2016 Annual Scientific Symposium

June 3 & 4

DoubleTree Hotel - Airport

Grand Rapids

Review of Basic Requirements/Recommendations for Positive and Negative Controls in IHC

Søren Nielsen
Project coordinator & Scheme Manager
NordiQC
Aalborg University Hospital, Denmark

NordiQC

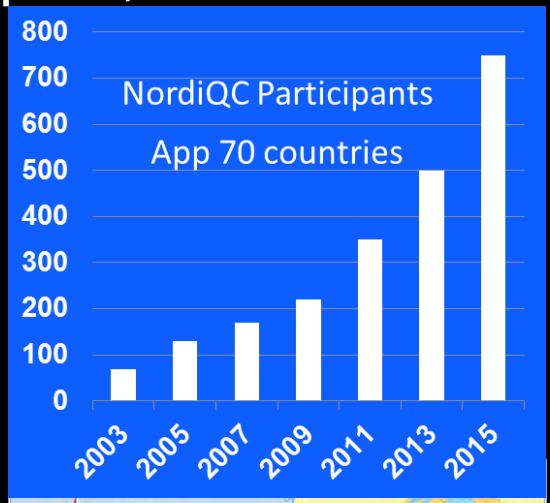
IHC – Biomarker controls

IHC project coordinator at Institute of Pathology, Aalborg, Denmark & Scheme manager NordiQC

- > 70.000 IHC slides annually
  - BenchMark Ultra, Ventana
  - Autostainer Link 48, Dako
  - Omnis, Dako
  - Bond III, Leica
- IHC cooperation partners
  - Biocare
  - Cell Marque
  - Dako / Agilent
  - Leica
  - Thermo Fisher
  - Ventana / Roche
  - + Ad hoc projects/partners

NordiQC Perspective

- International academic IHC proficiency testing program
- Founded 2003 by Nordic pathologists
- Independent non-profit organisation
- Institute of Pathology, Aalborg University Hospital, DK



- General module: 3 runs/year
  - 15-18 different markers
- Breast cancer IHC module: 2 runs/y
  - 3-5 different markers (HER2, ER, PR,..)
- HER-2 ISH module: 2 runs/year
  - BRISH, FISH (breast cancer)
- Pilot runs ongoing
  - ALK (lung), PD-L1 (lung)

www.nordiqc.org

NordiQC Perspective

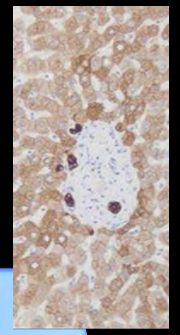
NordiQC EQA program – The short version:

Unstained slides of FFPE TMAs are circulated to participating labs

Labs perform IHC/ISH with their normal protocol and return slides

The stained slides are evaluated by NordiQC assessors

Results are sent back to labs and overall data published at www.nordiqc.org



Access to protocols giving optimal results and recommended controls

NordiQC

Central assessment with consensus between experienced pathologists and histotechnologists

- Correlate IHC staining results with central protocol parameters in order to identify
  - Successful and less successful Abs
  - Appropriate and inappropriate protocol settings
  - Staining platform issues
  - Reliable control tissues

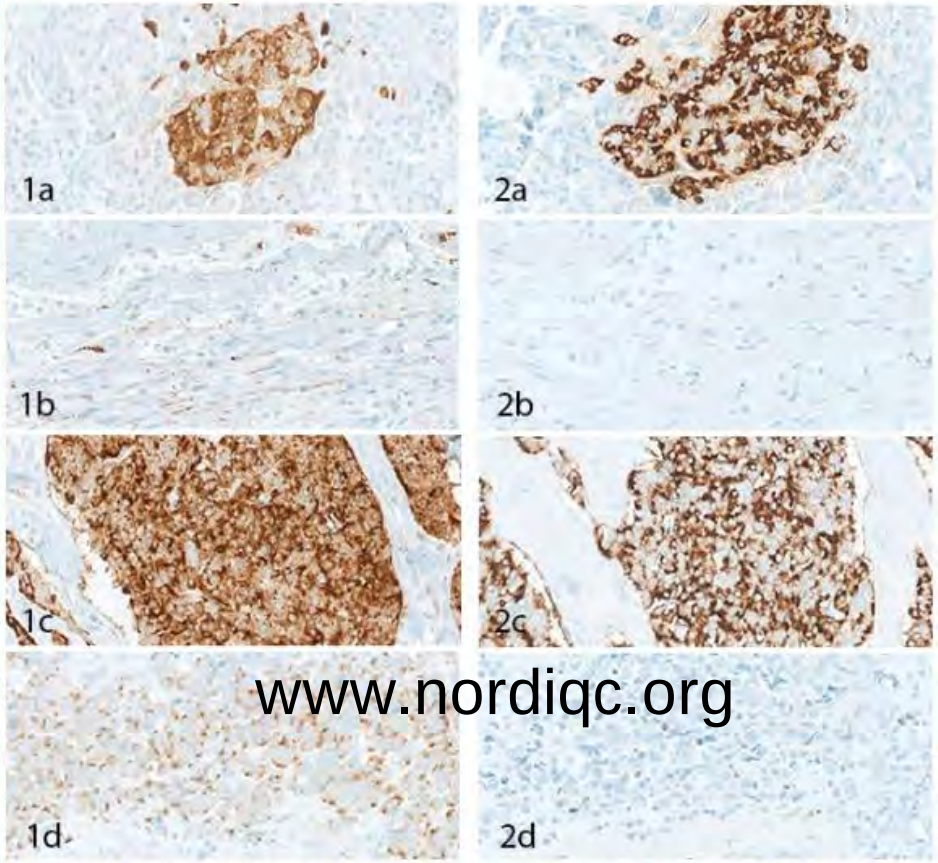
- Publish general results on an open website

- E-mail individual results to the participants
  - Specific explanations for insufficient results
  - Tailored recommendations for improvement

Nordic immunohistochemical Quality Control

NordiQC runs
Run 46 (general), **B21** (breast cancer) **and H9** (HER-2 ISH): Results are available, see **Newsletter**
Run 47 (general module): The website is opens for protocol submission: **Participation**
NordiQC teaching events
NordiQC Workshop in Diagnostic Immunohistochemistry Aalborg, Denmark, 19-21 September 2016
NordiQC Course in Diagnostic Immunohistochemistry for Pathologists Krakow, Poland, 12 - 14 October 2016

Figure: CGA assays with mAb LK2H10, 1a-d with HIER (as recommended by NordiQC), 2a-d without HIER (as recommended by vendor). a) pancreas islet, b) appendix muscularis with axons, c) endocrine carcinoma, d) small cell lung carcinoma. Witout HIER, the axons and the small cell carcinoma are false negative.



www.nordiqc.org

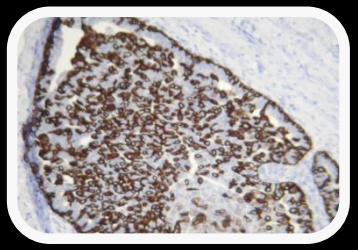
Update: 2016.04.21

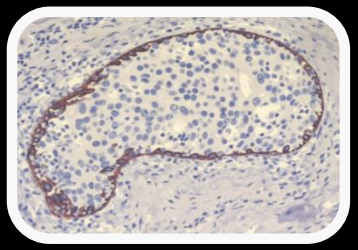
NordiQC is an independent scientific organization, promoting the quality of immunohistochemistry by arranging schemes for pathology laboratories, assessing tissue based assays, giving guidelines for improvement and providing good protocols.

IHC – Biomarker controls

Hyperplasia or In-situ

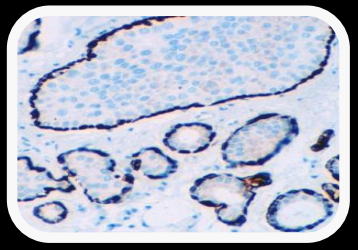
CK5, CK14, Heavy chain myosin, p63

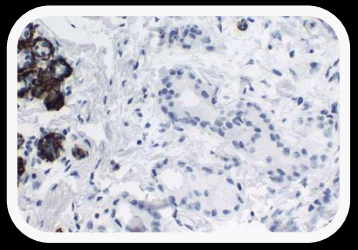




In-situ or invasive

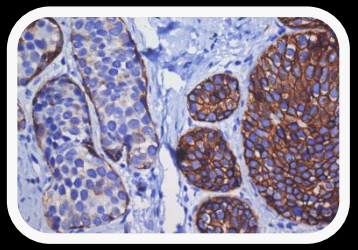
CK5, CK14, Heavy chain myosin, p63

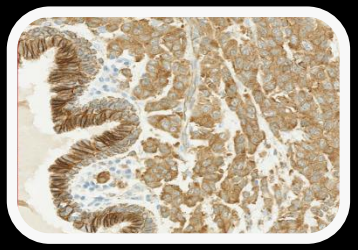




Lobular or ductal lesion

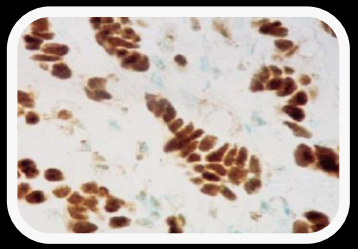
E-cadherin, p120

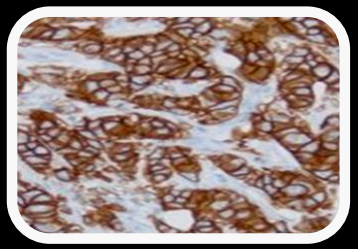




Predictive - Prognostic

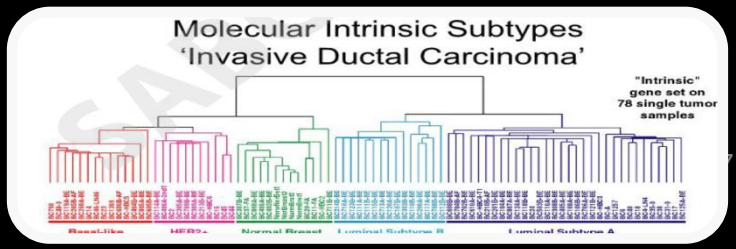
ER, PR, HER2, Ki67





Intrinsic subtype

PAM50 – *ER, PR, HER2, Ki67, CK5*



IHC – Biomarker controls

Original nomenclature and grouping of IHC tests:

- Class I IHC tests: Interpreted in the context of histo- or cytomorphologic and clinical data. Results interpreted and used by pathologists. E.g. CD45, TTF1, SOX10, CDX2, p40 etc

- Class II IHC tests: Stand-alone tests being interpreted (largely) to provide predictive and prognostic information. Results interpreted by pathologists and used by clinicians to give tailored treatment. E.g. ER, PR, HER2, CD117 etc .


AJCP / Special Article

Am J Clin Pathol 2010;133:354-365

Canadian Association of Pathologists–Association canadienne des pathologistes National Standards Committee/Immunohistochemistry

Best Practice Recommendations for Standardization of Immunohistochemistry Tests*

Emina Emilia Torlakovic, MD, PhD,[1] Robert Riddell, MD, FRCPath, FRCPC,[2] Diponkar Banerjee, MBChB, FRCPC, PhD,[3] Hala El-Zimaity, MD, MS, FRCPC,[4] Dragana Pilavdzic, MD, FRCPC,[5] Peter Dawe, MS,[6] Anthony Magliocco, MD, FRCPC,[7] Penny Barnes, MD, FRCPC,[8] Richard Berendt, MD, FRCPC,[9] Donald Cook, MD, FRCPC,[10] Blake Gilks, MD, FRCPC,[11] Gaynor Williams, MD, PhD,[12] Bayardo Perez-Ordonez, MD, FRCPC,[13] Bret Wehrli, MD, FRCPC,[14] Paul E. Swanson, MD,[15] Christopher N. Otis, MD,[16] Søren Nielsen, HT, CT,[17] Mogens Vyberg, MD,[17] and Jagdish Butany, MBBS, MS, FRCPC[13]

CME/SAM

IHC – Biomarker controls

Class II (Class III, US), IHC companion diagnostics:

IHC test	Demonstration	Application
ER	Estrogen receptor protein	Breast cancer
HER2	Overexpression of HER2 protein	Breast cancer, gastric cancer
CD117	Protein second to gene mutation	GIST
EGFR	Overexpression of HER1 protein	Colorectal cancer
ALK	Fusion protein second to gene rearrangement	NSCLC
PD-L1	PD-L1 protein expression	NSCLC, Melanoma,….
…….		

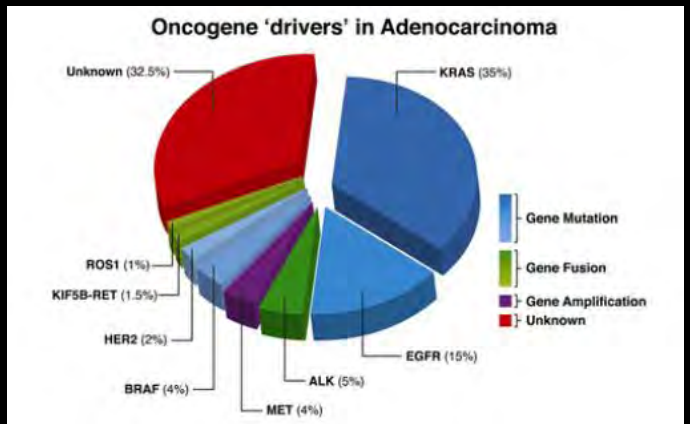
IHC – Biomarker controls

In practice more and more IHC tests become Class II tests: Directly indicated

	Area	Class I	Class II	Comment
CD20	Lymphoma	B-cell origin	Mabthera	Evaluation of theraphy
CD30	Lymphoma	HL, ALCL	Brentuximab	
CD56	Carcinoma	Neuroendo.	Lorvotuzumab	Class II: Lung SCLC
ALK	Lymphoma	ALCL	Crizotinib	Class II: Lung NSCLC

Indirectly indicated typically due to personalized treatment e.g.

	Area	Class I	Class II	Comment
p40 - lung	Carcinoma	Squamous		
TTF1- lung	Carcinoma	Adeno	Crizotinib,….	ALK, EGFR, ROS1…



IHC – Biomarker controls

... The biomarker protocol trap – Caution: not for faint-hearted lab personel !!!!!

Fixation
Time, Type, Volume

Decalcification
Preparation

Pre-analytic

Tissue
Type, Dimension,
Laser resection,
De-differentiation

Section
Thickness
Storage
Drying

Pre-treatment

Primary antibody
Clone, Dilution
Buffer, Time, Temp

Manual
Stainer

Analytic

Visualization
Sensitivity, Specificity

Development
Sensitivity,
Localization

With 3 choices for 5 variables in each phase = > 4 million protocols….

Controlment

Quantification
Reporting

Post-analytic

Interpretation
Localization
Positive/Negative - cut-off level

The right control material will expose right or wrong choices

IHC – Biomarker controls

- What is an IHC control in diagnostic IHC ?
- What is recommended and best practice ?
- What are the pitfalls for the use of IHC controls ?
- How can IHC controls be used by laboratories & EQA ?
- How to use IHC controls to implement new markers.

IHC – Biomarker controls

REVIEW ARTICLE

Appl Immunohistochem Mol Morphol . Volume 22, Number 4, October 2014

Standardization of Negative Controls in Diagnostic Immunohistochemistry: Recommendations From the International Ad Hoc Expert Panel

Emina E. Torlakovic, MD, PhD,†‡ Glenn Francis, MBBS, FRCPA, MBA, FFSc (RCPA),§‖¶ John Garratt, RT,†‡# Blake Gilks, MD, FRCPC,†‡** Elizabeth Hyjek, MD, PhD,* Merdol Ibrahim, PhD,†† Rodney Miller, MD,‡‡ Søren Nielsen, HT, CT,§§‖‖ Eugen B. Petcu, MD, PhD,§ Paul E. Swanson, MD,¶¶ Clive R. Taylor, MD, PhD,## and Mogens Vyberg, MD§§‖‖*

REVIEW ARTICLE

Appl Immunohistochem Mol Morphol • Volume 23, Number 1, January 2015

Standardization of Positive Controls in Diagnostic Immunohistochemistry: Recommendations From the International Ad Hoc Expert Committee

Emina E. Torlakovic, MD, PhD,† Søren Nielsen, HT, CT,‡§ Glenn Francis, MBBS, FRCPA, MBA, FFSc (RCPA),‖¶# John Garratt, RT,†** Blake Gilks, MD, FRCPC,† †† Jeffrey D. Goldsmith, MD,‡‡ Jason L. Hornick, MD, PhD,*§§ Elizabeth Hyjek, MD, PhD,* Merdol Ibrahim, PhD,‖‖ Keith Miller, FIBMS,‖‖ Eugen Petcu, MD, PhD,‖ Paul E. Swanson, MD,¶¶## Xiaoge Zhou, MD,***††† Clive R. Taylor, MD, PhD,‡‡‡ and Mogens Vyberg, MD‡§*

Abstract: Diagnostic immunohistochemistry (dIHC) has been practiced for several decades, with an ongoing expansion of applications for diagnostic use, and more recently for detection of prognostic and predictive biomarkers. However, stand-

mittee has clarified definitions of IHC assay sensitivity and specificity, with special emphasis on how these definitions apply to positive controls. Recommendations for "best laboratory practice" regarding positive controls for dIHC are specified. The first set of immunohistochemistry critical assay performance

IHC – Biomarker controls

Diagnostic Cytopathology, Vol 39, No 4

2011

Documentation of Immunocytochemistry Controls in the Cytopathologic Literature:
A Meta-Analysis of 100 Journal Articles

Carol Colasacco, M.L.I.S., S.C.T.(A.S.C.P.), C.T.(I.A.C.),[1*] Sharon Mount, M.D.,[1,2] and Gladwyn Leiman, M.B.B.C.H., F.I.A.C., F.R.C.Path.[1,2]

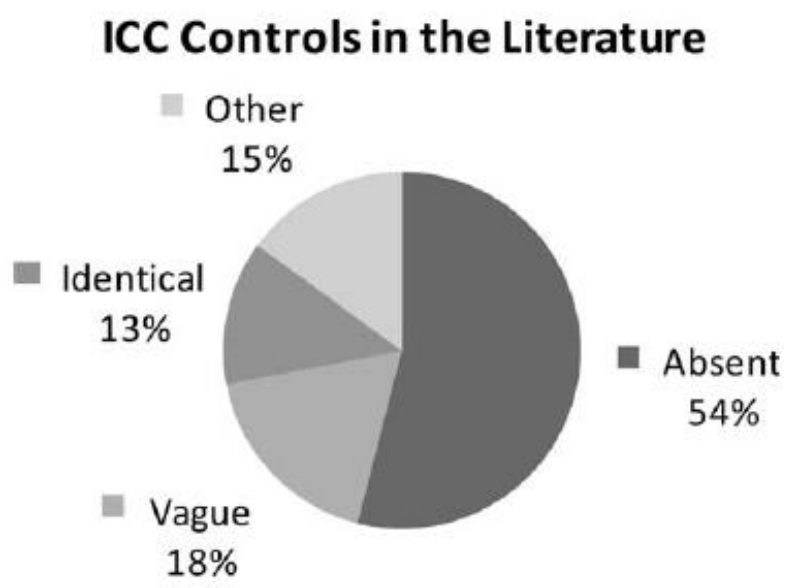


Fig. 1. Description of immunocytochemistry controls in articles reviewed.

Absent: Controls were not mentioned.

Vague: Statement such as "appropriate positive and negative controls were included."

Identical: Controls identical to study samples were described.

Other: Controls were dissimilar or partially similar (i.e., tissue control with smears or tissue control with cell block and ThinPrep samples run), or samples were too scant to include controls.

> 70 % of publications based on IHC do not describe controls used to verify data and conclusions....

IHC – Biomarker controls

J Neurooncol (2014) 119:39–47
DOI 10.1007/s11060-014-1459-5

1′ publication with this finding

LABORATORY INVESTIGATION

Till 2014; EpCAM not seen in glioma

The overexpression of Epithelial cell adhesion molecule (EpCAM) in glioma

Xin Chen · Wei-Yuan Ma · Shang-Chen Xu · Yu Liang · Yi-Bing Fu · Bo Pang · Tao Xin · Hai-Tao Fan · Rui Zhang · Jian-Gang Luo · Wen-Qing Kang · Min Wang · Qi Pang

"Immunohistochemistry results showed EpCAM was widely expressed in glioma (90.8 %).

The overall survival of WHO III and IV glioma patients with EpCAM overexpression was obviously lower than that without EpCAM overexpression. EpCAM overexpression was an independent prognostic factor for overall survival in glioma patients.

This study firstly shows that EpCAM overexpression correlates significantly with malignancy (WHO grades), proliferation (Ki67), angiogenesis (MVD), and prognosis in gliomas."

IHC – Biomarker controls

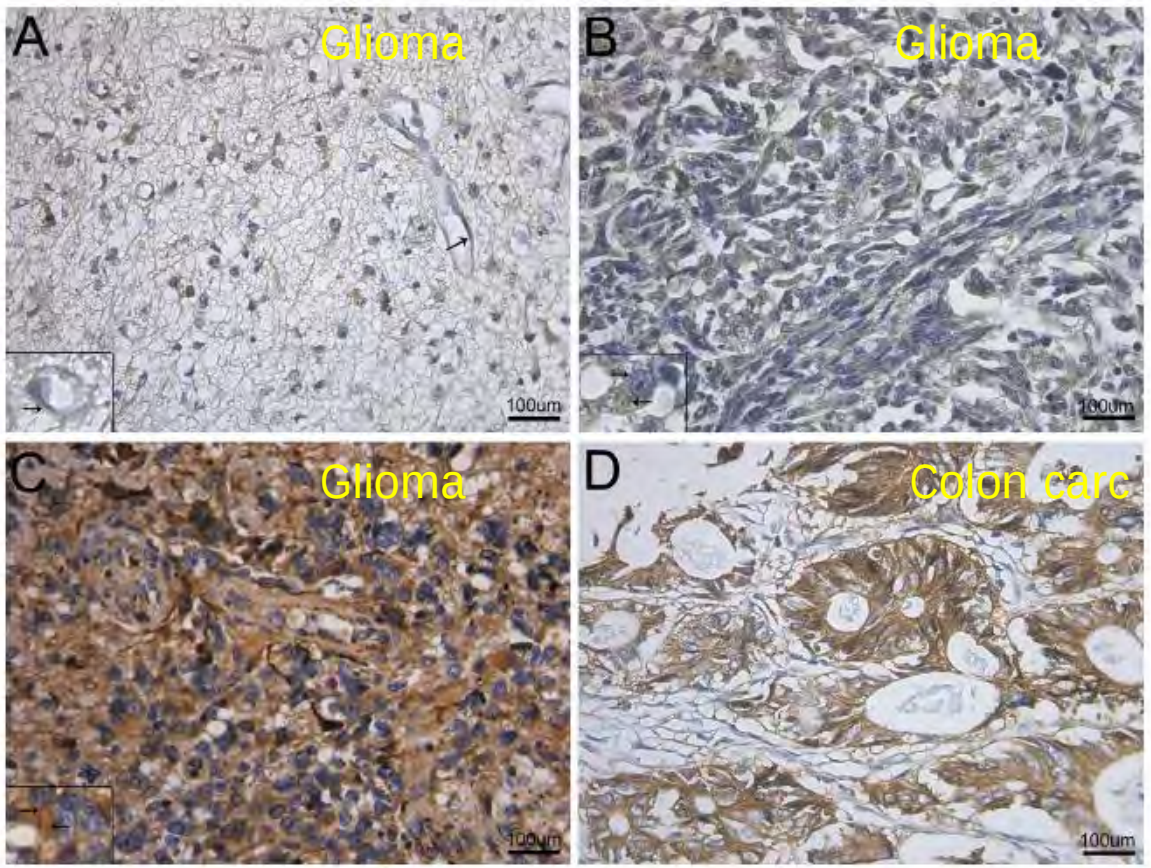


Fig. 1 Representative immunohistochemical staining for EpCAM (400×). Membranous and Cytoplasmic staining of EpCAM was observed in (a–c); a WHO grade II malignant glioma with weak EpCAM expression(TIS = 4), slant arrow shows EpCAM staining on epithelial cell; b WHO grade III malignant glioma with moderate EpCAM expression(TIS = 8); c WHO grade IV with intense EpCAM expression(TIS = 12). d intense membranous staining in intestine adenocarcinoma was showed as a positive control. Inserts show representative staining; Left-to-right arrows show membranous staining and right-to-left arrows show cytoplasmic staining. *WHO*, World Health Organization, *EpCAM* epithelial cell adhesion molecule, *TIS* total immunostaining score

Method – sensitivity, specificity – antibody, retrieval etc ?
Material – handling, processing, selected etc?
Interpretation – cut-off values, localization etc ?

IHC – Biomarker controls

Methods:

Polyclonal antibody towards EpCAM – Abcam ab 71916

- HIER Citrate pH 6 for 20 min. At 98°C
- 1:100, 16 hours incubation at 4°C
- 3-step polymer based detection system

Positive (tissue) control: Colon adenocarcinoma

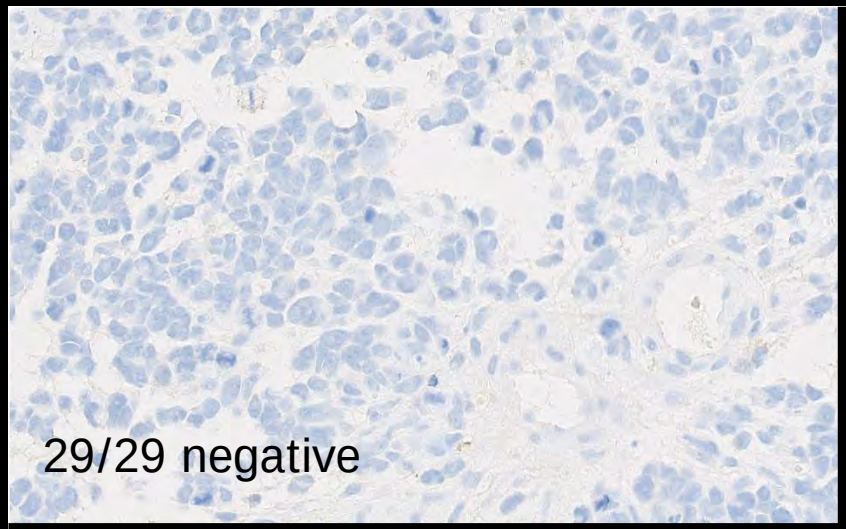
Negative (reagent) control: Omission of primary antibody

”Immunohistochemistry results showed EpCAM was widely expressed in glioma (90.8 %).”

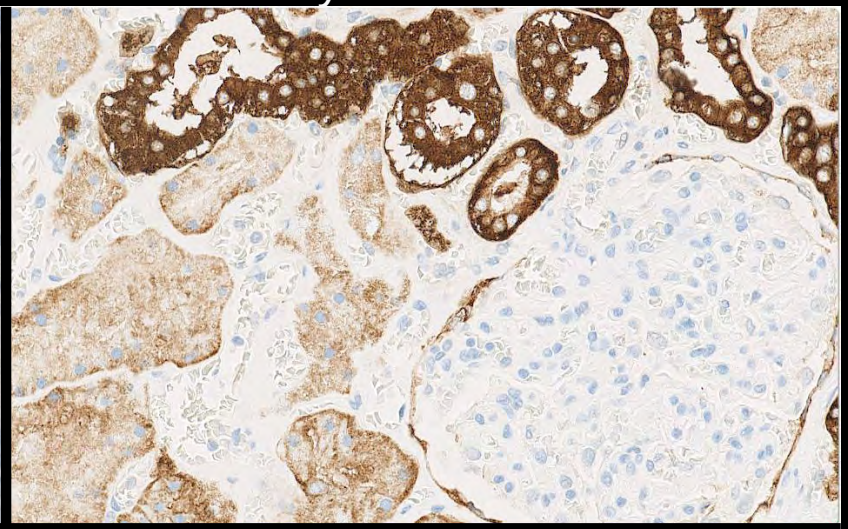
IHC – Biomarker controls

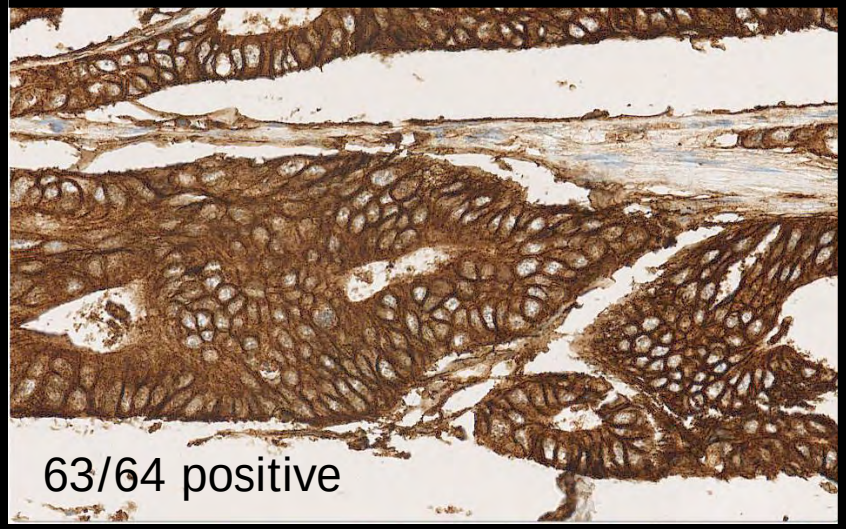
Ref. NordiQC: Ber-EP4: 1:50, HIER TRS pH 6.1, 3-step polymer

Glioma

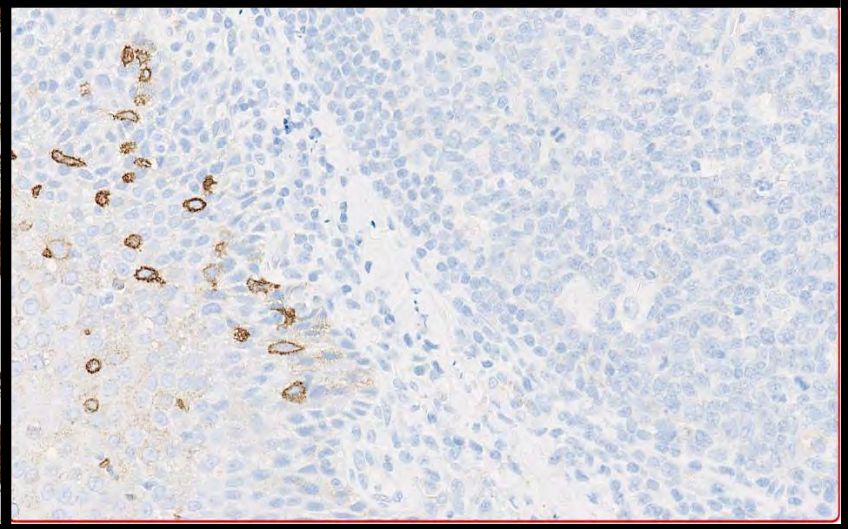


Kidney





Colon ad. carc.

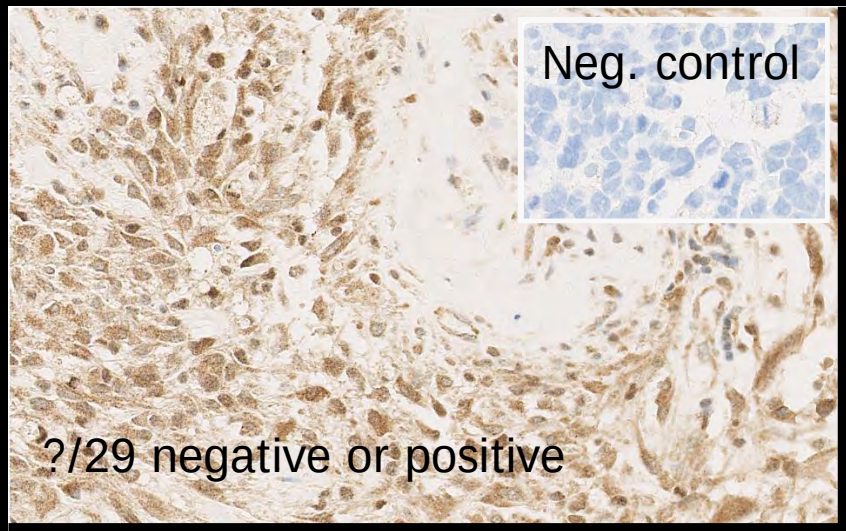


Tonsil

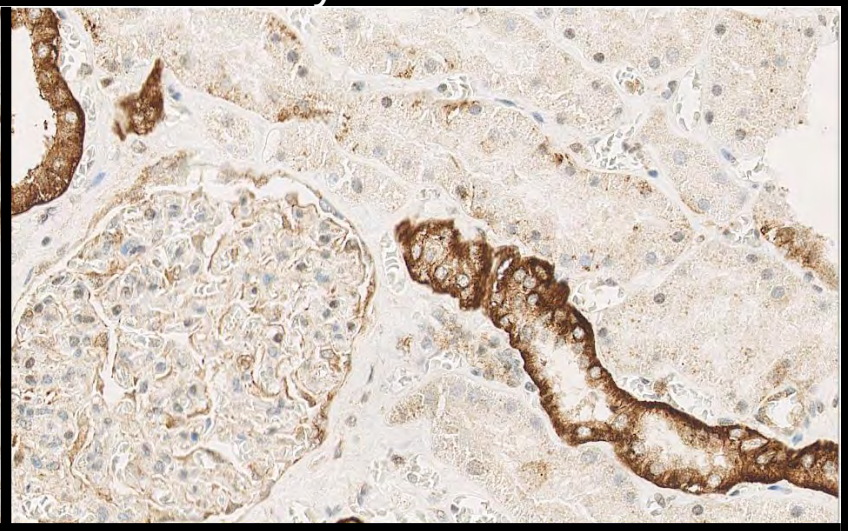
IHC – Biomarker controls

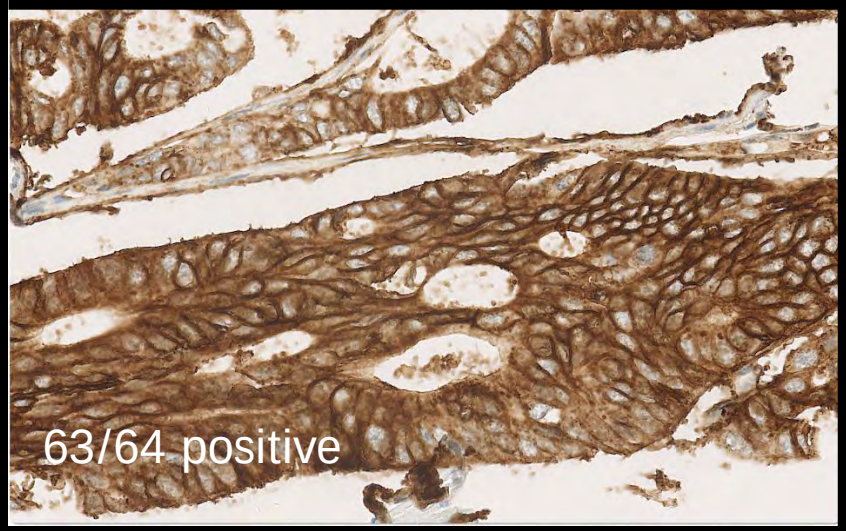
Study: Abcam ab 71916: 1:100, HIER TRS pH 6.1, 3-step polymer

Glioma



Kidney





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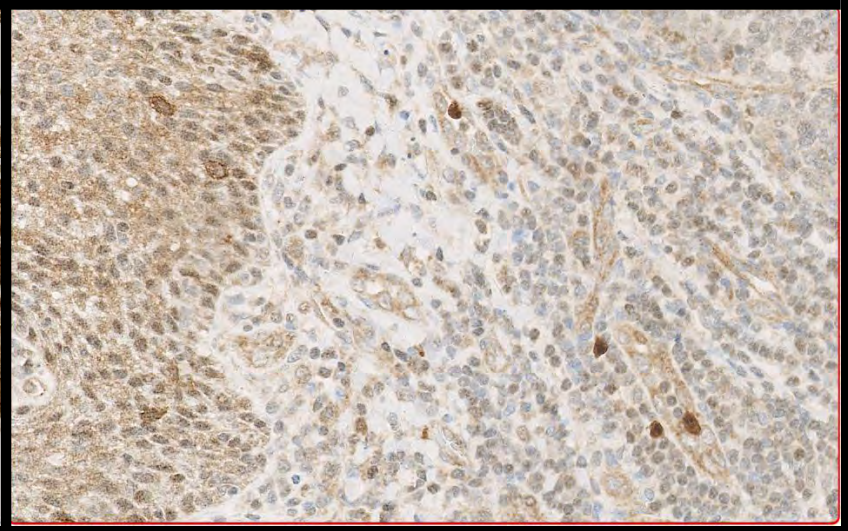
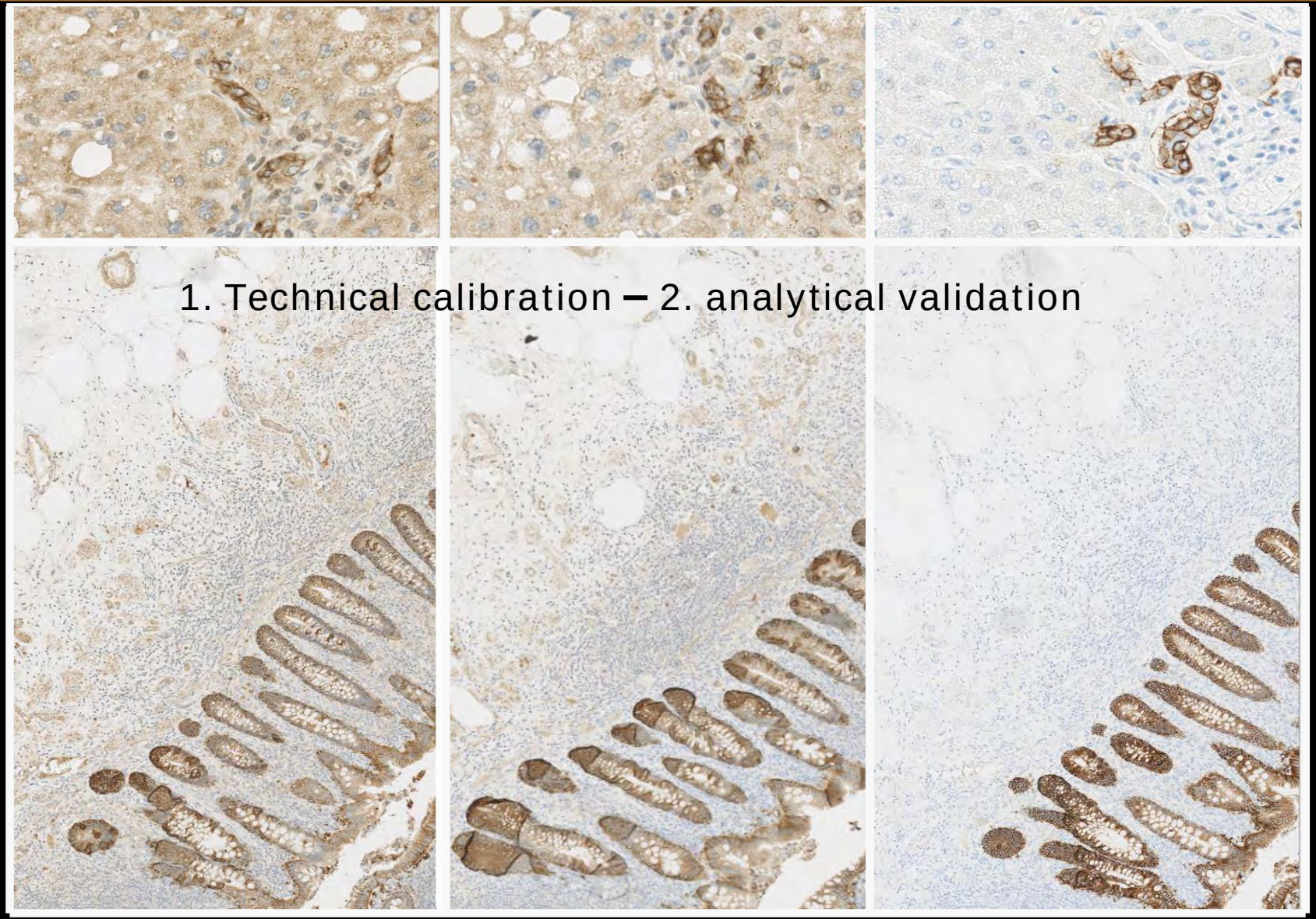


Tonsil

IHC – Biomarker controls



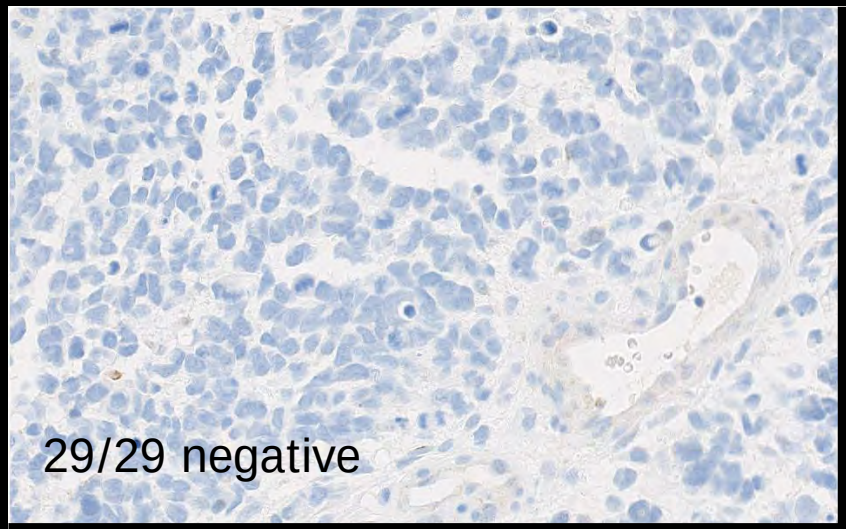
1:100 1:250 1:600

pAb ab71916 – 20 min. RT – HIER 20 min. Low pH – 3-step pol.

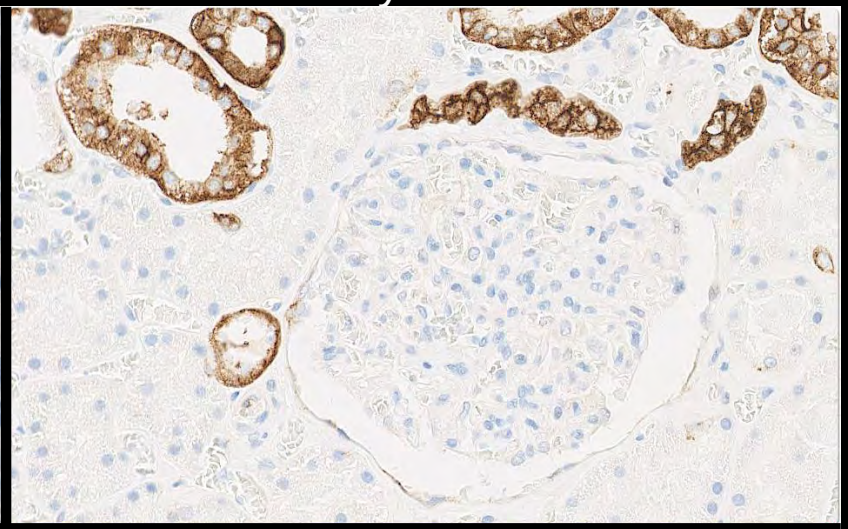
IHC – Biomarker controls

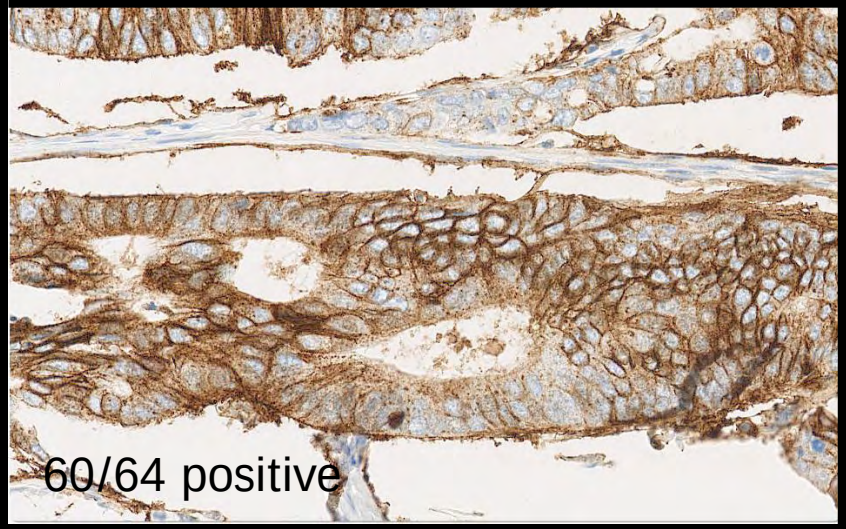
Abcam ab 71916: 1:600, HIER TRS pH 6.1, 3-step polymer

Glioma



Kidney





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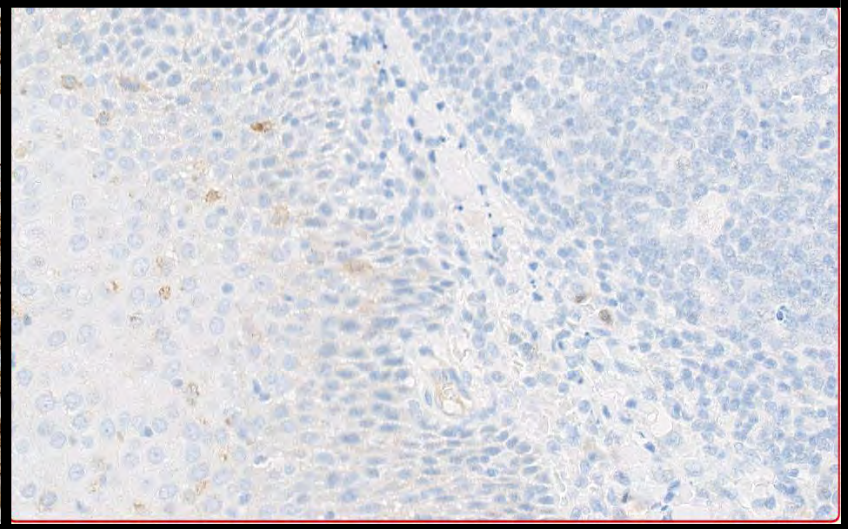


Tonsil

IHC – Biomarker controls

Methods:

Polyclonal

- HIER Citr
- 1:100, 16
- 3-step pc

Positive (ti

Negative (

Int J Clin Exp Pathol 2014;7(11):7907-7914
www.ijcep.com /ISSN:1936-2625/IJCEP0002589

Original Article

Overexpression of EpCAM and Trop2 in pituitary adenomas

Xin Chen[1,2*], Bo Pang[2*], Yu Liang[1,2], Shang-Chen Xu[1], Tao Xin[1], Hai-Tao Fan[1], Yan-Bing Yu[3], Qi Pang[1]

[1]Department of Neurosurgery, Shandong Provincial Hospital Affiliated to Shandong University, Jinan 250021, P. R. China; [2]Shandong University School of Medicine, Jinan 250012, P. R. China; [3]Department of Neurosurgery, China-Japan Friendship Hospital, Beijing 100029, P. R. China. [*]Equal contributors.

Received September 17, 2014; Accepted November 1, 2014; Epub October 15, 2014; Published November 1, 2014

All data based on inadequately calibrated protocol, inadequate controls and thus false positive results

J Neurooncol (2014) 119:39–47
DOI 10.1007/s11060-014-1459-5

LABORATORY INVESTIGATION

The overexpression of Epithelial cell adhesion molecule (EpCAM) in glioma

Xin Chen · Wei-Yuan Ma · Shang-Chen Xu · Yu Liang · Yi-Bing Fu · Bo Pang · Tao Xin · Hai-Tao Fan · Rui Zhang · Jian-Gang Luo · Wen-Qing Kang · Min Wang · Qi Pang

IHC – Biomarker controls

Main aim with IHC controls

To confirm that the IHC result can be trusted and subsequently used to analyze our specimen.

Guidance to level of analytical sensitivity
Guidance to level o analytical specificity

The selection of right controls is crucial

IHC – Biomarker controls

3 main practical areas of controls in diagnostic IHC

1. Calibration of IHC assay and identification of best practice protocol – clone, titre, retrieval etc
"Evaluation of the robustness – impact on pre-analytics.

2. Analytical validation – diagnostic potential
Sensitivity / specificity.

3. IHC performance controls – to monitor that the established level of detection is obtained in each test performed in daily practice – method transfer.

Virtually always; external tissue control

IHC – Biomarker controls

- Reagent and tissue controls are necessary for the validation of immunohistochemical staining results.

- Reagent controls typically used to validate specificty of the primary and secondary antibodies – to show that the antibody-antigen reaction is due to expression of the target of interest.
  - Often referred as negative controls

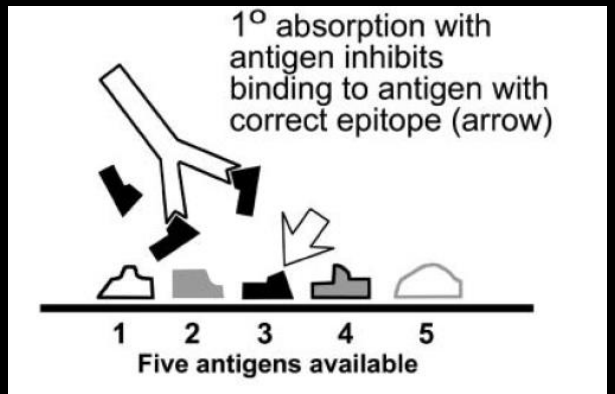
- Tissue controls typically used to show that the IHC staining was successful and capeable to demonstrate the target of interest
  - Often referred as positive controls

IHC – Biomarker controls

- Reagent and tissue controls are necessary for the validation of immunohistochemical staining results.
- Reagent control of the primary antibody is crucial for the producer to validate specificity and can include
  - Primary ab tested on knock-out mice
  - Primary ab tested on cell lines +/- antigen of interest
  - Primary ab tested by western blotting
  - Primary ab tested by antigen absorbtion
  - Primary ab tested on wide range of tissues/neoplasias

To secure specificity of primary ab -

Both by launch and new ab lots.



IHC – Biomarker controls

- Reagent and tissue controls are necessary for the validation of immunohistochemical staining results.

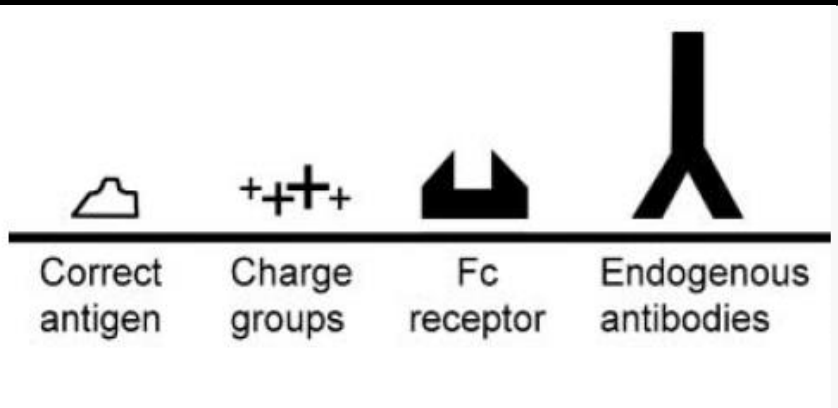
- Reagent (negative) control is for the laboratories of limited use and ″impossible″ to perform correctly.
  - Primary ab control – *negative reagent control*
    - Each primary ab must have its own negative control serum, and thus all the IHC slides performed will be doubled

IHC – Biomarker controls

- Reagent control is of limited use and impossible to perform correctly.
  - e.g. mAb clone PS1 CD3, IgG1a, Ig. conc 80 ug. Primary Ab is diluted 1:100
  - Neg control mouse serum, IgG1a, Ig conc 120 ug. Must be diluted 1:150 to match CD3

By a work-load of 25.000 slides = 50.000 slides.

By a price pr test of 6 euro the total increase will be 150.000 euro...



IHC – Biomarker controls

- Reagent and tissue controls are necessary for the validation of immunohistochemical staining results.

- Reagent (negative) control is for the laboratories of limited use and ”impossible” to perform correctly.
  - Secondary ab control – *negative reagent control*
    - The primary ab is substituted by e.g. diluent in order to monitor binding of the detection system to the tissue. In principle each of all retrieval methods applied in a diagnostic case must have its own negative diluent control.
    - Question – what is the value ?

IHC – Biomarker controls

- Reagent and tissue controls are necessary for the validation of immunohistochemical staining results.

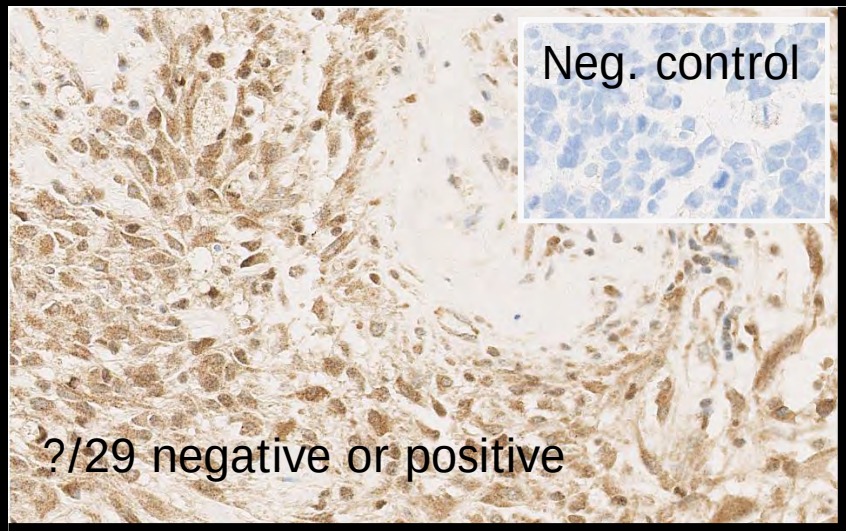
- Reagent (negative) control is for the laboratories of limited use and ″impossible″ to perform correctly.
  - Primary ab control – negative reagent control
    - *Ig subtype precisely calibrated*
  - Secondary ab control – negative reagent control
    - *Diluent or buffer*

WILL NOT EXPOSE IF WRONG, POOR CALIBRATED OR CONTAMINATED PRIMARY AB WAS APPLIED!!!!!

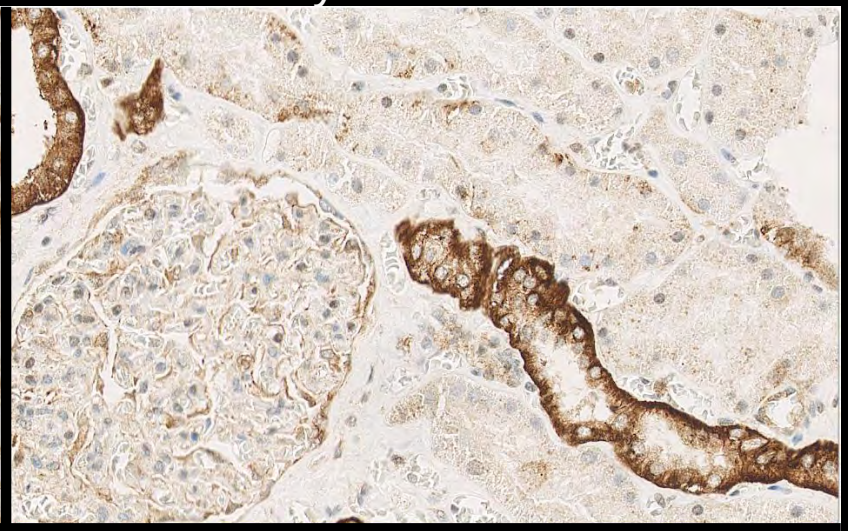
IHC – Biomarker controls

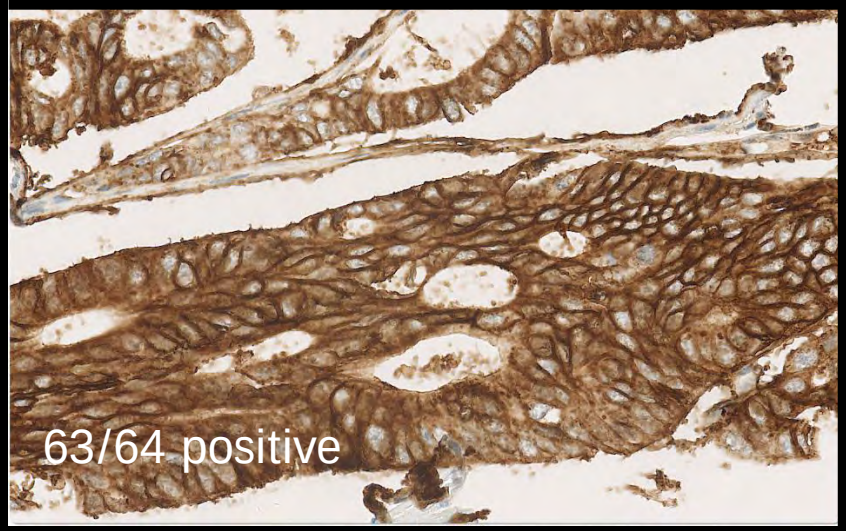
Study: Abcam ab 71916: 1:100, HIER TRS pH 6.1, 3-step polymer

Glioma

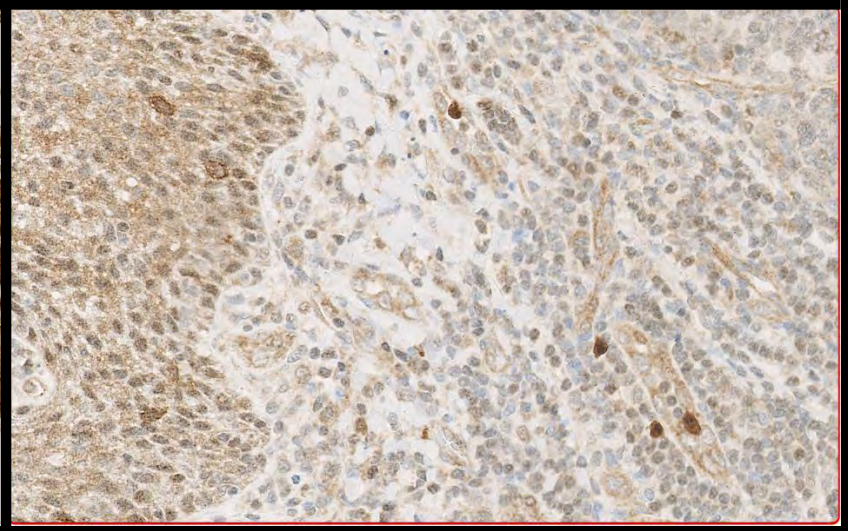


Kidney



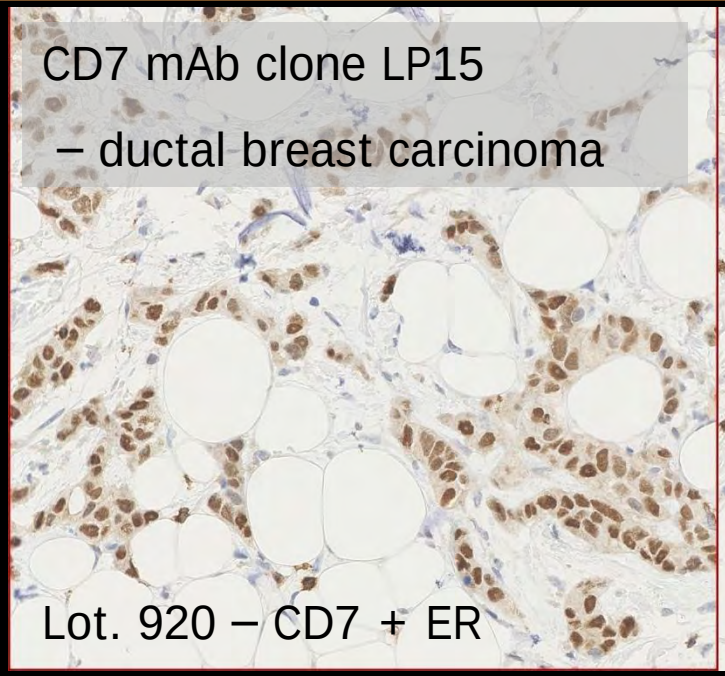


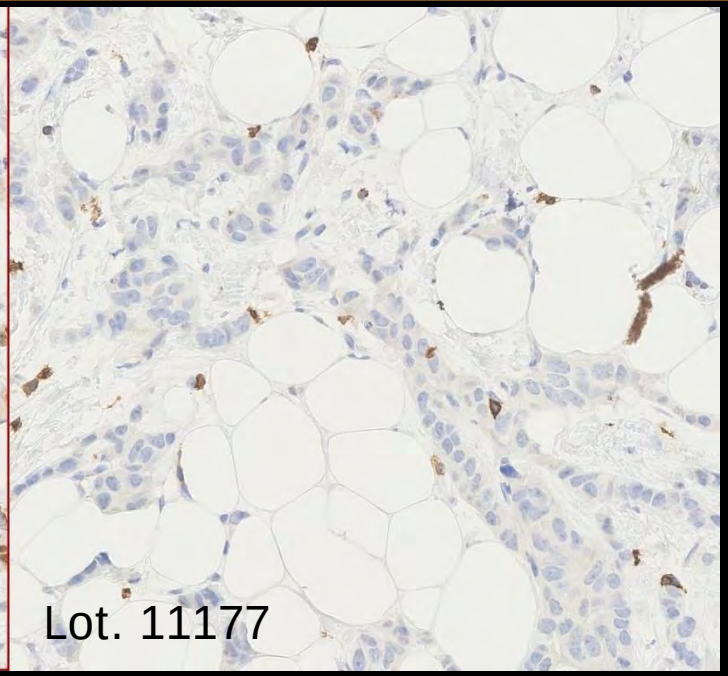
Colon ad. carc.

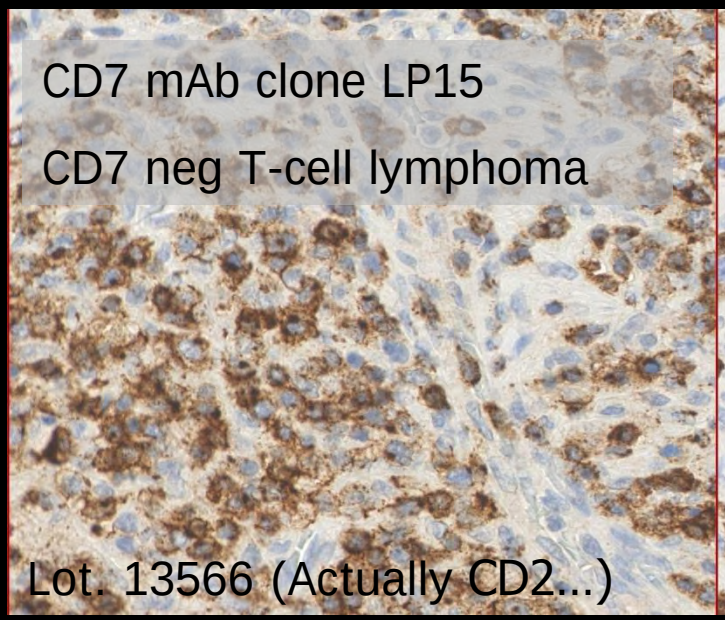


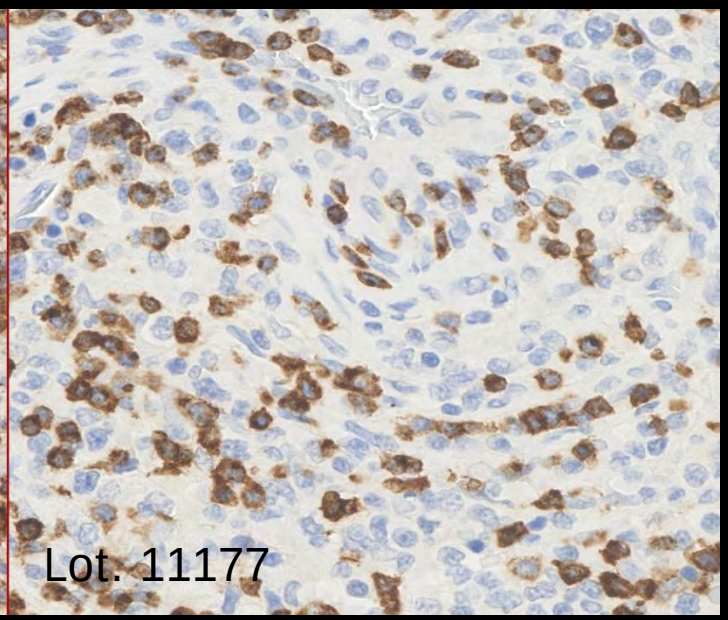
Tonsil

IHC – Biomarker controls









FP staining reactions

Not identified by negative reagent controls

The antibody giving the FP would be substituted by control serum and no staining seen giving a ”false security”

IHC – Biomarker controls

BSAP rmAb clone SP34

– NordiQC run 41, 2014

FP staining reactions
Not identified by negative reagent controls or other controls by 3 vendors and 5 laboratories

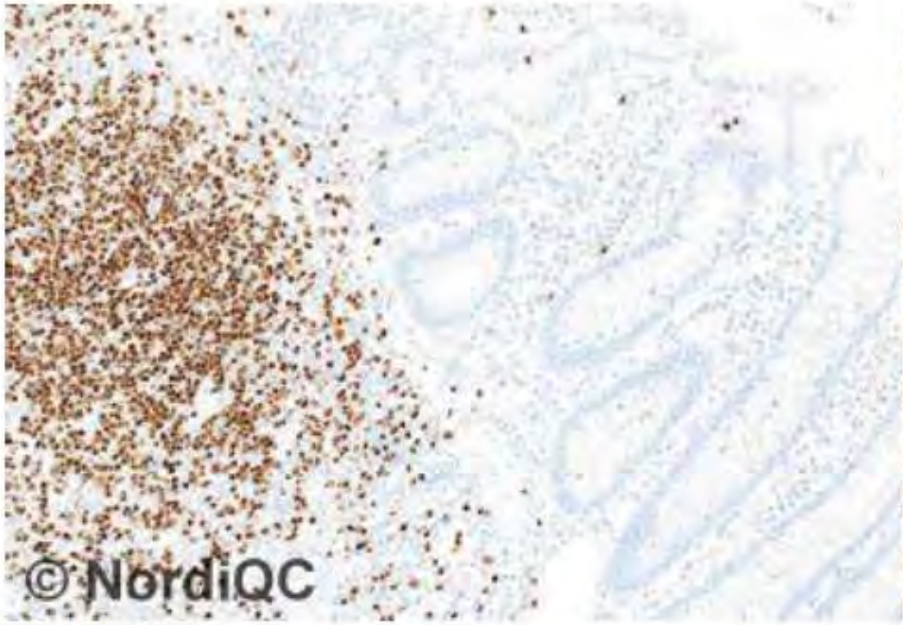


Fig.4a (X200)
Optimal BSAP staining of the appendix using same protocol as in Figs. 1a - 3a. The peripheral B-cells show a strong nuclear staining reaction, while the epithelial cells are negative.

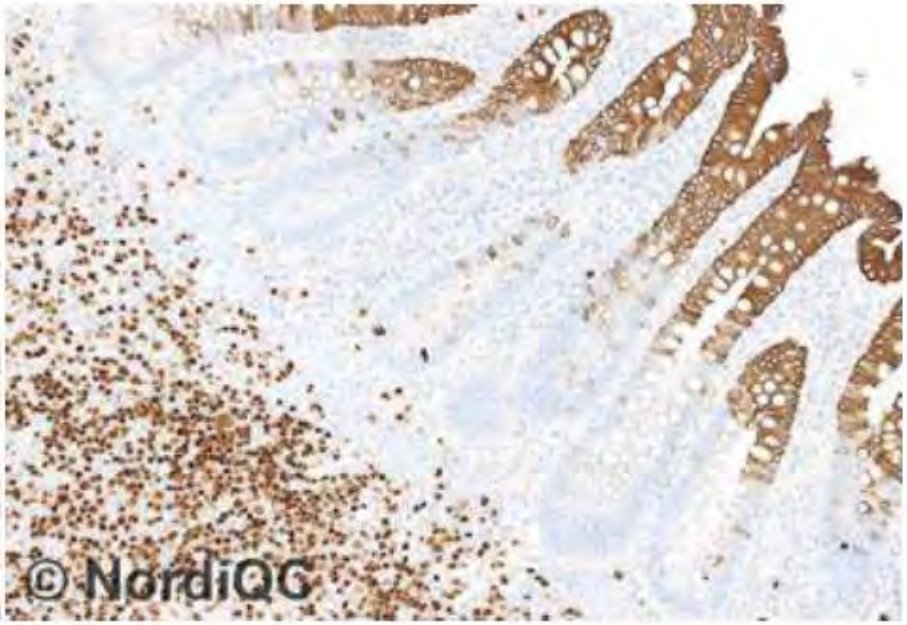


Fig. 4b (X200)
Aberrant BSAP staining of the appendix. In addition to the expected staining result for BSAP of the B-cells, the epithelial cells display a staining reaction corresponding to CK20. This aberrant staining result was frequently seen, when the rmAb clone SP34 was used as a concentrate and most likely caused by a contamination of the raw material of the clone. The staining reaction was seen in products from all companies providing the clone as a concentrate (see table 1).

IHC – Biomarker controls

Negative reagent control (diluent):

Must: Biotin based detection systems
Class II / III assays

Can: Pigmented tumours
Frozen sections
(No internal or external negative tissue structures)

REVIEW ARTICLE

(*Appl Immunohistochem Mol Morphol* 2014;22:241–252)

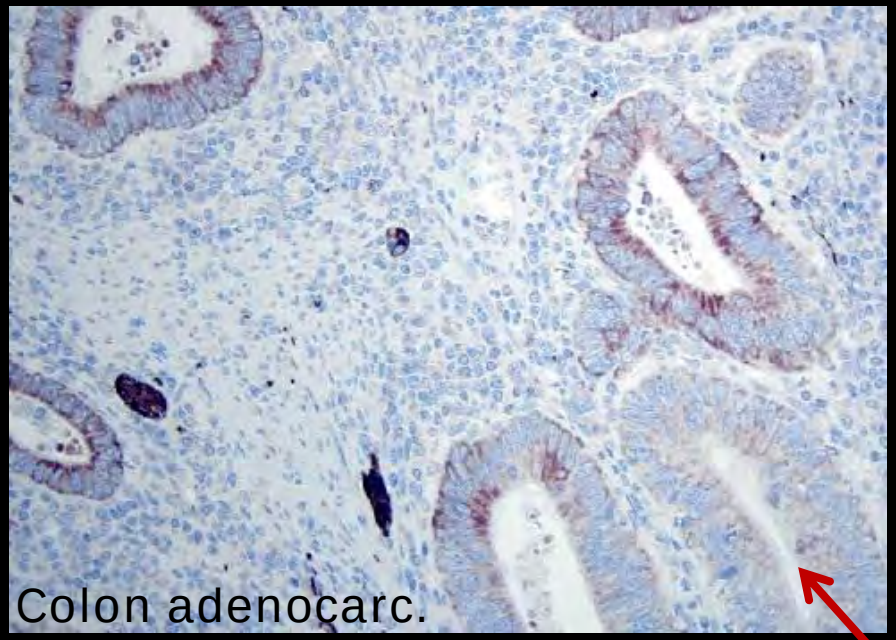
Standardization of Negative Controls in Diagnostic Immunohistochemistry: Recommendations From the International Ad Hoc Expert Panel

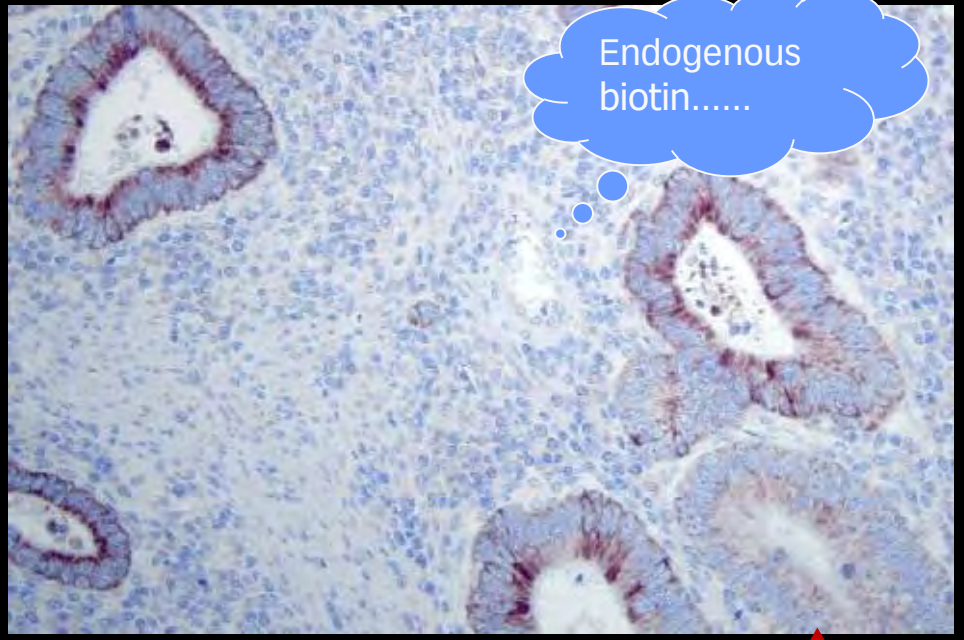
Emina E. Torlakovic, MD, PhD,†‡ Glenn Francis, MBBS, FRCPA, MBA, FFSc (RCPA),§‖¶ John Garratt, RT,†‡# Blake Gilks, MD, FRCPC,†‡** Elizabeth Hyjek, MD, PhD,* Merdol Ibrahim, PhD,†† Rodney Miller, MD,‡‡ Søren Nielsen, HT, CT,§§‖‖ Eugen B. Petcu, MD, PhD,§ Paul E. Swanson, MD,¶¶ Clive R. Taylor, MD, PhD,## and Mogens Vyberg, MD§§‖‖*

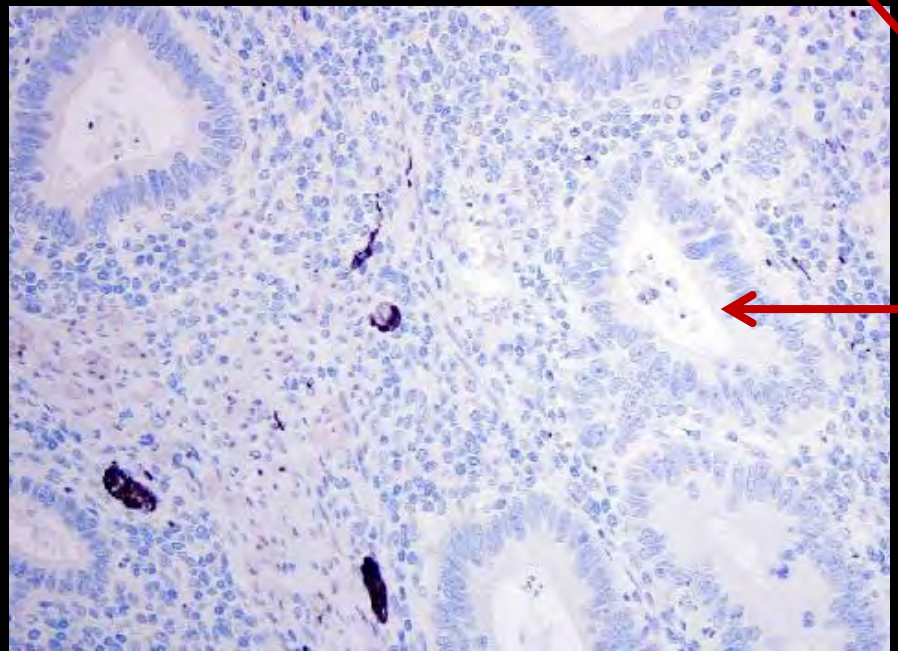
TABLE 2. Recommendations for Use of Negative Controls in Diagnostic Immunohistochemistry

Type of Control	CAP-ACP Clinical Use IHC Test Class I: FDA IHC Device Class I	CAP-ACP Clinical Use Class II Tests: FDA IHC Device Class II	CAP-ACP Clinical Use Class II Tests: FDA IHC Device Class III	Comments
Negative reagent control (NRC)				
NRC-primAb—replace primary Ab with "nonspecific" Ig	Recommended for initial antibody validation, and for use with avidin-biotin detection Not recommended for routine daily use of validated protocol using polymer-based detection Can be ordered by pathologist in specific situations (see text)	Recommended as per published guidelines When no guidelines exist, the NRC antibody control is recommended where results may dictate definitive treatment (ie, ER, PR), and are not confirmed by other aspects of pathology testing	Use negative reagent controls as per approved guidelines	When panels of several antibodies are used on serial sections, negative staining elements in the different sections serve as a negative reagent controls, obviating the need for a separate negative reagent control in most instances of class I testing Also, pathologists' interpretation of IHC-SE determines if NRC-primAb is required May require multiple controls if several different retrieval methods are in use
NRC-detSys (supplementary negative controls)		Use where unexpected staining is observed in the NRC antibody negative control slide (Table 1)		May require multiple controls for different components of detection system and if different retrieval methods are in use
Negative tissue control (NTC)				
Internal NTC—evaluate tissue elements that should be negative in test section of the patient's sample	Recommended	Recommended	Use negative and positive controls tissue as per approved guidelines	If test section does not include elements that serve as negative controls, then, external tissue control may be informative
External NTC—evaluate tissue elements in control tissue that should be negative	Recommended	Recommended		Control tissues may be derived from archived diagnostic tissue as single sections, or tissue microarrays. Cell lines prepared as cell blocks, if processed in the same way as patient samples can be also be used (see text)

IHC – Biomarker controls







Labelled Steptavidin-Biotin system

Labelled Streptavidin-Biotin system – neg control

Multimer / Polymer based system

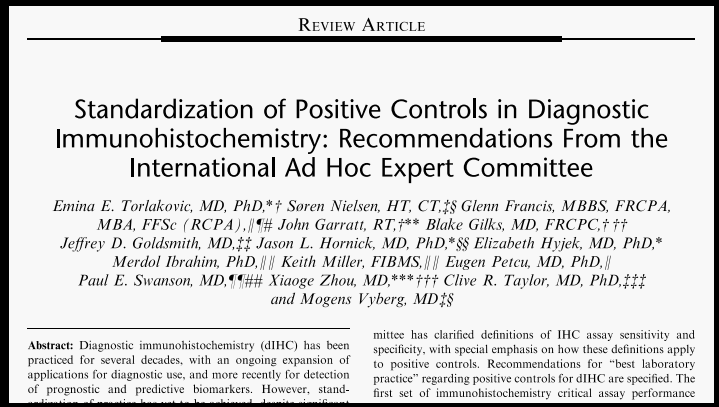
Synaptophysin mAb clone 27G12

HIER & biotin-based assays a challenge....

IHC – Biomarker controls

- Reagent and tissue controls are necessary for the validation of immunohistochemical staining results.

- Tissue controls are the most valueable tool to monitor the specificity and sensitivity for IHC
  - Internal positive and negative tissue control
    - Cells/structures within the patient slide
  - External positive and negative tissue control
    - Slide next to patient slide



REVIEW ARTICLE

Standardization of Positive Controls in Diagnostic Immunohistochemistry: Recommendations From the International Ad Hoc Expert Committee

Emina E. Torlakovic, MD, PhD,† Søren Nielsen, HT, CT,‡§ Glenn Francis, MBBS, FRCPA, MBA, FFSc (RCPA),‖¶# John Garratt, RT,†** Blake Gilks, MD, FRCPC,†† Jeffrey D. Goldsmith, MD,‡‡ Jason L. Hornick, MD, PhD,*§§ Elizabeth Hyjek, MD, PhD,* Merdol Ibrahim, PhD,‖‖ Keith Miller, FIBMS,‖‖ Eugen Petcu, MD, PhD,‖ Paul E. Swanson, MD,¶¶## Xiaoge Zhou, MD,***††† Clive R. Taylor, MD, PhD,‡‡‡ and Mogens Vyberg, MD‡§*

Abstract: Diagnostic immunohistochemistry (dIHC) has been practiced for several decades, with an ongoing expansion of applications for diagnostic use, and more recently for detection of prognostic and predictive biomarkers. However, stand-

mittee has clarified definitions of IHC assay sensitivity and specificity, with special emphasis on how these definitions apply to positive controls. Recommendations for "best laboratory practice" regarding positive controls for dIHC are specified. The first set of immunohistochemistry critical assay performance

IHC – Biomarker controls

- Reagent and tissue controls are necessary for the validation of immunohistochemical staining results.

- Tissue controls are the most valueable tool to monitor the specificity and sensitivity for IHC
  - Internal negative tissue control
    - Cells / structures to be negative
      - E.g. T-cells for CD19, CD20, CD79a...
      - Mantle zone B-cells for Ki67, Bcl-6...
      - Epithelial cells for CD3, CD5, MUM1,...

    Information of primary ab / assay specificity

IHC – Biomarker controls

NordiQC run 35, CD19

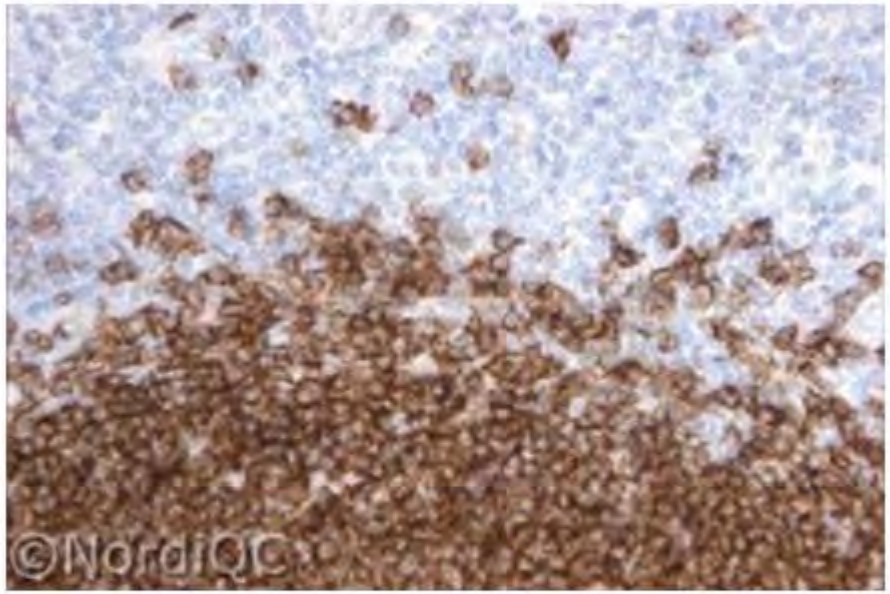


Fig. 1a. Normal tonsil showing an optimal staining for CD19 using the mAb clone LE-CD19 from Dako, diluted 1:50, on the Autostainer platform. HIER was performed using TRS pH 9 (3-in-1) (Dako). A strong and distinct membranous staining reaction is seen in virtually all B-cells. T-cells are negative.

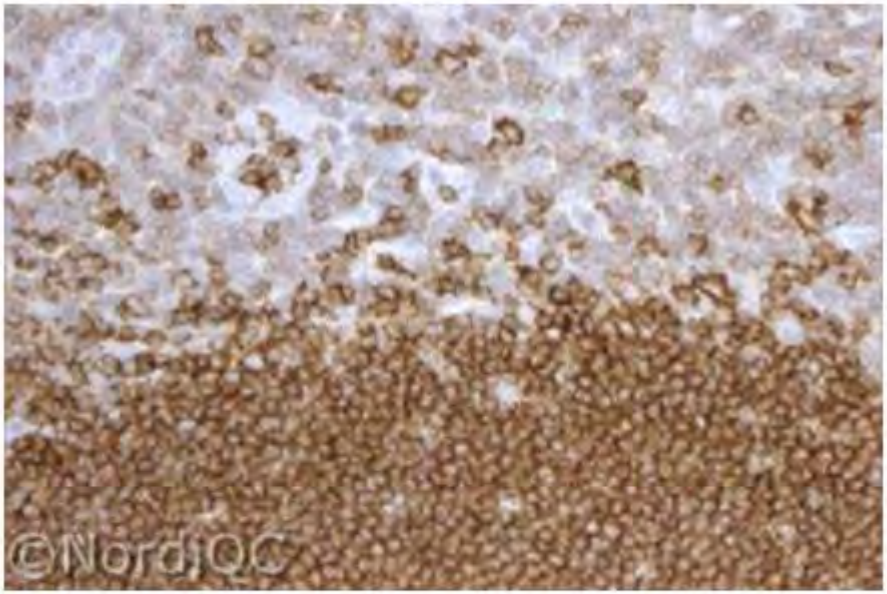
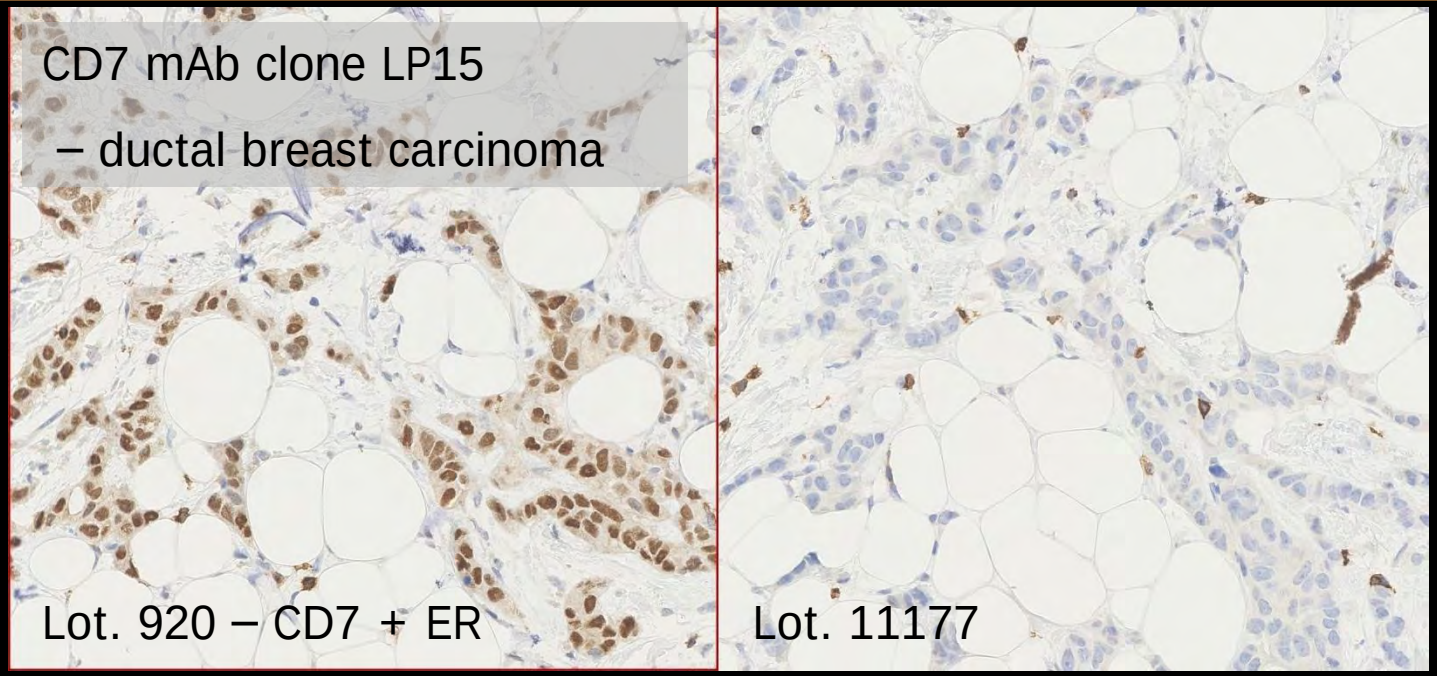


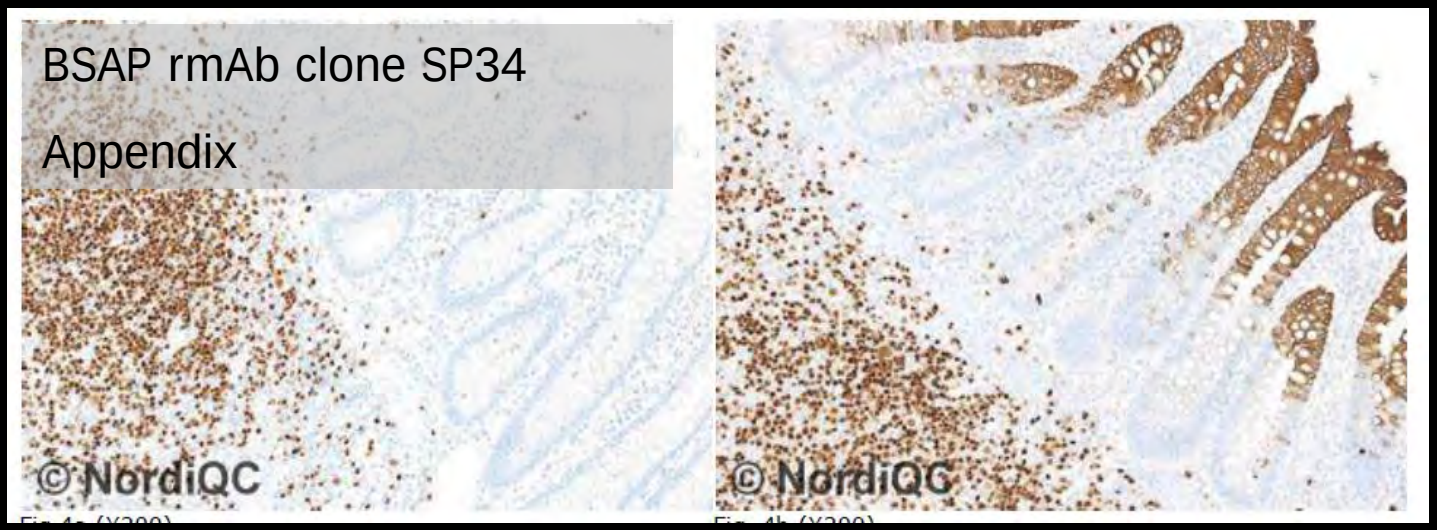
Fig. 1b. Normal tonsil showing an insufficient staining for CD19 using the mAb clone LE-CD19 from Serotec, diluted 1:500, on the Autostainer platform. HIER was performed using Citrate pH 6. In addition to a moderate to strong staining reaction in the normal B-cells (albeit weaker than that seen in Fig 1a), the majority of T-cells shows a false positive staining reaction.

mAb clone LE-CD19

Dako: B-cells positive, T-cells negative
Serotec: B-cells positive, T-cells <u>false</u> positive

IHC – Biomarker controls





FP staining reactions

Not identified by negative reagent controls

The antibody giving the FP would be substituted by control serum and no staining seen giving a ”false security”

IHC – Biomarker controls

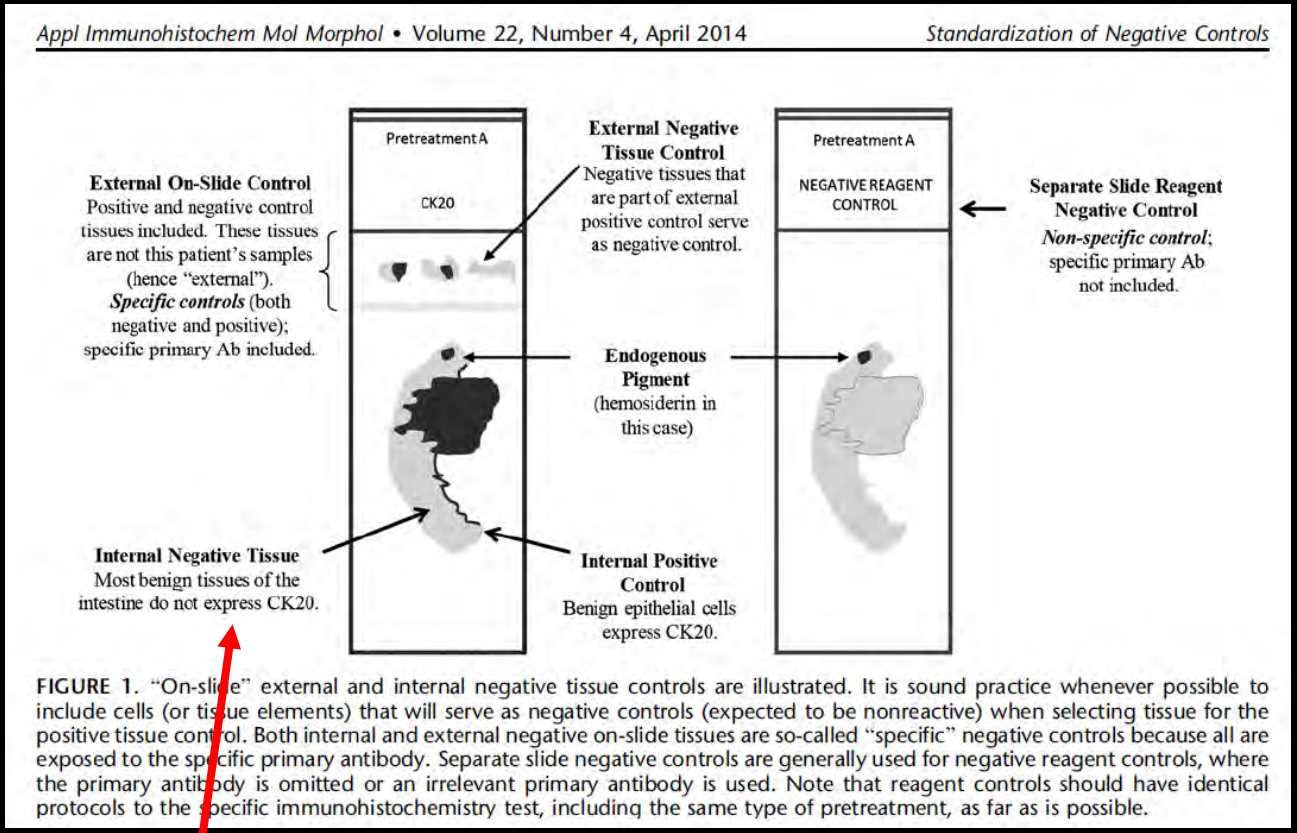


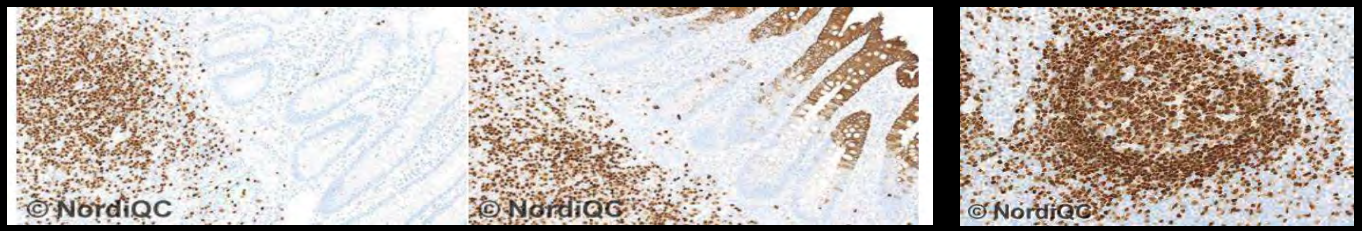
FIGURE 1. "On-slide" external and internal negative tissue controls are illustrated. It is sound practice whenever possible to include cells (or tissue elements) that will serve as negative controls (expected to be nonreactive) when selecting tissue for the positive tissue control. Both internal and external negative on-slide tissues are so-called "specific" negative controls because all are exposed to the specific primary antibody. Separate slide negative controls are generally used for negative reagent controls, where the primary antibody is omitted or an irrelevant primary antibody is used. Note that reagent controls should have identical protocols to the specific immunohistochemistry test, including the same type of pretreatment, as far as is possible.

What about internal positive tissue controls ???

Internal neg tissue control: Identification of false-positive staining reaction of structures known not to express the target antigen.

Limitation: Not all elements will be available to expose a potential false positive result

PAX5…. 3 vendors



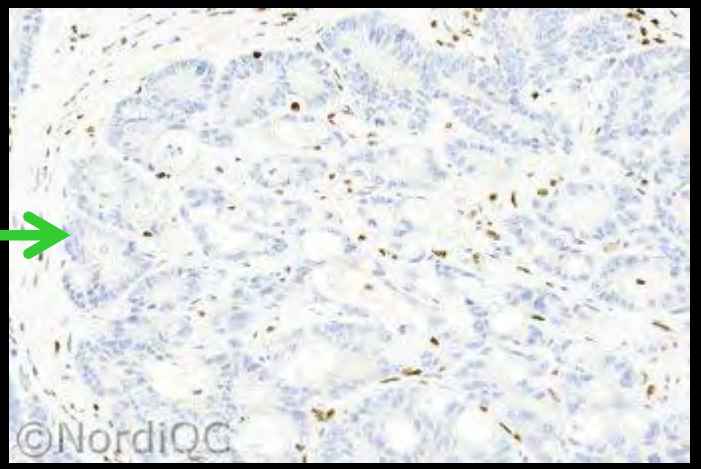
IHC – Biomarker controls

TABLE 2. Examples of IHC Assays Where Preferential Use of Internal Positive Controls Recommended

IHC Assay	Use	Comments
Cytokeratin 5	Demonstration of basal cells in glandular structures of prostate to differentiate between benign (positive) and malignant (negative) glands	Interpretation of the results in the tumor directly depends on clear demonstration of internal positive control Tested sample may be completely negative if no normal tissue is present
Mismatch repair proteins (MLH1, MSH2, PMS2, MSH6)	Absence of expression in the cells of colon or endometrial adenocarcinoma is abnormal; patients referred for molecular testing to rule out Lynch Syndrome	Interpretation of the results in the tumor directly depends on clear demonstration of internal positive control
SMAD4/Dpc4	Ubiquitously expressed tumor suppressor Ag that is inactivated in about 55% of pancreatic adenocarcinomas	Interpretation of the results in the tumor directly depends on clear demonstration of internal positive control
PTEN	Ubiquitously expressed; loss of expression is associated with carcinogenesis, cancer progression, and drug resistance	Interpretation of the results in the tumor directly depends on clear demonstration of internal positive control

Internal postive tissue controls;

Principally ideal as processed identically to patient relevant material evaluated



If internal positive control is neg or dubious – test is repeated

IHC – Biomarker controls

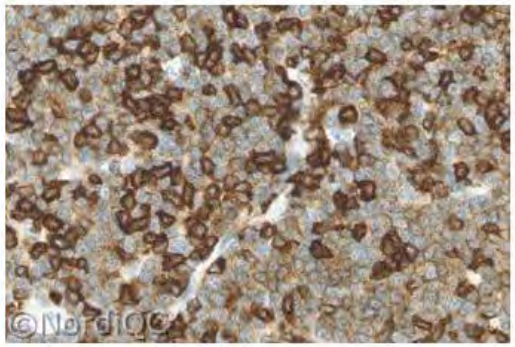


Fig. 4a. Optimal staining for CD5 of the B-CLL no. 5 using same protocol as in Figs. 1a - 4a.
The majority of the neoplastic cells show a moderate and distinct staining reaction, while the infiltrating normal T-cells show a strong staining reaction.

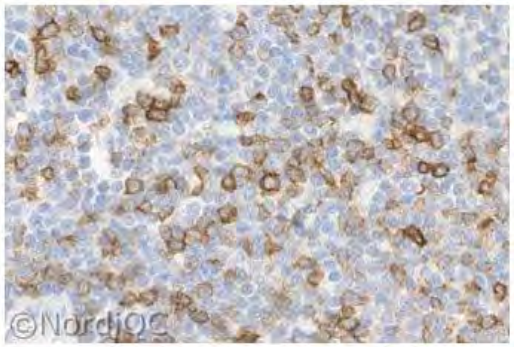


Fig. 4b. Insufficient staining for CD5 of the B-CLL using same protocol as in Figs. 1b - 3b – same field as in Fig. 4a.
The neoplastic cells are virtually negative and only the normal T-cells are clearly demonstrated.

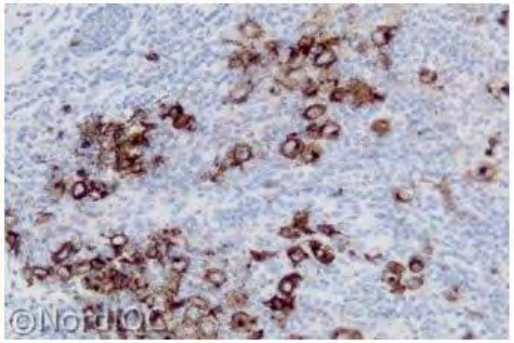


Fig. 2a. Optimal CD15 staining of the Hodgkin lymphoma no 2 (NS) using same protocol as in Fig. 1a. The Reed-Sternberg and Hodgkin cells show a strong membranous staining and a dot-like positivity.

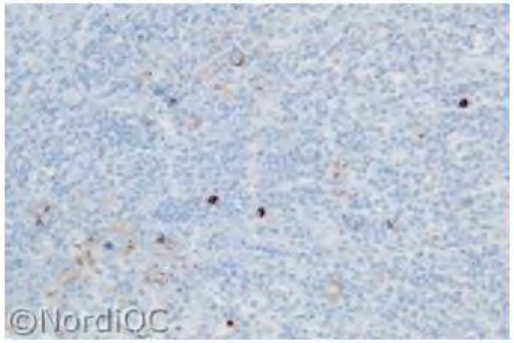


Fig. 2b. CD15 staining of the Hodgkin lymphoma no 2 (NS) using same protocol as in Fig. 1b. Only few Reed-Sternberg and Hodgkin cells show a weak staining - same field as in Fig. 2a.

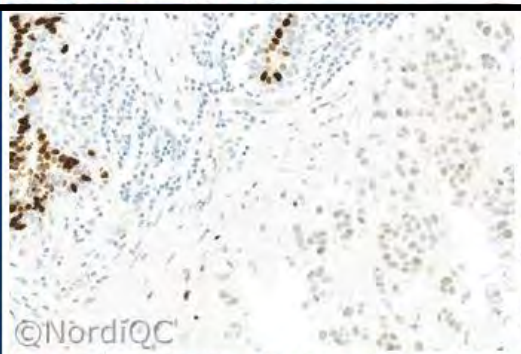


Fig. 3a. Optimal ER staining of the breast ductal carcinoma no. 3 with 60 – 80 % cells positive. A weak but distinct nuclear staining is seen in the appropriate proportion of the

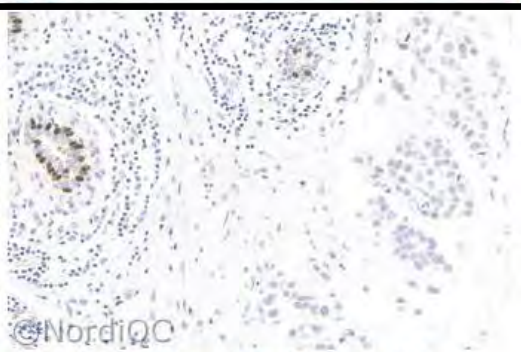
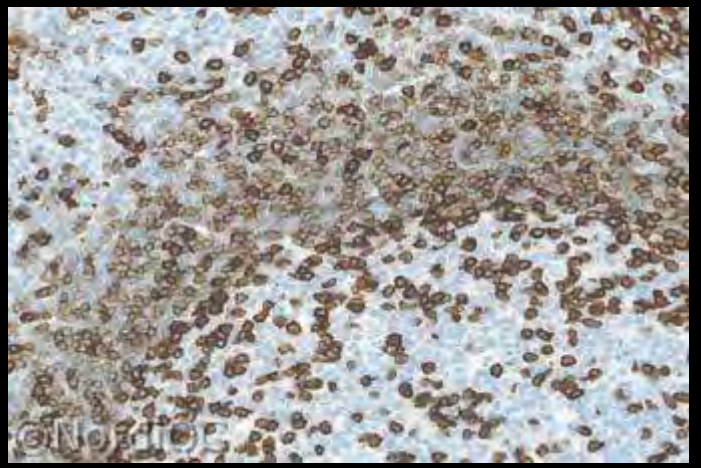


Fig. 3b. Insufficient ER staining of the breast ductal carcinoma no. 3 with 60 – 80 % cells positive using same protocol as in Figs. 1b and 2b – same field as in Fig. 3a.

Internal positive tissue controls;

In general not applicable as positive controls due to levels of expression may not be relevant for level of test calibration

e.g. CD5, CD15, CD34, CD45, CD56, S100, ER, PR etc



IHC – Biomarker controls

- Reagent and tissue controls are necessary for the validation of immunohistochemical staining results.
- Conclusions – Internal tissue controls
  - Internal positive tissue control
    - Indicative of *"successful"* IHC result
    - Cannot be recommended as generally reliable for evaluation of appropriate sensitivity
      - Essential for interpretation of MMR
      - Valueable for CK-HMW in prostate
  - Internal negative tissue control
    - Can provide valueable information of specificity of the primary antibody/assay

IHC – Biomarker controls

- Reagent and tissue controls are necessary for the validation of immunohistochemical staining results.

- Tissue controls are the most valueable tool to monitor the specificity and sensitivity for IHC
  - External positive and negative tissue control
    - Appropriate sensitivity of the IHC assay
    - Appropriate specificity of the IHC assay

The central tool to monitor the technical IHC quality, diagnostic utility and consistency.

IHC – Biomarker controls

Issues to be adressed :

1. Calibration of IHC assay and identification of best practice protocol – clone, titre, retrieval etc
2. ″Evaluation of the robustness of the IHC assay – impact on pre-analytics
3. Evaluation of the analytical sensitivity/specificity
4. Identification of IHC performance controls providing information that the established level of detection is obtained in each test performed in daily practice.

<u>Tissue controls are key element</u>

IHC – Biomarker controls

Issues to be adressed :

1. Calibration of IHC assay and identification of best practice protocol – clone, titre, retrieval etc
    - Concentrated formats
        - Full test comprising various titres, retrieval settings, detection systems (+/- different stainer platform)
    - Ready-To-Use formats
        - Confirmatory test primarily using official recommendations and if needed modifications e.g. incubation times, detection system etc

IHC – Biomarker controls

Concentrated antibodies – Aalborg Hospital (app. 200 Abs) – VMS ULTRA

	1:25	1:100	1:400
A	None	None	None
B	Enzyme P1, 4 min	Enzyme P1, 4 min	Enzyme P1, 4 min
C	HIER CC1 pH 8.5*	HIER CC1 pH 8.5	HIER CC1 pH 8.5
D	HIER CC2 pH 6.0*	HIER CC2 pH 6.0	HIER CC2 pH 6.0
(E)	CC1 + Enzyme P3, 8 min	CC1 + Enzyme P3, 8 min	CC1 + Enzyme P3, 8 min
(F)	Enzyme P3, 8 min + CC1	Enzyme P3, 8 min + CC1	Enzyme P3, 8 min + CC1

*HIER time 48 min. at 99°C
OptiView DAB, Ventana BenchMark Ultra

Protocol A: 2 %
Protocol B: 3 %
Protocol C: 90 %
Protocol E: 3 %
Protocol F: 1 %
Others : 2 % (E.g. prolonged HIER, prolonged proteolysis)

IHC – Biomarker controls

Issues to be adressed :

1. Calibration of IHC assay and identification of best practice protocol – clone, titre, retrieval etc
2. ″Evaluation of the robustness of the IHC assay – impact on pre-analytics
3. Evaluation of the analytical sensitivity/specificity
4. Identification of most robust controls providing information that the established level of detection is obtained in each test performed in daily practice.

<u>Tissue controls are key element</u>

IHC – Biomarker controls

MB Ab test 1

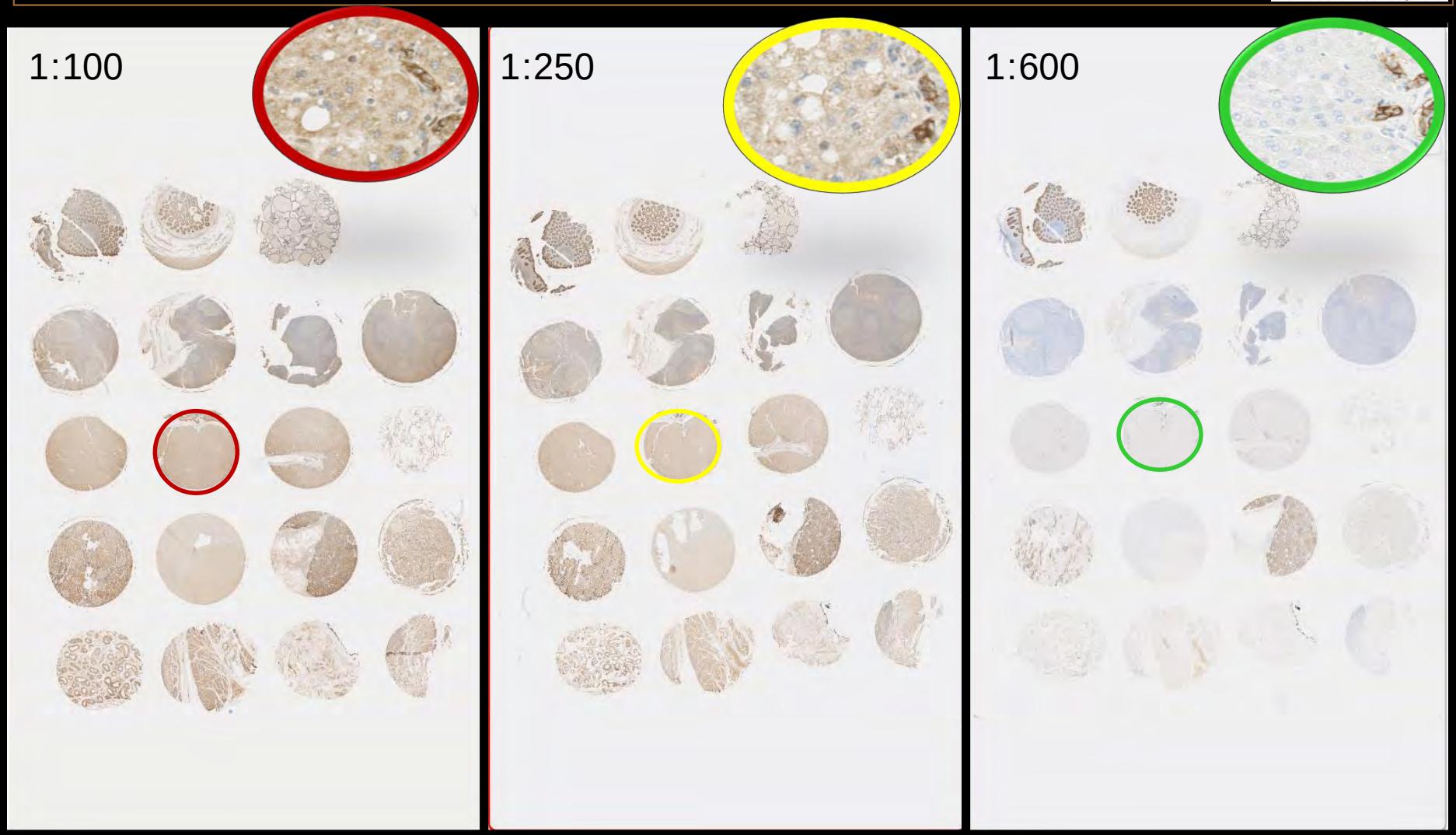
Calibration

Robustness

Appendix	Kidney	Thyroid	
Ton 6h	Ton 24h	Ton 72h	Ton 168h
Liver 6h	Liver 24h	Liver 72h	Lung
Kidney	Brain	Pancreas	Placenta
Testis	Prostate	Esophagus	Ton 24h + decalc.

Protocol set-up: used as primary material for the calibration of 130 of 195 routine diagnostic markers, Aalborg University Hospital

IHC – Biomarker controls



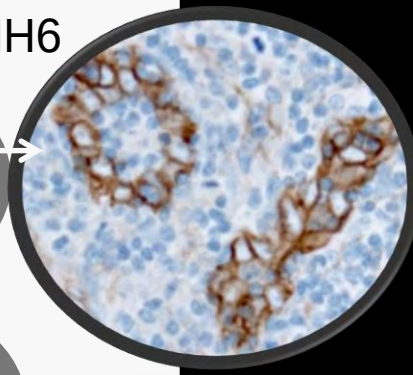
EPCAM calibration

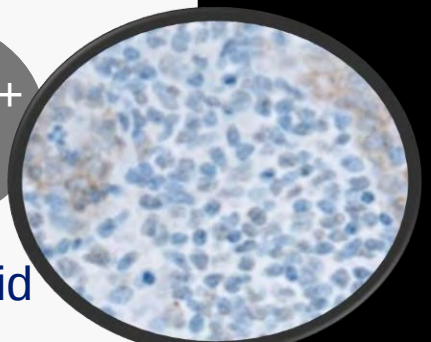
Tissue cores are used to identify best practice protocol providing highest signal-to-noise ratio for qualitative IHC markers

IHC – Biomarker controls

Photos by Ole Nielsen

Appendix	Appendix	Thyroid	MB Ab test 1 CD105, SNH6
Ton 6h	Ton 24h	Ton 72h	Ton 168h
Liver 6h	Liver 24h	Liver 72h	Pancreas
Placenta	Lung	Prostate	Breast
Skin	Brain	Tongue	Ton 24h + decalc.

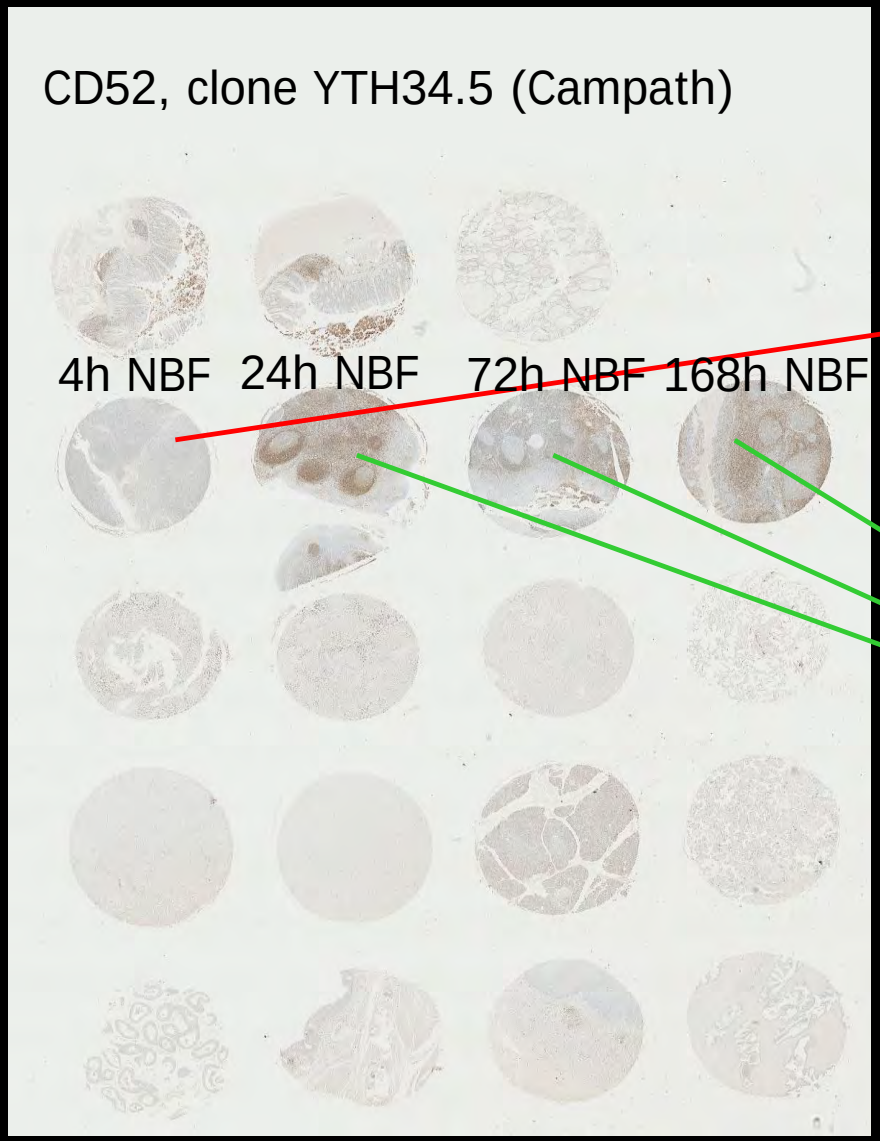


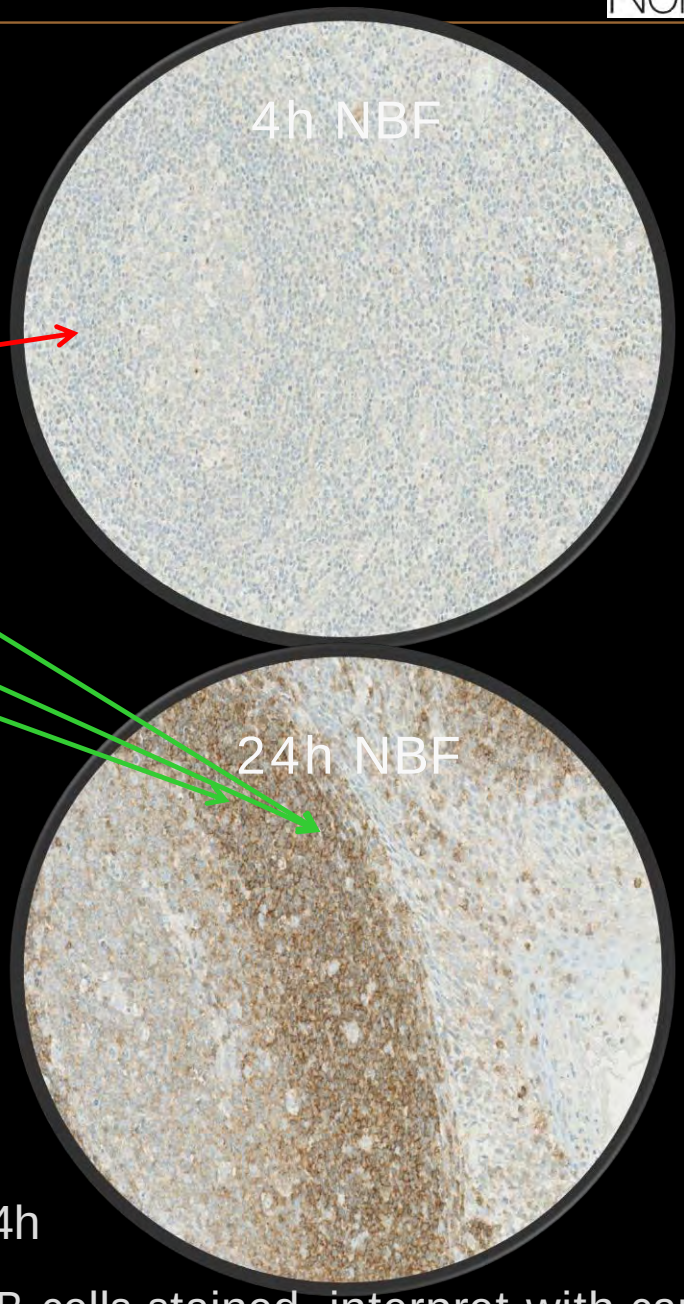


Protocol set-up:

Formic acid

IHC – Biomarker controls

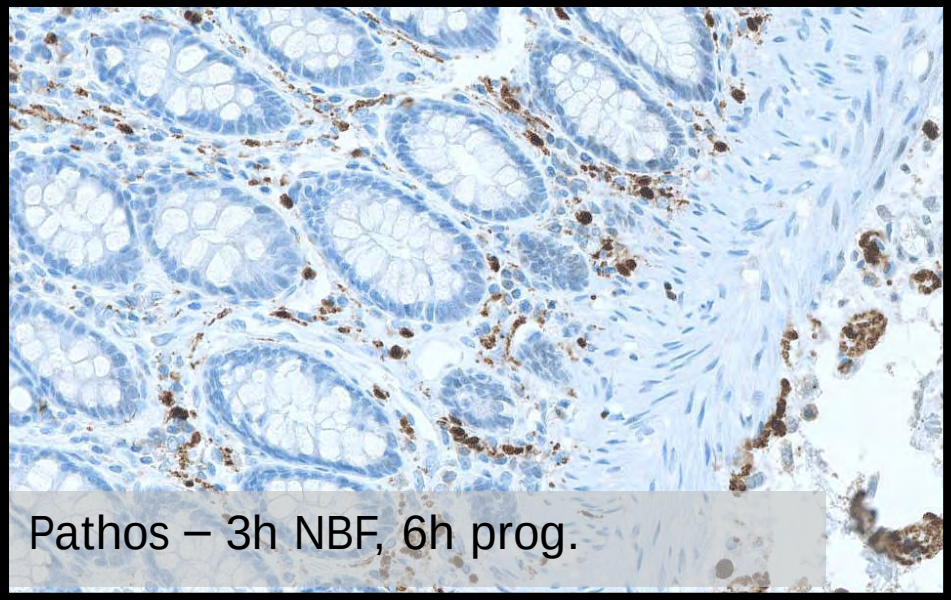




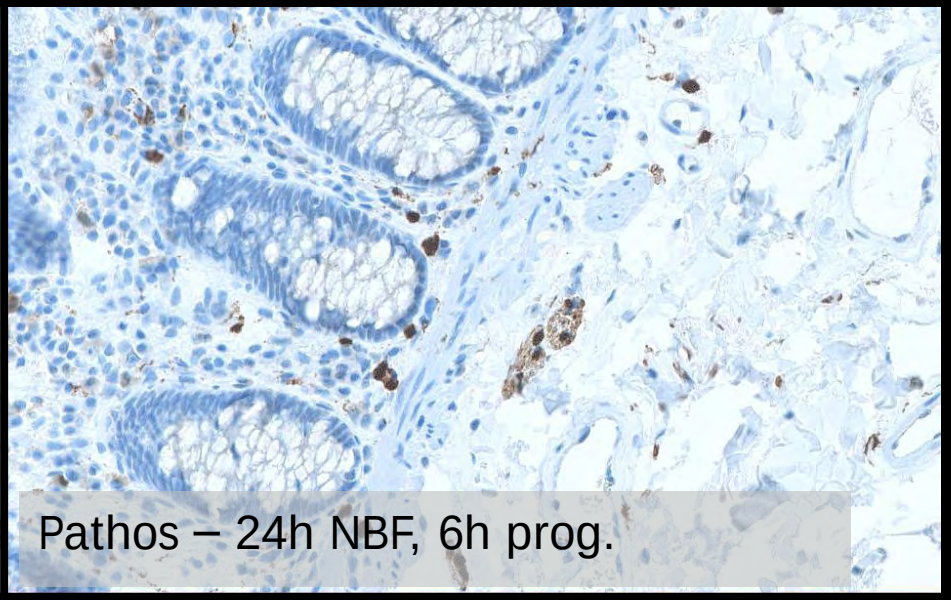
1. Influenced by fixation time – reduced in <24h
2. IHC protocol, 3. Control; Tonsil – cave if no B-cells stained, interpret with caution

IHC – Biomarker controls

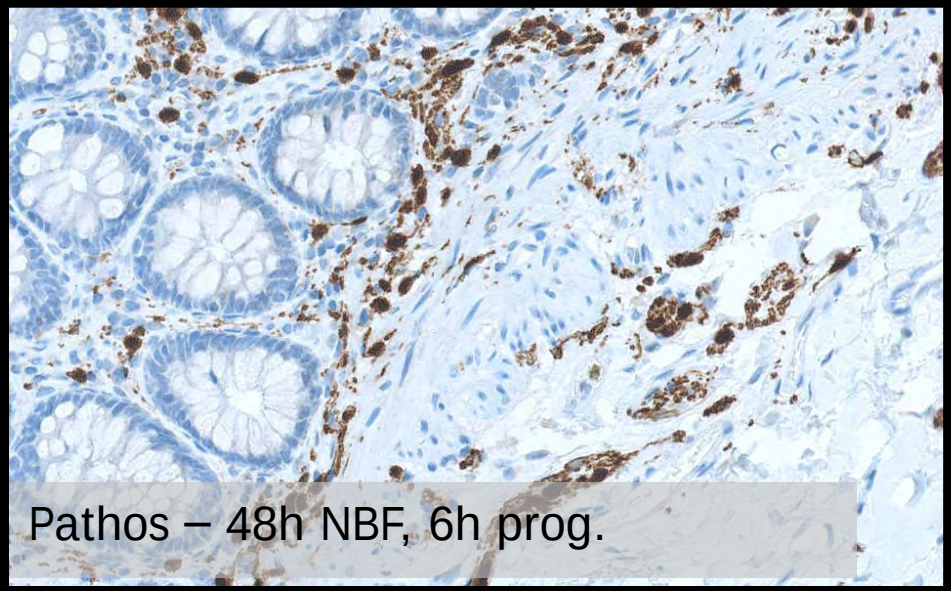
Colon: S100, polyclonal



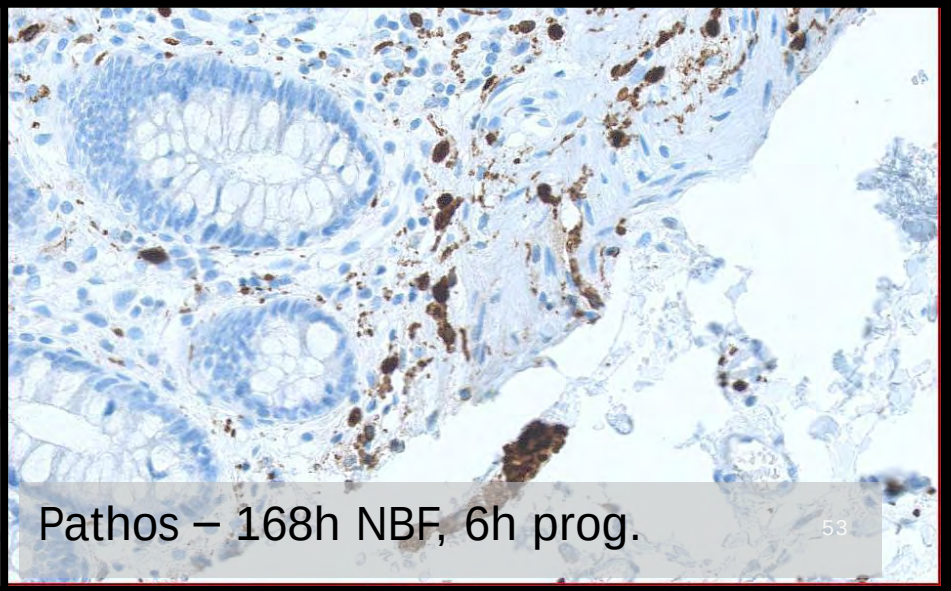
Pathos – 3h NBF, 6h prog.



Pathos – 24h NBF, 6h prog.



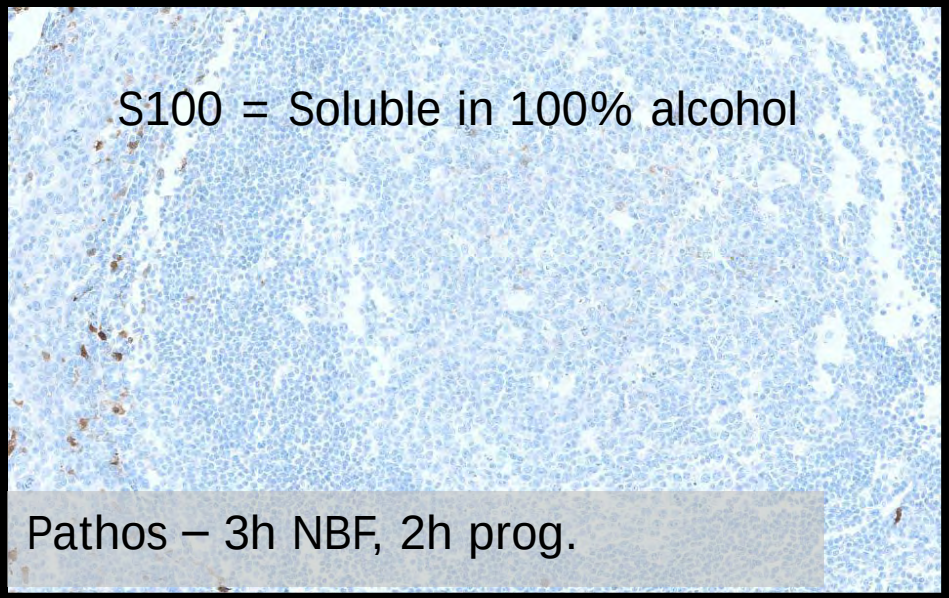
Pathos – 48h NBF, 6h prog.



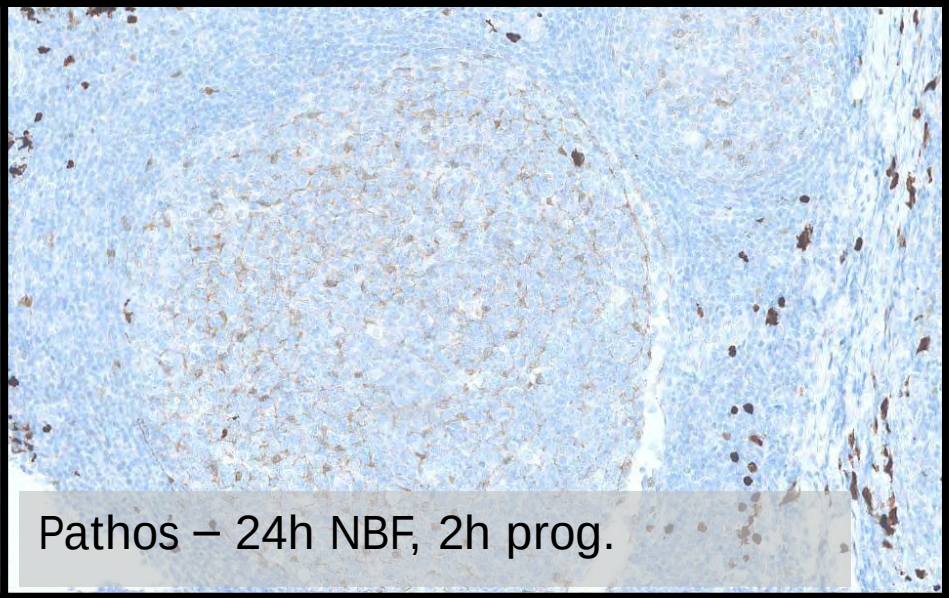
Pathos – 168h NBF, 6h prog.

IHC – Biomarker controls

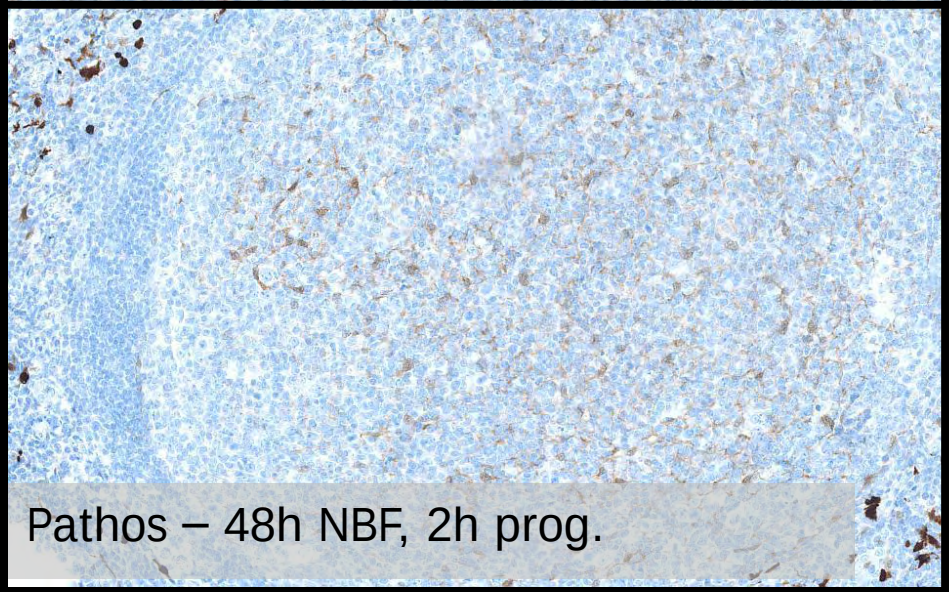
Tonsil: S100, polyclonal



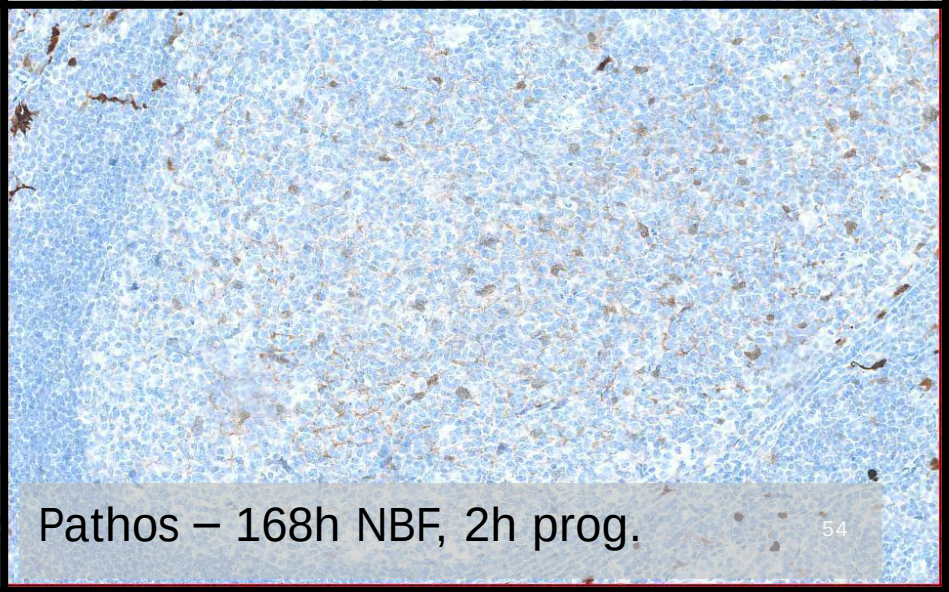
Pathos – 3h NBF, 2h prog.



Pathos – 24h NBF, 2h prog.



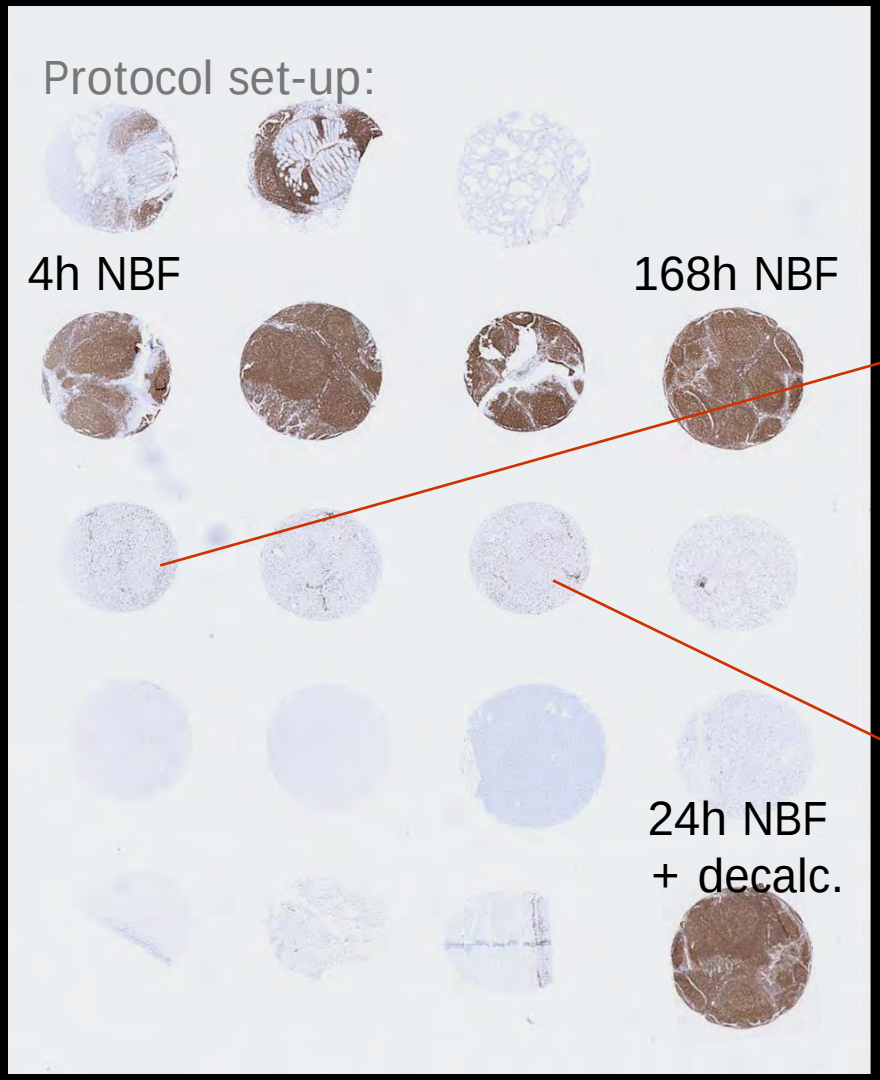
Pathos – 48h NBF, 2h prog.

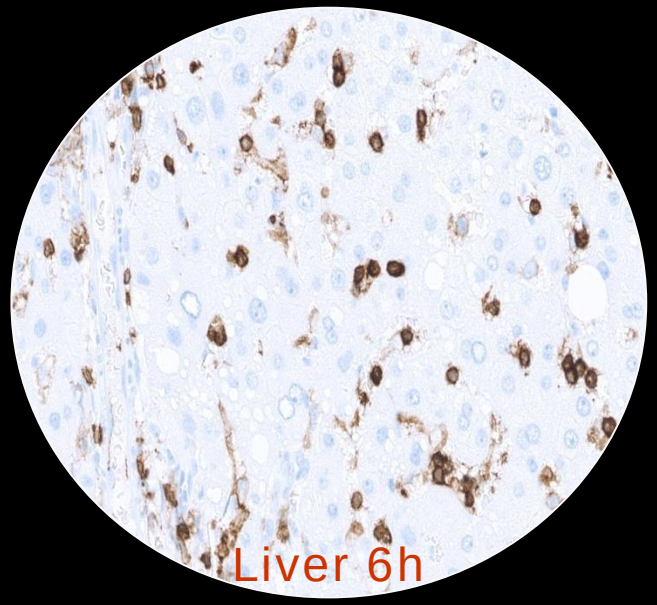


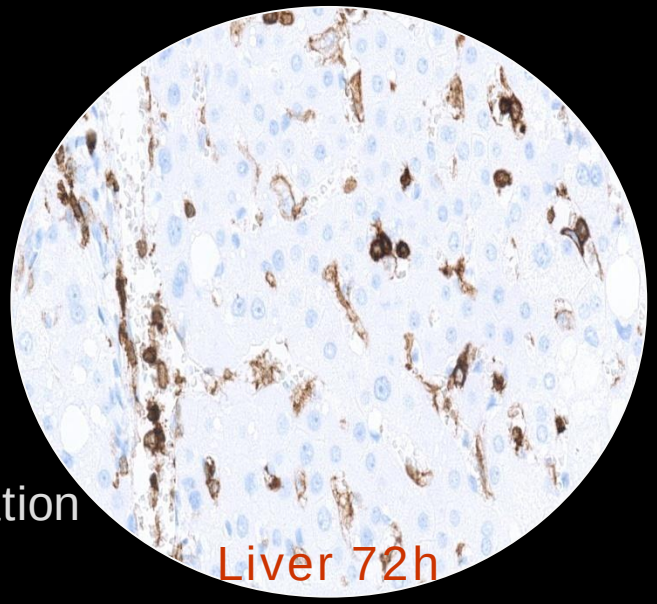
Pathos – 168h NBF, 2h prog.

IHC – Biomarker controls

Anti-CD45 test:

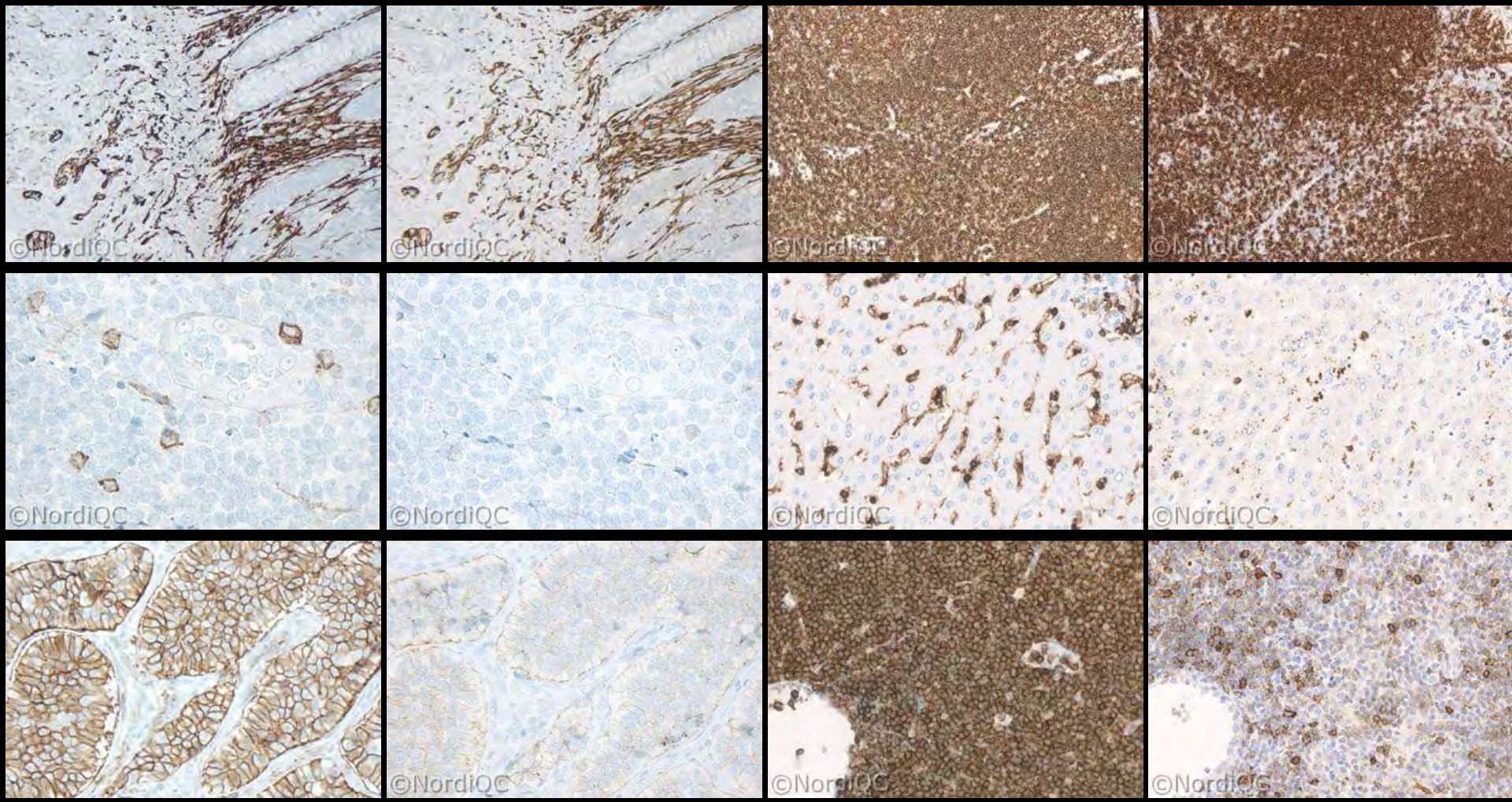






1. Not NBF dependent or influenced by decalcification
2. Liver as control, 3. IHC protocol

IHC – Biomarker controls

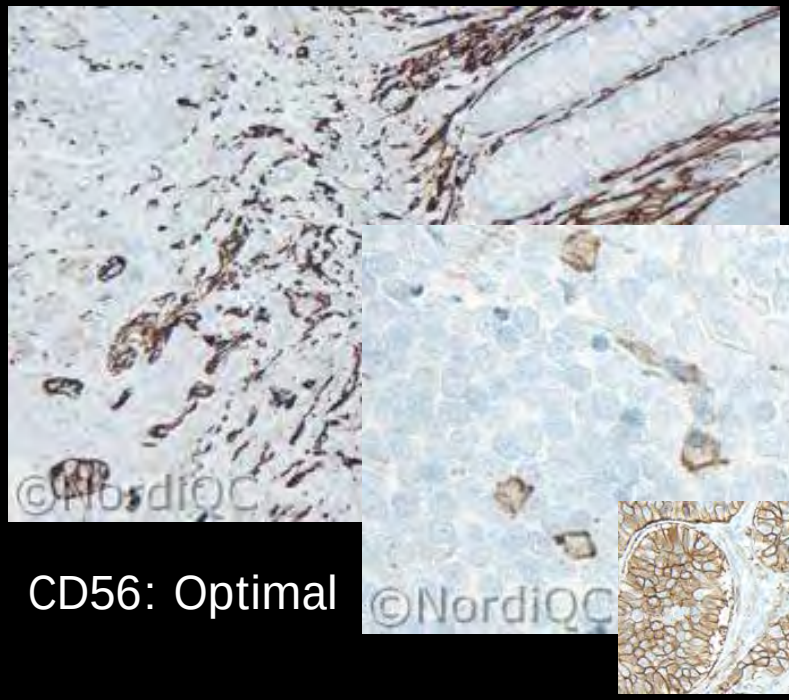


CD56
App – Tonsil – Neuroendocrine carc.

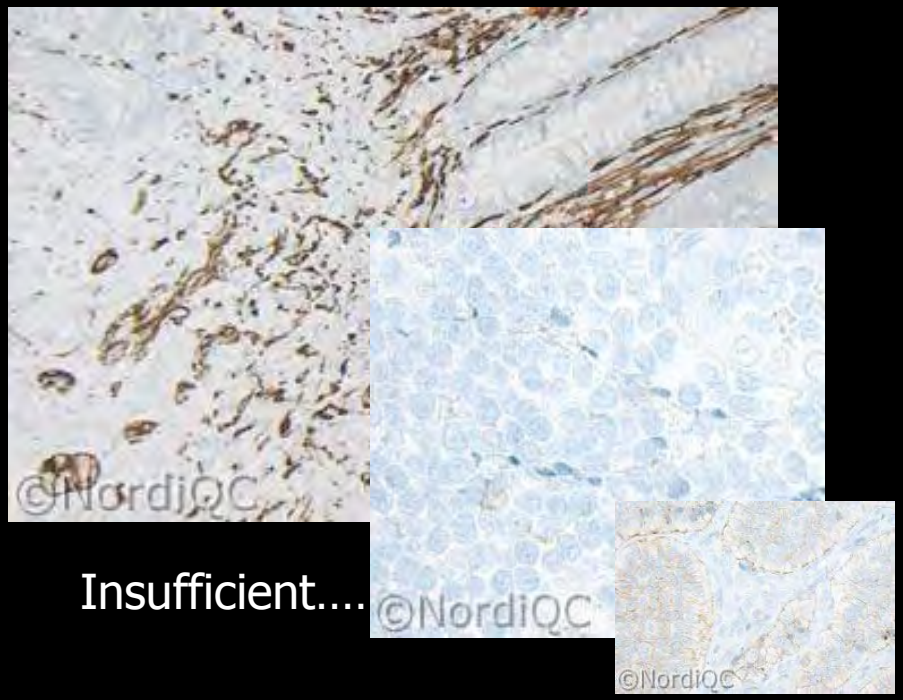
CD45
Tonsil – Liver – B-CLL.

Protocol A Protocol B Protocol A Protocol B

IHC – Biomarker controls



CD56: Optimal



Insufficient....

Tissues/cells with only high expression will not identify:

1. A poorly calibrated IHC assay
2. A reduced sensitivity in an optimally calibrated IHC assay

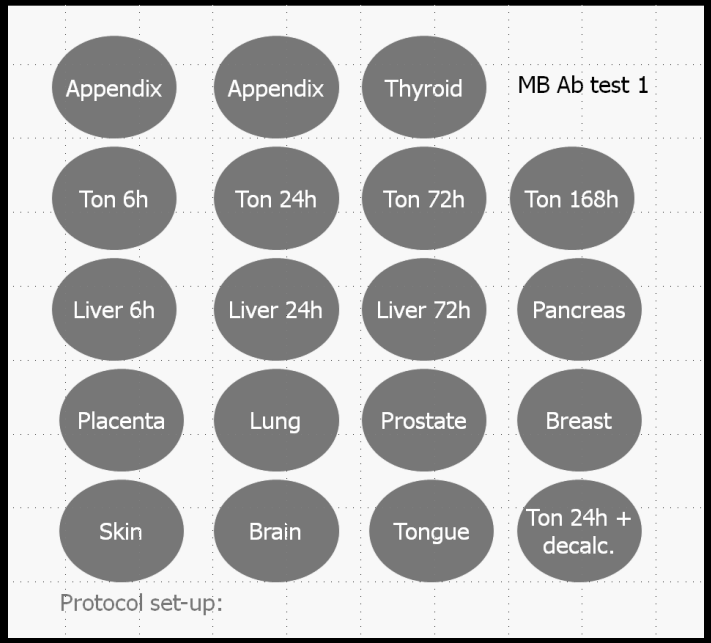
If an IHC test is used to demonstrate the target antigen being expressed at different levels, the controls must reflect this !

IHC – Biomarker controls

Concentrated antibodies – Aalborg Hospital (app. 200 Abs) – VMS ULTRA

	1:25	1:100	1:400
A	None	None	None
B	Enzyme P1, 4 min	Enzyme P1, 4 min	Enzyme P1, 4 min
C	HIER CC1 pH 8.5*	HIER CC1 pH 8.5	HIER CC1 pH 8.5
D	HIER CC2 pH 6.0*	HIER CC2 pH 6.0	HIER CC2 pH 6.0
(E)	CC1 + Enzyme P3, 8 min	CC1 + Enzyme P3, 8 min	CC1 + Enzyme P3, 8 min
(F)	Enzyme P3, 8 min + CC1	Enzyme P3, 8 min + CC1	Enzyme P3, 8 min + CC1

*HIER time 48 min. at 99°C
OptiView DAB



1. Technical calibration

2. Diagnostic / analytical evaluation

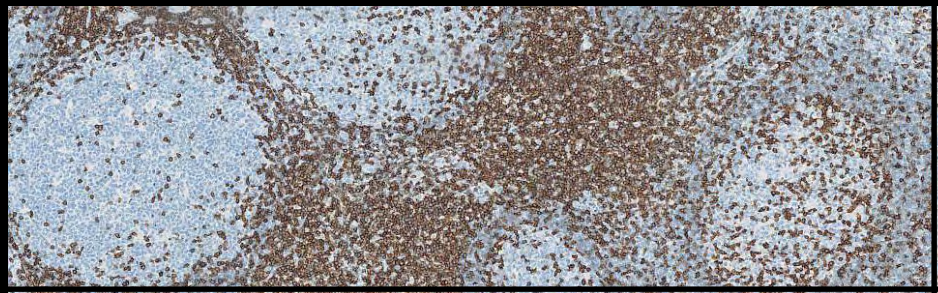
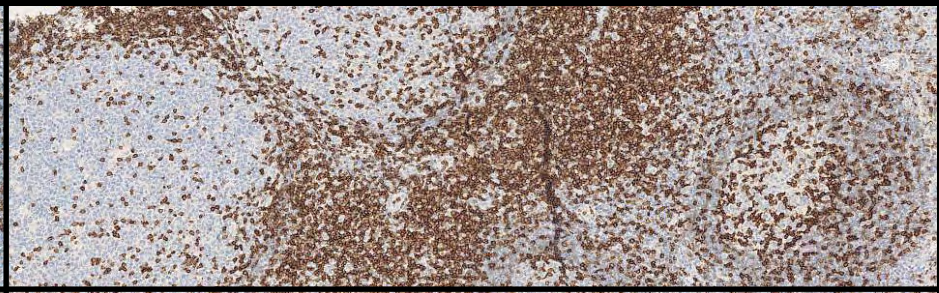
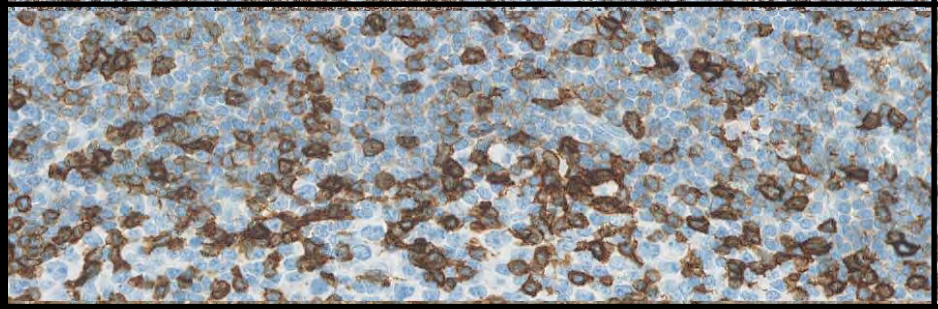
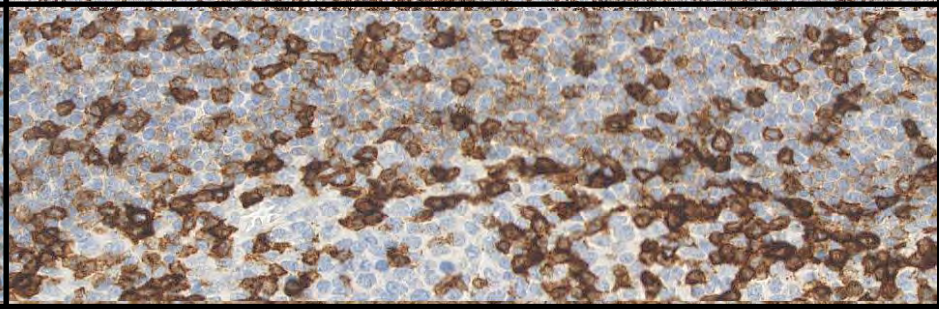
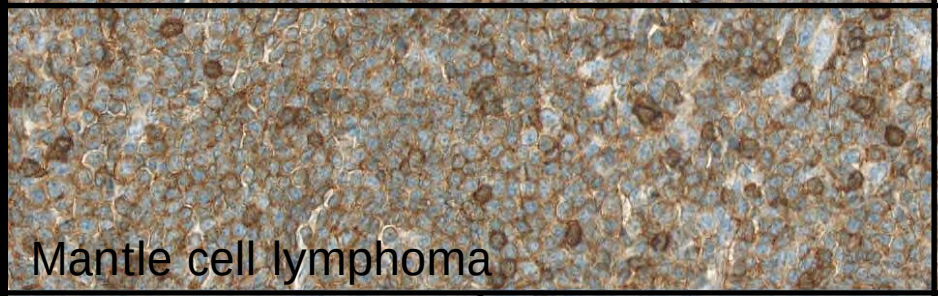
IHC – Biomarker controls

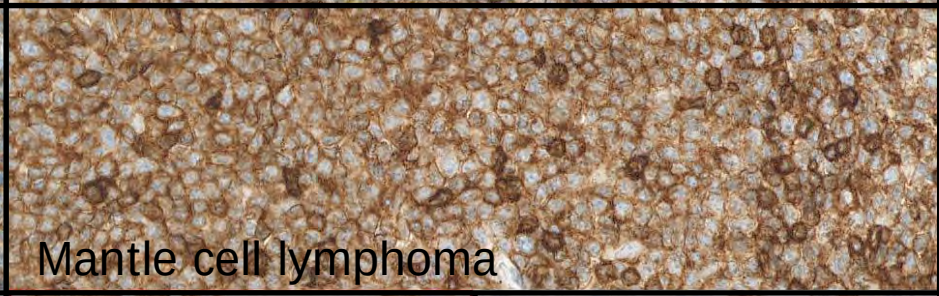
- Analytical validation
  - Laboratory developed tests (concentrates and RTU formats being applied modified to official protocol)
  - Non-predictive markers (- ER, PR, HER-2..)
    - CLSI: 20 cases per entity relevant (pos, neg)
    - CAP: 10 positive, 10 negative

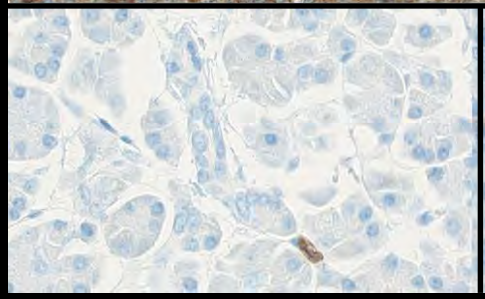
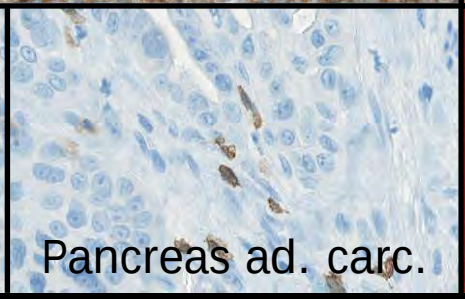
      The validation set should include high and low expressors for positive cases when appropriate and should span the expected range of clinical results (expression levels) for markers that are reported quantitatively.
    - Ad-Hoc: 10 strongly pos, 10 interm. to low, 5 neg.

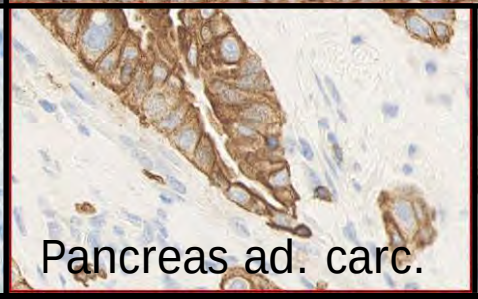
Number less important compared to use of tissue with full range of expression patterns reflecting the diagnostic use

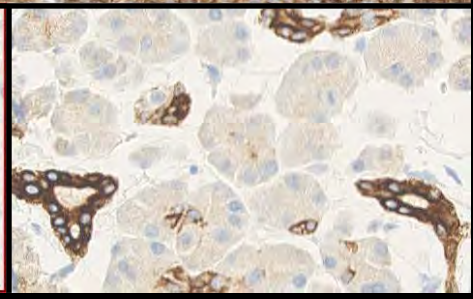
IHC – Biomarker controls



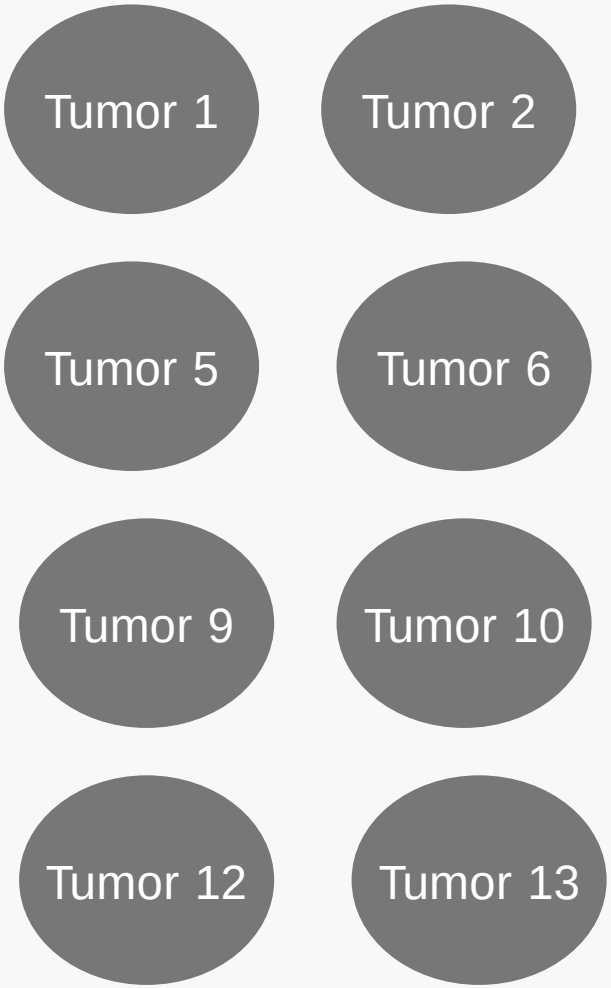




CD5 - rmAb SP19

CD5 - mAb 4C7

IHC – Biomarker controls



Diagnostic potential:

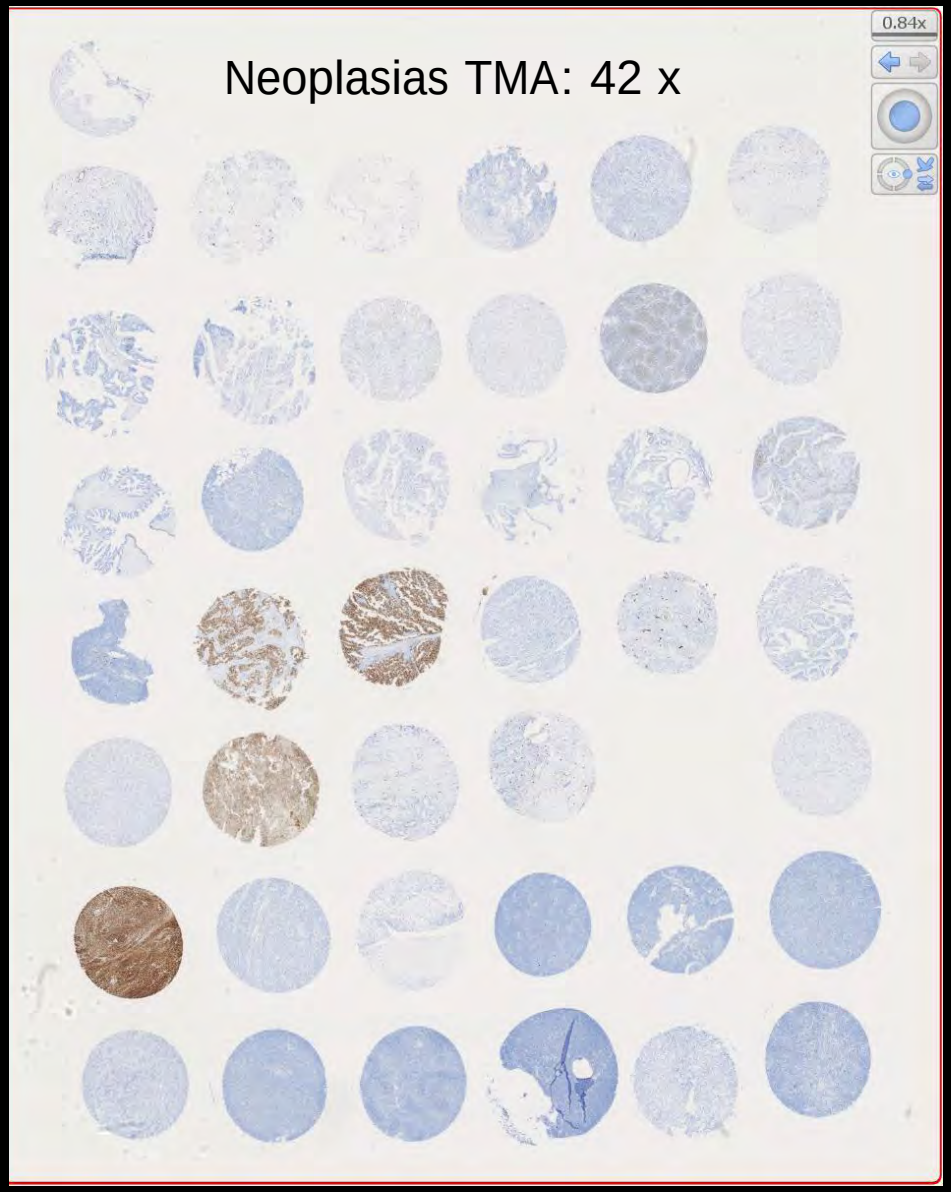
TMA Neoplasia

Liver					
Mamma ductal carc.	Mamma ductal carc.	Mamma Lobular carc.	Lung adeno carc.	Lung adeno carc.	Lung squam. carc.
Colon adeno carc.	Colon adeno carc.	Kidney clear c carc.	Kidney clear c carc.	Thyroid. follic. carc.	Thyroid. Medul. carc.
Ovary. Serous I carc.	Ovary. Serous I carc.	Ovary. Clear carc.	Ovary. Endom. carc.	Corpus Uteri Endom. carc.	Cerxix Uteri adeno carc.
Tonsil	Testis Semin.	Testis Semin.	Prostate adeno carc.	Prostate adeno carc.	Intest Carcinoid
Melanom	Melanom	Pancr. adeno carc.	Pancr. adeno carc.	Uroth. carc.	Uroth. carc.
GIST	Leio myo sarcoma	Rhabdo myo sarcoma	Hodgkin Classic	Hodgkin mixed	Diffuse large B lymph.
Diffuse large B lymph.	B-CLL	Follik. lymph	Mantle cell lymph.	T-cell lymph. perip.	T-cell lymph. Anapl.

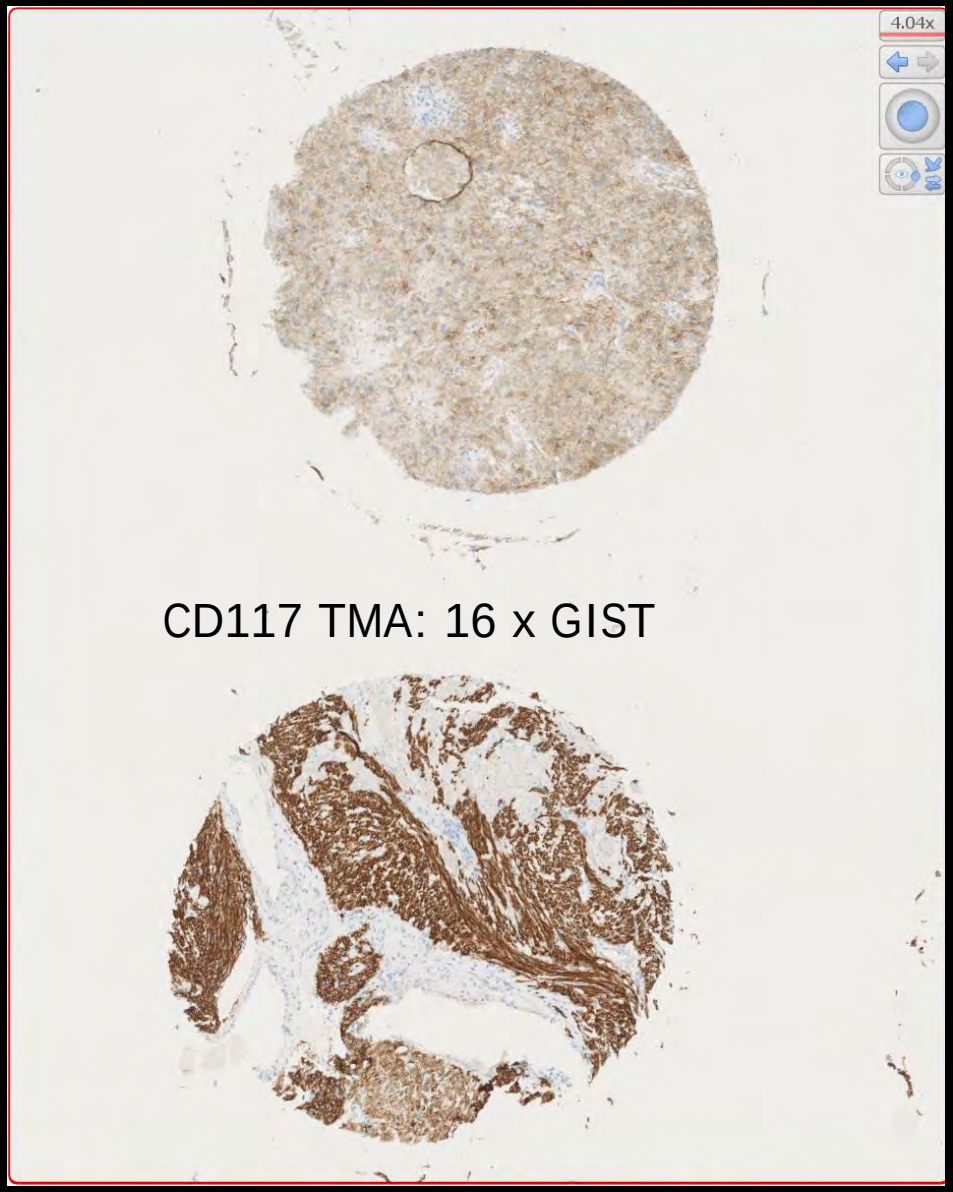
IHC – Biomarker controls

CD117 TMA: 16 x GIST

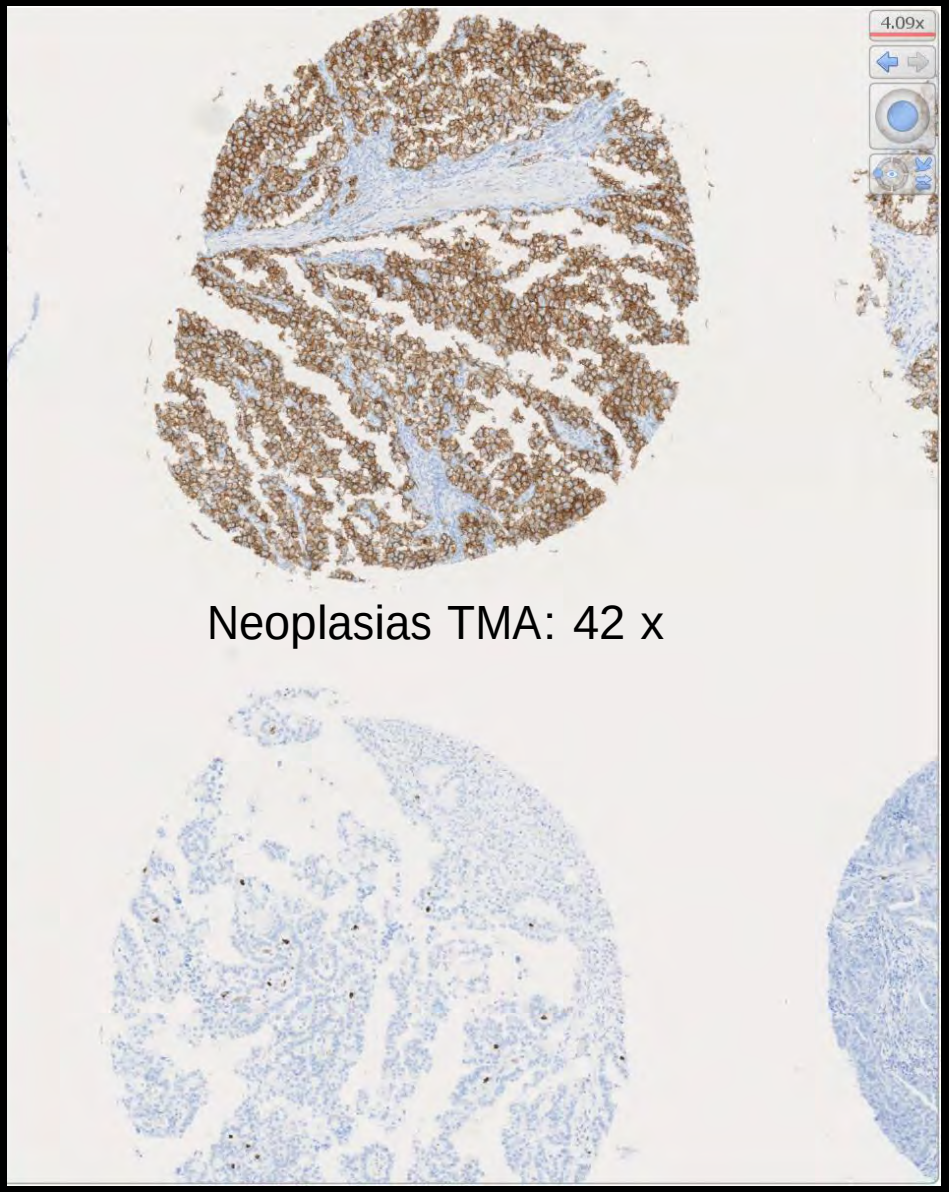
Neoplasias TMA: 42 x



IHC – Biomarker controls



CD117 TMA: 16 x GIST

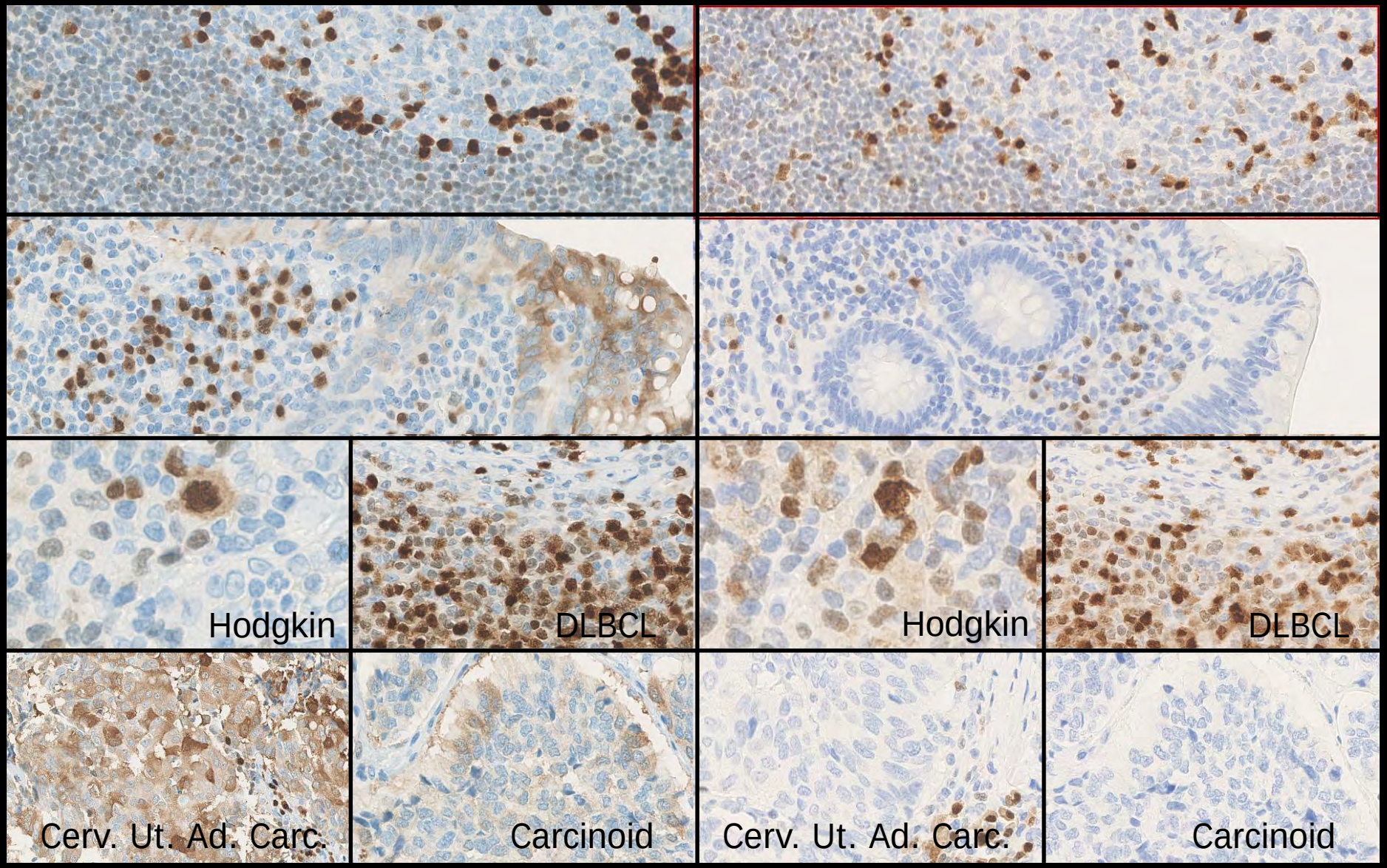


Neoplasias TMA: 42 x

NordiQC – Antibodies giving different patterns

Antigen	Clone	High expressor	Low expressor	Non expressor
CD3	LN10, 2GV6	√	√	–
CD3	Poly A0452	√	√	(+) – (epith.)
CD5	SP19	√	√	–
CD5	4C7	√	√	(+) – (epith.)
CD8	4B11,C8/144B	√	√	–
CD8	SP57	√	√	(+) – (epith.)
MUM1	EUA32, MUM1p,	√	√	–
MUM1	MRQ-43	√	√	(+) – (epith.)
OCT 3/4	C10, N1NK	√	√	–
OCT 3/4	MRQ-10	√	√	+ – (neuroendo.)
PLAP	NB10	√	√	–
PLAP	8A9	√	√	+ – (muscle)
WT1	WT49	√	√	–
WT1	6F-H2	√	√	+ – (epith.)

IHC – Biomarker controls



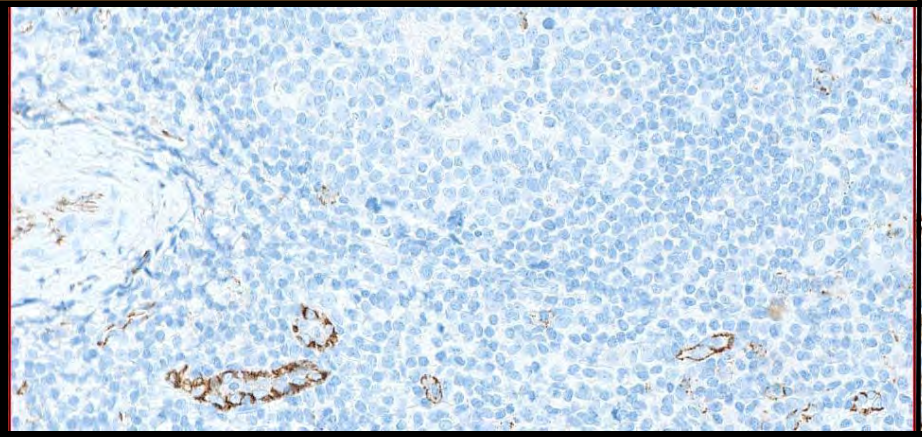
MUM1 - mAb MRQ-43

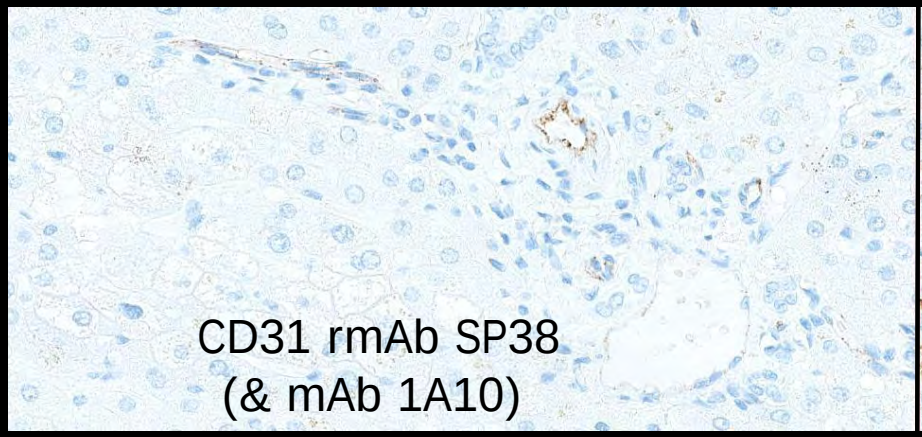
MUM1 - mAb EUA32 & MUM1p

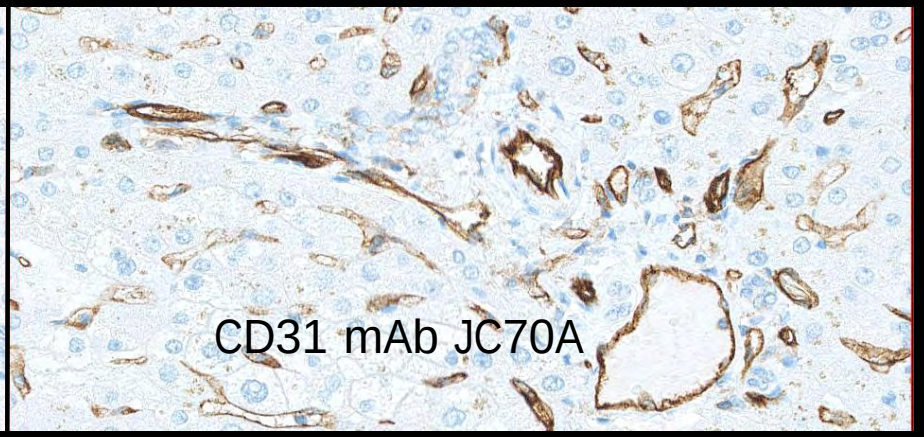
NordiQC – Less successful antibodies

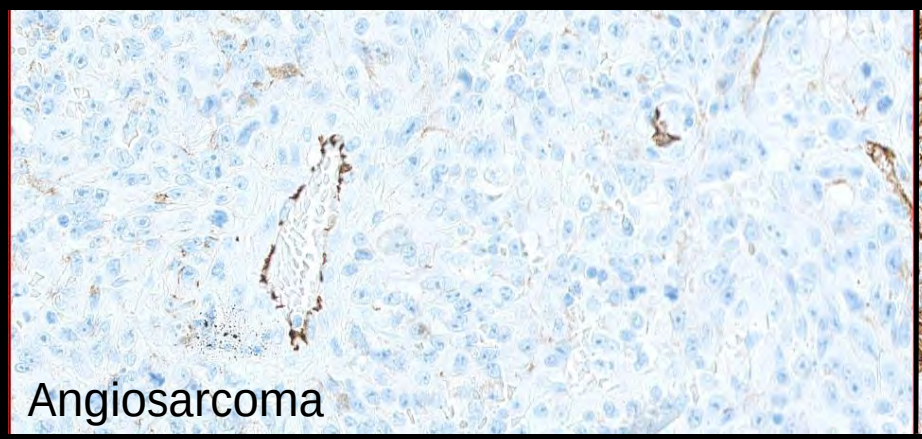
Antigen	Clone	High expressor	Low expressor	Non expressor
CD5	CD5/54/F6	√	FN	–
CD23	MHM6	√	FN	–
CD31	1A10	(√)	FN	–
CD31	SP38	(√)	FN	–
CD138	5F7	(√)	FN	–
CDX2	SP54	(√)	FN	FP
CEA	TF-3H8-1	√	√	FP
CGA	DAK. A3	√	FN	–
CK20	PW31	√	(√)	–
CK-LMW	35BH11	√	FN	–
MLH1	EPR3894	√	√	FP
MSH2	EPR3943	√	√	FP
MSH6	44	√	FN	XB
SYP	SY38	√	FN	XB

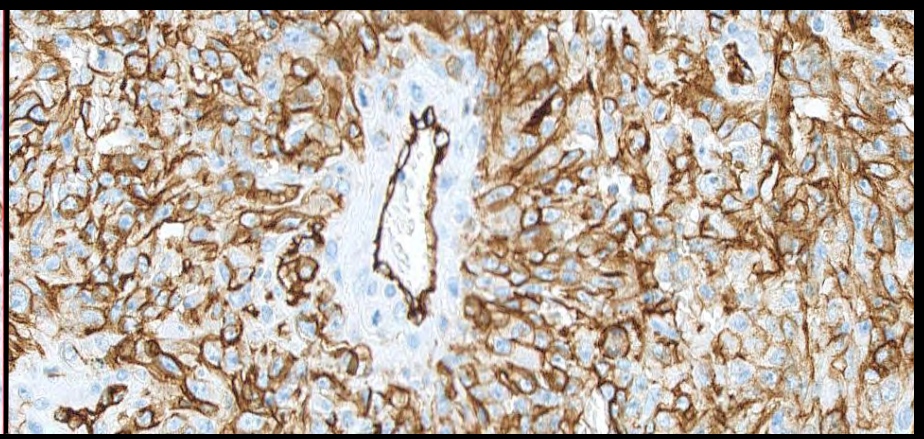
IHC – Biomarker controls











IHC – Biomarker controls

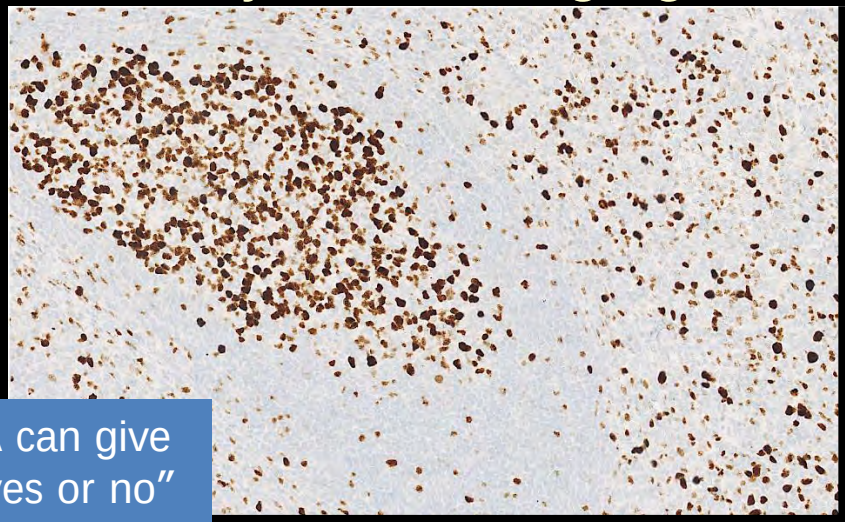
- Analytical validation – Challenges
  - Identification of tissue with expected level of high, low and absence can be difficult to comply with e.g.
    - New marker not tested previously
      - No reference as e.g. for change of clone etc
    - New IHC system changing the range
      - Next Generation, Dako – TSA amplification, VMS
  - Number of samples
    - TMA or whole sections (homogenous / heterogenous)
    - Normal tissues or neoplasias
    - Rare positive cases (ALK lung carcinoma)

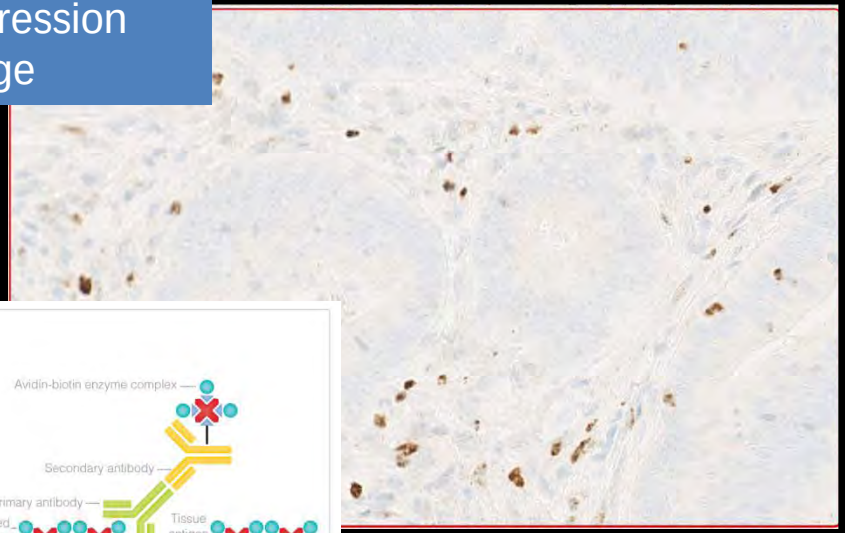
IHC – Biomarker controls

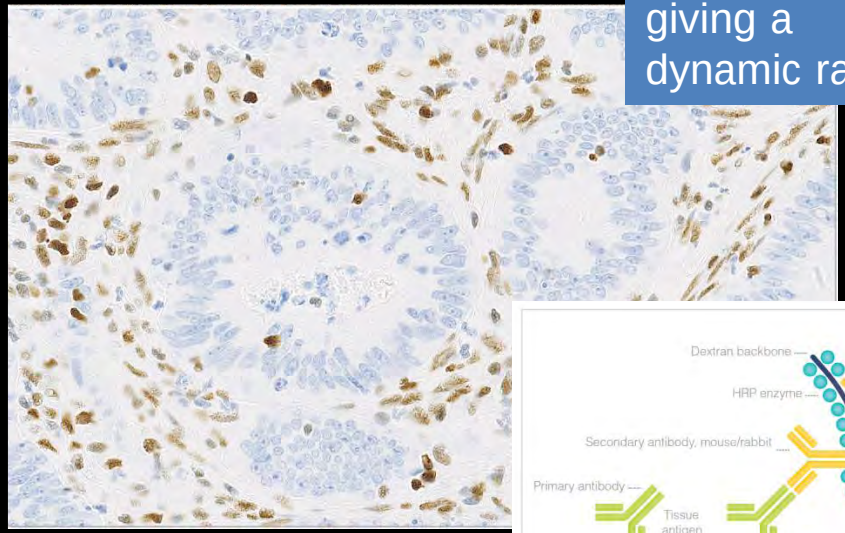
New IHC system changing the range – TSA based (OptView+A)

TSA can give a ”yes or no” expression range

”Standard” 2- & 3- step polymer giving a dynamic range







MSH6 rmAb EP49

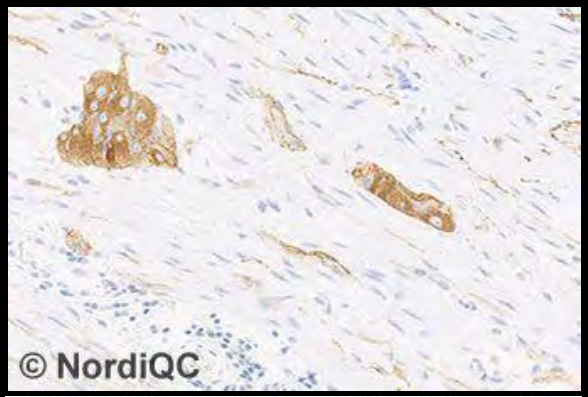
1:200 OptiView + Amp

1:50 OptiView

IHC – Biomarker controls

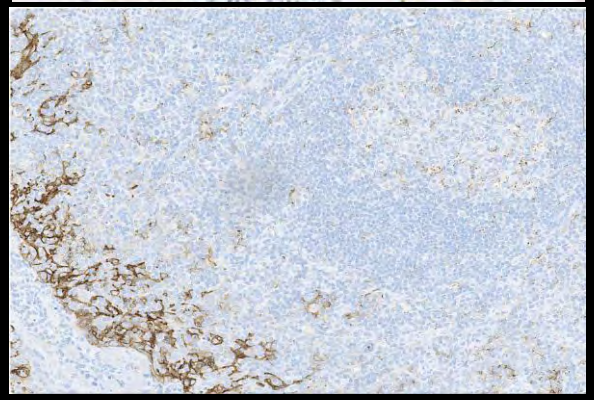
Challenge: Rare in cancers and/or in benign cells

- ALK, ROS1, PD-L1 etc and many molecular derived targets
- Needed to verify IHC method is working
- *ALK lung; 30 cancers used to find 1 pos case…..*



ALK
Appendix / Colon:

Peripheral nerves – axons and ganglion cells



PD-L1
Tonsil:
Germinal centre macrophages

Precision and metrics of test to be confirmed

IHC – Biomarker controls

Cell lines/Histoids:

A high valueable supplement to tissue controls:

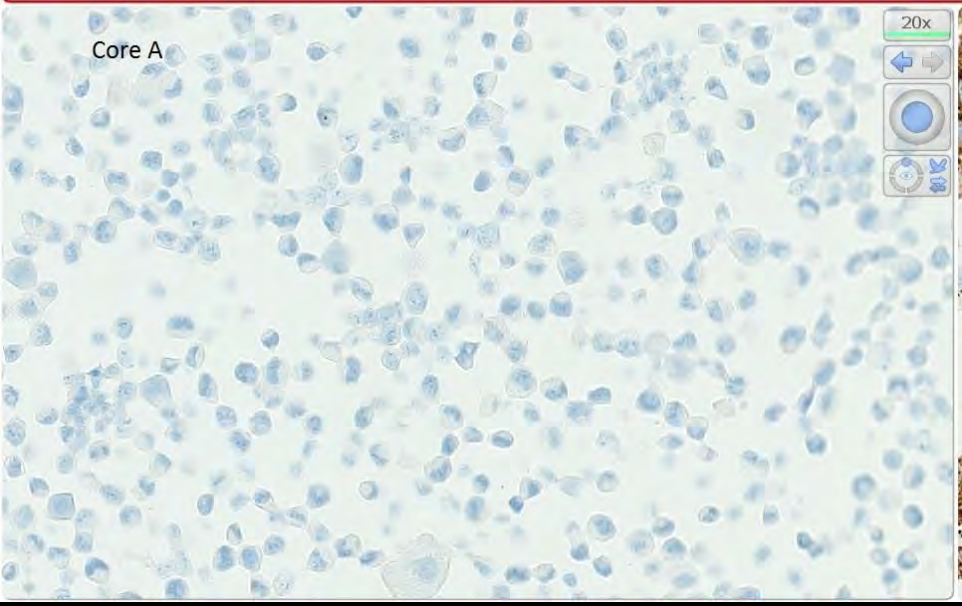
- Rare and/or not normal occuring targets
  - ALK, ROS1, BRAF, etc and other molecular derived targets
- Quantitative targets
  - ER, PR, HER2, PD-L1

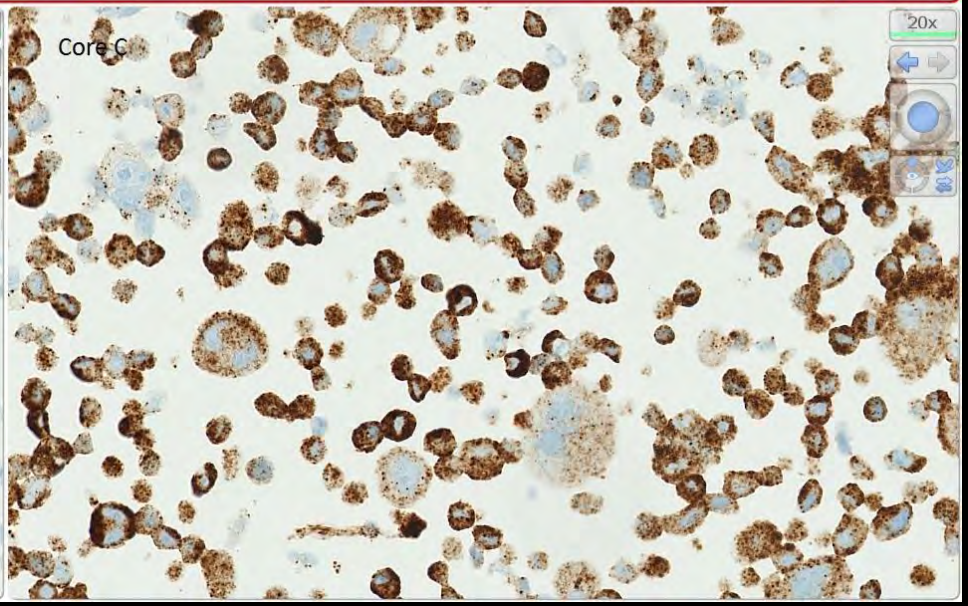
Cave-out – tissue processing and biological environment different compared to histological specimen and has to be encountered

www.horizondiscovery.com

www.histiocyte.com

IHC – Biomarker controls





IHC – Biomarker controls

3 main practical areas of controls in diagnostic IHC

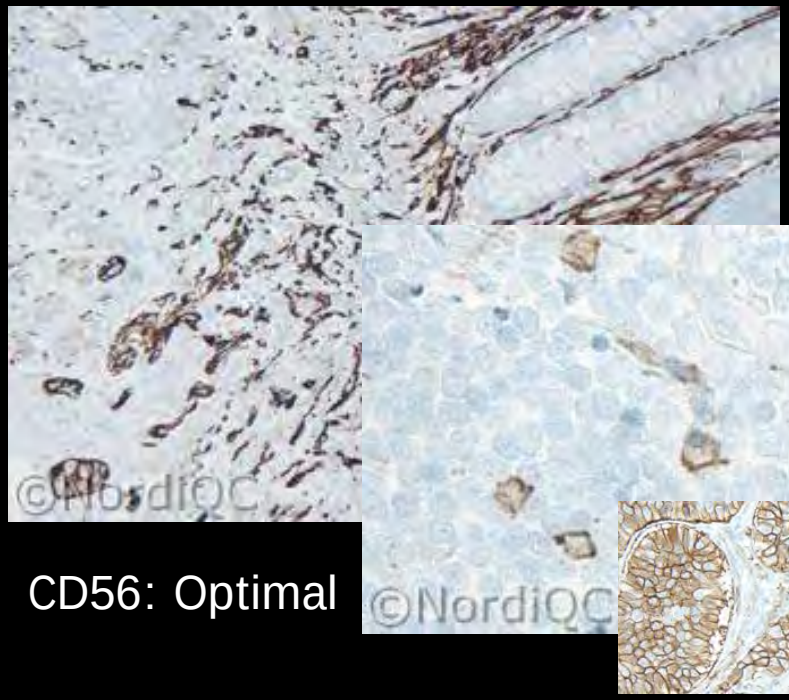
1. Calibration of IHC assay and identification of best practice protocol – clone, titre, retrieval etc
"Evaluation of the robustness – impact on pre-analytics.

2. Analytical validation – diagnostic potential
Sensitivity / specificity.

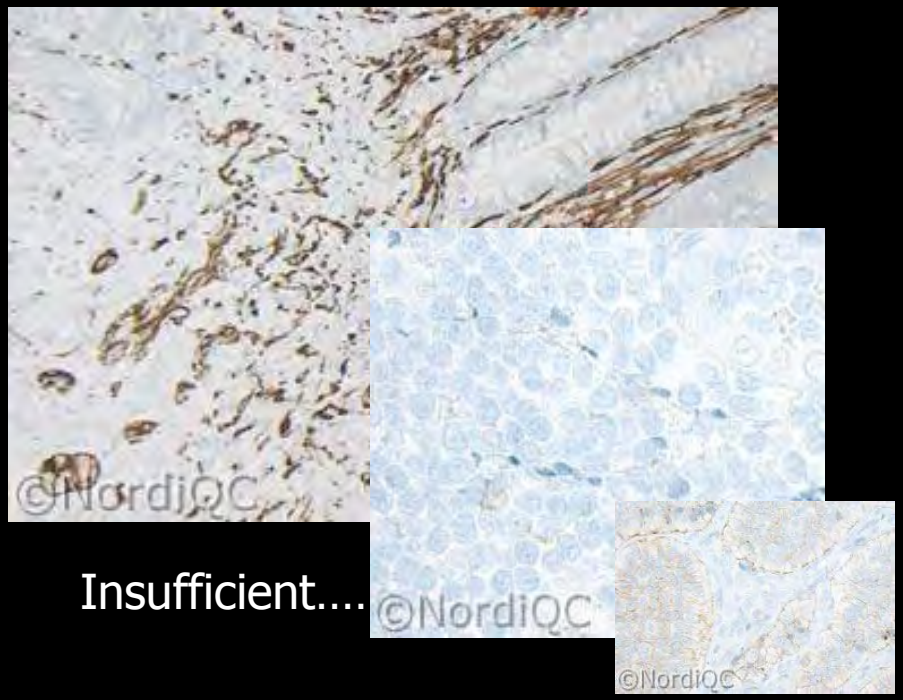
3. IHC performance controls – to monitor that the established level of detection is obtained in each test performed in daily practice – method transfer.

Virtually always; external tissue control

IHC – Biomarker controls



CD56: Optimal



Insufficient....

Tissues/cells with only high expression will not identify:

1. A poorly calibrated IHC assay
2. A reduced sensitivity in an optimally calibrated IHC assay

If an IHC test is used to demonstrate the target antigen being expressed at different levels, the controls must reflect this !

IHC – Biomarker controls

IHC Critical Assay Performance Controls (iCAPCs)

Which tissues are recommended ?

What is the expected staining pattern ?

Which tissues / cells are critical ?

Right antibody
Appropriate level of sensitivity
Guidance level of specificity

REVIEW ARTICLE

Standardization of Positive Controls in Diagnostic Immunohistochemistry: Recommendations From the International Ad Hoc Expert Committee

Emina E. Torlakovic, MD, PhD,† Søren Nielsen, HT, CT,‡§ Glenn Francis, MBBS, FRCPA, MBA, FFSc (RCPA),‖¶# John Garratt, RT,†** Blake Gilks, MD, FRCPC,†† Jeffrey D. Goldsmith, MD,‡‡ Jason L. Hornick, MD, PhD,*§§ Elizabeth Hyjek, MD, PhD,* Merdol Ibrahim, PhD,‖‖ Keith Miller, FIBMS,‖‖ Eugen Petcu, MD, PhD,‖ Paul E. Swanson, MD,¶¶## Xiaoge Zhou, MD,***††† Clive R. Taylor, MD, PhD,‡‡‡ and Mogens Vyberg, MD‡§*

IHC – Biomarker controls

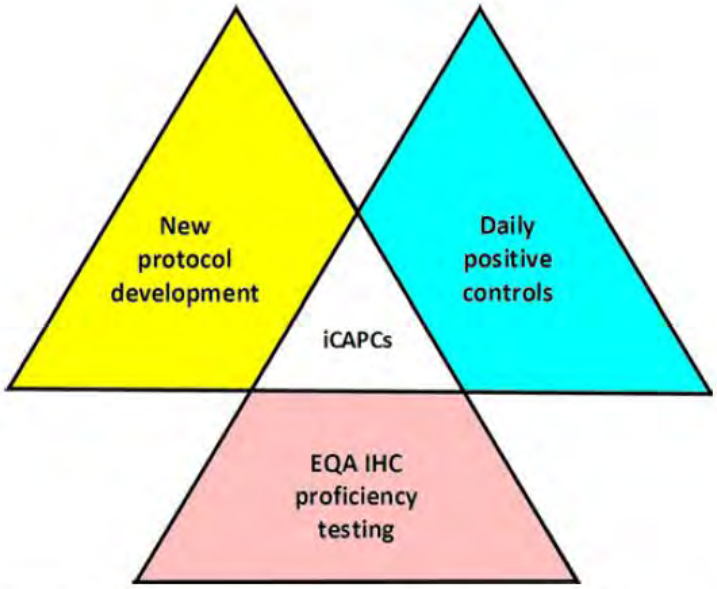


FIGURE 19. The roles of iCAPCs in clinical immunohistochemistry (IHC) laboratories. iCAPCs are an essential part of new protocol development, daily quality controls, and proficiency testing. EQA indicates External Quality Assurance; iCAPC, immunohistochemistry critical assay performance controls.

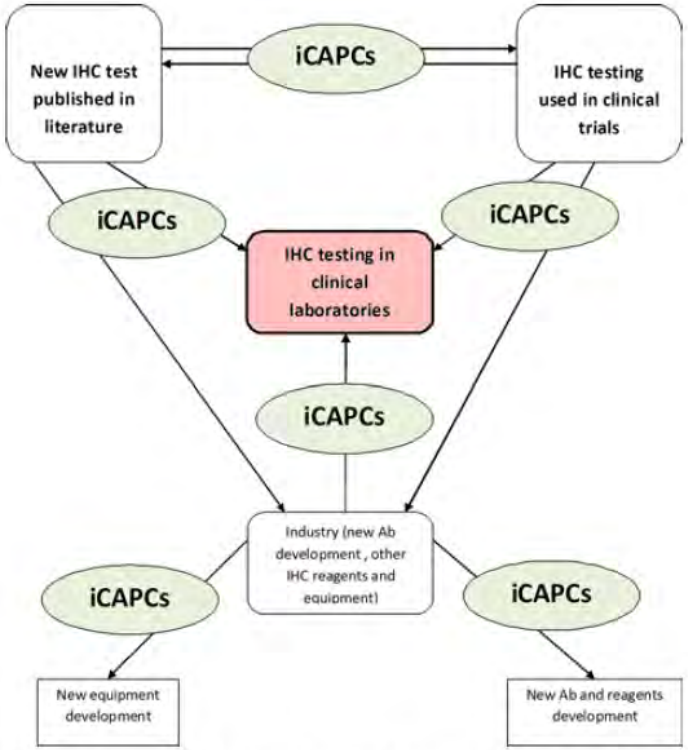


FIGURE 20. iCAPCs and Methodology Transfer. iCAPCs are proposed as important elements for harmonization of immunohistochemistry (IHC) testing between clinical research, product development, and clinical IHC testing. iCAPCs enable IHC harmonization of protocol transfer between research, industry, and clinical laboratories. iCAPC indicates immunohistochemistry critical assay performance controls.

iCAPS to be used as central element for evaluation of quality;

Expected level – calibration
Analytical sensitivity and specificity

IHC – Biomarker controls

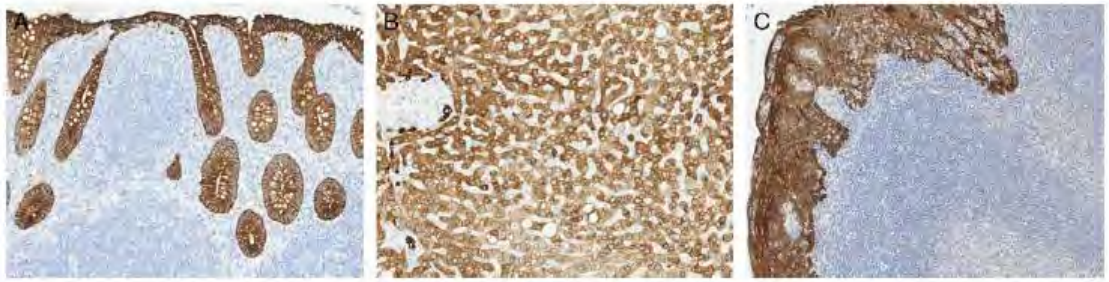


FIGURE 1. Pan-keratin iCAPC. A, Appendix: virtually all columnar epithelial cells must show a moderate to strong predominantly cytoplasmic staining reaction (a membranous accentuation will typically be seen). B, Liver: the vast majority of hepatocytes must show at least weak to moderate cytoplasmic staining reaction with a membranous accentuation (LLOD). C, Tonsil: all squamous epithelial cells must show a moderate to strong cytoplasmic staining reaction. Cytokeratin (CK)-positive interstitial reticulum cells (CIRCs) with dendritic/reticular pattern can show a weak to moderate cytoplasmic staining reaction (LLOD). iCAPC indicates immunohistochemistry critical assay performance controls; LLOD, low limit of detection.

FIGURE 7. TTF-1 iCAPC. A, Thyroid: virtually all epithelial cells must show a strong nuclear staining reaction. B, Lung: virtually all pneumocytes and basal cells of terminal bronchi must show a moderate to strong nuclear staining reaction. Columnar epithelial cells of terminal bronchi must show an at least weak nuclear staining reaction (LLOD). C, Tonsil: no staining reaction must be seen. iCAPC indicates immunohistochemistry critical assay performance controls; LLOD, low limit of detection.

FIGURE 8. CDX-2 iCAPC. A, Appendix: virtually all epithelial cells must show a strong nuclear staining reaction. A weak cytoplasmic staining reaction in addition to strong nuclear staining is often present. B, Pancreas: the majority of epithelial cells of intercalated ducts must show a weak to moderate nuclear staining reaction (LLOD). C, Tonsil: no staining reaction must be seen. iCAPC indicates immunohistochemistry critical assay performance controls; LLOD, low limit of detection.

Examples for 17 markers

Generel expected patterns

High expression
(Right antibody)

Low expression
(Appropriate sensitivity)

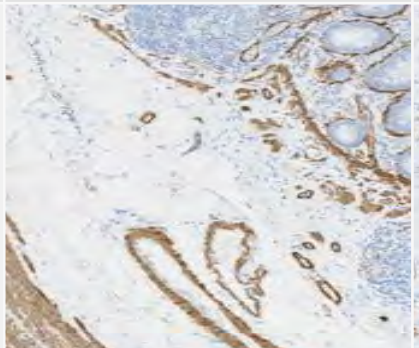
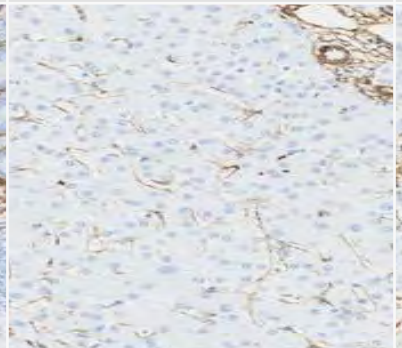
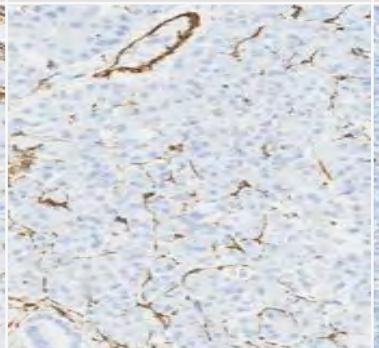
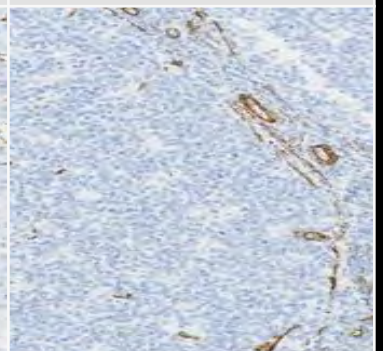
No expression
(Appropriate specificity)

Which tissue
Which cells
Which extension
Which intensity

IHC – Biomarker controls

	High express.	Low ex. (iCAPs)	Non express.	Comment
CK-PAN	Appendix	Liver	Tonsil	
CK-LMW	Appendix	Liver	Tonsil	
CK-HMW	Tonsil	Pancreas	Liver	
CK7	Liver	Pancreas	Tonsil	
CK20	Appendix	Appendix	Tonsil	Different comp.
CD3	Tonsil	Appendix	Tonsil	
CD20	Tonsil	Appendix	Appendix	
CD31	Tonsil	Liver	Appendix	
Vimentin	Appendix	Liver	Liver	Different comp.
Desmin	Appendix	Tonsil	Appendix	Different comp.
ASMA	Appendix	Liver	Appendix	
SYP	Appendix	Appendix	Tonsil	Different comp.
CGA	Appendix	Appendix	Tonsil	Different comp.
TTF1	Thyroid	Lung	Tonsil	
CDX2	Appendix	Pancreas	Tonsil	
S100	Appendix	Tonsil	Appendix	Different comp.
Ki67	Tonsi[1]	Tonsil	Tonsil	Different comp.

IHC – Biomarker controls

ASMA (C)	Appendix	Liver	Pancreas	Tonsil
High expression (right ab)	A moderate to strong staining reaction in virtually all smooth muscle cells in muscularis mucosae	A moderate to strong staining reaction in the smooth muscle cells in vessels	A moderate to strong staining reaction in the smooth muscle cells in vessels	A moderate to strong staining reaction in the smooth muscle cells in vessels
Low expression iCAPS (right sens.)	-	An at least weak to moderate, staining reaction of the majority of the perisinusoidal cells	-	-
Non expression (right spec.)	No staining reaction in the epithelial cells	No staining in the hepatocytes (except lipofuscin)	No staining reaction in the epithelial cells	No staining reaction in lymphocytes
				

IHC – Biomarker controls

- The NordiQC focus areas
  - Central protocol elements for an optimal staining
    - Antibody selected
    - Antibody dilution range / Ready-To-Use
    - Epitope retrieval
    - IHC detection system & stainer platforms
  - Recommendable control and identification of critical quality stain indicators / iCAPCs
    (Which tissue ? Which cells ?, How must they look ?)

IHC – Biomarker controls

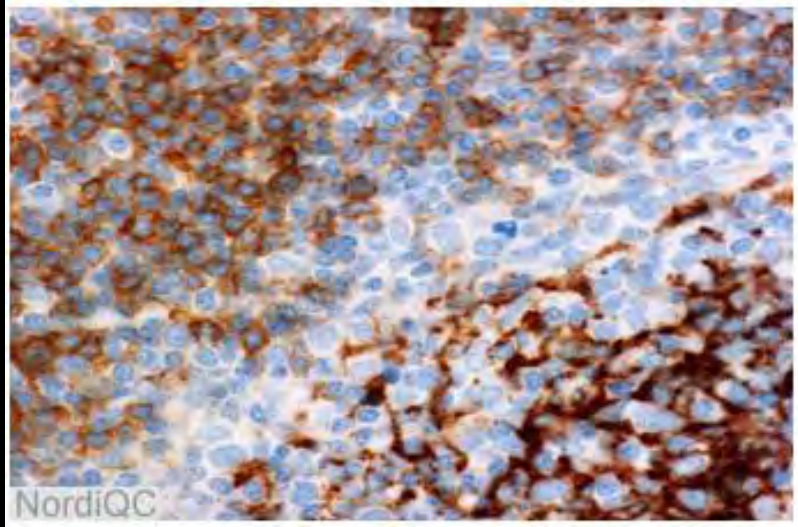


Fig. 2a. High magnification of the optimal staining in Fig 1a of the secondary follicle in the tonsil. The activated B-cells show a distinct continuous membranous reaction.

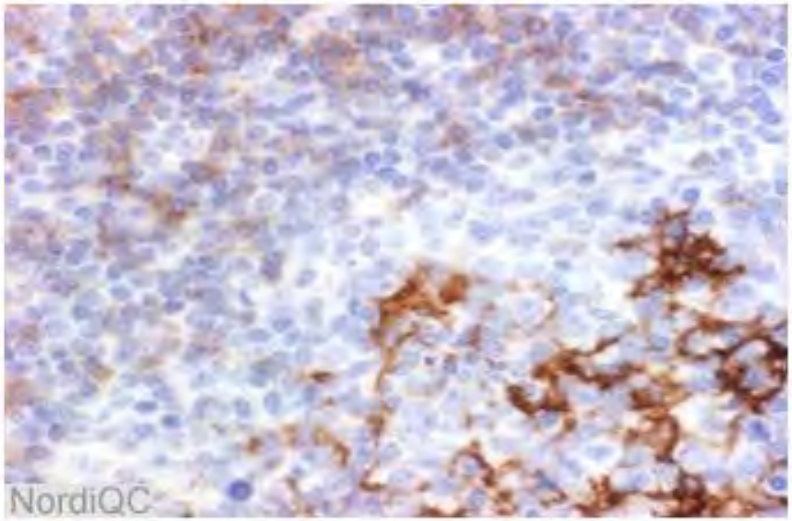


Fig. 2b. High magnification of the insufficient staining in Fig 1b of the secondary follicle in the tonsil (same field as in Fig 2a). The activated B-cells only show a weak imprecise reaction.

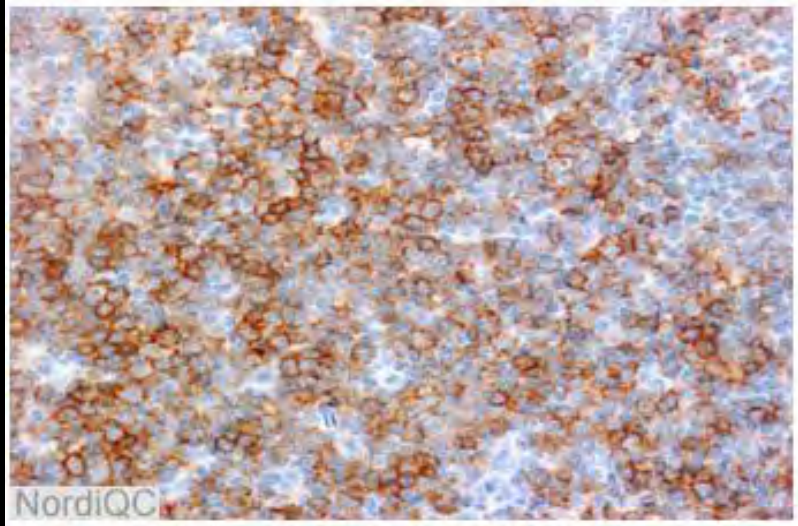


Fig. 3a. Optimal staining for CD23 of the B-CLL no 4 using same protocol as in Fig 1b and 2 b. The majority of the neoplastic cells show a strong and distinct membranous staining.

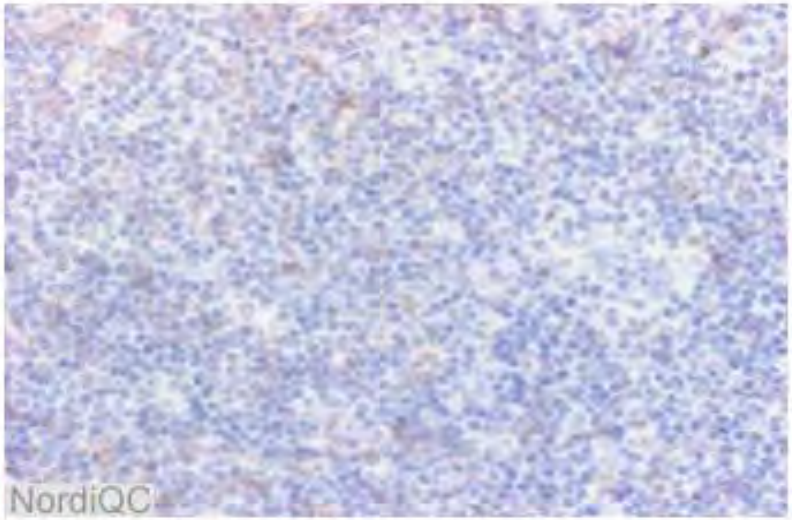


Fig. 3b. Insufficient staining for CD23 of the B-CLL no 4 using same protocol as in Fig 1b and 2 b. The neoplastic cells are virtually negative.

CD23

iCAPCs:

Activated B-cells in mantle z.

IHC – Biomarker controls

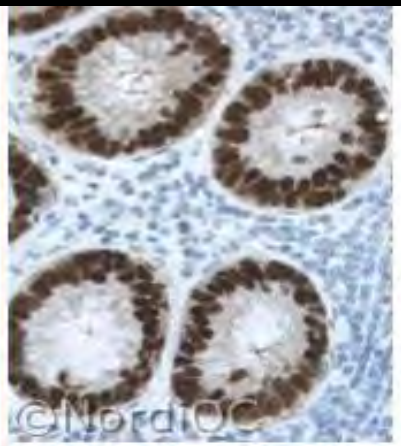


Fig. 1a. Optimal staining for CDX2 using the mAb clone CDX2-88.
Left, colon: A strong nuclear staining is seen in all the enterocytes with a minimal cytoplasmic reaction.
Right, pancreas: A weak to moderate staining is seen in the majority of the ductal epithelial cells.

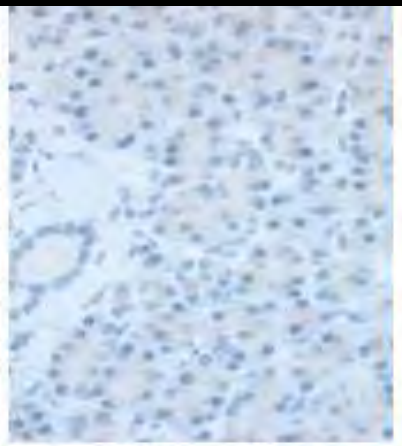
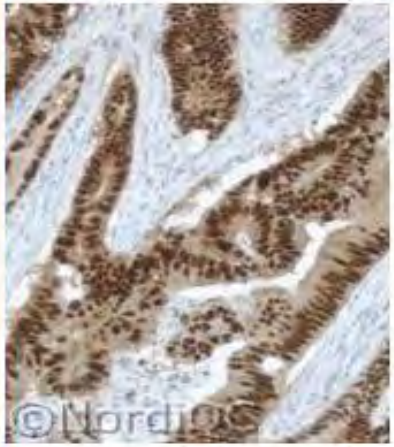


Fig. 1b. Staining for CDX2 using the mAb clone CDX2-88 with an insufficient protocol.
Left, colon: A moderate to strong nuclear staining is seen in all the enterocytes.
Right, pancreas: No nuclear staining is seen in the ductal epithelial cells. Also compare with Fig 2b – same protocol.



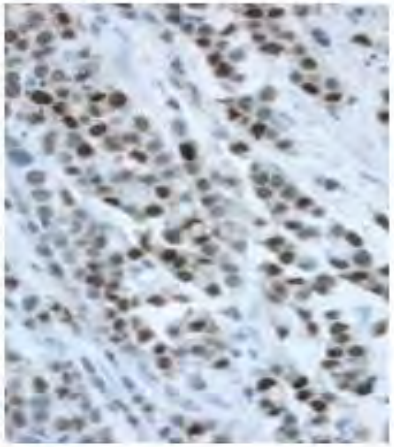
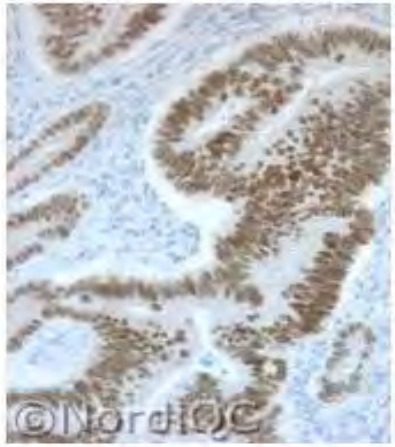


Fig. 2a. Optimal staining for CDX2 using same protocol as in Fig. 1a.
Left: Colon adenocarcinoma with high expression of CDX2: The nuclei of the neoplastic cells show an intense staining while the cytoplasmic compartment is weakly stained.
Right: Colon adenocarcinoma with low expression of CDX2: The majority of the neoplastic cells show a moderate to strong nuclear reaction.



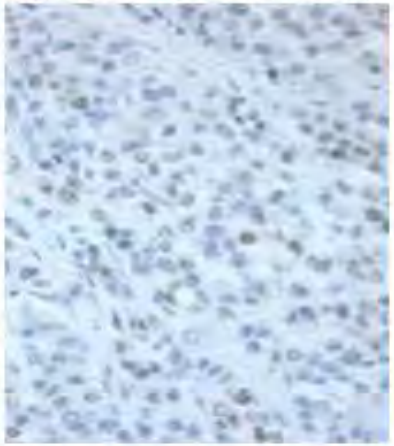


Fig. 2b. Insufficient staining for CDX-2 using same protocol as in Fig. 1b.
Left: Colon adenocarcinoma with high expression of CDX2: The nuclei of the neoplastic cells show a moderate staining, while the cytoplasmic compartment is almost negative.
Right: Colon adenocarcinoma with low expression of CDX2: Only scattered neoplastic cells show a weak nuclear reaction.

CDX2

iCAPCs:

Pancreatic duct ep. cells

IHC – Biomarker controls

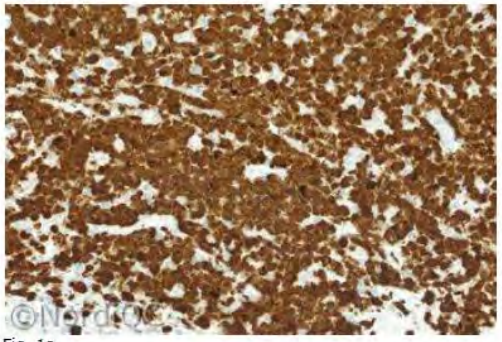


Fig. 1a.
Optimal ALK staining of the ALCL with ALK rearrangement using the rmAb clone D5F3 as RTU format (Ventana), showing an intense nuclear and cytoplasmic staining reaction. Despite the intense staining reaction, a high signal-to-noise ratio is provided and no background staining is seen. Also compare with Figs. 2a - 4a, same protocol.

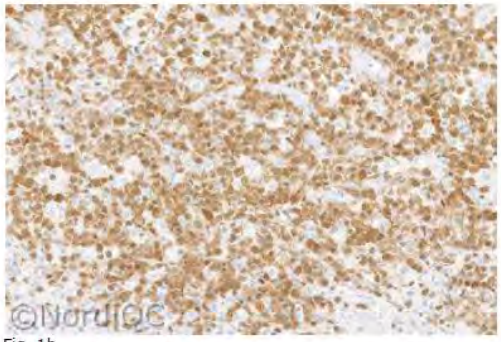


Fig. 1b
Insufficient ALK staining of the ALCL with ALK rearrangement using a protocol based on the mAb clone ALK1 - same field as in Fig. 1a. The vast majority of the neoplastic cells are demonstrated, however also compare with Fig. 2b, same protocol.

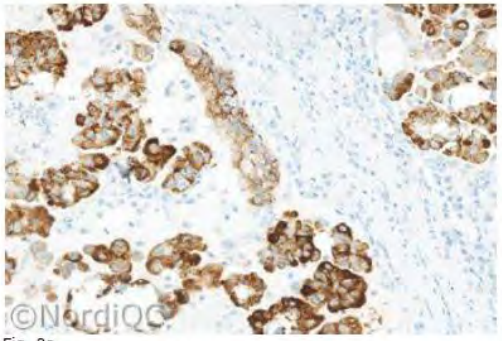


Fig. 2a
Optimal ALK staining of the lung adenocarcinoma with ALK rearrangement using same protocol as in Fig. 1a.
The majority of the neoplastic cells show a moderate to strong granular cytoplasmic staining reaction. No background staining is seen.

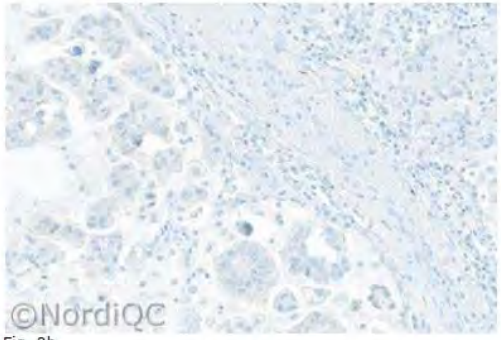
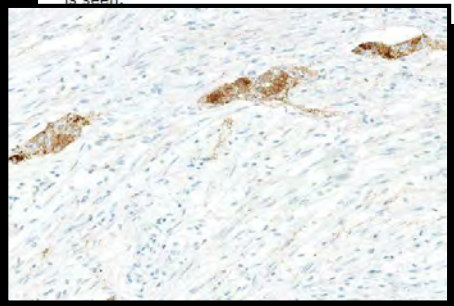


Fig. 2b
Insufficient ALK staining of the lung adenocarcinoma with ALK rearrangement using same protocol as in Fig. 1b - same field as in Fig. 2a. Only scattered neoplastic cells show a weak cytoplasmic staining reaction, while the vast majority are negative.



ALK:

Depending on application

iCAPCs:

Lung;

Lung ad.carc.

Colon

Lymphoma;

ALCL

IHC – Biomarker controls

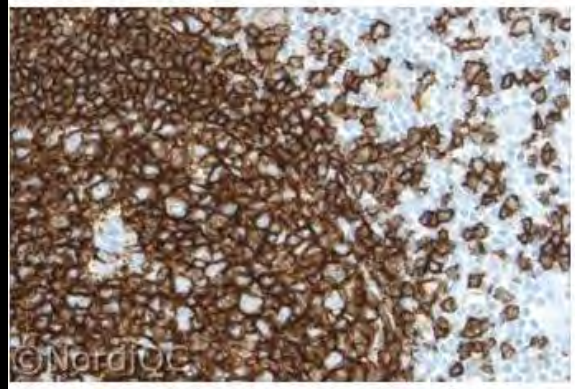


Fig. 1a. Lymphatic tissue in the appendix showing an optimal staining reaction for CD20 using the mAb clone L26 in a RTU format on the BenchMark platform. HIER was performed using Cell Conditioning 1. A very strong membranous staining reaction is seen in virtually all the B-cells.

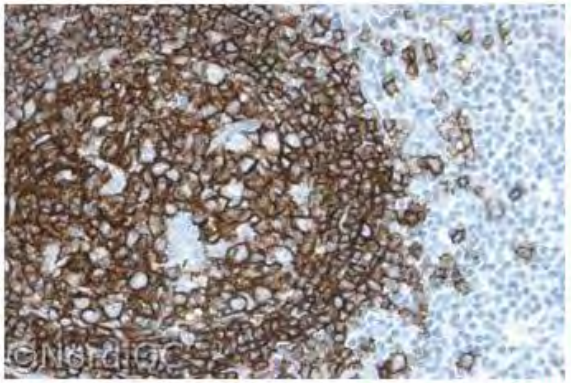


Fig. 1b. Lymphatic tissue in the appendix. Same field as in Fig. 1a. Insufficient staining for CD20 using the mAb clone L26 in a RTU format at the BenchMark platform. No HIER was performed. A moderate to strong staining reaction is seen in virtually all the B-cells. The normal B-cells are high expressors of CD20, hence the relatively strong reaction. Even so, the staining intensity should be improved in order to detect low expressors of CD20 (e.g. B-CLL in Fig. 2a and 2b).

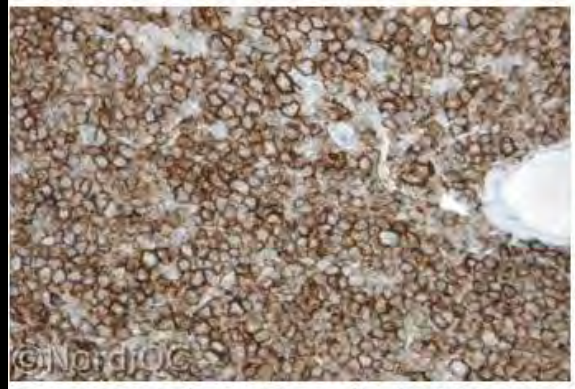


Fig. 2a. B-CLL. Optimal staining reaction for CD20. Same protocol as in Fig. 1a. A moderate to strong membranous staining is seen in virtually all the neoplastic cells.

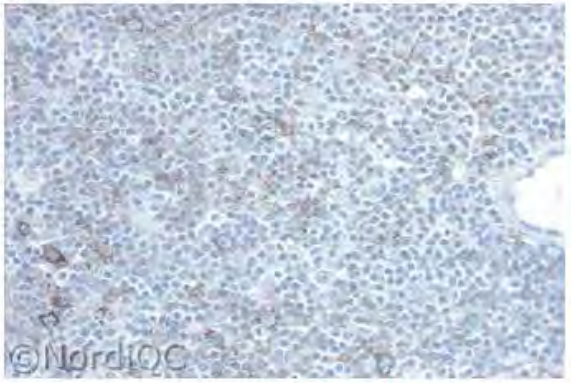


Fig. 2b. B-CLL. Insufficient staining for CD20 using the same protocol as in Fig. 1b. Omitting HIER, only scattered cells are positive. The majority of the neoplastic cells are negative. Compare with the optimal result in Fig. 2a, same field.

CD20:

iCAPCs:

????

ASAP….

As strong as possible…

IHC – Biomarker controls

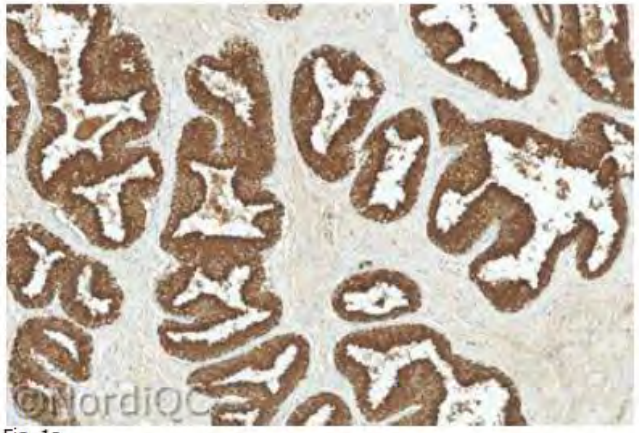


Fig. 1a
Optimal PSA staining of the prostate hyperplasia using the mAb 35H9 carefully calibrated and with HIER in an alkaline buffer (x100). All the epithelial cells of the prostatic glands show a strong cytoplasmic staining reaction. A weak stromal staining reaction is seen, which has to be expected and accepted in prostate tissue. Also compare with Figs. 2a - 5a, same protocol.

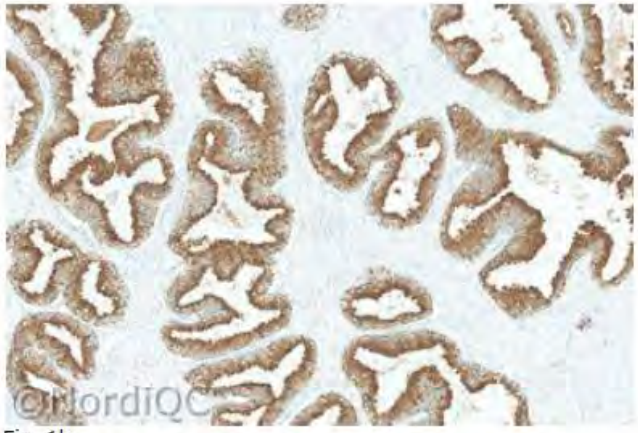


Fig. 1b
Staining for PSA of the prostate hyperplasia using an insufficient protocol based on the pAb A0452 with protocol settings giving a too low sensitivity.Too low concentration of the primary Ab and omission of HIER - same field as in Fig. 1a (x100). The epithelial cells are demonstrated, but a reduced intensity compared to the result seen in Fig. 1a is seen. Also compare with Figs. 2b – 4b, same protocol.

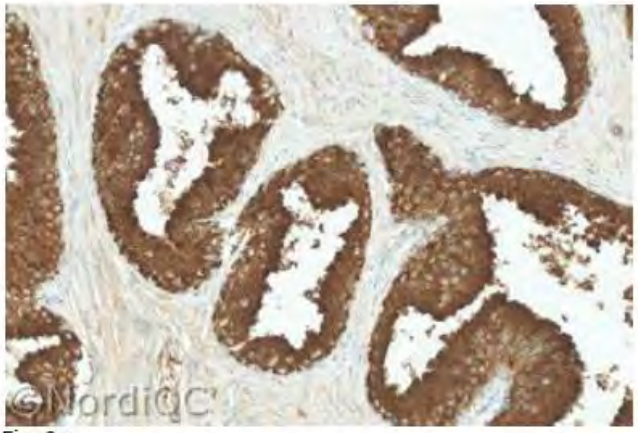


Fig. 2a
High magnification (x200) of the PSA staining of prostate hyperplasia in Fig. 1a. A weak to moderate stromal staining reaction is seen. However no general background staining or poor signal-to-noise ratio is seen, as no staining in the appendix is seen – see Fig. 5a.

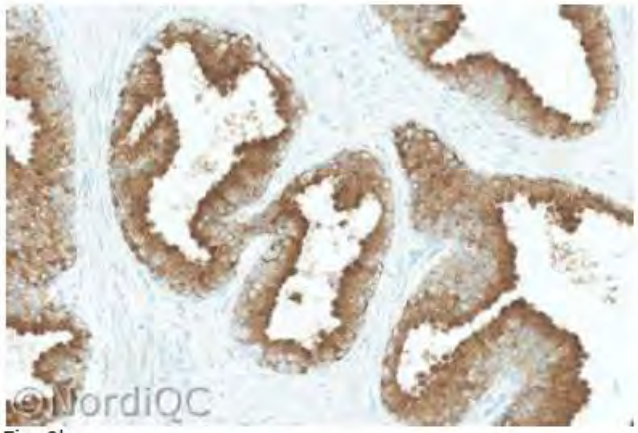


Fig. 2b
High magnification (x200) of the PSA staining of the prostate hyperplasia in Fig. 1b. Also compare with Figs. 2b – 4b, same protocol.

PSA:

iCAPCs:

????

ASAP….

As strong as possible…

IHC – Biomarker controls

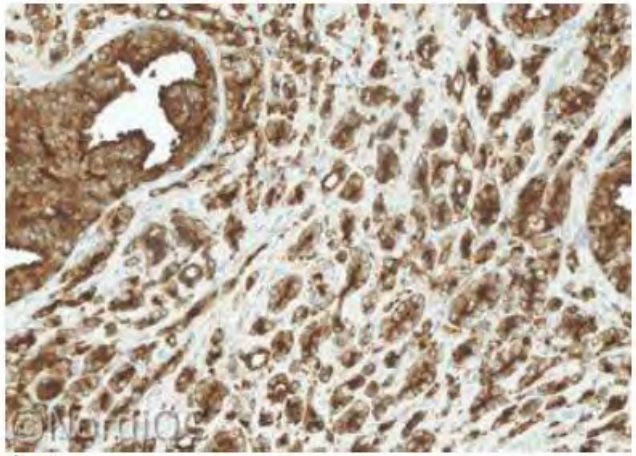


Fig. 3a
Optimal staining for PSA of the prostate adenocarcinoma no. 4 using same protocol as in Figs. 1a and 2a. Virtually all the neoplastic cells show a moderate to strong cytoplasmic staining reaction.

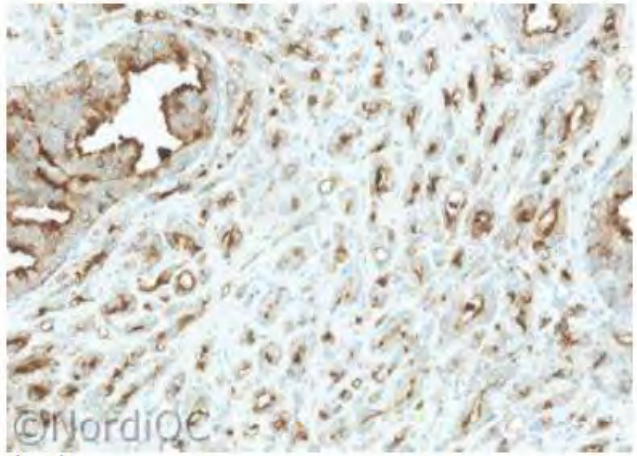
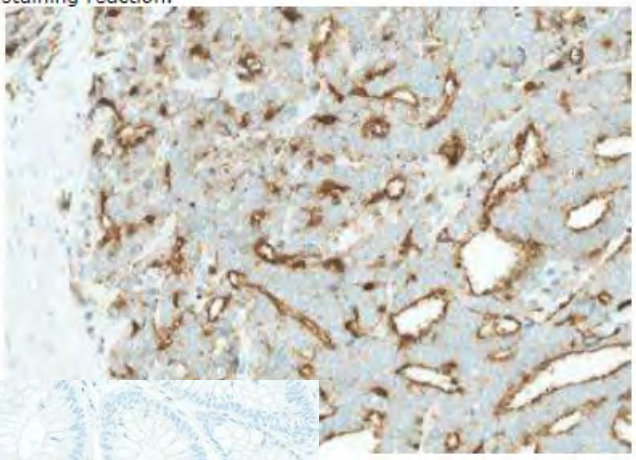


Fig. 3b
Staining for PSA of the prostate adenocarcinoma no. 4 using same protocol as in Figs. 1b and 2b - same field as in Fig. 3a. The majority of the neoplastic cells are demonstrated, but with significantly reduced intensity compared to the level expected.



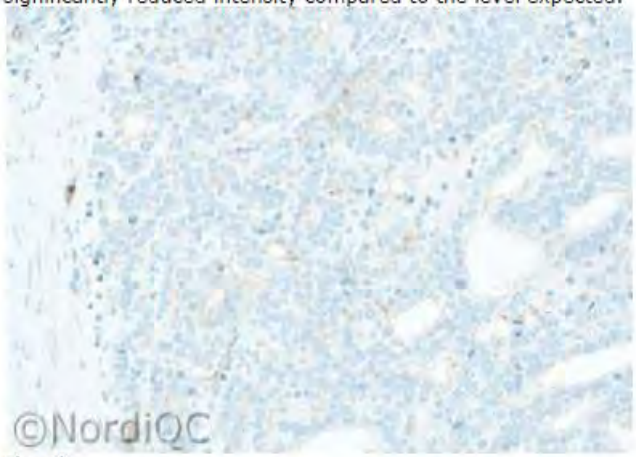


Fig. 4b
Insufficient staining for PSA of the prostate adenocarcinoma no. 5 using same protocol as in Figs. 1b – 3b. Only scattered cells show a faint and dubious staining reaction.

tate adenocarcinoma no. 5
1a - 3a. The majority of the
moderate cytoplasmic staining.

"neg tissue control"

PSA:

iCAPCs:

????

ASAP….

As strong as possible…

IHC – Biomarker controls

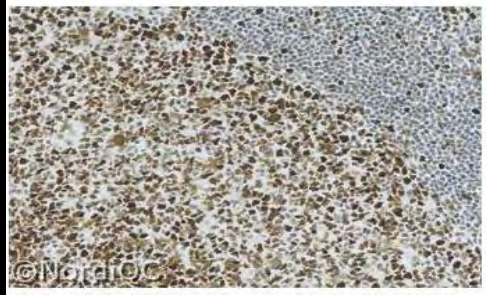


Fig. 1a. Optimal staining for MSH6 of the tonsil using the rmAb clone EP49 optimally calibrated, HIER in an alkaline buffer and a 3-step polymer based detection system.
Virtually all the mantle zone B-cells show a distinct, moderate to strong nuclear staining, while the germinal centre B-cells show a strong nuclear staining.

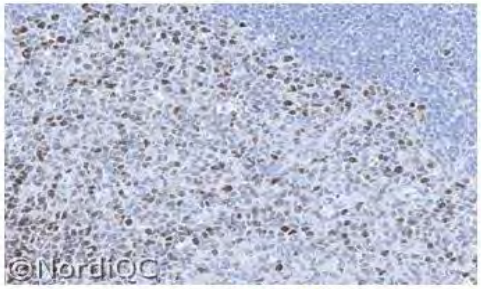


Fig. 1b. Insufficient staining for MSH6 of the tonsil using the mAb clone 44. by a protocol with a too low sensitivity (2-step polymer and too low. conc. of the primary Ab), same field as in Fig. 1a.
Only the germinal centre B-cells are demonstrated, while the mantle zone B-cells expressing limited MSH6 are virtually unstained.
Also compare with Figs. 2b. & 3b., same protocol.

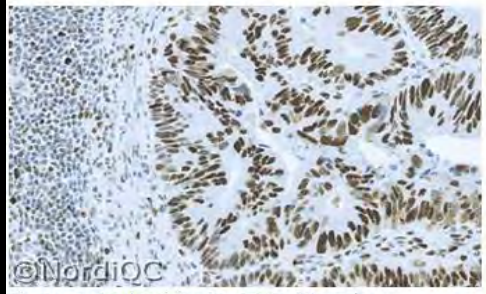


Fig. 2a. Optimal staining for MSH6 of the colon adenocarcinoma no. 3 with intact MSH6 protein using same protocol as in Fig. 1a.
The majority of the epithelial and the stromal cells show a moderate to strong nuclear staining. No background staining is seen.

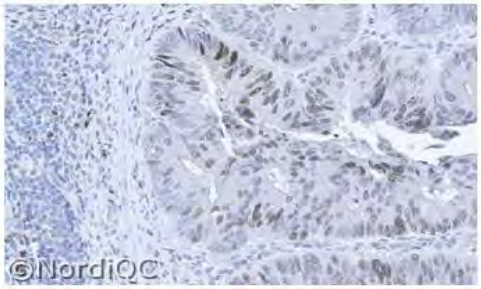


Fig. 2b. Insufficient staining for MSH6 of the colon adenocarcinoma no. 3 with intact MSH6 protein using same protocol as in Fig. 1b., same field as in Fig. 2a.
The proportion of positive cells and the intensity of the staining reaction is significantly reduced compared to the result in Fig. 2a.
Also compare with Fig. 3b., same protocol.

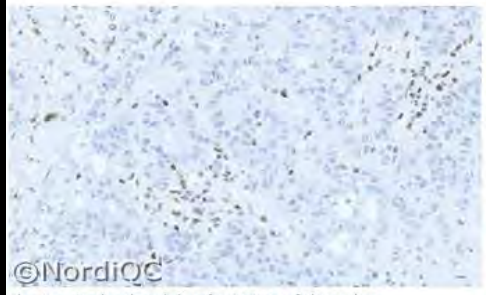


Fig. 3a. Optimal staining for MSH6 of the colon adenocarcinoma no. 5 with loss of MSH6 protein using same protocol as in Figs. 1a. & 2a.
The neoplastic cells are negative, while the remnants of entrapped lymphocytes and stromal cells show a distinct nuclear staining, serving as internal positive control.

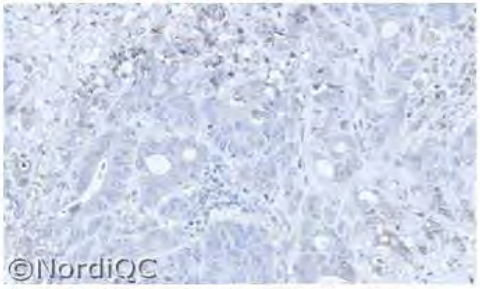


Fig. 3b. Insufficient staining for MSH6 of the colon adenocarcinoma no. 5 with loss of MSH6 protein using same protocol as in Figs. 1b. & 2b., same field as in Fig. 3a.
No nuclear staining reaction is seen in the neoplastic cells, but as virtually no nuclear staining reaction is seen in the normal cells as stromal cells, the staining pattern can not reliably be interpreted. Also note the weak cytoplasmic staining complicating the interpretation.

MMR:

iCAPCs:

Mantle zone B-cells in tonsil

++++++++

(internal control)

Stromal cells!!

IHC – Biomarker controls

Requirements to tissue control library / catalogue:

- Recommendations for virtually all markers used
  - Qualitative markers ” Class I” – yes / no
  - Quantitative markers ” Class II” – how much
  - *”Research” markers / not established markers*

IHC – Biomarker controls

Requirements to tissue control library / catalogue:

- Recommendations for virtually all markers used
  - Qualitative markers ”Class I”– yes / no
    - Will typically comprise 80-90% of IHC markers
    - E.g. CDs, CKs, lineage markers, etc
    - Even though binary - a degree of quantification is observed and used.

IHC – Biomarker controls

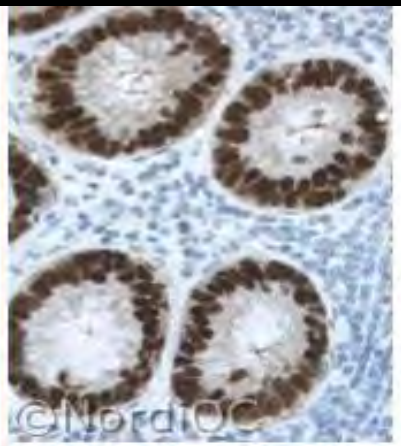


Fig. 1a. Optimal staining for CDX2 using the mAb clone CDX2-88.
Left, colon: A strong nuclear staining is seen in all the enterocytes with a minimal cytoplasmic reaction.
Right, pancreas: A weak to moderate staining is seen in the majority of the ductal epithelial cells.

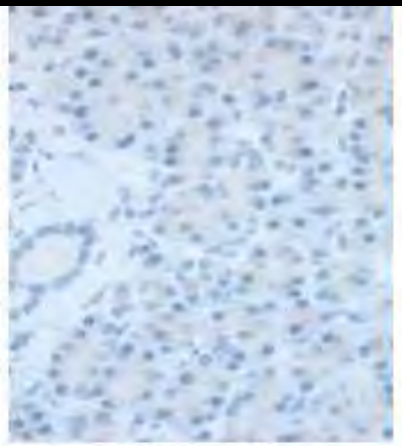
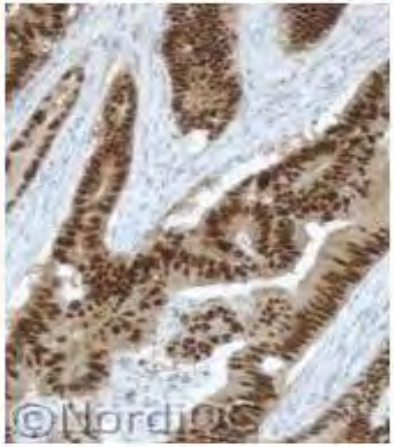


Fig. 1b. Staining for CDX2 using the mAb clone CDX2-88 with an insufficient protocol.
Left, colon: A moderate to strong nuclear staining is seen in all the enterocytes.
Right, pancreas: No nuclear staining is seen in the ductal epithelial cells. Also compare with Fig 2b – same protocol.



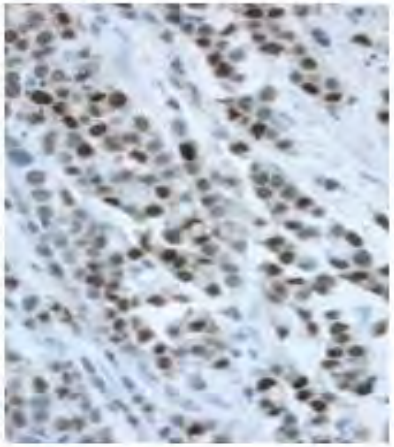
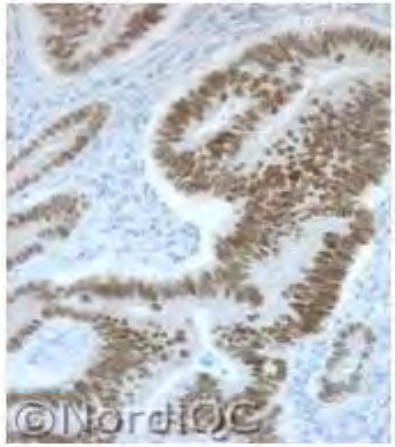


Fig. 2a. Optimal staining for CDX2 using same protocol as in Fig. 1a.
Left: Colon adenocarcinoma with high expression of CDX2: The nuclei of the neoplastic cells show an intense staining while the cytoplasmic compartment is weakly stained.
Right: Colon adenocarcinoma with low expression of CDX2: The majority of the neoplastic cells show a moderate to strong nuclear reaction.



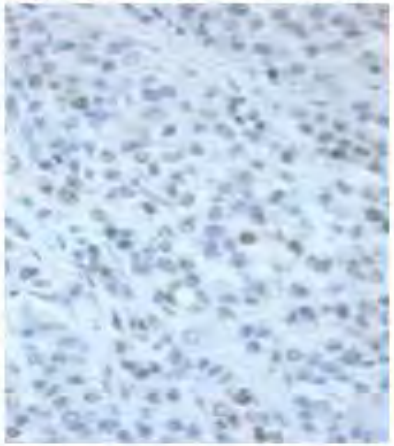


Fig. 2b. Insufficient staining for CDX-2 using same protocol as in Fig. 1b.
Left: Colon adenocarcinoma with high expression of CDX2: The nuclei of the neoplastic cells show a moderate staining, while the cytoplasmic compartment is almost negative.
Right: Colon adenocarcinoma with low expression of CDX2: Only scattered neoplastic cells show a weak nuclear reaction.

CDX2

iCAPCs:

Pancreatic duct ep. cells

IHC – Biomarker controls

B1: Appendix, Hepar, Tonsil, Pancreas

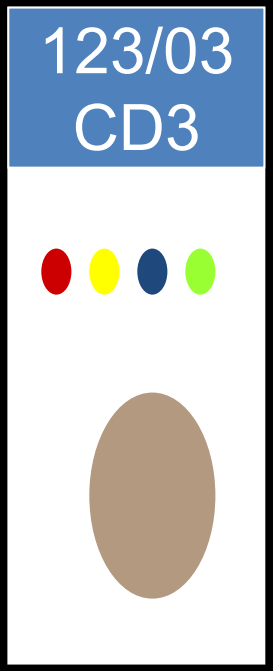
Appendix	Hepar	Tonsil		Pancreas
CD2	ASMA	BCL2	MMR	CDX2
CD3	CD4	BCL6	S100	CGA
CD19	CD31	CD2		SYP
CD34	CD34	CD3		CK7
CD117	CD45	CD4		PP
CEA	CD68	CD5		SMAD4
CGA	CK Pan	CD8		SYP
CK20	CK LMW	CD10		
DOG1	CK8	CD20		
MMR	CK18	CD21		
S100	HEPA	CD23		
SYP	Arginase	CD38		
		CD56		
		CD79a		
		CD138		
		CK Pan		
		CyD1		
		EMA		

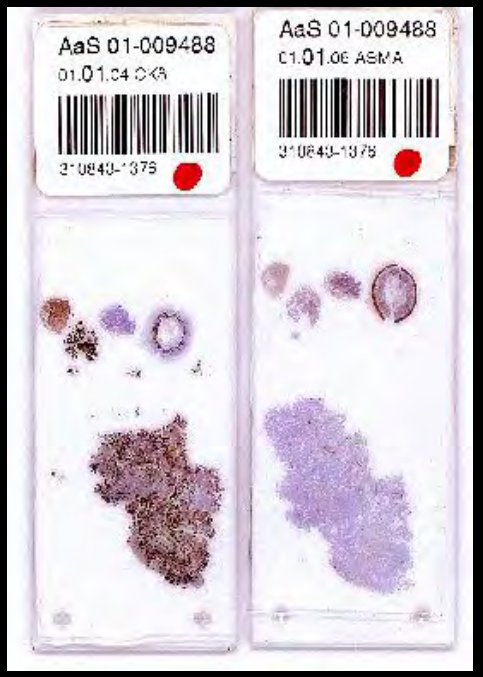
Used together inclusive:
HE
LE
NE

IHC – Biomarker controls

"Ideal" daily control for the majority of routine markers:

Appendix
Liver
Pancreas
Tonsil





Each slide stained and evaluated has essential information of the obtained sensitivity and specificity

In contrast only using 1 external tissue control, no information is available for the single slide evaluated

IHC – Biomarker controls

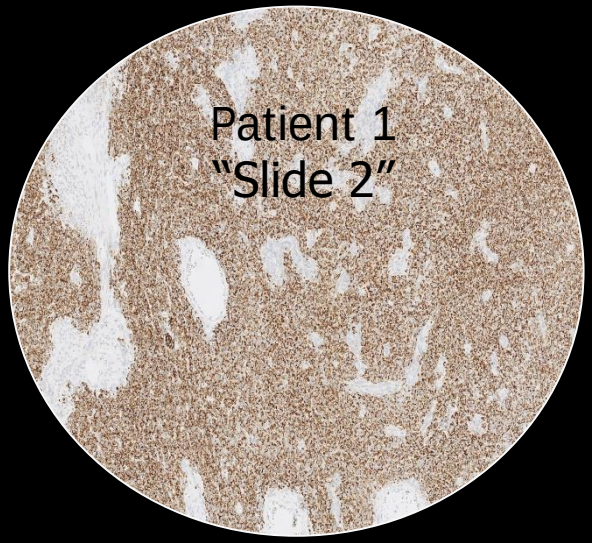
	TMA control on all slides	One batch control	Remarks
Missing reagent FN in patient test	Yes	No – only control slide	Potential internal pos. control only indicator of protocol performed
Wrong antibody FP in patient test	Yes	No – only control slide	
Inappropriate protocol performance - Drying out etc FN / FP in patient test	Yes	No – only control slide	
	Errors seen for all IHC automated and semi-automated IHC platforms		

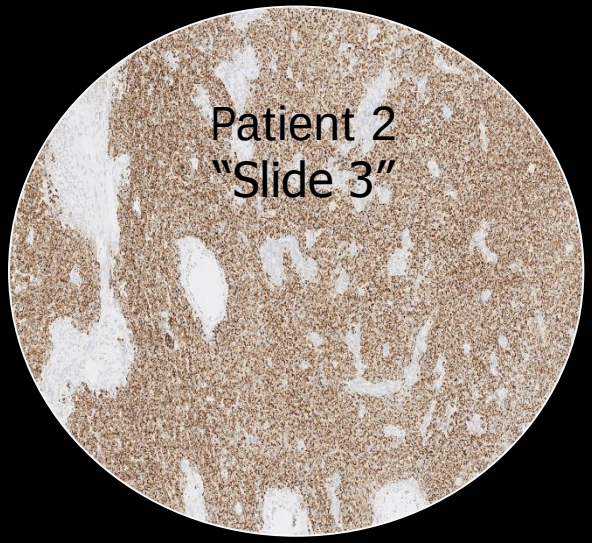
IHC – Biomarker controls

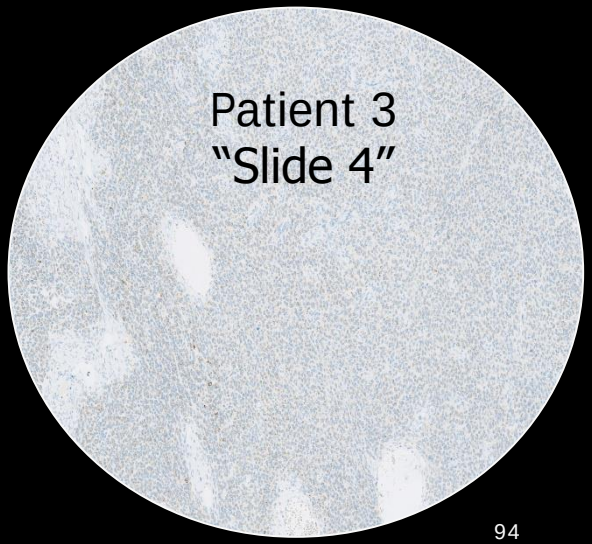
"Patient" 3 IHC assay level could be related to:

1. Biology
2. Tissue processing
3. Missing reagent or other technical issue

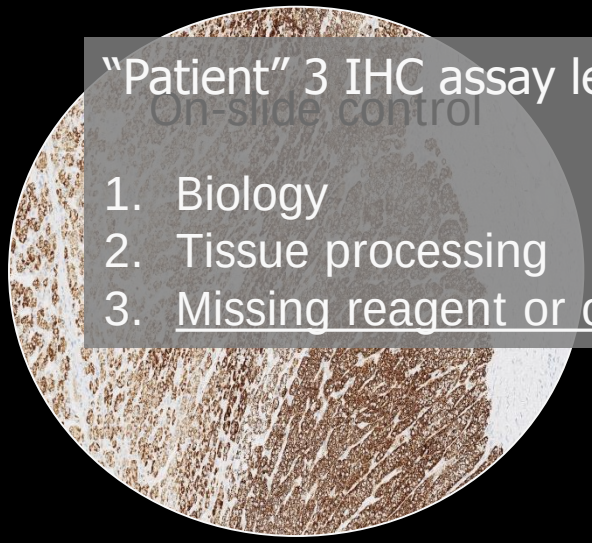
Melan-A in sex cord tumours





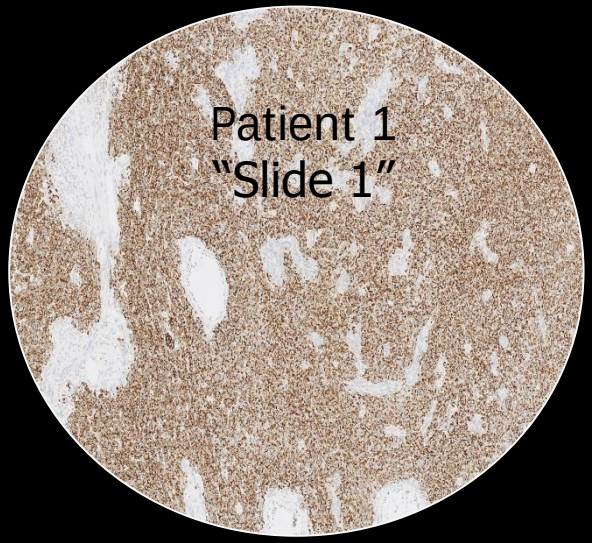


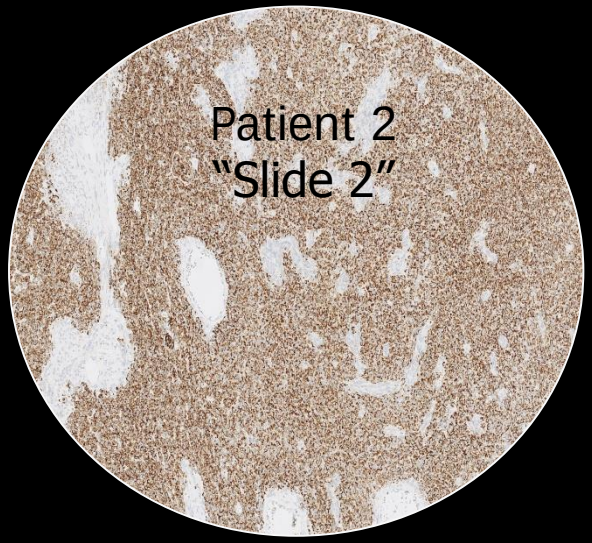
IHC – Biomarker controls

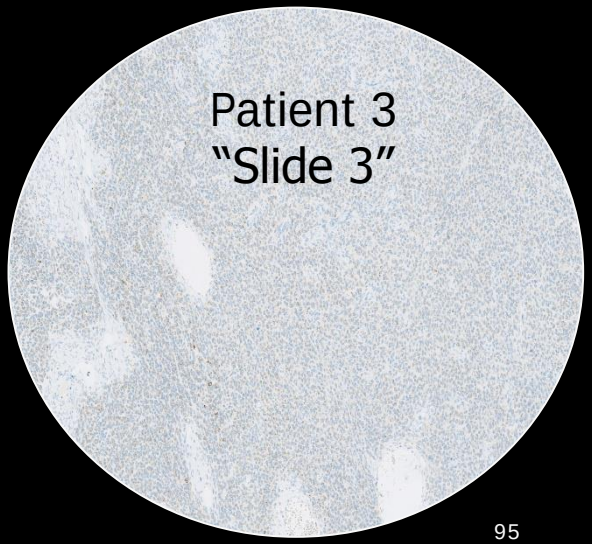


"Patient" 3 IHC assay level could be related to:

1. Biology
2. Tissue processing
3. Missing reagent or other technical issue

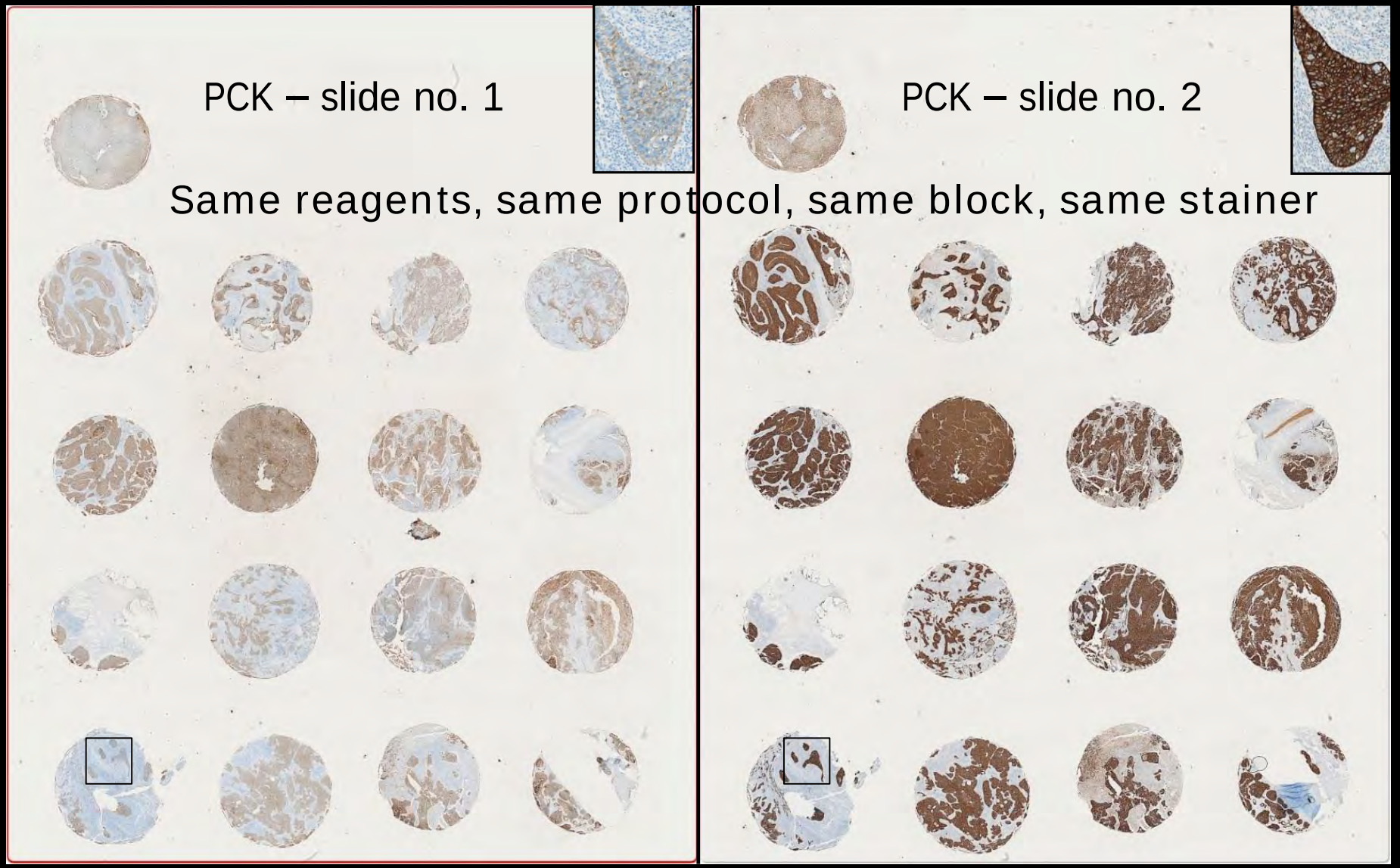






IHC – Biomarker controls

Consider each slide position / chamber on the IHC stainer as an individual stainer and use appropriate on-slide controls



IHC – Biomarker controls

Requirements to tissue control library / catalogue:

- Recommendations for virtually all markers used
  - Qualitative markers ”Class I”– yes / no
  - Quantitative markers ”Class II” – how much
  - ”Research” markers / not established markers

IHC – Biomarker controls

Central issues to adress for control material of HER2 IHC test

1. Control material for initial calibration and validation
e.g 100 samples ranging 0, 1+, 2+, 3+.

Optimally all samples confirmed by ISH

Metrix can be generated and test implemented.

2. Control material to monitor consistent and right level of sensitivity as identified by calibration – transfer of method – is obtained in each test performed.

Focus: The issue to identify and use proper control material to monitor consistency of test

IHC – Biomarker controls

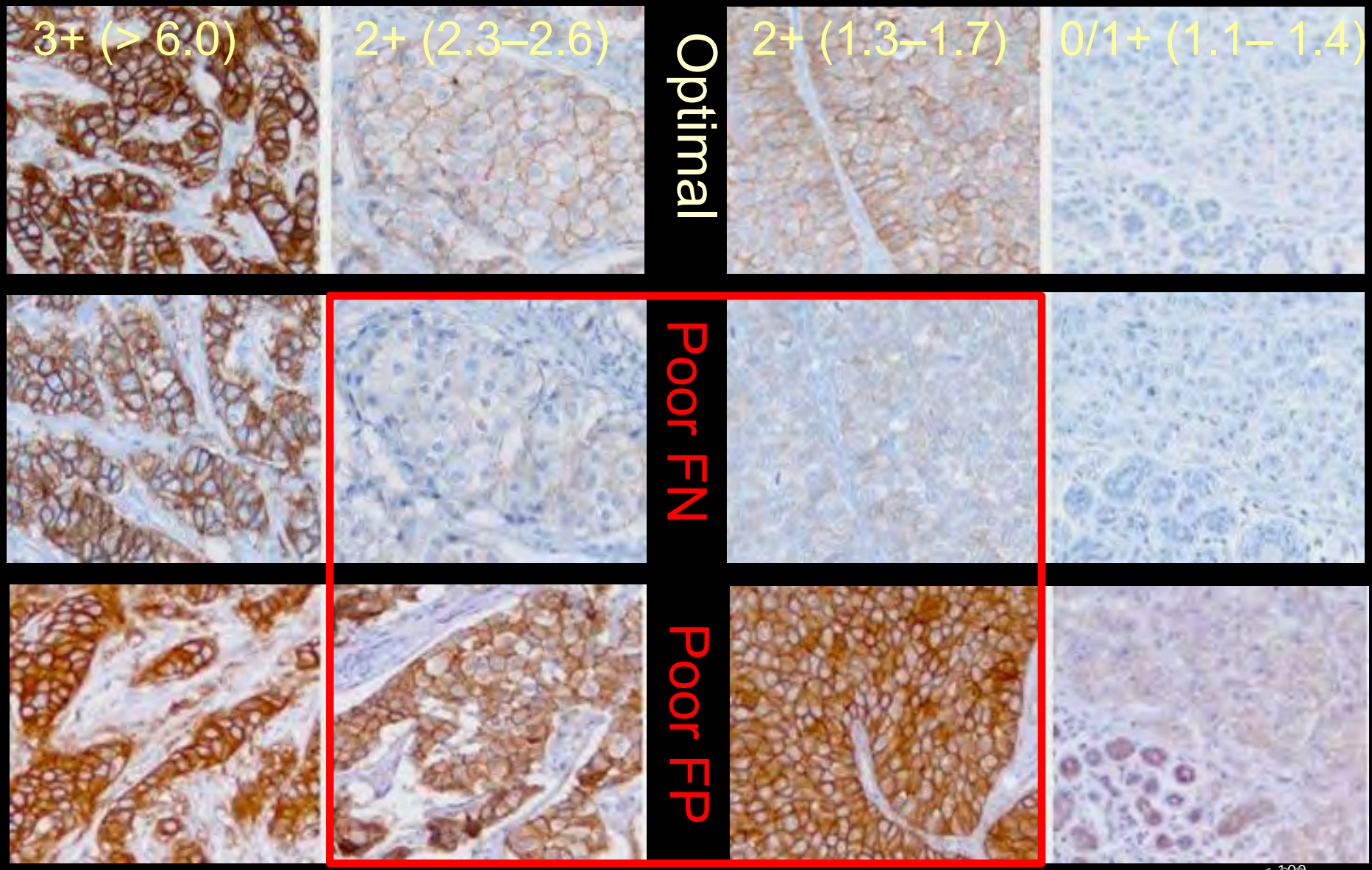
Central issues to adress for control material of HER2 IHC test

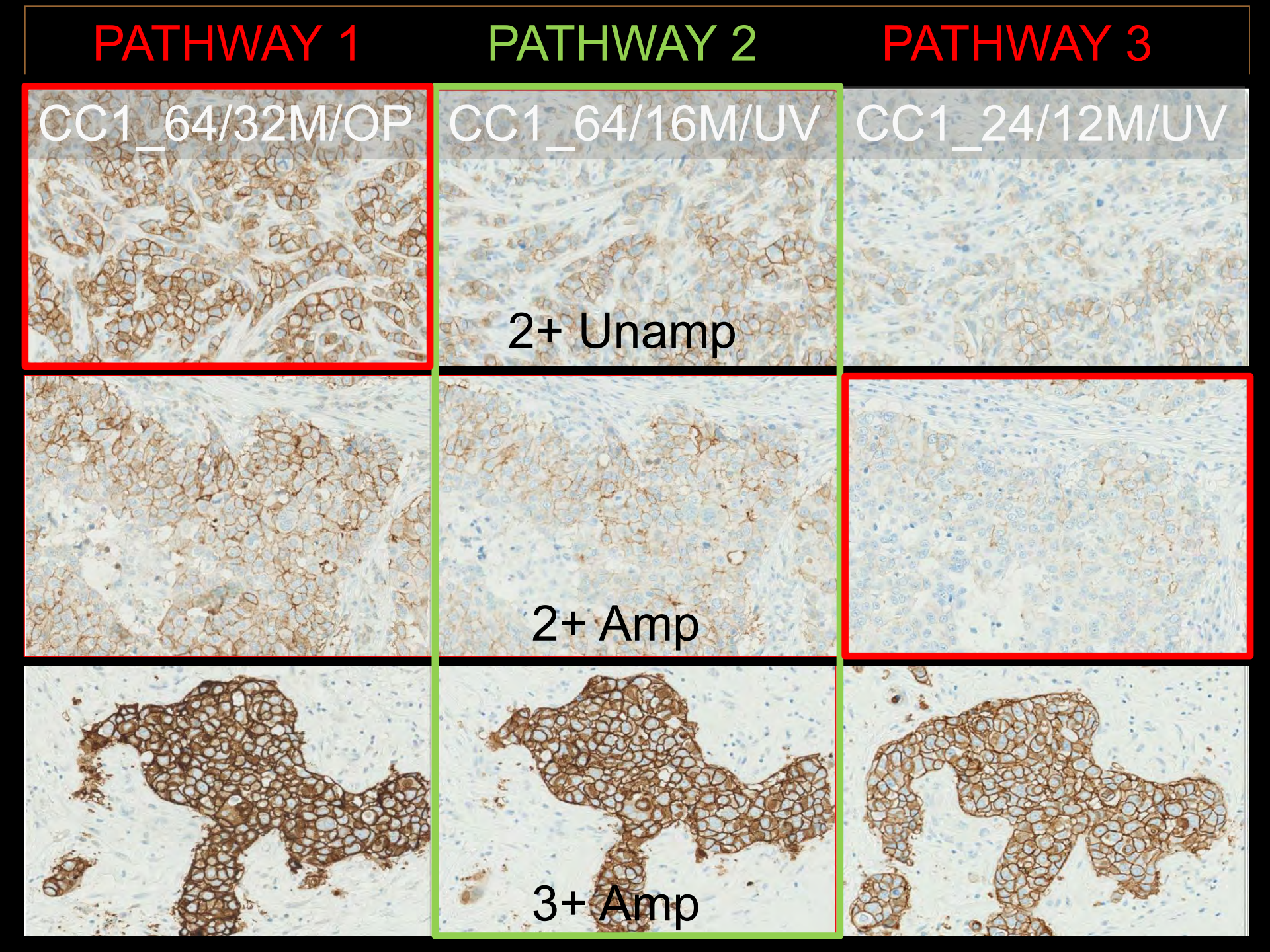
In NordiQC app. 60-70% of laboratories use a 3+ tumour as routine positive control for HER2 IHC

Question:

Is this reliable to monitor a consistent level of HER2 assay ?

IHC – Biomarker controls




PATHWAY 1
PATHWAY 2
PATHWAY 3
CC1_64/32M/OP
CC1_64/16M/UV
CC1_24/12M/UV
2+ Unamp
2+ Amp
3+ Amp

IHC – Biomarker controls

Central issues to adress for control material of HER2 IHC test

In NordiQC app. 60-70% of laboratories use a 3+ tumour as positive control for HER2 IHC

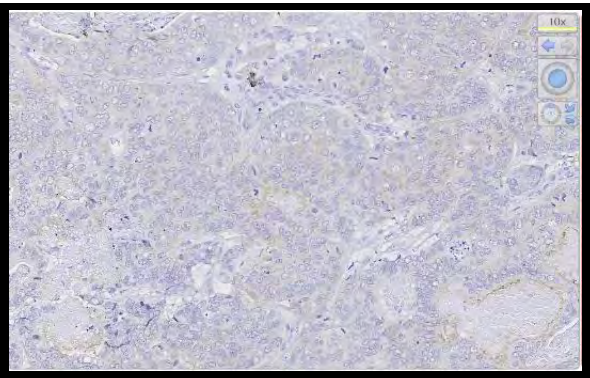
Optimally:

Use small TMA with 1+, 2+ & 3+ mounted on same slide as pt material for daily control of HER2 IHC assay

IHC – Biomarker controls

IHC scoring system according to the 2013 ASCO/CAP guidelines

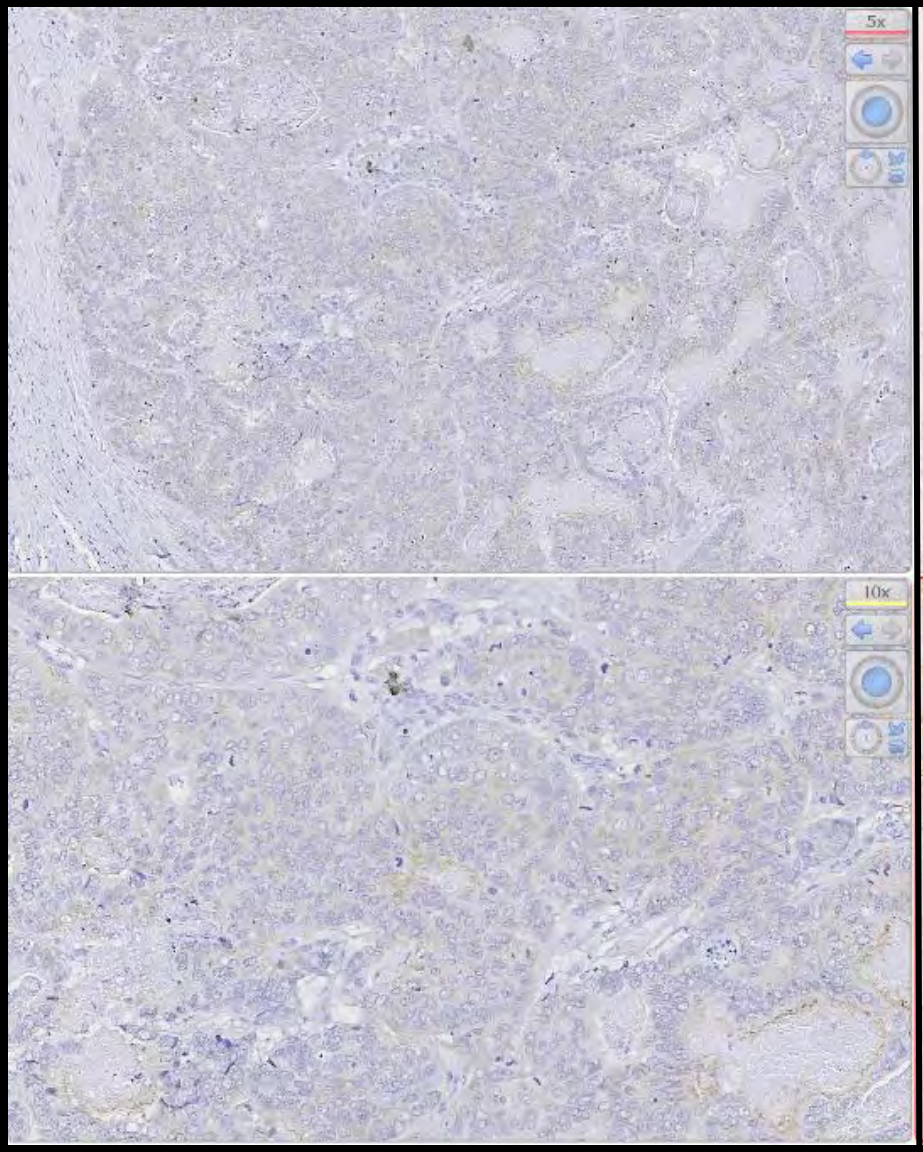
Score 0	No staining is observed or incomplete membrane staining is observed in ≤ 10% of the tumour cells.
Score 1+	A faint perceptible and incomplete membrane staining is observed in more than 10% of the tumour cells.
Score 2+	A weak to moderate circumferential incomplete membrane staining is observed in more than 10% of the tumour cells or an intense circumferential complete membranous staining in ≤ 10% of the tumour cells.
Score 3+	An intense circumferential complete membrane staining is observed in more than 10% of the tumour cells.



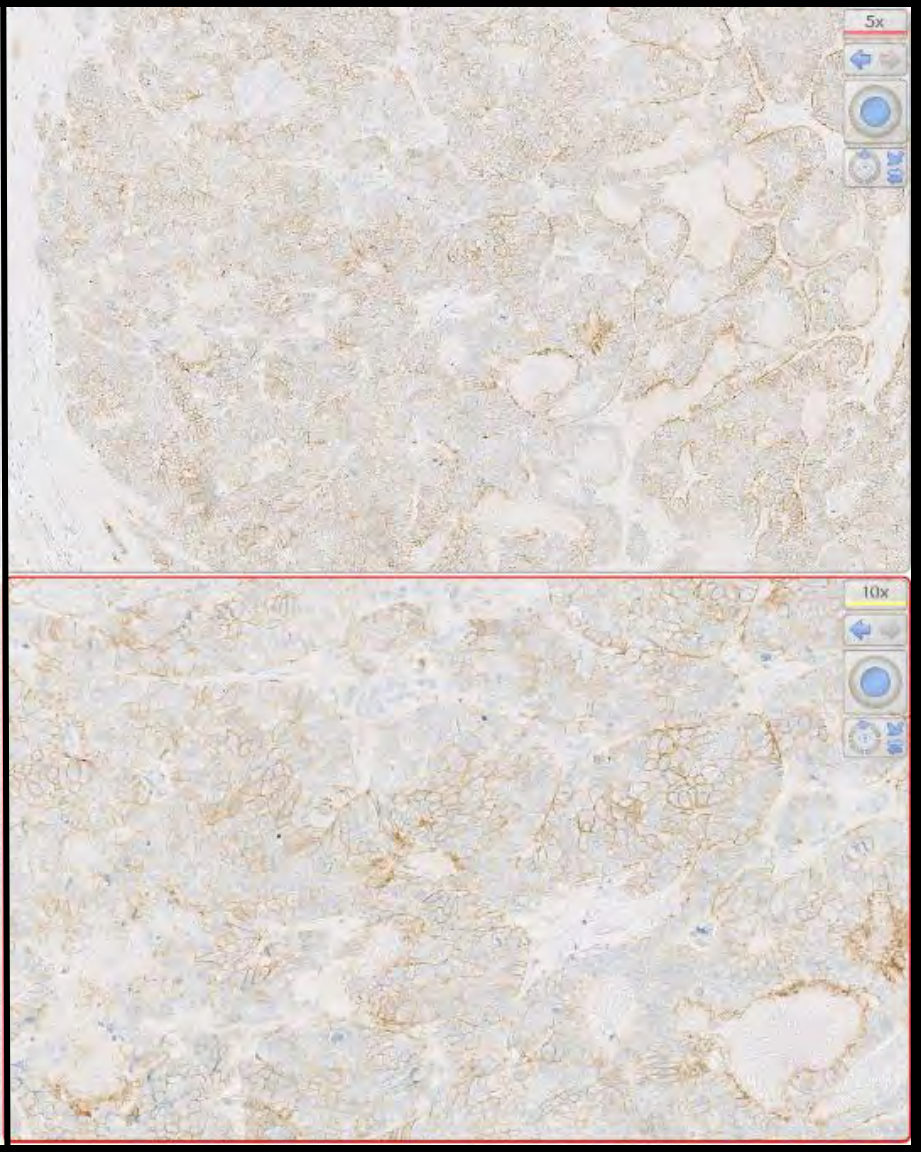
- What is faint ?
- What is weak ?

Up to 20-40% HER2 IHC tests are reflexed to ISH due to expanded criteria for 2+ (internal data)

IHC – Biomarker controls



Lab 1; scored 2+



Lab 2; scored as 2+

IHC – Biomarker controls

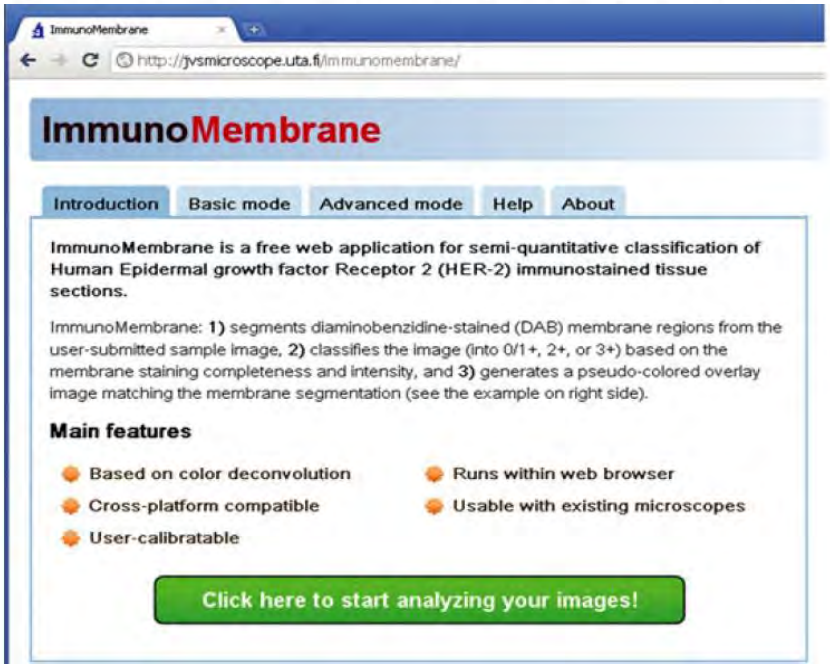
Histopathology

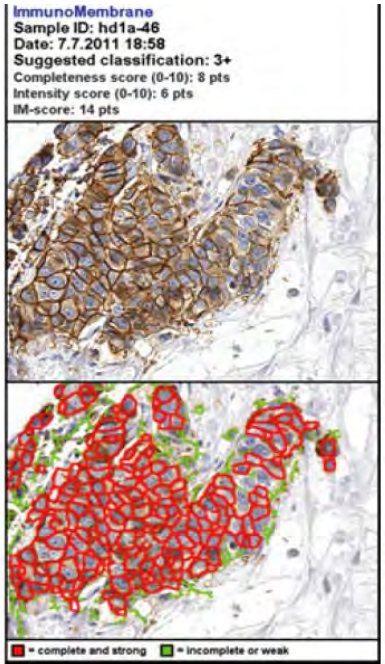
Histopathology 2012, **60**, 758–767. DOI: 10.1111/j.1365-2559.2011.04142.x

ImmunoMembrane: a publicly available web application for digital image analysis of HER2 immunohistochemistry

Vilppu J Tuominen,[1] Teemu T Tolonen[1,2] & Jorma Isola[1]

[1]*Institute of Biomedical Technology, University of Tampere, Tampere, Finland, and* [2]*Department of Pathology, Centre for Laboratory Medicine, Tampere University Hospital, Tampere, Finland*





Digital computer assisted analysis to be integrated.

IHC – Biomarker controls

ASCO/CAP score	Connectivity Range
0	0.00
1+	]0.00 - 0.40]
2+	]0.40 - 0.64]
3+	]0.64 - 1.00]

Breast Cancer Res Treat (2012) 132:41–49
DOI 10.1007/s10549-011-1514-2

PRECLINICAL STUDY

Digital image analysis of membrane connectivity is a robust measure of HER2 immunostains

Anja Brügmann · Mikkel Eld · Giedrius Lelkaitis · Søren Nielsen · Michael Grunkin · Johan D. Hansen · Niels T. Foged · Mogens Vyberg

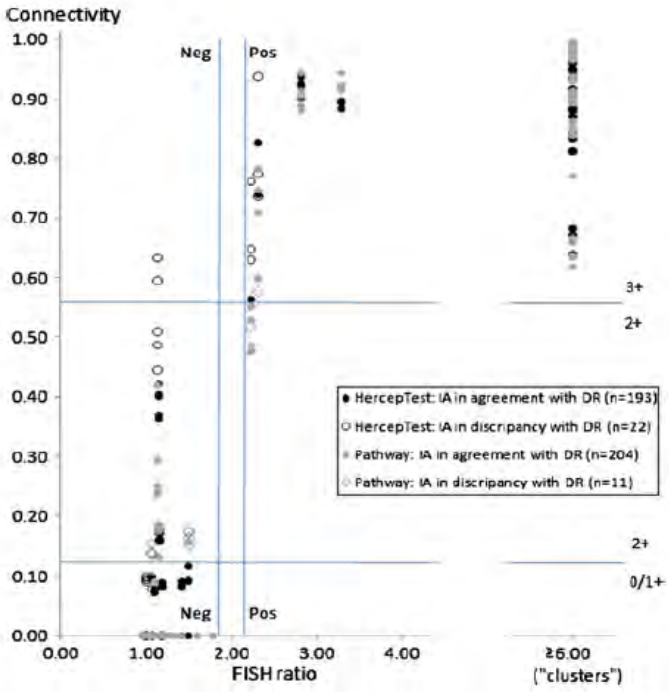
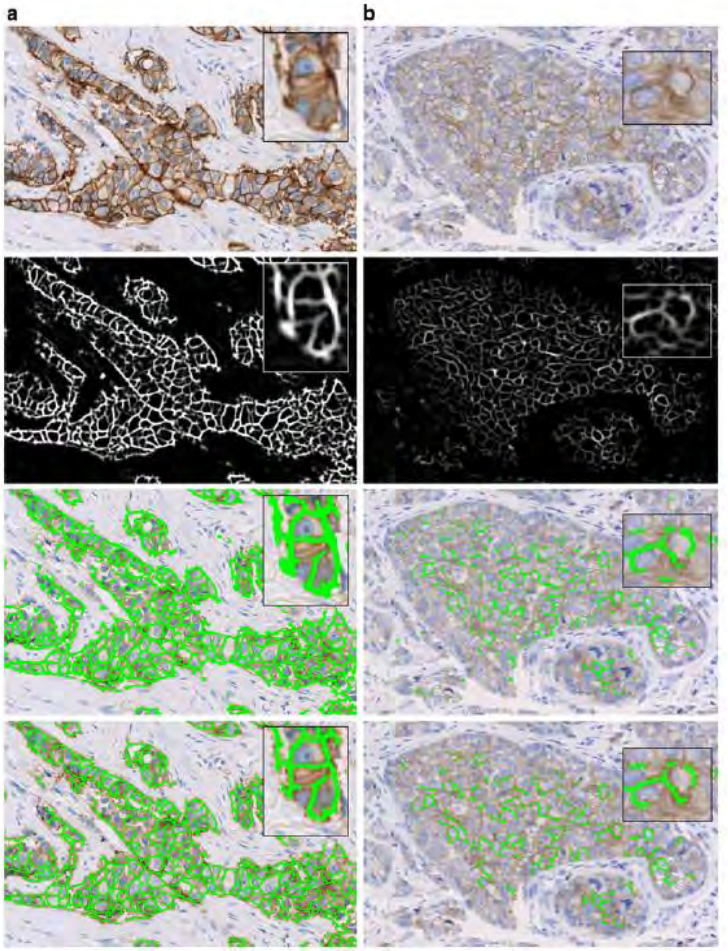


Fig. 4 HER2 connectivity versus FISH ratio. The blue lines on the *x* axis define the "equivocal" category with FISH ratio between 1.8 and 2.2. The FISH ratios lower than 1.8 are negative (Neg) and above 2.2 are positive (Pos). On the *y* axis the blue lines define the connectivity cut-off levels separating the IHC score categories

Fig. 2 Stepwise processing of digital images by the HER2-CONNECT™ algorithm. Digital images of two fields of view from a positive (3+) (a) and an equivocal (2+) (b) sample. The major steps were pre-processing quantifying for each pixel its contribution to brown linear structure (white: high contribution, black: low contribution); the segmentation classifying the pixels which constitute brown linear structures (green overlay label), and the post-processing skeletonizing and merging the green overlay label, when a few pixels are missing in a linear structure, and removing small objects of green overlay label

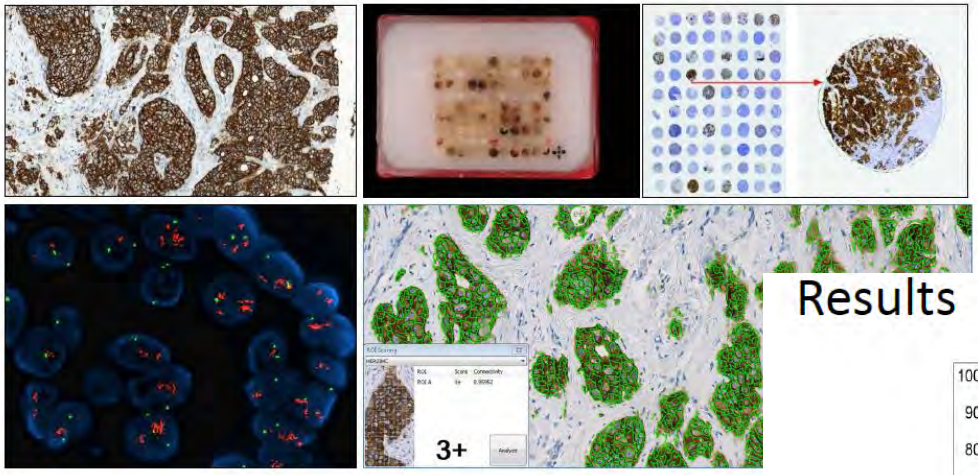


IHC – Biomarker controls

Automated image analysis is superior to manual reading of HER2 expression in breast cancer

Rossing HH, Talman ML, Vainer B
Department of Pathology, Rigshospitalet, University of Copenhagen, Denmark

Aim: Validate digital, automated image analysis algorithm HER2-CONNECT, with a goal to minimizing the number of inconclusive HER 2+ scores.



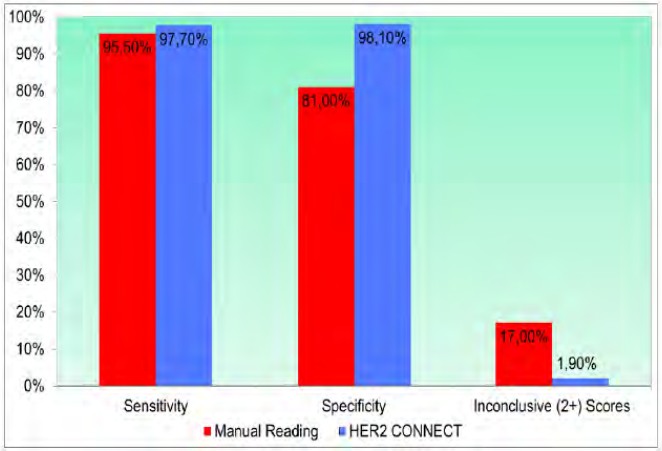
156 patients in 12 TMA

To improve consistency

To reduce cohort of 2+

To serve as internal QC

Results



Automated image analysis HER2-CONNECT algorithm for HER2 protein expression decreased the need for supplementary FISH testing by almost 90%

HER2-CONNECT will make the assessment of HER2 fully automatic, fast and objective to the benefit of breast cancer patients.

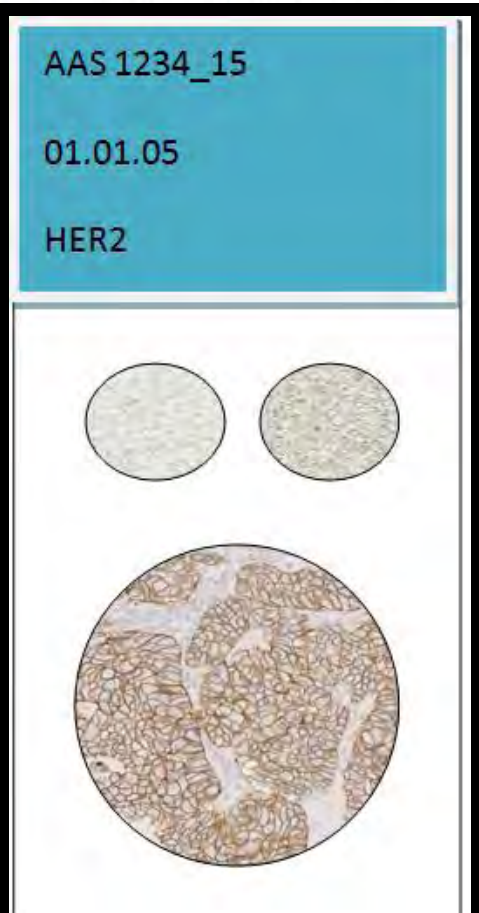
IHC – Biomarker controls

visiopharm
TURNING IMAGES INTO KNOWLEDGE

HER2-CONNECT™ CE

EU: For in vitro diagnostics use

- PATHWAY
- HercepTest
- Oracle

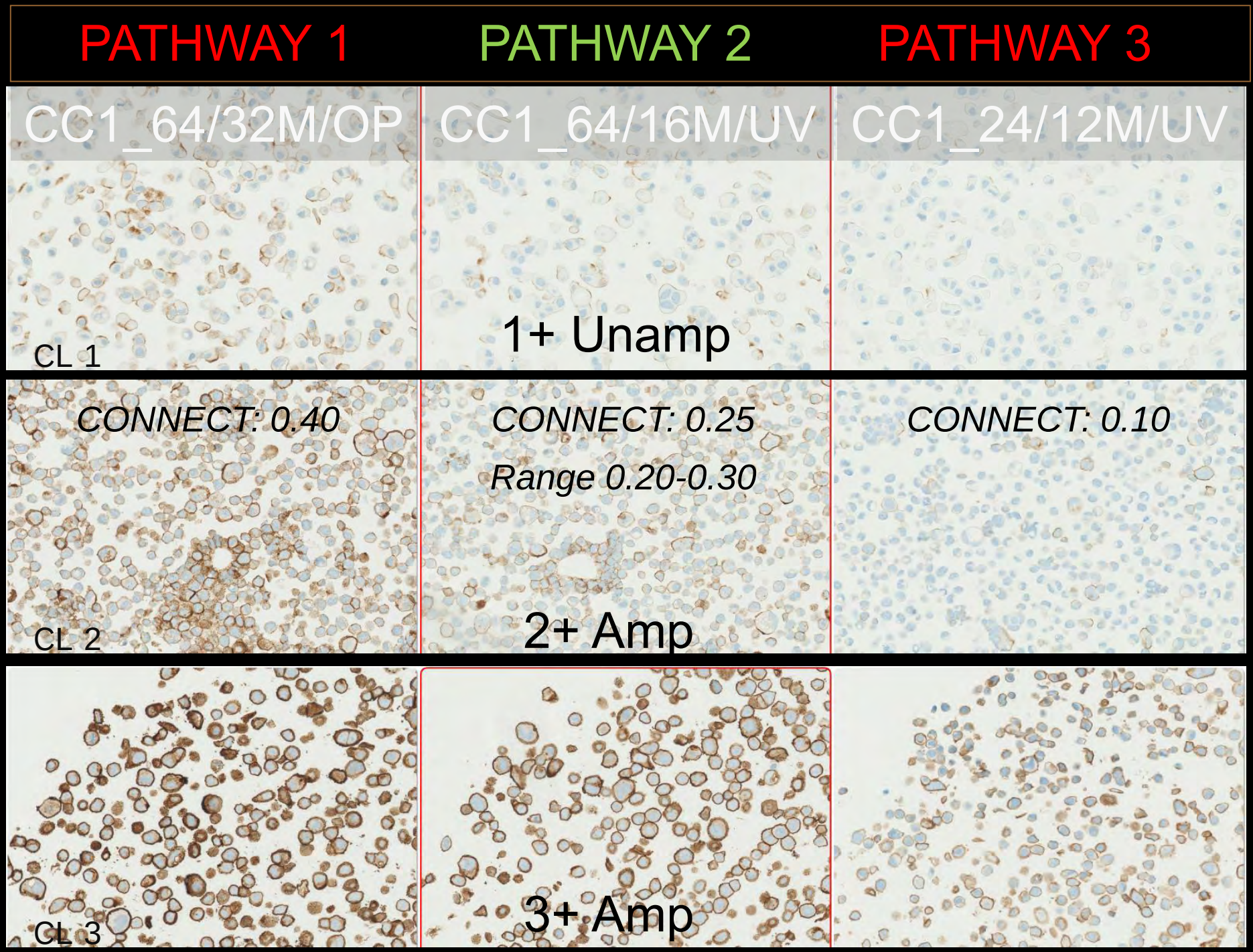


	FN	Optimal	FP
CL1	< 0.40	0.40-0.50	> 0.50
CL2	< 0.20	0.20-0.30	> 0.40

Cell line HER2

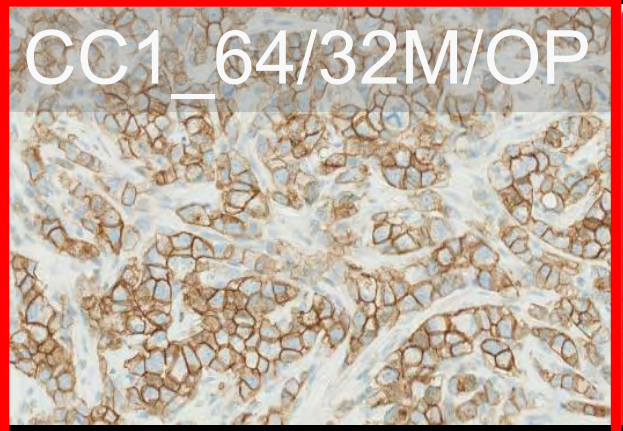
Pt HER2

ASCO/CAP score	Connectivity Range
0	0.00
1+	]0.00 - 0.40]
2+	]0.40 - 0.64]
3+	]0.64 - 1.00]

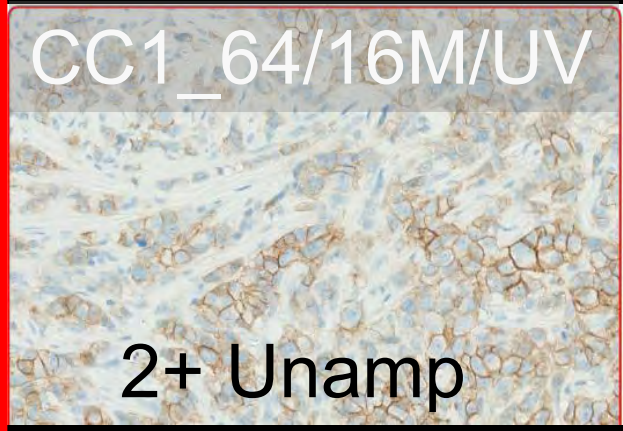

PATHWAY 1
PATHWAY 2
PATHWAY 3
CC1_64/32M/OP
CC1_64/16M/UV
CC1_24/12M/UV
CL 1
1+ Unamp
CONNECT: 0.40
CONNECT: 0.25
Range 0.20-0.30
CONNECT: 0.10
CL 2
2+ Amp
CL 3
3+ Amp

PATHWAY 1 PATHWAY 2 PATHWAY 3

CC1_64/32M/OP

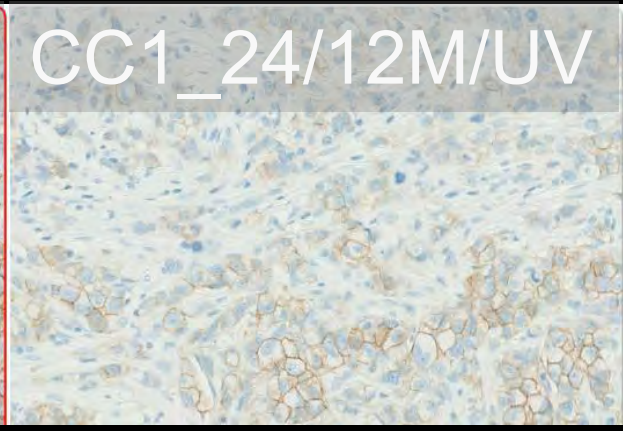


CC1_64/16M/UV



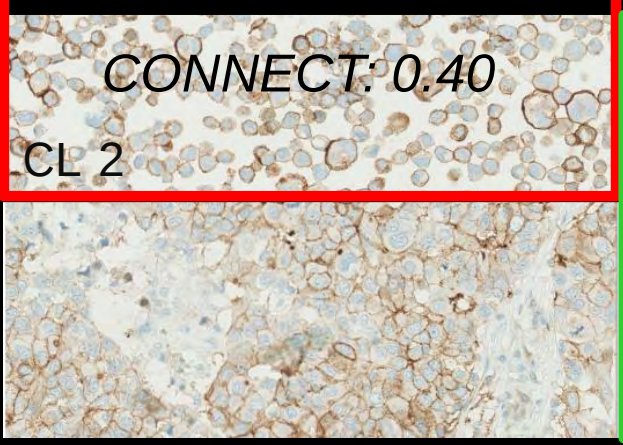
2+ Unamp

CC1_24/12M/UV



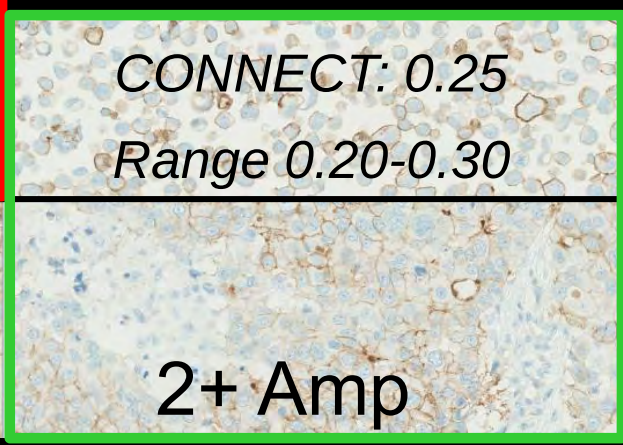
CONNECT: 0.40

CL 2



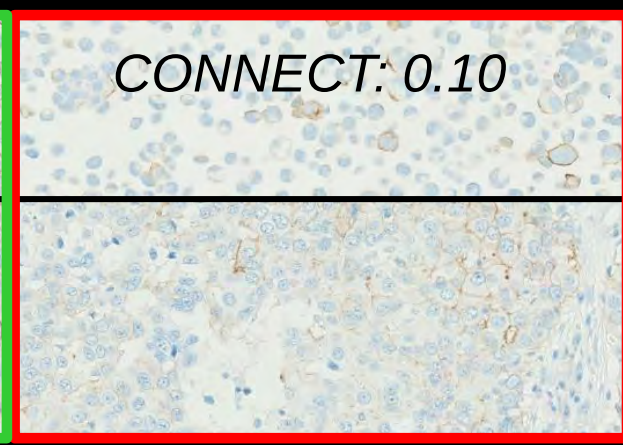
CONNECT: 0.25

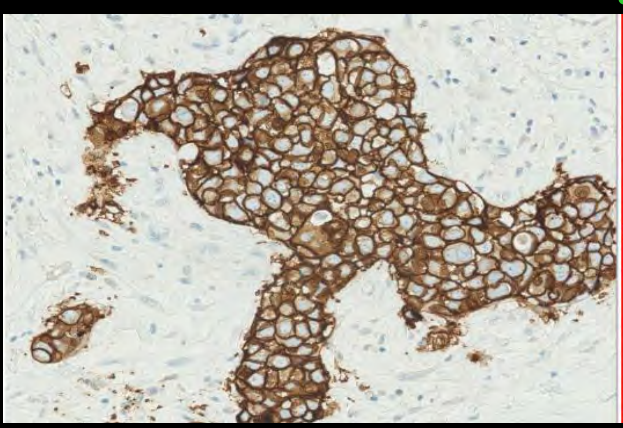
Range 0.20-0.30

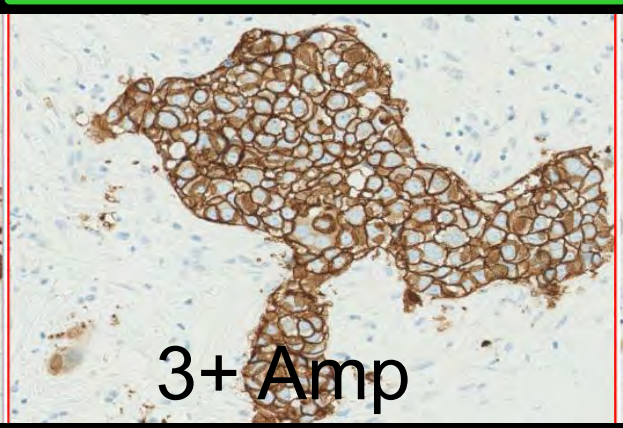


2+ Amp

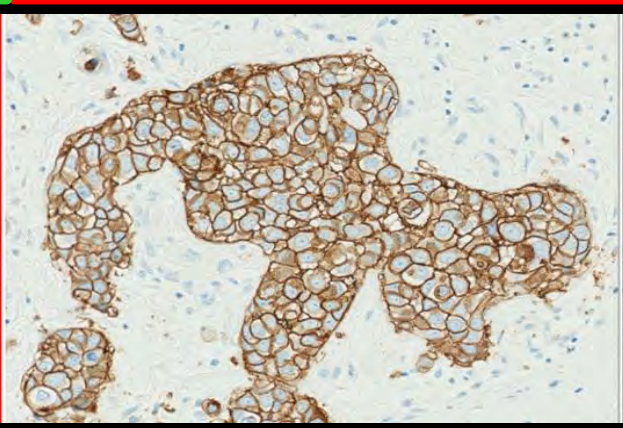
CONNECT: 0.10







3+ Amp



IHC – Biomarker controls

Requirements to tissue control library / catalogue:

- Recommendations for virtually all markers used
  - Quantitative markers – how much
    - External tissue controls to confirm right ab
    - External tissue controls to guide level of detection is acceptable
    - External cell lines with documented reference values as final QC

IHC – Biomarker controls

Conclusions:

Controls are essential to evaluate IHC results:

- Tissue controls used to calibrate IHC assay
- Tissue controls processed by variables applied in the laboratory is needed to evaluate on robustness
- Tissue controls to evaluate analytical potential
- Tissue controls to monitor consistency of IHC assay

IHC – Biomarker controls

Conclusions:

Focus on external tissue controls are central to standardize and optimize IHC:

- External tissue control ”catalogue” (normal preferable) with describtions of HE, LE and NE
- Accepted and developed by KOL, EQA, Industry, Labs
- Used to validate/verify IHC studies and publications
- Used for both internal and external IHC QC

IHC – Biomarker controls

Conclusions:

Focus on external tissue controls is central to standardize and optimize IHC:

- On-slide TMA controls are preferable to 1 bacth control
- Internal tissue controls are of limited value
- Negative reagent controls are only essential for biotin-based detection systems
- Negative reagent controls can be valueable for non-biotin based systems e.g. If pigment, frozen sections..

IHC – Biomarker controls

Requirements to tissue selection for external controls:

- Preferable normal and homogenous tissue
- Optimally prospectively collected patient material grossed simultanously with diagnostic material
- Must be fixed and processed by well defined standards
- *Retrospectively collected material can be used – some degree of uncertainty of processing conditions*

IHC – Biomarker controls

Thank You for the attention and.....

IHC – Biomarker controls