Nordic immunohistochemical Quality Control

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I

Immunohistochemical double stainings – overview, considerations and applications

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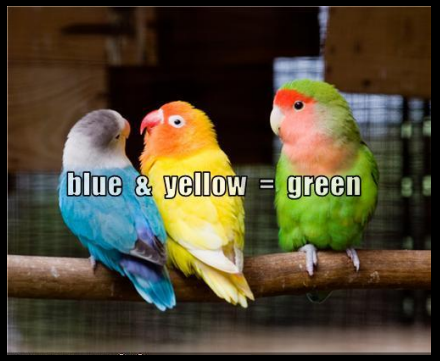
Double or multi-staining techniques (IHC)

Multiple staining can be defined as the detection of two or more antigenic epitopes on one slide

- ❑ Determine whether targets are present in different cells
- ❑ Determine whether targets are present in the same cell
- ❑ Determine whether targets are present in the same cellulare compartment



Double or multi-labeling techniques - Advantages:

Save tissues due to increasing demand for less invasive sampling techniques (smaller and fewer specimens available)

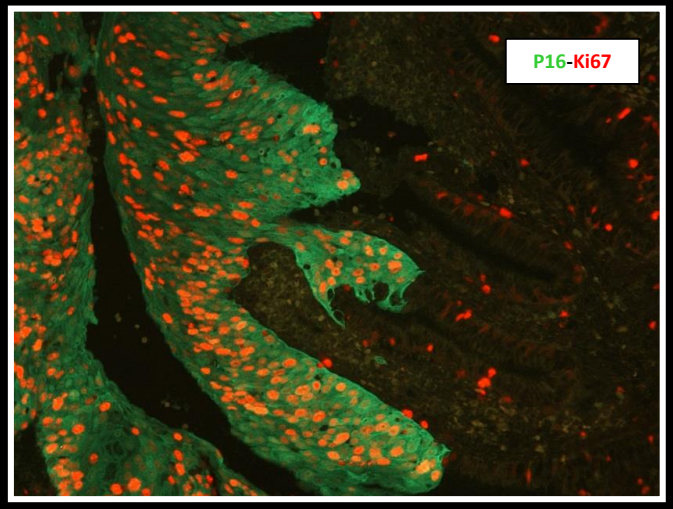
Saves time (quick overview)

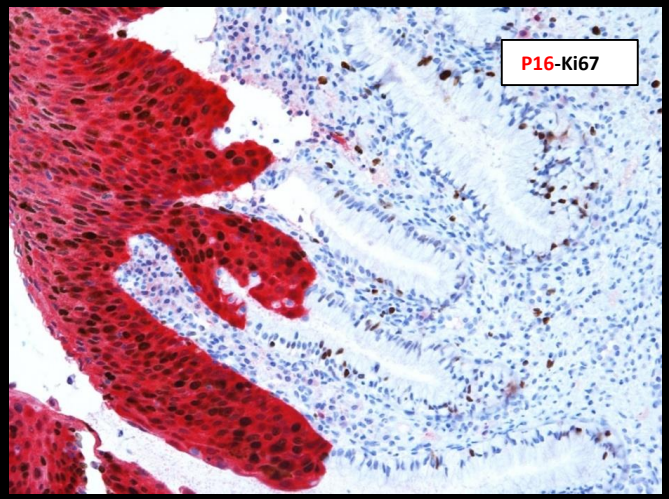
Saves “reagents”

Overcomes some of the problems related to performing serial sections

- ➢ Cellulare structures or small foci of tumor cells may be lost during processing.

Cervix / dysplasia





Double labelling techniques (IHC)

Basic procedures:

- ❑ Cocktails single staining technique (e.g. PAN-CK, AE1/AE3; PIN)
- ❑ Sequential double-staining technique
- ❑ Simultaneous double-staining technique
- ❑ Double (multi) sequential & erasing staining technique (SIMPLE)
- ❑ Virtual double (multi) staining technique

Immuno-enzymatic techniques

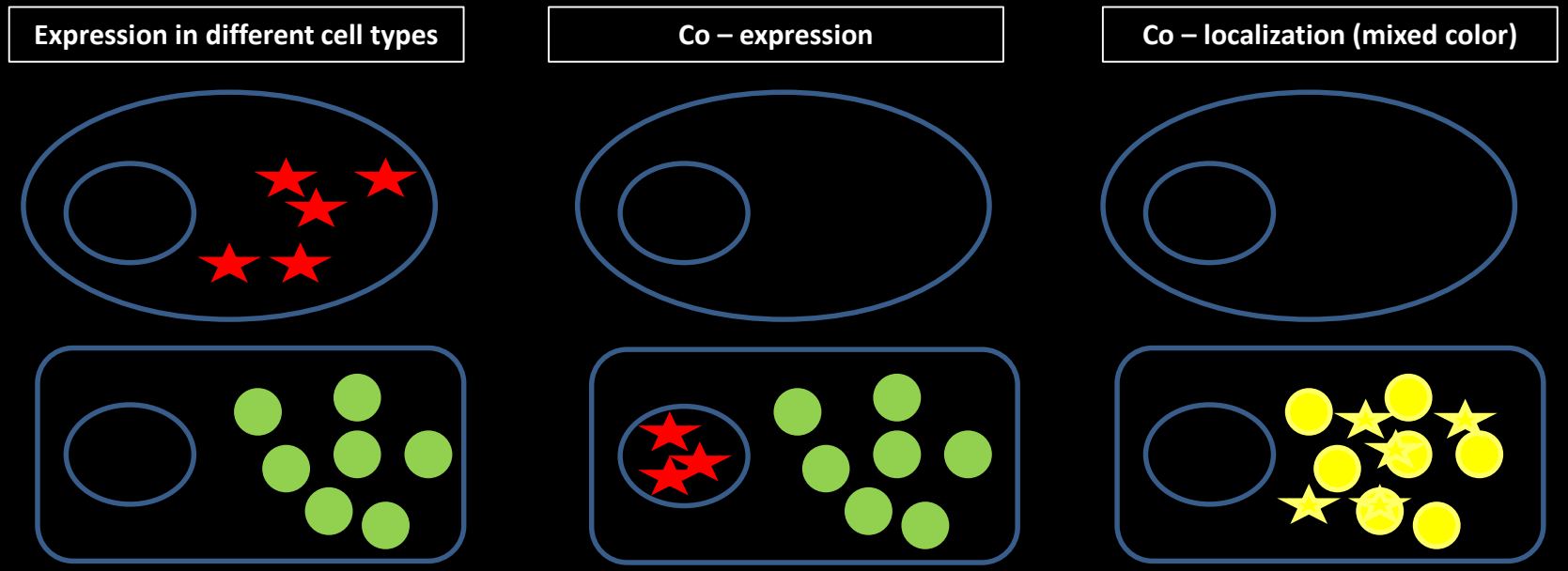
Immuno- fluorescence techniques

Double or multi-staining techniques (IHC)

Requirements ("traditional double-staining techniques") :

- Two different visualization systems showing no cross-reactivity
- Two different chromogens showing high color contrast and allowing mixed colors at sites of co-localization

Expression patterns:



The Minimal Carcinoma Triple Stain Is Superior to Commercially Available Multiplex Immunohistochemical Stains

Breast Triple Stain and LC/DC Breast Cocktail

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From the Department of Pathology and Laboratory Medicine, Weill Cornell Medical College, New York, NY.

Key Words: Breast carcinoma; Needle core biopsy; Immunohistochemistry; E-cadherin; Triple stain; Multiplex; Cocktail; Lobular; Ductal

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ABSTRACT

Objectives: *The Minimal Carcinoma (MC) Triple Stain is a tri-chromogen multiplex immunostain (CK7, p63, and E-cadherin) helpful in classifying morphologically ambiguous and/or small carcinomas as either ductal or lobular and/or in situ or invasive. We compared the utility of this stain with two commercially available duplex/multiplex immunostains: Breast Triple Stain (BTS) (Clarient, Aliso Viejo, CA; CK5, p63, and CK8/18) and LC/DC Breast Cocktail (LCDC) (Biocare, Concord, CA; E-cadherin and p120).*

Methods: *Ninety-seven mammary carcinomas stained with the MC Triple Stain, BTS, and LCDC were compared.*

Results: *The MC Triple Stain, LCDC, and BTS were diagnostic in 90 (93%) of 97, 82 (85%) of 97, and 85 (88%) of 97 of cases, respectively. All stains showed decreased diagnostic utility due to variability in tissue integrity, quality of the staining, and/or ease of interpretation. In cases where all immunostains were interpretable, the MC Triple Stain yielded the most information.*

Conclusions: *When technically sufficient, all three immunostains demonstrated relative strengths and weaknesses in their ability to provide diagnostic information with the highest consistency and ease of use. Many cases stained with LCDC were technically insufficient due to a suboptimal staining protocol provided by the company. Overall, the MC Triple Stain outperformed BTS and LCDC by more consistently providing more diagnostic information. The MC Triple Stain is a viable alternative to other multiplex immunostains in evaluating small foci of carcinoma, particularly when both the histologic type and extent of disease (in situ vs invasive) require clarification.*

From a technical point of view: Challenging assays and stainings should be interpreted cautiously

Challenges or considerations performing double or multi-staining techniques:

Which double or multi-staining technique should I use ?

Pre-treatment - do the antigens of interest require the same Epitope retrieval procedure ?

Is the primary Abs available and in which form (important using simultaneous labelling techniques) ?

Is the primary Abs made in different host (e.g. mouse, rabbit) ~ If not
Is the primary Abs of different Immunoglobulin type (e.g. IgG, IgM) or subclass (e.g. IgG1, IgG2) ~ If not
Is the primary Abs available as conjugate (e.g FITC, biotin)
Do the primary Abs have the right specificity and sensitivity

Is the detection systems of choice specific and sensitive - Interspecies cross reactivity (specificity) ?

Is secondary Abs (app. conjugate) available matching choice of the primary Abs (important using simultaneous techniques)
Interspecies cross reactivity between primary Abs and secondary Abs (detection system) – pre-absorbed ?
Interspecies cross reactivity between secondary Abs (secondary Abs made in different host`s) – pre-absorbed ?

Which chromogens /fluorochroms should I use - depending on:

Type of conjugate (e.g. HRP, AP, Fluorochrom)
Color compatibility and allowing mixed color of chromogens (spectral differentiation) at sites of co-localization
Equipment (bright field versus fluorescence microscopy)

How can I inactivate the first set of antibody reagents (important using sequential labelling techniques) ?

Elution of immuno-reagents (Abs) / Heat inactivation
Take advantage of that DAB, DAB based or metallic deposit can shelter /block for following immunoreagents
How can I control the efficiency of the inactivating protocol

Controls

iCAPs - compare individual staining results of the multi labelling technique with optimal single staining procedure (routine)

A simple approach to double immuno-staining

Are antigens of interest located in the same cellular compartment (e.g. the nuclei`s) ?

NO

Use a DAB based sequential or a simultaneous technique

Enzyme conjugates

Conventional bright field microscopy

Include controls

YES

Use a immuno-fluorescence simultaneous technique.

Fluorochrome conjugates

Fluorescence microscopy

Mixed colors will be present at sites of co-localization

Include controls

❑ Double staining using sequential technique (Immuno-enzymatic)

- Preferred method to detect antigenic epitopes in different cell types or in different cellulare compartments (e.g. nuclei and cytoplasma) ~ not suitable for detection of co-localized signal.

- The primary antibodies is applied in sequence , detected and visualized individually with two different detection (HRP and/or AP conjugates) & chromogen systems (e.g. DAB and/or Fuchin-Red).

- Each antibody: antigen reaction will yield a specific color of staining that can be easily identified.

- Primary antibodies may be of either the same or different host , Ig-type/subclass(e.g. mouse/IgG1)

- Visualization with DAB, DAB based chromogens or EnzMet (metallic silver) is always included in the first sets of the detection procedure as the deposit from these chromogens shields for un-specific reaction of the following primary antibodies or detection reagents.

Double staining using sequential technique (Immuno-enzymatic)

Sequential procedure (Immuno-emzymatic):

Pre-treatment (Antigen Retrieval)

First primary Ab (same or different host, Ig-type or subclass) (20 min).
Detection with Quanto/HRP (10+10 min).
Visualization with Deep Space Black (DAB/Ni ?) (10 min).

Second primary Ab (same or different host, Ig-type or subclass) (20 min).
Detection with Hi-Def /AP (10+10 min).
Visualization with Warp Red (Fuchin-Red) (7-20 min).

Counterstain, dehydration and mounting.

DAB reaction product of the first set of immunoreagents create a barrier that is impermeable for the second set of immunoreagents ~ no cross reactivity

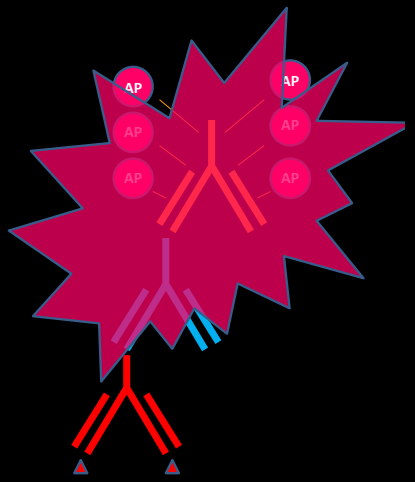
Sternberger LA, Joseph SA. J Histochem Cytochem 27:1424, 1979

Valnes K and Brandtzaeg P . J Histochem Cytochem 1982; 30(6) 518-524.

Metallic deposits (e.g. EnzMET) has the same ability

Inactivation step or blocking procedures not needed

1.Set of Immuno-reagents
Deep Space Black
DAB/Ni ?



2. Set of Immuno-reagents
Warp Red
Fast Red / Fuchin-Red

Optimizing double immuno-staining protocol

Sequential staining : Either DAB based (shielding properties) or with blocking procedures between reaction series A & B

Testing:

- Antibody A (dilution series) → Detection A → Chromogen A
- Antibody B (dilution series) → Detection B → Chromogen B

→ Calibrate titre (both primary antibody A & B) according to iCAPs

- Antibody A → Detection A → Chromogen A
- Diluent → Detection B → Chromogen B

→ Control 1 (only reaction for A should be observed – no co-localized signals)

- Diluent → Detection A → Chromogen A
- Antibody B → Detection B → Chromogen B

→ Control 2 (only reaction for B should be observed – no co-localized signals)

- Antibody A → Detection A → Chromogen A
- Antibody B → Detection B → Chromogen B

→ Double staining

Remember:

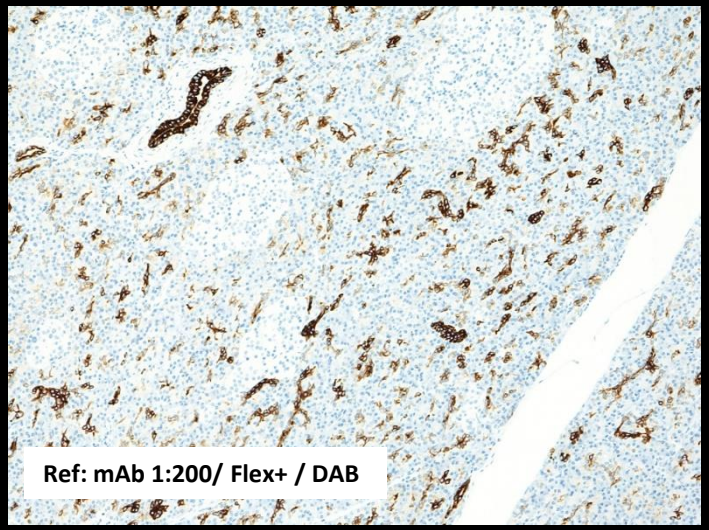
Not suitable for detection of co-localized signal as a DAB based chromogen is involved in the first set of immuno-reactions (Sequence A).

Typical end-result including controls: Optimizing the protocol

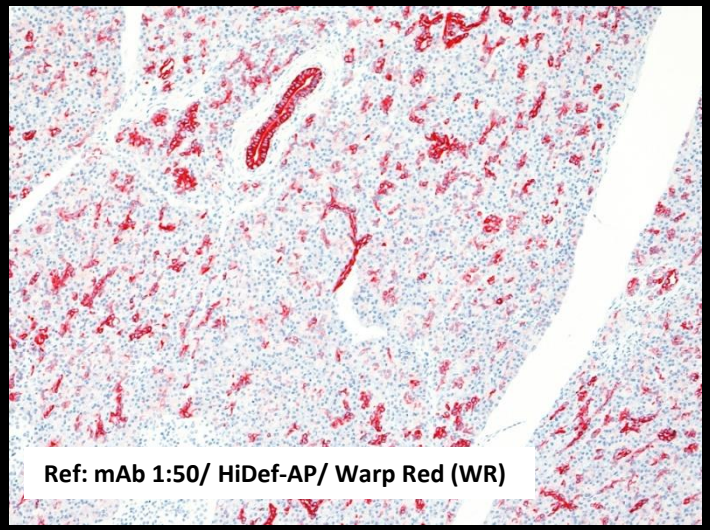
CDX2 (EP25) + CK7 (OV-TL 12/30)

Pancreas

CK7, OV-TL12/30

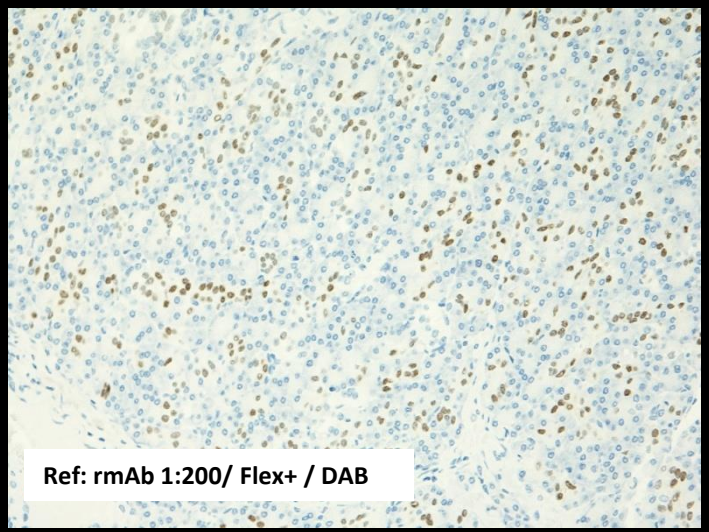


Ref: mAb 1:200/ Flex+ / DAB

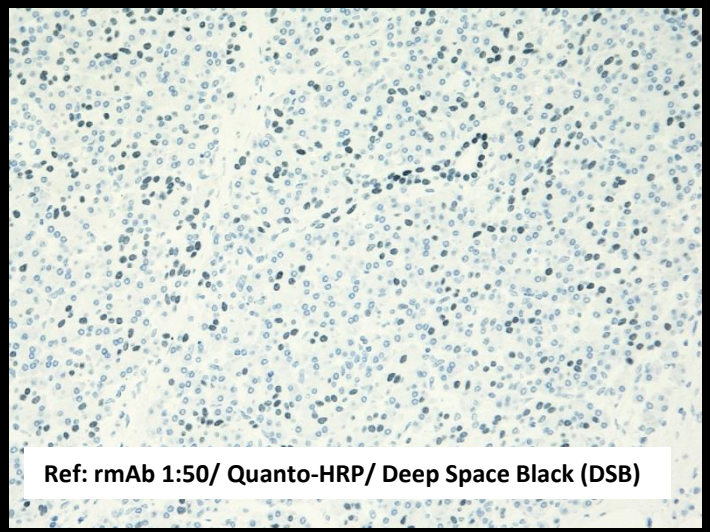


Ref: mAb 1:50/ HiDef-AP/ Warp Red (WR)

CDX2, EP25



Ref: rmAb 1:200/ Flex+ / DAB

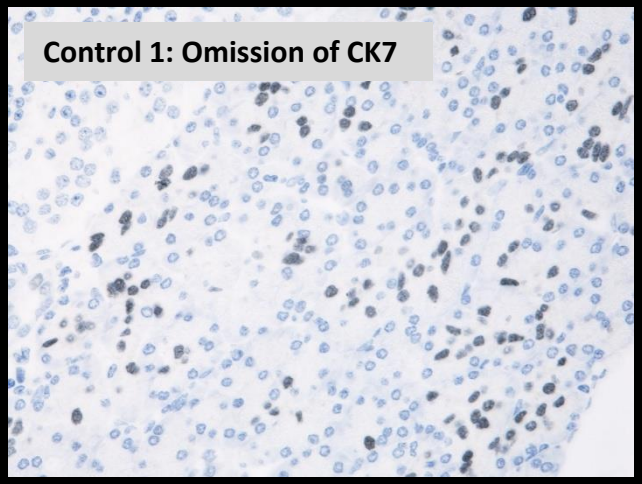


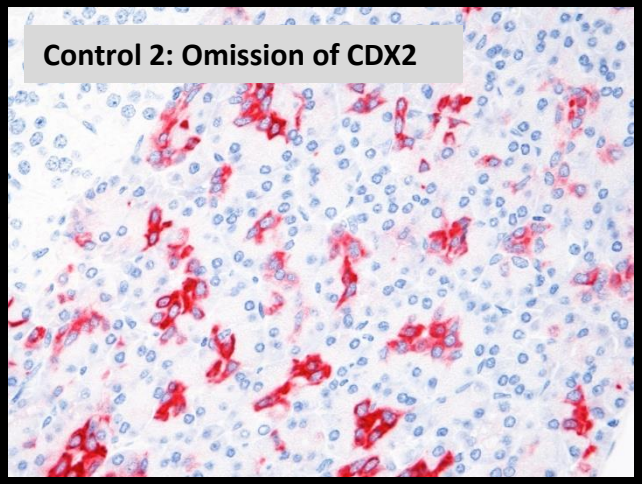
Ref: rmAb 1:50/ Quanto-HRP/ Deep Space Black (DSB)

Typical end-result including controls: Optimizing the protocol

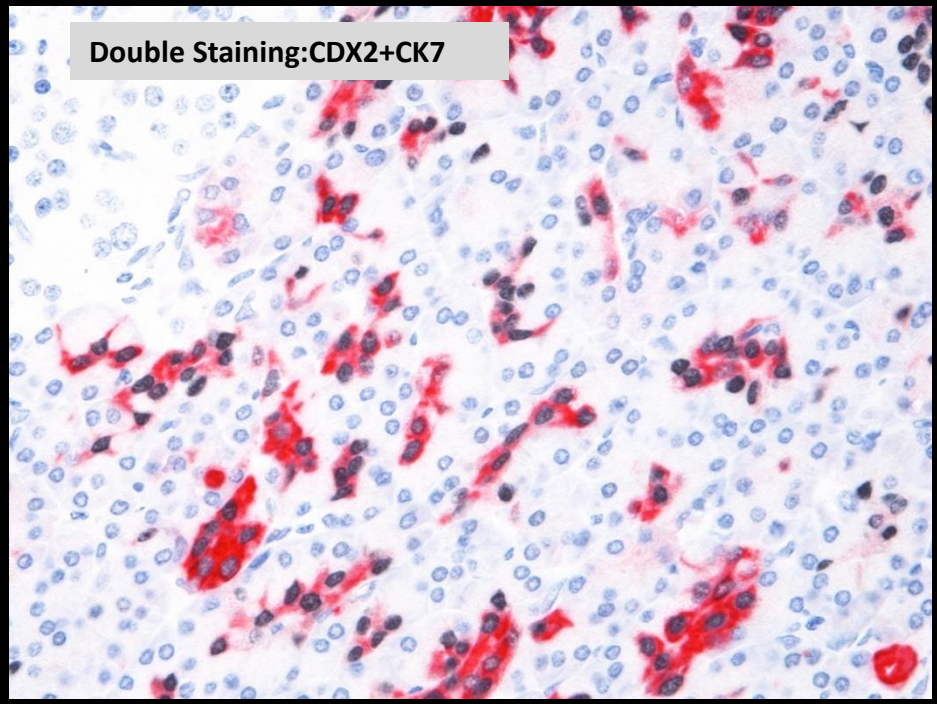
Optimal dilutions of CDX2 (EP25 1:50) and CK7 (OV-TL 12/30 1:50)

Sequential staining : Quanto-HRP/ DSB followed by HiDef-AP/WR





Pancreas

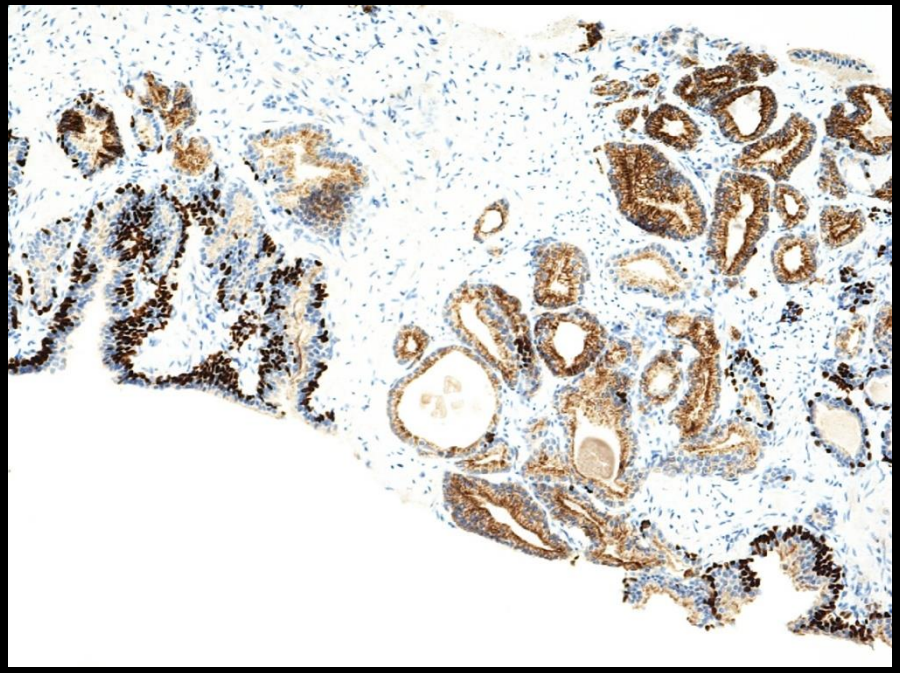


Note : Efficient sheltering capacity of Deep Space Black

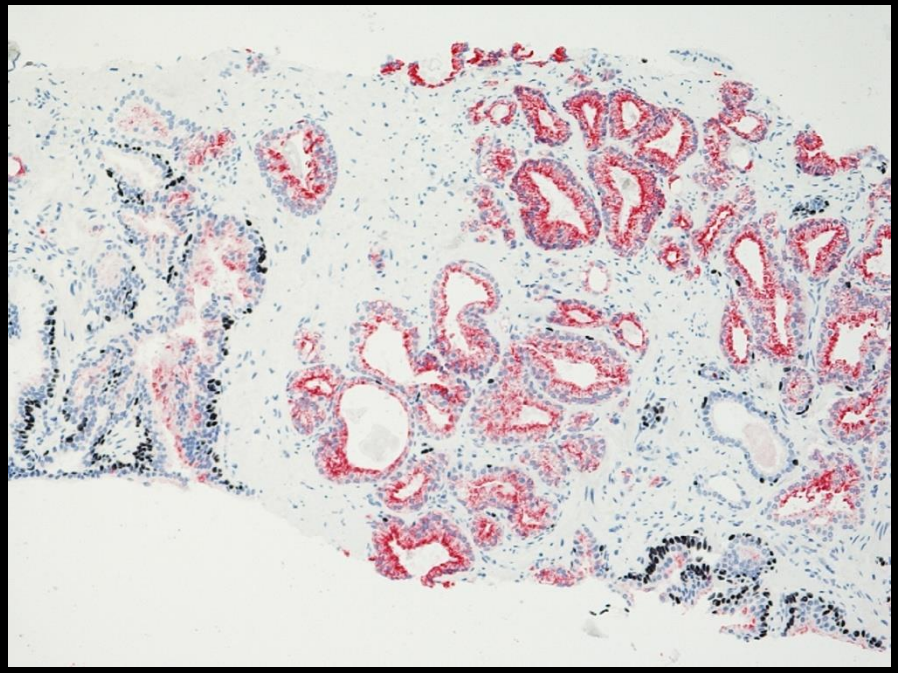
No mixed colors or double colors within controls and in the double staining for CDX2+CK7 (Intercalating ducts of the pancreas are positive for both markers)

Double staining using sequential technique (Immuno-enzymatic)

PIN [cocktail P63 (4A4) + P504s (13H4)] (Mab+Rab)



PIN [P40 (BC28) + P504s (13H4)] / Sequential staining (Mab+Rab)

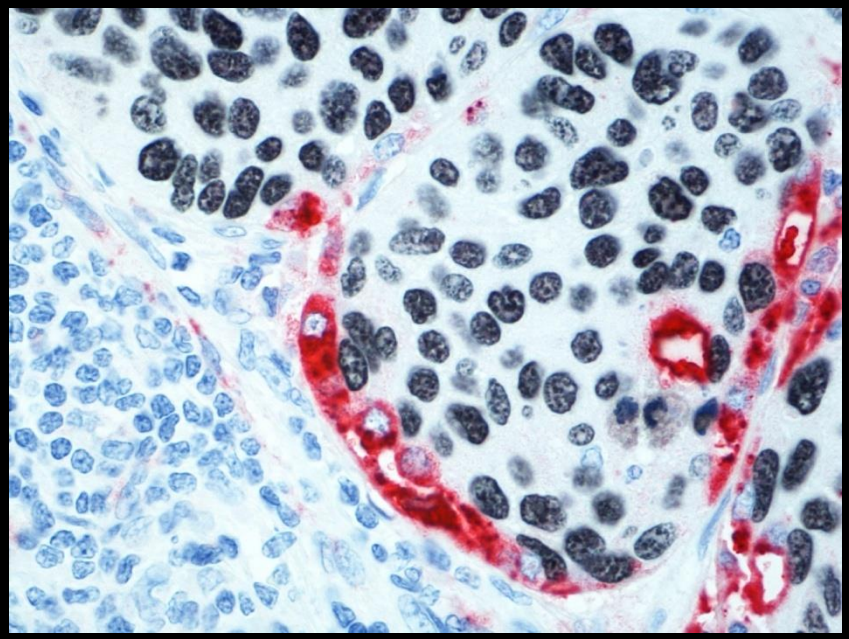


Prostate: Adenocarcinoma

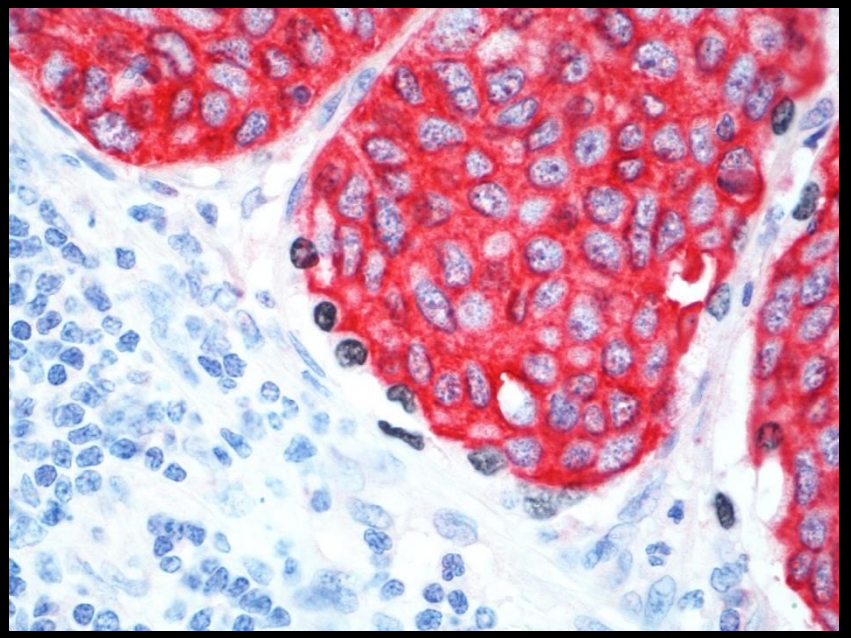
Other useful combinations: : P63 + P504s and/or CK5 (HMw CK) + P504s

Double staining using sequential technique (Immuno-enzymatic)

P40, BC28 (1:25) + Napsin, IP64 (1:100) (Mab+Mab)



TTF1, SPT24 (1:25) + CK5,XM26 (1:100) (Mab+Mab)

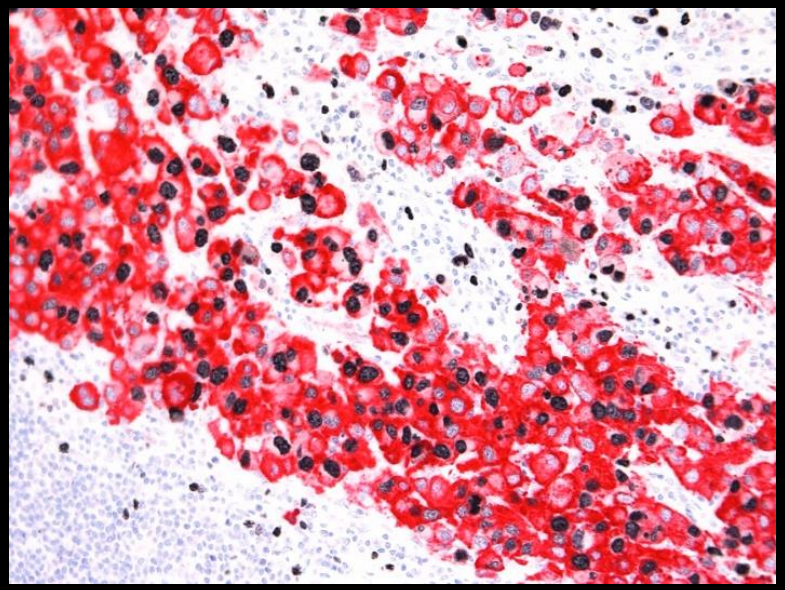


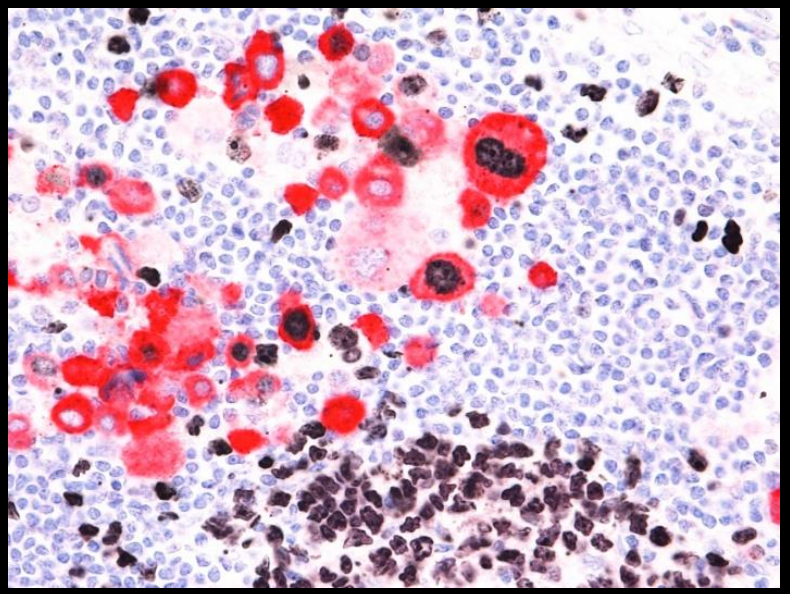
Lung : Squamous cell carcinoma

Other useful combinations: P40 + CK5 and/or TTF-1+ Napsin A

Double staining using sequential technique (Immuno-enzymatic)

Melanoma (Lymph node)





Ki67 , SP6 (1:100) + Melan A, A103 (1:100) (Rab+Mab)

Other useful combinations: Sox-10 + MLA and/or Sox-10 + S100

Double staining using sequential technique (Immuno-enzymatic)

The order of primary antibodies

In general:

- Nuclear markers before cytoplasmic or membranous markers
- Membranous markers before cytoplasmic

A reversed applications of the primary antibodies may un-ravel that :

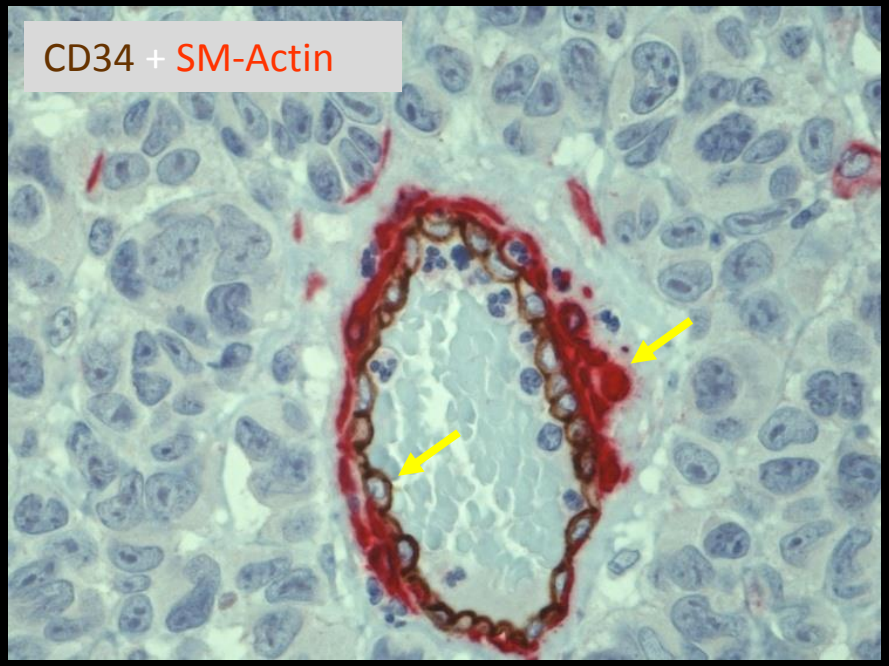
DAB deposit shields for the second antigen of interest - first and second antigen are in close proximity with each other (false negative result)

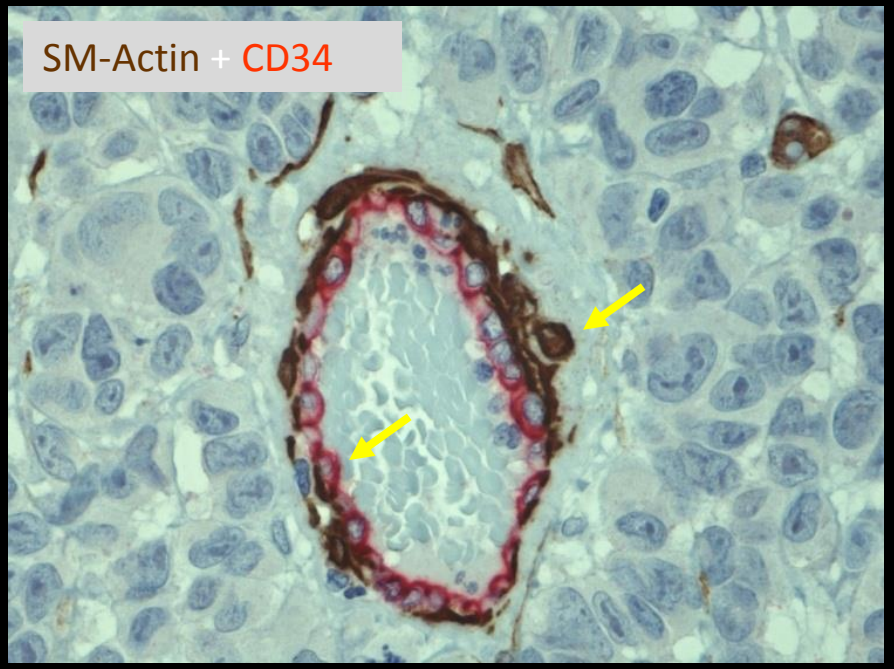
Co-localized signals is present due to expression of the two antigens of interest in the same cellular compartment

Double Immunostaining

CD34 (Q-BEND 10) + SM-Actin (1A4)

Melanoma



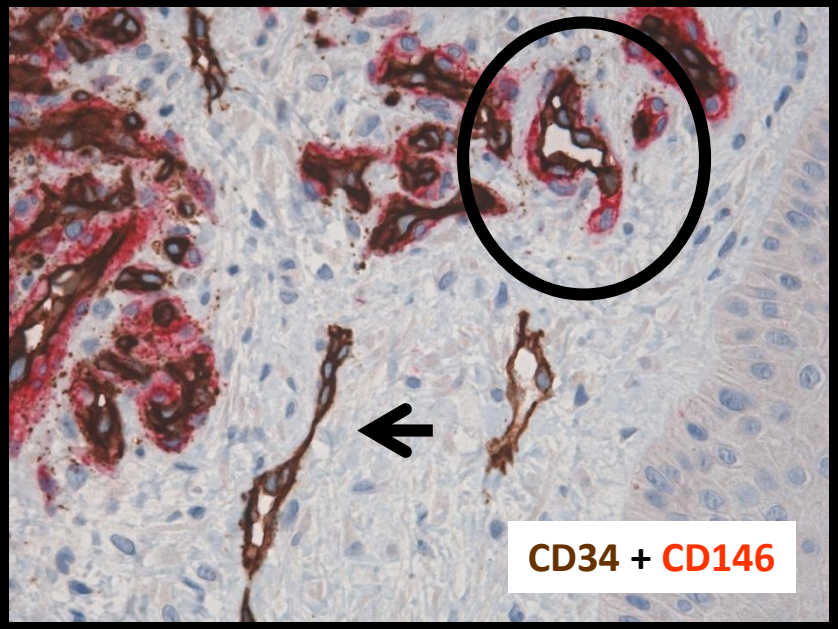


Note: No co-localization of either endothelium or smooth muscles (arrows)

Double Immuno-staining – reversal stainings

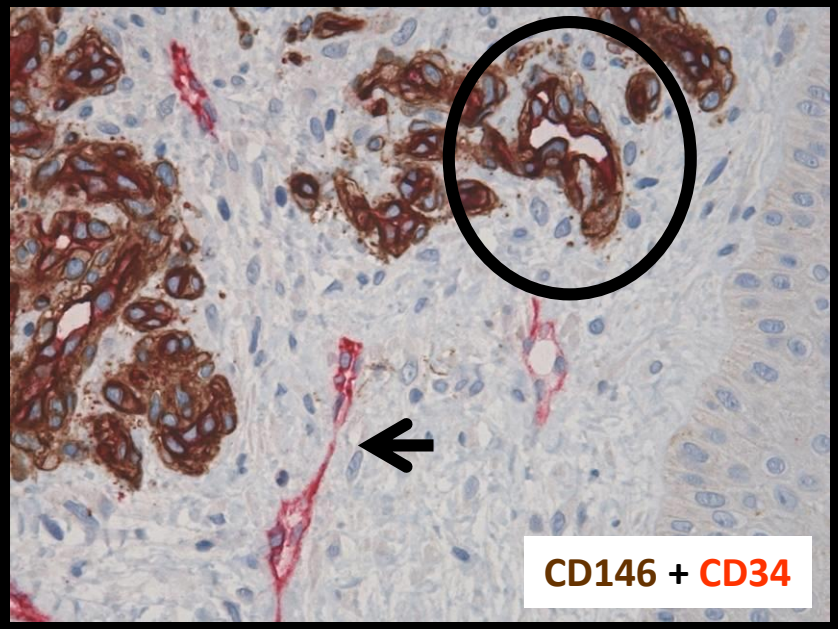
CD34 (Q-BEND 10) + CD146 (EPR3208)

Hemangioma



The order of primary antibodies

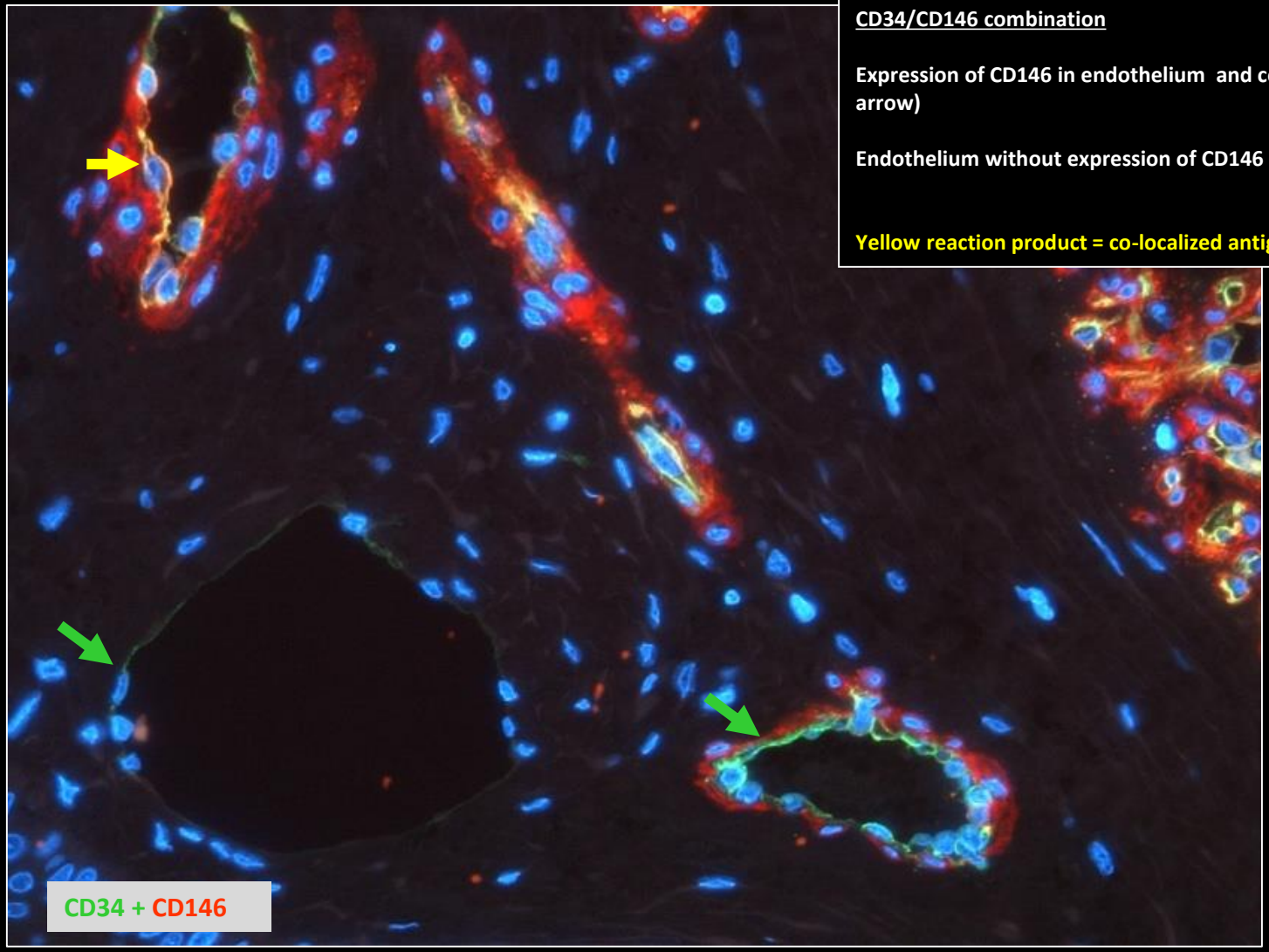
The brown deposit (DAB) from the first set of immuno-reagents hinder the second set of immuno-reagents (the red subsequent staining – compare the two images).



Co-localization - non of the combinations is useful ?

Double immunofluorescence staining of vascular structures

Hemangioma



CD34/CD146 combination

Expression of CD146 in endothelium and co-localization with CD34 (yellow arrow)

Endothelium without expression of CD146 (green arrow)

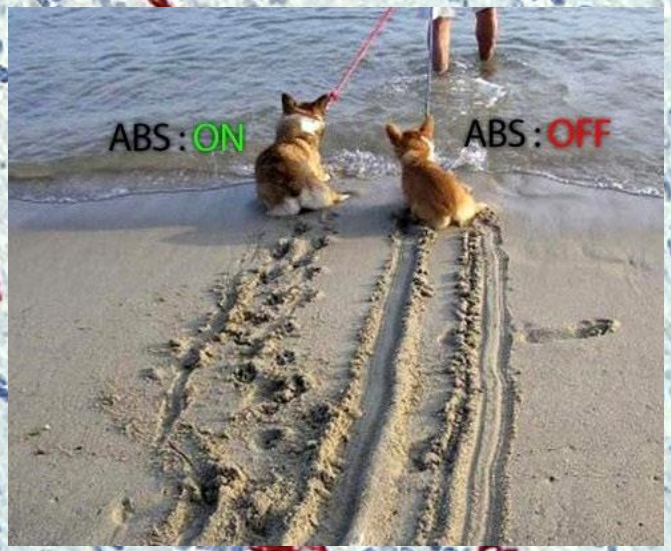
Yellow reaction product = co-localized antigens

CDX-2 (DAK-CDX2) + CK 8/18 (5D3)

Problems ?

The DAB deposit may cause problems as it may block for the next set of immuno-reagents - arrows.

Tumor cells with infiltrative growth pattern (buddings) show expression of CK 8/18 due to downregulation of CDX2 – arrowheads.

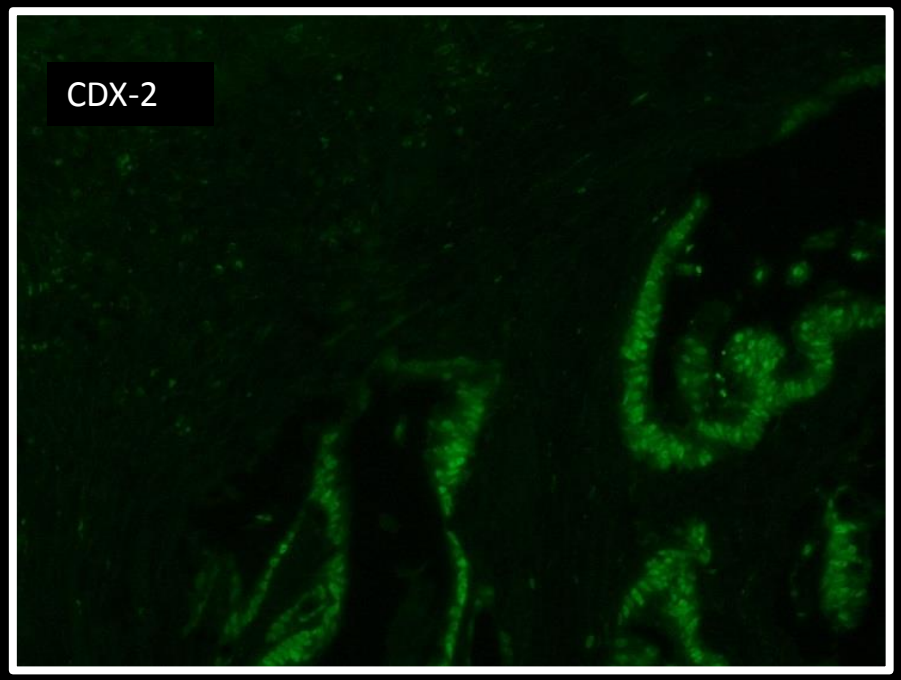


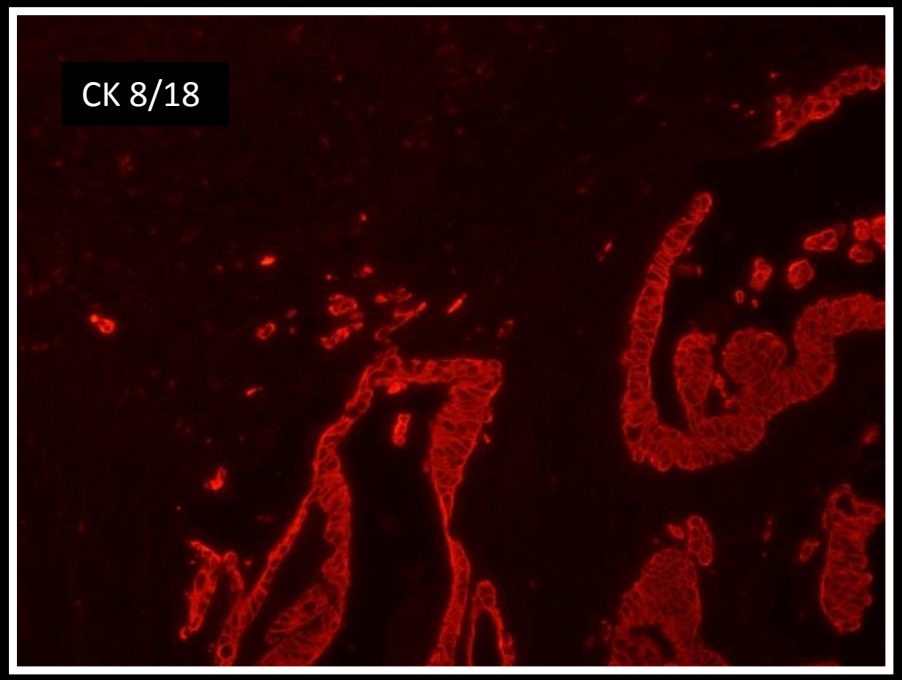
Colon Adenocarcinoma

CDX-2 (EP25) + CK 8/18 (5D3)

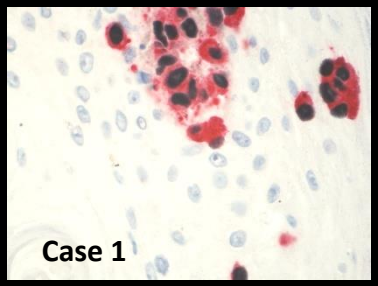
Double immuno-fluorescence

Colon Adenocarcinoma





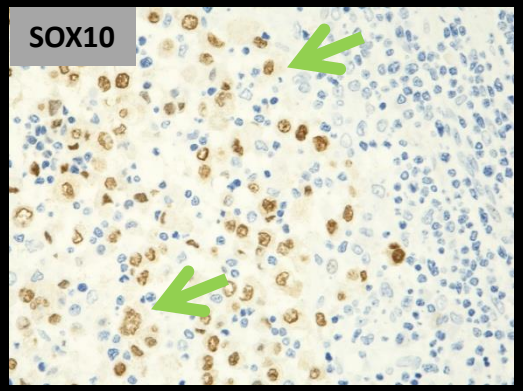
Note expression of CK 8/18 in all tumor cells

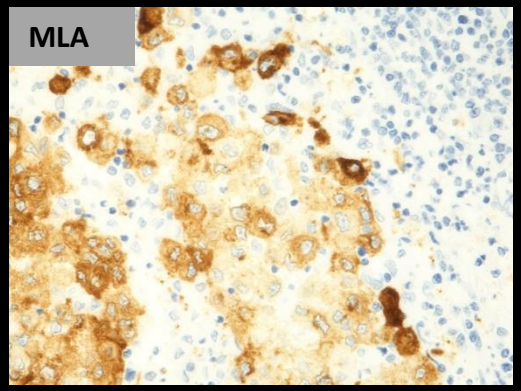


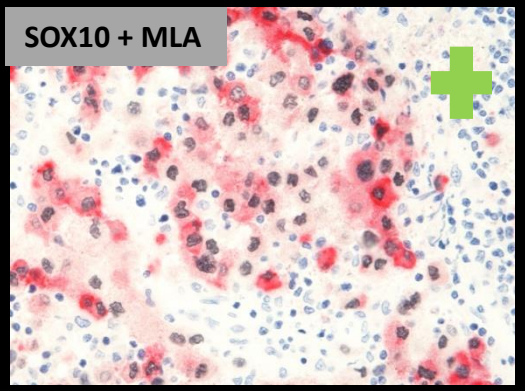
Sequential double immunostaining: Sox-10, BC34 (1:20) + MLA, A103 (1:25)

Melanomas

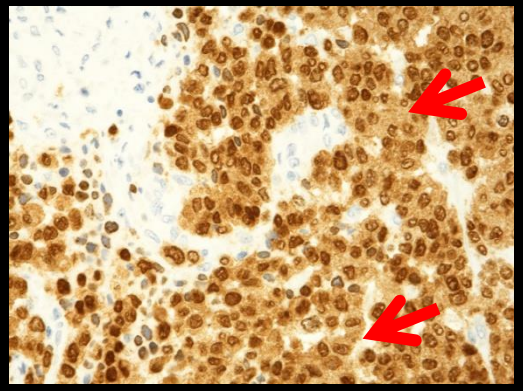
Case 2

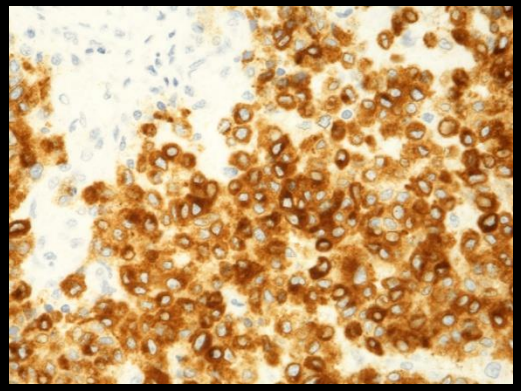


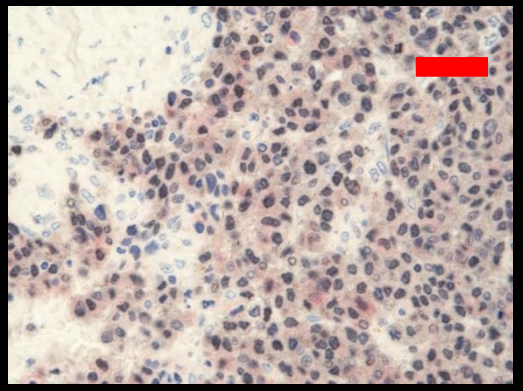




Case 3







Problems ?

DAB/DAB based chromogens shields for second set of immuno-reagents (red arrows showing moderate-strong "un-specific" cytoplasmic staining reaction with SOX-10 in melanoma case 3)

Double/Multiple staining using sequential technique (Immuno-enzymatic)

Limitations using DAB based chromogens

- ✓ The DAB products is NOT suitable for demonstration of co-localized signals (e.g. two cytoplasmic antigens in the same cell

- ✓ If two antigens are in close proximity with each other – DAB based chromogens shields for 2nd antigen of interest

- ✓ Now, what if unexpected color mixing occur due to cross reactivity with the 1th set of reagents (mainly a problem related to other than DAB or DAB based chromogens) ?

Inactivation of 1th set of immuno-reagents :

- ❑ Elution methods (High salt, extreme pH values and strong oxidizing agents)
- ❑ HIER using Citrate pH6 (10 min)

Applied between the 1th and 2nd set of immuno-reagents

Double staining using sequential technique (Immuno-enzymatic)

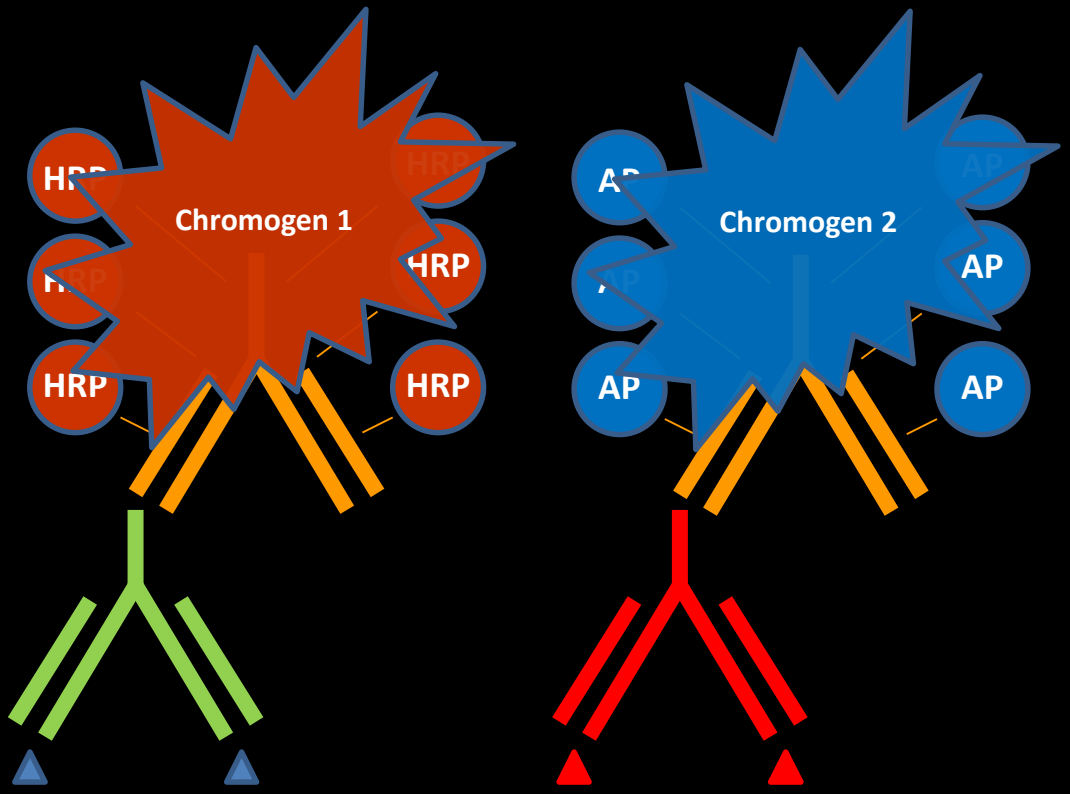
Inactivation of the first set of antibody reagents

Elution step

Glycine-HCL pH 2.2 (Nakane PK, 1968)

HCL pH 2 (Nakane PK, 1968)
$KMnO_4$ (Tramu G,1978)
Glycine-SDS pH2 (Pirici D, 2009)

And many more



Limitations: High affinity antibodies difficult to elute

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Journal of Histochemistry & Cytochemistry
http://www.jhc.org

ARTICLE

Antibody Elution Method for Multiple Immunohistochemistry on Primary Antibodies Raised in the Same Species and of the Same Subtype

Daniel Pirici, Laurentiu Mogoanta, Samir Kumar-Singh, Ionica Pirici, Claudiu Margaritescu, Cristina Simionescu, and Radu Stanescu

Control studies

Table 1 Primary antibodies used in this study

Antigen	IgG isotype, species	Specificity	Working dilution	Antigen retrieval	Source
CD20cy	IgG2a, Ms	B lymphocytes	1:100	10 mM citrate buffer, pH 6	Dako
CD45Ro	IgG2a, Ms	T lymphocytes	1:50	10 mM citrate buffer, pH 6	Dako
CD3	Pab, Rbb	T lymphocytes	1:100	10 mM citrate buffer, pH 6	Dako
CD31	IgG1, Ms	Endothelial cells	1:50	10 mM citrate buffer, pH 6	Dako
CD34	IgG1, Ms	Endothelial cells	1:50	10 mM citrate buffer, pH 6	Dako
CD105	Pab, Rbb	Endothelial cells	Ready to use	10 mM citrate buffer, pH 6	Abcam
CD105	IgG1, Ms	Endothelial cells	1:1000	–	Dako
Collagen IV	IgG1, Ms	Basal membranes	1:25	10 mM citrate buffer, pH 6	Dako
F VIII	IgG1, Ms	Endothelial cells	1:50	10 mM citrate buffer, pH 6	Dako
CD68	IgG1, Ms	Macrophages	1:100	10 mM citrate buffer, pH 6	Dako
S100	Pab, Rbb	Antigen presenting cells	1:2000	10 mM citrate buffer, pH 6	Dako
PCNA	IgG2a, Ms	Cell proliferation marker	1:50	10 mM citrate buffer, pH 6	Dako
Ki67	Pab, Rbb	Cell proliferation marker	1:250	10 mM citrate buffer, pH 6	Abcam
Ki67	IgG1, Ms	Cell proliferation marker	1:100	10 mM citrate buffer, pH 6	Dako

Abcam; Cheminkpress, Craiova, Romania.

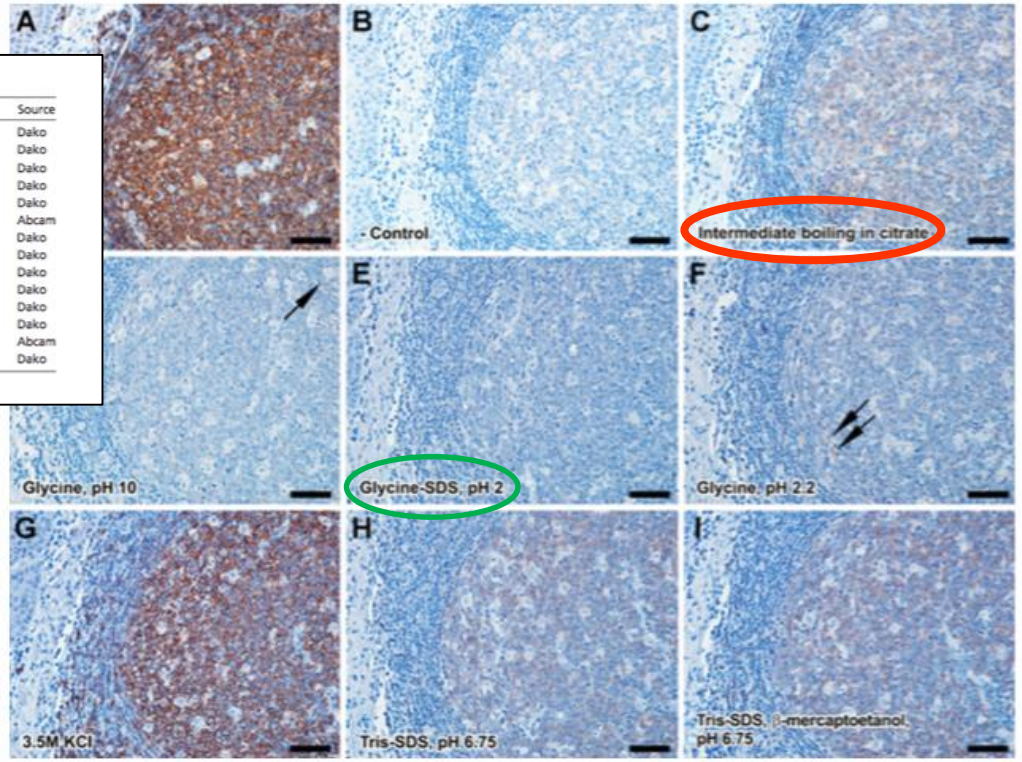


Figure 1 Choosing the optimum antibody-elution protocol. The procedure involved incubation with a primary antibody (follicular B-cell marker CD20cy here), applying the elution procedure, and detecting the remaining primary antibody. Positive and negative controls for this reaction were obtained by skipping the elution step (A) or not adding the primary antibody (B). Boiling in citrate buffer (C), incubating in glycine pH 10 (D), glycine-HCl, pH 2.2 (F), 3.5 M KCl (G), Tris-SDS, pH 6.75 (H), and Tris-SDS β-mercaptoethanol, pH 6.75 (I) showed insufficient signal reduction, whereas the glycine-SDS pH 2 protocol (E) showed a complete antibody elution. All elutions involved a 30-min incubation under agitation at 50C. Arrows indicate sites with faint remnant signal. Bar = 50 μm.

Demonstrated that:

Elution, using a glycine SDS pH 2 solution, of the antibodies after the first round of immunolabelling was superior in regard of eliminate cross reactivity with the second set of immuno-reagents compared to other methods tested .

This also includes an intermediate HIER step using Citrate pH6

Double staining using sequential technique (Immuno-enzymatic)

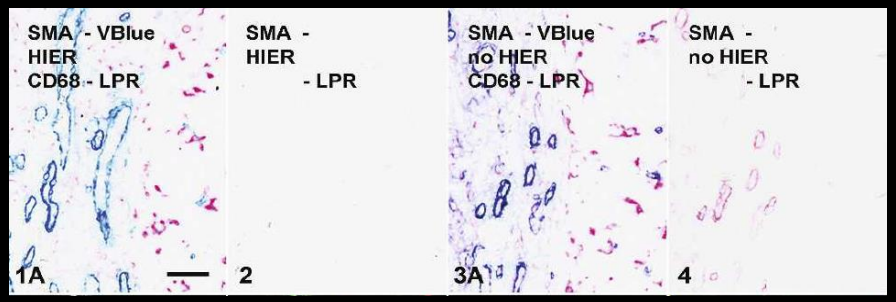
Inactivation of the first set of antibody reagents

Van der Loos CM et al. J Histotechnol; 31: 119-127

A Generally Applicable Sequential Alkaline Phosphatase Immunohistochemical Double Staining

Table 3. Sequential Double AP Immunohistochemical Staining on FFPE Tissue Sections Using SMA and CD68 as Primary Antibodies: Different Orders of AP Chromogens, with and without HIER, and Controls (*Experiment 1*)

First primary	*First detection*	*First chrom.*	*HIER step*	*Second primary*	*Second detection*	*Second chrom.*	*Figure*
SMA	Anti-Ms polymer/AP	LPRed	+	CD68	Anti-Ms polymer/AP	VBlue	5
SMA	—	LPRed	+	—	Anti-Ms polymer/AP	VBlue	6
SMA	Anti-Ms polymer/AP	LPRed	–	CD68	Anti-Ms polymer/AP	VBlue	7
SMA	—	LPRed	–	—	Anti-Ms polymer/AP	VBlue	8
SMA	Anti-Ms polymer/AP	VBlue	+	CD68	Anti-Ms polymer/AP	LPRed	1
SMA	—	VBlue	+	—	Anti-Ms polymer/AP	LPRed	2
SMA	Anti-Ms polymer/AP	VBlue	–	CD68	Anti-Ms polymer/AP	LPRed	3
SMA	—	VBlue	–	—	Anti-Ms polymer/AP	LPRed	4



Blocking of first set of detection with a HIER step - select primary Abs where no co-localization is expected

Inactivation by HIER:

HIER in Ci pH 6, (Lan HY ,1995)

Limitations:

Require Heat stable chromogens - DAB, VBlue, VRed and LPR (Dako)

Always efficient ?

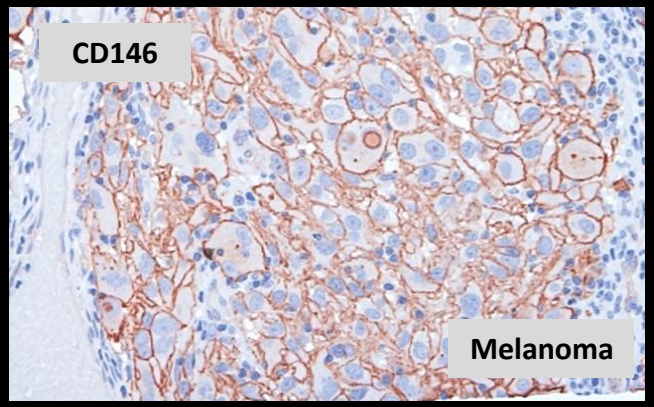
Inactivating of the first set of immuno-reagent using HIER

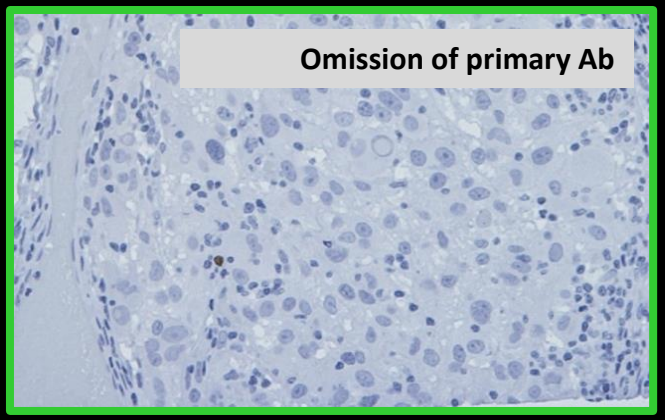
CD146, EPR3208/Flex+ HRP/ AEC (first cycle) → omission of primary Ab /Flex+ HRP/ DAB (second cycle)

CD15, Carb-3/Flex+ HRP/ AEC (first cycle) → omission of primary Ab /Flex+ HRP/ DAB (second cycle)

Control

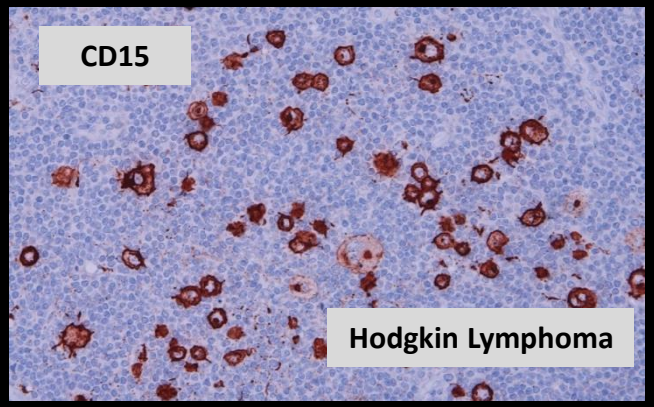
De-coverslip, Acetone, blocking (HIER Ci pH 6/10min), omission of primary Ab and repeated second cycle with DAB





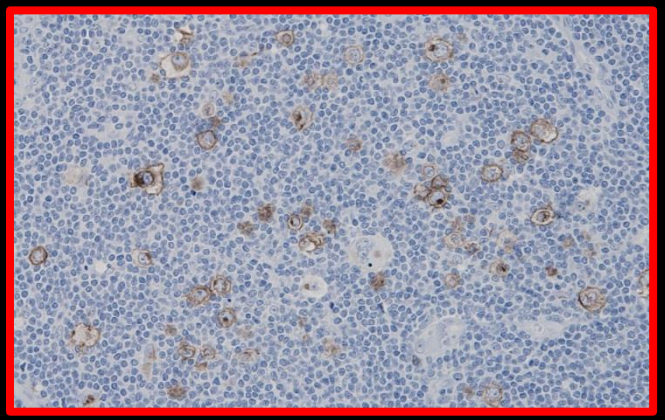
Problem:

CD15, Carb-3 / IgM /Ig-isotype ?
High affinity Abs ?
Antigen density ?
Inefficient HIER Temp or time ?



Control

De-coverslip, Acetone, blocking (HIER Ci pH 6/ 30min), omission of primary Ab and repeated second cycle with DAB



Double immuno-enzymatic staining

Simultaneous technique

Simultaneous Double Immuno-labelling procedure

Polymer/multimer procedure

Pre-treatment (Antigen Retrieval)

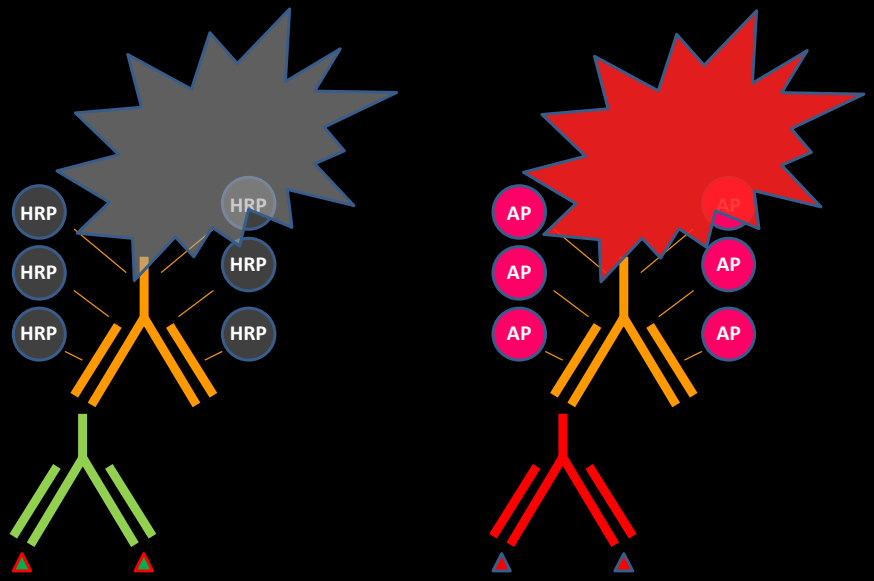
Incubation with mix of primary Abs (Rab+Mab)

Incubation with polymer/multimer mix (anti -Mab/HRP + anti -Rab/AP)

Incubation with HRP substrate (e.g. DAB or DSB)

Incubation with AP substrate (e.g. Fast Red or Warp Red)

Counter stain, mounting and microscopy



Commercial kits (Naestved Lab):

Lab Vision™ MultiVision Polymer Detection System: anti-Mouse-AP and anti-Rabbit-HRP (TL-012-MARH)

Lab Vision™ MultiVision Polymer Detection System: anti-Mouse-HRP and (TL-012-MHRA)

Biocare MACH2 Double Stain 1: anti-Mouse-AP and anti-Rabbit-HRP (MRCT523)

Biocare MACH2 Double Stain 2: anti-Mouse-HRP and anti-Rabbit-AP (MRCT525)

Double enzymatic staining using simultaneous technique

- Primary antibody cocktails is applied to the tissue at the same time (simultaneously)

- Primary antibodies must be of different host (e.g. mouse and rabbit) or at least different Ig-types, subclass or one primary Ab with a conjugate (e.g. biotin).

- Reactions are detected with a mix of secondary antibodies conjugated with two different enzymes (e.g. HRP and AP) and applied to the tissue at the same time (simultaneously).

  To prevent cross-reaction between secondary antibodies raised against different species, it is recommended to apply second-step antibodies raised in the same host – if not possible, use pre-absorbed secondary antibodies.

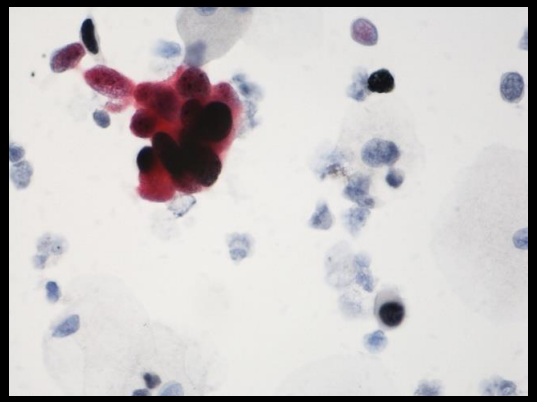
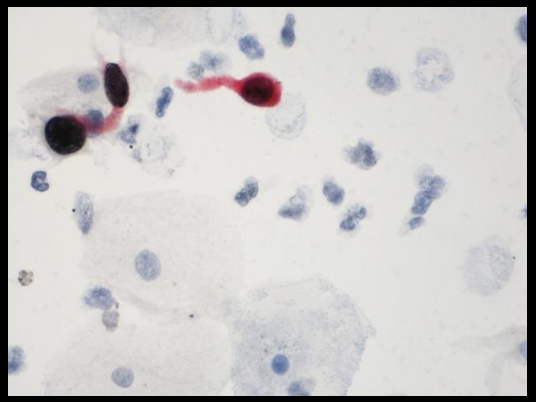
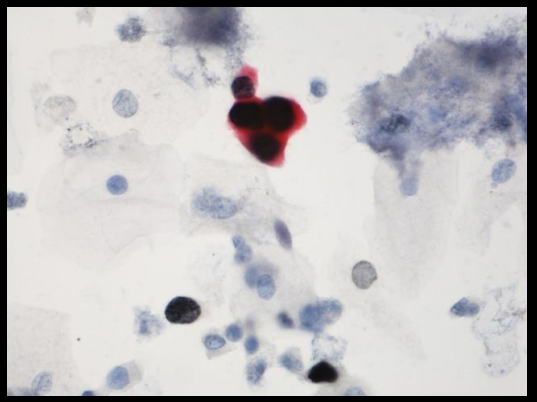
- Each antibody: antigen reaction will yield a specific color of staining that can be easily identified.

MACH2 Double Staining : Ki-67, SP6 (1:25) + P16, E6H4 (RTU,Ventana)

Dysplasia / Cervix (Cytology)

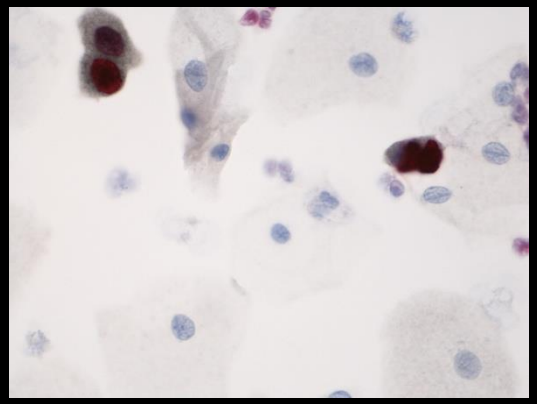
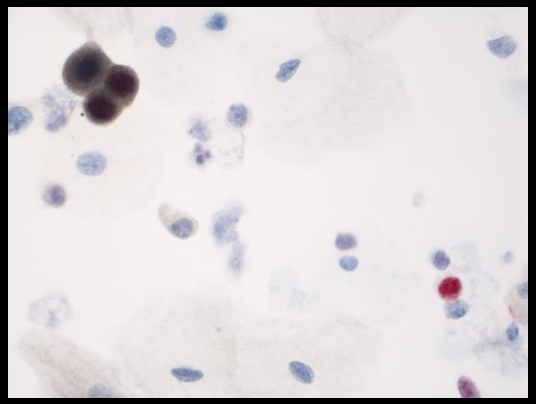
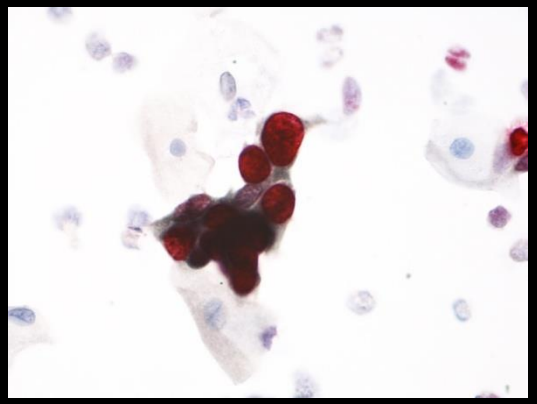
Double Stain 1 (Biocare)

Anti-Rab/HRP
Anti-Mab/AP

Double Stain 2 (Biocare)

Anti-Rab/AP
Anti-Mab/HRP

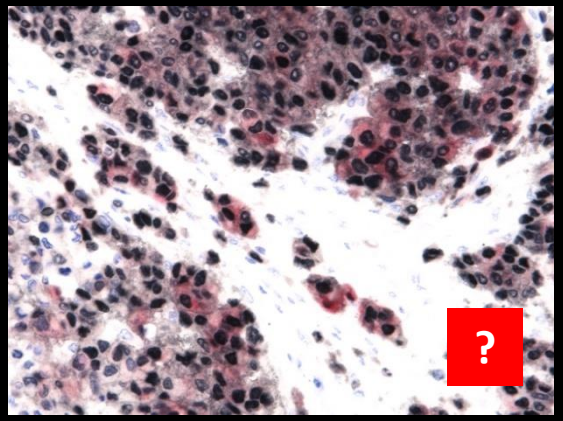
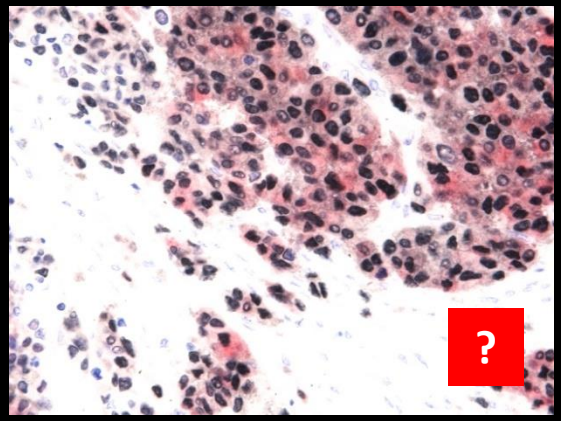
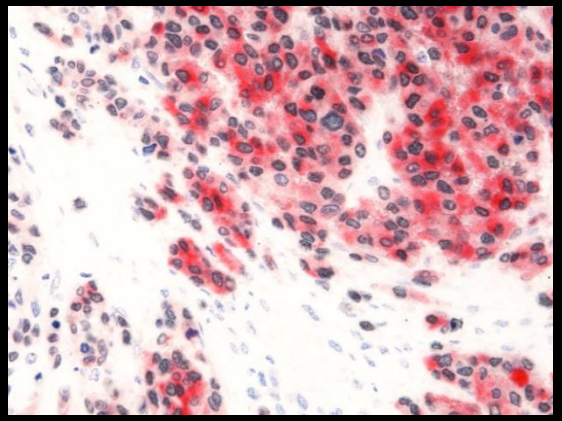
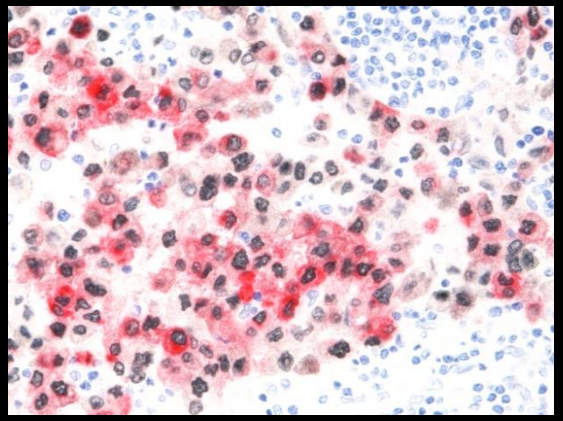
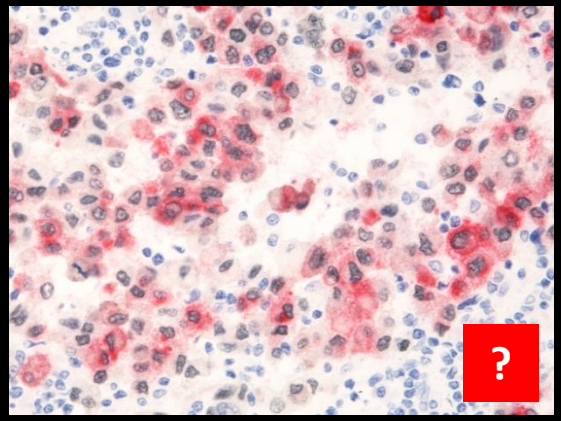
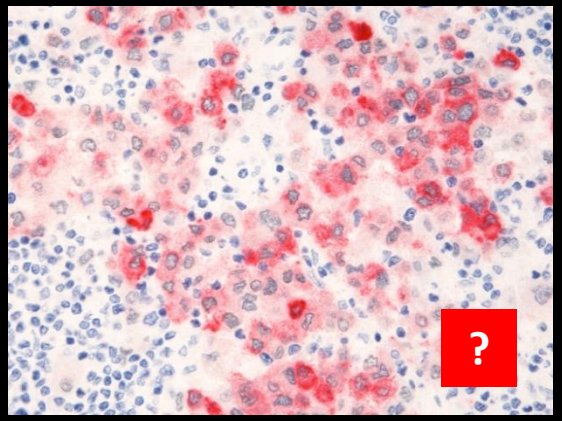
Cytological specimens fixed in NBF 30` → 96 alk. 10` → Wash buffer → HIER/TE 20`

MACH2 Double Staining 1: Sox-10, EP238 + MLA, A103

Deep Space Black 5` + Warp Red 2x8`

Problems ?

Melanomas

	SOX-10, EP258 (1:25) + MLA, A103 (1:25)	SOX-10, EP258 (1:100) + MLA, A103 (1:25)	SOX-10, EP258 (1:400) + MLA, A103 (1:25)
Case 3			
Case 2			

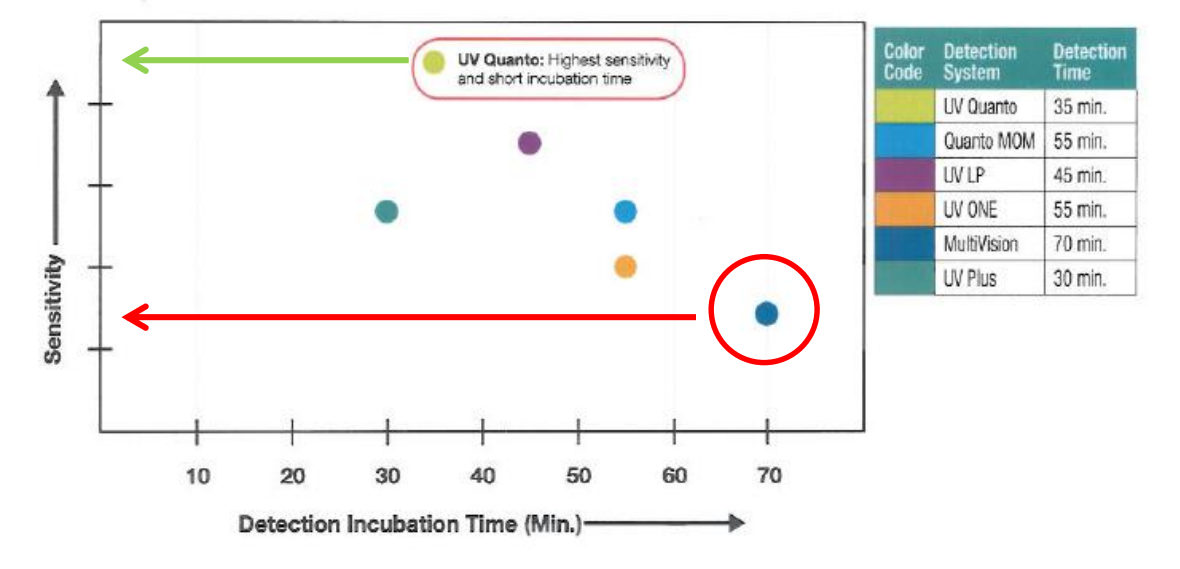
Double immuno-enzymatic techniques
Sequential or simultaneous methods

Some final comments to :

Detection systems, Chromogens and Automated platforms

Detection systems and sensitivity

Double (MultiVision) versus single immuno-staining protocols



Color Code	Detection System	Detection Time
	UV Quanto	35 min.
	Quanto MOM	55 min.
	UV LP	45 min.
	UV ONE	55 min.
	MultiVision	70 min.
	UV Plus	30 min.

www.thermoscientific.com/labvision

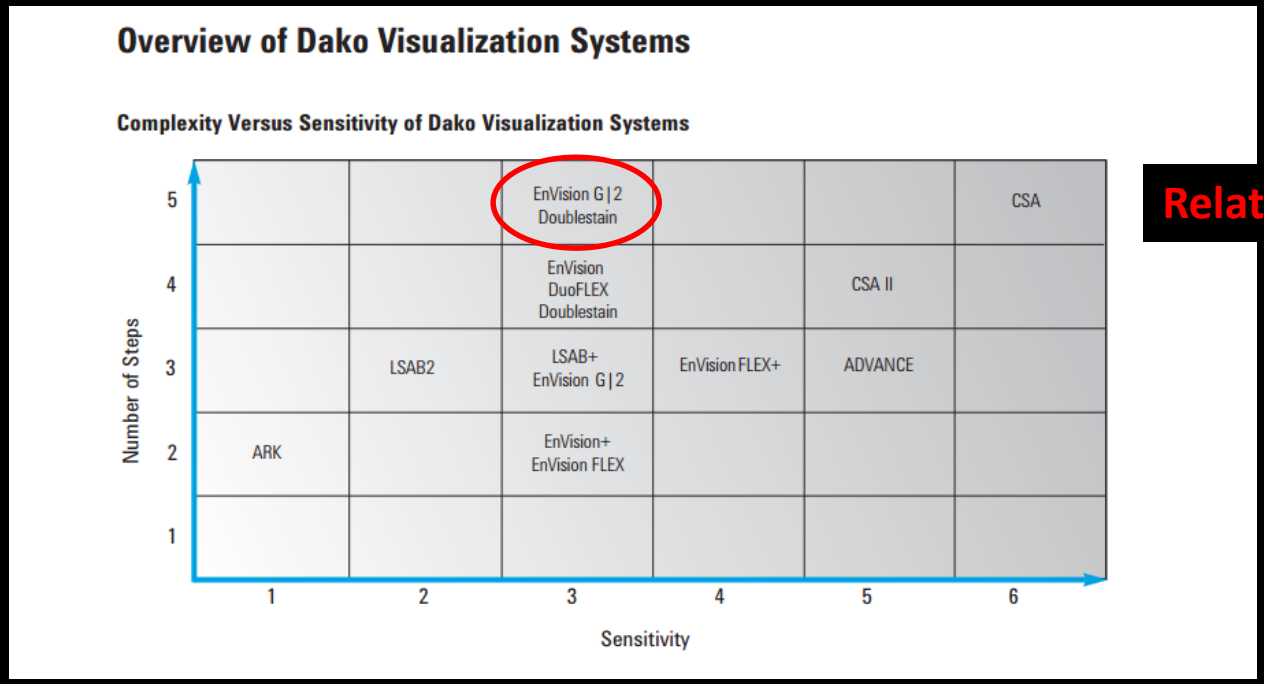
<u>Important questions using double immuno-staining systems:</u>

Critical Staining Quality Indicators (iCAPs) ?

Challenging primary antibodies – how will they perform ?

Detection systems and sensitivity

EnVision G/2 Doublestain versus single immuno-staining protocols



Relative low sensitivity

<u>Important questions using double immuno-staining systems:</u>

Critical Staining Quality Indicators (iCAPs) ?

Challenging primary antibodies – how will they perform ?

End result depending on the chosen platform or detection system?

Ki-67, SP6 + MART1, EP43

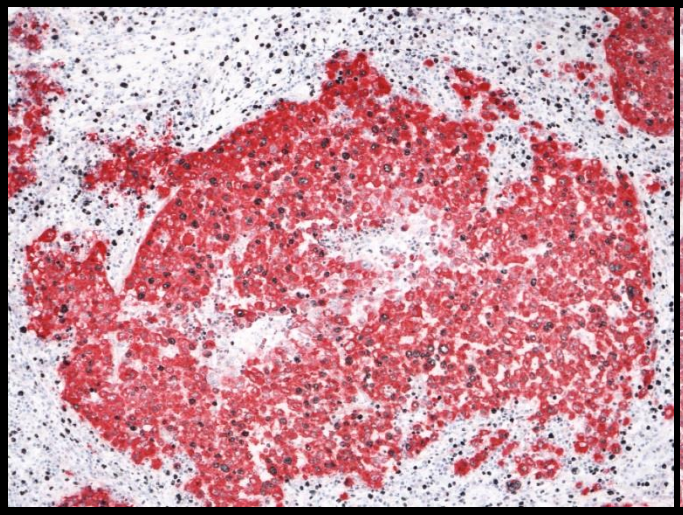
HIER in TRS pH9

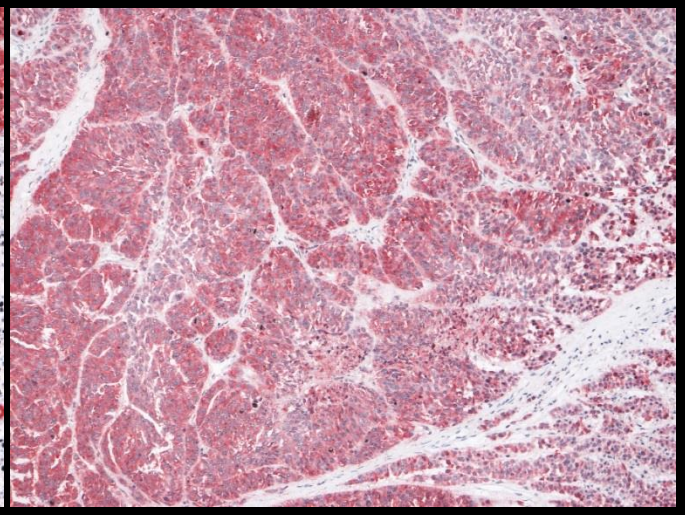
Autostainer

Open system

Ki67, SP6 (1:50)
MART1, EP43 (1:50 RR)

Quanto-HRP 10` (DSB 2x5`)
HiDef-AP 10 ` (WR 20`)





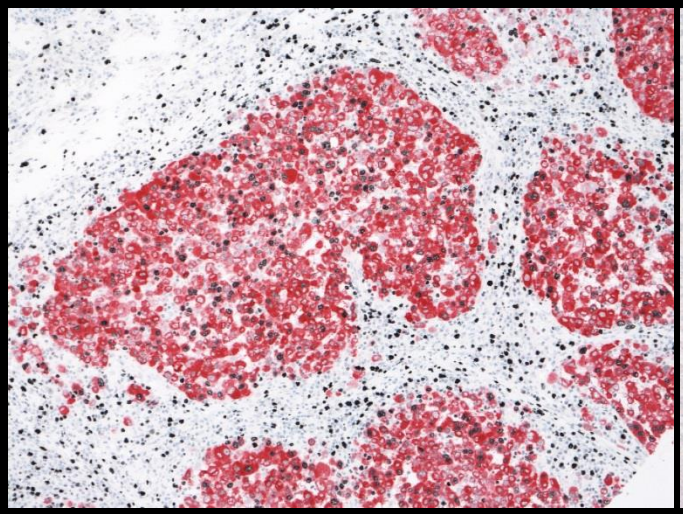
Omnis

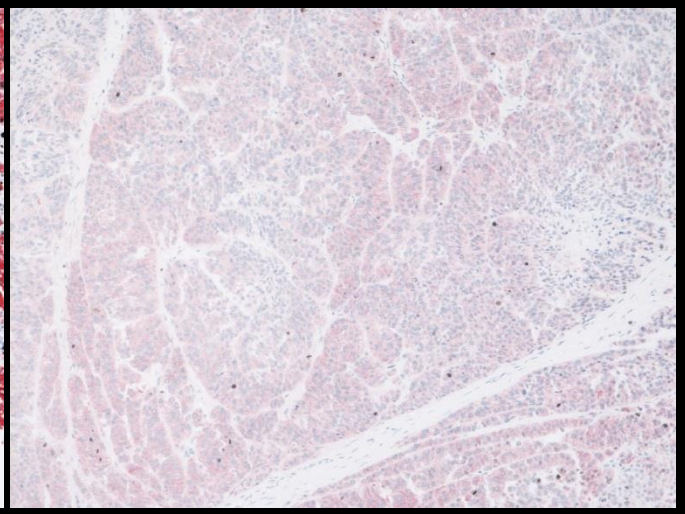
"closed system"

Ki67, SP6 (1:50)
MART1, EP43 (1:25 RR)

EnVision G2- double stain

DSB 2x5`
WR 20` or PR 20`





G/2 double stain versus In-House develop protocol

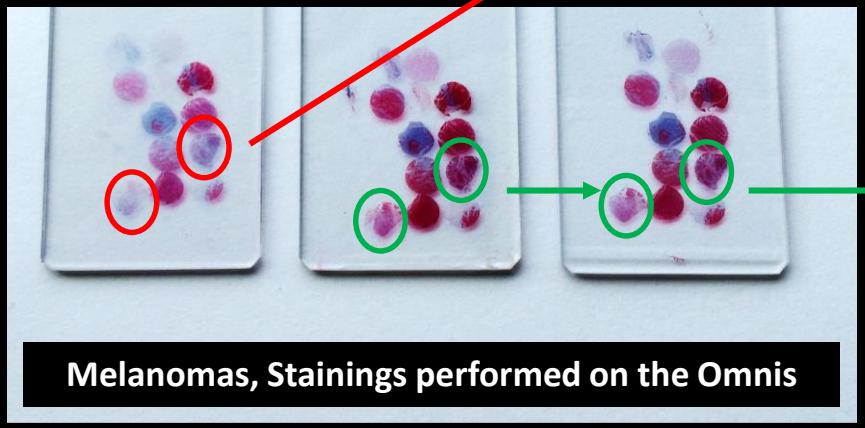
Omission of first primary ab + MART1, EP43 (1:30 RR)

Substitution of the second set of immuno-reagents (Detection system & Chromogen)

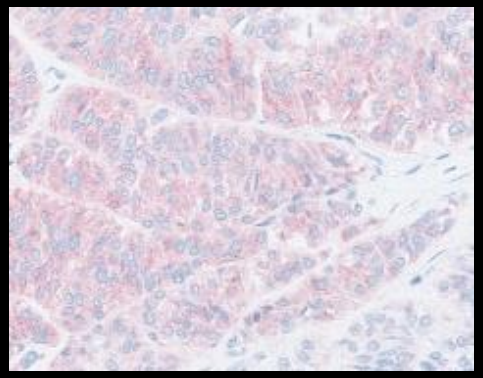
G/2 double stain + PR

HiDEF /AP + PR

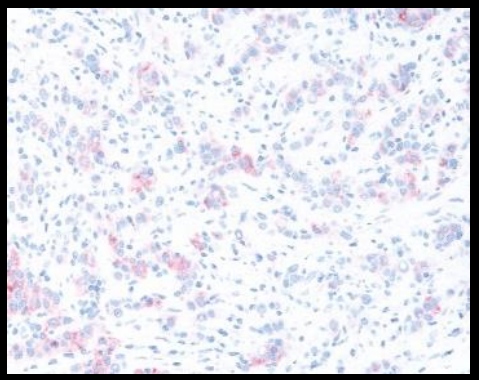
HiDEF /AP + WR



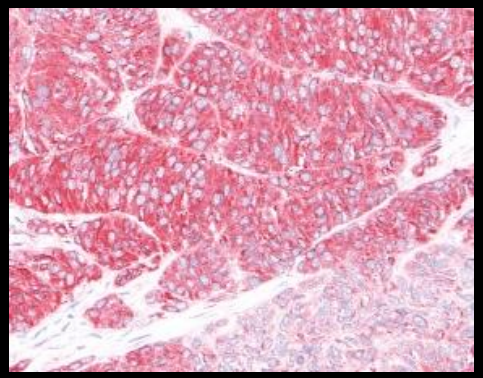
Melanomas, Stainings performed on the Omnis

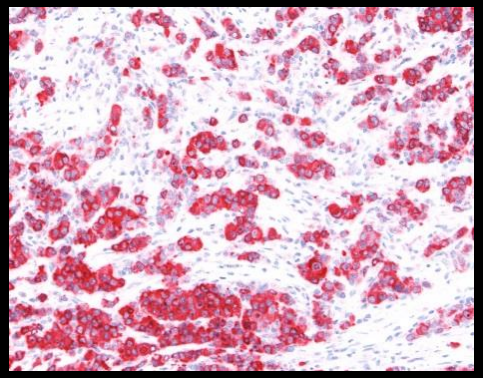


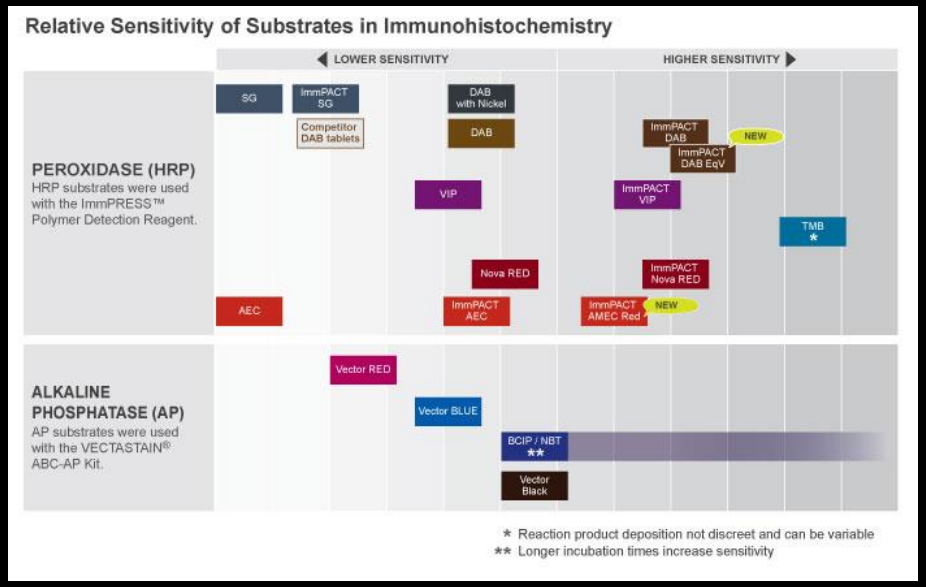
Melanoma 1



Melanoma 2







Second Substrate / First Substrate	Vector® Red (magenta) SK-5100	Vector® Blue (blue) SK-5300	BCIP/NBT (indigo) SK-5400	VIP/ ImmPACT™ VIP (purple) SK-4605, SK-4600	DAB/ ImmPACT™ DAB (brown) SK-4105, SK-4100	DAB-Ni (gray-black) SK-4100	NovaRED™/ ImmPACT™ NovaRED™ (red) SK-4805, SK-4800	SG/ ImmPACT™ SG (blue-gray) SK-4705, SK-4700	AEC/ ImmPACT™ AEC (red) SK-4205, SK-4200	ImmPACT™ AMEC Red (red) SK-4285
Vector® Red (magenta) Cat. No. SK-5100		–	–	–	+	+	–	+	–	–
Vector® Blue (blue) Cat. No. SK-5300	+		–	+	+	+	+	+	+	+
BCIP/NBT (indigo) Cat. No. SK-5400	+	–		+	+	+	+	+	+	+
VIP/ImmPACT™ VIP (purple) Cat. No. SK-4605, SK-4600	–	+	–		+	+	–	+	–	–
DAB/ImmPACT™ DAB (brown) Cat. No. SK-4105, SK-4100	+	+	+	+		–	–	+	+	+
DAB-Ni (gray-black) Cat. No. SK-4100	+	–	–	+	+		+	–	–	–
NovaRED™/ImmPACT™ NovaRED™ (red) Cat. No. SK-4805, SK-4800	–	+	+	–	+	+		+	–	–
SG/ImmPACT™ SG (blue-gray) Cat. No. SK-4705, SK-4700	+	–	–	+	+	–	–		+	+
AEC/ImmPACT™ AEC (red) Cat. No. SK-4205, SK-4200	–	–	–	–	+	–	–	+		–
ImmPACT™ AMEC Red (red) SK-4285	–	–	–	–	+	–	–	+	–	

Key: Alkaline Phosphatase | Peroxidase | + Indicates good contrast | – Indicates incompatiblity of substrates for various reasons

Chromogen reaction products

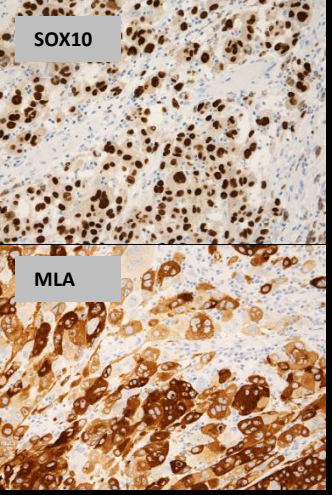
- ❑ Precise (specific)
- ❑ Sensitive
- ❑ Quick / Fast (short incubation time)
- ❑ Compatible with and easy to distinguish from counter staining
- ❑ Stabile and unaffected of the mounting procedure
- ❑ In double immune experiments: High color contrast between chromogens and allowing mixed colors at sites of co-localization

Efficiency depends on:

- ❑ Enzyme conjugate (e.g. HRP and/or AP)
- ❑ Chromogen type (e.g. BCIP/NBT)
- ❑ Incubation time
- ❑ Incubation temperature

MACH2 Double Staining: Sox-10, EP238 (1:25) + MLA, A103 (1:25)

Melanoma

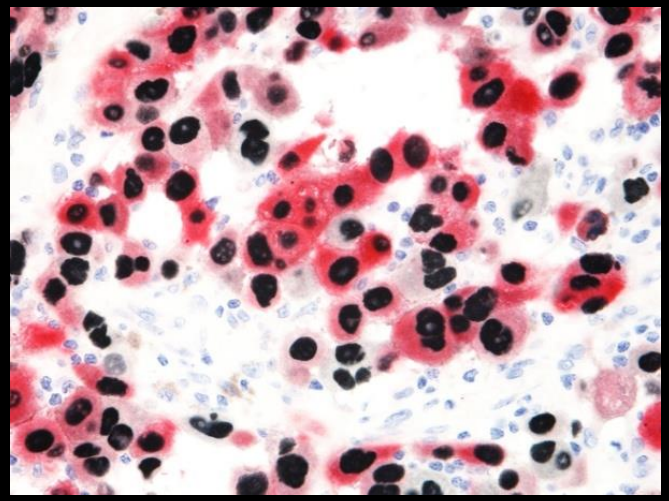


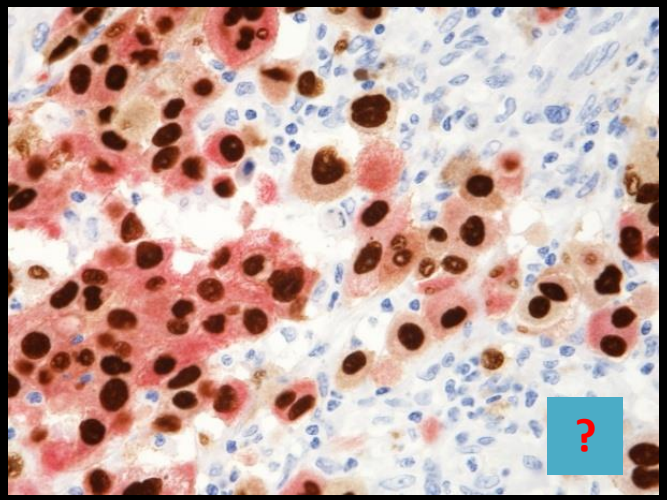
Deep Space Black 5` + Warp Red 2x8`

DAB (BZ) 5` + Warp Red 7`
Recommended protocols (Vendor)

Double stain 1

Anti-Rab/HRP + Anti-Mab/AP

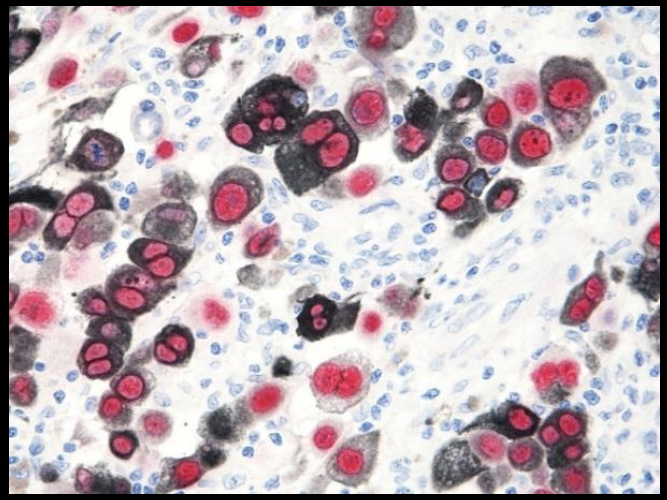


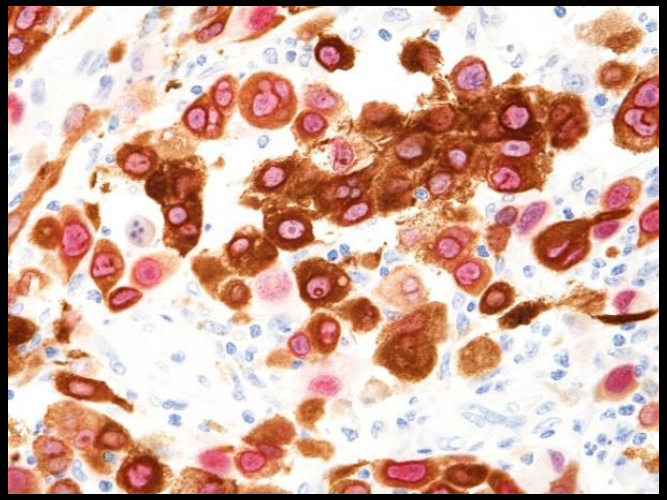


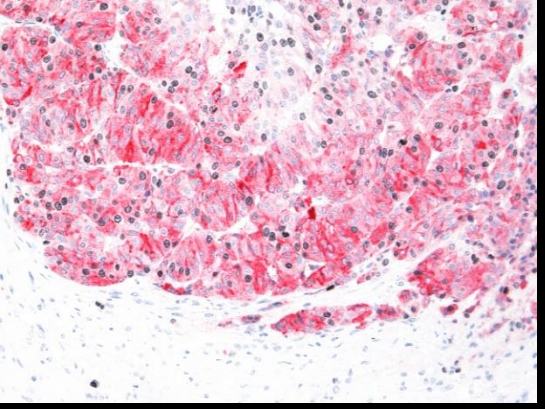
Color contrast`s ?

Double stain 2

Anti-Rab/AP + Anti-Mab/HRP



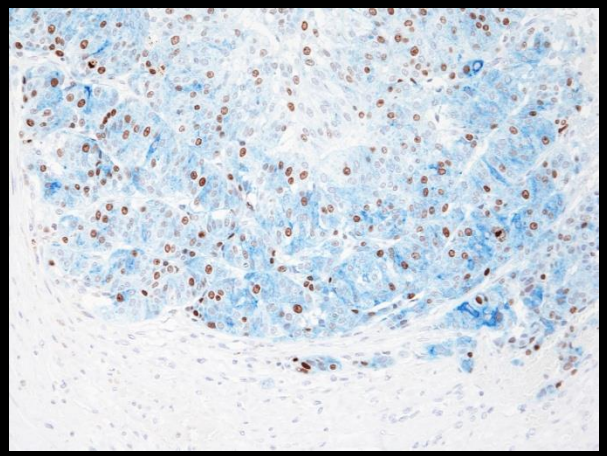
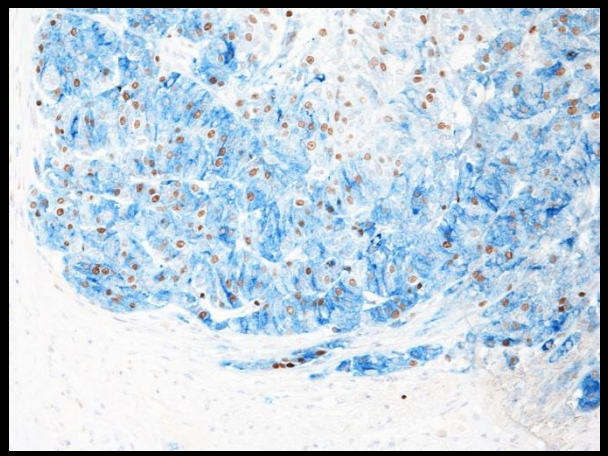
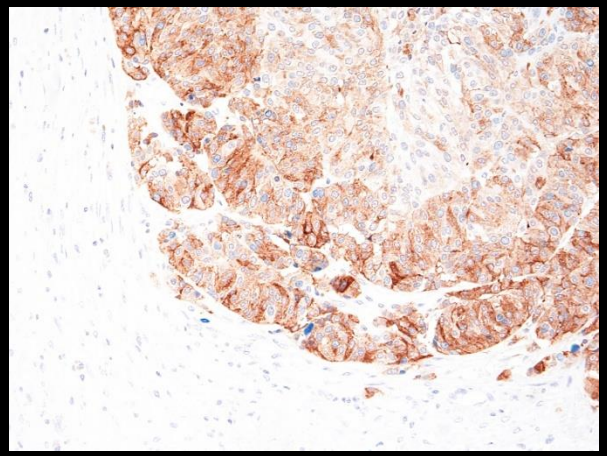
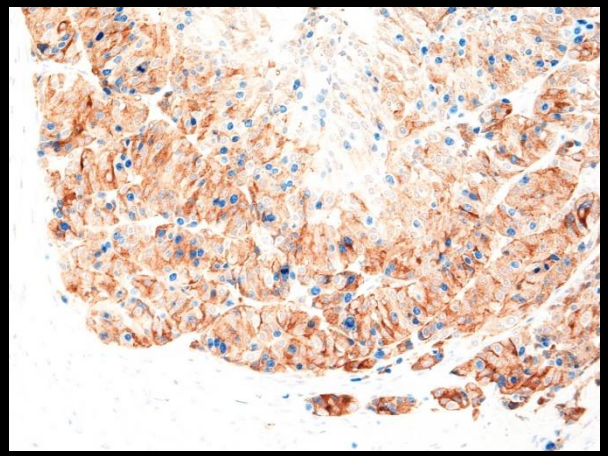




Sensitivity of the detection and visualization systems

Ki67 (SP6) 1:25 + MLA (A103) 1:25

Melanoma

	LVBlue , 10min at RT	LVBlue , 2x10min at RT
MultiVision 1 / TS (simultaneous) Rabbit-HRP/Mouse-AP LVBlue &LVRed		
MultiVision 2 / TS (simultaneous) Rabbit-AP/Mouse-HRP LVBlue &LVRed		

A simple approach to double immuno-staining

Are antigens of interest located in the same cellular compartment (e.g. the nuclei`s) ?

NO

Use a DAB based sequential or a simultaneous technique

Enzyme conjugates

Conventional bright field microscopy

Include controls

YES

Use a immuno-fluorescence simultaneous technique.

Fluorochrome conjugates

Fluorescence microscopy

Mixed colors will be present at sites of co-localization

Include controls

Co-localization using double immuno-enzymatic techniques ?

Only a few chromogen combinations fulfill the criteria of a good visual contrast between the basic colors and a good contrasting mixed color at sites of co-localization:

- Red–blue combination, composed of HRP activity visualized with amino-ethyl carbazole (AEC) and alkaline phosphatase (AP) activity visualized with naphthol- AS-MX-phosphate/Fast Blue BB

- Vector NovaRed (Vector Laboratories) and Vector Blue (VBlue) for HRP and AP activities, respectively (alternatively - MultiVision kit system for rabbit and mouse primaries including all chromogen reagents by Thermo Fisher Scientific (LabVision))

- Red–blue combination, composed of alkaline phosphatase (AP) activity visualized with VBlue and AP activity visualized with Liquid Permanent Red (Dako) – sequential technique with intermediary HIER step to block first set of immuno-reagents

- Red–turquoise color combination composed of AP and beta-galactosidase activities, respectively. (Compared with most HRP and AP reaction products, beta-galactosidase activity visualized by X-gal/ferro-ferri cyanide is relatively insensitive/inefficient and also quite diffusely localized)

Van der Loos CM. The J Histotechnol 33 (1): 31-40, 2010

Van der Loos CM et al. J Histotechnol; 31: 119-127, 2008

Simultaneous double immuno-enzymatic technique

Simultaneous Double Immuno-labelling procedure

MultiVision procedure (Labvision)

Pre-treatment (Antigen Retrieval)

Incubation with mix of primary Abs (Rab+Mab)

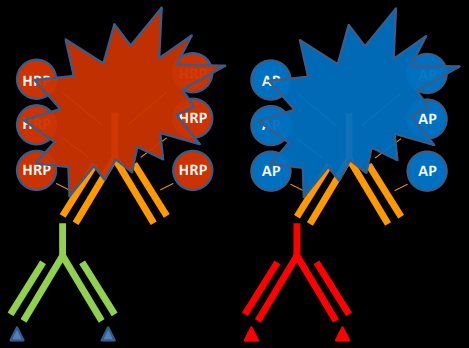
Incubation with Multivision (anti -Mab/HRP + anti -Rab/AP)

Incubation with HRP substrate + Chromogen LVRed

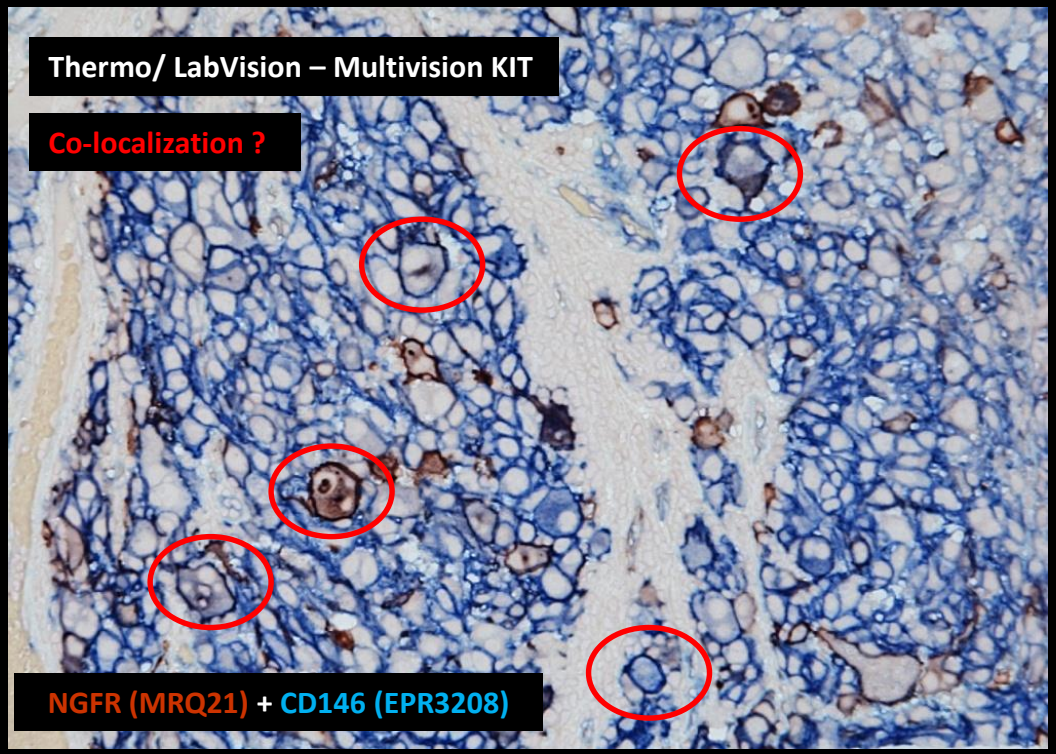
Incubation with AP substrate + Chromogen (LBV Blue)

Counter stain, mounting and Imaging / Interpretation

MultiVision HRP and AP polymer cocktail



Melanoma



J Pathol. 2000 Aug;191(4):452-61.

Double immunofluorescence labelling of routinely processed paraffin sections.

Mason DY, Micklem K, Jones M.

Argued that double immuno-enzymatic labelling of routinely processed tissue are :

- Time-consuming
- Prone to background staining
- Rarely suitable for detecting two antigens present at the same site- since one label tends to obscure the other.

Demonstrated that double immunofluorescence labelling :

- Is more rapid than enzyme-based techniques
- Avoids the problems of interpreting two antigens present at the same site
- Only minimal tissue autofluorescence was observed.

The double immunoflourescence procedure may represent the optimal technical approach to the co-localization of pairs of antigens in routinely processed tissue samples.

❑ Double immunofluorescence staining using simultaneous technique – Næstved LAB

- **Primary antibody cocktail is applied to the tissue at the same time (simultaneously)**

- **Primary antibodies must be of different host (e.g. mouse and rabbit) or at least different Ig-types, subclass or one primary Ab with a conjugate (e.g. biotin).**

- **Reactions is detected with a mix of secondary antibodies conjugated with two different fluorescent dyes (e.g. A488 and A594) and applied to the tissue at the same time (simultaneously).**

  To prevent cross-reaction between secondary antibodies raised against different species, it is recommended to apply second-step antibodies raised in the same host – if not possible, use pre-absorbed secondary antibodies.

- **Each antibody: antigen reaction will yield a specific color of staining that can be easily identified (Fluorescence microscopy with appropriate single, double or triple filters).**

- **Preferred method to detect targets in the same cell compartment (co-localized signal)**

Double immuno-fluorescence staining using simultaneous technique

Simultaneous procedure (Immuno-Fluorescence):

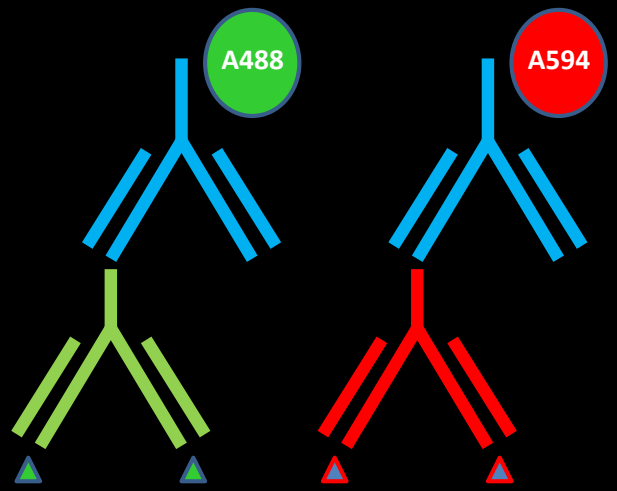
Pre-treatment (Antigen Retrieval)

Incubation with Primary Ab mix (e.g different host/ Mab+Rab) (1h).

Incubation with Secondary Ab mix (Goat -anti Mab+Rab) (1h).

Alexa Fluor 488 anti mouse Ig + Alexa Fluor 594 anti rabbit Ig

Dehydration + air-drying + coverslipping with Vectashield (+ DAPI)



- Goat anti mouse IgG (H+L):
  - Alexa Fluor 488 (Cat.no. A11001 / Molecular Probes)
  - TSA-Alexa 488 (Cat.no. T20912 / Molecular Probes)
- Goat anti rabbit IgG (H+L):
  - Alexa Fluor 594 (Cat.no. A11012 / Molecular Probes)
  - TSA-Alexa 594 (Cat.no. T20927 / Molecular Probes)

If required – increase sensitivity with Tyramide Signal Amplification (TSA-Alexa Fluor 488 anti mouse Ig **)**

NGFR (MRQ21) + CD146 (EPR3208)

NGFR (MRQ21) + CD146 (EPR3208)

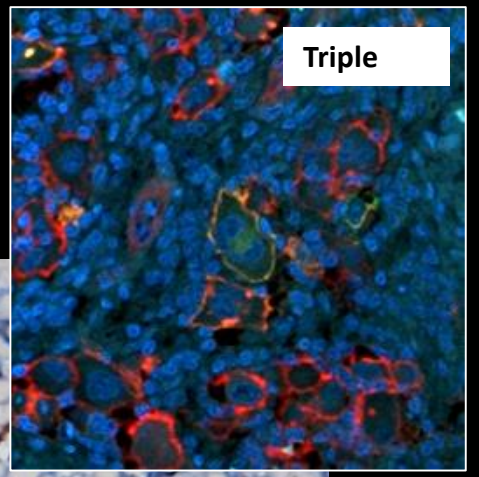
Double immuno-fluorescence technique

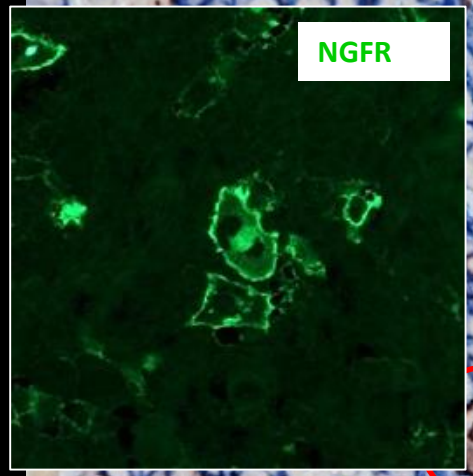
Melanoma

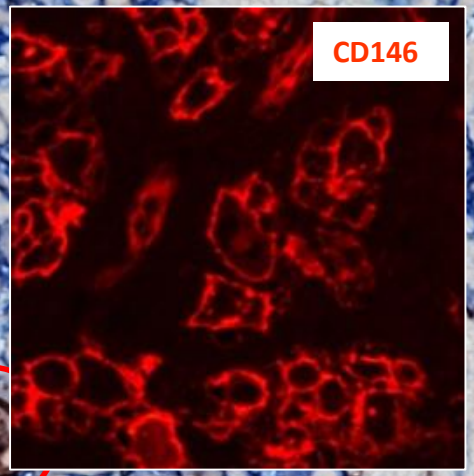
Melanoma

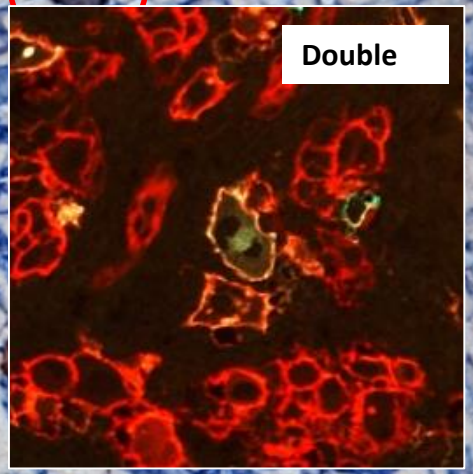
Thermo/ LabVision – Multivision KIT

Co-localization ?





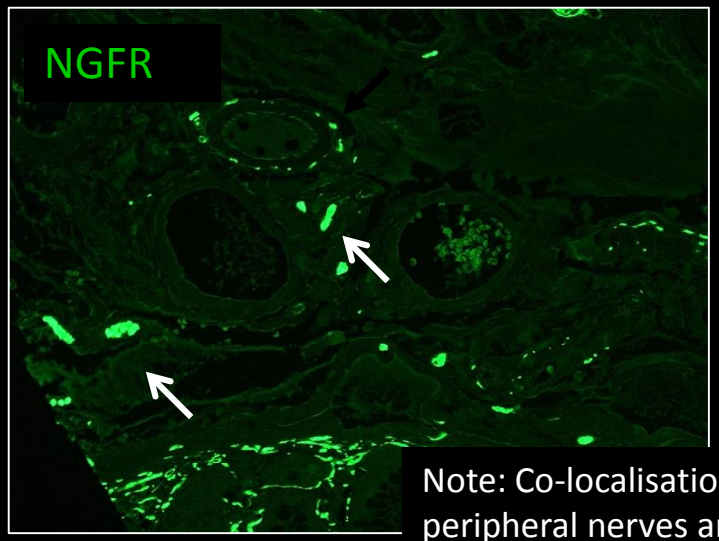


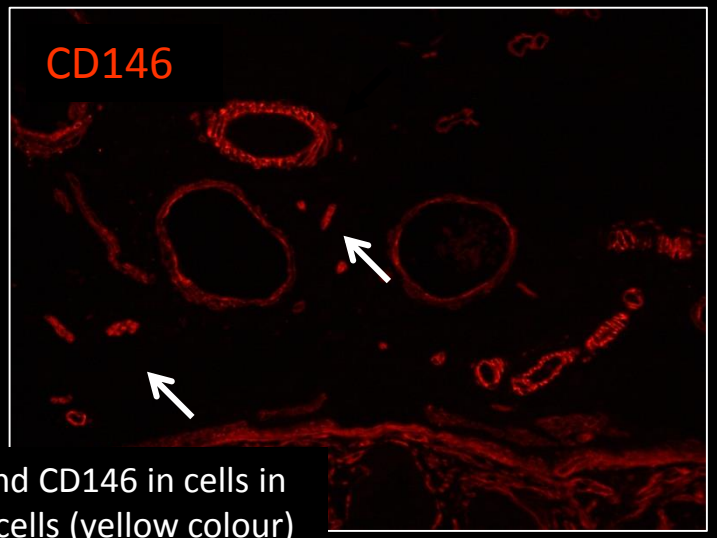


Note: Co-expression in tumour cells from melanoma (yellow colour).

NGFR (Mab, MRQ-21) + CD146 (Rab, EPR3208)

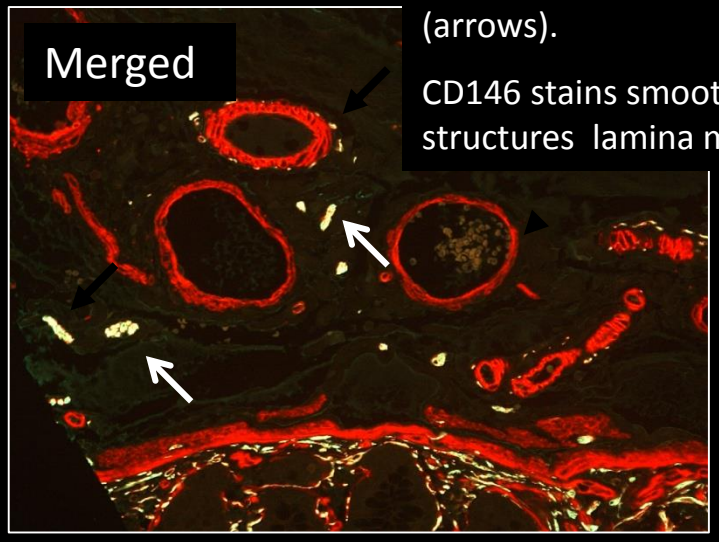
Bowell

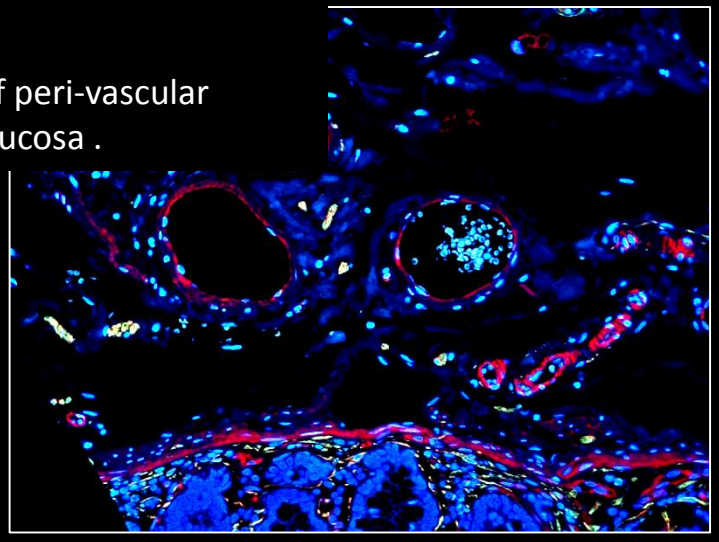




Note: Co-localisation of NGFR and CD146 in cells in peripheral nerves and ganglion cells (yellow colour) (arrows).

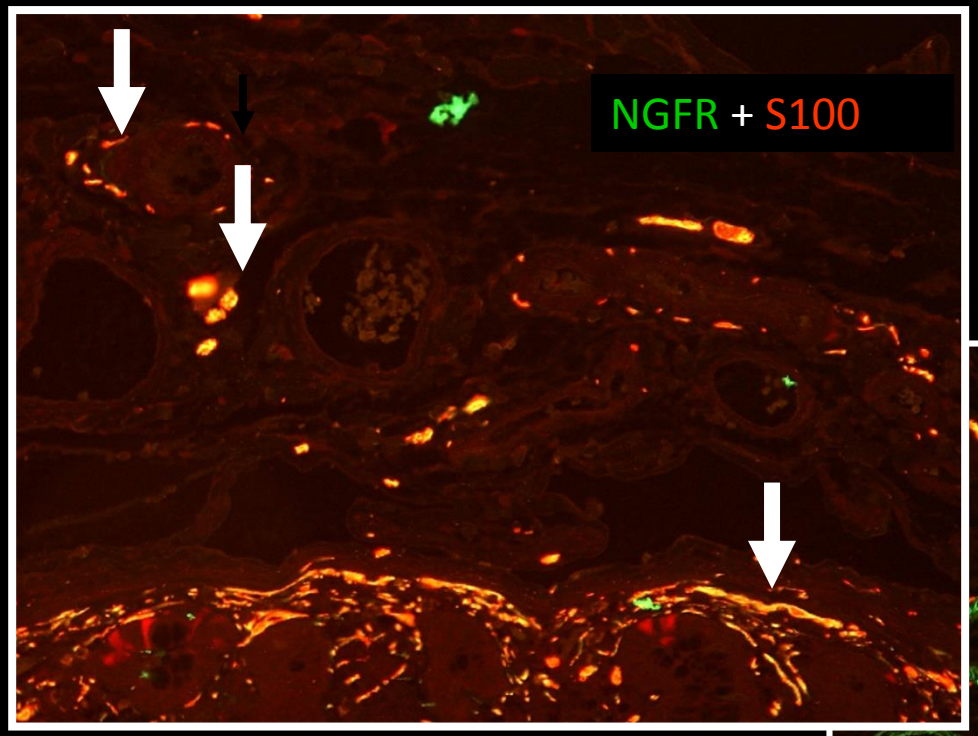
CD146 stains smooth muscles of peri-vascular structures lamina muscularis mucosa .



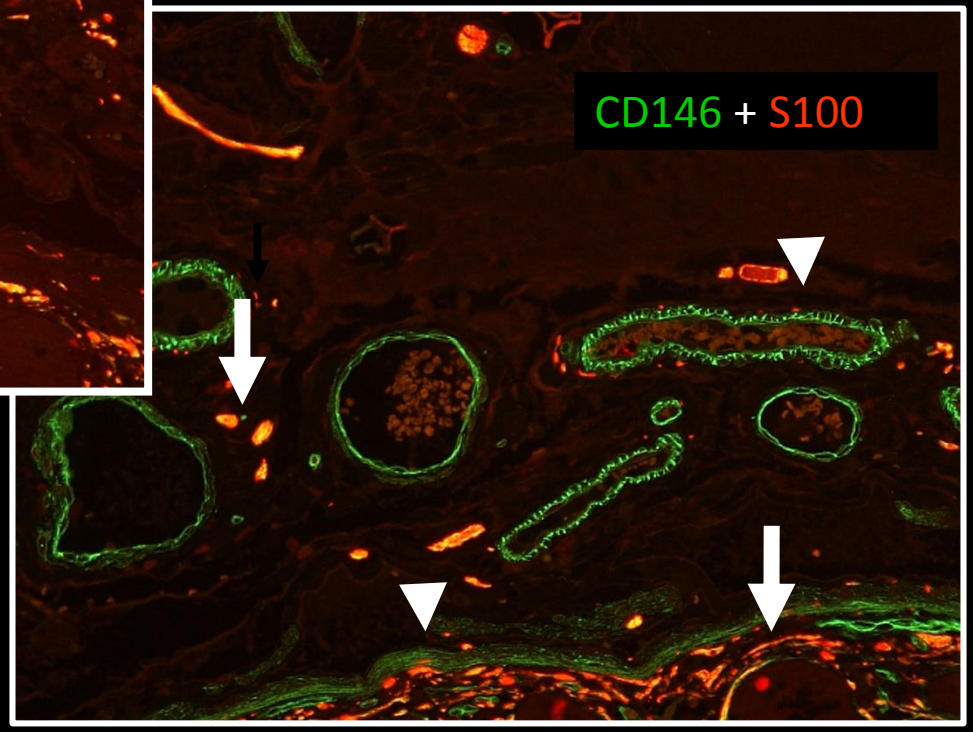


S100(Rab, poly) + CD146 (Mab, N1238) / NGFR (Mab, MRQ-21)

Bowell

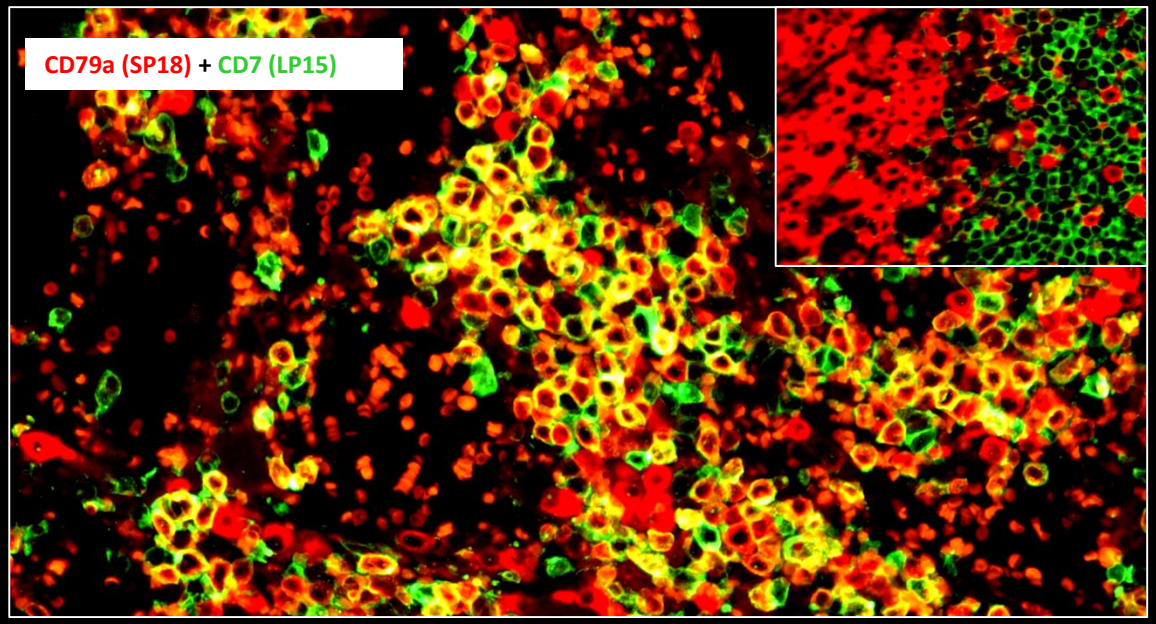


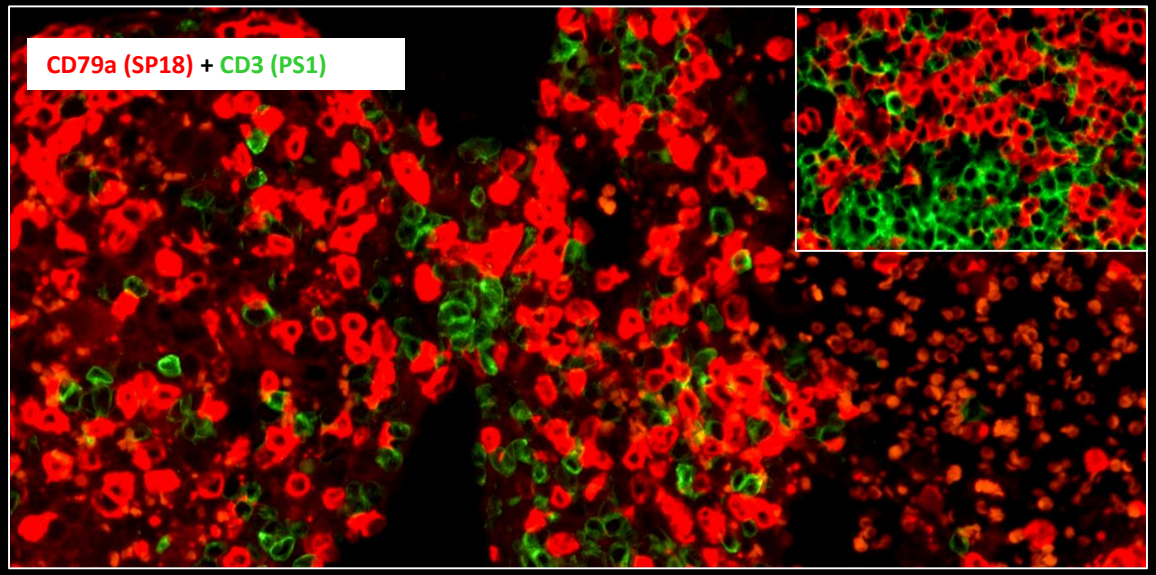
Note: Co-localization of NGFR and S100 in peripheral nerves and ganglion cells (arrows) (yellow colour) .



Note: Co-localization of CD146 and S100 in peripheral nerves and ganglion cells (arrows).

Also, CD146 stains peri-vascular structures and smooth muscles of lamina muscularis mucosa (arrowheads).





PT : ALL (B-type)

Flowcytometry showed aberrant expression of CD7 in neoplastic B-cells

Note: Aberrant co-localization between CD79a (B-cell marker) and CD7 (T-cell marker) of the neoplastic B-cells in the ALL

The reaction pattern between CD79 and CD3 showed expression of the respective markers in different cell types (neoplastic B-cells and T-cells).

Tonsil (insert`s)

Normal expression of the B-cell marker (CD79a) and the T-cell markers (CD7 or CD3). No co-localized signals

Double immuno-fluorescence staining using simultaneous technique

LAB Næstved

Simultaneous procedure using TSA amplification:

Exactly the same method as the "normal" double immufluorescence procedure except for introducing HRP in the secondary Ab mix and a final amplification step using Alexa Fluor 488 conj. Tyramide reagent.

Mechanism of Tyramide amplification:

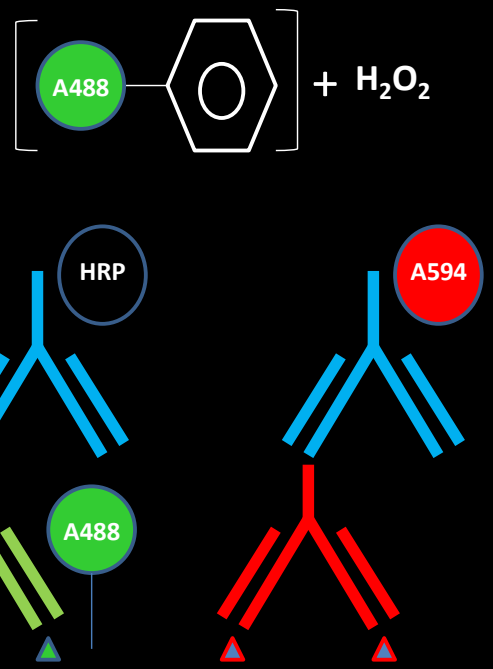
-Introducing HRP
-Incubation with A488 conjugated Tyramide and H_2O_2

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

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

- Deposit of A488 in close vicinity of Ab/Ag reactions

-Visualization of A488 deposit under fluorescence microscopy

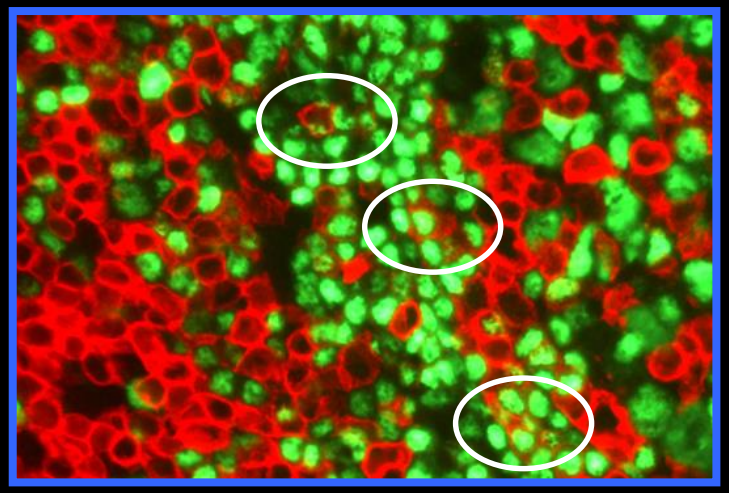


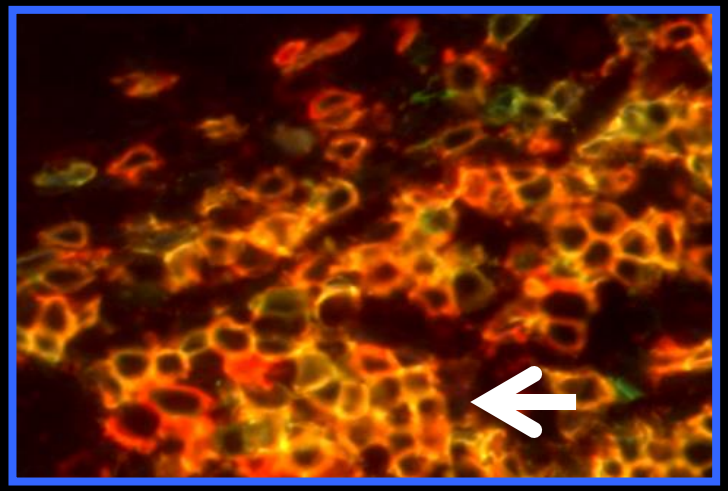
PAX-5, 1EW (TSA amp.) + CD5, SP19

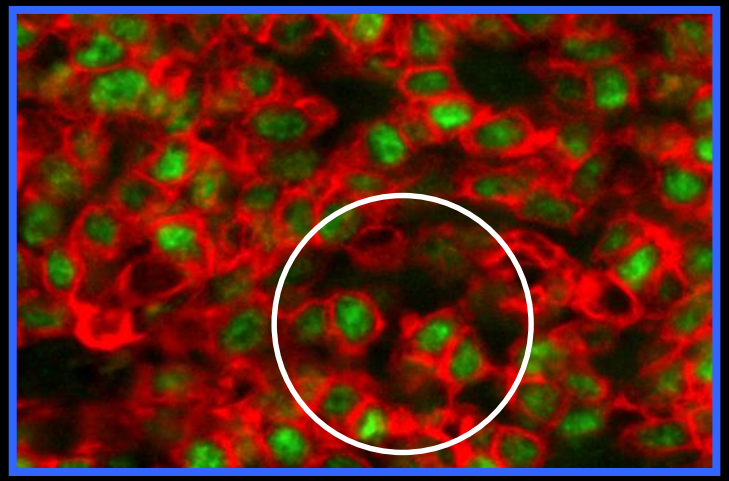
CD3, PS1 + CD5, SP19

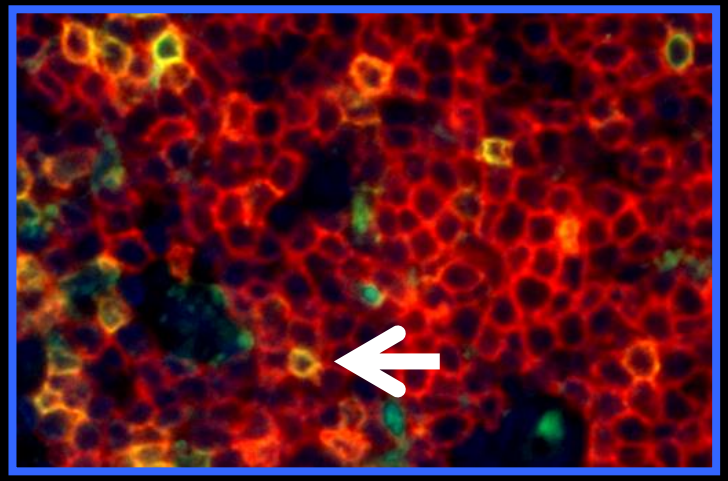
Tonsil

B-CLL









Note : Co-localization of normal T-cells in the tonsil and in the B-CLL (CD3+CD5) and co-expression of the neoplastic B-cell (Pax-5 and CD5) in the B-CLL and normal B-Cells in the mantle zone of the tonsil

Some final remarks to double immunofluorescence labeling using simultaneous technique:

Control of cross-reactivity between secondary Abs and primary Abs:

Test on tissue specimens in which two antigens is expressed in different cells and easy to differentiate (e.g. SMA and CD68)

Incubate with primary Ab-X followed by detection with secondary antibody anti-Y
= Negative

Incubate with primary Ab-Y followed by detection with secondary antibody anti-X
= Negative

Omission of primary Ab-X followed by detection with secondary antibody cocktails (anti-X and anti-Y)
= Only antigen Y should display a positive signal without any signs of co-localized signal

Omission of primary Ab-Y followed by detection with secondary antibody cocktails (anti-X and anti-Y)
= Only antigen X should display a positive signal without any signs of co-localized signal

Drawbacks of immunofluorescence techniques:

Fading of fluorescence signal upon storage

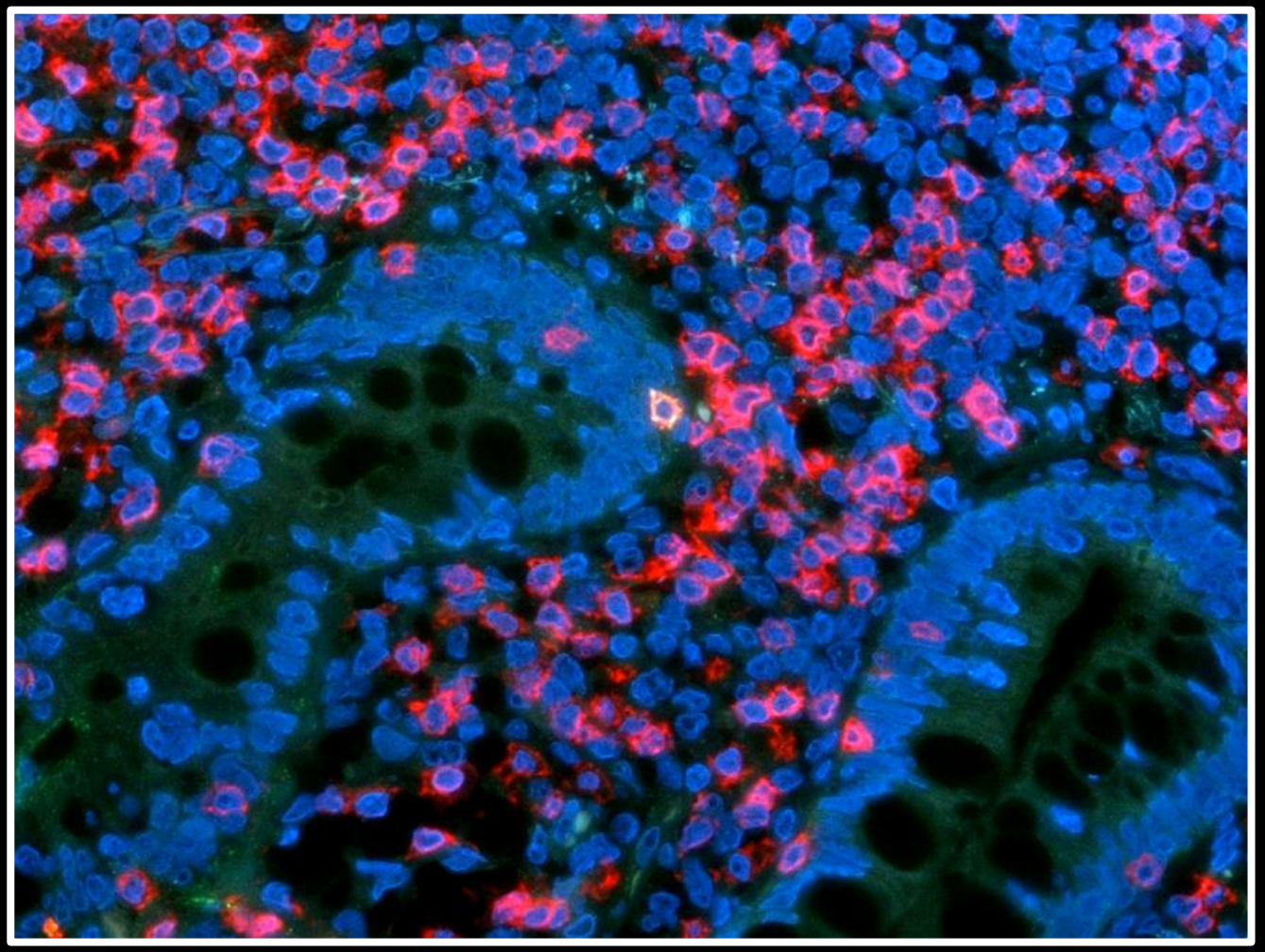
Quenching of fluorescence signal at excitation (fluorescence microscopy)

Auto fluorescence cause by formaldehyde fixation (especially connective tissue – collagen fibers)

Double immuno-staining - The basal fundament for a technical “optimal” performance:

- **Appropriate tissue fixation and processing**
  - Garbage in – Garbage out
- **Appropriate and efficient epitop retrieval**
  - Do the antigenic epitopes of interest require the same pre-treatment ?
- **Appropriate choice of antibody pairs / clones and dilutions**
  - Do the primary antibody pairs provide app. sensitivity & specificity depending on the chosen detection method ?
  - Is primary antibody pairs available in app. format depending on the chosen detection method ?
- **Robust, specific & sensitive double immuno - detection and visualization systems**
  - Sequential or simultaneous double immuno-staining techniques ?
  - For the routine: Is a double immuno - detection and visualization system (with appropriate sensitivity) always available ?
- **Appropriate choice of control material**
  - Calibrating the primary antibody concentrations carefully according to Critical Staining Quality Indicators (iCAPs)
    - will it work in practice or do I face new problems ?

Thank you for your attention



TCR γ (Mab,3.20) /TSA amp. + CD3 (Rab,SP19)

Triple staining using sequential technique (Immuno-enzymatic)

LAB Næstved

Sequential procedure (Immuno-emzymatic):

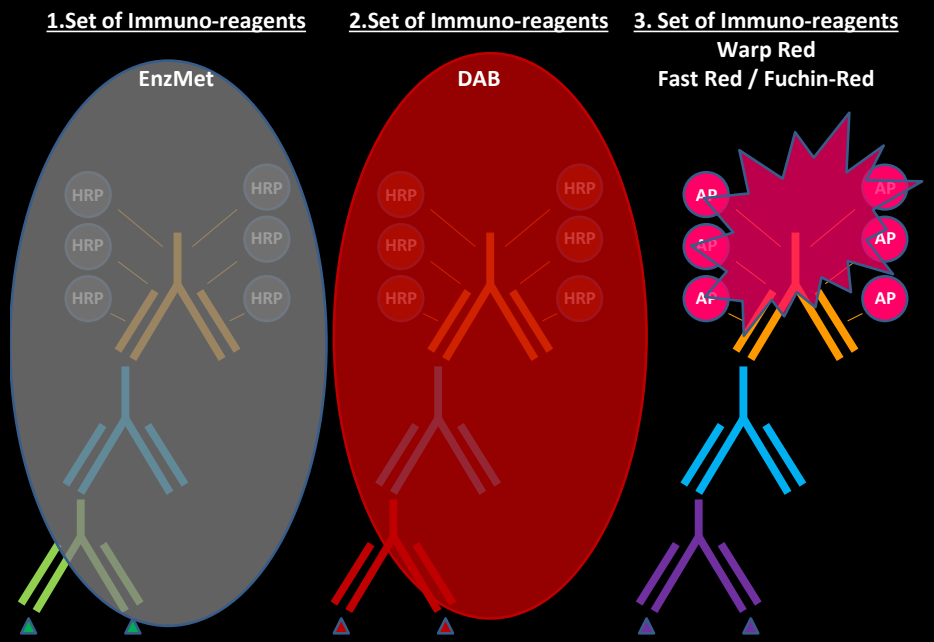
Pre-treatment (Antigen Retrieval)

First primary Ab (same or different host, Ig-type or subclass) (20 min).
Detection with Quanto/HRP (10+10 min).
Visualization with EnZMet (5 min).

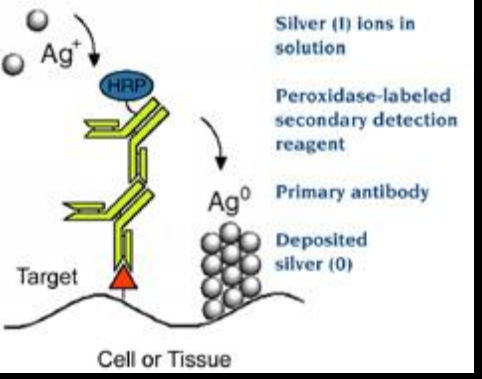
Second primary Ab (same or different host, Ig-type or subclass) (20 min).
Detection with Quanto/HRP (10+10 min).
Visualization with DAB (7min).

Third primary Ab (same or different host, Ig-type or subclass) (20 min).
Detection with Hi-Def /AP (10+10 min).
Visualization with Warp Red (Fuchin-Red) (7min).

Counterstain, dehydration and mounting.



EnzMet (silver) and DAB deposit (after 1. and 2. set of immuno-reagens) shelter / shields for un-specific reaction of the following primary antibodies and/or detection reagents.

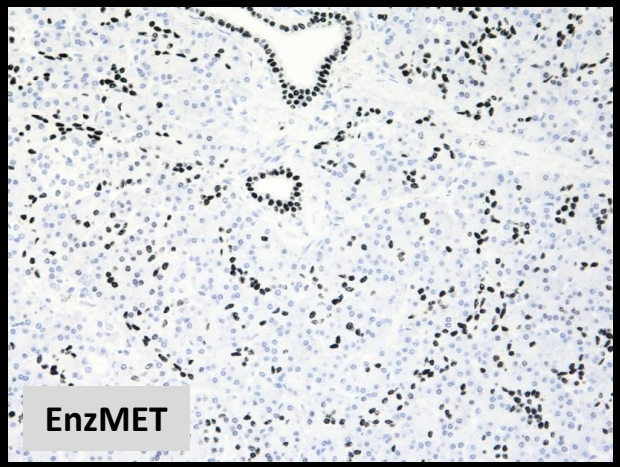


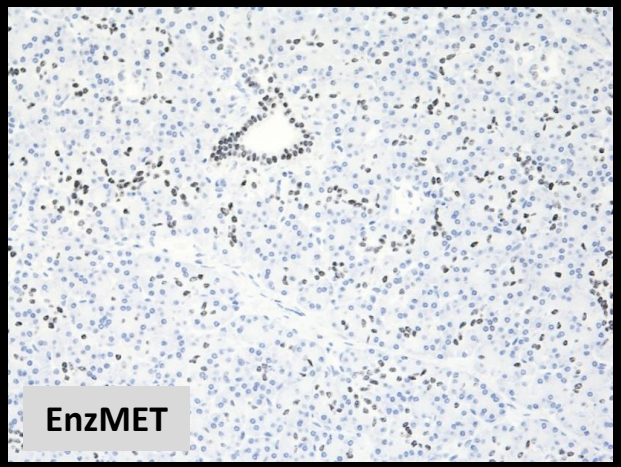
EnzMET versus DAB

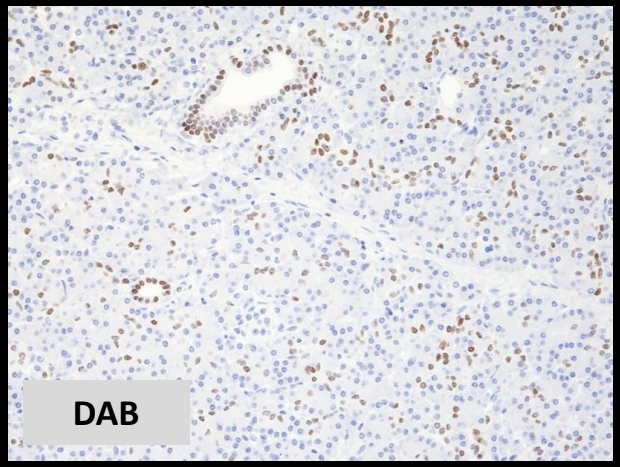
CDX-2, DAK-CDX2

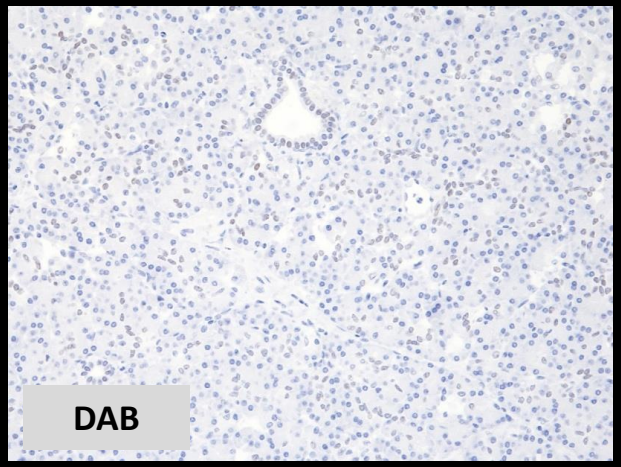
Pancreas

1:25 **1:100**



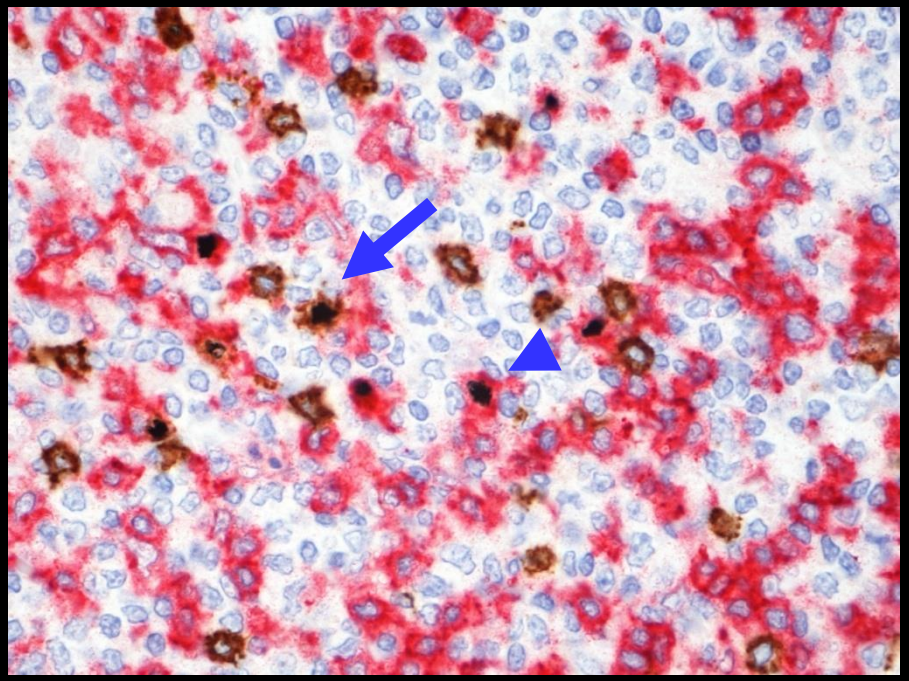


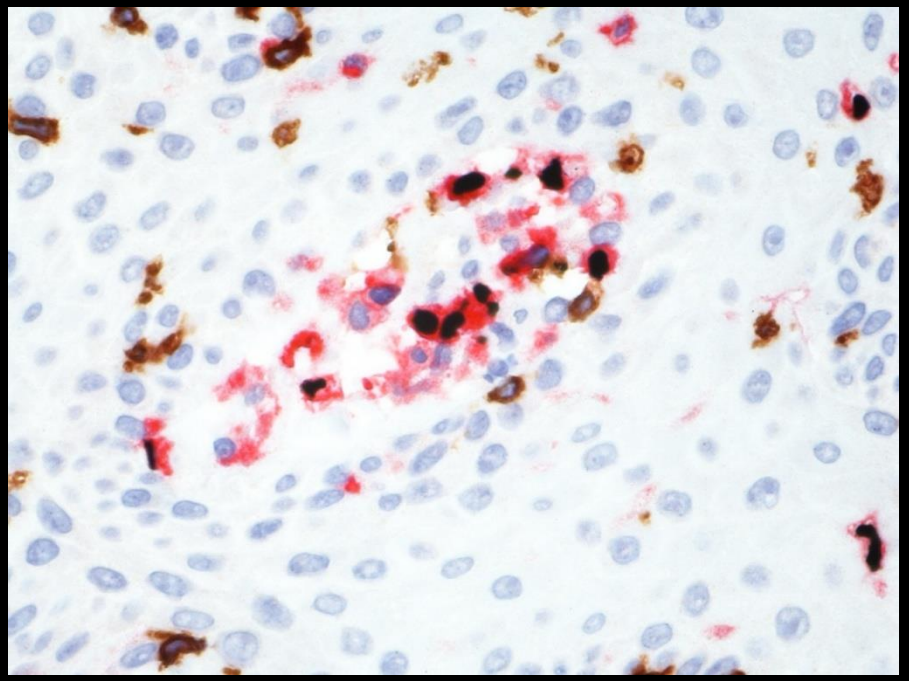




Triple staining using sequential technique (Immuno-enzymatic)

FoxP3 (236/E7) + CD8 (C8/144B) + CD4 (EPR6855) (Mab+Mab+Rab)





Tonsil

FoxP3 (black nuclear staining)

CD8 (brown membraneous/cytoplasmic staining, arrow)

CD4 (red membraneous/cytoplasmic staining, arrow-head)

Skin

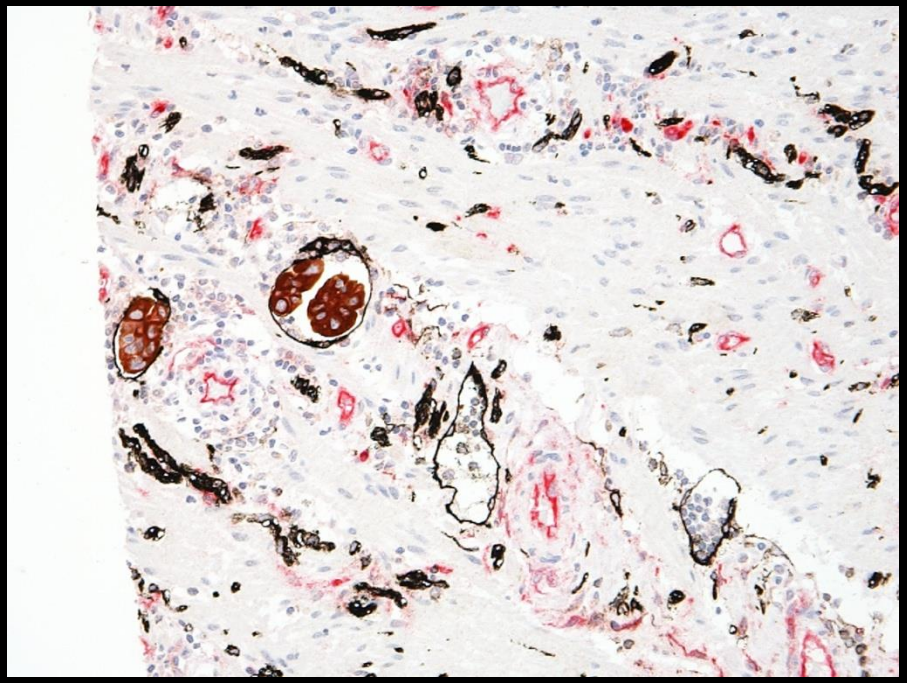
FoxP3 (black nuclear staining)

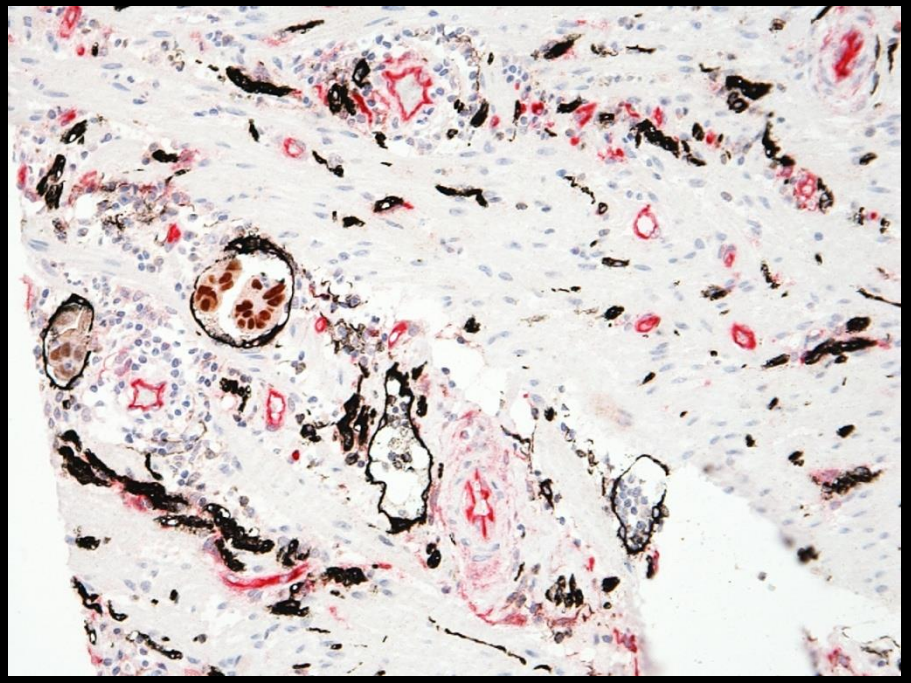
CD8 (brown membraneous/cytoplasmic staining, arrow)

CD4 (red membraneous/cytoplasmic staining, arrow-head)

Triple staining using sequential technique (Immuno-enzymatic)

Podoplanin (D2-40) + CDX-2(DAK-CDX2) or CK20 (K_s20.8) + CD34 (QBEND10) / (Mab x3)





Adenocarcinoma colon:

D2-40 (black lymph-endotheliale staining)

CK20 (brown cytoplasmic staining of the tumor cells)

CD34 (red membraneous endotheliale staining)

Adenocarcinoma colon:

D2-40 (black lymph-endotheliale staining)

Cdx-2 (brown nuclear staining of the tumor cells)

CD34 (red membraneous endotheliale staining)