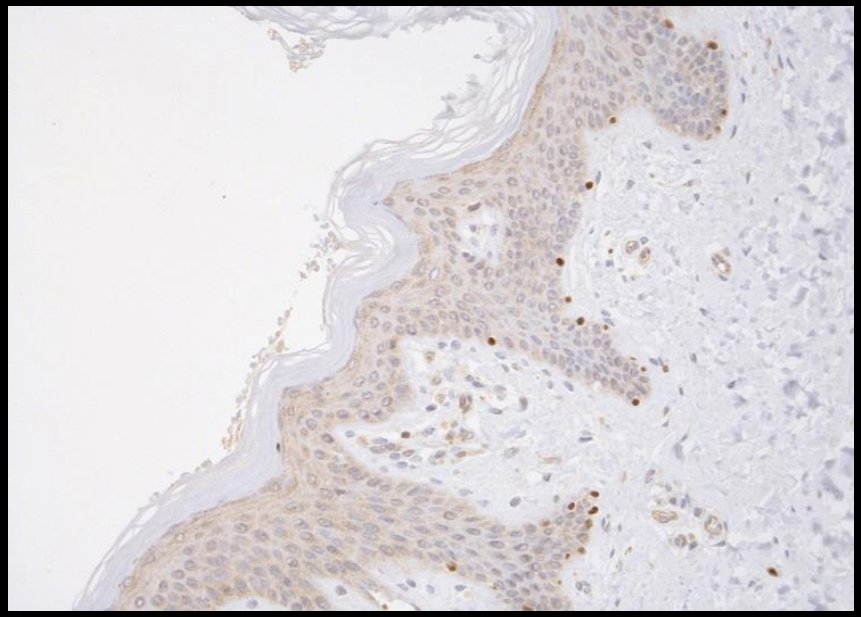
	Melanoma 1 High expressor	Melanoma 2 Low expressor	Melanoma 3 Low expressor
Sox-10, polyclonal 1:125			
Sox-10, BC 34 1:40			

Is it to possible to increase the concentration of the primary Ab Sox-10, polyclonal ?

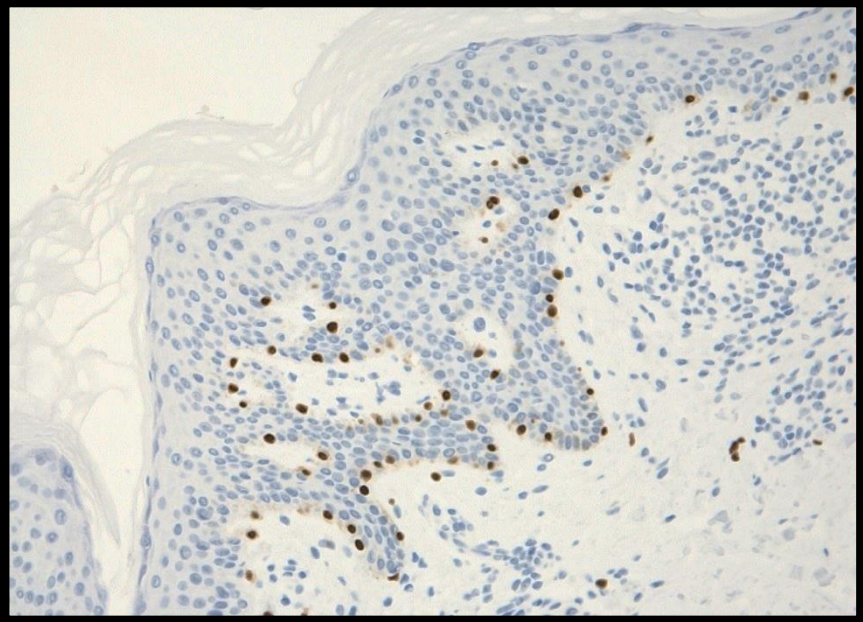
High pH / Flex+/Autostainer

Antibody-Antigen reaction – Antibody choice / Sensitivity

Sox-10, polyclonal (Cell Marque) 1:125



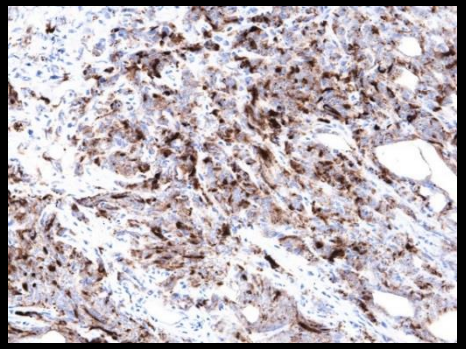
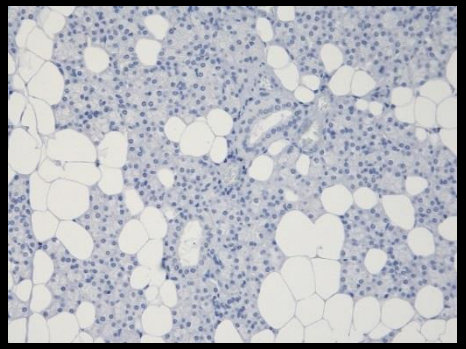
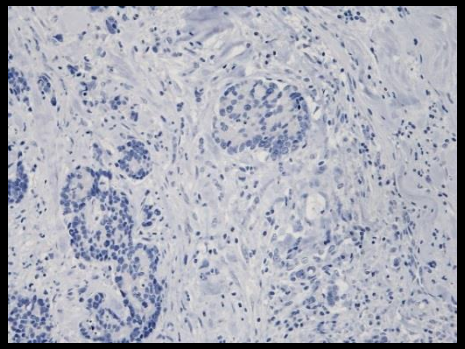
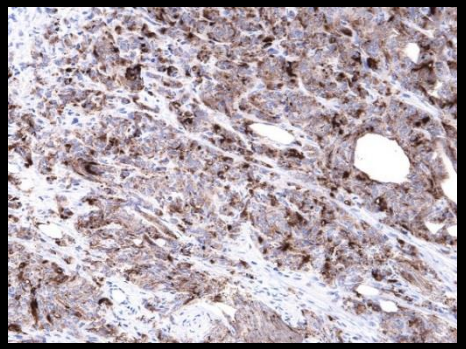
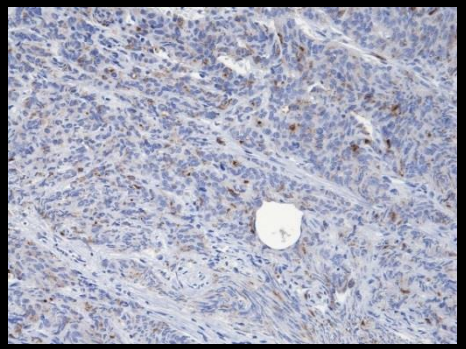
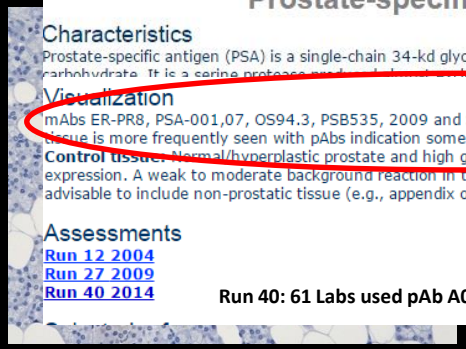
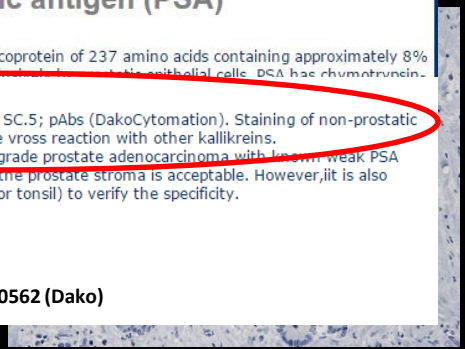
Sox-10, BC34 (Biocare) 1:40



Note: Proportion of positive normal melanocytes is higher with SOX-10, BC34 and no background staining is observe in contrary to the polyclonal Ab from Cell Marque ~ increased concentration of the polyclonal AB (CM) will cause poor to signal noise ratio

High pH / Flex+/Autostainer

Antibody-Antigen reaction – Antibody choice / Specificity

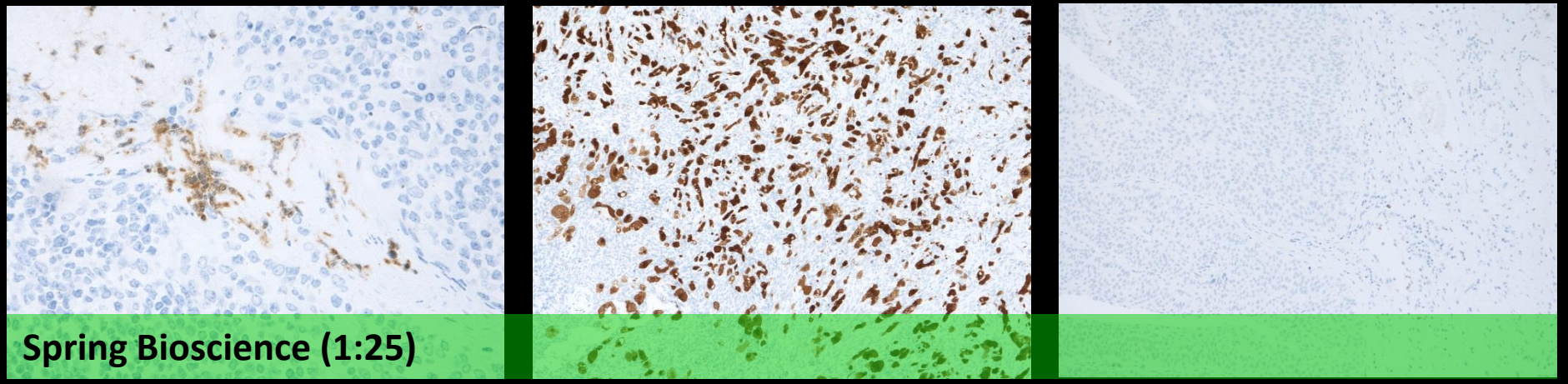
	Prostate Adenocarc.	Salivary Gl.	Carcinoid / Appendix
PSA, 35H9 1:50			
PSA , Poly (Dako) 1:5000			
PSA , Poly (Dako) 1:40000			

High pH / Super Sensitive/ Biogenex i6000

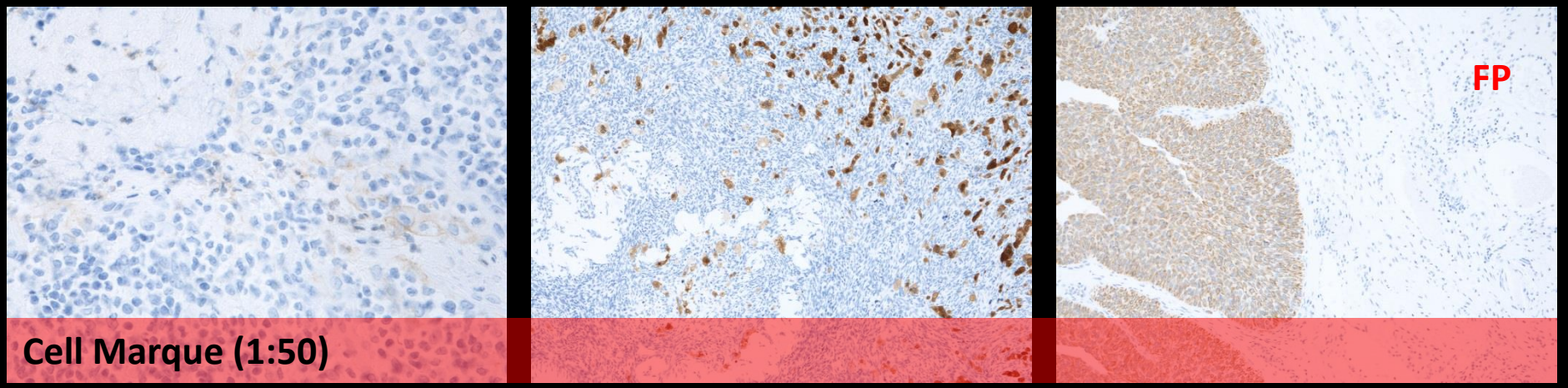
ARG1 clone SP156

Different vendors and specificity ?

Tonsil | Hepatocellular carc. | Bladder carc.



Spring Bioscience (1:25)



Cell Marque (1:50)

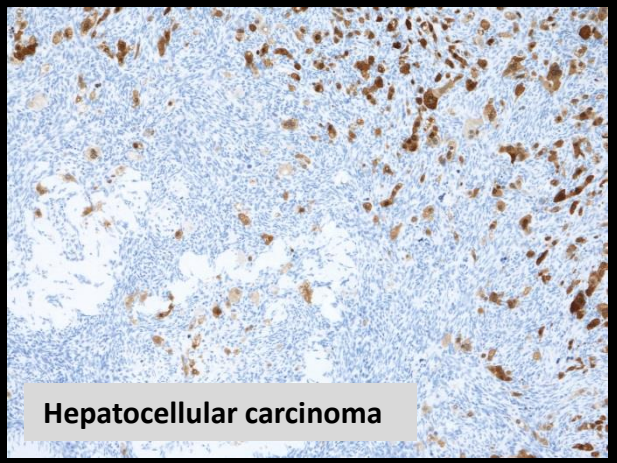
HIER High pH 24`; Flex+ Rabbit linker

In collaboration with Ole Nielsen, Department of Pathology, Odense

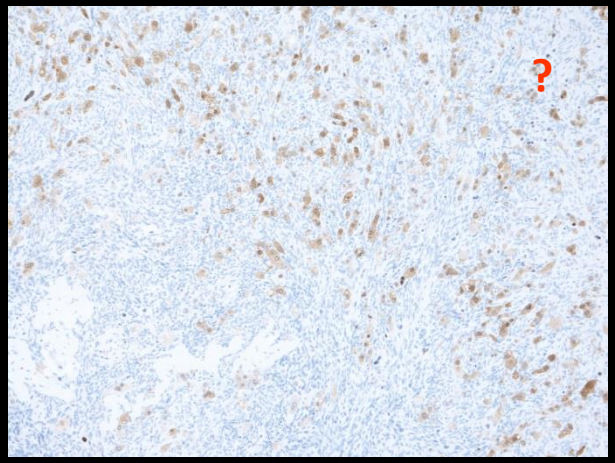
ARG1 clone SP156

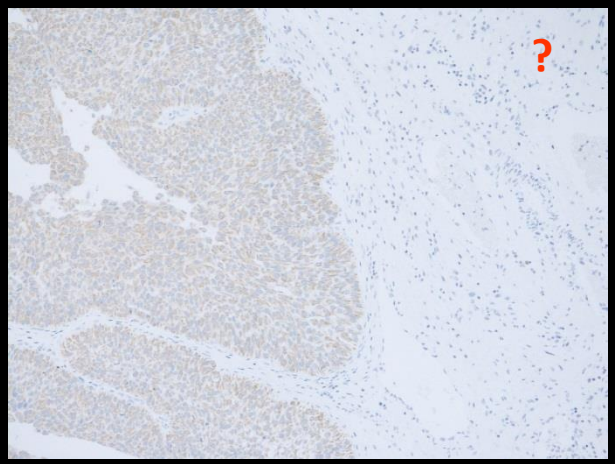
Different vendors and specificity ?

ARG1, SP156 Cell M 1:50

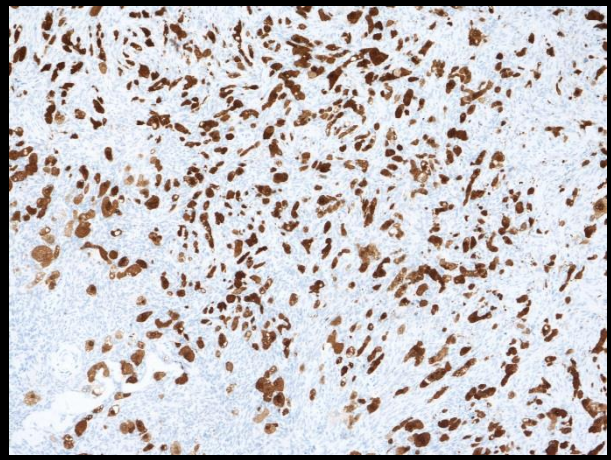


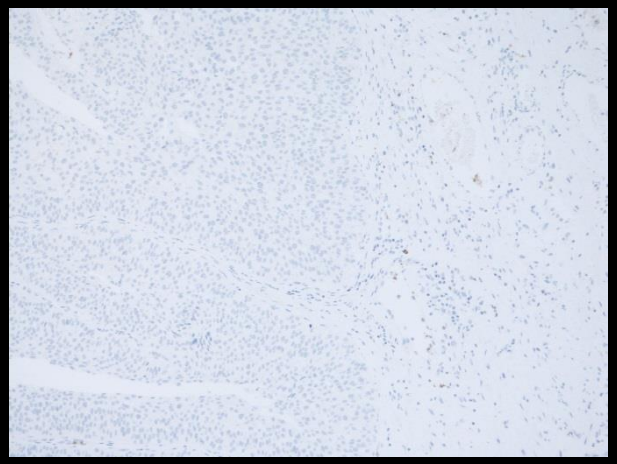
ARG1, SP156 Cell M 1:100





ARG1, SP156 Spring B. 1:25



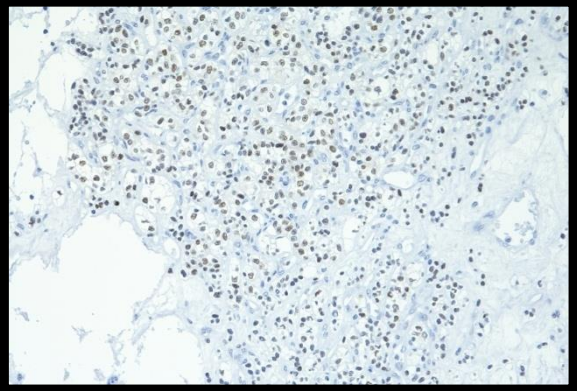


HIER High pH 24`; Flex+ Rabbit linker

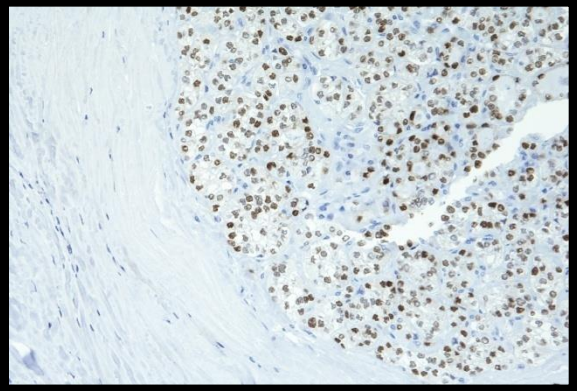
In collaboration with Ole Nielsen, Department of Pathology, Odense

Which antibody ?

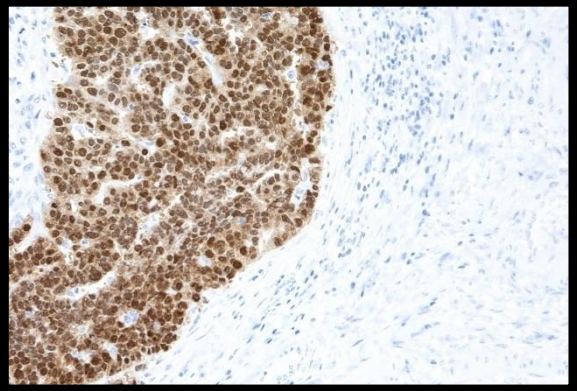
Renal Cell Carcinoma (Hn)



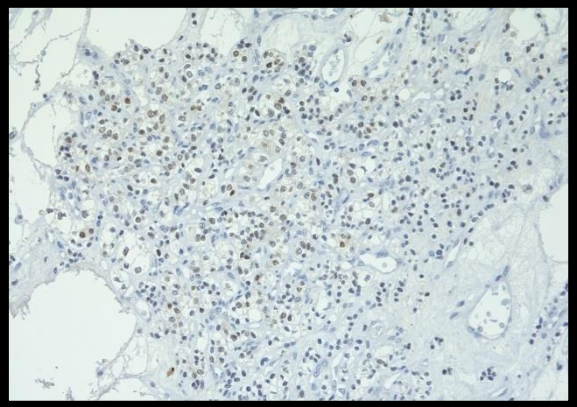
Thyroid Carcinoma (Pa)

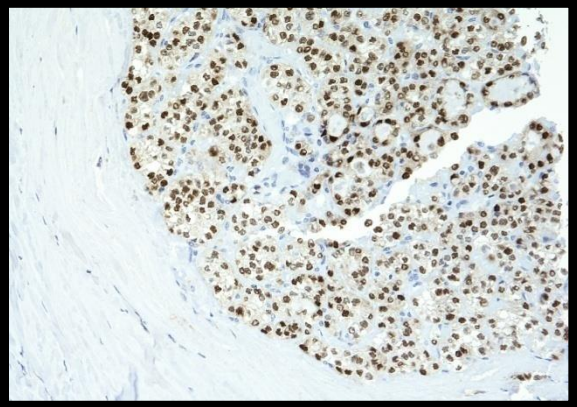


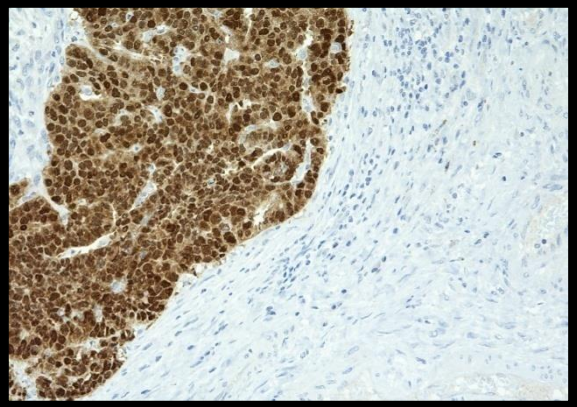
Ovary Carcinoma (Se)



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







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

The Diagnostic Utility of PAX8 for Neuroendocrine Tumors: An Immunohistochemical Reappraisal

Jau-Yu Liau, MD,† Jia-Huei Tsai, MD,*† Yung-Ming Jeng, MD, PhD,*† Kuan-Ting Kuo, MD,* Hsin-Yi Huang, MD, PhD,*† Cher-Wei Liang, MD,*† and Ching-Yao Yang, MD, PhD‡*

Appl Immunohistochem Mol Morphol . Ahead of print, Post Author Corrections: February 21, 2015

Material and Methods:

115 neuroendocrine tumors (NET`s) of various organs

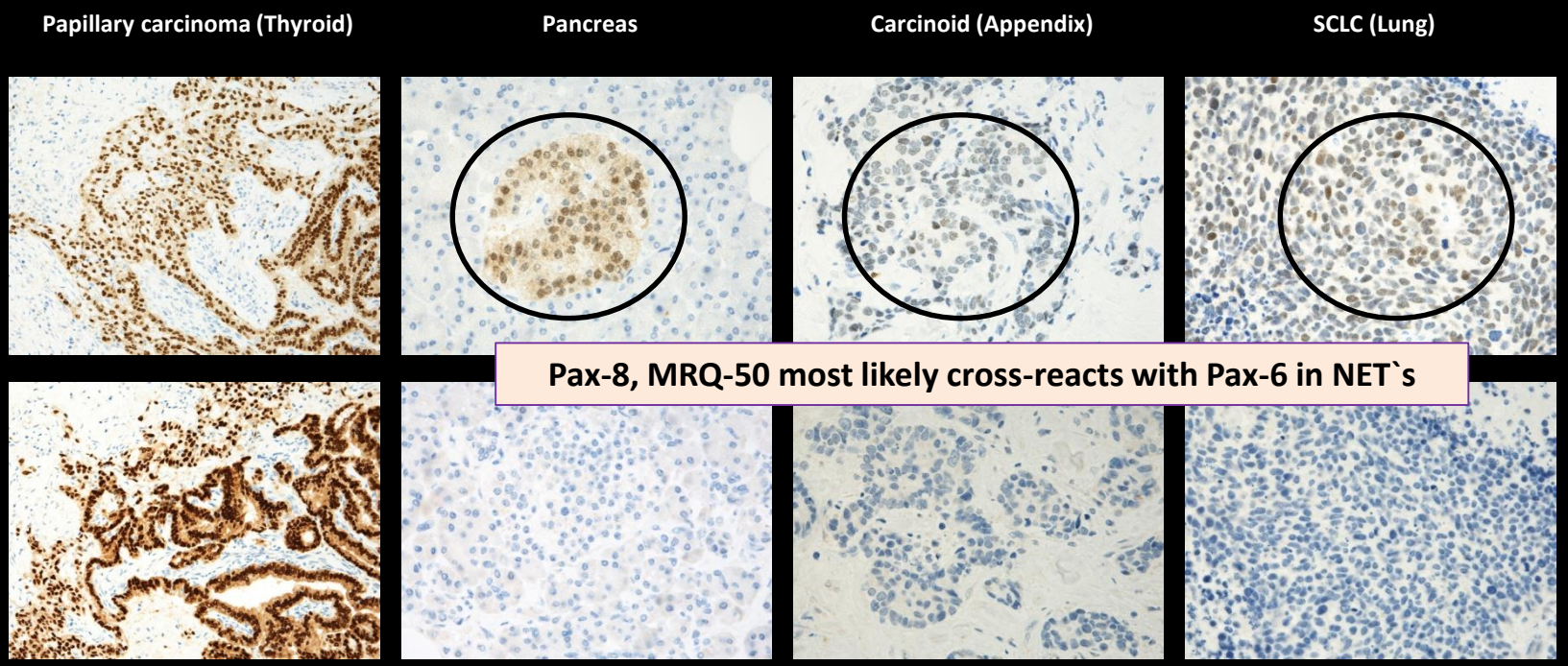
Four PAX8 antibodies (Proteintech polyclonal , MRQ50, PAX8R1 & BC12)

Demonstrated that:

- NET`s from a large variety of organs were immuno-reactive to the two less specific antibodies cross-reacting with other PAX proteins (Proteintech & MRQ50)

- All NET`s were immuno-negative to the two monoclonal antibodies specific to the less conserved C-terminal proportion of PAX8 (PAX8R1 & BC12).

Antibody-Antigen reaction – Antibody choice / Specificity



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

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

Question: 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 ?

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

Concentrated antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	Suff. OPS[2]
mAb clone **MRQ-50**	33	Cell Marque	19	8	6	0	82%	81%
mAb clone **BC12**	7	BioCare	1	3	1	2	57%	-
mAb clone **ILQ-150**	1	Immunologic	1	0	0	0	-	-
mAb clone **PAX8R1**	1	Abcam	0	1	0	0	-	-
rmAb clone **ZR-1**	1 1 1	Abcam Zeta Zhongshan	2	0	0	1	-	-
pAb, **363A**	11	Cell Marque	0	4	7	0	36%	-
pAb, **10336-1-AP**	11	Protein Tech	5	5	0	1	91%	100%
pAb, **CP379**	4	Biocare	1	2	1	0	-	-
pAb, **RBK047**	2	Zytomed Systems	0	1	1	0	-	-
pAb, **HPA030062**	1	Atlas Antibodies	0	0	0	1	-	-
pAb, **ILP3633-C05**	1	Immunologic	0	1	0	0	-	-
pAb, **ABE671**	1	Millipore	0	0	1	0	-	-
pAb, **NBP1-32440**	1	Novus	1	0	0	0	-	-

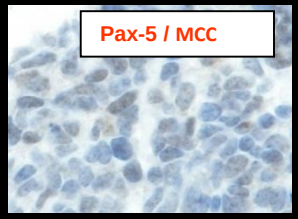
Cross react with other Pax proteins in the family e.g. PAX5

Question:

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 ?

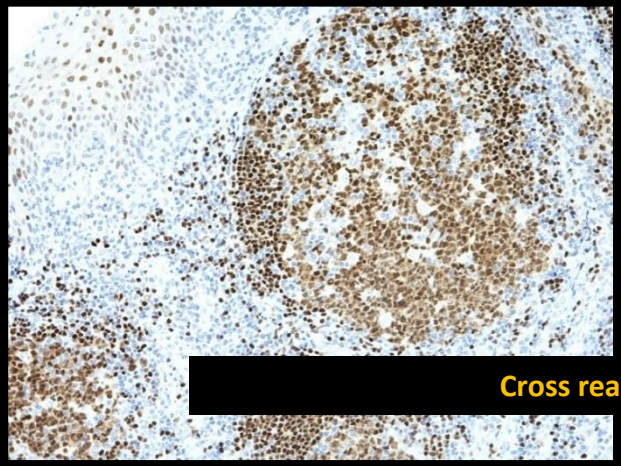
Antibody-Antigen reaction – Antibody choice / Specificity

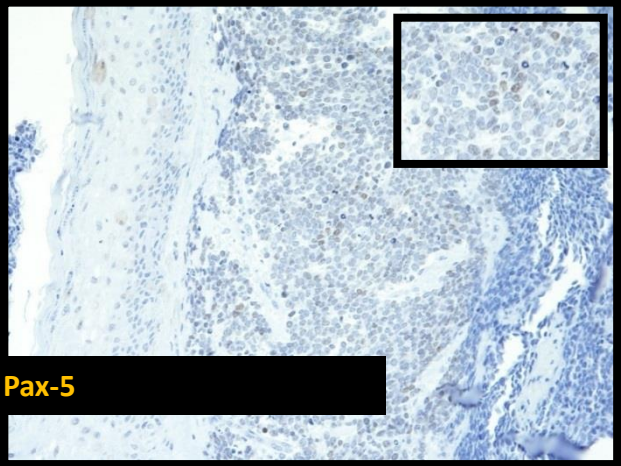


Tonsil

Merkel cell carcinoma

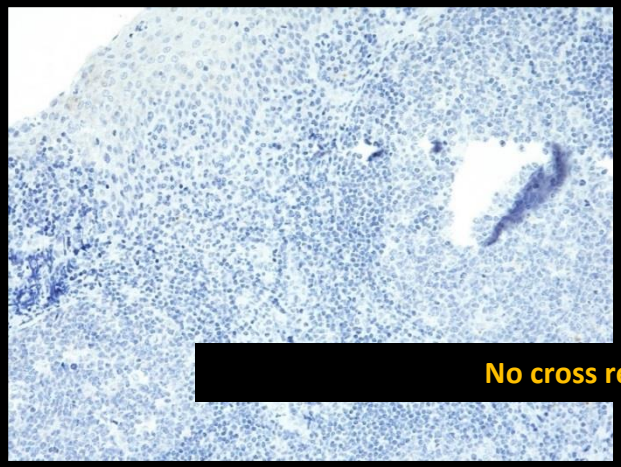
Pax-8, MRQ-50

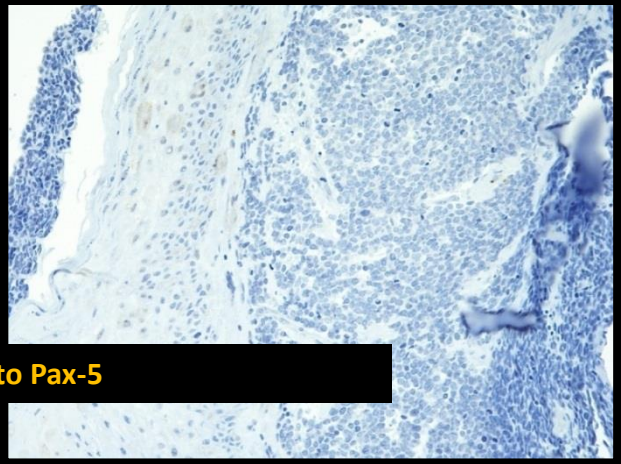




Cross reactivity to Pax-5

Pax-8, BC12





No cross reactivity to Pax-5

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

Demonstrated that an N-terminal PAX-8 polycl. 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.

Antibody-Antigen reaction - Antibody Titer & Dilutions

Titer is the highest dilution of the primary antibody resulting in strong specific staining with the least amount of background

<u>NordiQC assessments (LAB`s using inapp. titer causing insufficient results)</u>

- **Primary antibody used in too low concentration / FN (95%)**
- **Primary antibody used in too high concentration / FP (5%)**

The question is - how should I calibrate antibody concentration and determinate correct titer ?

Tissue is the key element

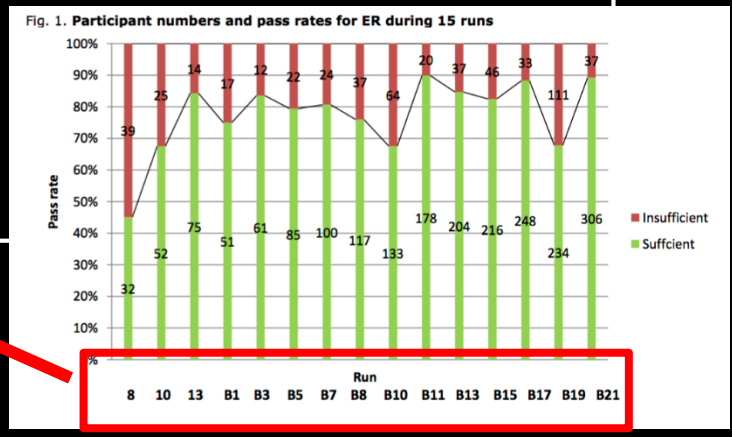
Antibody-Antigen reaction - Antibody Titer & Dilutions

Estrogen Receptor (ER), NQC Run B21		Optimal	Good	Borderl.	Poor	Suff
Total protocols assessed	343	210	96	26	11	-
Proportion		61 %	28 %	8%	3%	89%

* All Ab clones and protocol settings

The most frequent causes of insufficient staining reactions were:

- Insufficient HIER (too short efficient HIER time)
- Less successful primary Ab
- Too low concentration of the primary Ab.

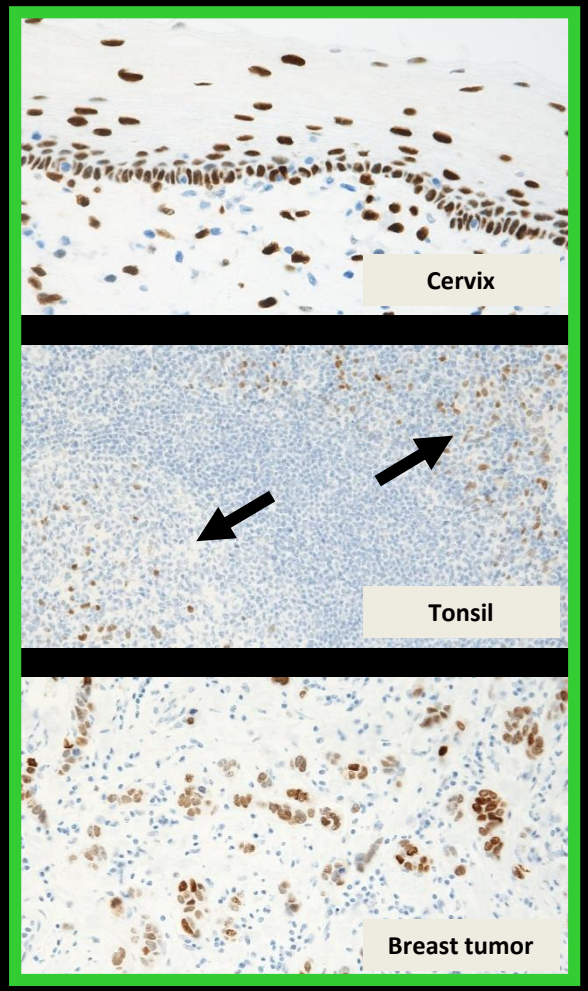


Correct control tissue for ER ?

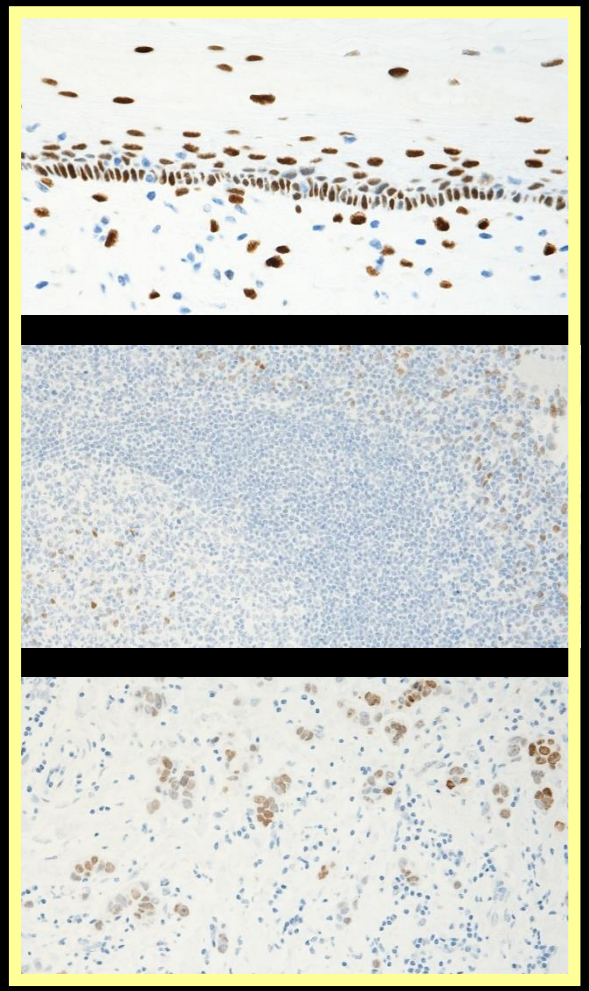
- Cervix (Normal tissue – high and non-expressors)
- Breast tumor´s x3 (non-, low and medium/high expressors)
- Tonsil (Normal tissue – low and non-expressors)

Antibody-Antigen reaction - Antibody Titer & Dilutions

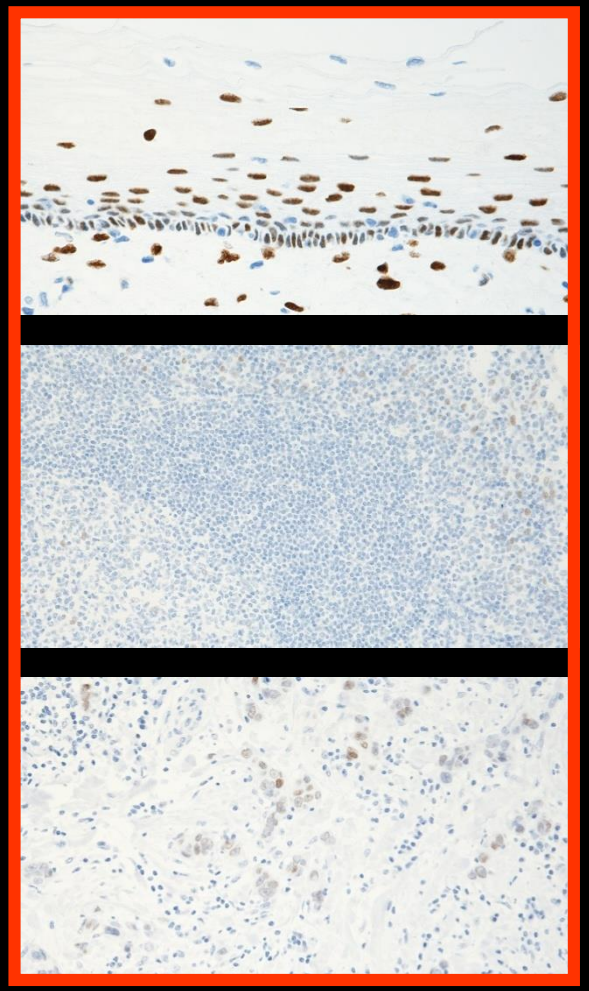
ER clone 6F11 / 1:100



ER clone 6F11 / 1:200



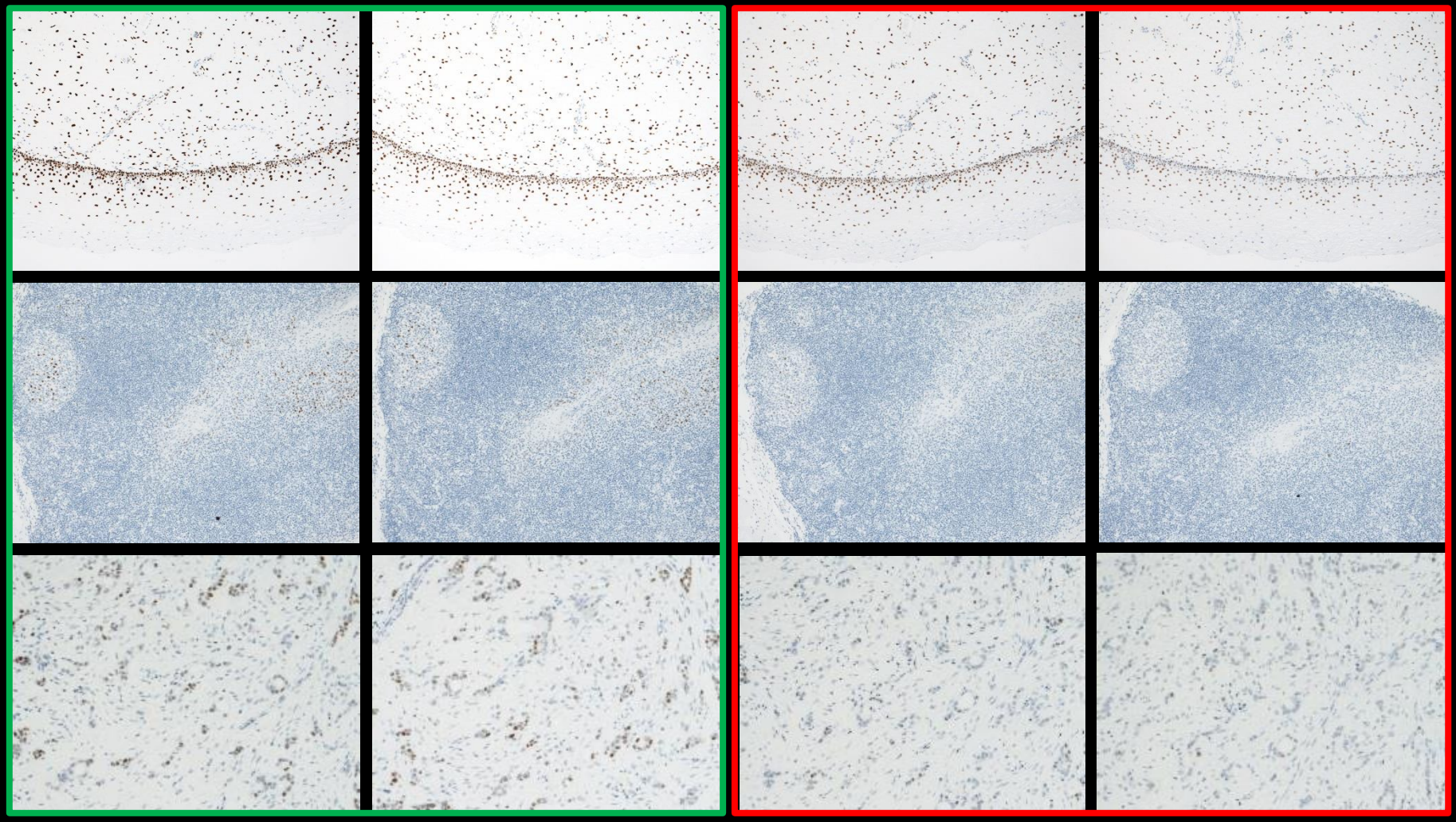
ER clone 6F11 / 1:400



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

High pH 20`, Flex+Mouse

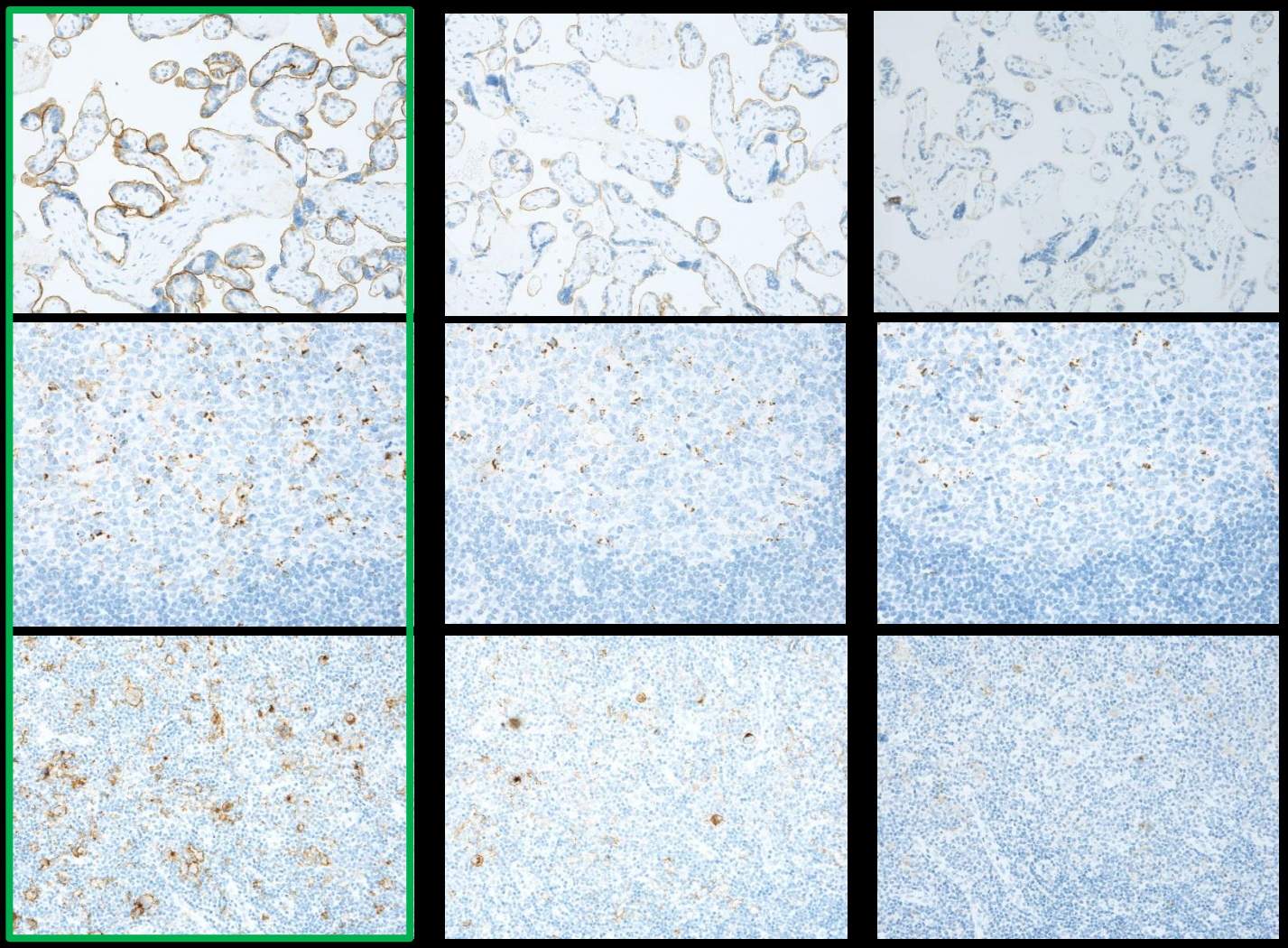
Antibody-Antigen reaction - Antibody Titer & Dilutions



Reduced intensity and proportion of cells expected to be stained

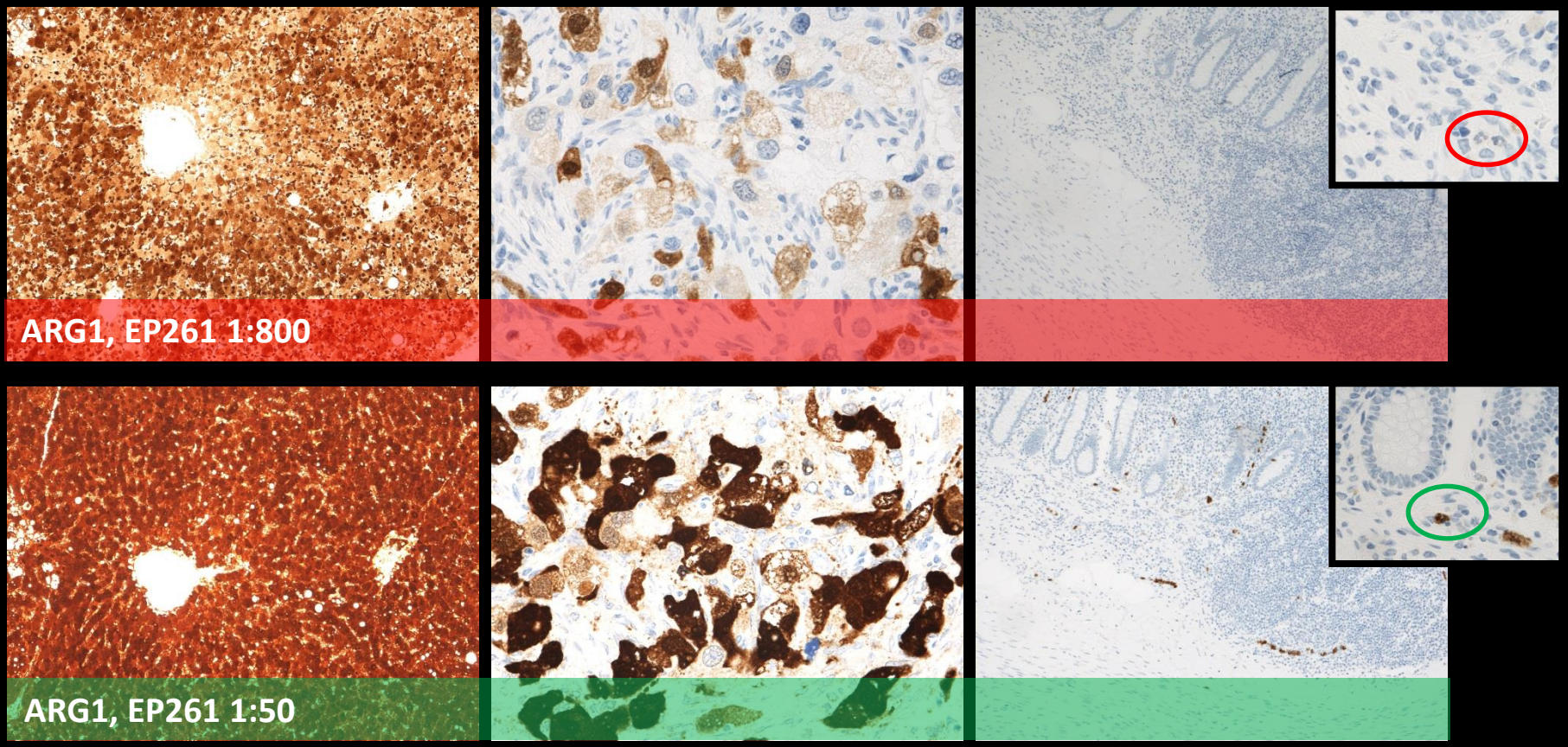
High pH 24`, Flex+Rabbit

Antibody-Antigen reaction - Antibody Titer & Dilutions



High pH 24`, Flex+Rabbit

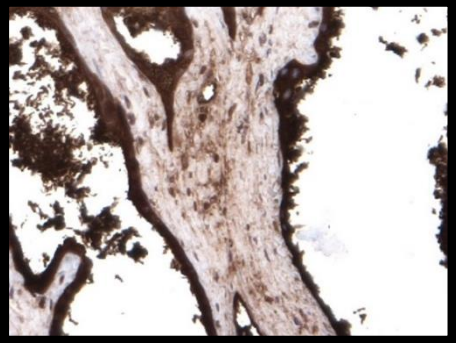
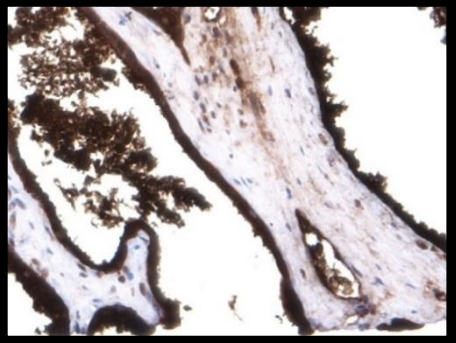
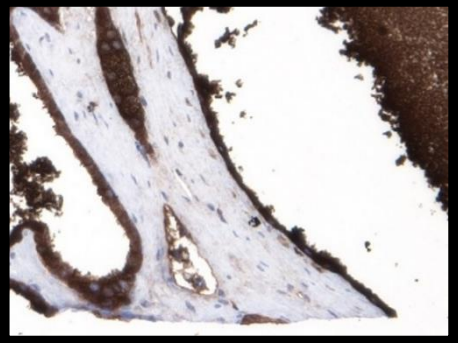
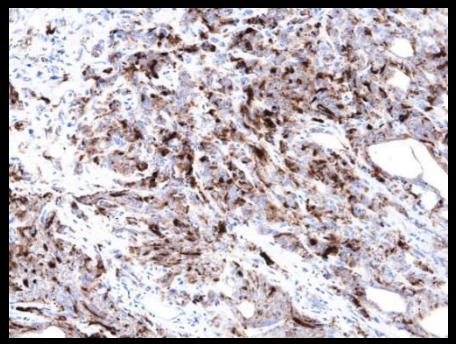
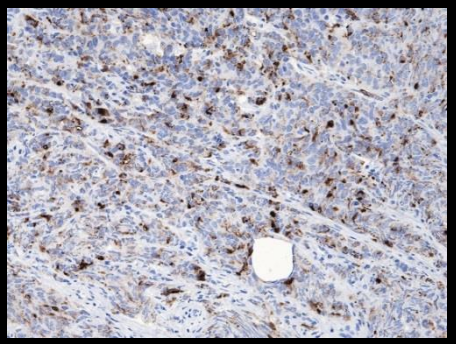
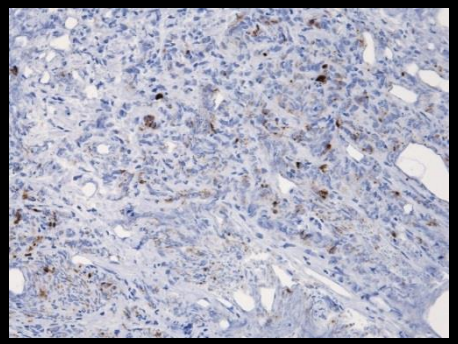
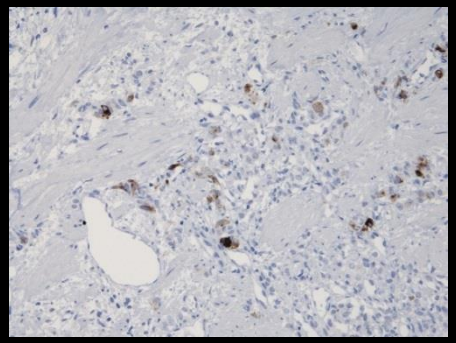
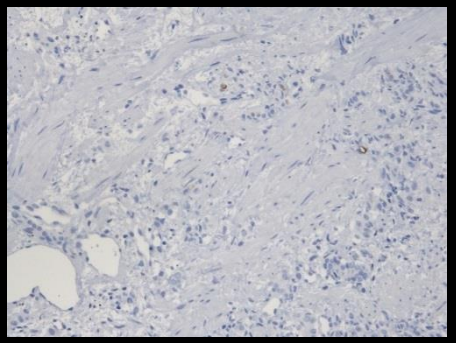
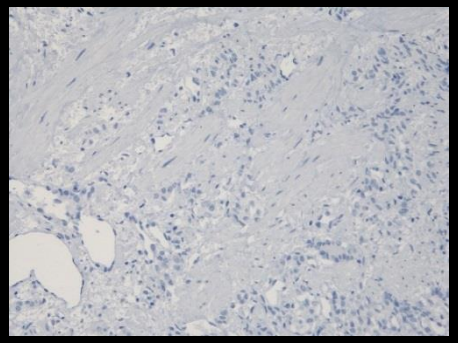
ARG1 clone EP261



HIER High pH 24`; Flex+ Rabbit linker

In collaboration with Ole Nielsen, Department of Pathology, Odense

Antibody-Antigen reaction - Antibody Titer & Dilutions

	PSA, 35H9 1:50	PSA, 35H9 1:200	PSA, 35H9 1:800
Prostate Hyperplasia High expressor			
Prostate Adenocarc.			
Prostate Adenocarc. Low expressor			

All other tissue components or neoplasms except prostate and prostate neoplasm tested were negative

HIER High pH 20`, Flex+

Antibody-Antigen reaction – Sensitive to the chosen automated platform

Implementing a new platform has been a challenge (Næ)

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
Calreticulin (mut specific Ab)
.....

Changing the primary Ab

Changing Ab-Ag reaction microenvironment (Diluent)

Low affinity primary antibodies

Omnis ?

IHC – The Technical Test Approach

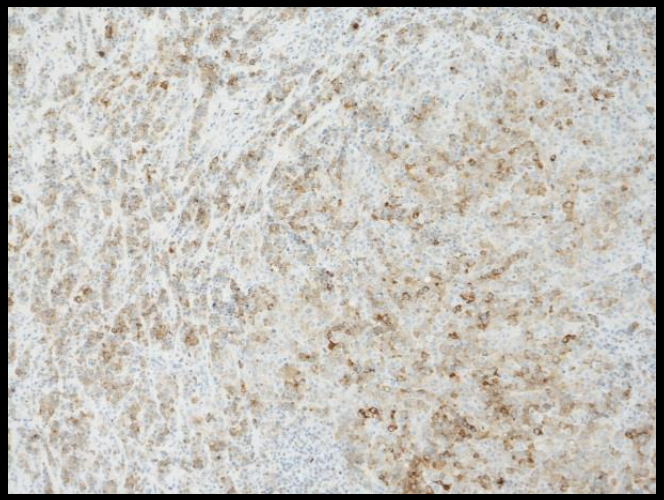
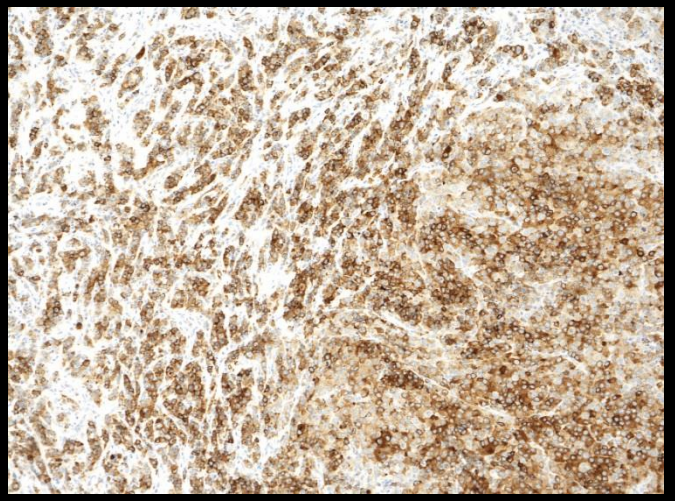
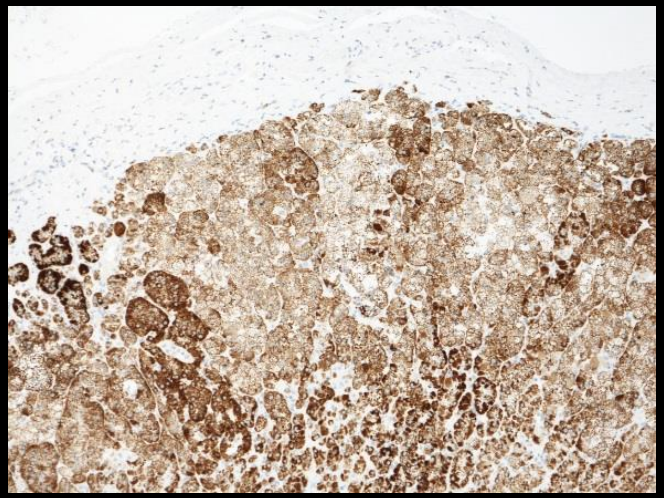
Antigen	Clone	XT / Ultra	Autostainer	Bond-max
CD4	~~1F6, 4B12~~	FN (3%H2O2)	√	√
CD4	SP35 EP204	√	√	√
CD5	4C7	FP	√	√
CD5	SP19	√	√	√
CD79a	JCB117	Weak	√	√
CD79a	SP18	√	√	√
ASMA	~~1A4~~ BS66	(√) Weak	√	√
BSAP	~~24~~ DAK-Pax5	FN	√	(- Weak)
BSAP	SP34	√	√	√
BCL6	PG-B6p ★	FN (3%H2O2)	√	√
BCL6	"GI191E/A8 "	√	√	√
Oct-2	OCT-207	FN	√	?
Oct-2	MRQ-2	√	?	? 131

"IHC-Platform" depending markers"

★ Cocktail with LN22

Courtesy Søren Nielsen

Antibody-Antigen reaction – Sensitive to the chosen automated platform

	Melan A, A103 1:25 / Omnis	Melan A, A103 1:25 / Autostainer
Melanoma Lymph Node		
Adrenal Gland		
	HIER High pH 24`, Flex+	HIER High pH 20`, Flex+

Antibody-Antigen reaction – Sensitive to the chosen automated platform

Table 1. **Antibodies and assessment marks for MLA, run 42**

Concentrated Abs:	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	Suff. OPS[2]
mAb clone **A103**	71 13 1 3 1 4 1 1 5	Dako Leica/Novocastra NeoMarkers Monosan Biogenex Cell Marque Immunologic Genemed Thermo Scientific	33	27	32	8	60%	63%
mAb clone **M2-7C10**	1	Zytomed	1	0	0	0	-	-
mAb clone cocktail **M2-7C10+M2-9E3**	2 1	Master Diagnostica Biocare	2	1	0	0	-	-
mAb clone **M2-7C10+M2-9E2**	1	Master Diagnostica	1	0	0	0	-	-
mAb clone cocaktail **M2-7C10 + M2-9E3 PM077**	1	Biocare	0	1	0	0	-	-
Total	198		80	55	52	11	-	
Proportion			40%	28%	27%	5%	68%	

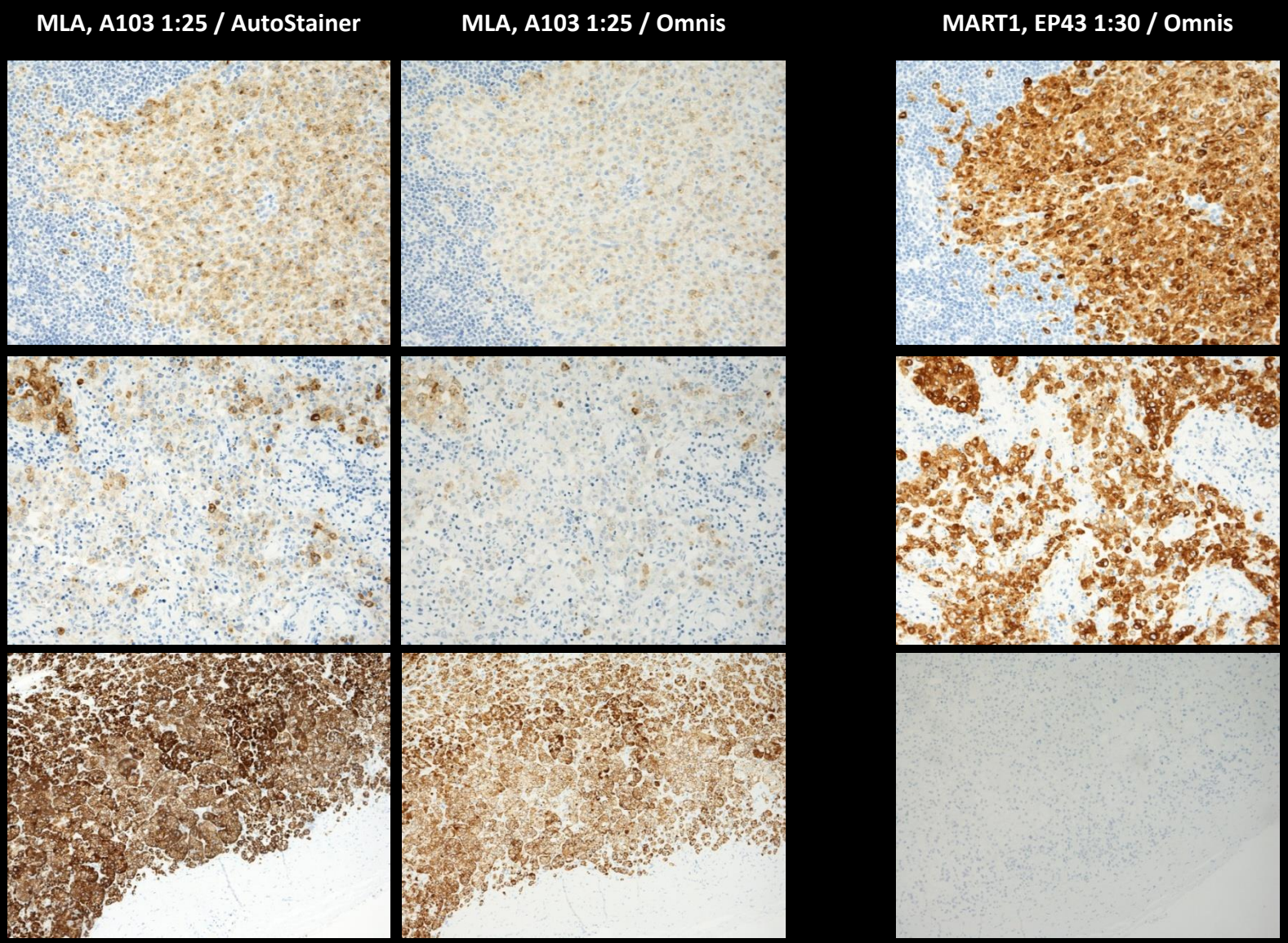
1) Proportion of sufficient stains (optimal or good),
2) Proportion of sufficient stains with optimal protocol settings only, see below.

Melan A (MLA) / MART-1:

200 participants ~ 95% used clone A103

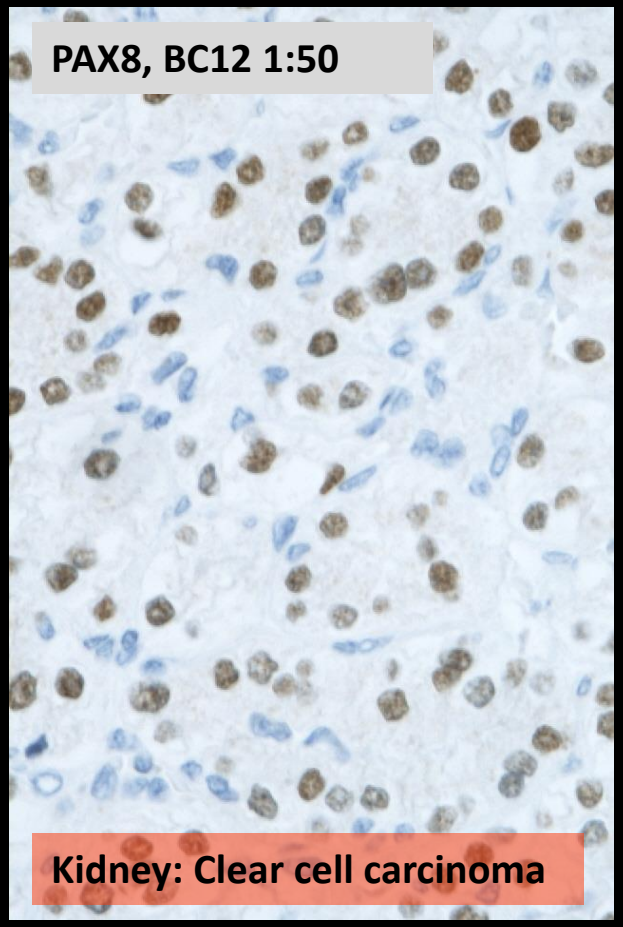
Question: Is MLA , A103 the best primary Ab ?

Antibody-Antigen reaction – Sensitive to the chosen automated platform



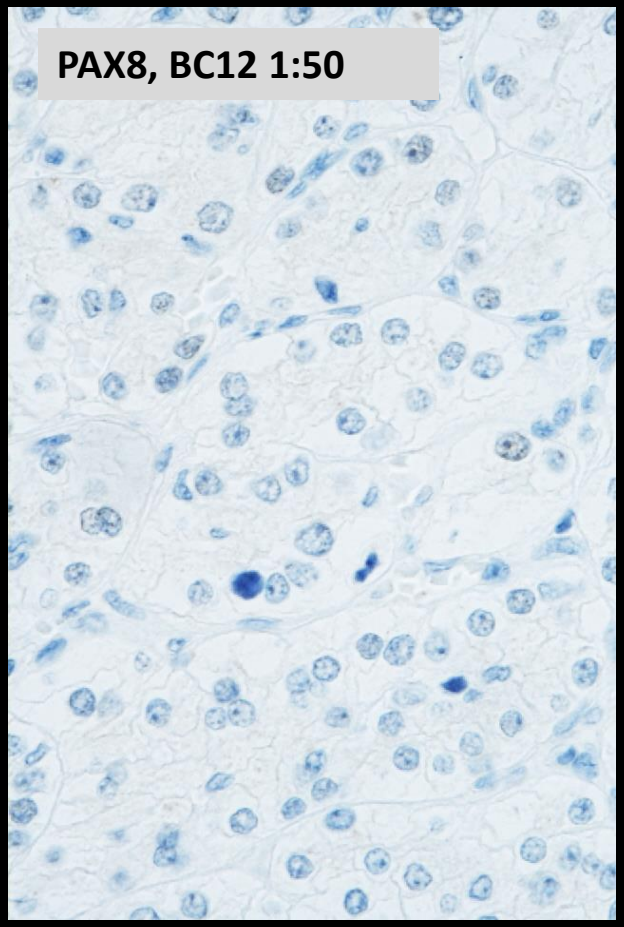
Antibody-Antigen reaction – Sensitive to the chosen automated platform

Autostainer



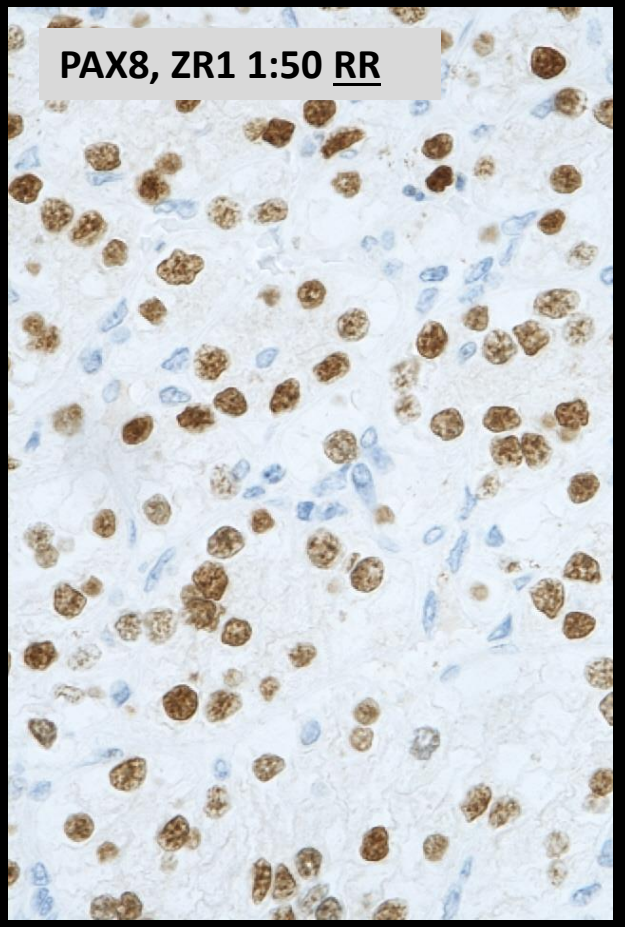
HIER High pH 20`, Flex+ (10+20)

Omnis



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

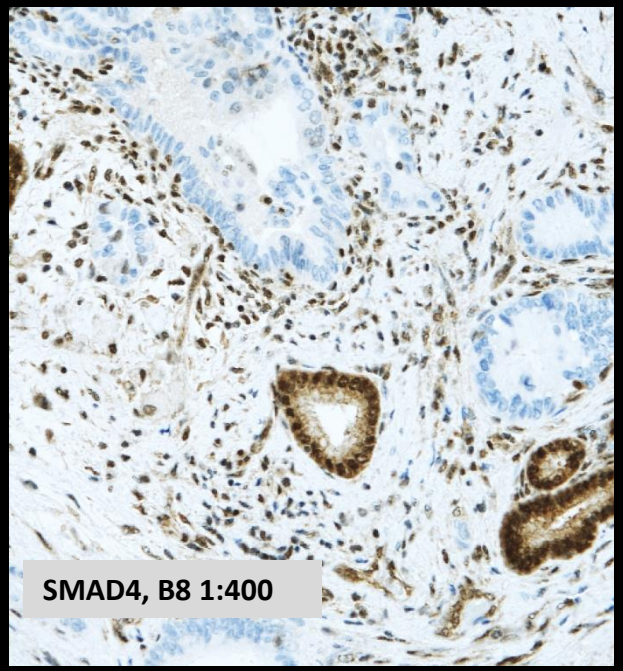
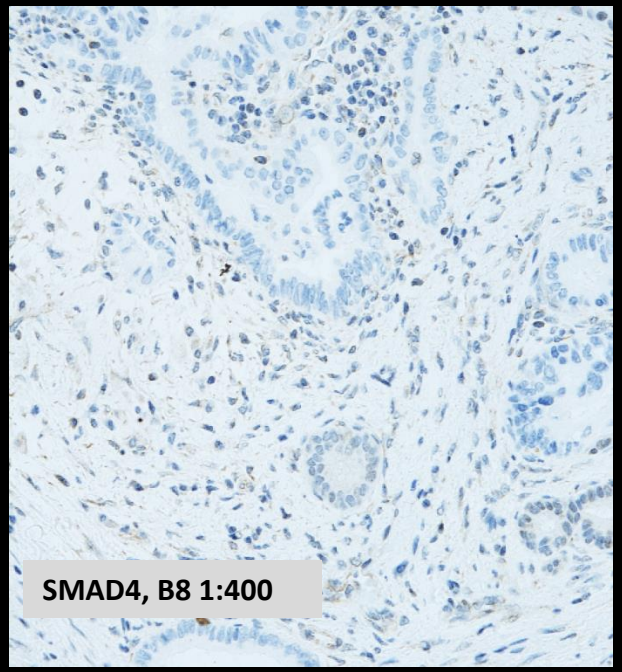
Omnis



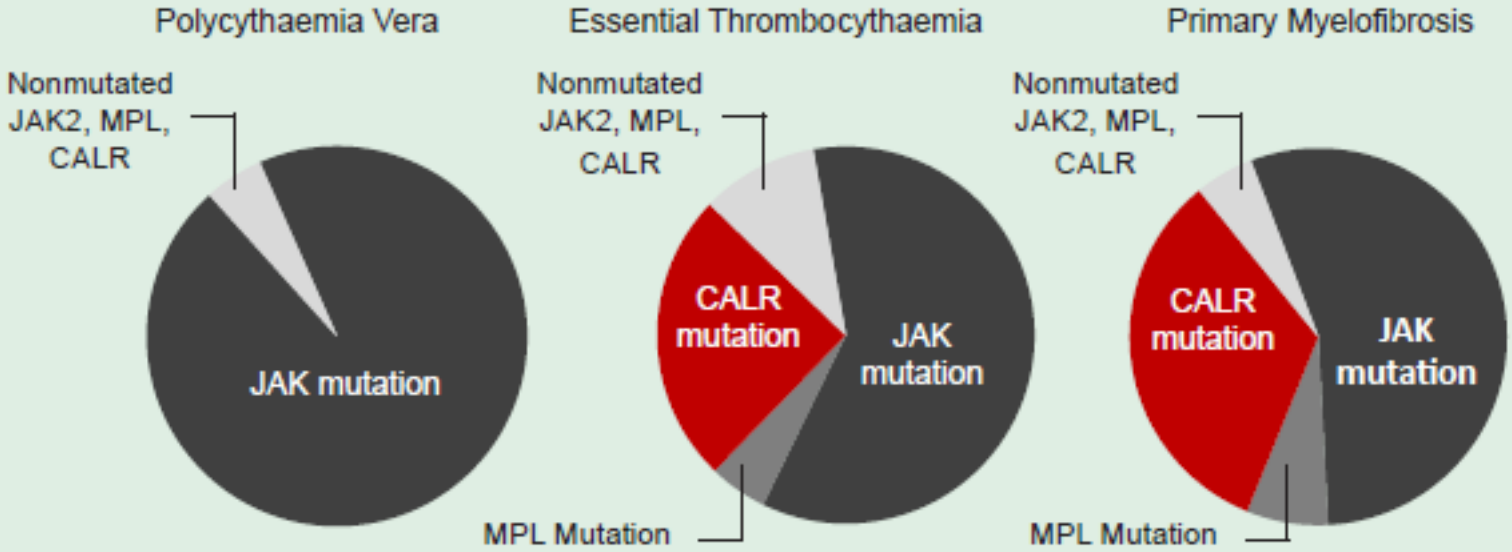
HIER High pH 48`, Flex+ (10+20)

Antibody-Antigen reaction – Sensitive to the chosen automated platform

Pancreatic Adenocarcinoma

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)

Diagnostic significance of *CALR* mutations in relation to *JAK2* and *MPL* mutations in MPNs



CALR mutations are detectable in 67% of ET and 88% of PMF cases with non-mutated JAK2 or MPL. It is mutually exclusive with mutations of *JAK2* or *MPL* in MPNs: The detection of *CALR* mutations fills a diagnostic gap in ET and PMF patients harboring non-mutated *JAK2/MPL*.

References:

Klampfl T et al. Somatic Mutations of Calreticulin in Myeloproliferative Neoplasms
N Engl J Med 369(25): 2379-2390, 2013.

Nangalia J et al. Somatic CALR Mutations in Myeloproliferative Neoplasms with Nonmutated JAK2.
N Engl J Med 369(25): 2391-2405, 2013.

OPEN

Leukemia (2016) 30, 131–135

www.nature.com/leu

ORIGINAL ARTICLE

A new monoclonal antibody (CAL2) detects CALRETICULIN mutations in formalin-fixed and paraffin-embedded bone marrow biopsies

H Stein[1], R Bob[1], H Dürkop[1], C Erck[2], D Kämpfe[3], H-M Kvasnicka[4], H Martens[2], A Roth[5] and A Streubel[5]

Table 1. Correlation between CALR mutations detected by Sanger Sequencing and CAL2-immunohistochemistry in samples obtained from bone marrow of patients with myeloproliferative neoplasms or other disorders and from control tissues

Disease type	*No. of samples*	*No. of cases with detected mutations*	
		Sanger sequencing	*CAL2 IHC*
MPN NOS	17	12	12
PMF	52	20	20
ET	59	20	20
PV	19	0	0
Myeloid neoplasms other than PV, ET and PMF	8		
RARS-T	1	0	0
MDS with fibrosis	1	0	0
RAEB-1	1	0	0
CNL	1	0	0
CML	1	0	0
aCML	1	0	0
Mastocytosis	2	0	0
BM with non-myeloid neoplasm	8		
CLL	3	0	0
MCL	1	0	0
HCL	1	0	0
PTCL	1	0	0
cHL	1	0	0
MGUS	1	0	0
Non-neoplastic tissue	10		
BM in Iron deficiency	1	0	0
BM in idiopathic thrombocyopenia	1	0	0
Normal BM	4	0	0
Tonsils	4	0	0
Total No	173	52	52

Abbreviations: aCML, atypical chronic myeloid leukaemia, BCR-ABL1 negative; BM, bone marrow; CALR, CALRETICULIN; cHL, classical Hodgkin lymphoma; CLL, chronic lymphocytic leukaemia; CML, chronic myelogenous leukaemia, CNL, chronic neutrophilic leukaemia; ET, essential thrombocythaemia; HCL, hairy cell leukaemia; IHC, immunohistochemistry; MCL, mantle cell lymphoma; MDS, myelodysplastic syndrom; MGUS, monoclonal gammopathy of undetermined significance; MPN NOS, myeloproliferative neoplasm not otherwise specified, that is, MPN cases where the differential diagnosis between prefibrotic PMF and ET was not possible; PMF, primary myelofibrosis; PTCL, peripheral T-cell lymphoma; PV, polycythaemia vera; RAEB-1, refractory anaemia with excess blasts-1, BCR-ABL1 positive; RARS-T, refractory anaemia with ring sideroblasts in transformation.

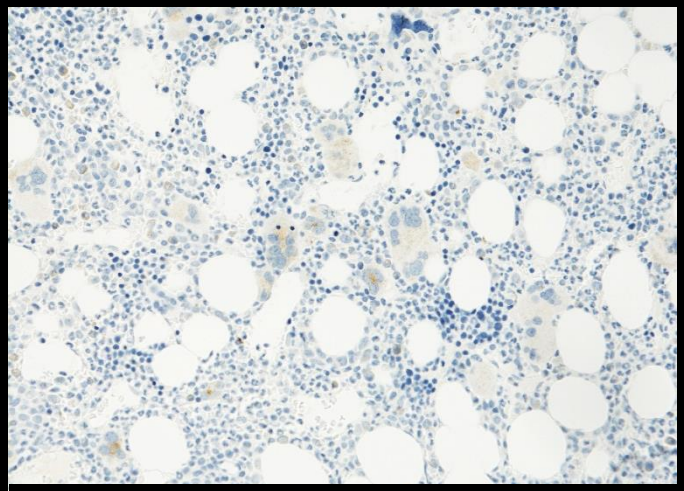
100% correlation between CALR mut (Sanger sequencing) and IHC (CAL2)

CALR mut specific Ab, CAL2 is raised against a C-neoterminus of the CALreticulin protein caused by all know fused CALreticulin mut (somatic deletions or insertion of exon9, chr19).

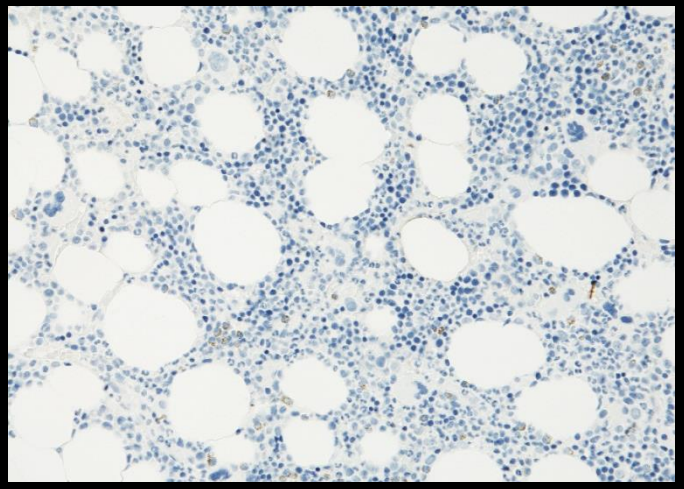
Antibody-Antigen reaction – Sensitive to the chosen automated platform

Calreticulin mut. specific Ab, Clone CAL2 (1:30 RR)

Omnis

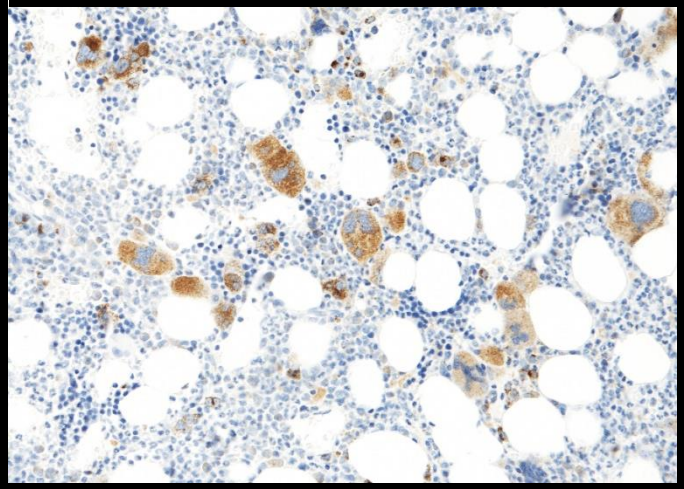


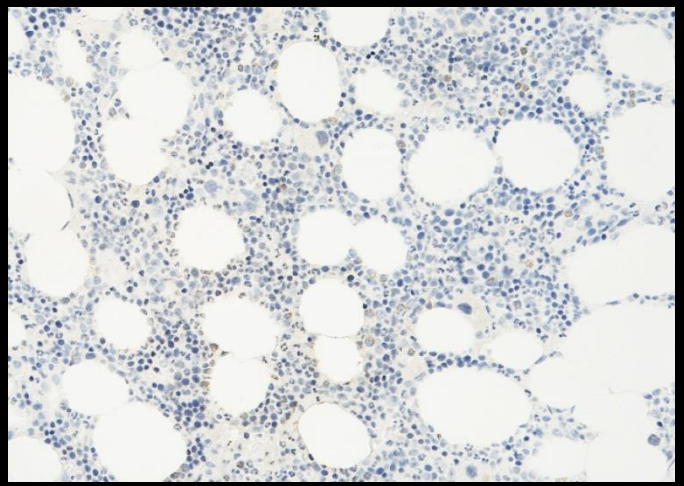
Calreticulin mutated



JAK2 mutated

Autostainer



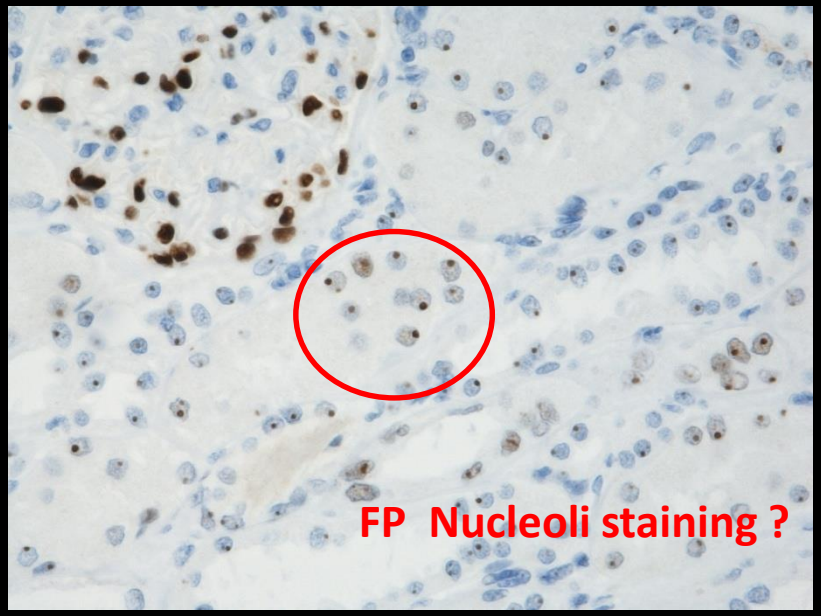


Omnis: HIER/HIGH pH 24` , Flex+ Mouse (10+20`)

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

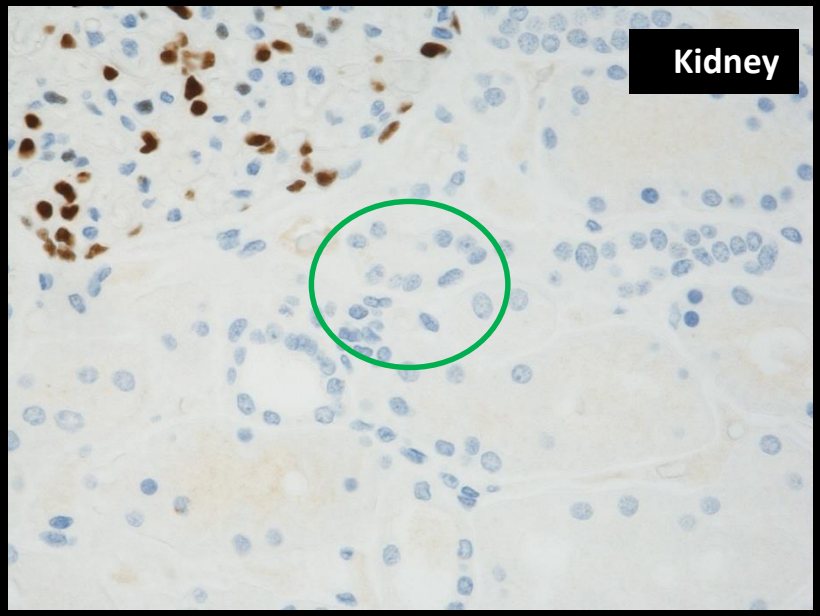
Antibody-Antigen reaction – Sensitive to the chosen automated platform

WT1, EP122 (1:100) Autostainer



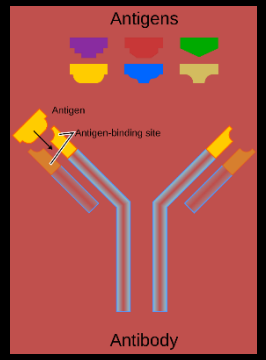
HIER/HIGH pH 20` , Flex+ Mouse (10+20`)

WT1, EP122 (1:100) Omnis



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

Antibody-Antigen reaction



Parameters affecting antibody-antigen reactions in tissue:

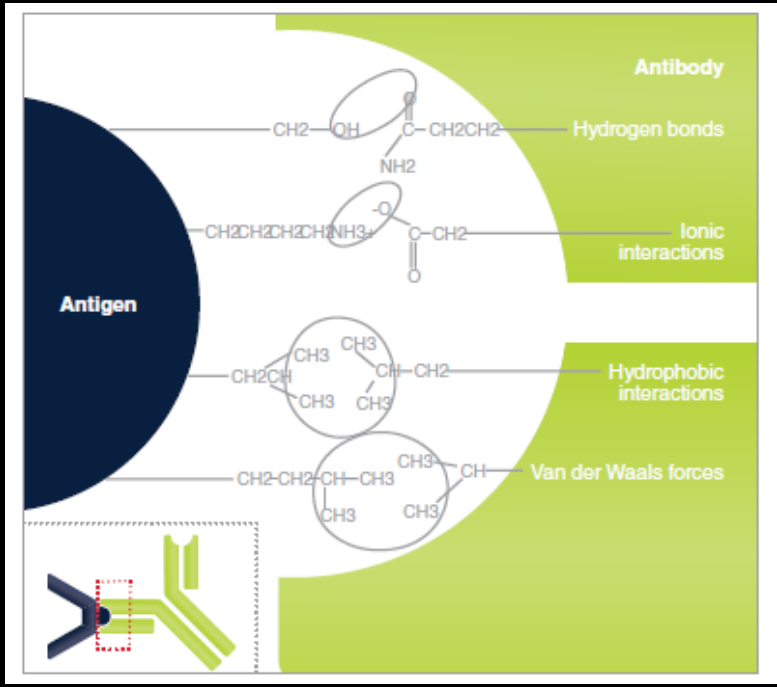
Antibody choice – specificity/sensitivity
Antibody Titer
Antibody performance related to the chosen automated platform

Antibody diluents

Incubation time
Incubation temperature
Sensitive to endogenous peroxidase blocking

Storage of concentrated primary antibodies
Storage of diluted primary antibodies

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

Antibody-Antigen reaction – 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 and that addition of NaCL (ionic strength) to the diluent negates most of the sensitivity gained through Antigen Retrieval (Table 3).

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

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

ND, not done.

Antibody-Antigen reaction – Antibody Diluents

Antibody Diluents	Description	Cat. No.
DaVinci Green	pH 7.3, Phosphate-based universal diluent	PD900 H, L, M
Renoir Red	pH 6.2, Tris-based solution	PD904 H, L, M
Van Gogh Yellow	pH 6.0, Phosphate based solution	PD902 H, L, M
Monet Blue	pH 7.9, Tris-based solution	PD901 H, L, M
VP Monet Blue	For Ventana® Systems	VPD901 H, L
HPV Diluent	For HPV Broad Spectrum	PD906 L
Background Sniper	For Antibodies that produce nonspecific background	?
Renaissance Background Reducing	For antibodies that produce nonspecific background	PD905 H, L

Standard Diluent pH 7.3 (Dako, K8006)

PAX8, BC12 (challenging on the Omnis)

Autostainer +
Omnis -

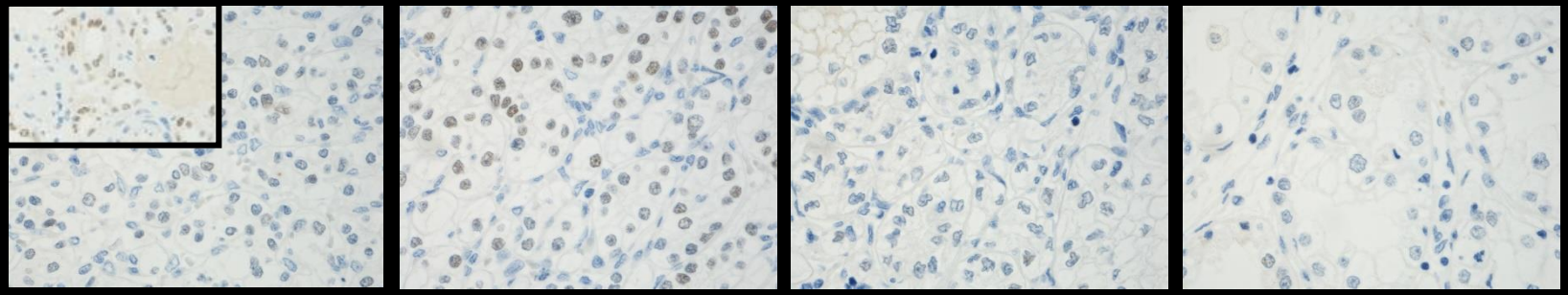
PAX8, Clone ZR1 (Omnis)

PK (2` at RT/ off-board) + HIER (Dako, S2367 pH9) (30` at 97°C)

Flex+ Rabbit (10+20`)

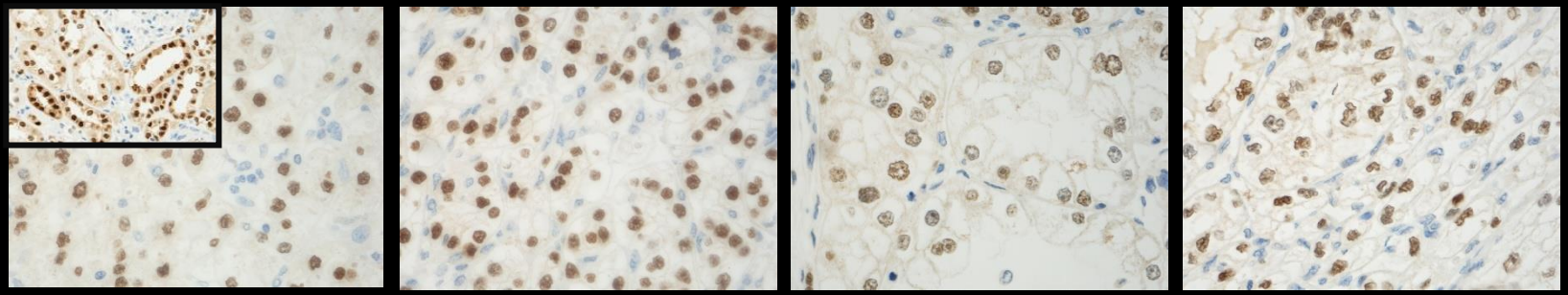
PAX8, ZR1 1:50

Dako Dil. pH7.3



PAX8, ZR1 1:50

Renoir R pH 6.2



4x Clear Cell Carcinomas (Kidney)

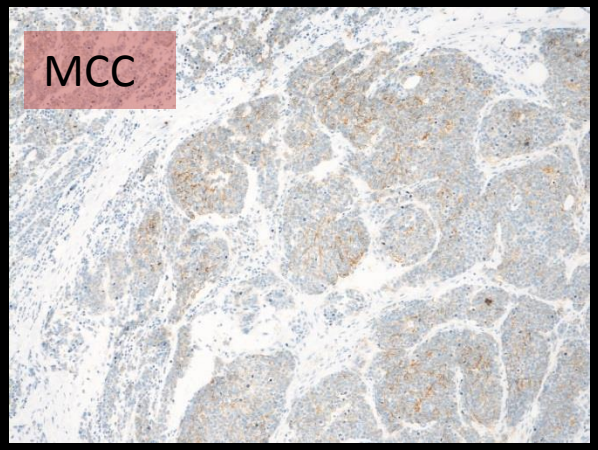
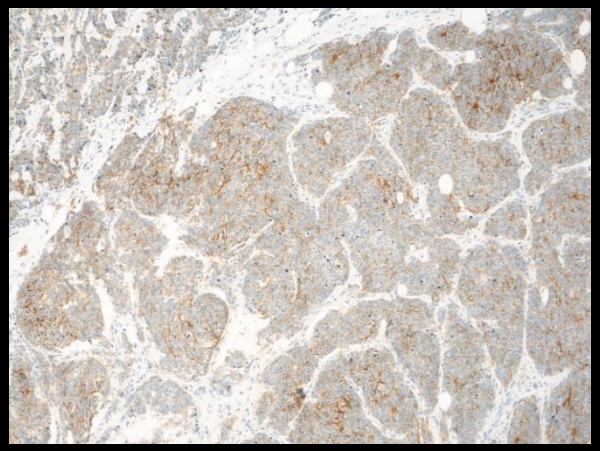
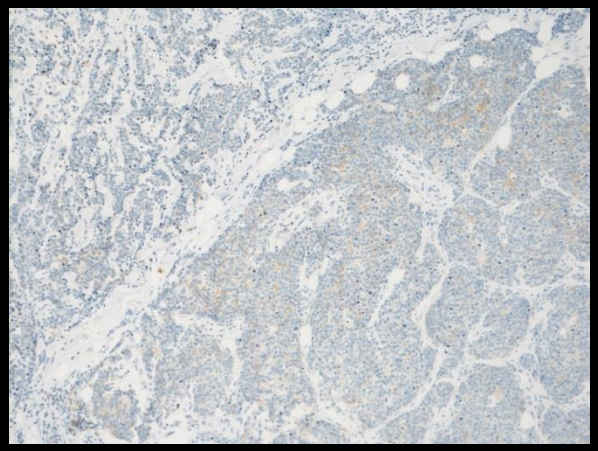
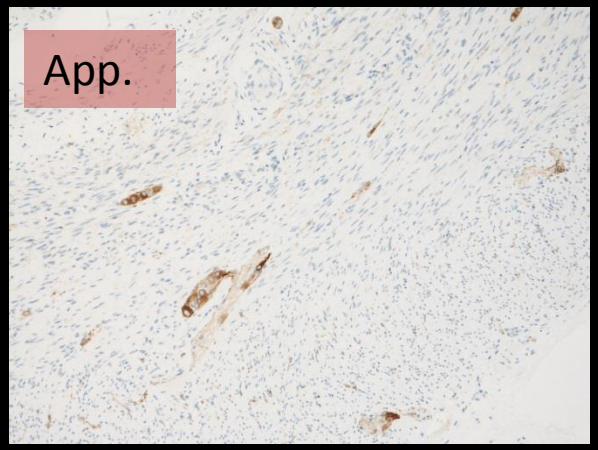
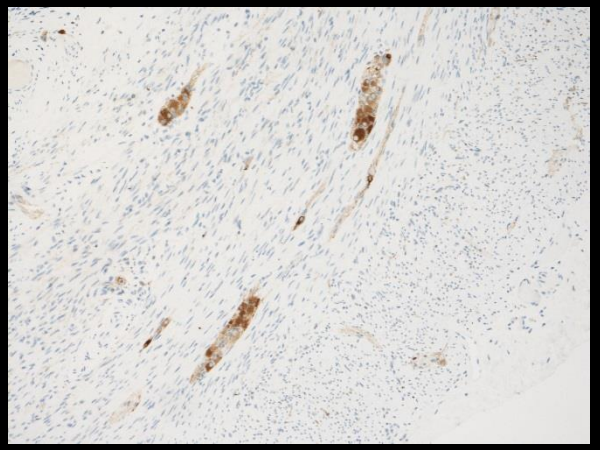
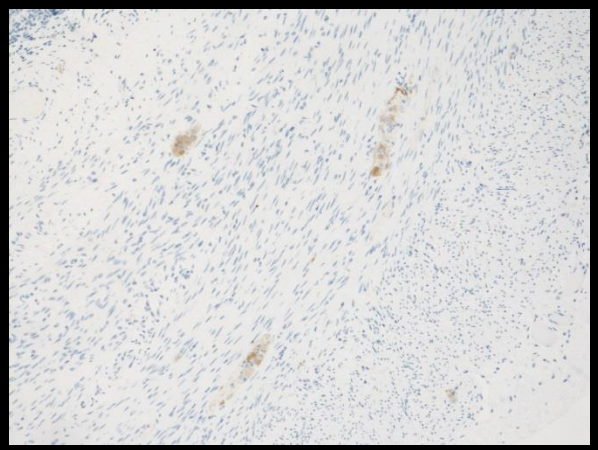
PK ~ Proteinase K Solution RTU (Dako cat.no.S3020) diluted 1:10 in TBS pH7.6 / 2 min at RT

ALK, 5A4 or D5F3 (challenging on the Omnis)

Autostainer +
Omnis -

ALK, 1A4 (Origene)

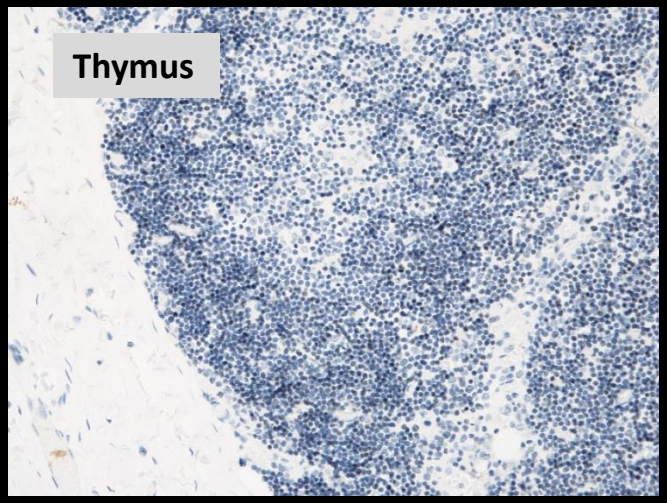
Omnis: HIER/HIGH pH 24` at 97°C, Flex+ Mouse (10+20`)

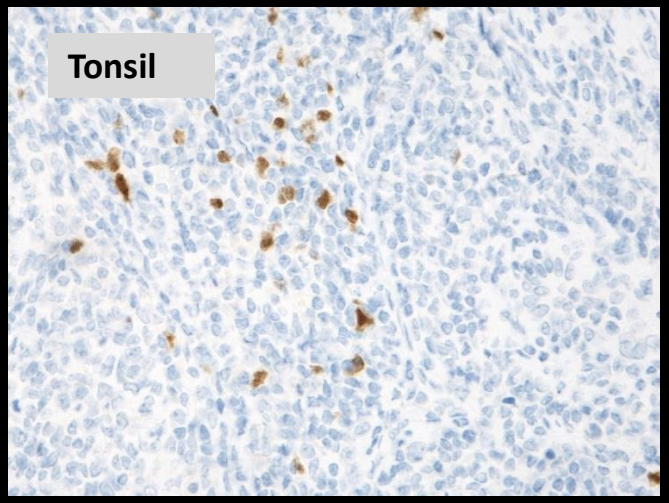
ALK, 1A4 1:300 / Dako Dil. pH 7.3	ALK, 1A4 1:1200 / Renoir R pH 6.2	ALK, 1A4 1:1200 / Dako Dil. pH7.3
		
		

Antibody-Antigen reaction – Antibody Diluents

TdT, Clone SEN28

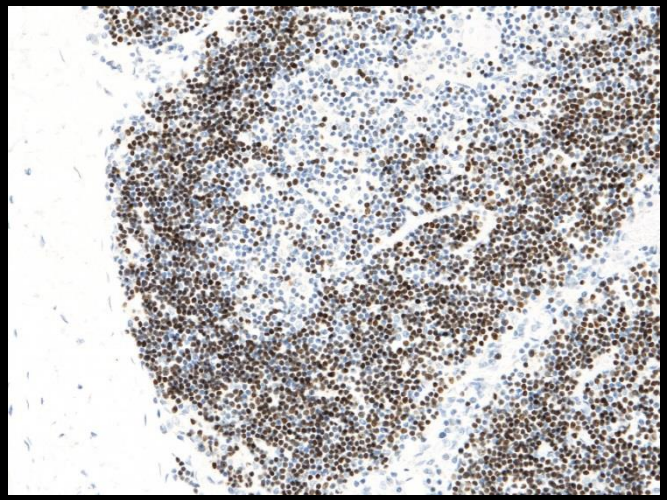
TdT, SEN28 1:50
Dako dil. pH 7.3

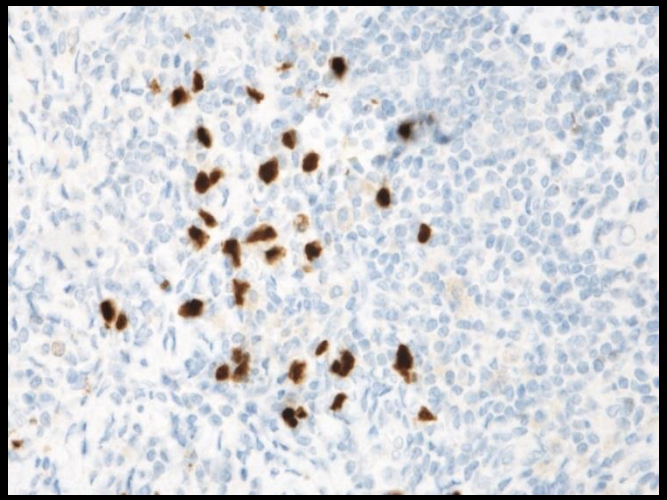




Omnis: HIER/HIGH pH 24`, Flex+ Mouse (10+20`)

TdT, SEN28 1:50
Renoir Red pH 6.2



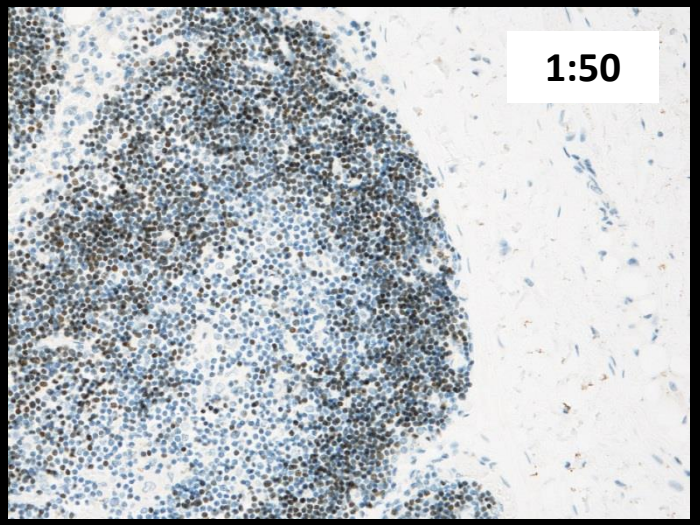


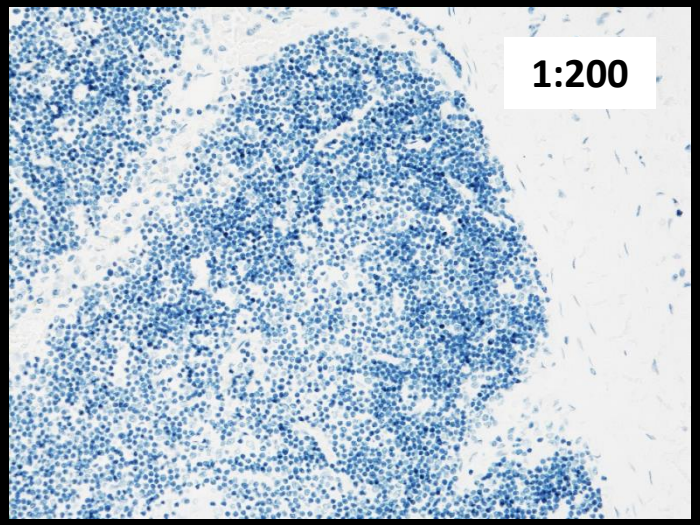
Antibody-Antigen reaction – Antibody Diluents

Thymus

TdT, Clone EP266

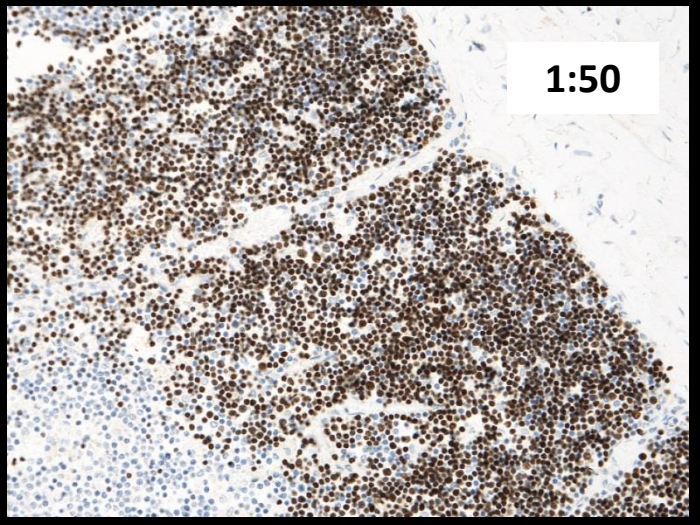
TdT, EP266
Dako dil. pH 7.3

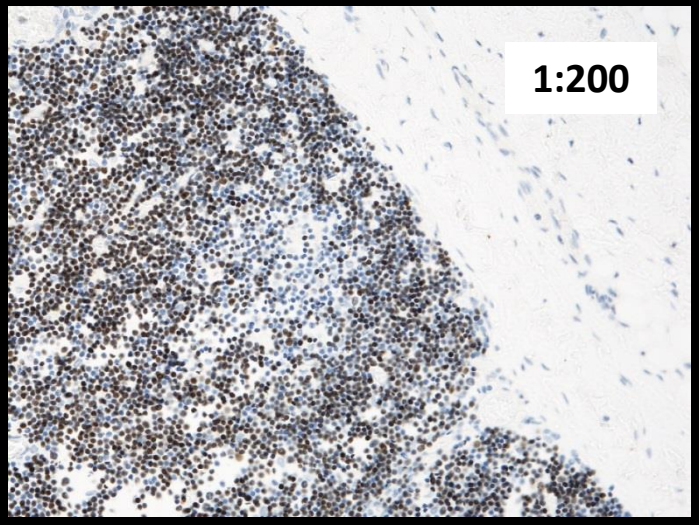




Omnis: HIER/HIGH pH 24`, Flex+ Rabbit (10+20`)

TdT, EP266
Renoir Red pH 6.2





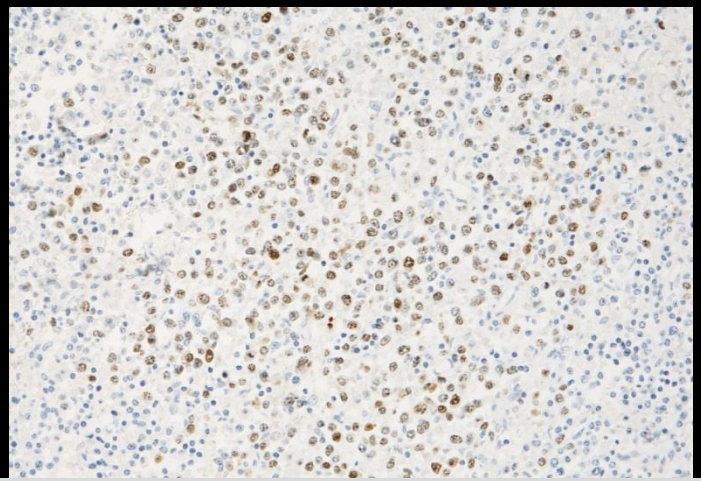
HHV8, clone 13B10

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

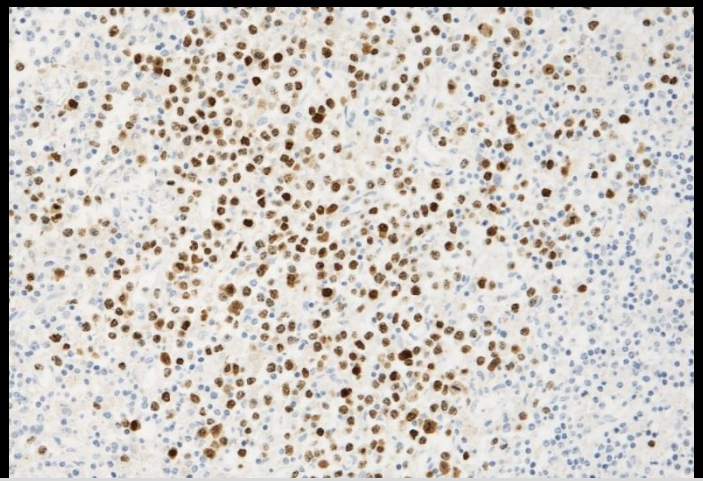
HHV8, 13B10 / 1:100 Renoir Red pH 6.2

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

Case 1

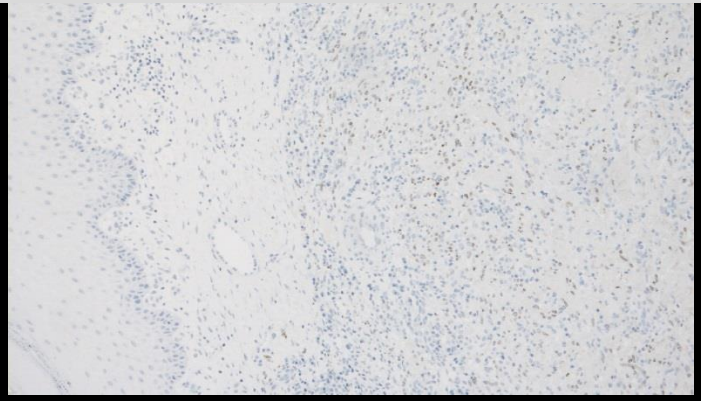


Flex+ Rabbit (10+20')

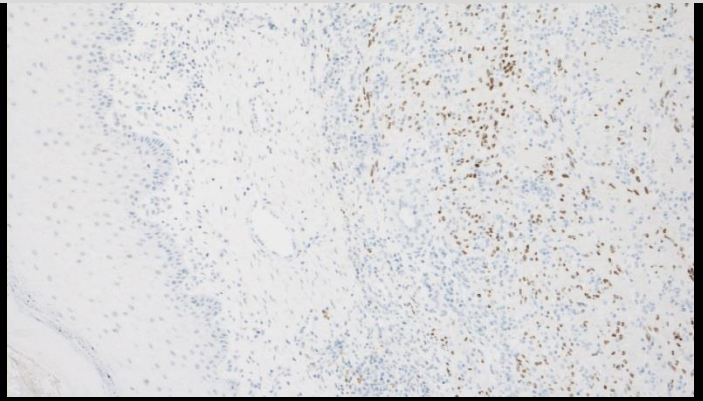


Renoir Red is not always the best antibody diluent
Remember to use a "antibody diluent test battery"

Case 2



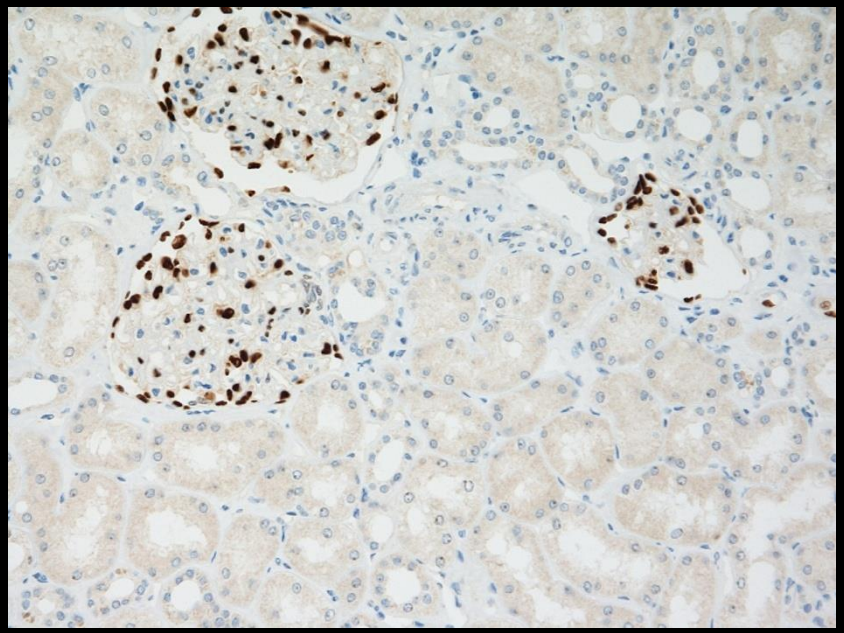
Omnis: HIER/HIG



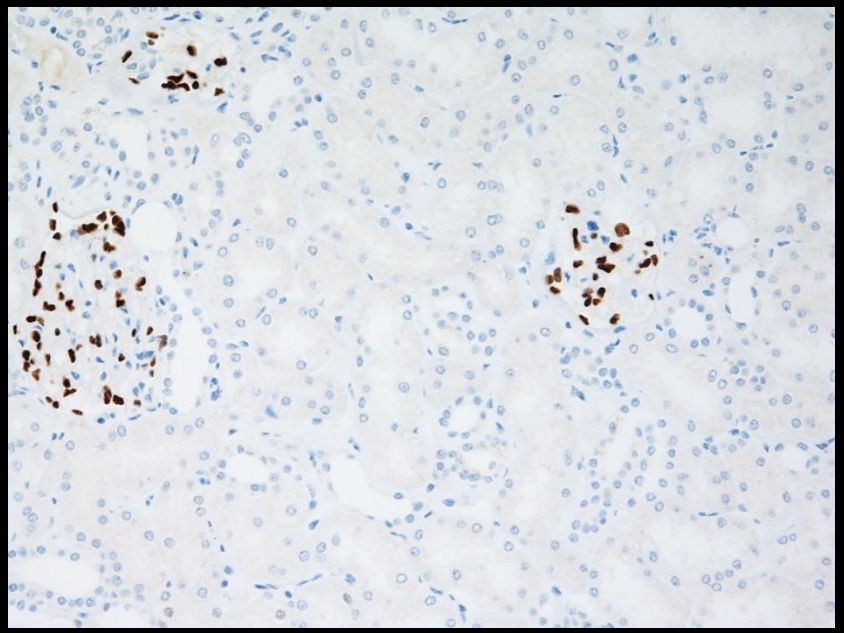
Antibody-Antigen reaction – Antibody Diluents

Kidney

WT1,EP122 1:25
Renoir Red (Biocare)



WT1,EP122 1:25
Background Sniper (Biocare)



HIER TRS pH9 (24` /97°C) + Pep © (3`)

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

Antibody-Antigen reaction – Antibody Diluents

Omnis (Department of Pathology, Naestved, Denmark)

Markers benefitting from dilution in Renoir Red pH 6.2 (improving signal):

ALK (1A4), CR (CAL6), CD4 (EP204), CD5 (SP19), CMYC (EP121), GATA3 (L20-823), GPC3 (1G12), IMP3 (69.1), MLH1 (ES05 & GM011), MSH2 (G219-1129), MSH6 (EP49), NKX 3.1 (poly), SALL4 (6E3), PAX8 (ZR1), PMS2 (EP51), SOX10 (EP268), SOX11 (C1 & MRQ58), TdT (SEN28 & EP266), UP-II (BC21), WT1 (WT49) and …….

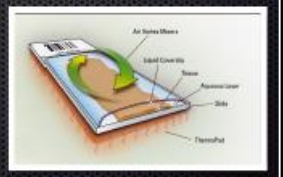
Markers that don`t benefit from dilution in Renoir Red pH 6.2:

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

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

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

pH during incubation

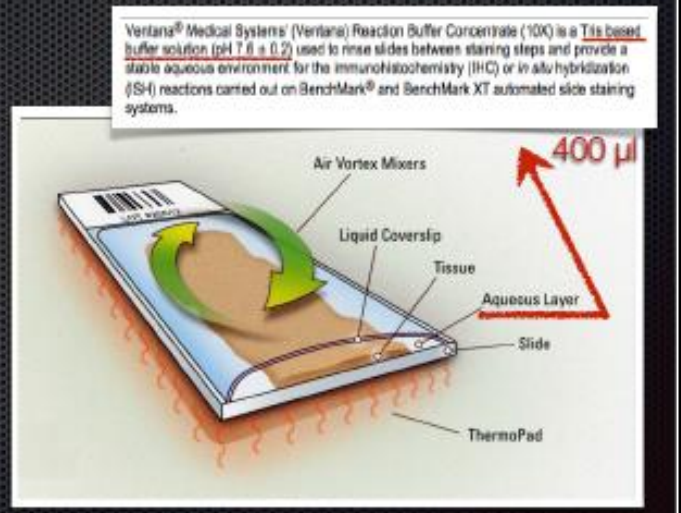


pH 6.20 + pH 7.50 = pH 7.25

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]

Major problems 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
- 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

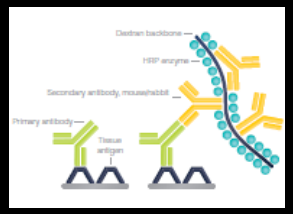
[c] Biotin-based detection kit for cytoplasmic epitopes, use of detection kits providing a too low sensitivity, or use of detection kits and chromogens giving imprecise localization of the staining signals complicating the interpretation

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

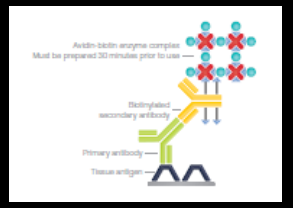
Polymer / multimer based systems

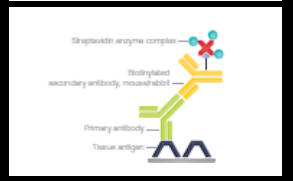
Advance
Envision
Envision Flex
Envision Flex+
Bond Refine
Power Vision
Power Vision +
Super picture
Impress
UltraVision One
UltraVision LP
MACH 2
MACH 3
MACH 4
UltraView
UltraView + Amp
Optiview
Optiview + Amp
Quanto
Hi Def
BrightVision
ZytoChem
ZytoChem plus
……



Biotin based systems

I-View
EpiPrecision
Vectors Elite ABC
Histostain+
……





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**

Polymer/Multimer detection systems used by NordiQC participants

Vendor	Detection System 2- Step	Detection System 3-step	Amplifier	Cat.no
Dako	EnVision EnVision +/Flex	Envision Flex+	Anti -Ms/Rb	K4001 K8000 /10 (K5007) K8002/12
Ventana	UltraView	UltraView + Amp OptiView Optiview + Amp	Anti -Ms/Rabbit Anti-Hapten Anti-Hapten + TSA	760-500 760-500 + 760-080 760-700 760-700 + 760-099
Leica	App. 90%	Bond Refine (PowerVision)	Anti-Ms (Rb?)	DS9800 (HRP); DS9390 (AP)
Biocare	MACH 2	MACH 3 MACH 4	Ms/Rb probe Ms probe (Rb?)	M2U522; MHRP520; RHRP520 M3M530; M3R531 M4U534
LAB Vision/TS	UltaVision One	Quanto	?	TL-125-HLJ TL-125-QHD /QHL
Immunologic	BrightVision (PowerVision)	BrightVision+	Anti-Ms/Rat (Rb ?)	DPVM (Anti-Ms)/DPVR (Anti-Rb) DPVO (Anti-Ms/Rb/Rat) DPVB ((Anti-Ms/Rb/Rat)
Master Diag.		Quanto	?	MAD-021881QK
ZytoMed System		ZytoChem Plus (PowerVision)	Anti-Ms (Rb?)	PolHRP-100
And a few more (Advance, GTVision……..)				

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

Detection systems

Skaland I et al : ***Appl Immunohistochem Mol Morphol 2010 Jan; 18(1) : 90-6***

Demonstrated that there are significant differences in sensitivity between 5 different polymer detection systems.

Also, two of the polymer detection systems showed weak background staining both in negative controls and at optimal primary antibody dilution.

Buchwalow I et al : ***Acta Histochemica 2013 (115) : 587-594***

Demonstrated that the AmpliStain™ detection system was more sensitive than EnVision+ - the difference in sensitivity was explained by the nature of the polymer backbone:

AmpliStain™ has higher penetration ability compared to EnVision+ due to the SnakeLinker™ technology – creating compact and metaplastic polymer conjugates (flexible/deformable).

Also, the antibody – HRP ratio of AmpliStain™ is significant higher than EnVision+ , app. 1: 12-24 of AmpliStain™ compared to 1:4 for EnVision+ detection system.

Polymer based detection systems - Detection system provide low sensitivity

Pass rate's correlated with the choice of polymer detection system

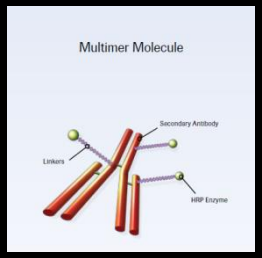
CR, NQC Run 33	Detection system type	Sufficient	Optimal
mAb DAK-Calret 1*	2-step polymer or multimer.	11/22 (50%)	1/50 (2%)
mAb DAK-Calret 1*	3-step polymer or multimer	14/14 (100%)	9/14 (64%)

*** 1:50-1:300, HIER in an alkaline buffer (pH 9)**

Pax-8, Run 34	Detection system type	Sufficient	Optimal
All primary Abs*	2-step polymer or multimer.	6/13(46%)	0/13 (0%)
All primary Abs*	3-step polymer or multimer	16/22(73%)	9/22 (41%)

*** All protocol settings**

Detection systems (Ventana/Roche)



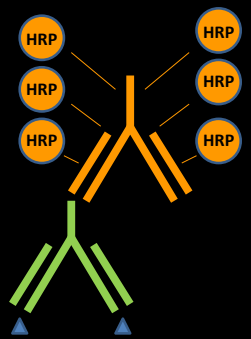
Universal Linker
Linker
Enhancer
Post Blocking
Amplifier

Increases sensitivity

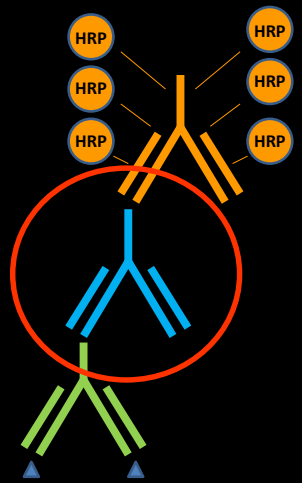
HQ = 3-HydroxyQuinoxaline ?

iVIEW

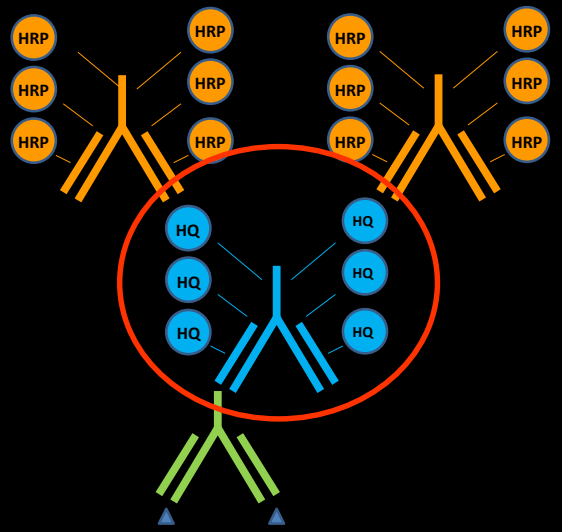
UltraView



UltraView /Amp



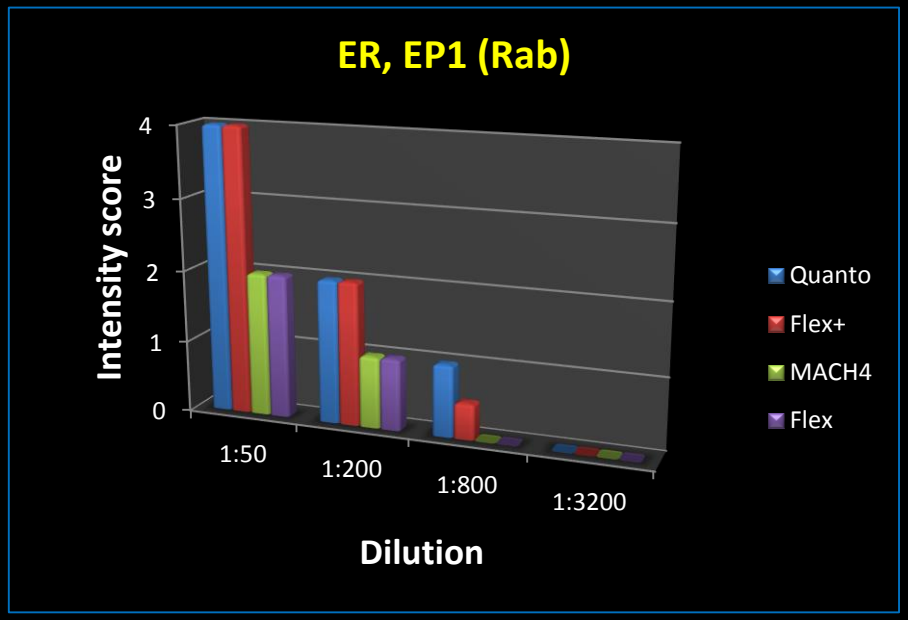
OptiView

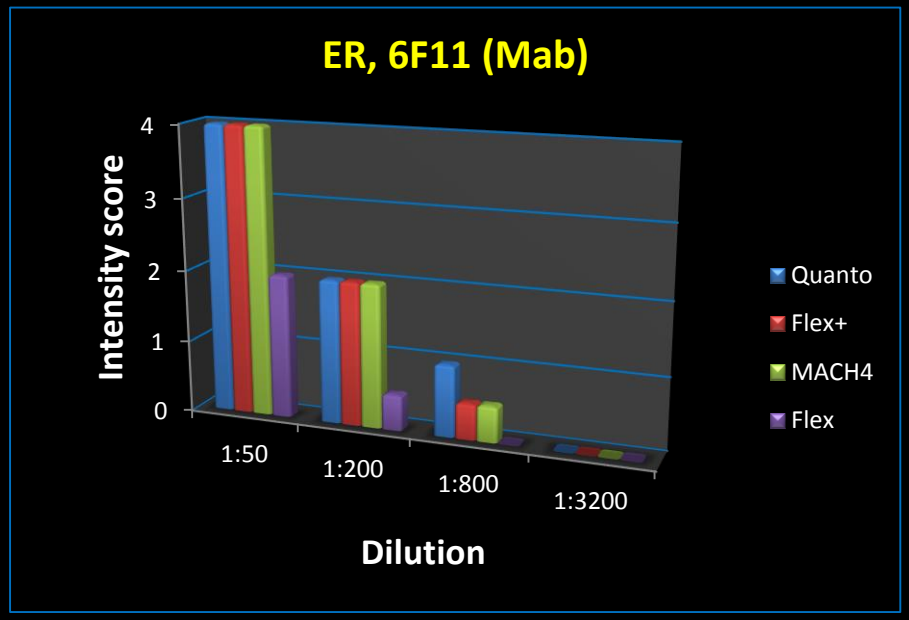


Sensitivity

Polymer based detection systems

Performance Testing using incubation times recommended by the vendors





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

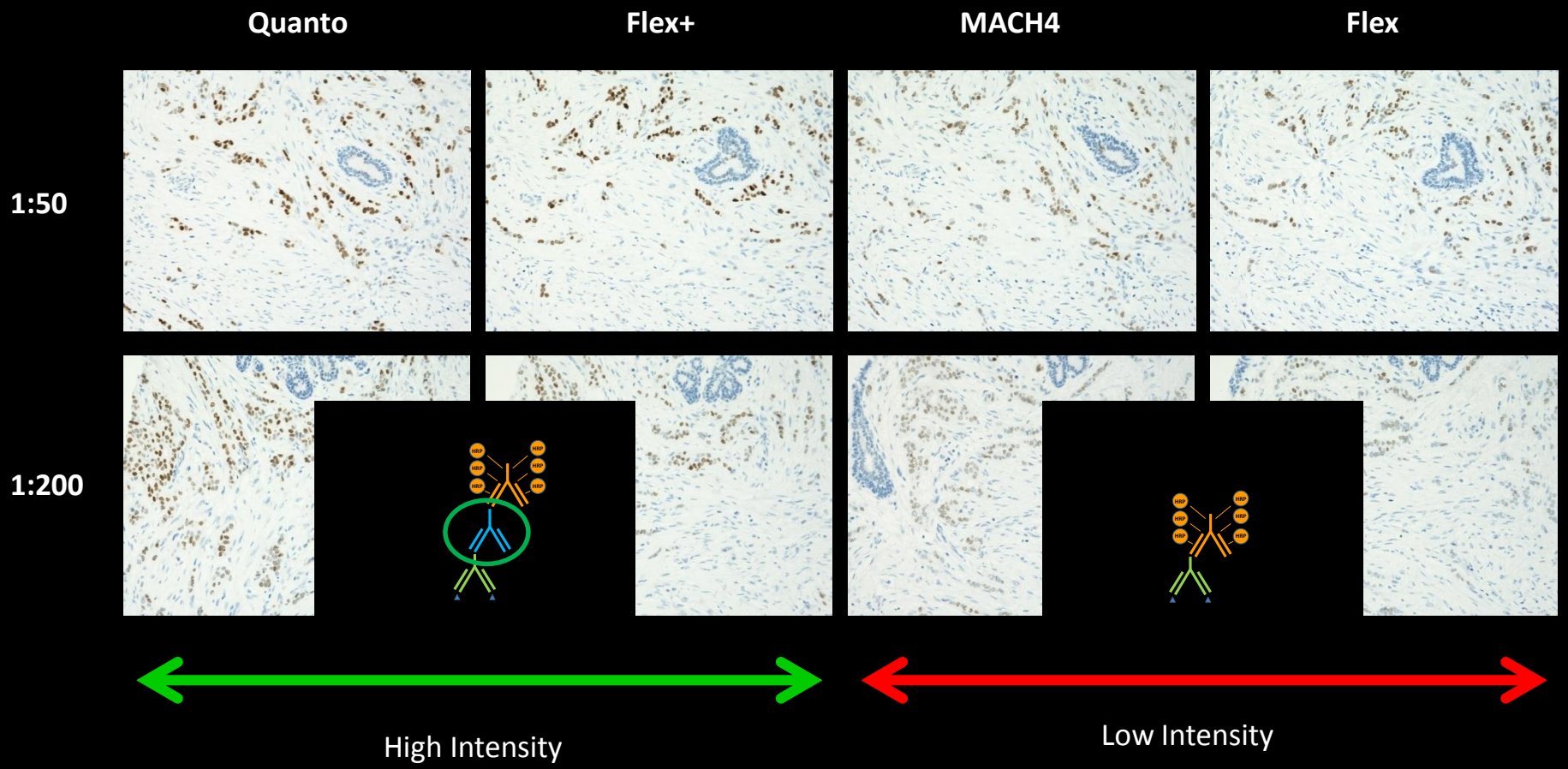
- The 3-step polymer detection systems Quanto and Flex+ - produced the overall highest intensity.
- High intensity could also be obtained with the 3-step polymer detection system MACH4, but only with the Mab (ER,6F11).
- The 2-step polymer detection system Flex produced the overall lowest intensity.
- Using the Rab (ER, EP1) - the "3-step polymer" detection system MACH4 provided similar intensity as Flex.
- "Optimal staining" was highly influenced by the concentration of the primary Abs and the nature of detection system.

Polymer based detection systems

Performance Testing using incubation times recommended by the vendors

ER, EP1 (Rab)

Breast tumor

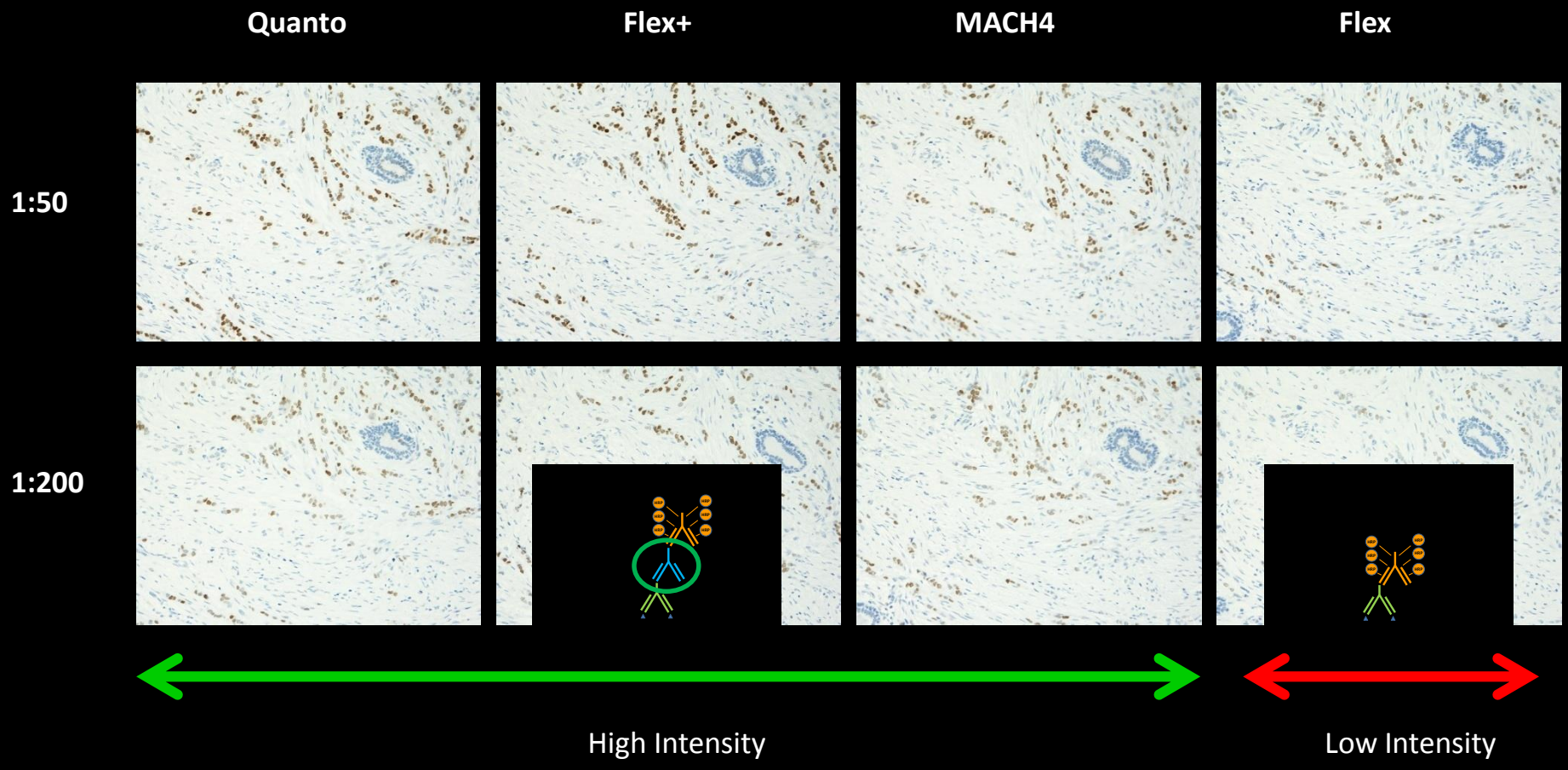


Polymer based detection systems

Performance Testing using incubation times recommended by the vendors

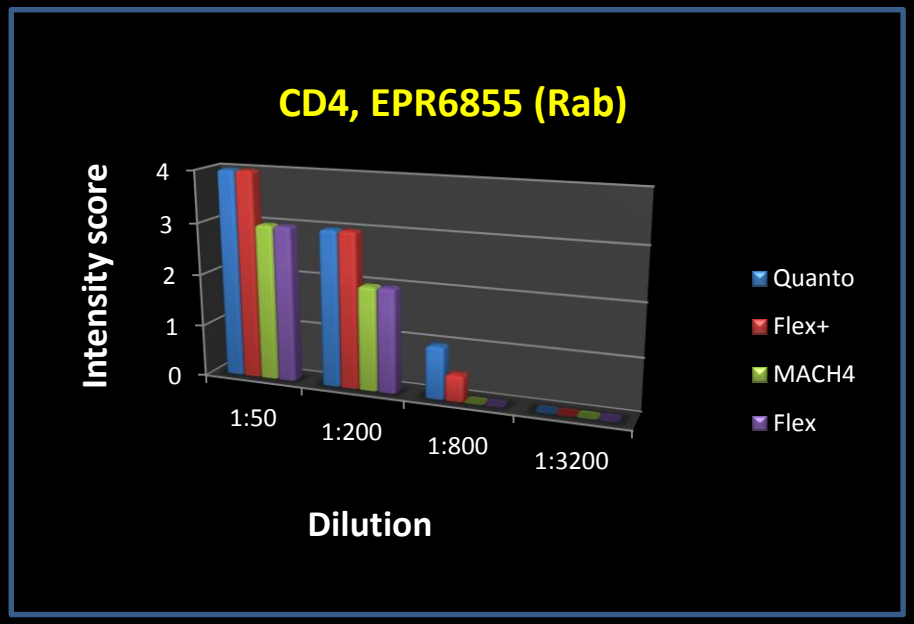
ER, 6F11 (Mab)

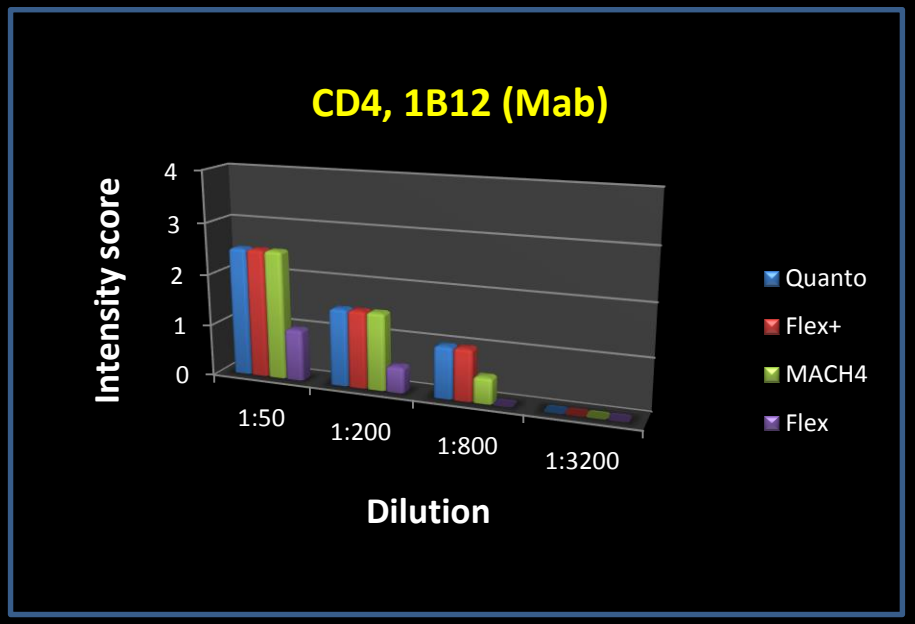
Breast tumor



Polymer based detection systems

Performance Testing using incubation times recommended by the vendors





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

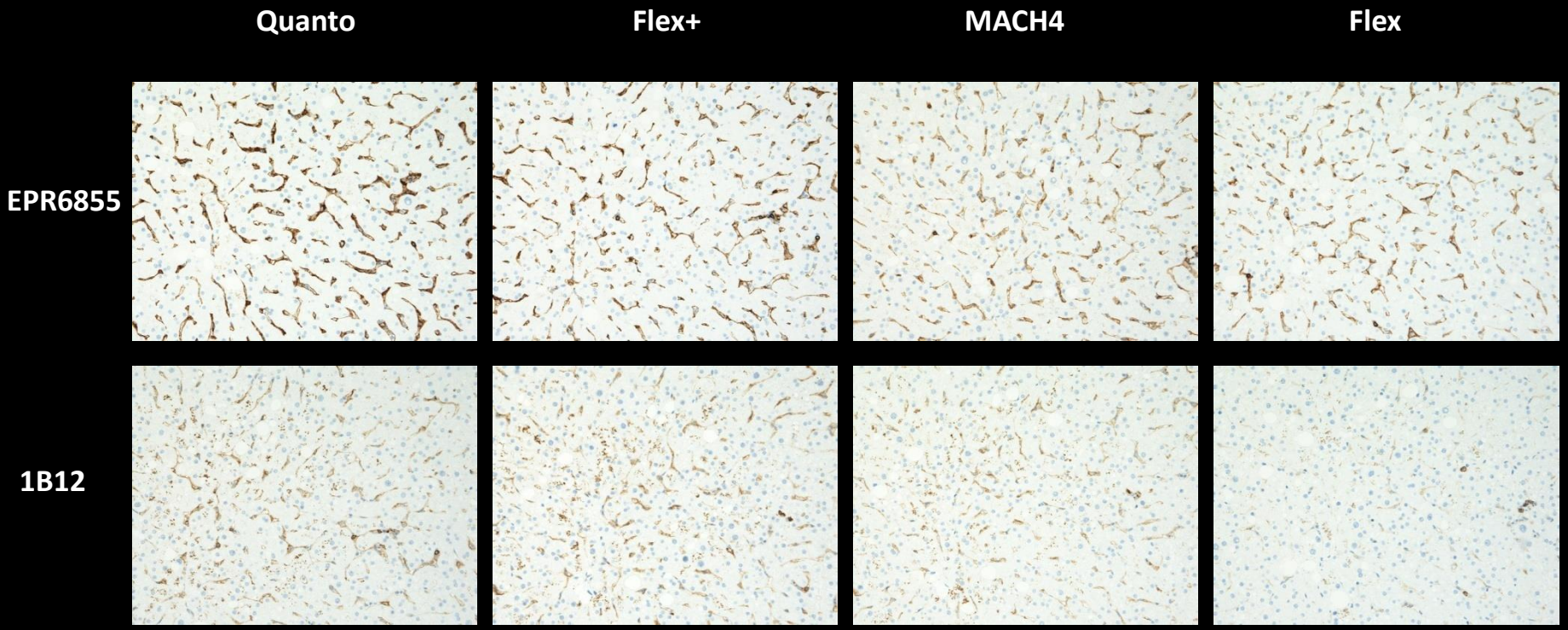
- **The 3-step polymer detection systems Quanto and Flex+ - produced the overall highest intensity.**
- **Using the Mab CD4, 1B12 - Comparable staining intensity could be obtained with all the 3-step polymer detection systems.**
- **The 2-step polymer detection systems Flex produced the overall lowest intensity.**
- **Using the Rab CD4, EPR6855 - the "3-step polymer" detection system MACH4 providing similar intensity as Flex.**
- **Intensity was highly influenced by the nature of primary Ab and "optimal" staining could only be obtained with the Rab (CD4, EPR6855) used in combination with the 3 step – polymer detection systems Quanto or Flex+.**
- **Intensity was higher with the Rab (CD4, EPR6855) at 1:50 with all of the detection systems tested compared to any intensity obtainable with the Mab (CD4, 1B12) in combination with the use of a 3 step polymer system (Quanto, Flex+ or MACH4)**

Polymer based detection systems

Performance Testing using incubation times recommended by the vendors

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

Liver



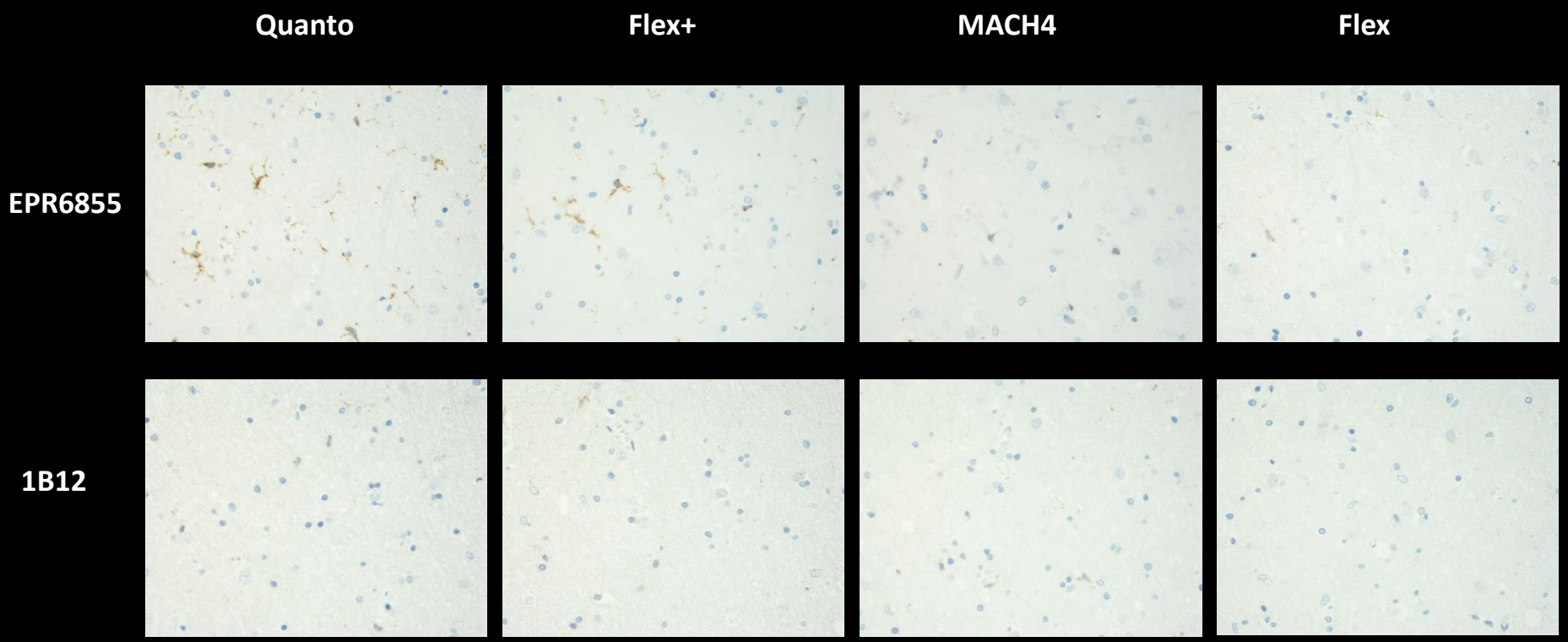
Note: Strong staining of hepatic endothelial cells and kupffer cells using the Rab (CD4, EPR6855) in combination with all the detection system tested (2-step or 3-step polymer systems) . Intensity is significantly reduced using the Mab (CD4, 1B12).

Polymer based detection systems

Performance Testing using incubation times recommended by the vendors

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

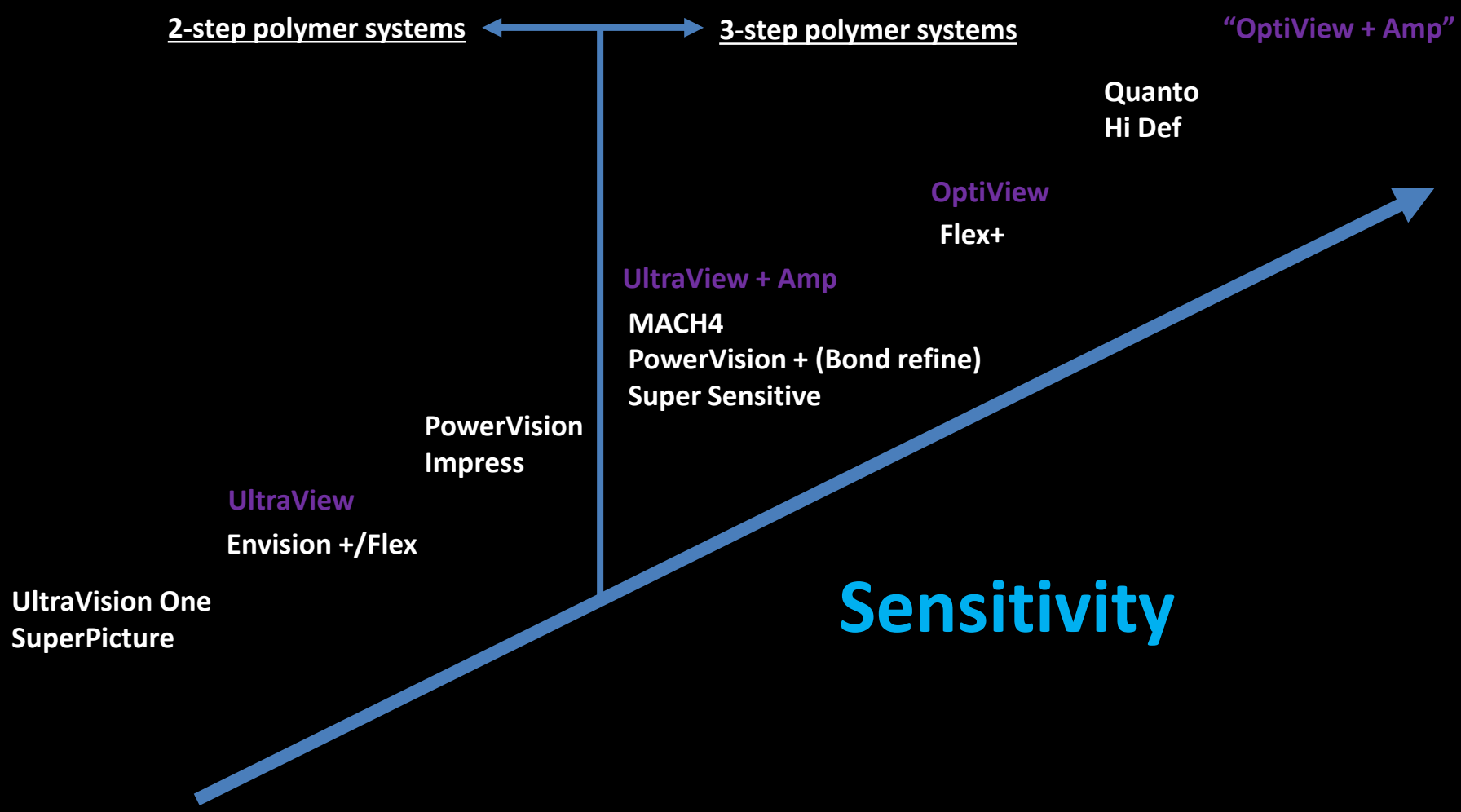
Brain



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

Detection systems

Polymer based detection systems tested in Dept. of Pathology, Naestved, DK



Biotin based detection systems

Miller RT*: Society for Applied Immunohistochemistry 2001 Annual Meeting, New York Cornell-Queens Hospital Medical Center, Flushing, NY.*

TECHNICAL IMMUNOHISTOCHEMISTRY: Achieving Reliability and Reproducibility of Immunostains.

Since the widespread use of heat induced epitope retrieval (HIER) techniques, endogenous biotin has become a much more serious problem that needs to be dealt with whenever avidin-biotin detection systems are employed.

Any laboratory employing HIER techniques should routinely include steps for blocking of endogenous biotin in any immunostain that is subjected to HIER

Vosse BA et al : *Appl Immunohistochem Mol Morphol 2007 Mar; 15(1) : 103-7*

Demonstrated that Avidin - Biotin based detection systems can result in high background staining due to endogenous biotin activity

Also, that endogenous biotin activity may be difficult to block

App. 5% of all NordiQC participants use a biotin based detection systems (iView)

Biotin based detection systems - provides low specificity

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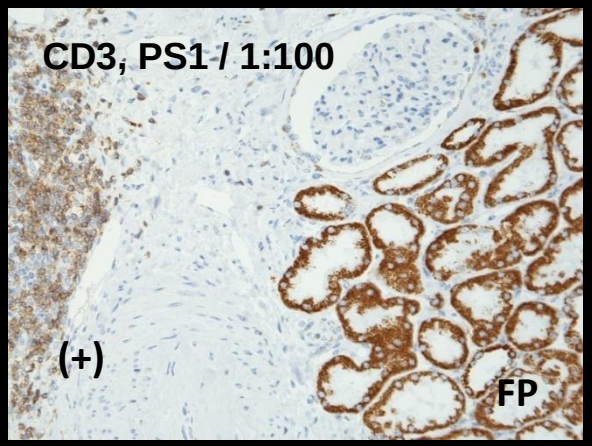
ORIGINAL ARTICLE

Expression of CD3 antigens in renal tubule epithelium and renal oncocytomas

Joseph Alroy[a,b,*], Angelo A. Ucci[b], Gisso Azabdoaftari[b], Barbara F. Banner[c], John C. Cheville[d]

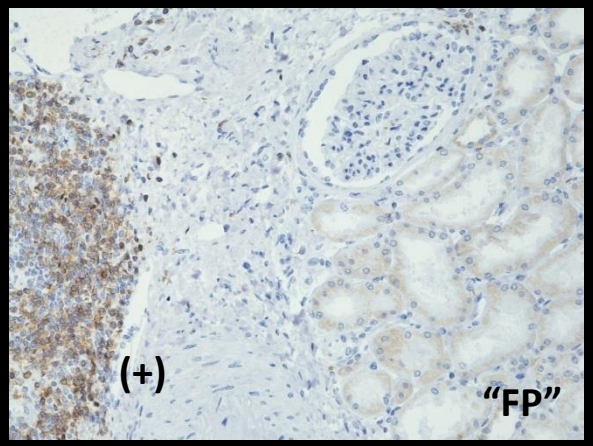
Biotin based detection systems should not be used - false positive due to endogenous biotin

Næstved LAB: CD3 staining using a biotin based detection system with and without blocking for E.B. (kidney)



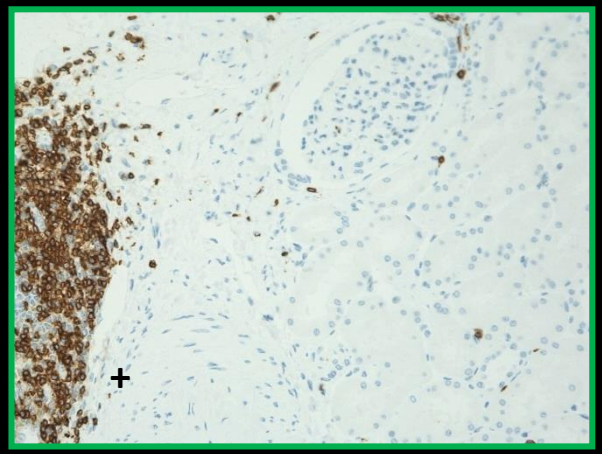
Histostain+ (Zymed) / LSAB

Without blocking of EB



Histostain+ (Zymed) / LSAB

With blocking of EB



Flex+ (Dako) / Polymer system

No need for blocking of EB

The basal fundament for a technical optimal performance is :

- **Appropriate tissue fixation and processing**

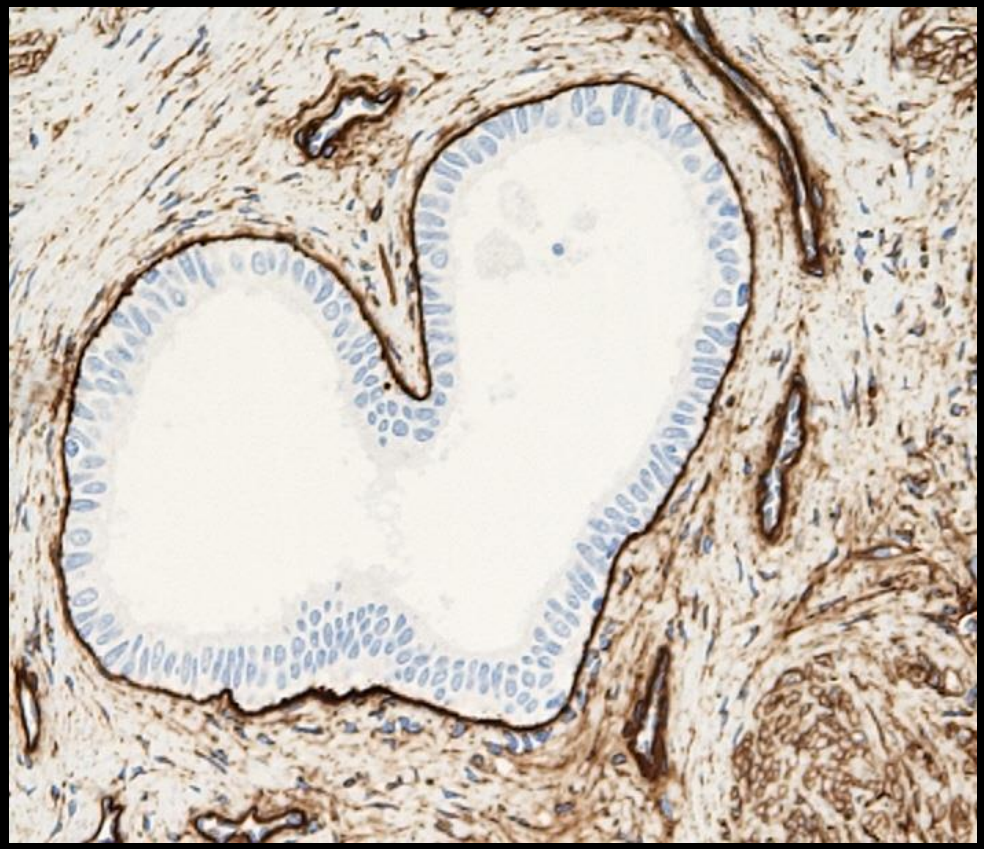
- **Appropriate and efficient epitop retrieval**
  - Remember - 95% of the Abs require HIER and app. 90% prefer high pH retrieval buffers.
  - Also, use efficient HIER temperature and time (app. 100°C for 20 - 40min).

- **Appropriate choice of antibody / clone, diluent and dilution**
  - If possible - compare different clones / Abs against the desired antigen before implementation
  - Calibrate the Ab concentration carefully in relation to Critical Staining Quality Indicators

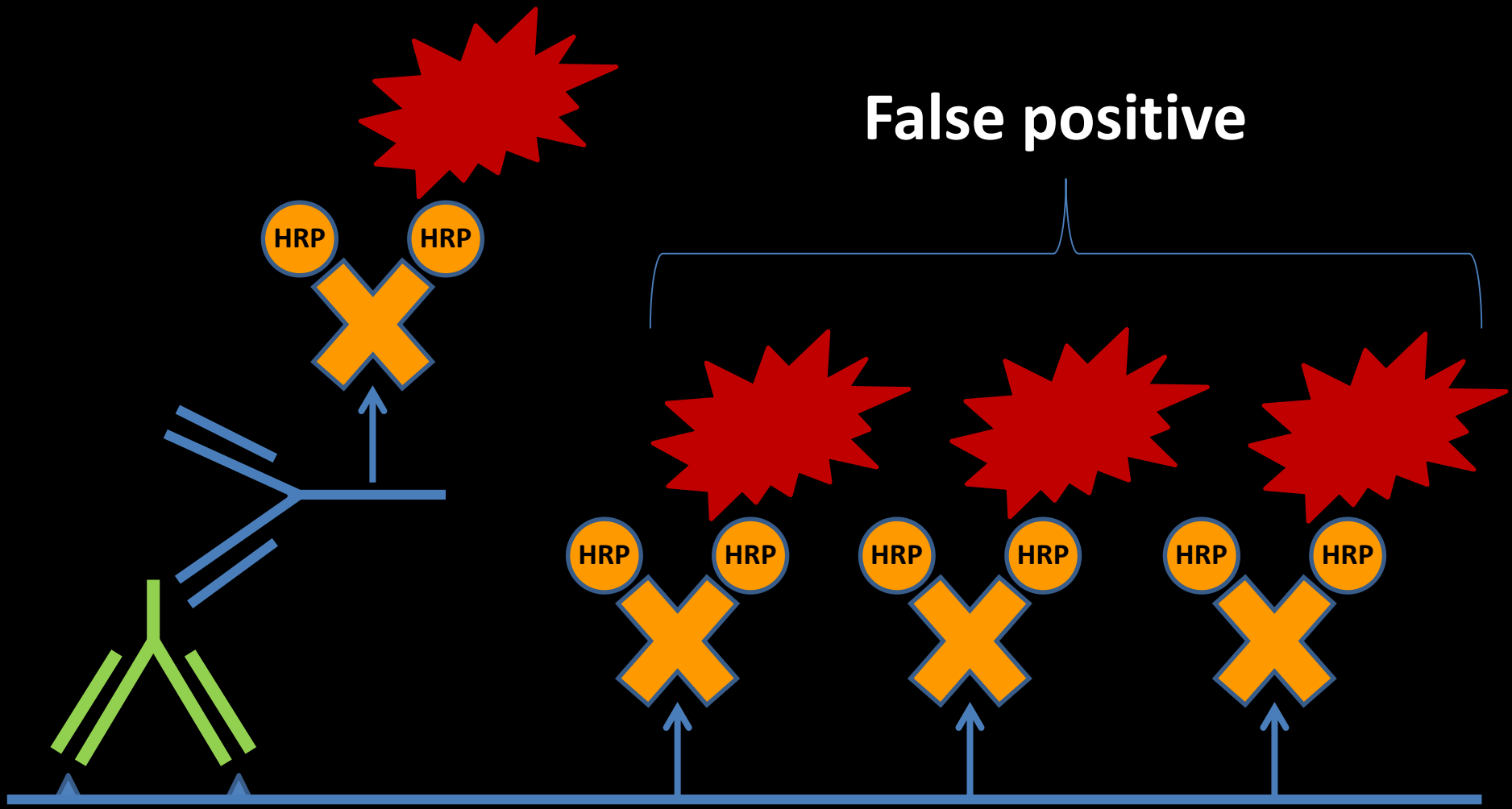
- **Robust, specific & sensitive detection system**
  - Use of a 3-step multimer/polymer system is preferable to a 2- step multimer/polymer system
  - Don`t use biotin-based detection systems

- **Appropriate choice of control material**
  - Important – include tissue material with low expressors, but also high and non-expressors

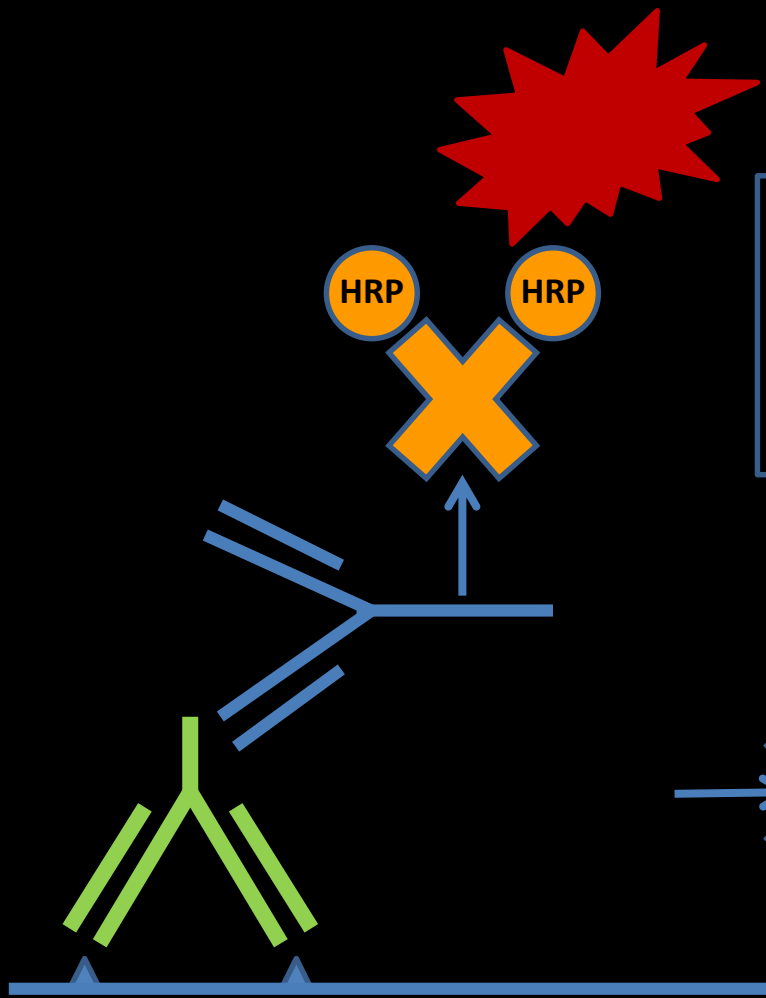
Thank you for your attention



Biotin based detection system and Endogenous Biotin



Biotin based detection system and Endogenous Biotin



Blocking endogenous biotin (commersial kits)

1. Ad avidin or steptavidin solution (interact with endogeneous biotin)

2. Ad biotin solution (saturates free avidin binding sites)