Quality in Pathology

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

NordiQC

Immunohistochemical principles The technical test approach

Post-analytical phase Controls

IHC – Biomarker controls

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

Fixation
Time, Type, Volume

Decalcification
Preparation

Pre-analytic

Pre-treatment

Primary antibody
Clone, Dilution
Buffer, Time, Temp

Tissue
Type, Dimension,
Laser resection,
De-differentiation

Section
Thickness
Storage
Drying

Manual
Stainer

Analytic

Visualization
Sensitivity, Specificity

Development
Sensitivity,
Localization

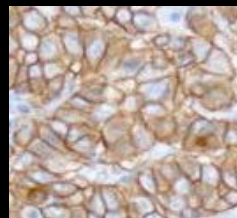
With 3 choices for 5 variables in each phase = > 4 million protocols….

Controlment

Post-analytic

Quantification
Reporting

Interpretation
Localization
Positive/Negative - cut-off level

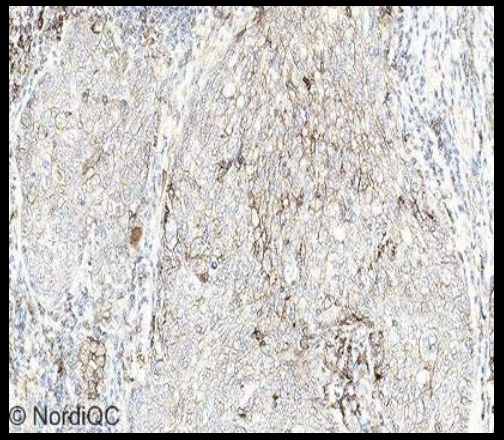


The right control material will expose right or wrong choices

IHC – Biomarker controls

The journey from antibody to a diagnostic IHC assay with a specific purpose

Based on external tissue controls



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

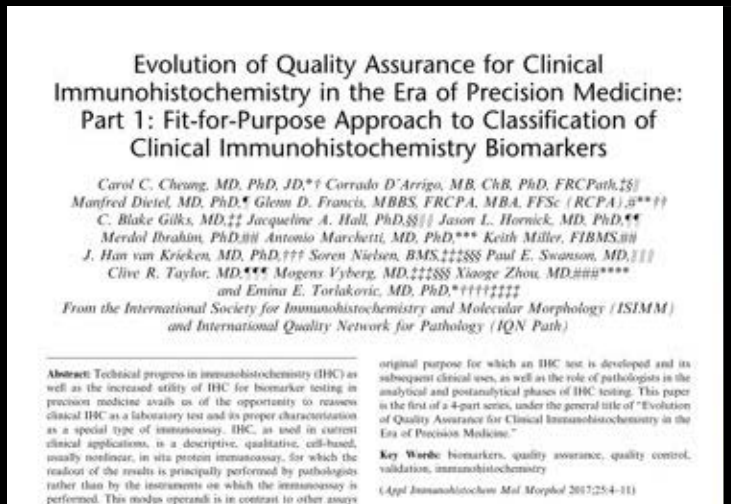
Emina E. Torlakovic, MD, PhD,† Søren Nielsen, HT, CT,‡§ Glenn Francis, MBBS, FRCPA, MBA, FFSc (RCPA),‖¶# John Garratt, RT,†** Blake Gilks, MD, FRCPC,† †† Jeffrey D. Goldsmith, MD,‡‡ Jason L. Hornick, MD, PhD,*§§ Elizabeth Hyjek, MD, PhD,* Merdol Ibrahim, PhD,‖‖ Keith Miller, FIBMS,‖‖ Eugen Petcu, MD,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

AIMM: January to April 2017



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

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

Abstract: Technical progress in immunohistochemistry (IHC) as well as the increased utility of IHC for biomarker testing in precision medicine avails us of the opportunity to reassess clinical IHC as a laboratory test and its proper characterization as a special type of immunoassay. IHC, as used in current clinical applications, is a descriptive, qualitative, cell-based, usually nonlinear, in situ protein immunoassay, for which the readout of the results is principally performed by pathologists rather than by the instruments on which the immunoassay is performed. This modus operandi is in contrast to other assays where the instrument also performs the readout of the test result (eg, nephelometry readers, mass spectrometry readers, etc). The readouts (results) of IHC tests are used either by pathologists for diagnostic purposes or by treating physicians (eg, oncologists) for patient management decisions, the need for further testing, or follow-up. This paper highlights the distinction between the original purpose for which an IHC test is developed and its subsequent clinical uses, as well as the role of pathologists in the analytical and postanalytical phases of IHC testing. This paper is the first of a 4-part series, under the general title of "Evolution of Quality Assurance for Clinical Immunohistochemistry in the Era of Precision Medicine."

Key Words: biomarkers, quality assurance, quality control, validation, immunohistochemistry

(*Appl Immunohistochem Mol Morphol* 2017;25:4–11)

In the era of precision medicine, biomarker testing using immunohistochemistry (IHC) has not only become more precise but also more complex.[1–6] Precision medicine requires precision results, which can only come about from precision testing. Because of increasing reliance on

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

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

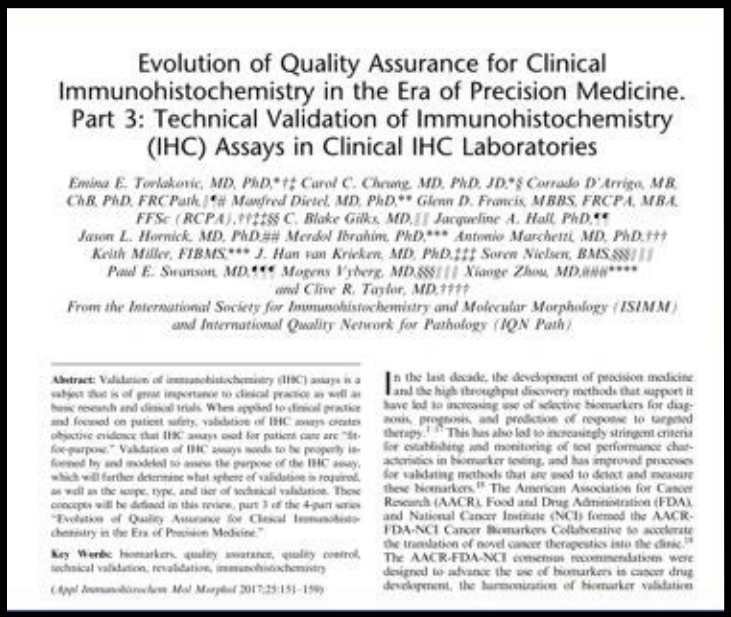
From the International Society for Immunohistochemistry and Molecular Morphology (ISIMM) and International Quality Network for Pathology (IQN Path)

Abstract: All laboratory tests have test performance characteristics (TPCs), whether or not they are explicitly known to the laboratorian or the pathologist. TPCs are thus also an integral characteristic of immunohistochemistry (IHC) tests and other in situ, cell-based molecular assays such as DNA or RNA in situ hybridization or aptamer-based testing. Because of their descriptive, in situ, cell-based nature, IHC tests have a limited repertoire of appropriate TPCs. Although only a few TPCs are relevant to IHC, proper selection of informative TPCs is nonetheless essential for the development of and adherence to appropriate quality assurance measures in the IHC laboratory. This paper describes the TPCs that are relevant to IHC testing and emphasizes the role of TPCs in the validation of IHC tests. This is part 2 of the 4-part series "Evolution of Quality Assurance for Clinical Immunohistochemistry in the Era of Precision Medicine."

Key Words: biomarkers, quality assurance, quality control, validation, immunohistochemistry, test performance characteristics

(*Appl Immunohistochem Mol Morphol* 2017;25:79–85)

Historically, immunohistochemistry (IHC) has for all practical purposes been considered a "special stain" similar to traditional histochemical preparations; how-



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

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

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

Abstract: Validation of immunohistochemistry (IHC) assays is a subject that is of great importance to clinical practice as well as basic research and clinical trials. When applied to clinical practice and focused on patient safety, validation of IHC assays creates objective evidence that IHC assays used for patient care are "fit-for-purpose." Validation of IHC assays needs to be properly informed by and modeled to assess the purpose of the IHC assay, which will further determine what sphere of validation is required, as well as the scope, type, and tier of technical validation. These concepts will be defined in this review, part 3 of the 4-part series "Evolution of Quality Assurance for Clinical Immunohistochemistry in the Era of Precision Medicine."

Key Words: biomarkers, quality assurance, quality control, technical validation, revalidation, immunohistochemistry

(*Appl Immunohistochem Mol Morphol* 2017;25:151–159)

In the last decade, the development of precision medicine and the high throughput discovery methods that support it have led to increasing use of selective biomarkers for diagnosis, prognosis, and prediction of response to targeted therapy.[1–7] This has also led to increasingly stringent criteria for establishing and monitoring of test performance characteristics in biomarker testing, and has improved processes for validating methods that are used to detect and measure these biomarkers.[8] The American Association for Cancer Research (AACR), Food and Drug Administration (FDA), and National Cancer Institute (NCI) formed the AACR-FDA-NCI Cancer Biomarkers Collaborative to accelerate the translation of novel cancer therapeutics into the clinic.[9] The AACR-FDA-NCI consensus recommendations were designed to advance the use of biomarkers in cancer drug development, the harmonization of biomarker validation

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

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

Abstract: The numbers of diagnostic, prognostic, and predictive immunohistochemistry (IHC) tests are increasing; the implementation and validation of new IHC tests, revalidation of existing tests, as well as the on-going need for daily quality assurance monitoring present significant challenges to clinical laboratories. There is a need for proper quality tools, specifically tissue tools that will enable laboratories to successfully carry out these processes. This paper clarifies, through the lens of laboratory tissue tools, how validation, verification, and revalidation of IHC tests can be performed in order to develop and maintain high quality "fit-for-purpose" IHC testing in the era of precision medicine. This is the final part of the 4-part series "Evolution of Quality Assurance for Clinical Immunohistochemistry in the Era of Precision Medicine."

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

(*Appl Immunohistochem Mol Morphol* 2016;00:000–000)

Before the decision to implement a new immunohistochemistry (IHC) test is made, several considerations relevant to test development and maintenance need to be contemplated (see parts 1 to 3 of the Evolution series). To introduce a new IHC test, a series of steps must be followed that require careful planning, from test development through to on-going quality monitoring. For this process to be successful, proper tissue tools, which are a cornerstone of quality for the modern day clinical

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]

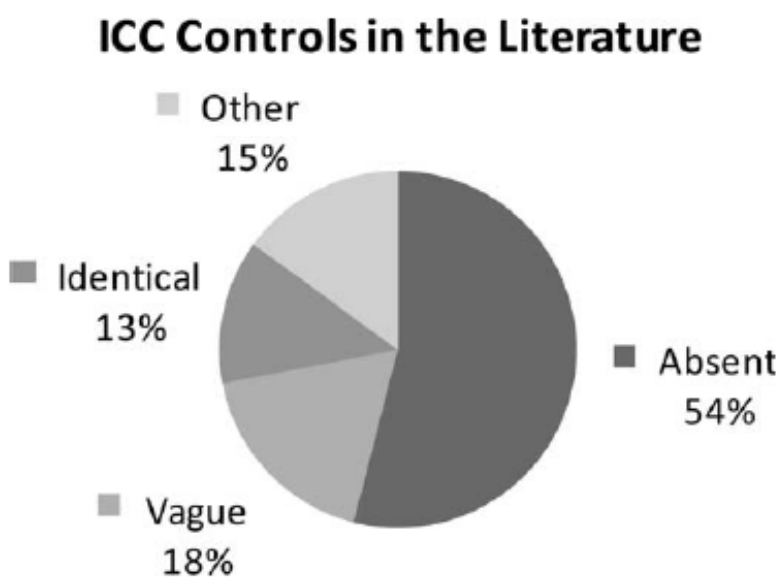


Fig. 1. Description of immunocytochemistry controls in articles reviewed.

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

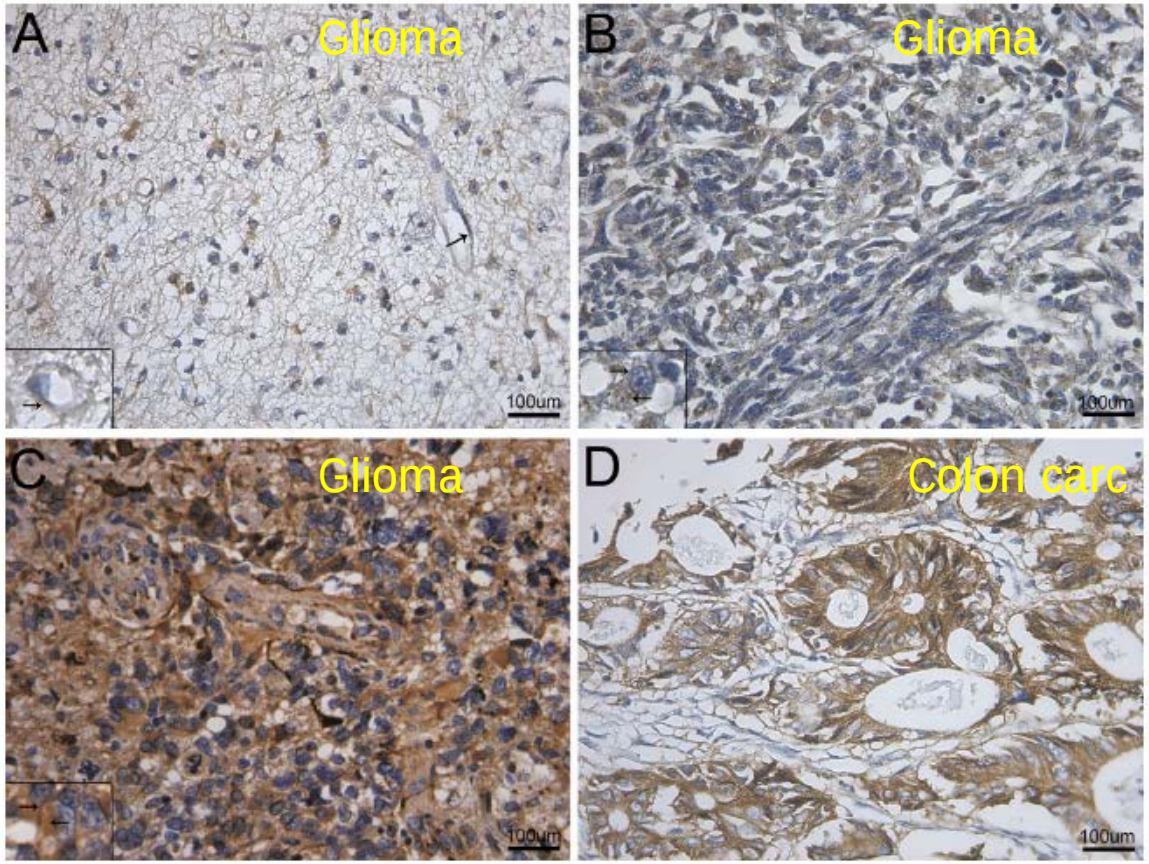


Fig. 1 Representative immunohistochemical staining for EpCAM (400×). Membranous and Cytoplasmic staining of EpCAM was observed in (a–c); a WHO grade II malignant glioma with weak EpCAM expression(TIS = 4), slant arrow shows EpCAM staining on epithelial cell; b WHO grade III malignant glioma with moderate 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

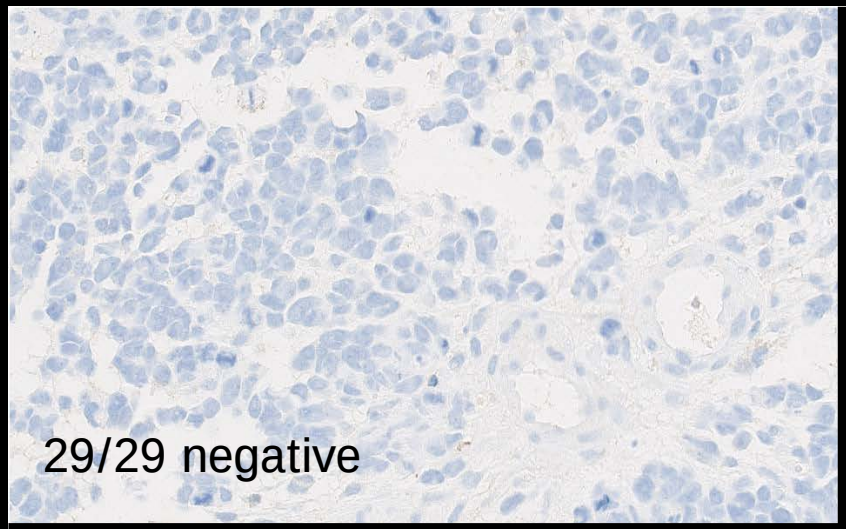
Cut-off was 1% positivity – any intensity; ”overexpression ≥ 10% and mod.

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

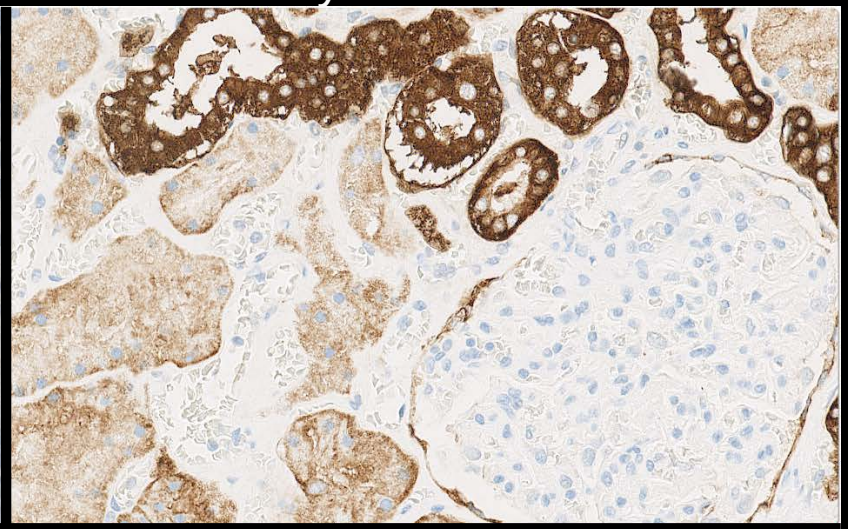
IHC – Biomarker controls

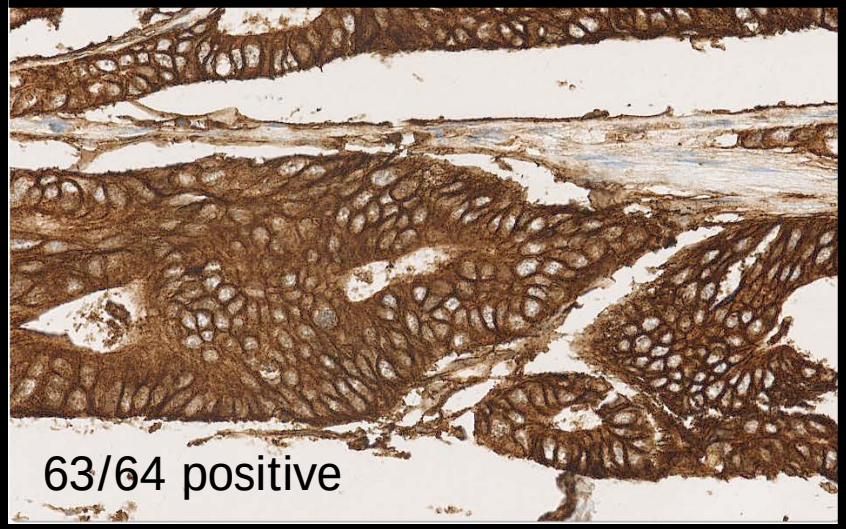
Ref. NordiQC: Ber-EP4: 1:50, HIER TRS pH 6.1, 3-step polymer

Glioma



Kidney





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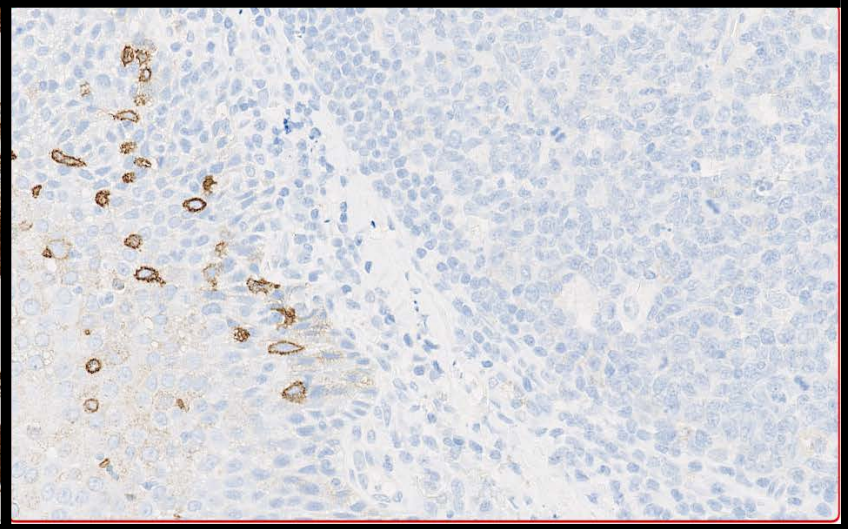


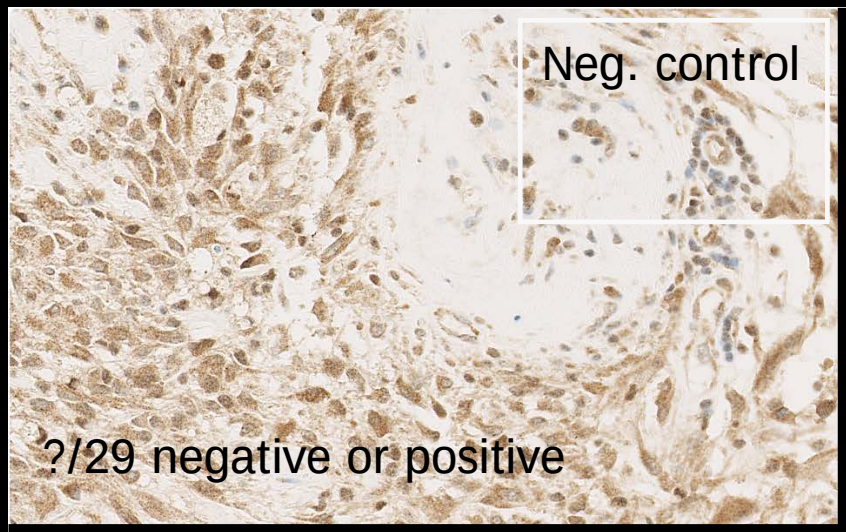
Tonsil

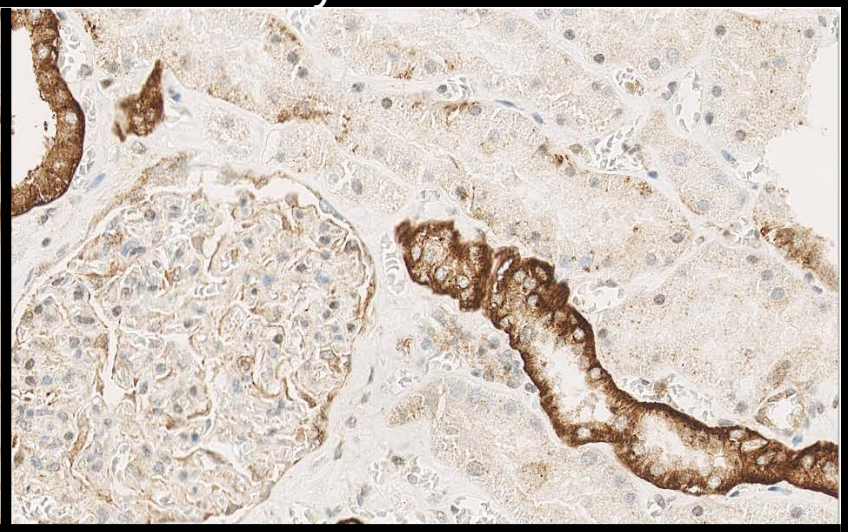
IHC – Biomarker controls

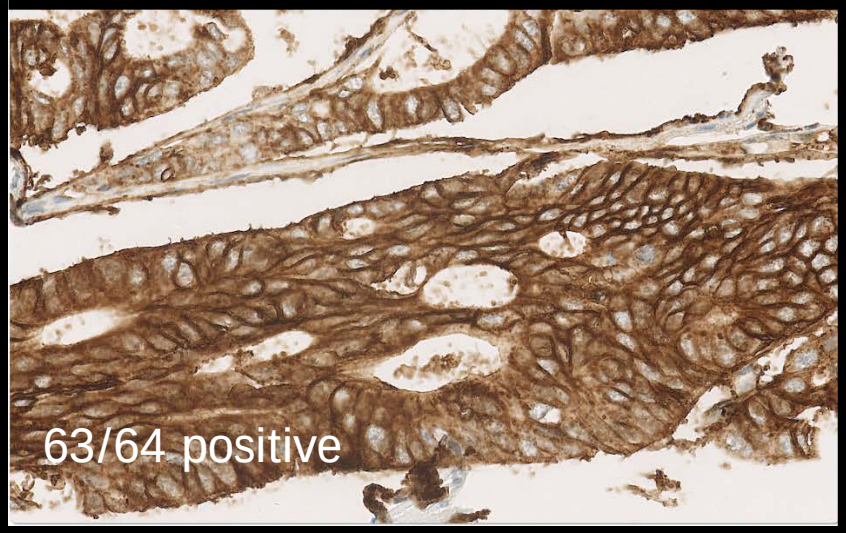
Study: Abcam ab 71916: 1:100, HIER TRS pH 6.1, 3-step polymer

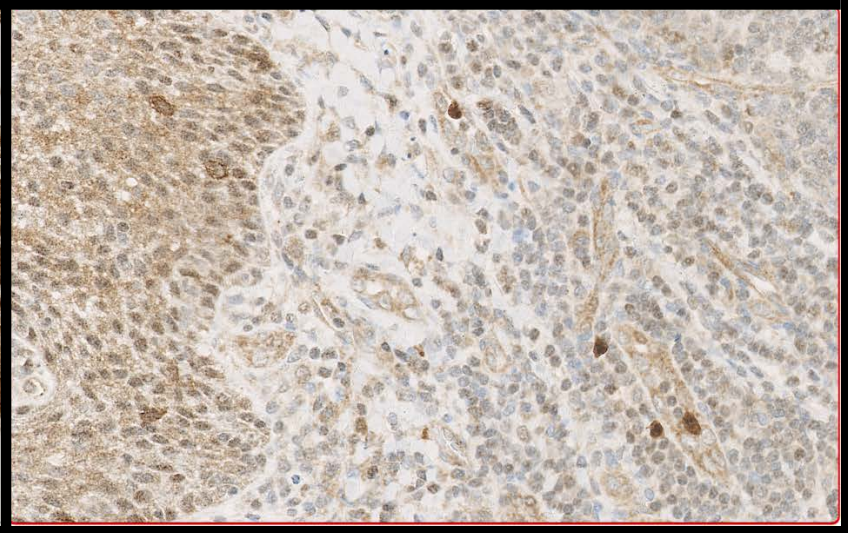
Glioma

Kidney









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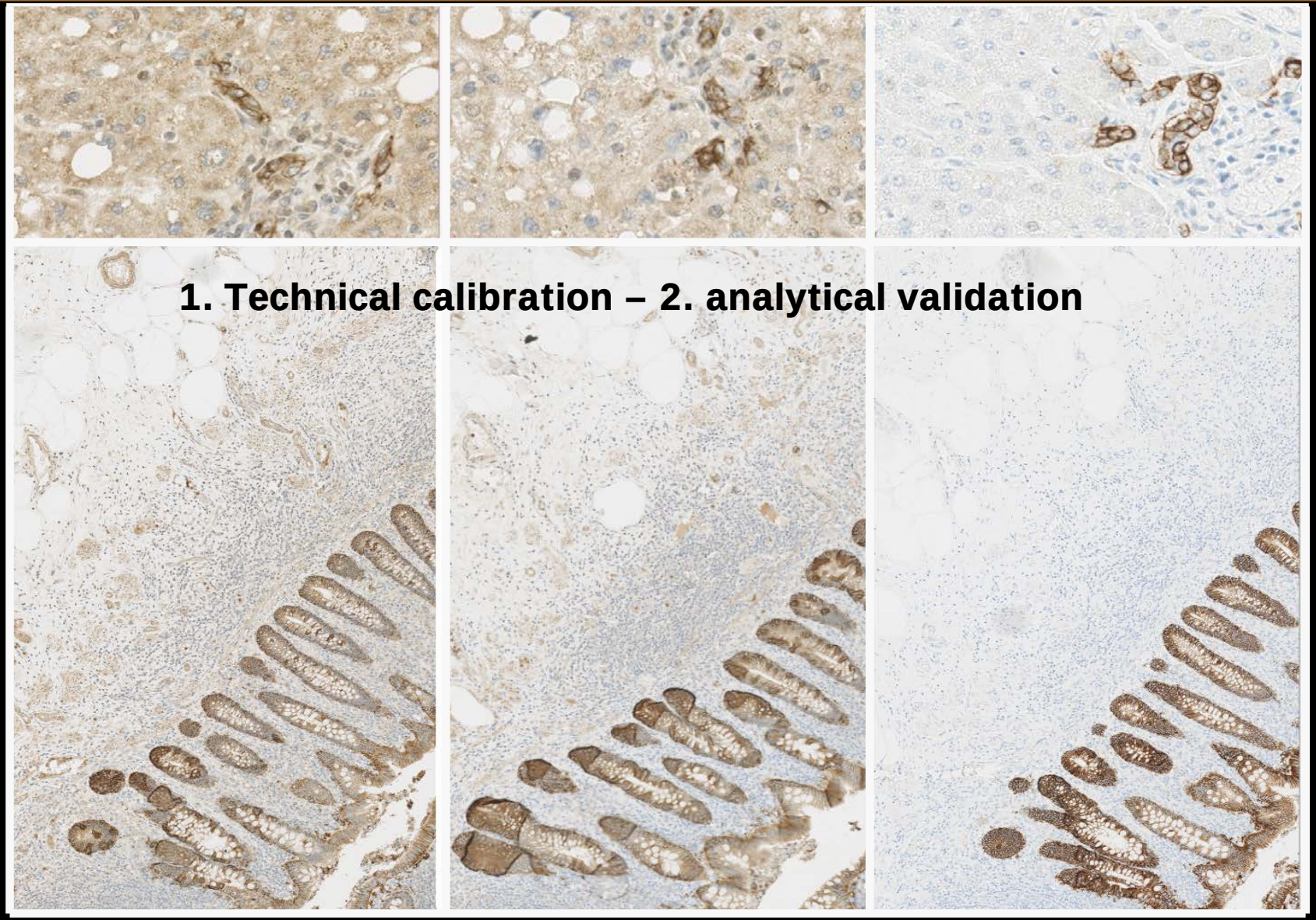
Tonsil

IHC – Biomarker controls



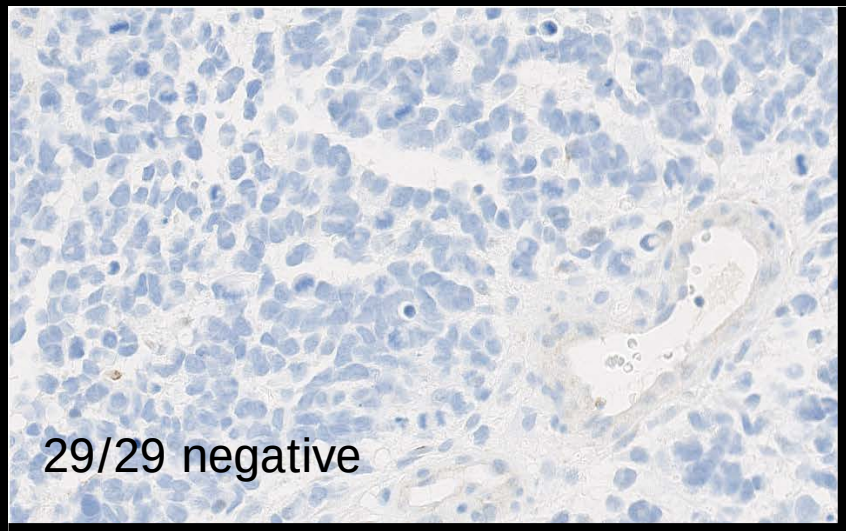
1:100 1:250 1:600

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

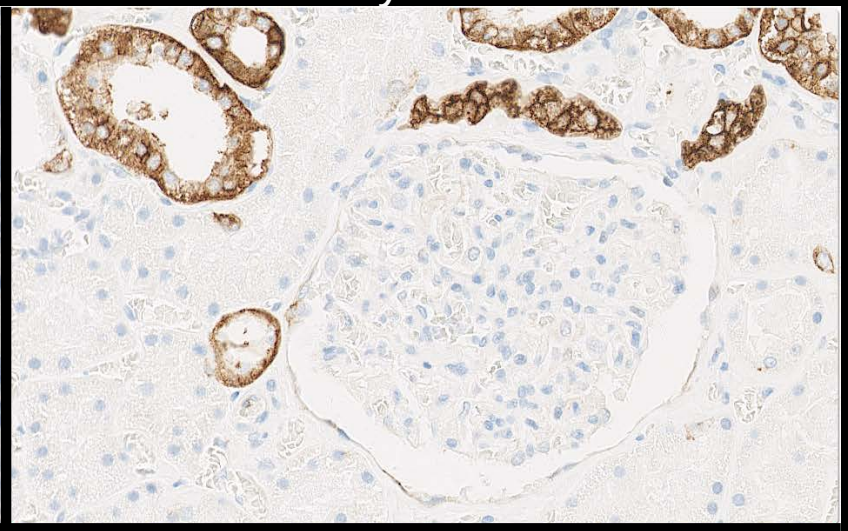
IHC – Biomarker controls

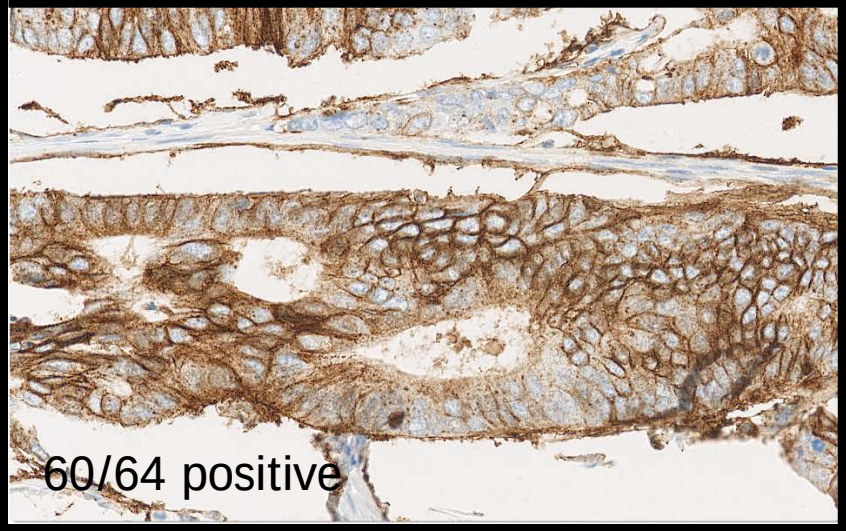
Abcam ab 71916: **1:600**, HIER TRS pH 6.1, 3-step polymer

Glioma



Kidney





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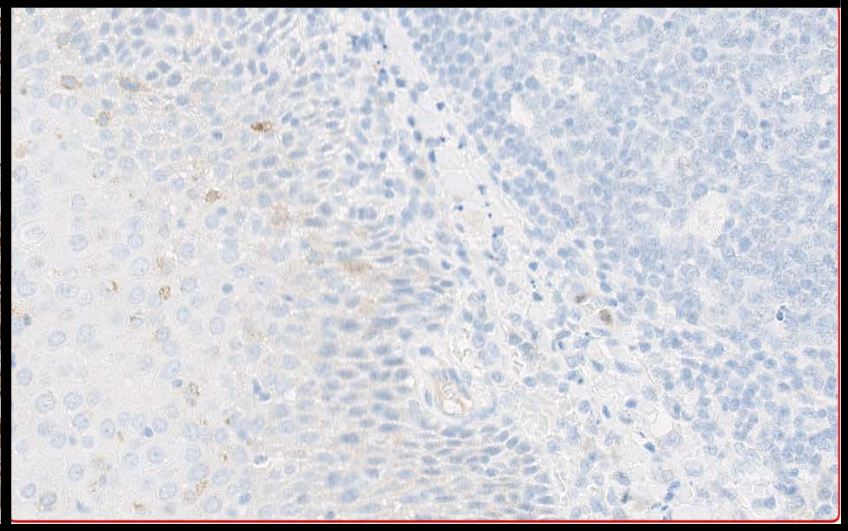

Tonsil

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

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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 analytical sensitivity
Guidance to analytical specificity

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

External tissue control tool-box:

Calibration TMA's			Analytical "Validation" TMA's		Lab QC TMA
iCAPC TMA	Specificity TMA	Pre-analyt. TMA	Accuracy TMA	Index TMA	"Daily QC" TMA
					
"Gold standard" tissue controls	"Normal" tissues	iCAPCs processed as lab procedures	"Lesional" tissues	"Lesional" tissues	iCAPCs + selected tissues
IHC critical assay performance controls	Maps Ab reaction pattern	Fixation time Fixative(s) Decalcification	Range of relevant expression levels	Range of relevant expression levels	Reproducibility Method of transfer proof
High expression Low expression No expression	With expression No expression		With expression No expression 20/40 of each Type I/II IHC	High expression Low expression No expression + relevant cut-off	

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.

- Negative reagent 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*

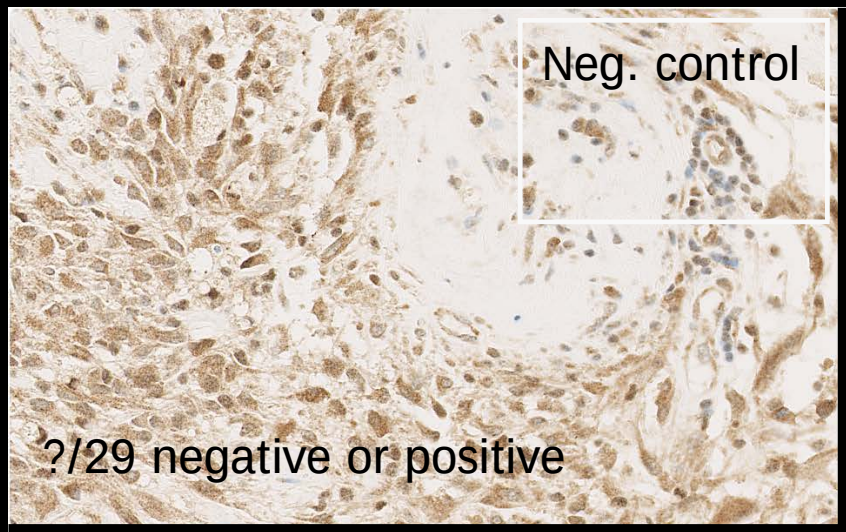
WILL NOT EXPOSE IF WRONG, POOR CALIBRATED OR CONTAMINATED PRIMARY AB WAS APPLIED!!!!!

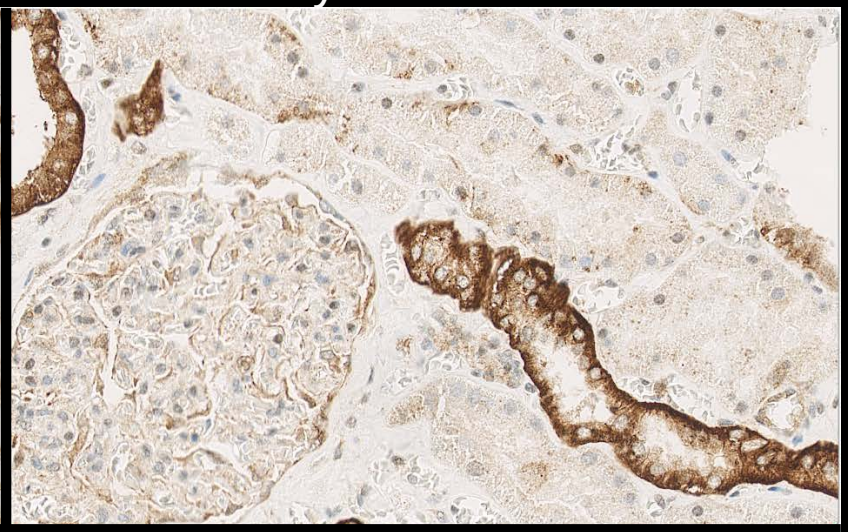
IHC – Biomarker controls

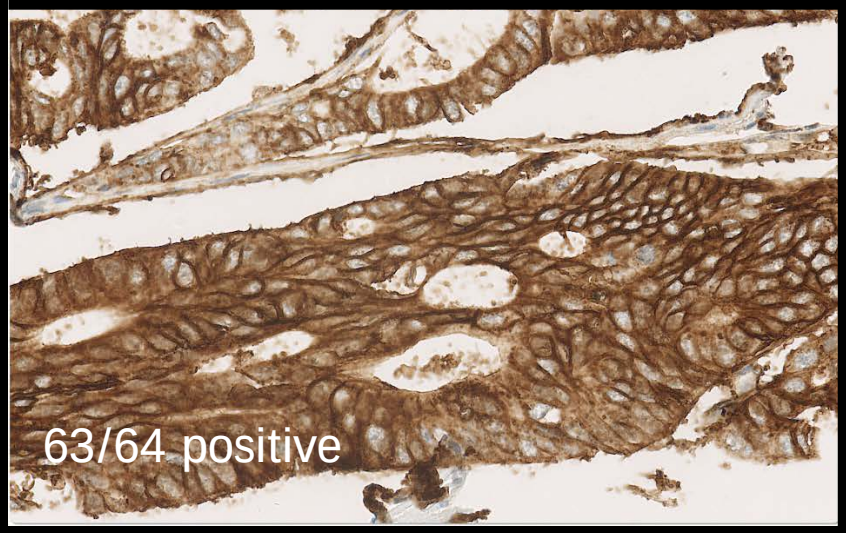
Study: Abcam ab 71916: 1:100, HIER TRS pH 6.1, 3-step polymer

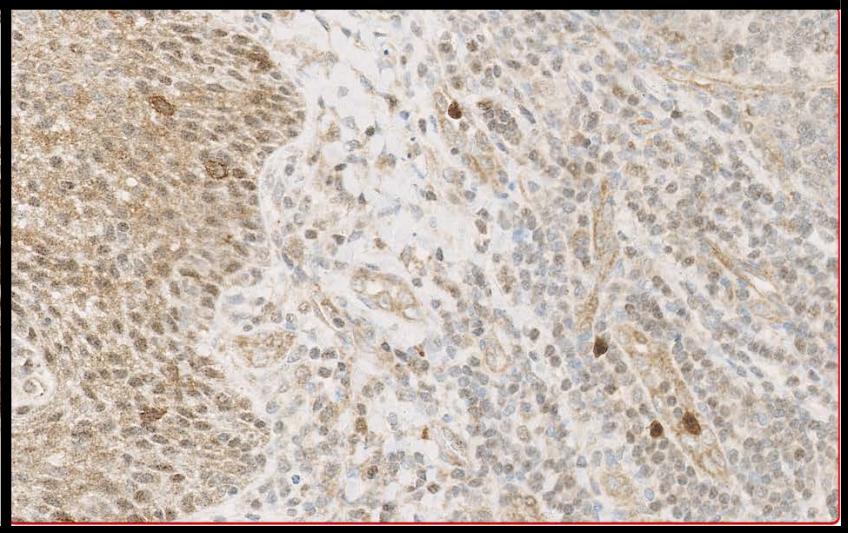
Glioma

Kidney









Colon ad. carc.

Tonsil

IHC – Biomarker controls

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

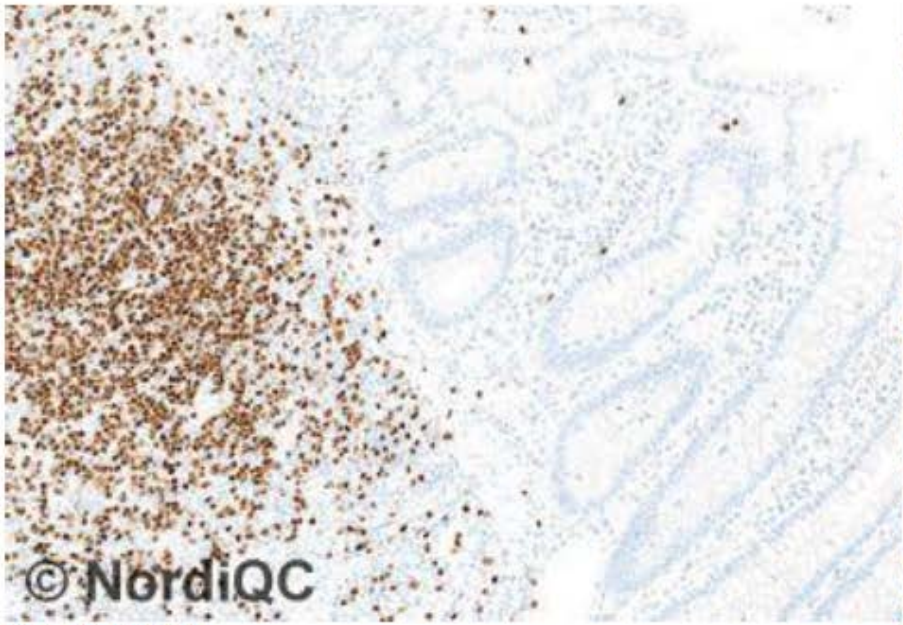


Fig.4a (X200)
Optimal BSAP staining of the appendix using same protocol as in Figs. 1a - 3a. The peripheral B-cells show a strong nuclear staining reaction, while the epithelial cells are negative.

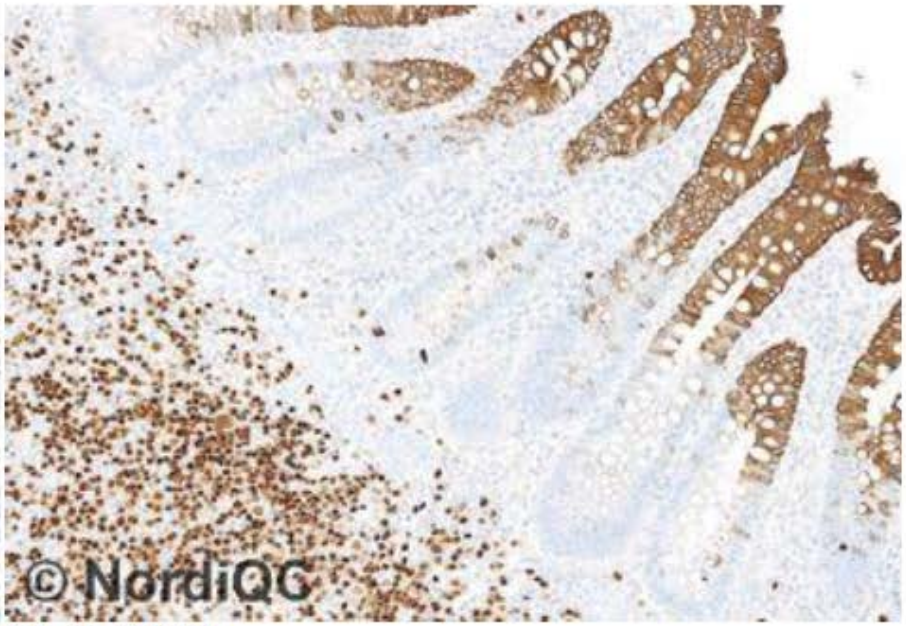


Fig. 4b (X200)
Aberrant BSAP staining of the appendix. In addition to the expected staining result for BSAP of the B-cells, the epithelial cells display a staining reaction corresponding to CK20. This aberrant staining result was frequently seen, when the rmAb clone SP34 was used as a concentrate and 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: 1. Biotin based detection systems
2. Certain class II / III assays

Can: 1. Pigmented tumours
2. Frozen sections
3. (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

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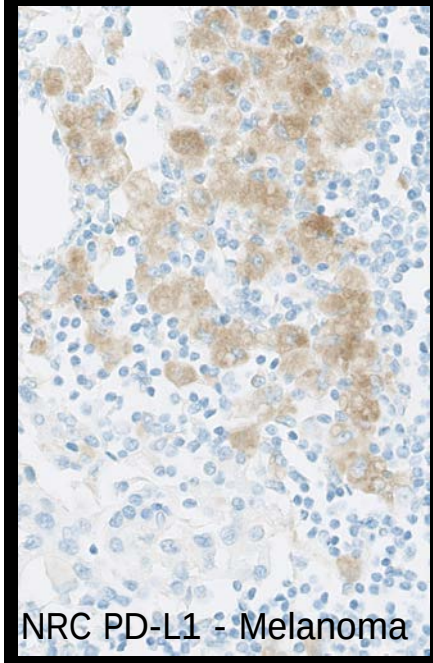
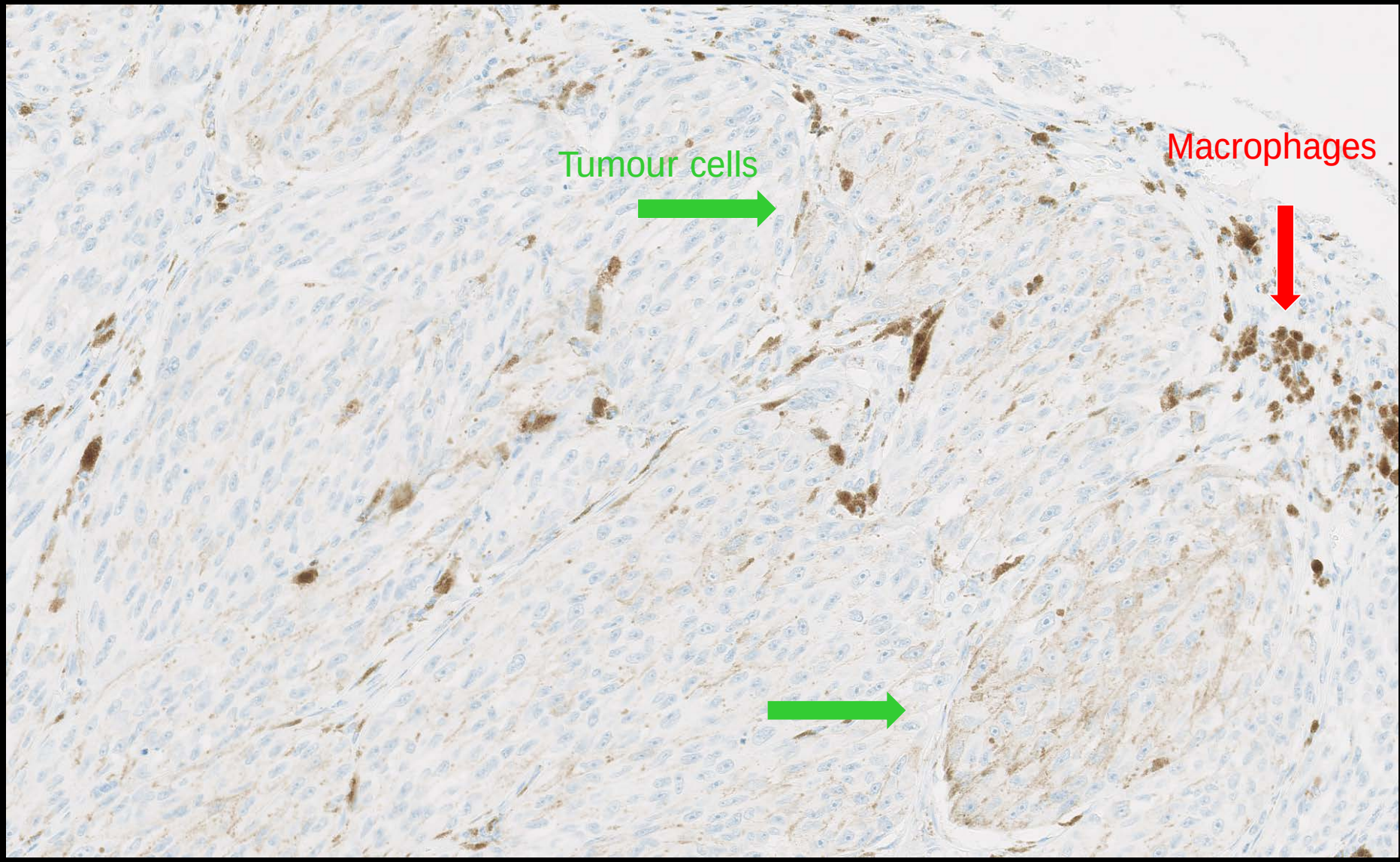
TABLE 2. Recommendations for Use of Negative Controls in Diagnostic Immunohistochemistry

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)



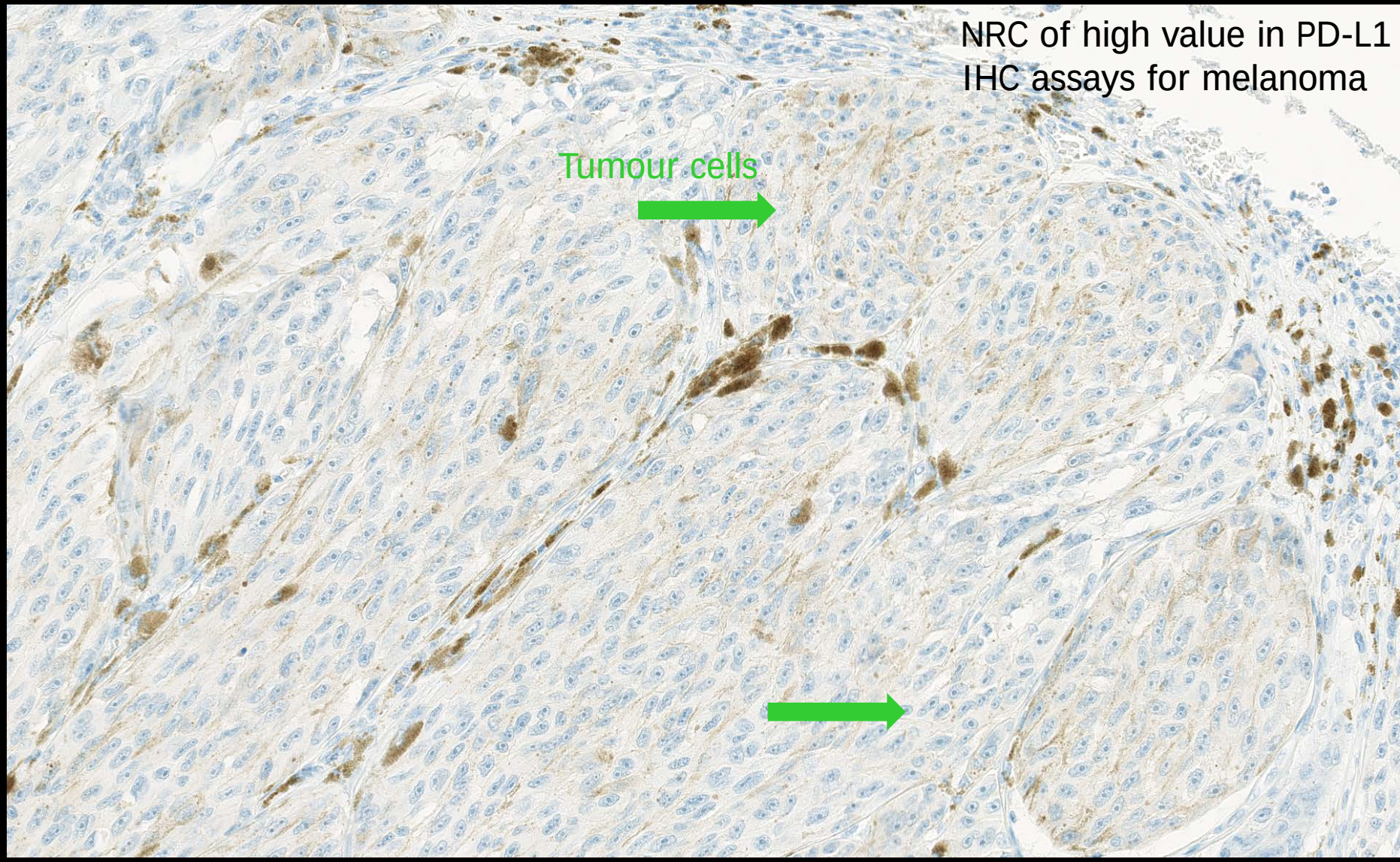
NRC PD-L1 - Melanoma

IHC – Biomarker controls



PD-L1 IHC 22C3 Class II/III test – malignant melanoma

IHC – Biomarker controls



PD-L1 IHC 22C3 Class II/III test - negative reagent control – malignant melanoma

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 material
  - External positive and negative tissue control
    - Slide next to patient material

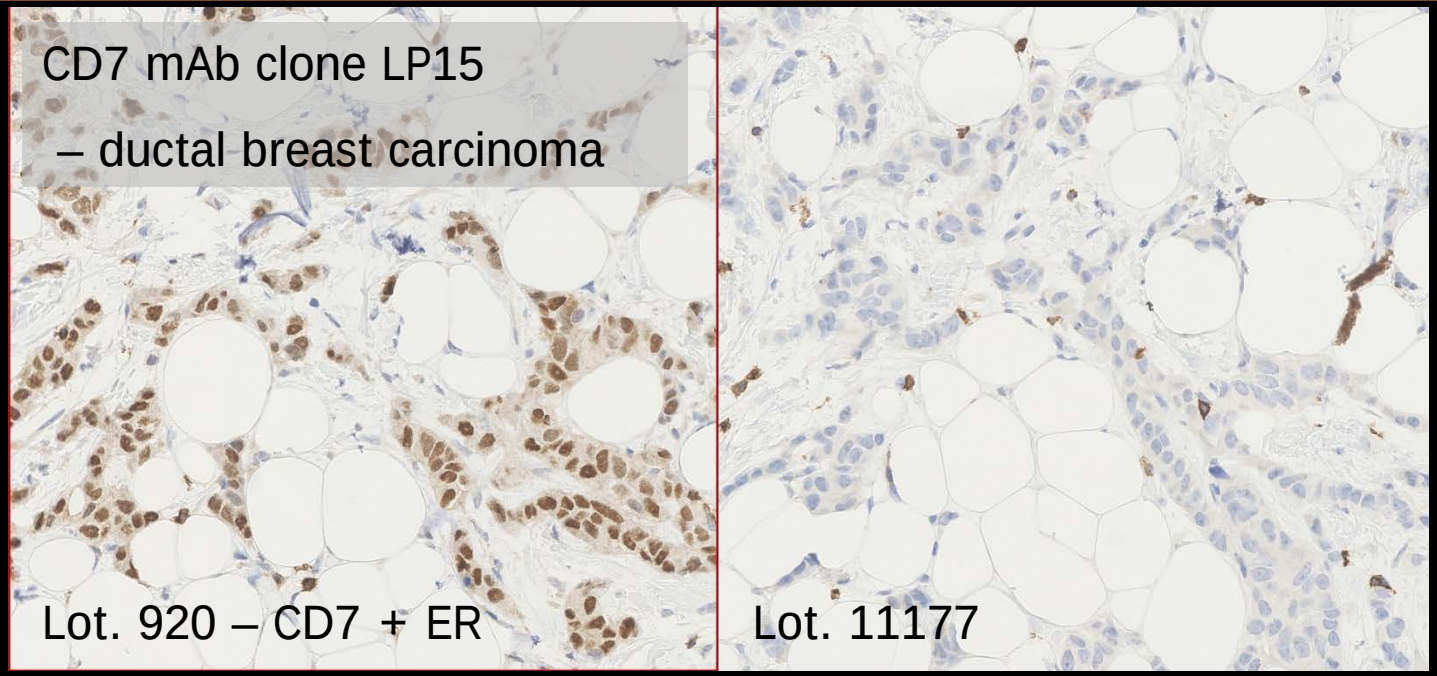
IHC – Biomarker controls

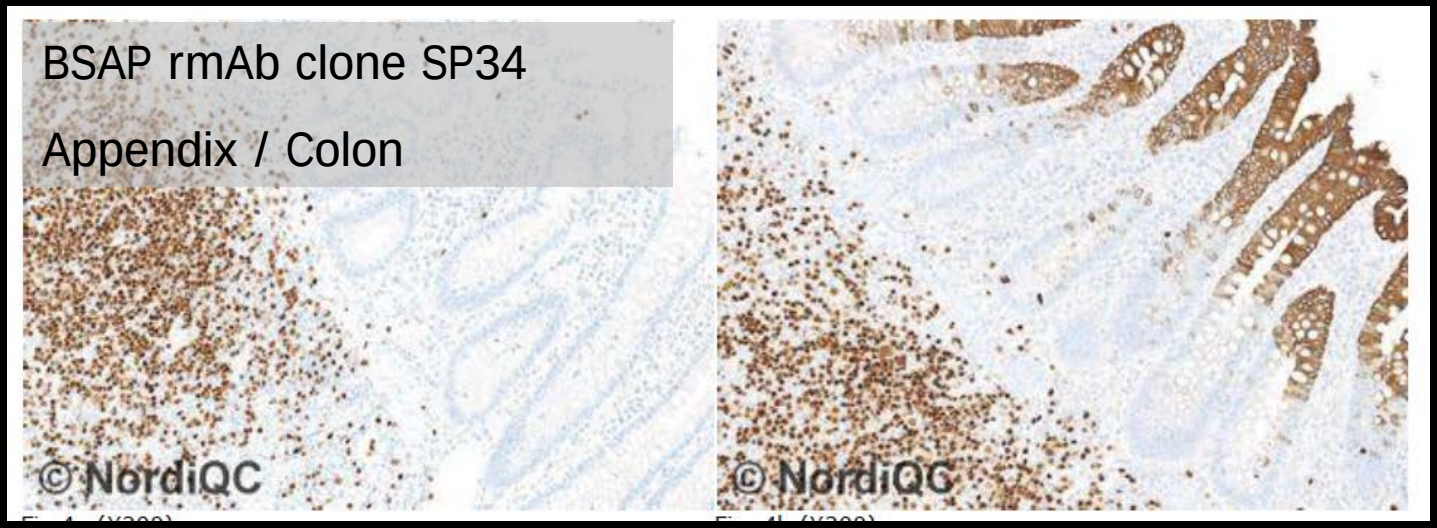
- Reagent and **tissue** controls are necessary for the validation of immunohistochemical staining results.

- 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



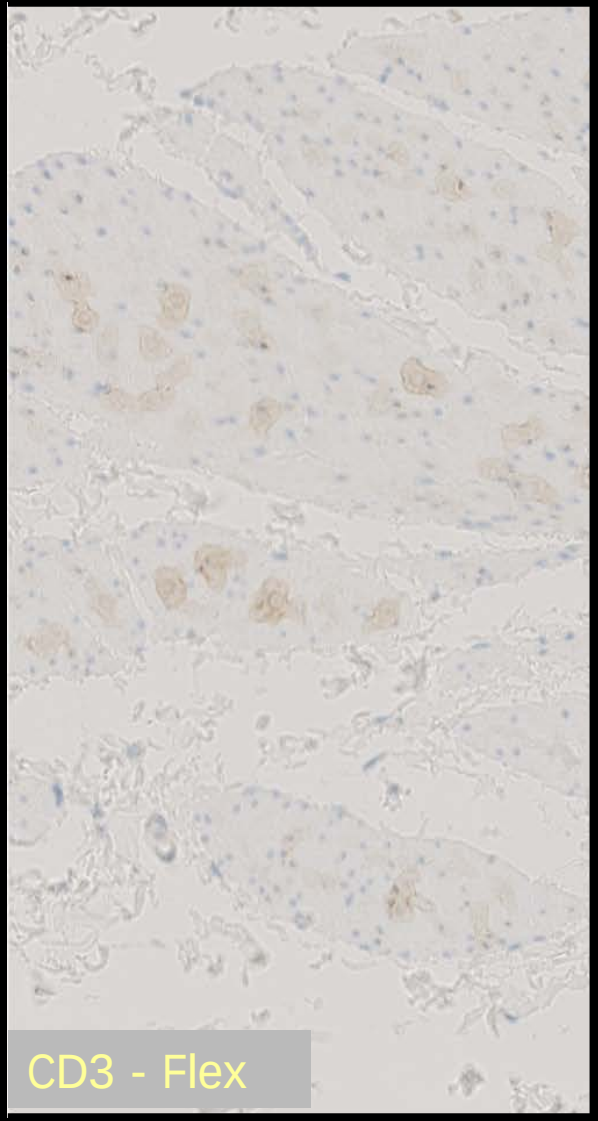


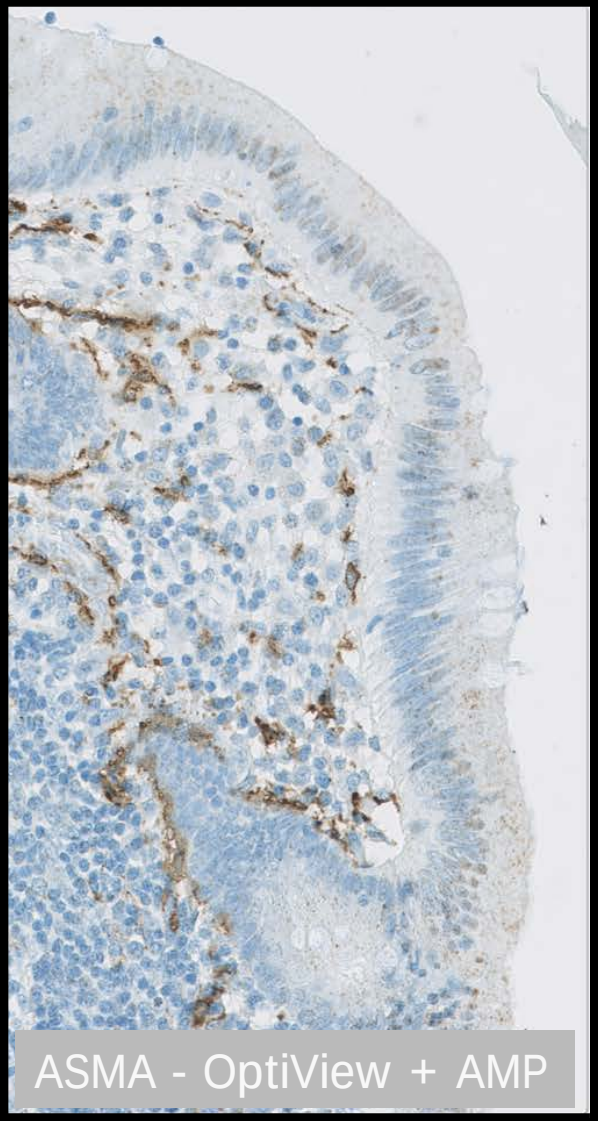
FP staining reactions

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





Internal structures used as negative tissue control for polymer/multimer based detection systems

IHC – Biomarker controls

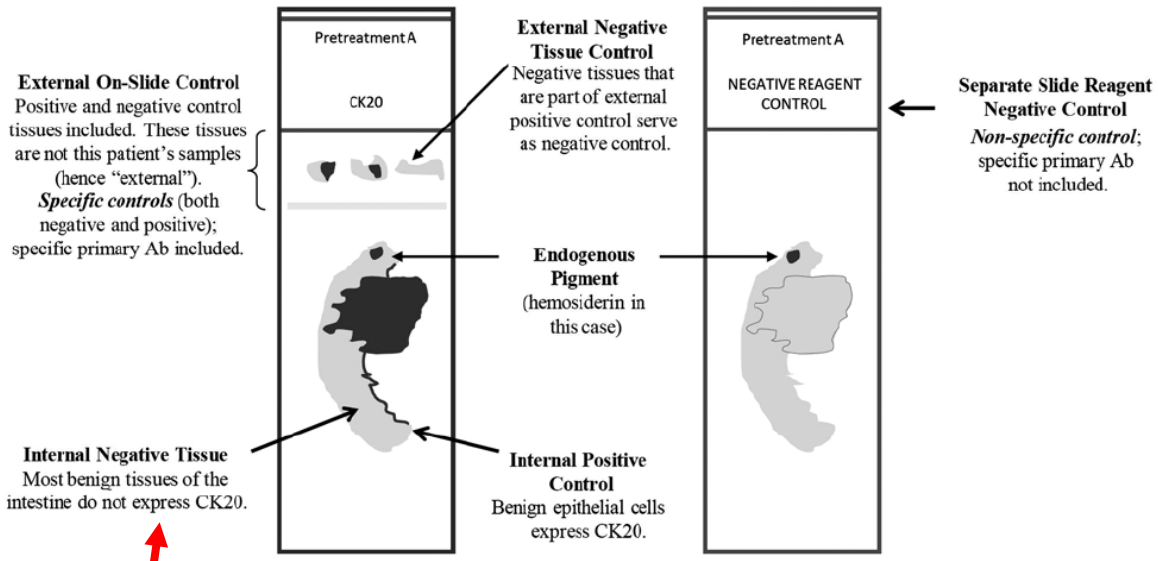


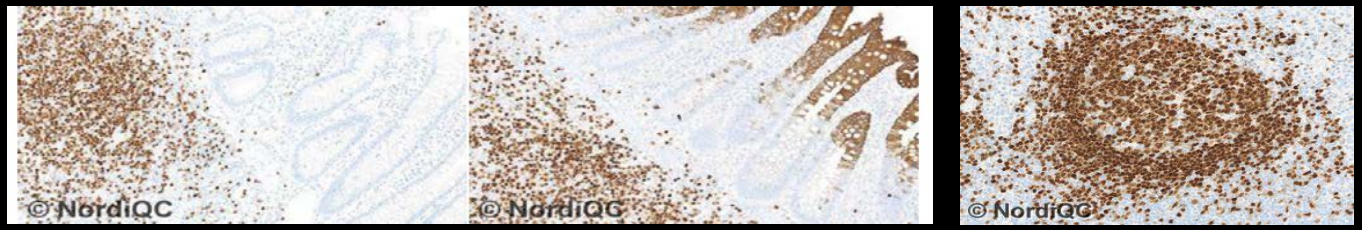
FIGURE 1. "On-slide" external and internal negative tissue controls are illustrated. It is sound practice whenever possible to include cells (or tissue elements) that will serve as negative controls (expected to be nonreactive) when selecting tissue for the positive tissue control. Both internal and 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.

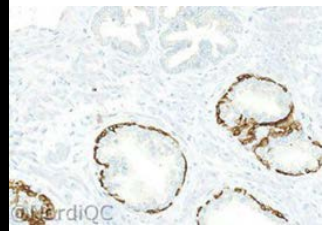
Limitation: Not all elements will be available to expose a potential false positive result

PAX5.... 3 vendors



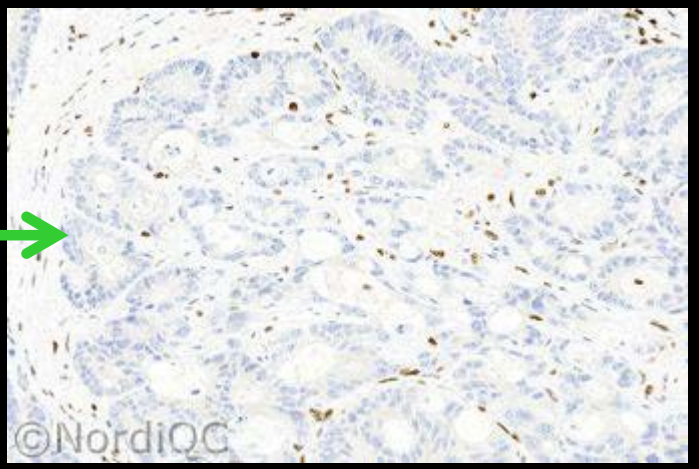
IHC – Biomarker controls

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

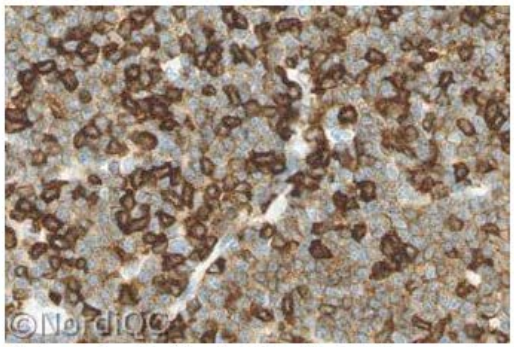


Fig. 4a. Optimal staining for CD5 of the B-CLL no. 5 using same protocol as in Figs. 1a - 4a.
The majority of the neoplastic cells show a moderate and distinct staining reaction, while the infiltrating normal T-cells show a strong staining reaction.

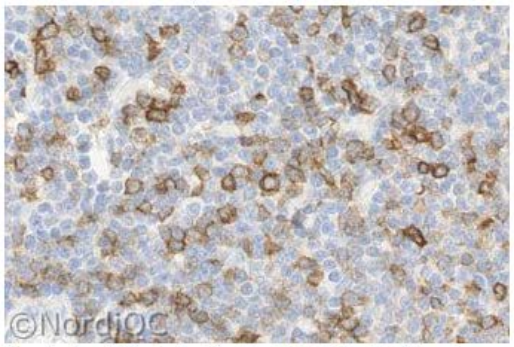


Fig. 4b. Insufficient staining for CD5 of the B-CLL using same protocol as in Figs. 1b - 3b – same field as in Fig. 4a.
The neoplastic cells are virtually negative and only the normal T-cells are clearly demonstrated.

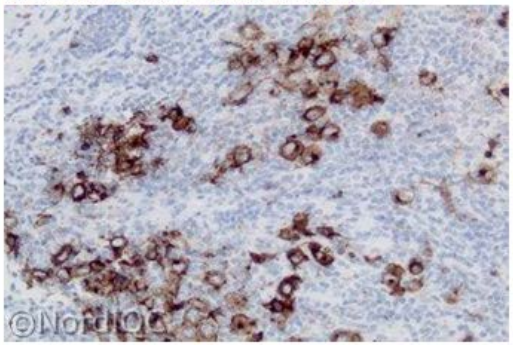


Fig. 2a. Optimal CD15 staining of the Hodgkin lymphoma no 2 (NS) using same protocol as in Fig. 1a. The Reed-Sternberg and Hodgkin cells show a strong membranous staining and a dot-like positivity.

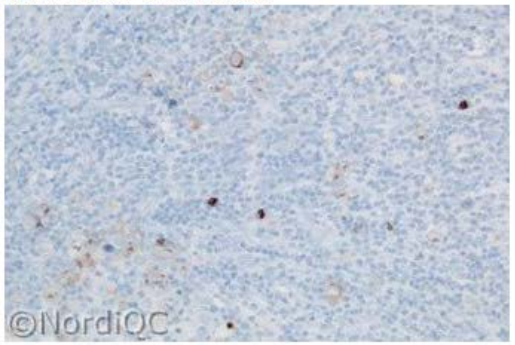


Fig. 2b. CD15 staining of the Hodgkin lymphoma no 2 (NS) using same protocol as in Fig. 1b. Only few Reed-Sternberg and Hodgkin cells show a weak staining - same field as in Fig. 2a.

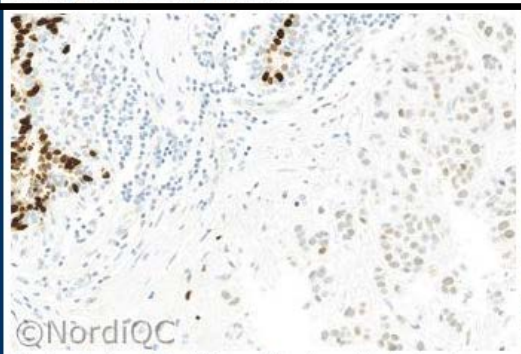


Fig. 3a. Optimal ER staining of the breast ductal carcinoma no. 3 with 60 – 80 % cells positive. A weak but distinct nuclear staining is seen in the appropriate proportion of the

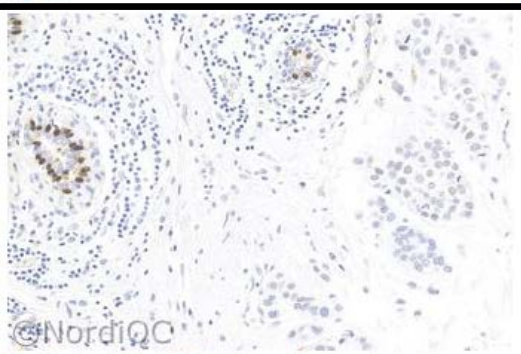
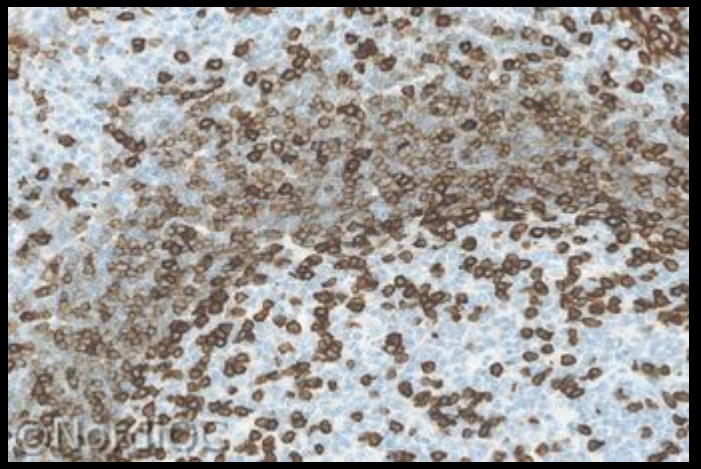


Fig. 3b. Insufficient ER staining of the breast ductal carcinoma no. 3 with 60 – 80 % cells positive using same protocol as in Figs. 1b and 2b – same field as in Fig. 3a.

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, ** HIER time 32 min. at 99°C
32 min in primary Ab, 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, amp. Kit....)

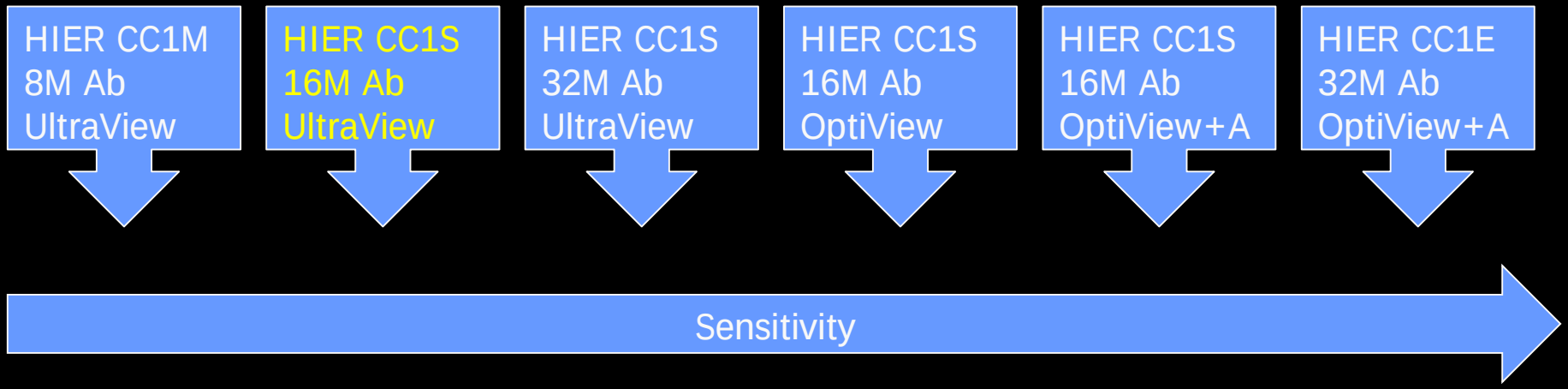
IHC – Biomarker controls

Ready-To-Use – VMS ULTRA

RTU

Typical protocol:

A: HIER in CC1 standard (64 min.), 16 min. Incubation time in primary Ab and UltraView-DAB



IHC – Biomarker controls

Issues to be adressed :

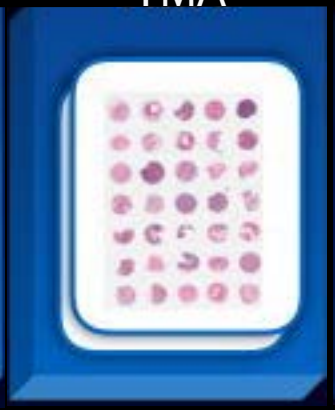
1. Calibration of IHC assay and identification of best practice protocol – clone, titre, retrieval etc
2. ”Evaluation of the robustness of the IHC assay – impact on pre-analytics
3. Evaluation of the analytical sensitivity/specificity
4. Identification of most robust controls providing information that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

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

IHC – Biomarker controls

External tissue control tool-box:

Calibration TMA's

iCAPC TMA	Specificity TMA	Pre-analyt. TMA
		
"Gold standard" tissue controls	"Normal" tissues	iCAPCs processed as lab procedures
IHC critical assay performance controls	Maps Ab reaction pattern	Fixation time Fixative(s) Decalcification
High expression Low expression No expression	With expression No expression	

Analytical "Validation" TMA's

Accuracy TMA	Index TMA
	
"Lesional" tissues	"Lesional" tissues
Range of relevant expression levels	Range of relevant expression levels
With expression No expression	High expression Low expression No expression
20/40 of each Type I/II IHC	+ relevant cut-off

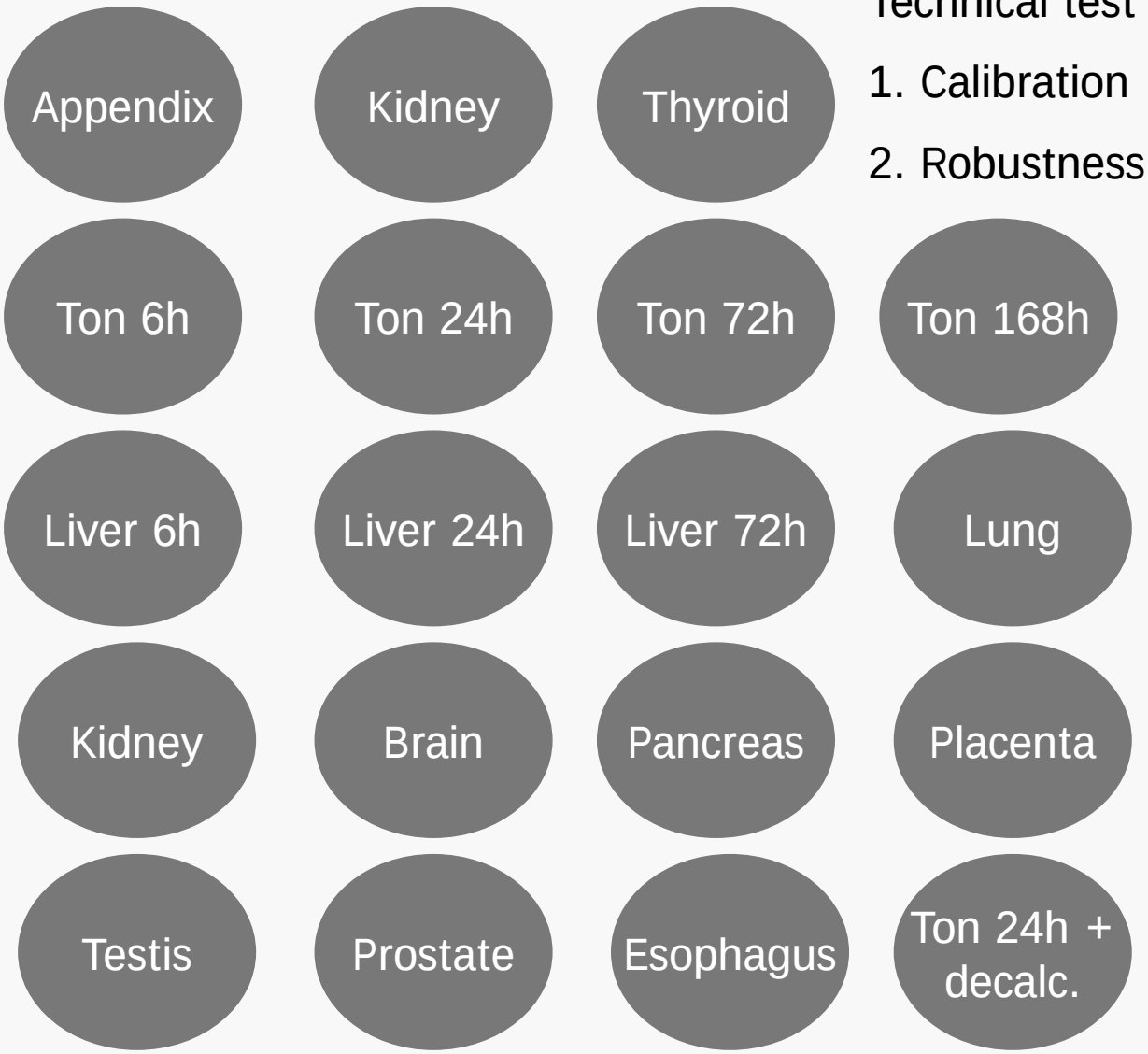
Lab QC TMA

"Daily QC" TMA

iCAPCs + selected tissues
Reproducibility Method of transfer proof

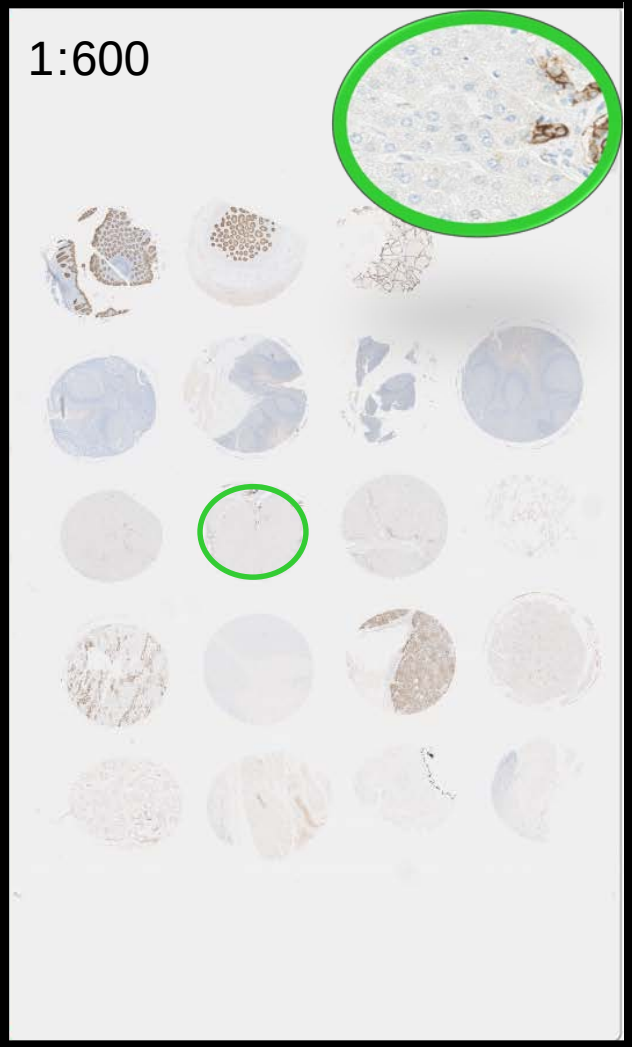
Tissue

IHC – Biomarker controls



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

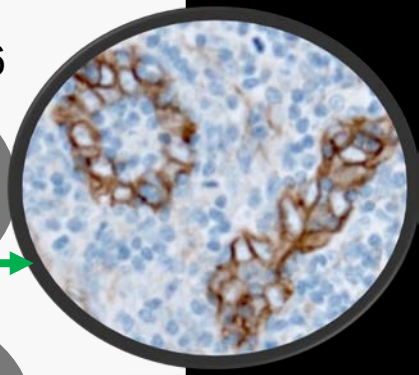
IHC – Biomarker controls

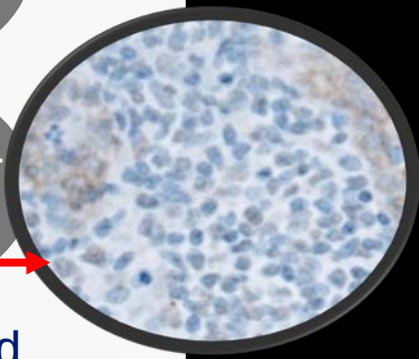
Technical test array

1. Calibration
2. Robustness

CD105, SNH6

Appendix	Appendix	Thyroid	
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.



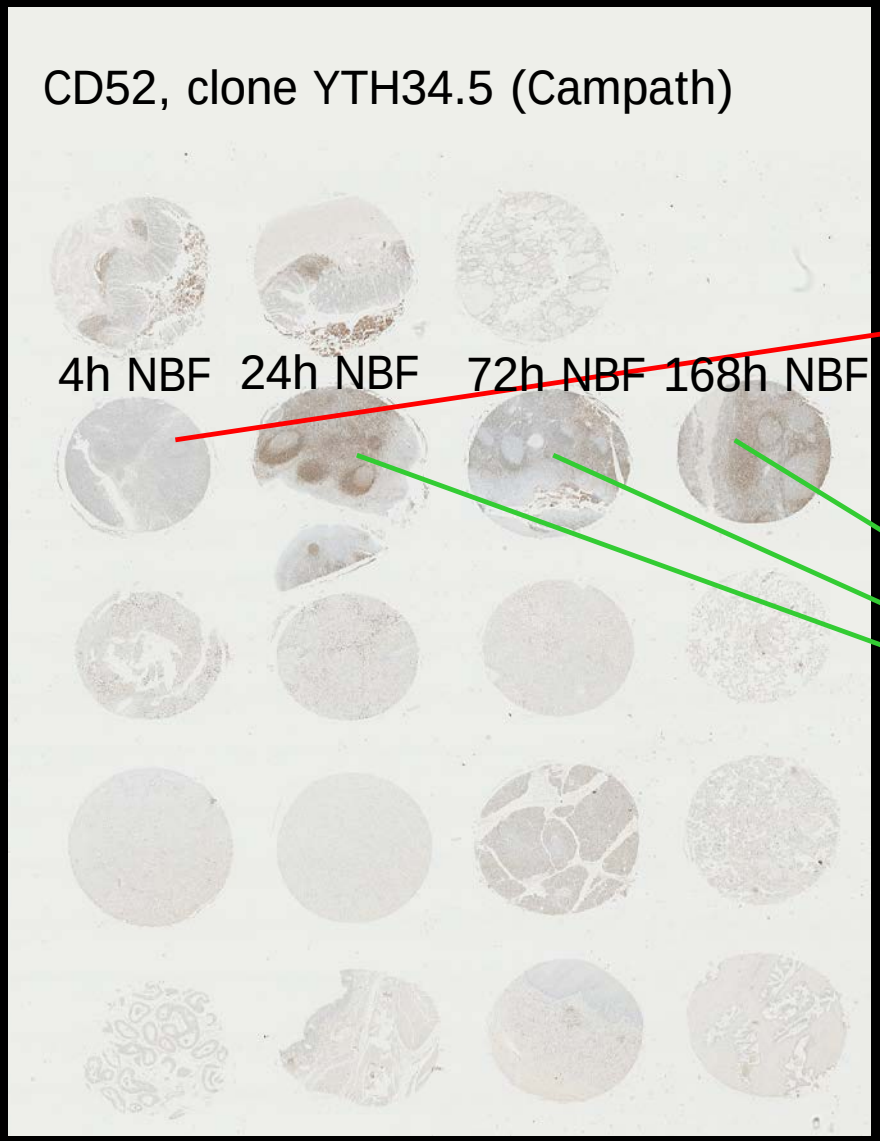


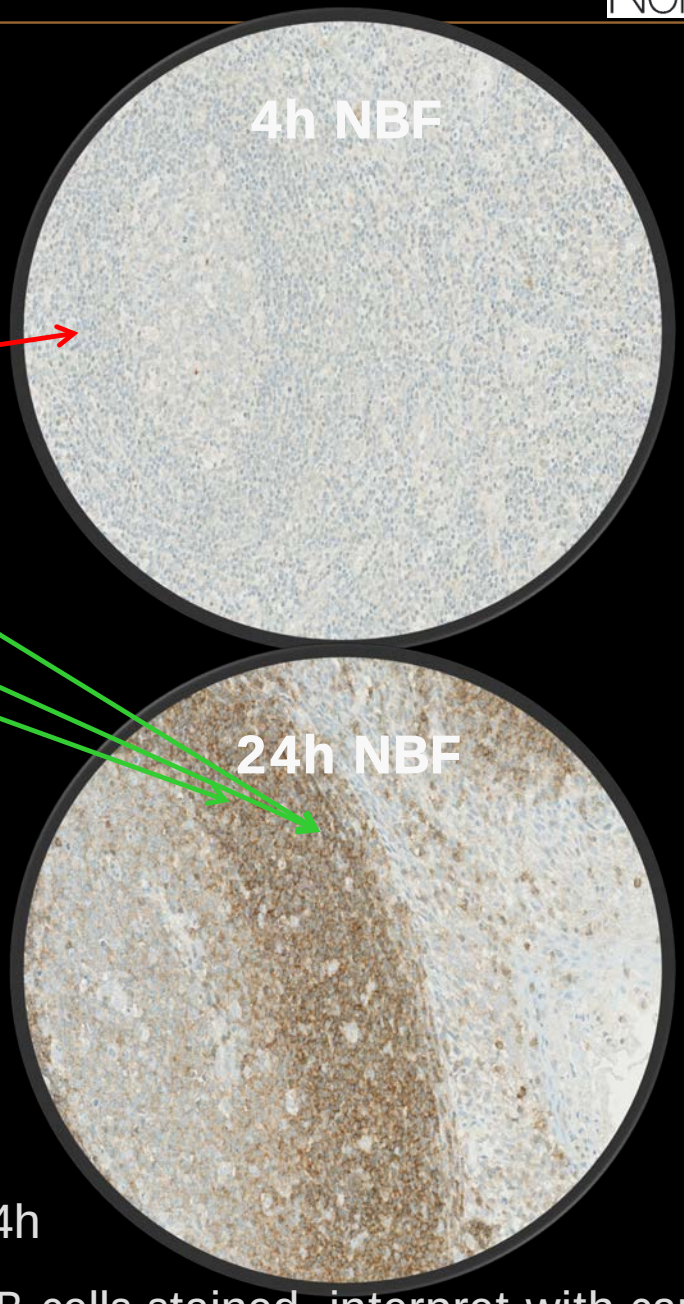
Formic acid

Protocol set-up:

Ole Nielsen, OUH DK

IHC – Biomarker controls

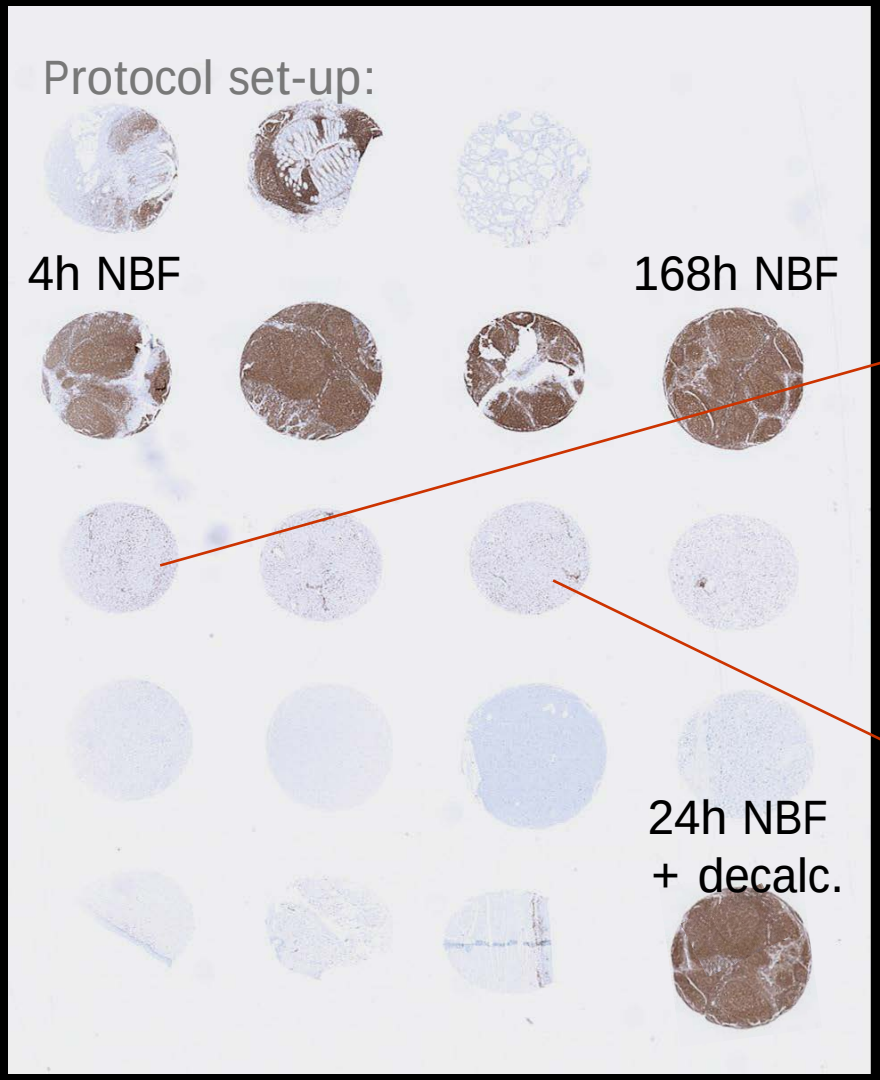


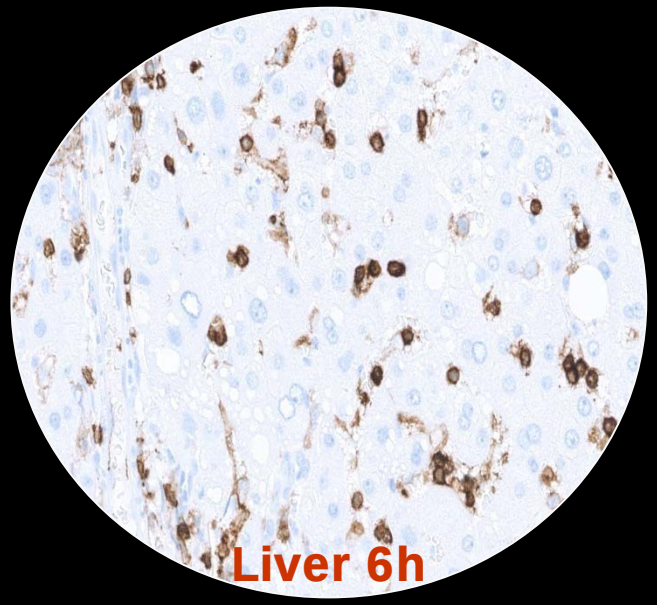


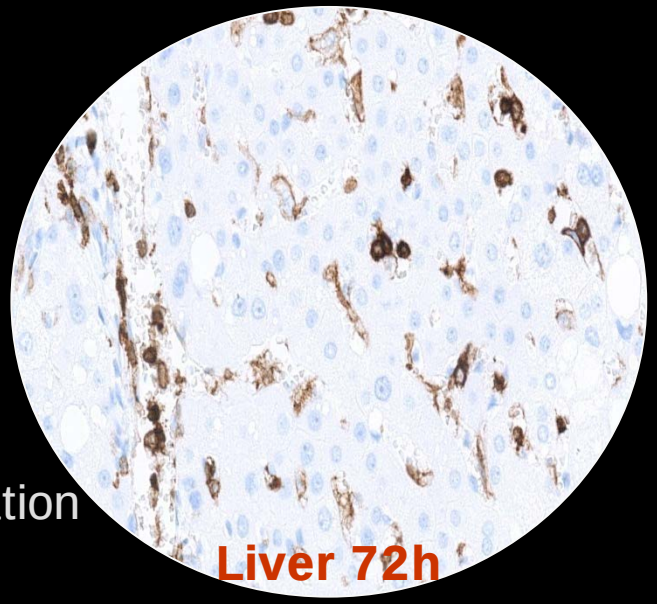
1. Influenced by fixation time – reduced in <24h
2. IHC protocol, 3. Control; Tonsil – cave if no B-cells stained, interpret with caution

IHC – Biomarker controls

Anti-CD45 test:

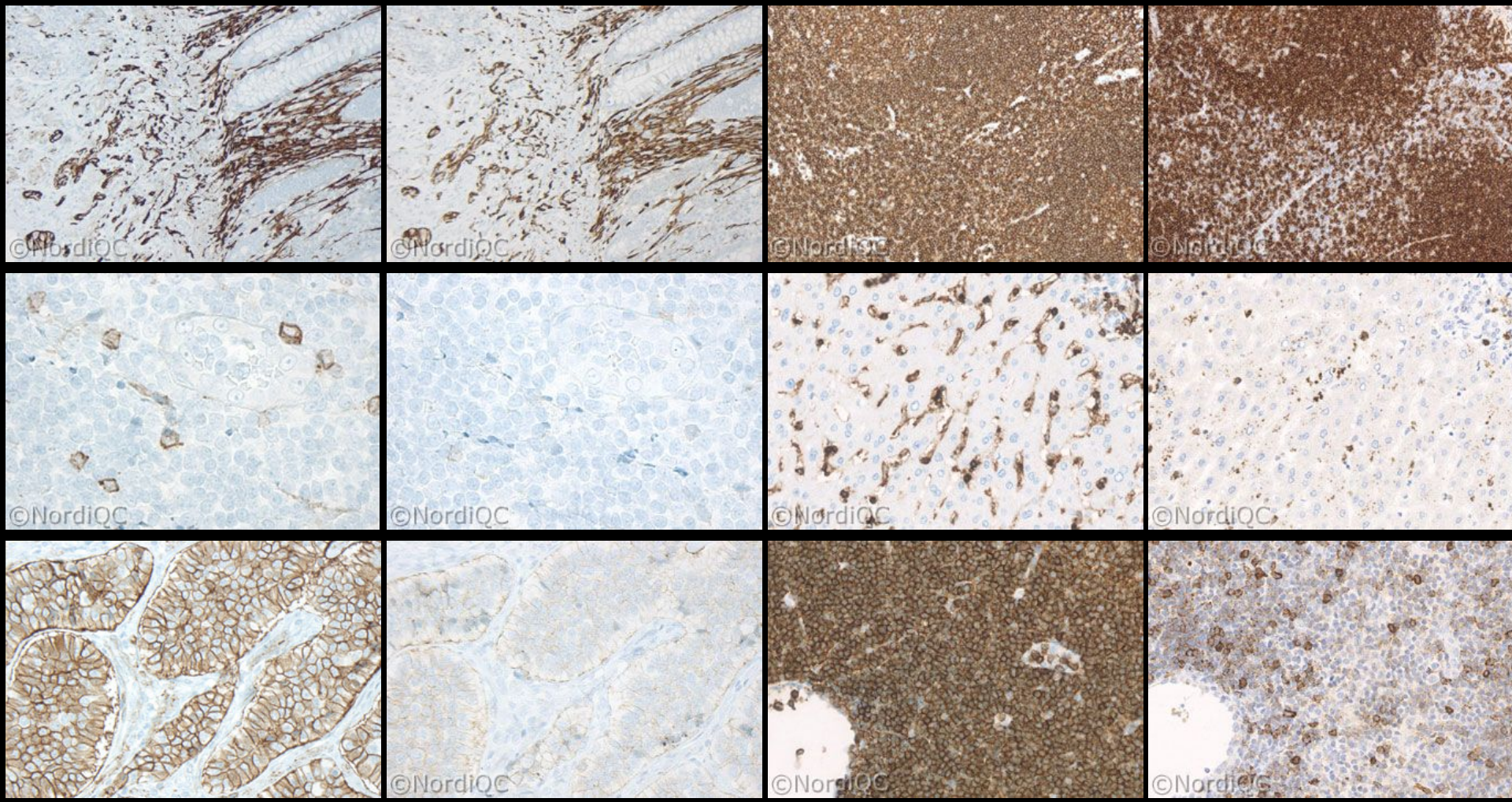






1. Not NBF dependent or influenced by decalcification
2. Liver as control, 3. IHC protocol

IHC – Biomarker controls

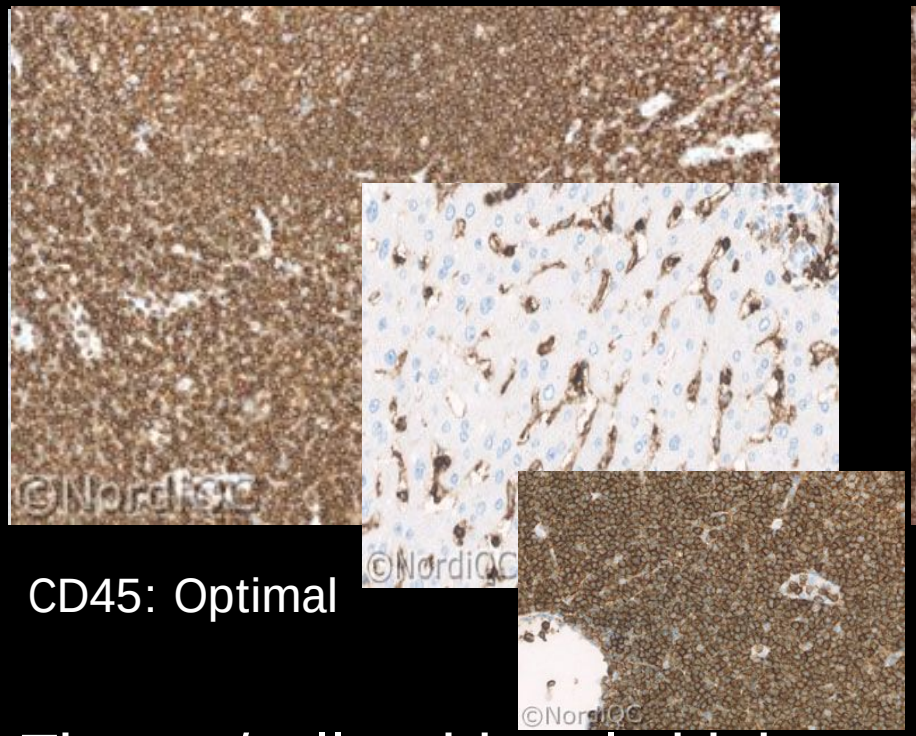


CD56
App – Tonsil – Neuroendocrine carc.

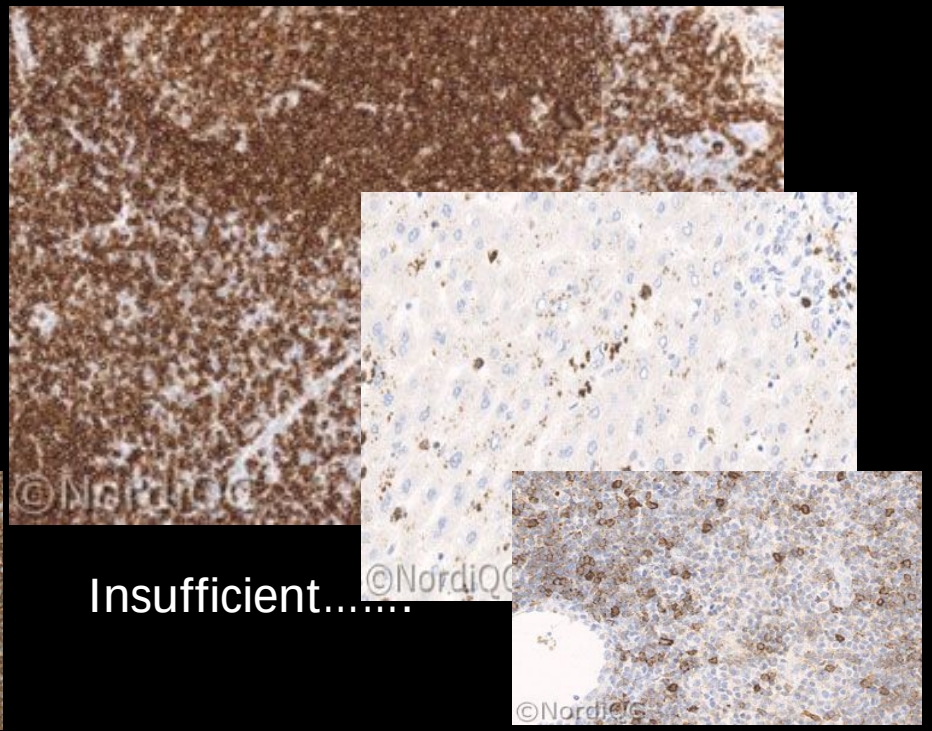
CD45
Tonsil – Liver – B-CLL.

Protocol A Protocol B Protocol A Protocol B

IHC – Biomarker controls



CD45: 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

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IHC – Biomarker controls

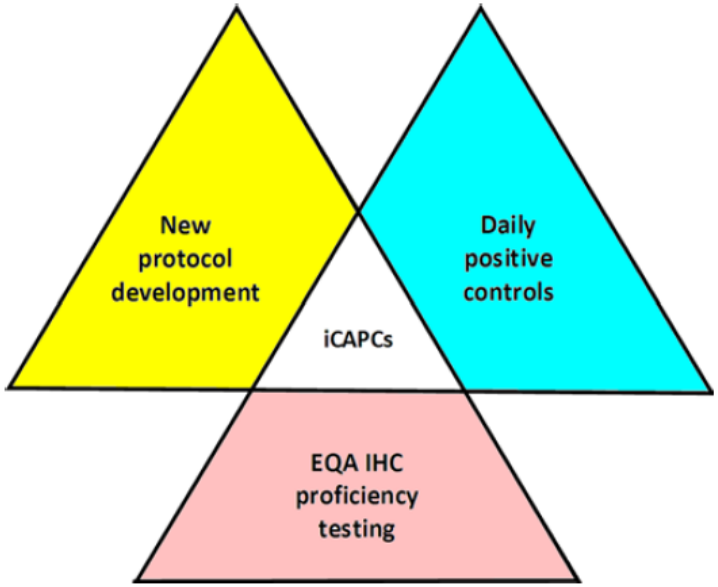


FIGURE 19. The roles of iCAPCs in clinical immunohistochemistry (IHC) laboratories. iCAPCs are an essential part of new protocol development, daily quality controls, and proficiency testing. EQA indicates External Quality Assurance; iCAPC, immunohistochemistry critical assay performance controls.

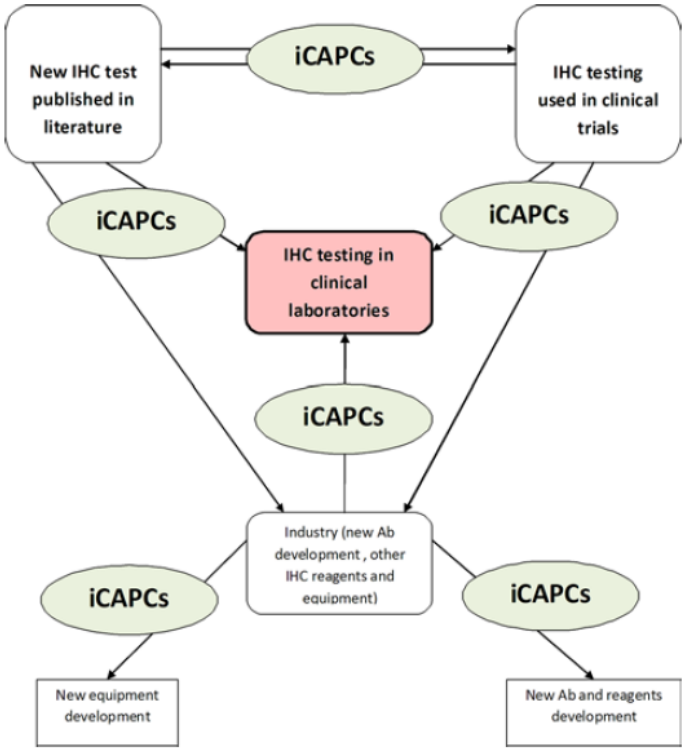


FIGURE 20. iCAPCs and Methodology Transfer. iCAPCs are proposed as important elements for harmonization of immunohistochemistry (IHC) testing between clinical research, product development, and clinical IHC testing. iCAPCs enable IHC harmonization of protocol transfer between research, industry, and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

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

Expected level – calibration
Analytical sensitivity and specificity

IHC – Biomarker controls

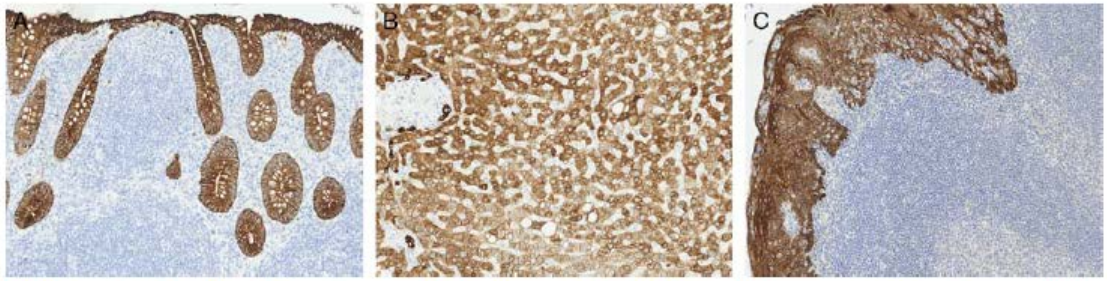


FIGURE 1. Pan-keratin iCAPC. A, Appendix: virtually all columnar epithelial cells must show a moderate to strong predominantly cytoplasmic staining reaction (a membranous accentuation will typically be seen). B, Liver: the vast majority of hepatocytes must show at least weak to moderate cytoplasmic staining 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.

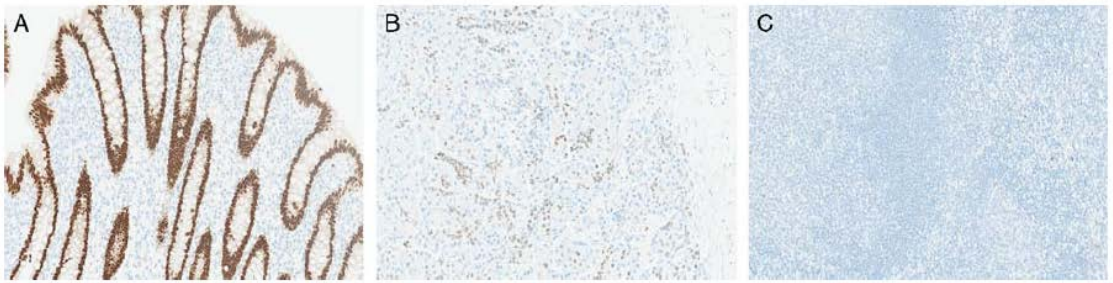


FIGURE 8. CDX-2 iCAPC. A, Appendix: virtually all epithelial cells must show a strong nuclear staining reaction. A weak cytoplasmic staining reaction in addition to strong nuclear staining is often present. B, Pancreas: the majority of epithelial cells of intercalated ducts must show a weak to moderate 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)

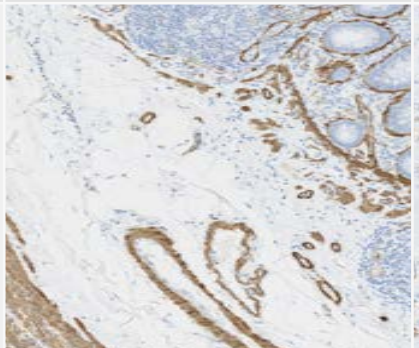
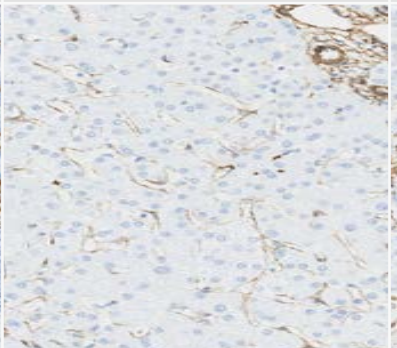
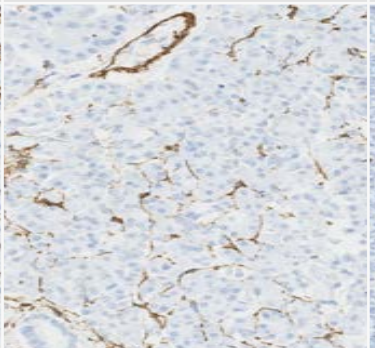
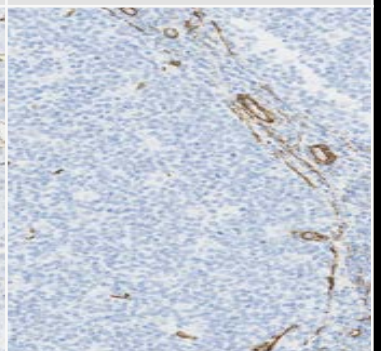
No expression
(Appropriate specificity)

Which tissue
Which cells
Which extension
Which intensity

IHC – Biomarker controls

	High express.	Low ex. (iCAPCs)	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	Different comp.
CD31	Tonsil	Liver	Appendix	
Vimentin	Appendix	Liver	Liver	Different comp.
Desmin	Appendix	Tonsil	Appendix	Different comp.
ASMA	Appendix	Liver	Appendix	Different comp.
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 iCAPCs (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

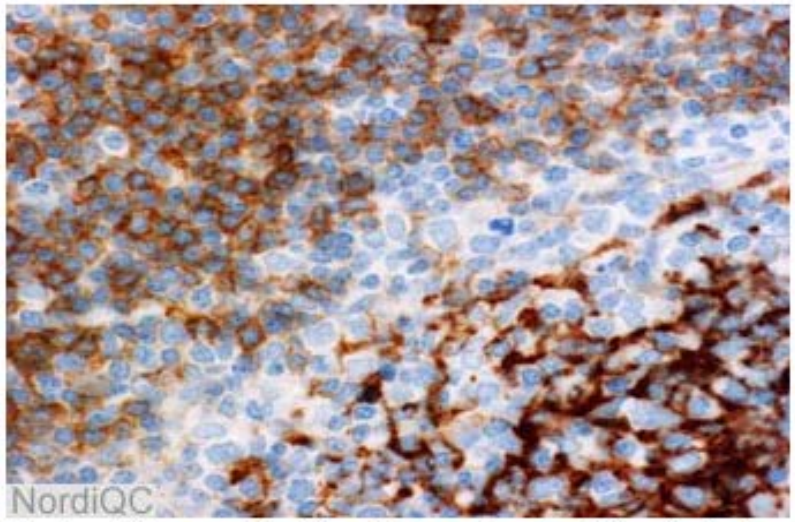


Fig. 2a. High magnification of the optimal staining in Fig 1a of the secondary follicle in the tonsil. The activated B-cells show a distinct continuous membranous reaction.

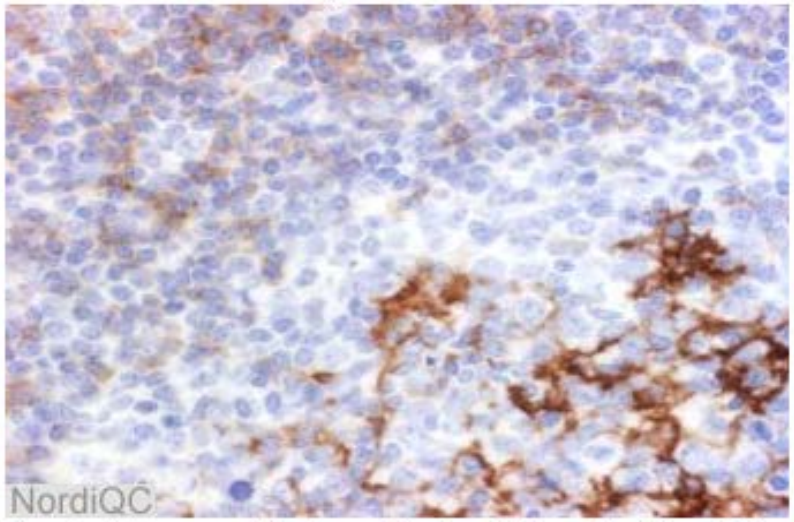


Fig. 2b. High magnification of the insufficient staining in Fig 1b of the secondary follicle in the tonsil (same field as in Fig 2a). The activated B-cells only show a weak imprecise reaction.

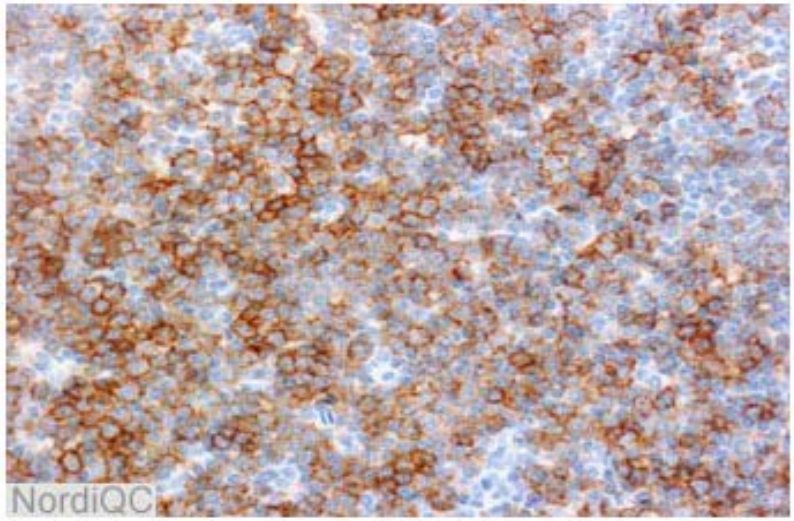


Fig. 3a. Optimal staining for CD23 of the B-CLL no 4 using same protocol as in Fig 1b and 2 b. The majority of the neoplastic cells show a strong and distinct membranous staining.

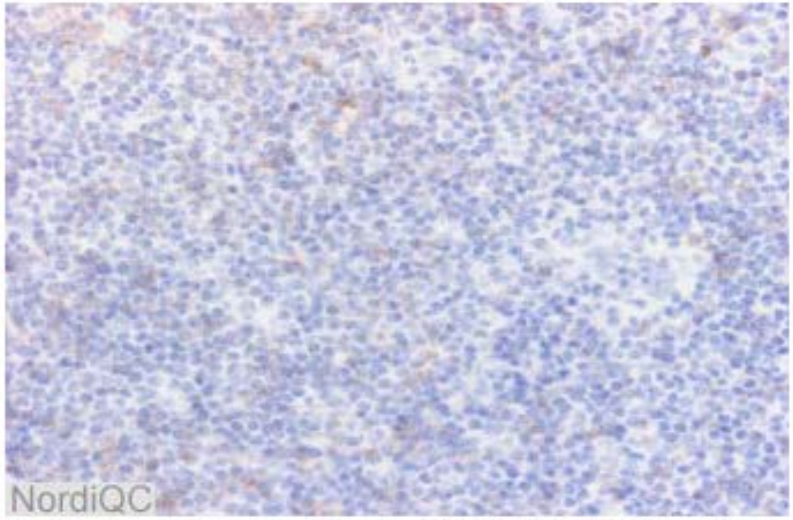


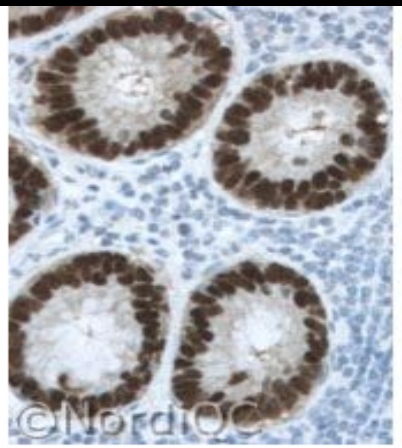
Fig. 3b. Insufficient staining for CD23 of the B-CLL no 4 using same protocol as in Fig 1b and 2 b. The neoplastic cells are virtually negative.

CD23

iCAPCs:

Activated B-cells in mantle z.

IHC – Biomarker controls



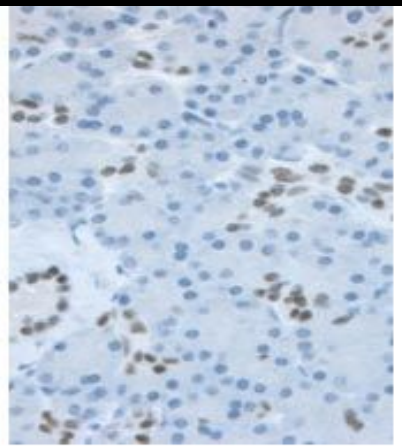


Fig. 1a. Optimal staining for CDX2 using the mAb clone CDX2-88.
Left, colon: A strong nuclear staining is seen in all the enterocytes with a minimal cytoplasmic reaction.
Right, pancreas: A weak to moderate staining is seen in the majority of the ductal epithelial cells.

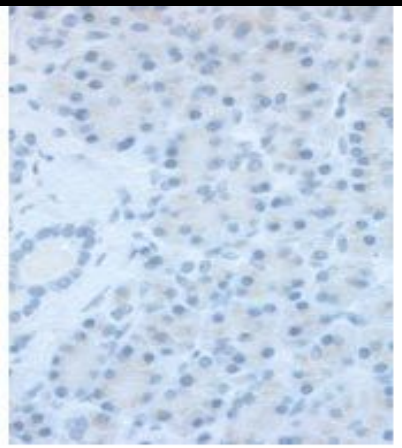
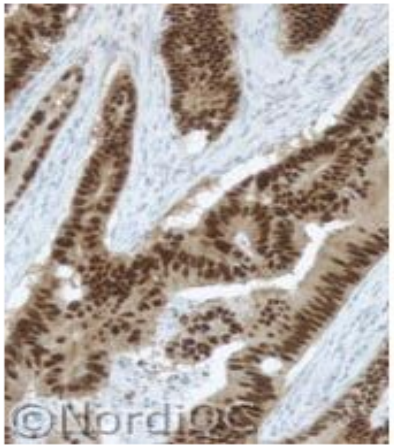


Fig. 1b. Staining for CDX2 using the mAb clone CDX2-88 with an insufficient protocol.
Left, colon: A moderate to strong nuclear staining is seen in all the enterocytes.
Right, pancreas: No nuclear staining is seen in the ductal epithelial cells. Also compare with Fig 2b – same protocol.



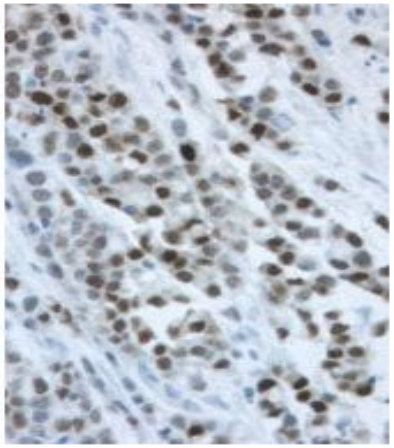
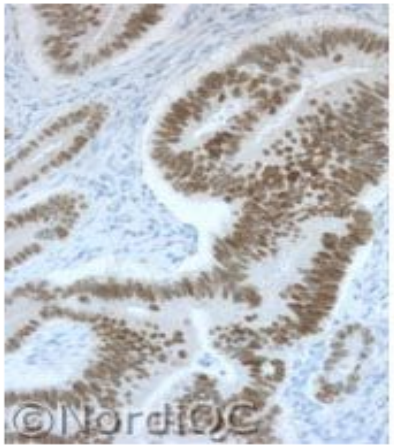


Fig. 2a. Optimal staining for CDX2 using same protocol as in Fig. 1a.
Left: Colon adenocarcinoma with high expression of CDX2: The nuclei of the neoplastic cells show an intense staining while the cytoplasmic compartment is weakly stained.
Right: Colon adenocarcinoma with low expression of CDX2: The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



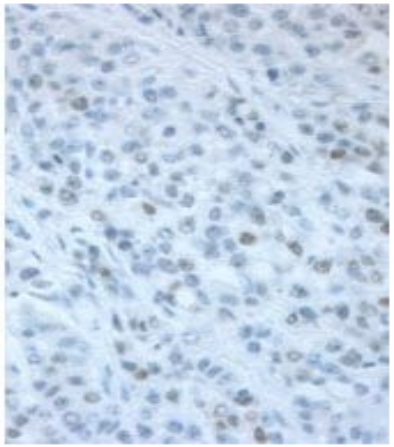


Fig. 2b. Insufficient staining for CDX-2 using same protocol as in Fig. 1b.
Left: Colon adenocarcinoma with high expression of CDX2: The nuclei of the neoplastic cells show a moderate staining, while the cytoplasmic compartment is almost negative.
Right: Colon adenocarcinoma with low expression of CDX2: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

CDX2

iCAPCs:

Pancreatic duct ep. cells

IHC – Biomarker controls

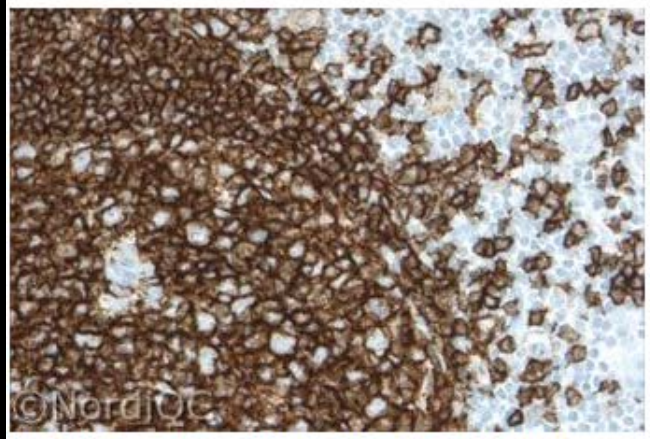


Fig. 1a. Lymphatic tissue in the appendix showing an optimal staining reaction for CD20 using the mAb clone L26 in a RTU format on the BenchMark platform. HIER was performed using Cell Conditioning 1. A very strong membranous staining reaction is seen in virtually all the B-cells.

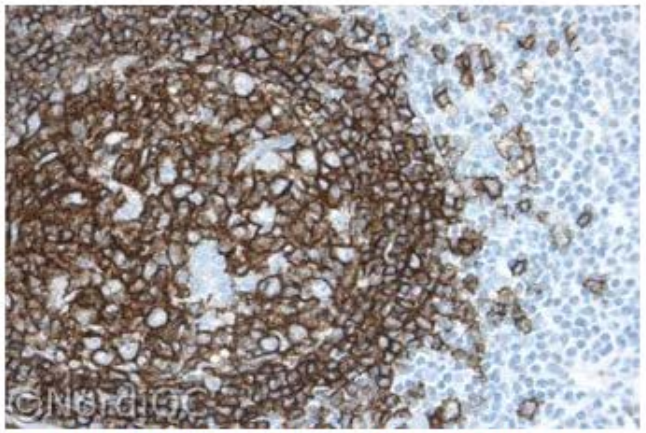


Fig. 1b. Lymphatic tissue in the appendix. Same field as in Fig. 1a. Insufficient staining for CD20 using the mAb clone L26 in a RTU format at the BenchMark platform. No HIER was performed. A moderate to strong staining reaction is seen in virtually all the B-cells. The normal B-cells are high expressors 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).

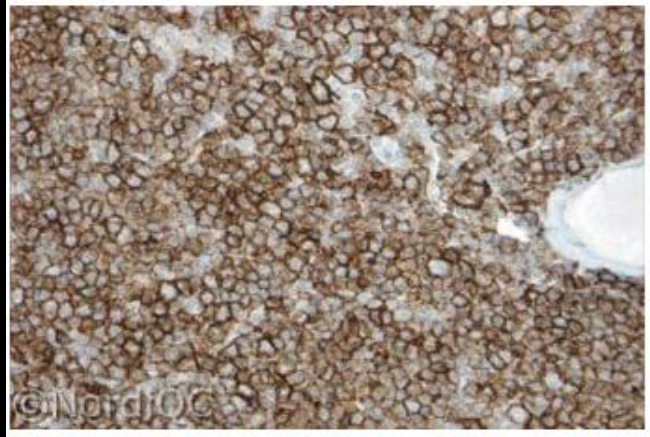


Fig. 2a. B-CLL. Optimal staining reaction for CD20. Same protocol as in Fig. 1a. A moderate to strong membranous staining is seen in virtually all the neoplastic cells.

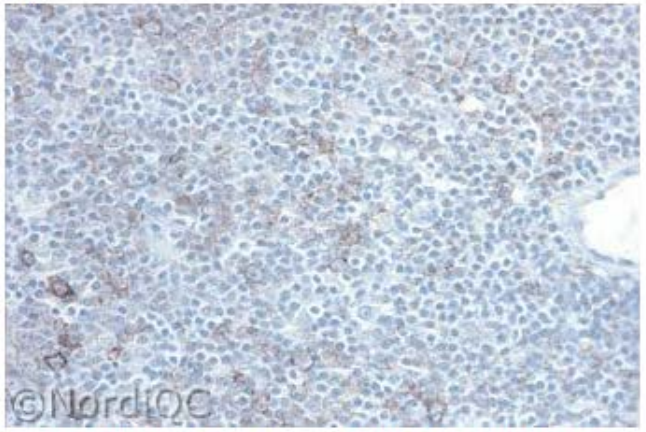


Fig. 2b. B-CLL. Insufficient staining for CD20 using the same protocol as in Fig. 1b. Omitting HIER, only scattered cells are positive. The majority of the neoplastic cells are negative. Compare with the optimal result in Fig. 2a, same field.

CD20:

iCAPCs:

????

ASAP….

As strong as possible…

IHC – Biomarker controls

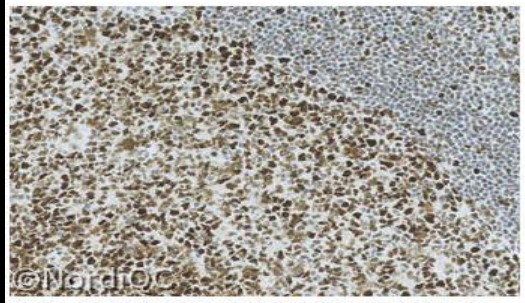


Fig. 1a. Optimal staining for MSH6 of the tonsil using the rmAb clone EP49 optimally calibrated, HIER in an alkaline buffer and a 3-step polymer based detection system.
Virtually all the mantle zone B-cells show a distinct, moderate to strong nuclear staining, while the germinal centre B-cells show a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

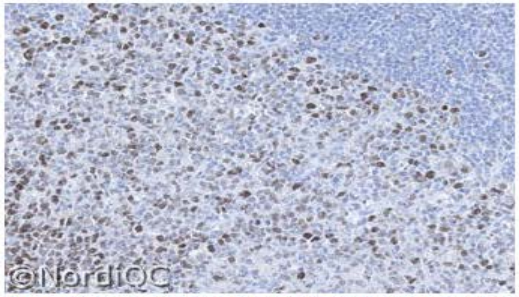


Fig. 1b. Insufficient staining for MSH6 of the tonsil using the mAb clone 44. by a protocol with a too low sensitivity (2-step polymer and too low. conc. of the primary Ab), same field as in Fig. 1a.
Only the germinal centre B-cells are demonstrated, while the mantle zone B-cells expressing limited MSH6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

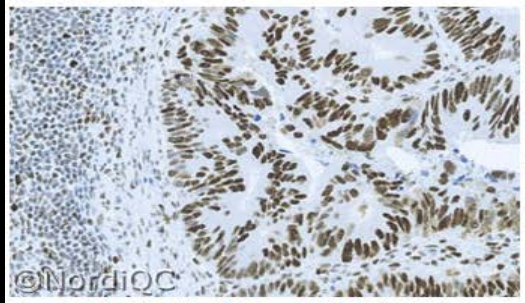


Fig. 2a. Optimal staining for MSH6 of the colon adenocarcinoma no. 3 with intact MSH6 protein using same protocol as in Fig. 1a.
The majority of the epithelial and the stromal cells show a moderate to strong nuclear staining. No background staining is seen.

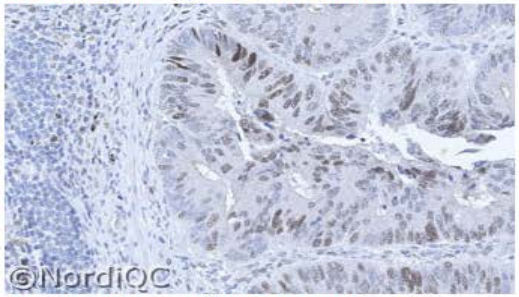


Fig. 2b. Insufficient staining for MSH6 of the colon adenocarcinoma no. 3 with intact MSH6 protein using same protocol as in Fig. 1b., same field as in Fig. 2a.
The proportion of positive cells and the intensity of the staining reaction is significantly reduced compared to the result in Fig. 2a.
Also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

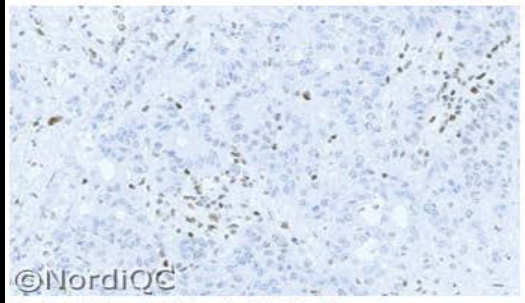


Fig. 3a. Optimal staining for MSH6 of the colon adenocarcinoma no. 5 with loss of MSH6 protein using same protocol as in Figs. 1a. & 2a.
The neoplastic cells are negative, while the remnants of entrapped lymphocytes and stromal cells show a distinct nuclear staining, serving as internal positive control.

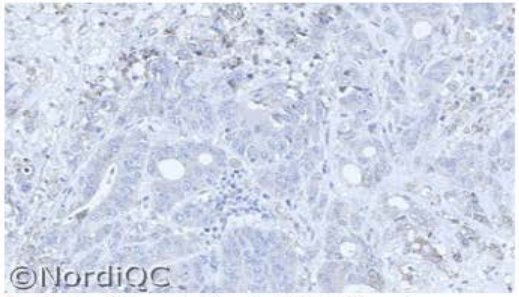


Fig. 3b. Insufficient staining for MSH6 of the colon adenocarcinoma no. 5 with loss of MSH6 protein using same protocol as in Figs. 1b. & 2b., same field as in Fig. 3a.
No nuclear staining reaction is seen in the neoplastic cells, but as virtually no nuclear staining reaction is seen in the normal cells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

MMR:

iCAPCs:

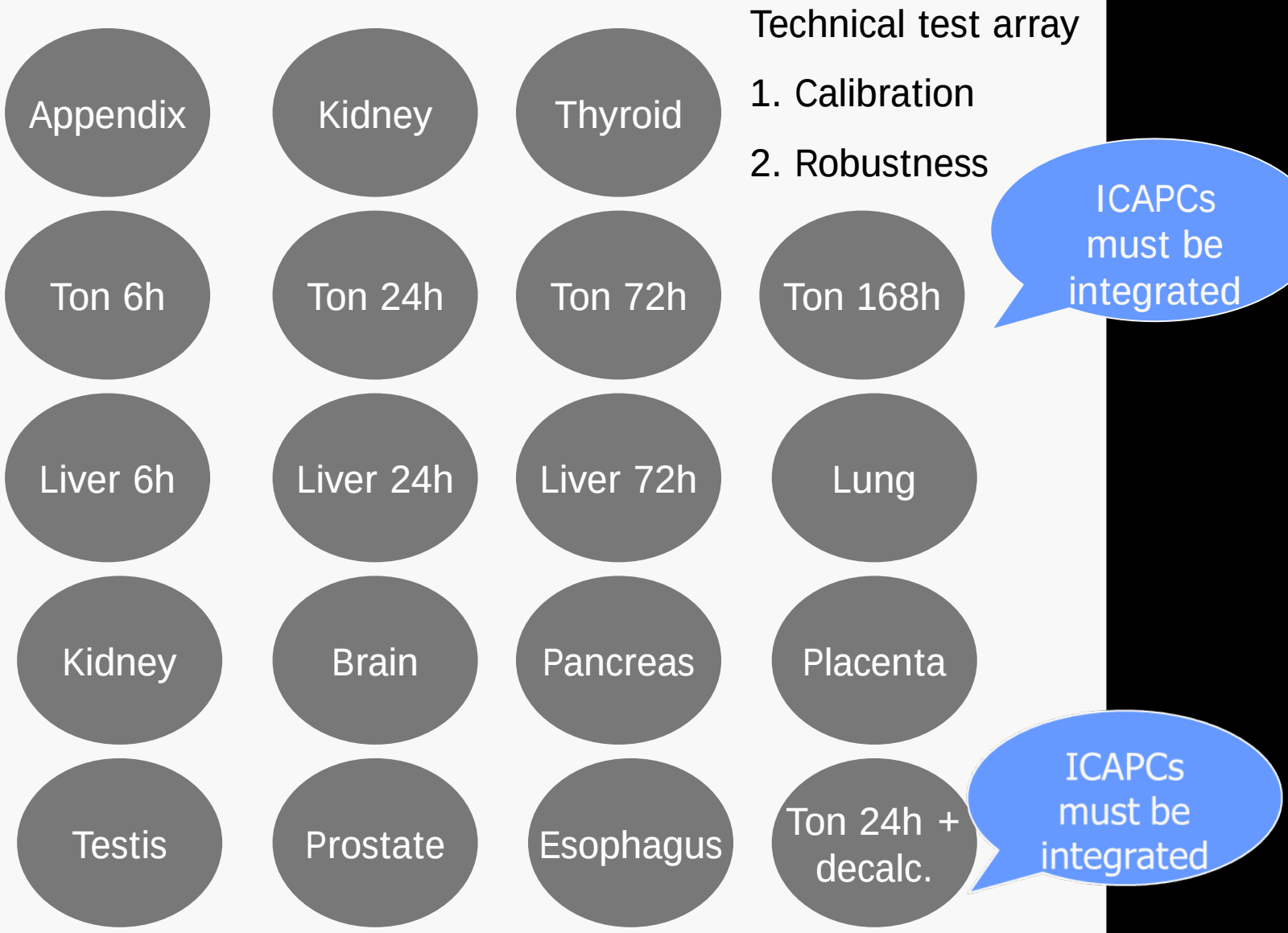
Mantle zone B-cells in tonsil

++++++++

(internal control)

Stromal cells!!

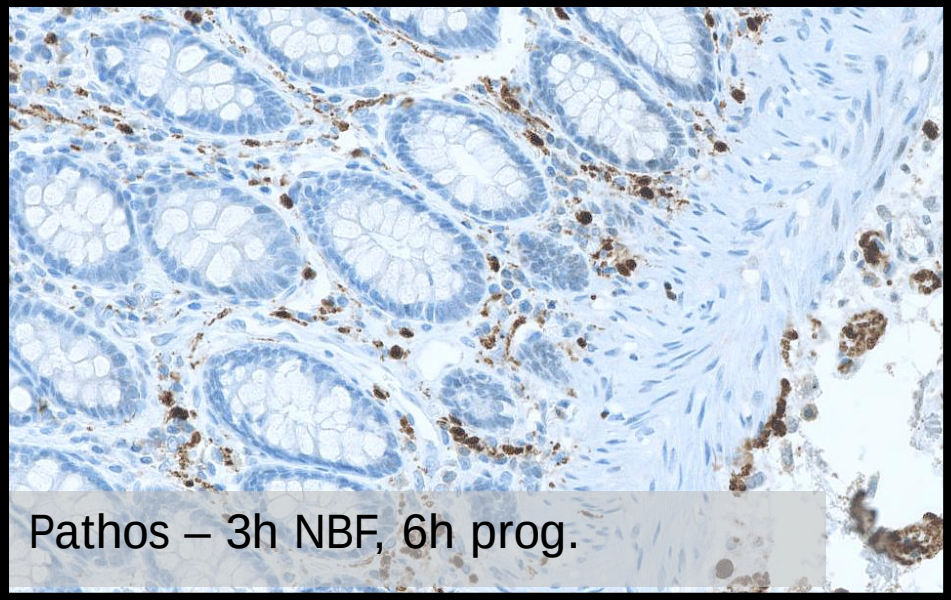
IHC – Biomarker controls



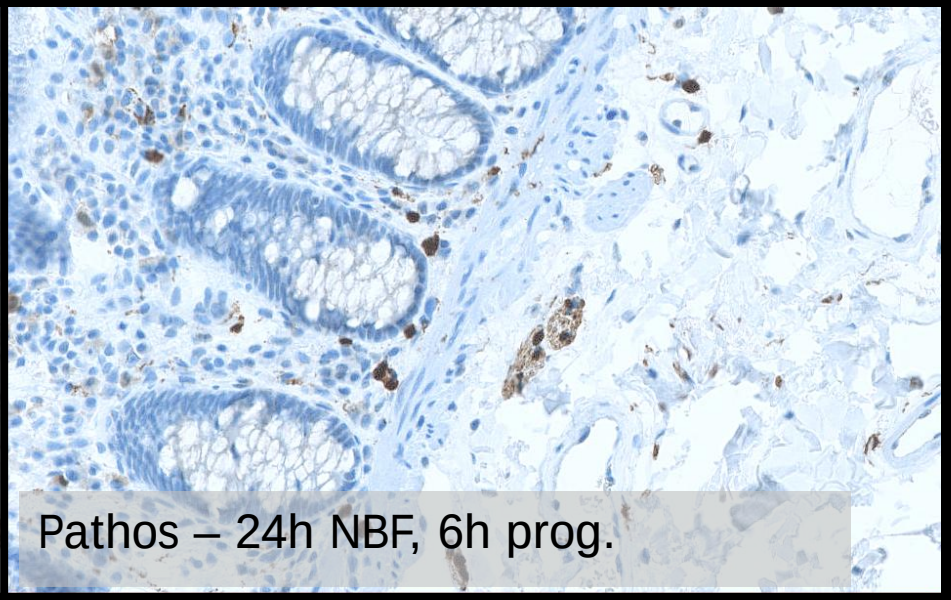
Protocol set-up: used as primary material for the calibration of 130 of 195 routine diagnostic markers, Aalborg University Hospital

IHC – Biomarker controls

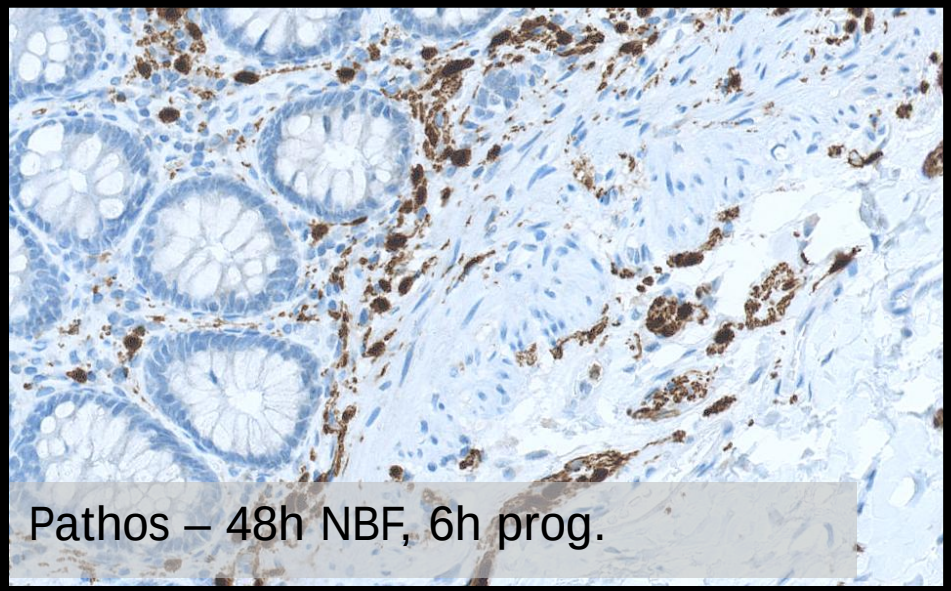
Colon: S100, polyclonal



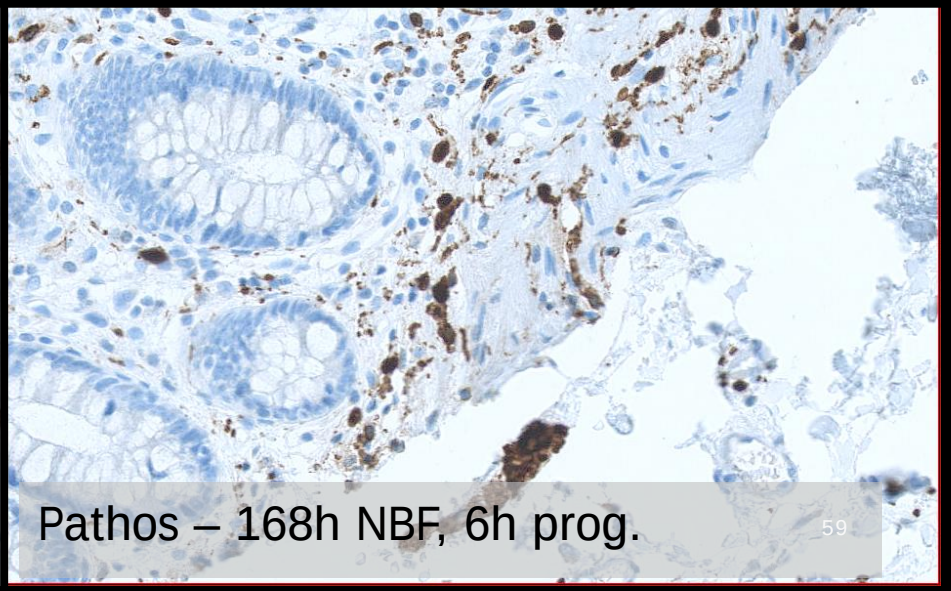
Pathos – 3h NBF, 6h prog.



Pathos – 24h NBF, 6h prog.



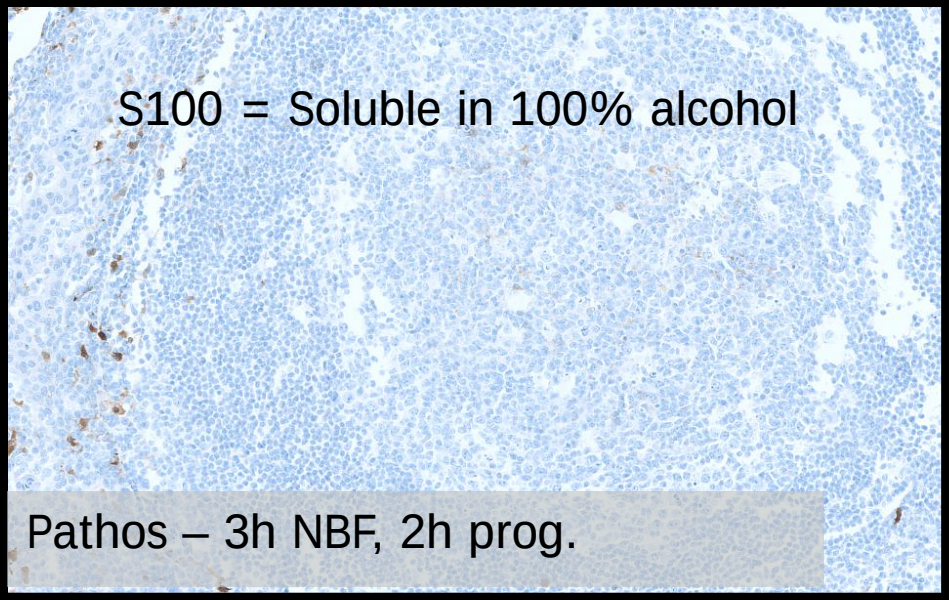
Pathos – 48h NBF, 6h prog.



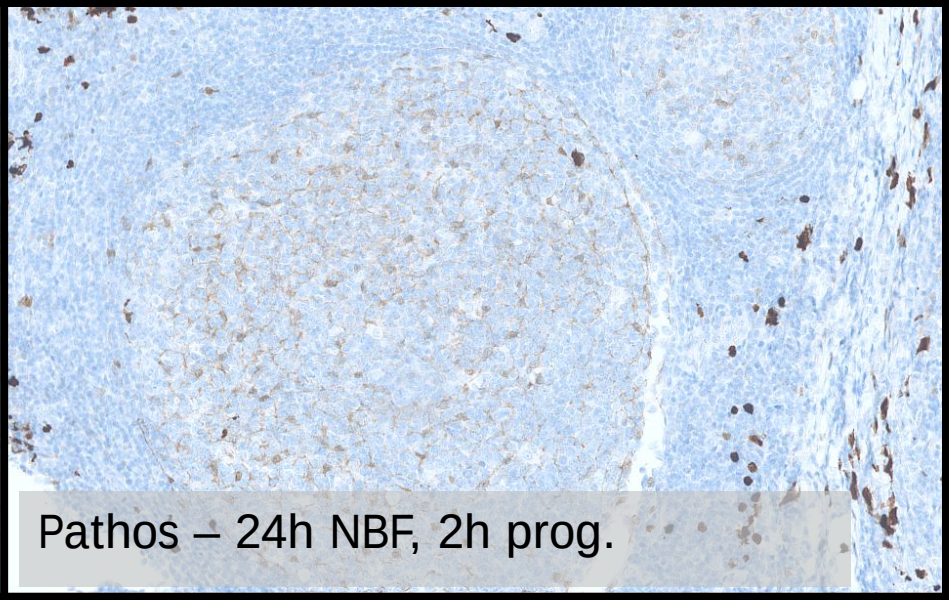
Pathos – 168h NBF, 6h prog.

IHC – Biomarker controls

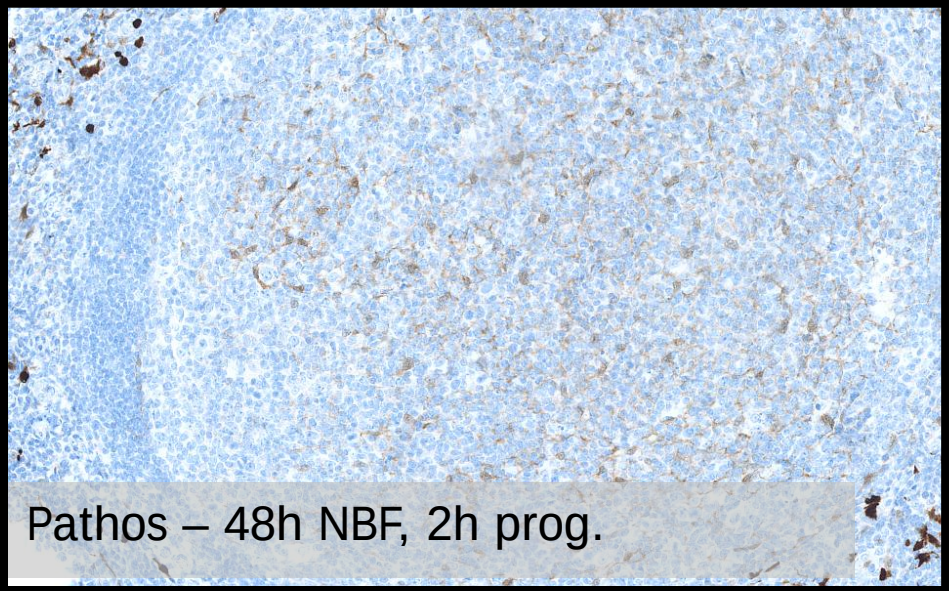
Tonsil: S100, polyclonal



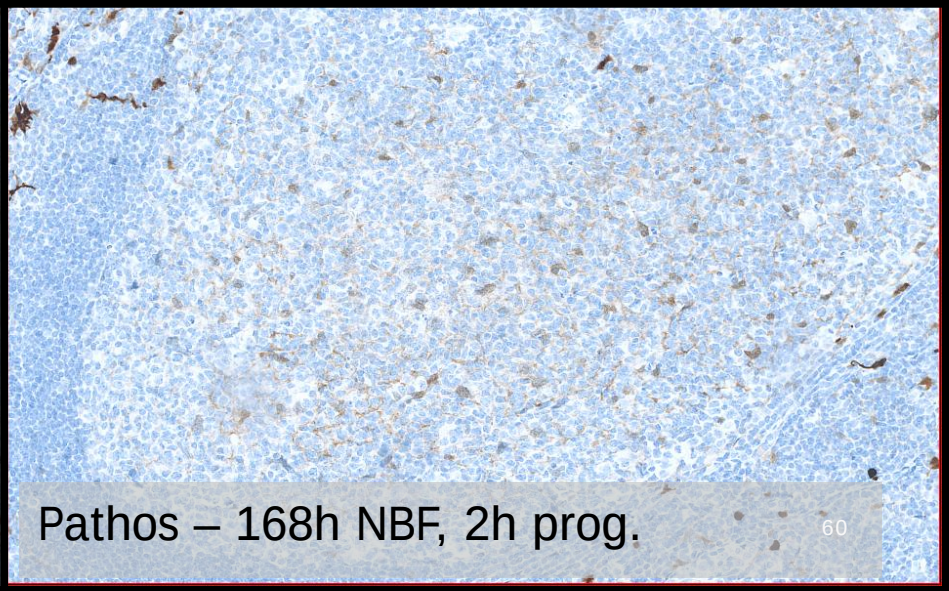
Pathos – 3h NBF, 2h prog.



Pathos – 24h NBF, 2h prog.



Pathos – 48h NBF, 2h prog.



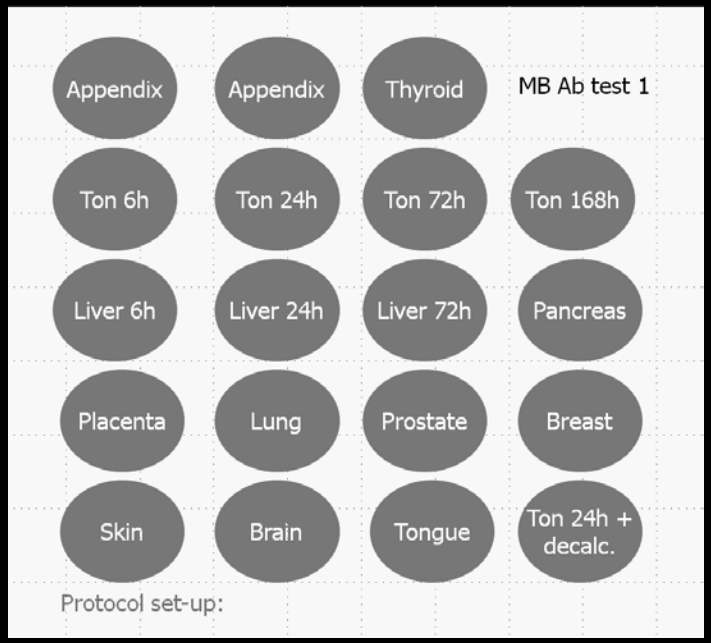
Pathos – 168h NBF, 2h prog.

IHC – Biomarker controls

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

External tissue control tool-box:

Calibration TMA's

iCAPC TMA	Specificity TMA	Pre-analyt. TMA
		
"Gold standard" tissue controls	"Normal" tissues	iCAPCs processed as lab procedures
IHC critical assay performance controls	Maps Ab reaction pattern	Fixation time Fixative(s) Decalcification
High expression Low expression No expression	With expression No expression	

Analytical "Validation" TMA's

Accuracy TMA	Index TMA
	
"Lesional" tissues	"Lesional" tissues
Range of relevant expression levels	Range of relevant expression levels
With expression No expression	High expression Low expression No expression
20/40 of each Type I/II IHC	+ relevant cut-off

Lab QC TMA

"Daily QC" TMA

iCAPCs + selected tissues
Reproducibility
Method of transfer proof

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.

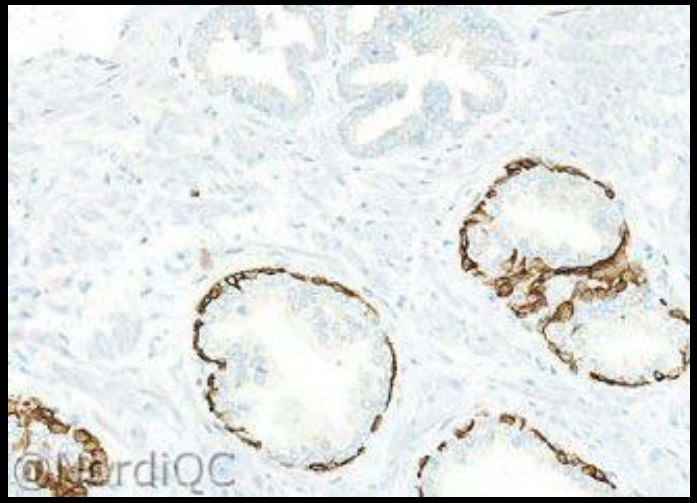
Number less important compared to use of tissue with full range of expression patterns reflecting the diagnostic use

IHC – Biomarker controls

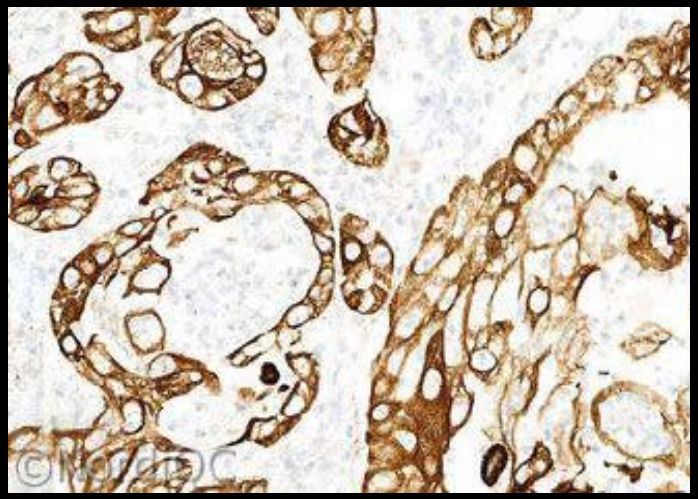
An IHC assay can have one or more purposes and it is crucial to secure the need is fulfilled

IHC for CK5

1. To differentiate prostate gland hyperplasia/PIN from prostate ad.carcinoma
2. Identify squamous cell differentiation in lung carcinomas
3. .....



Prostate sample



Lung sample

Same protocol applied for different purposes and meeting the requirements

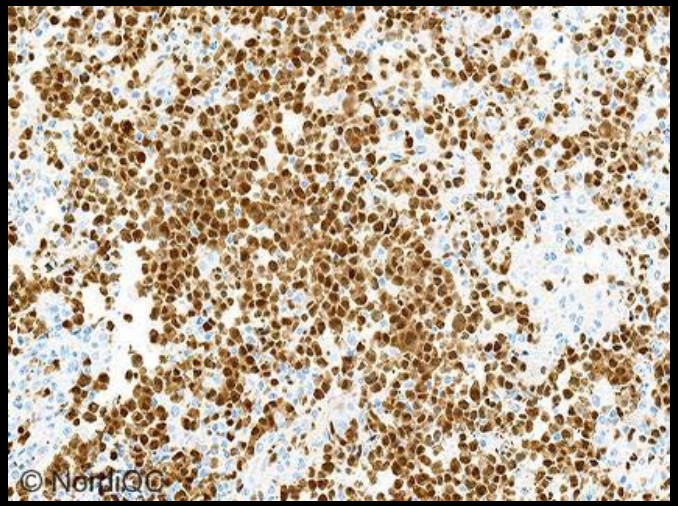
(www.nordiqc.org)

IHC – Biomarker controls

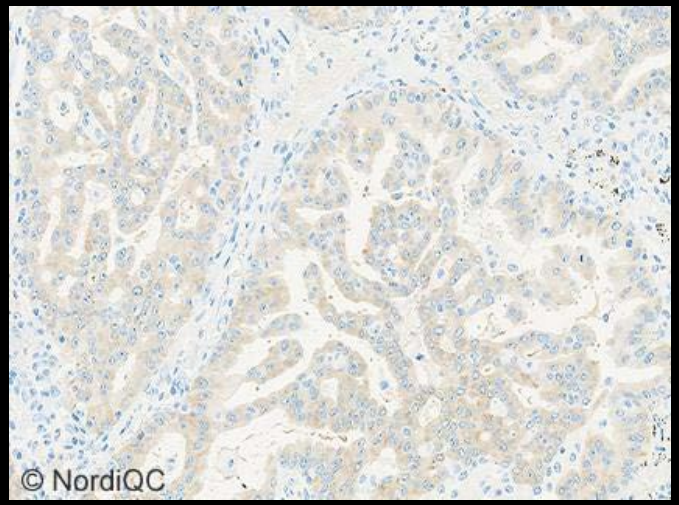
An IHC assay can have one or more purposes and it is crucial to secure the need is fulfilled

IHC for ALK

1. To identify anaplastic large cell cell lymphoma
2. To identify lung adenocarcinoma with ALK mutation
3. .....



ALCL



Lung ad. Carc with EML-ALK mutation

Same protocol applied for different purposes **NOT** meeting the requirements

(www.nordiqc.org)

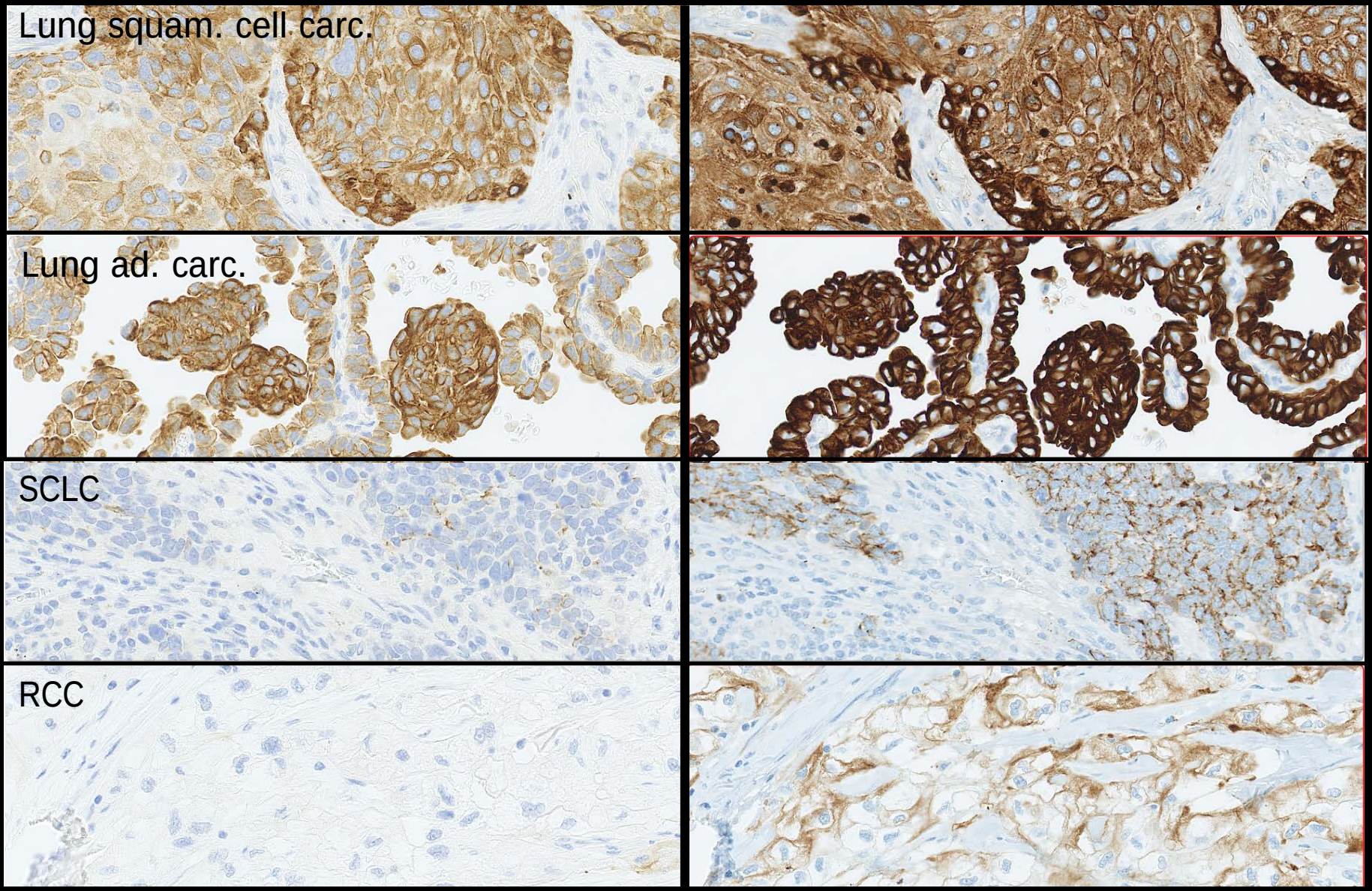
IHC – Biomarker controls

An IHC assay can have one or more purposes and it is crucial to secure the need is fulfilled

	Purpose I (HE)	Purpose II (LE)	Comments
CD34	Dermatofibrosarcoma protuberans	Stem cells / leukemia	Different pre-anal
CD56	Neuroendocrine differentiation	Lymphoma classification	
CD117	GIST	Stem cells / leukemia	Different pre-anal
IgK / IgL	Clonality myeloma (Cytopl)	Clonality lymphoma (Membrane)	
Melan A	Melanoma	Sex cord tumours	(mAb A103)
PAX5	B-cell lineage marker (Lymphoma)	Hodgkin	
.....			

In addition an extensive range within same purpose can be seen....
E.g. Pan-CK for carcinoma identification (primary panel)

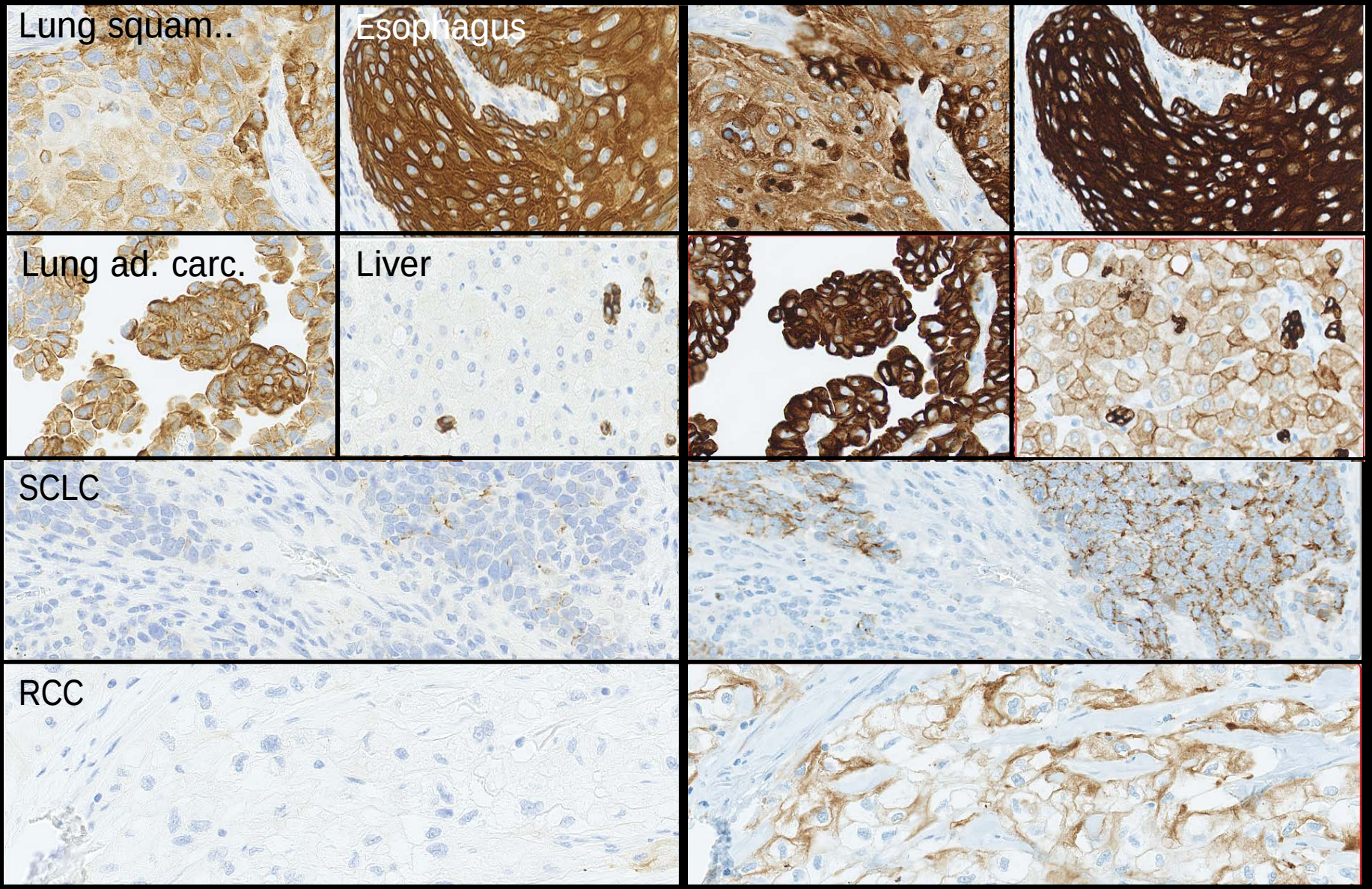
IHC – Biomarker controls



CK-PAN - mAb AE1/AE3 – Prot. 1

CK-PAN - mAb AE1/AE3 – Prot. 2

IHC – Biomarker controls



CK-PAN - mAb AE1/AE3 – Prot. 1

CK-PAN - mAb AE1/AE3 – Prot. 2

IHC – Biomarker controls

TMA Neoplasia

Analytical Index / sensitivity TMA

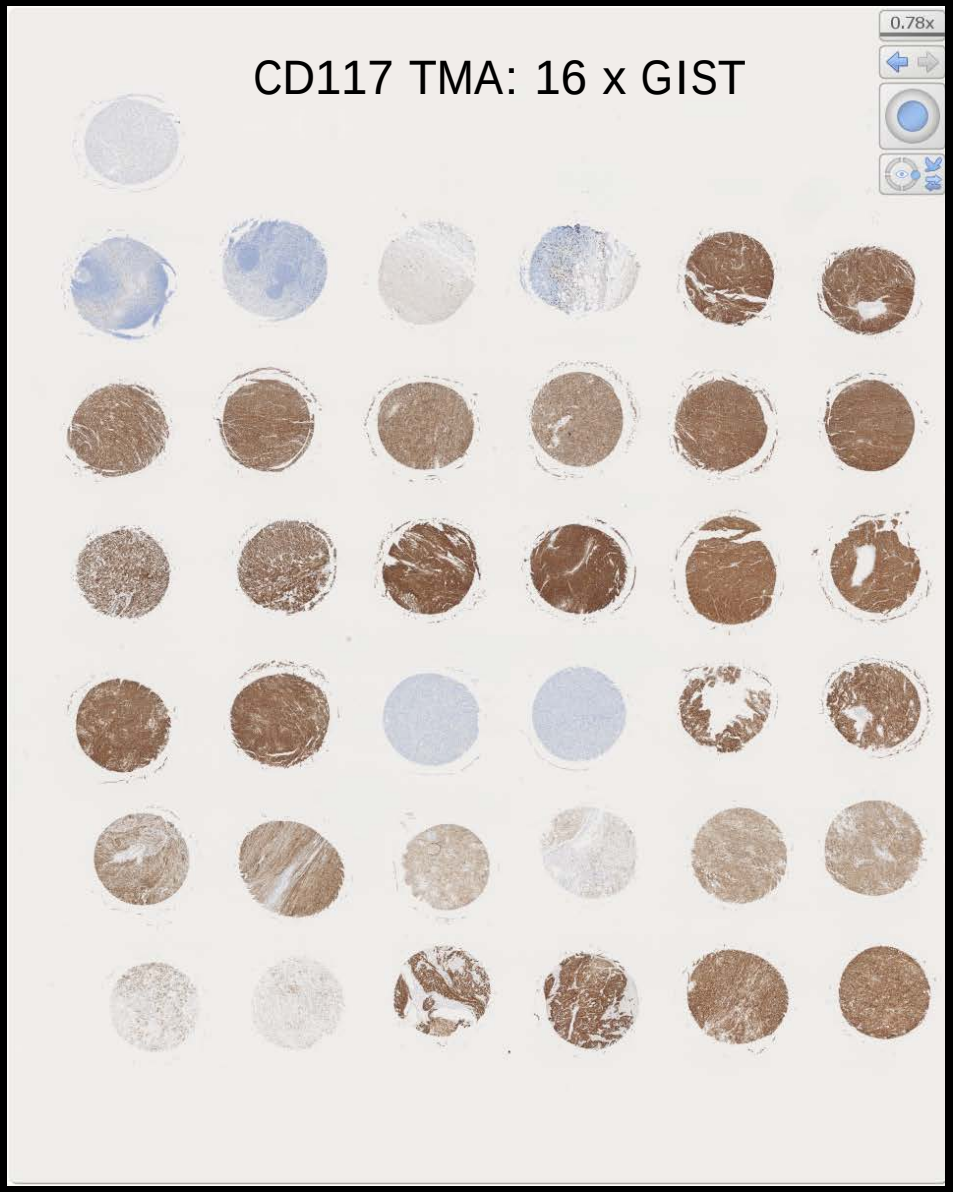
Diagnostic potential:

Index and accuracy TMA's

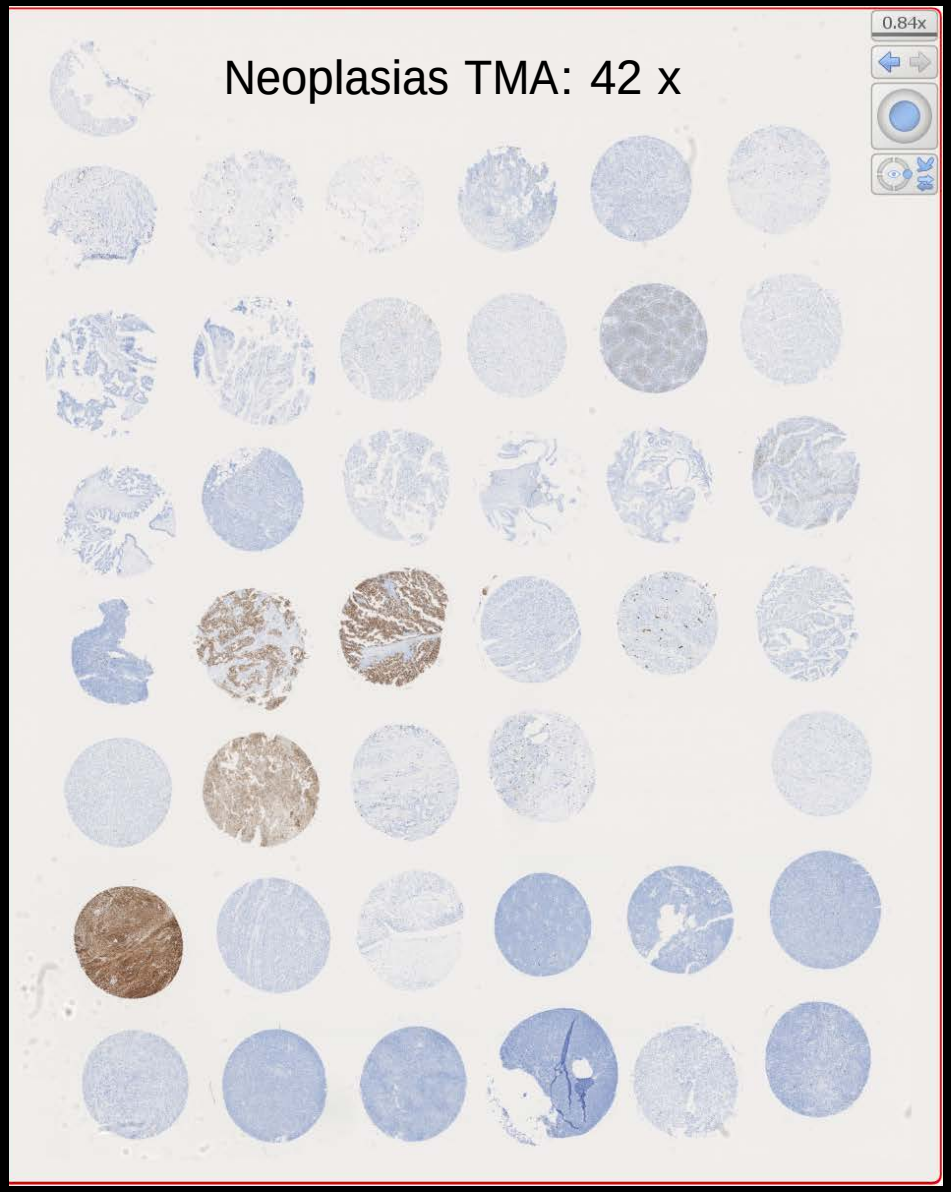
Analytical accuracy / specificity TMA

IHC – Biomarker controls

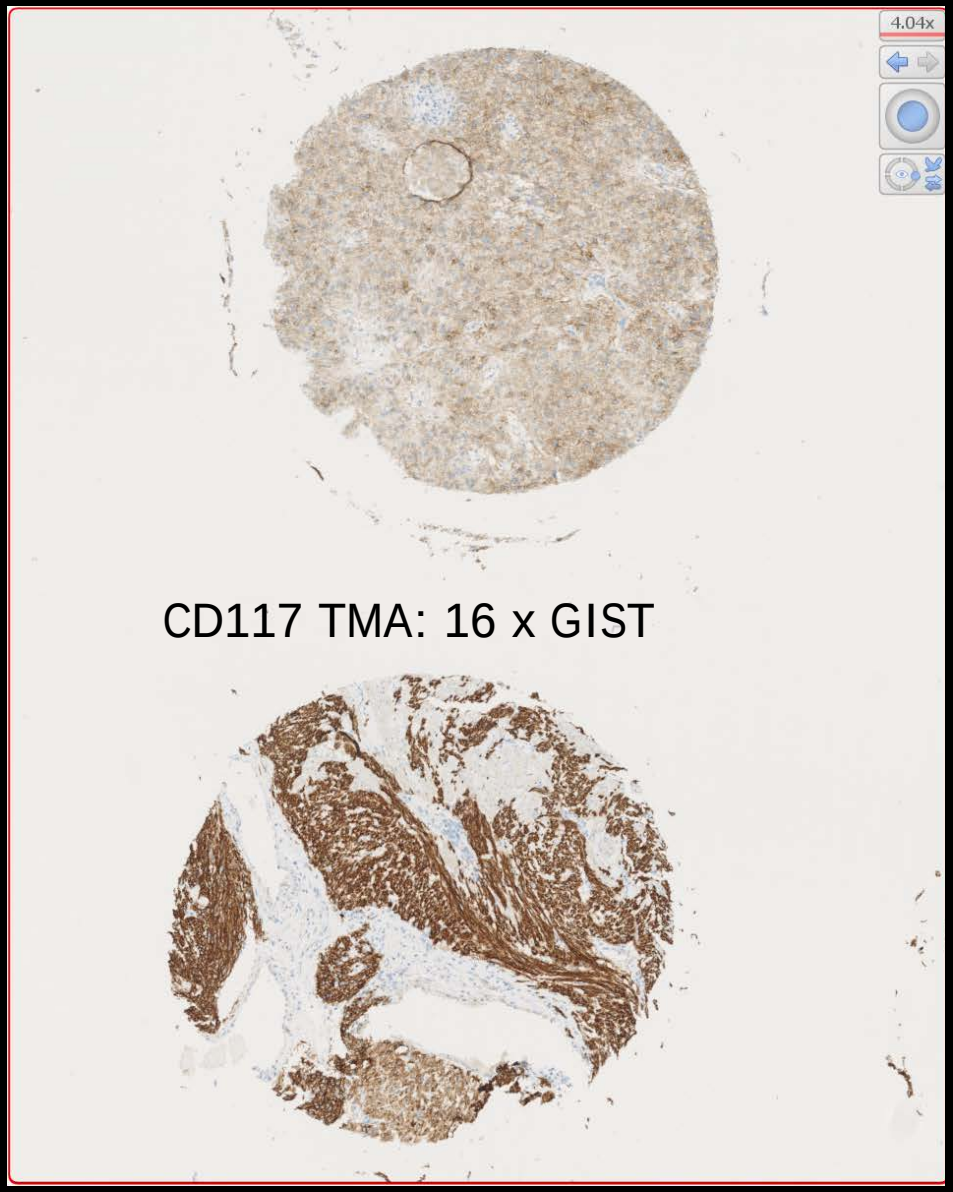
CD117 TMA: 16 x GIST



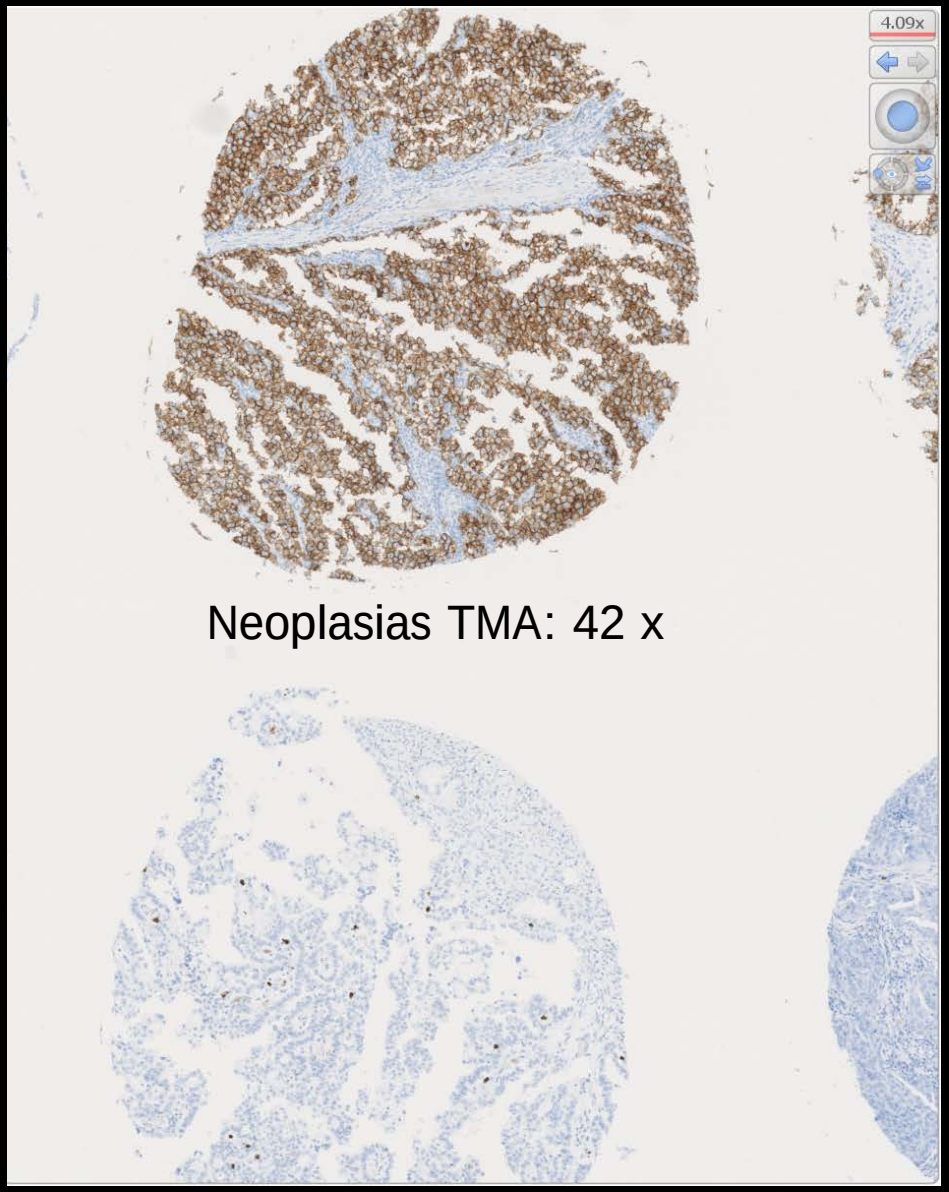
Neoplasias TMA: 42 x



IHC – Biomarker controls



CD117 TMA: 16 x GIST

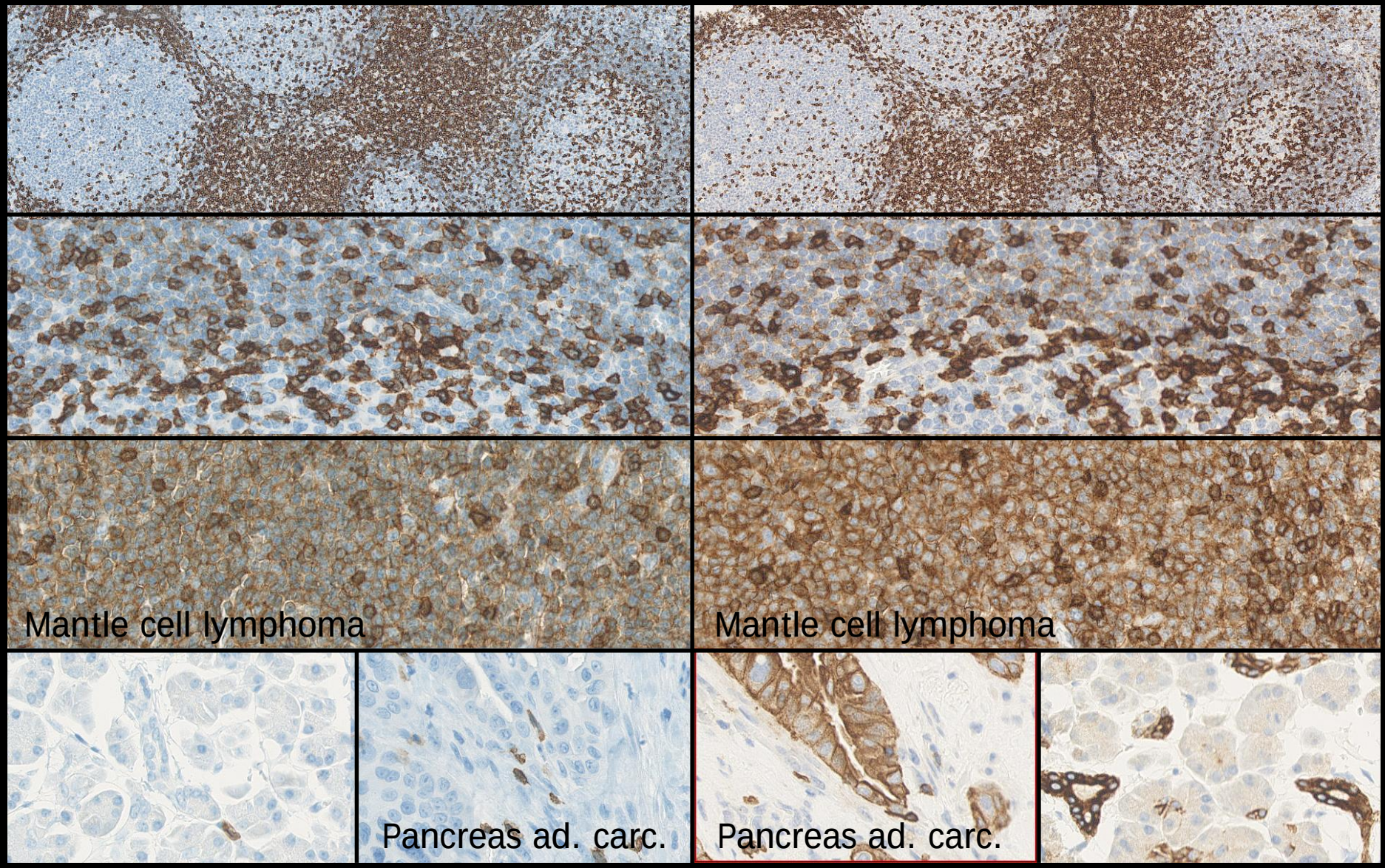


Neoplasias TMA: 42 x

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



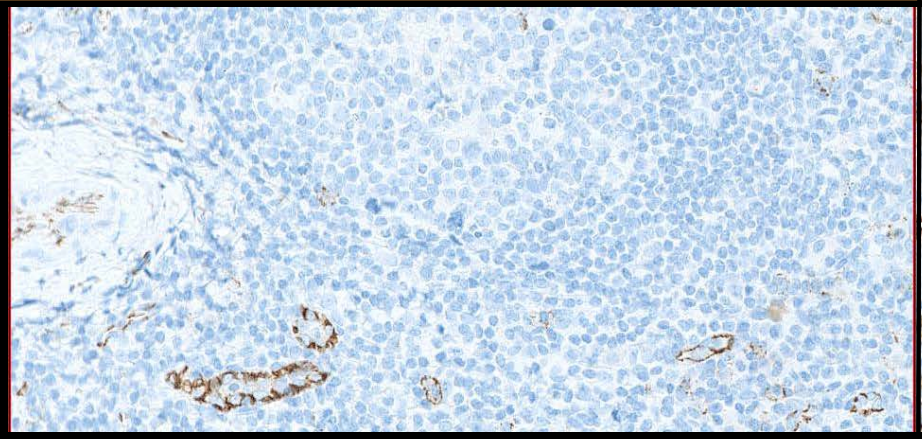
CD5 - rmAb SP19

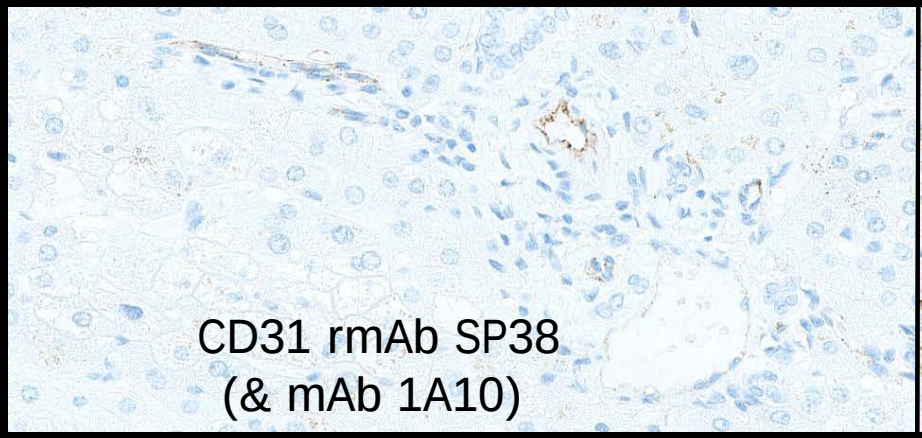
CD5 - mAb 4C7

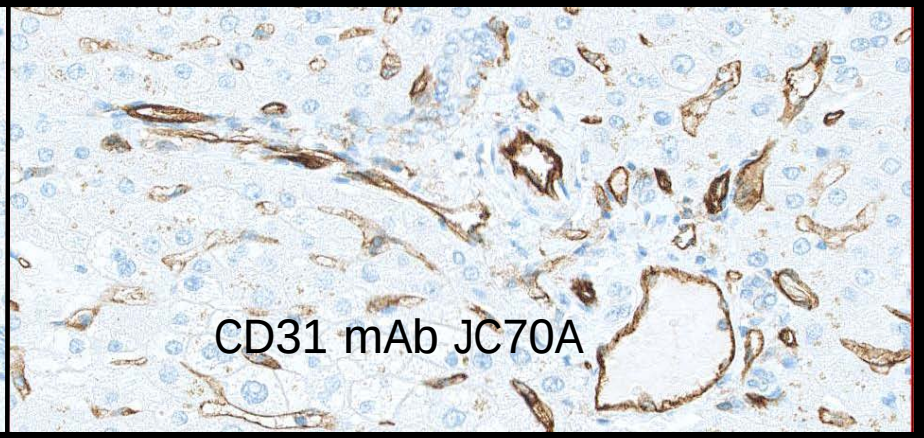
NordiQC – Less successful antibodies

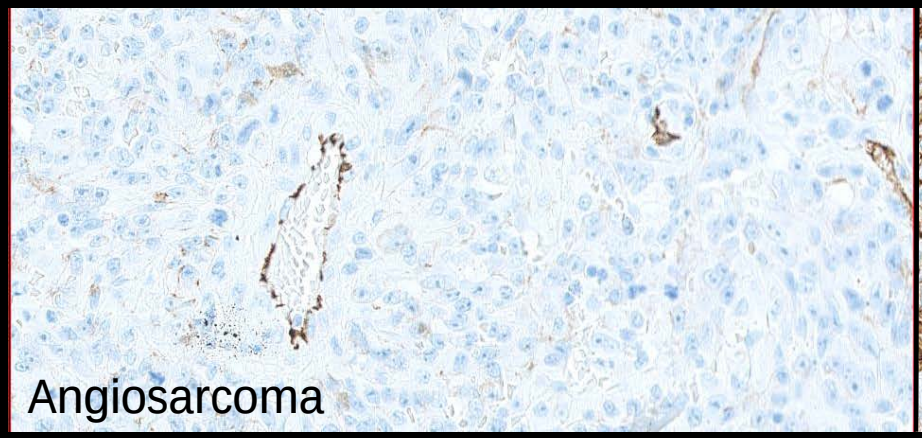
Antigen	Clone	High expressor	Low expressor	Non expressor
CD5	CD5/54/F6	√	FN	–
CD23	MHM6	√	FN	–
CD31	1A10	(√)	FN	–
CD31	SP38	(√)	FN	–
CD138	5F7	(√)	FN	–
CDX2	SP54	(√)	FN	FP
CEA	TF-3H8-1	√	√	FP
CGA	DAK. A3	√	FN	–
CK20	PW31	√	(√)	–
CK-LMW	35BH11	√	FN	–
MLH1	EPR3894	√	√	FP
MSH2	EPR3943	√	√	FP
MSH6	44	√	FN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

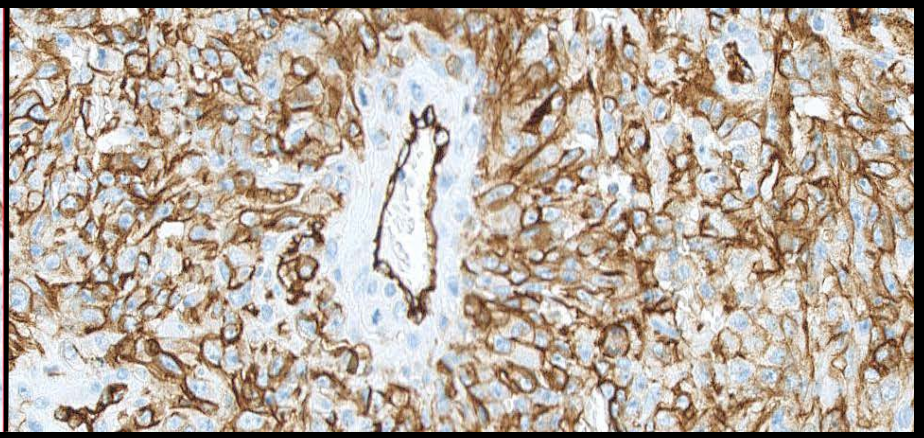
IHC – Biomarker controls



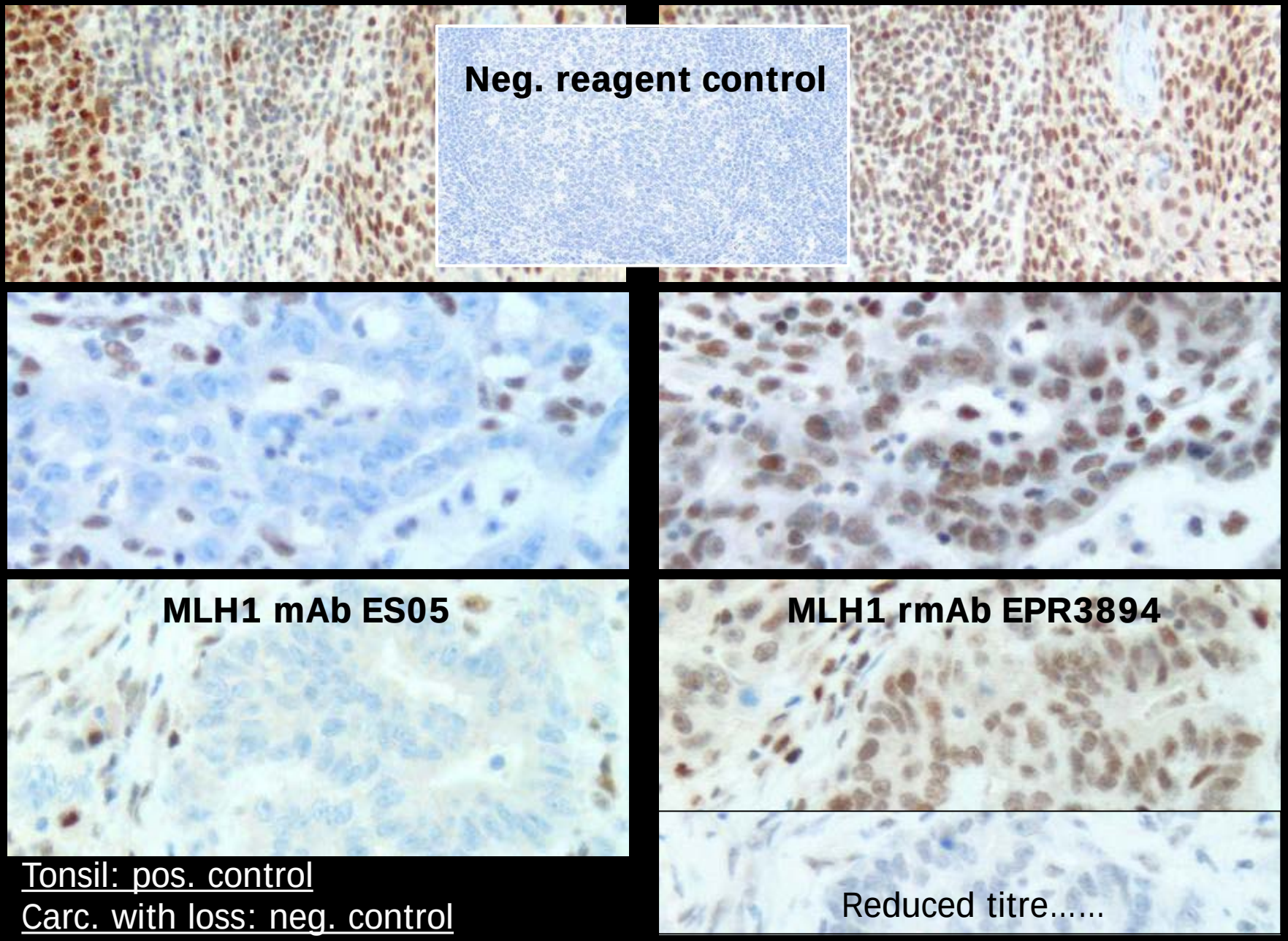








IHC – Biomarker controls



Tonsil: pos. control
Carc. with loss: neg. control

IHC – Biomarker controls

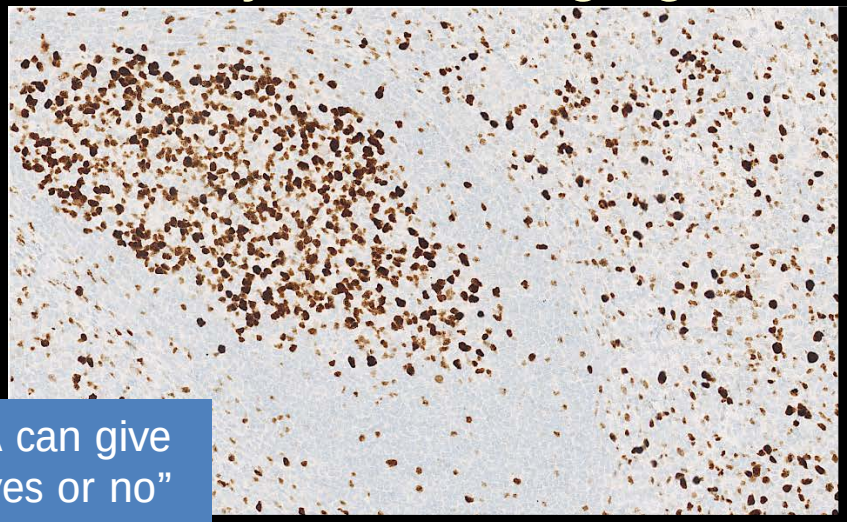
- Analytical validation – Challenges
  - Expected level of high, intermediate, low and absence can be difficult to comply with e.g.
    - New marker not tested previously
    - Binary expression – yes/no (CD20) – no dynamic range
    - New IHC system changing the range
      - Next Generation, Dako – TSA amplification, VMS
  - Number of samples
    - TMA or whole sections (homogenous / heterogenous)
    - Normal tissues or neoplasias
    - Rare positive cases (ALK lung carcinoma)

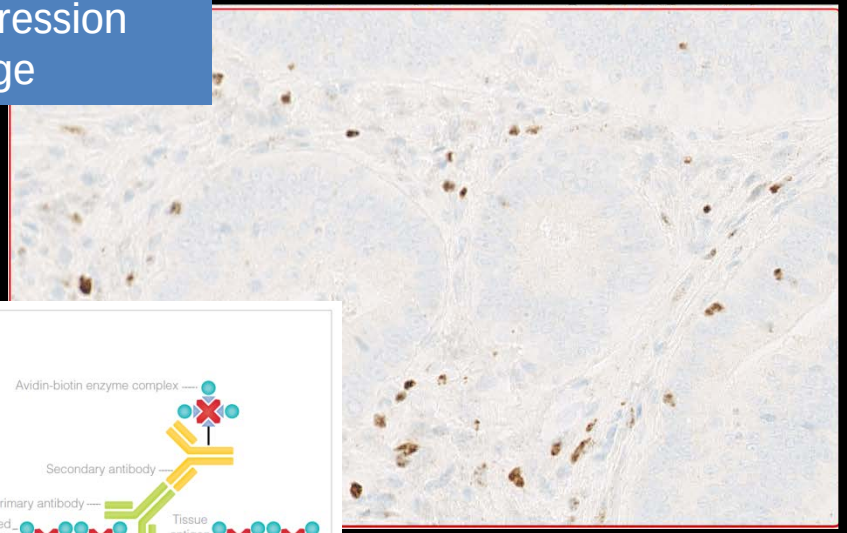
IHC – Biomarker controls

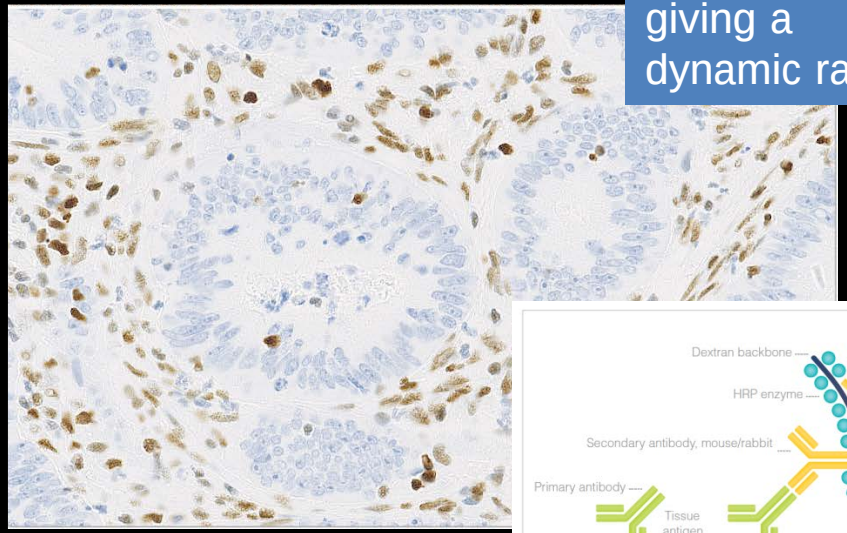
New IHC system changing the range – TSA based (OptView+A)

TSA can give a ”yes or no” expression range

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







MSH6 rmAb EP49

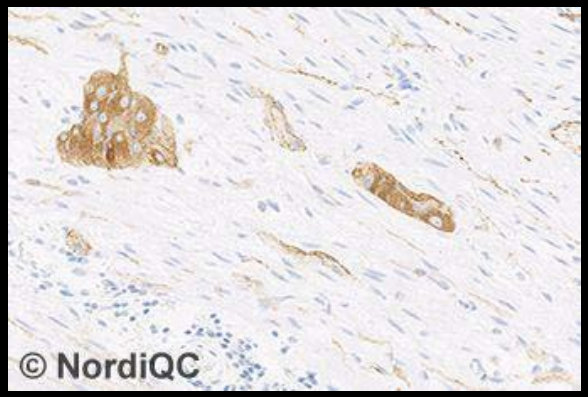
1:200 OptiView + Amp

1:50 OptiView

IHC – Biomarker controls

Challenge: Rare in cancers and/or in benign cells

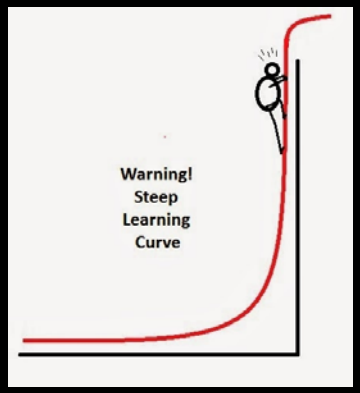
- ALK, ROS1, PD-L1 etc and many molecular derived targets
- Needed to verify IHC method is working
- *ALK lung; 30 cancers used to find 1 pos case…..*



ALK
Appendix / Colon:

Peripheral nerves – axons and ganglion cells

PD-L1
Tonsil:
Germinal centre macrophages



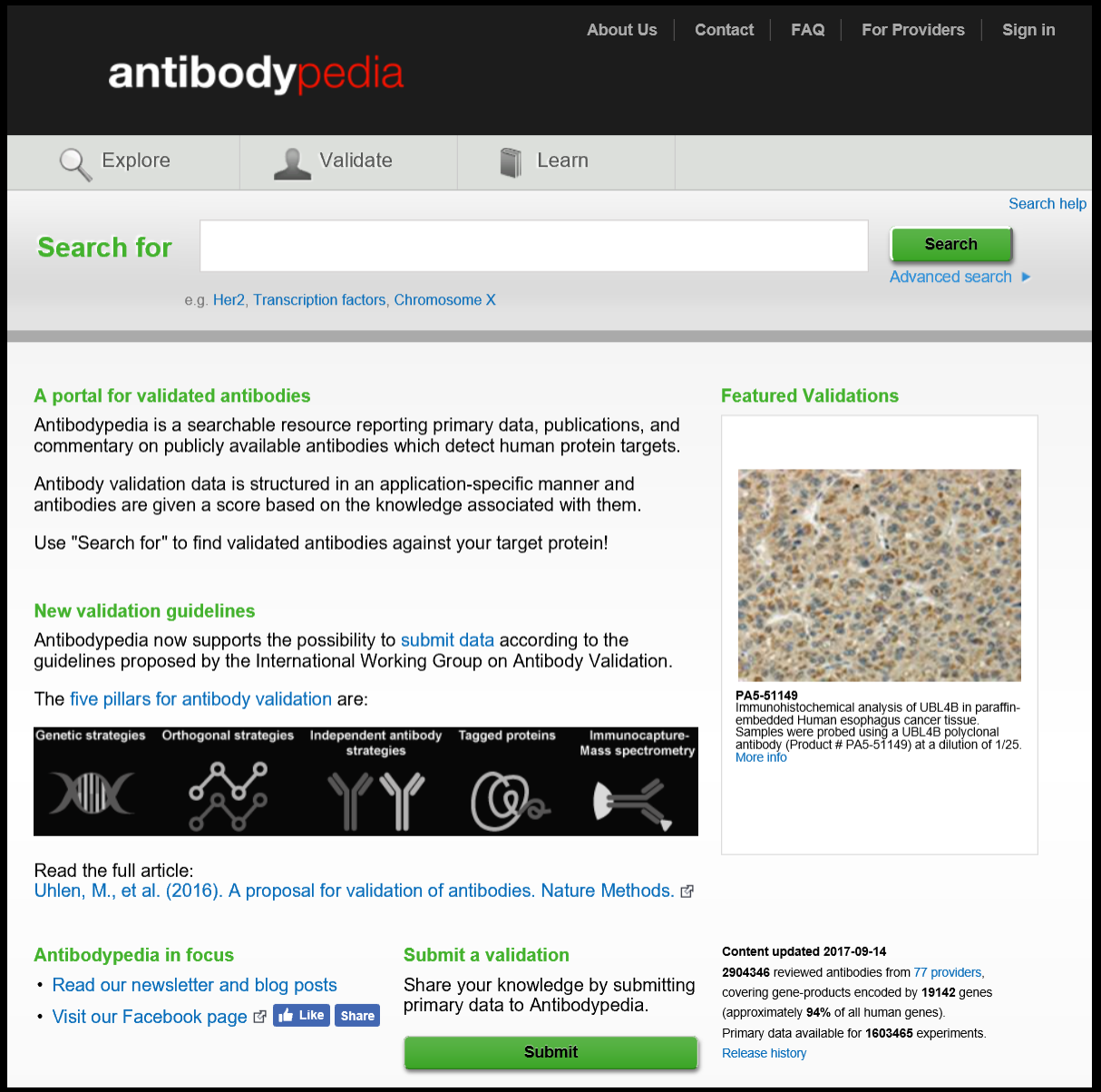
Precision and metrics of test to be confirmed

IHC – Biomarker controls

- Analytical validation – Challenges
  - New marker not tested previously
    - Search literature, pubmed etc
    - Identify tissues with and without expression
      - Normal or only neoplastic, Cell lines, etc
    - Localization – nuclear, membrane, etc
    - How to interpret – cut-off, qualitative, quantitative ?
    - Define potential clinical/diagnostic utility
    - Define other assays to be used for validation

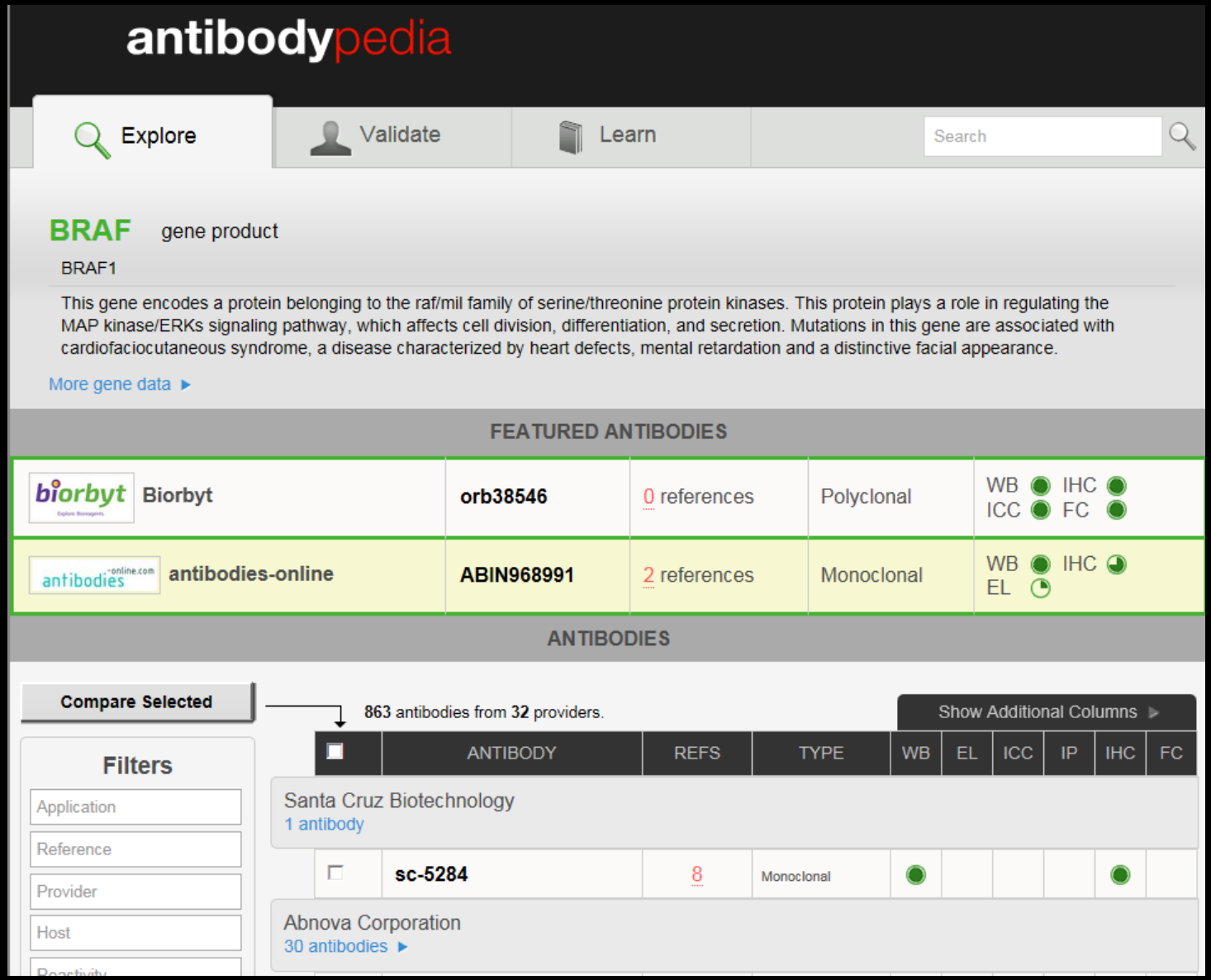
IHC – Biomarker controls

www.antibodypedia.com



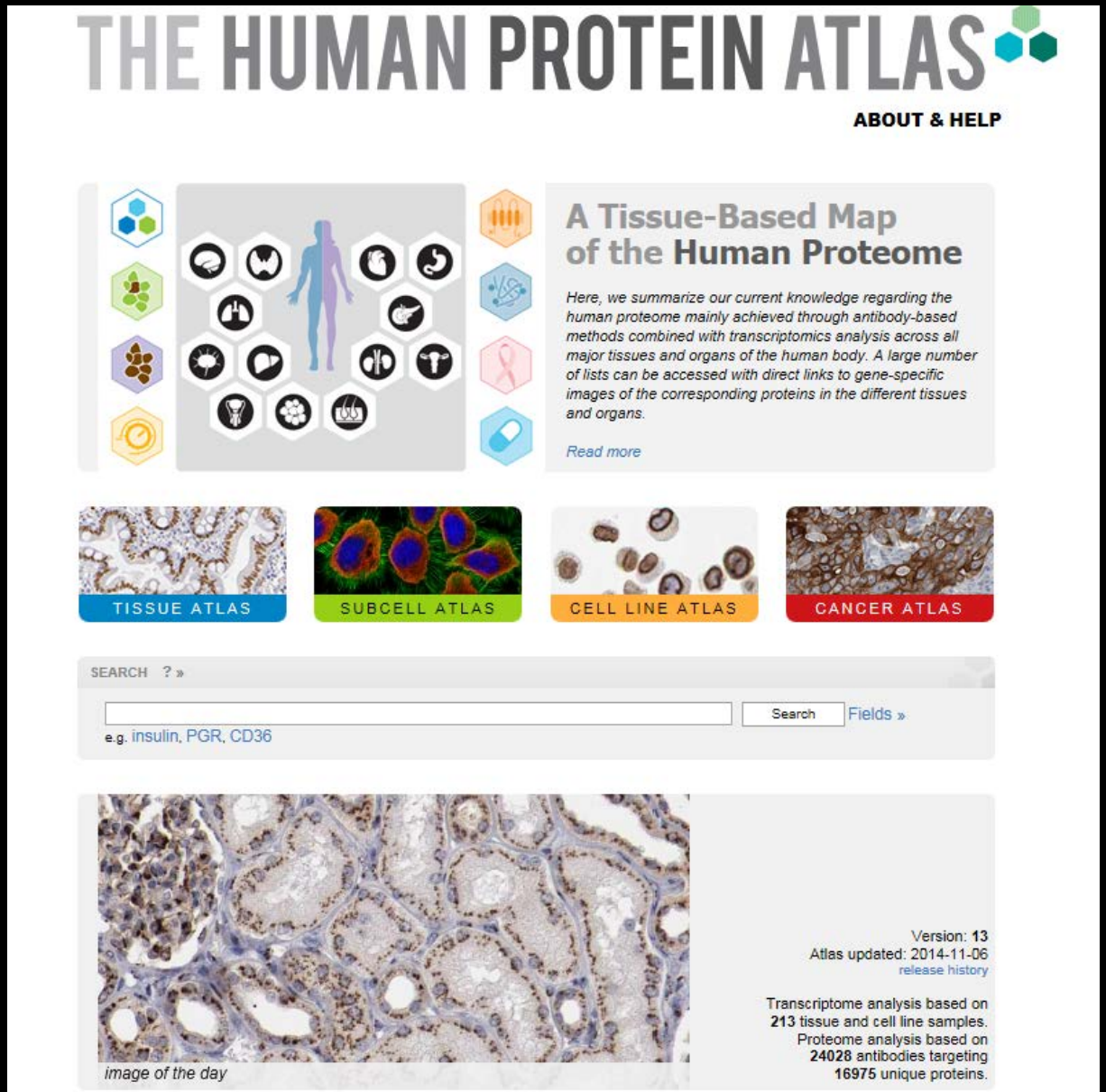
IHC – Biomarker controls

www.antibodypedia.com



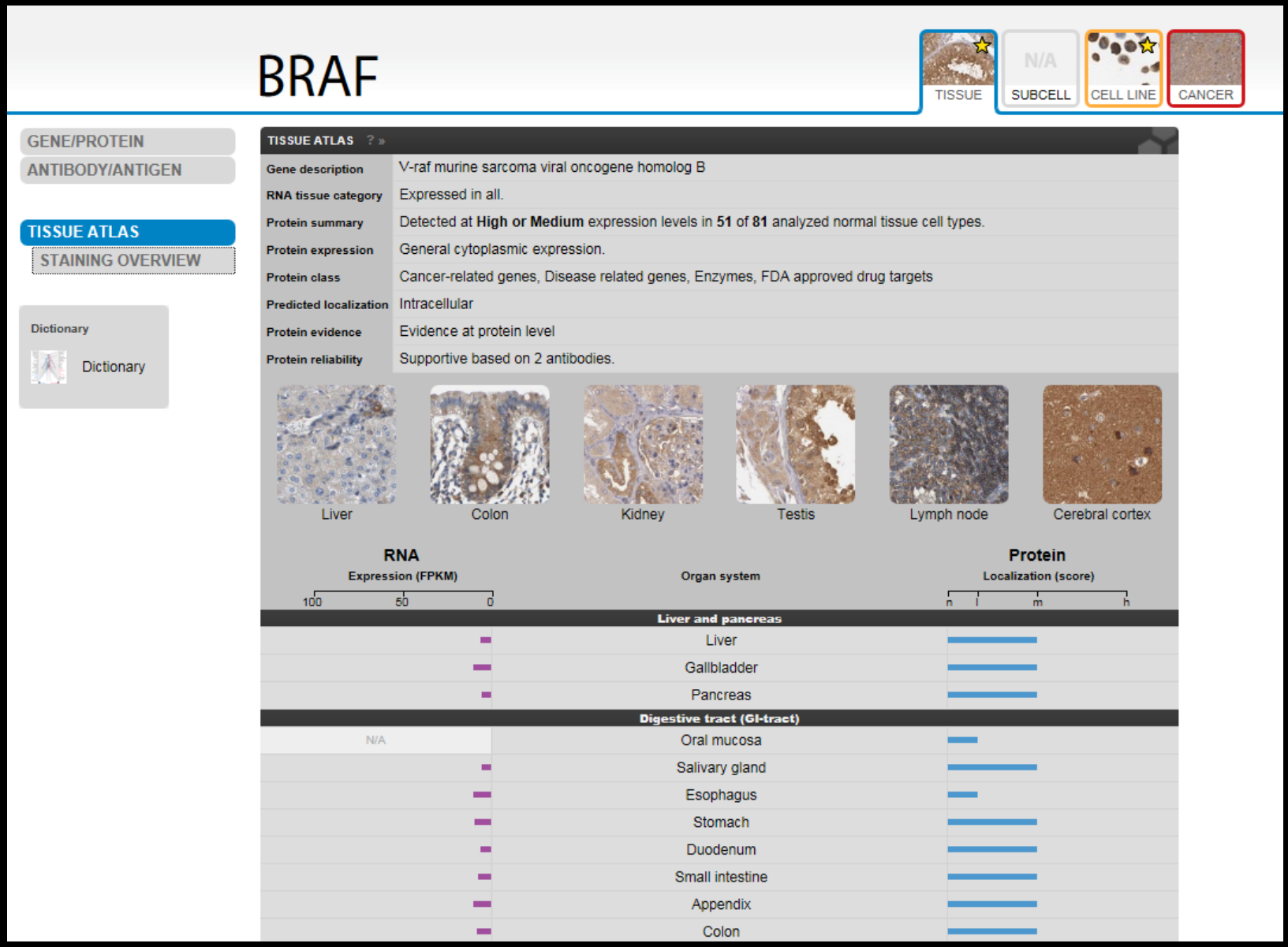
IHC – Biomarker controls

www.proteinatlas.org



IHC – Biomarker controls

www.proteinatlas.org



IHC – Biomarker controls

Cell lines/Histoids:

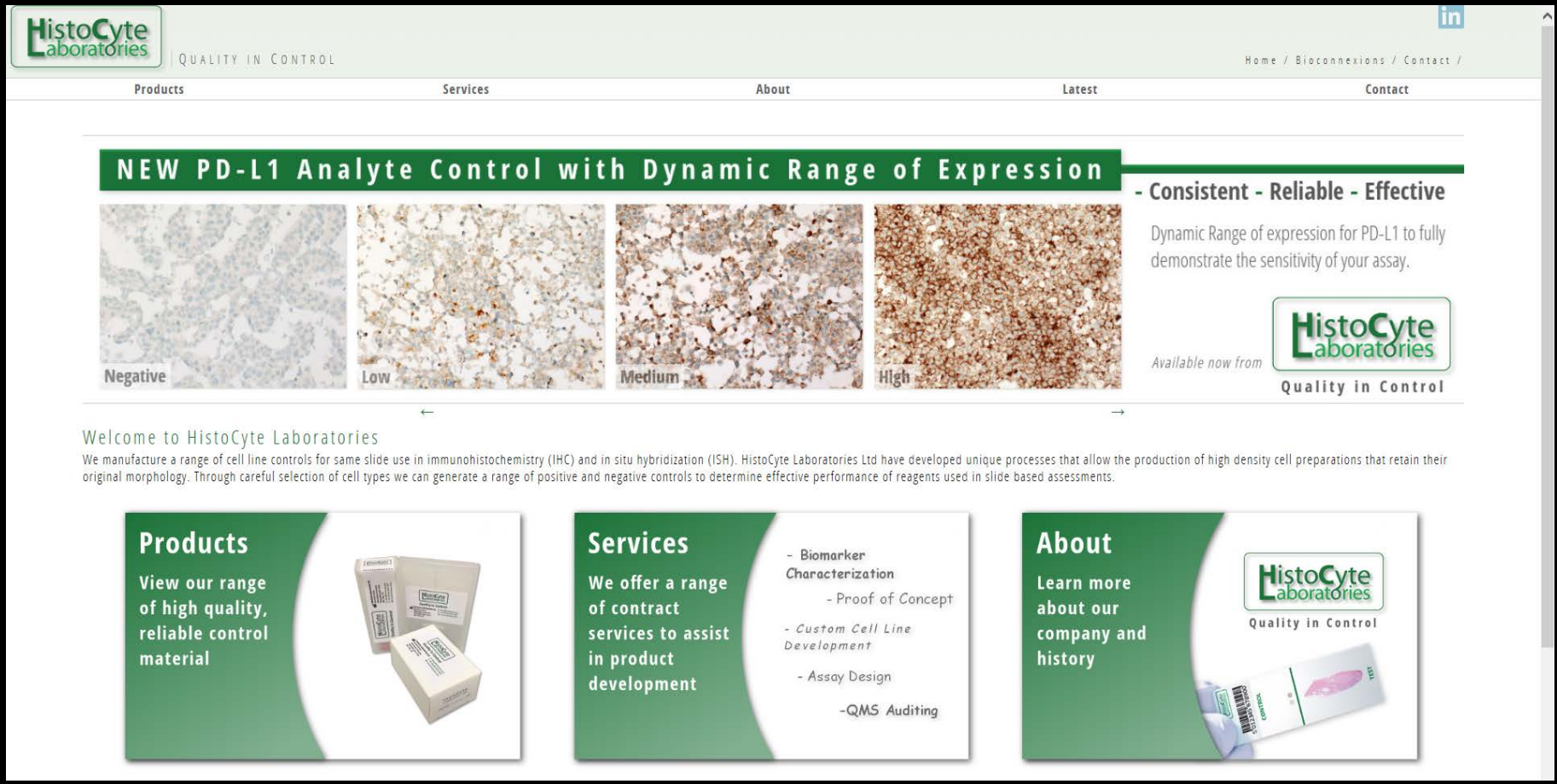
A high valueable supplement to tissue controls:

- 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

IHC – Biomarker controls

www.histiocyte.com



IHC – Biomarker controls

Conclusions –
technical calibration & analytical validation (IHC Class I)

1. IHC assay is calibrated (LD assay) / verfied (RTU – plug-and-play) on TMA with 16-30 different normal tissues. If access to ICAPCs these must be included and submitted to pre-analytical conditions applied in the laboratory.

2. IHC assay is validated on TMAs with e.g. 30-45 commonly seen neoplasias and on TMAs with the target of interest - 20/20 neoplasias expected to be pos./neg. (accuracy) covering the dynamic range of expression and cut-off's (index) – note not all markers are reliable if only TMA's are used (e.g. heterogene expression)

3. Results compared to literature, reference clone etc and conclusion made.

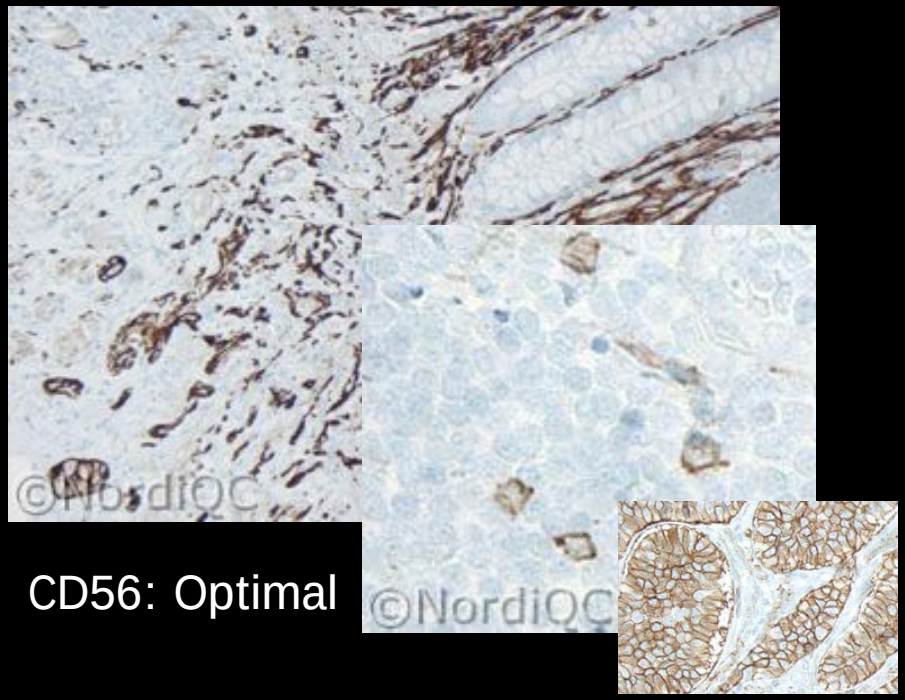
IHC – Biomarker controls

Issues to be adressed :

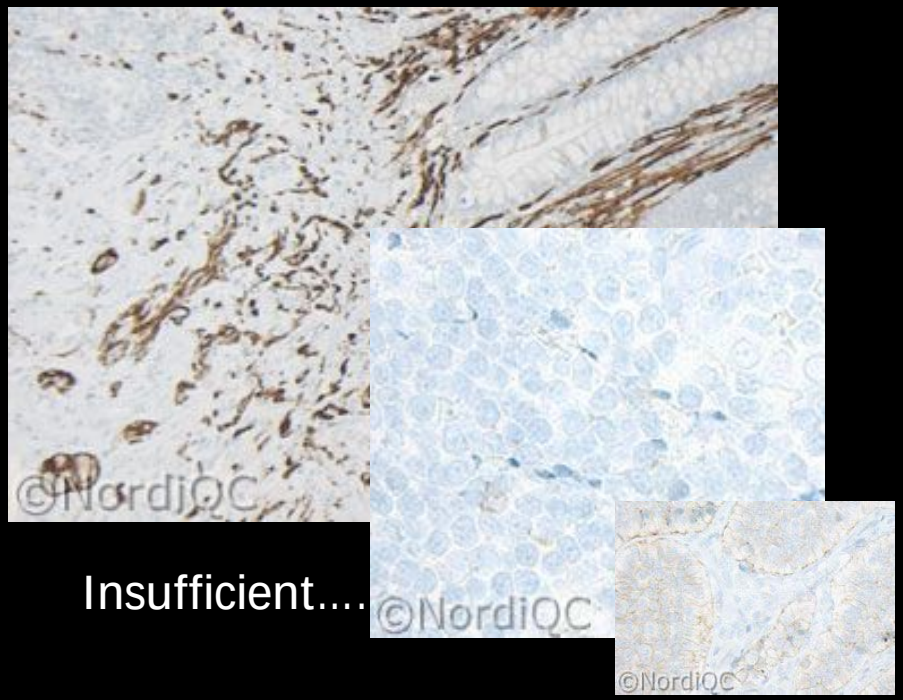
1. Calibration of IHC assay and identification of best practice protocol – clone, titre, retrieval etc
2. ”Evaluation of the robustness of the IHC assay – impact on pre-analytics
3. Evaluation of the analytical sensitivity/specificity
4. Identification of IHC performance controls providing information 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



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

External tissue control tool-box:

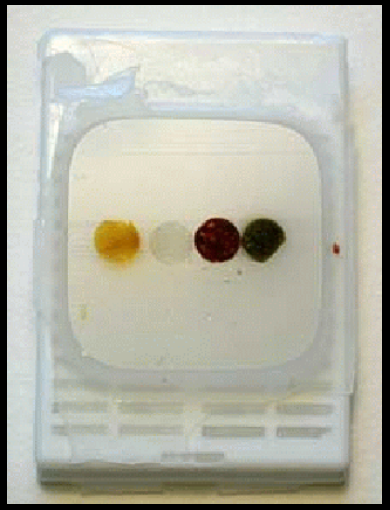
Calibration TMA's			Analytical "Validation" TMA's		Lab QC TMA
iCAPC TMA	Specificity TMA	Pre-analyt. TMA	Accuracy TMA	Index TMA	"Daily QC" TMA
					
"Gold standard" tissue controls	"Normal" tissues	iCAPCs processed as lab procedures	"Lesional" tissues	"Lesional" tissues	iCAPCs + selected tissues
IHC critical assay performance controls	Maps Ab reaction pattern	Fixation time Fixative(s) Decalcification	Range of relevant expression levels	Range of relevant expression levels	Reproducibility
					Method of transfer proof
High expression Low expression No expression	With expression No expression		With expression No expression	High expression Low expression No expression	
			20/40 of each Type I/II IHC	+ relevant cut-off	

IHC – Biomarker controls

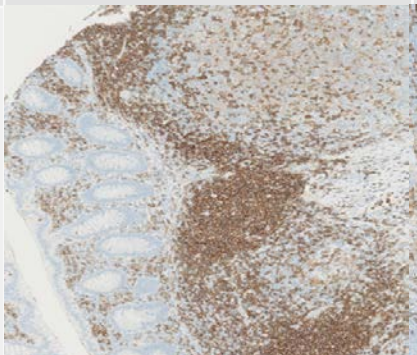
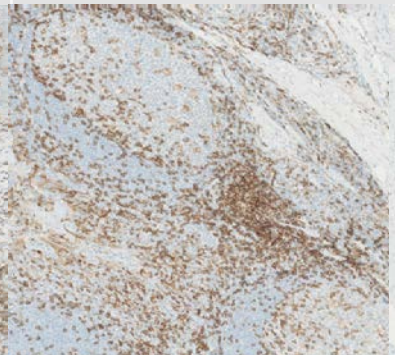
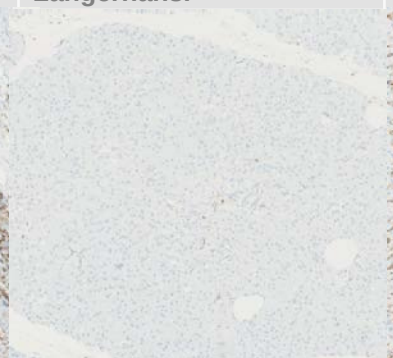
B1: Appendix, Hepar, Tonsil, Pancreas

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



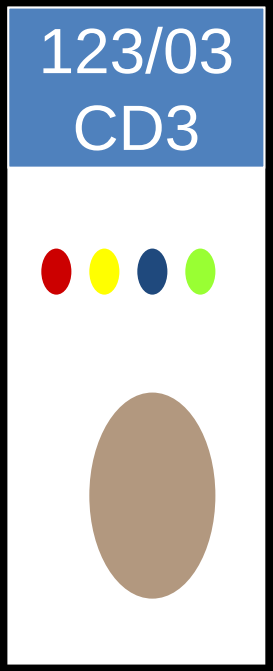
IHC – Biomarker controls

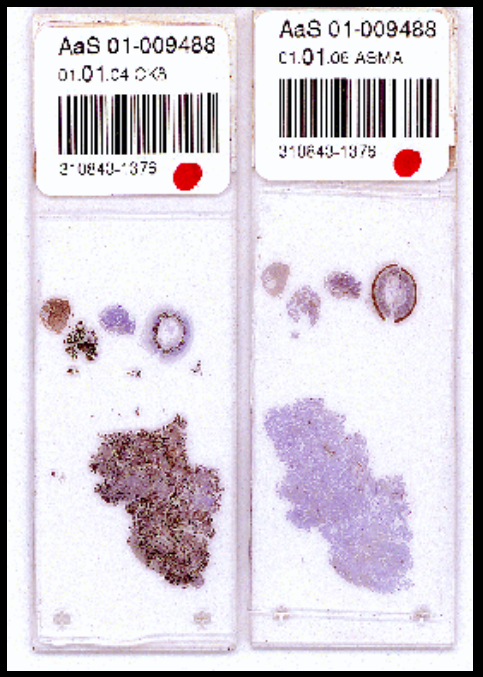
CD4 (M)	Appendix	Liver	Pancreas	Tonsil
High expression (right ab)	The majority of T-cells in lamina propria must show a moderate to strong, distinct predominantly membranous staining reaction.	The majority of T-cells, both in the interfollicular T-zones and in the germinal centres must show a moderate to strong, distinct, predominantly membranous staining reaction.	Dispersed T-cells must show a moderate to strong, distinct predominantly membranous staining reaction.	Dispersed T-cells and Kupffer cells must show a moderate to strong, distinct predominantly membranous staining reaction.-
Low expression iCAPCs (right sens.)	Dispersed intra-epithelial T-cells must show an at least weak to moderate, distinct predominantly membranous staining reaction.	**The germinal centre macrophages must show an at least weak to moderate predominantly membranous staining reaction.**	-	**The vast majority of the endothelial cells of the liver sinusoids must show an at least weak to moderate, distinct predominantly membranous staining reaction.**
Non expression (right spec.)	No staining reaction must be seen in the columnar epithelial cells.	No staining reaction must be seen in the mantle zone and germinal centre B-cells.	No staining reaction must be seen in the epithelial cells of the exocrine pancreas or the endocrine cells of the islets of Langerhans.	No staining reaction must be seen in the hepatocytes.
				

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 run control, no information is available for the single slide evaluated

IHC – Biomarker controls

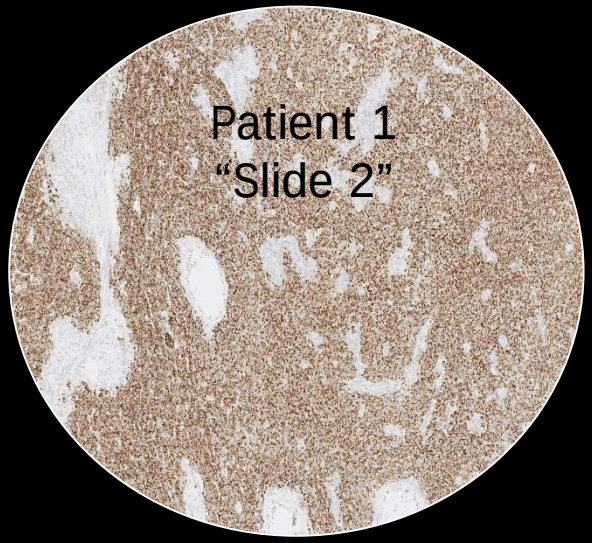
	TMA control on all slides	One batch control	Remarks
Missing reagent FN in patient test	Yes	No – only control slide	Potential internal pos. control only indicator of protocol performed
Wrong antibody FP in patient test	Yes	No – only control slide	
Inappropriate protocol performance - Drying out etc FN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

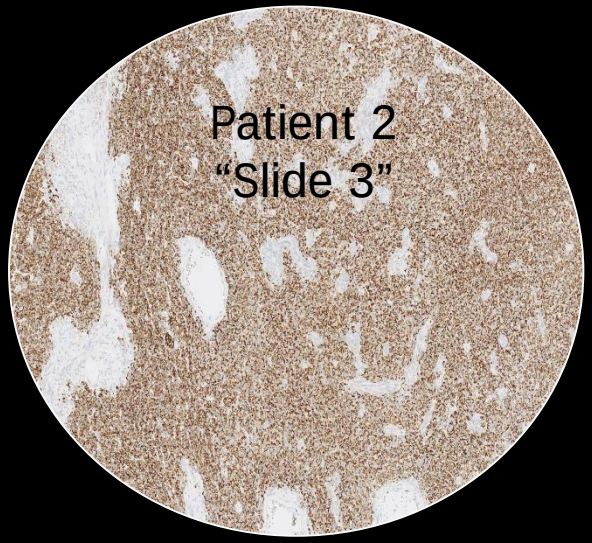
IHC – Biomarker controls

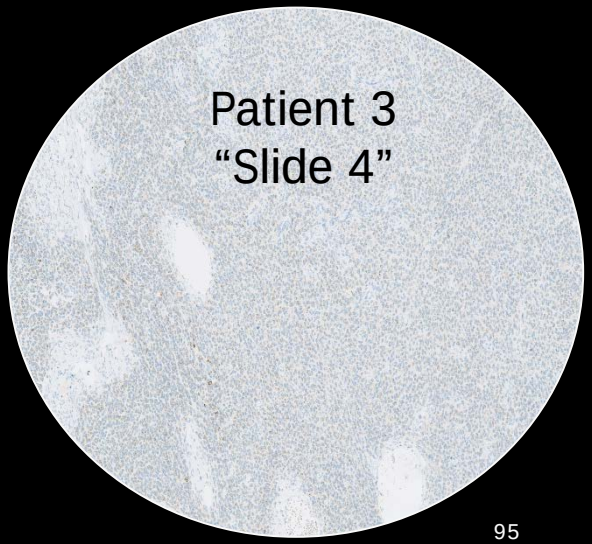
"Patient" 3 IHC assay level could be related to:

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

Melan-A in sex cord tumours



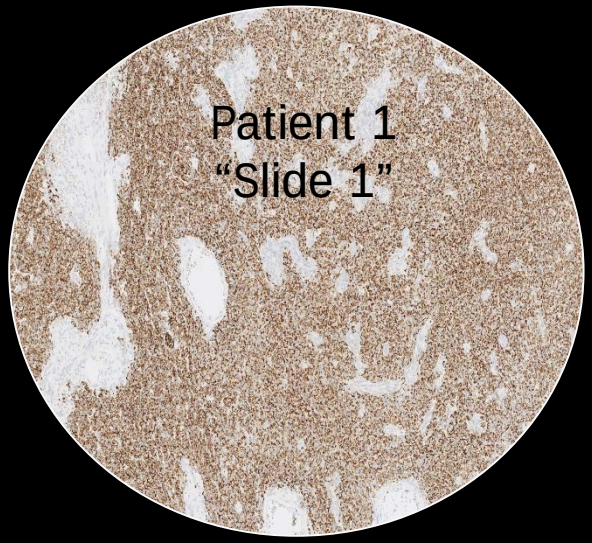


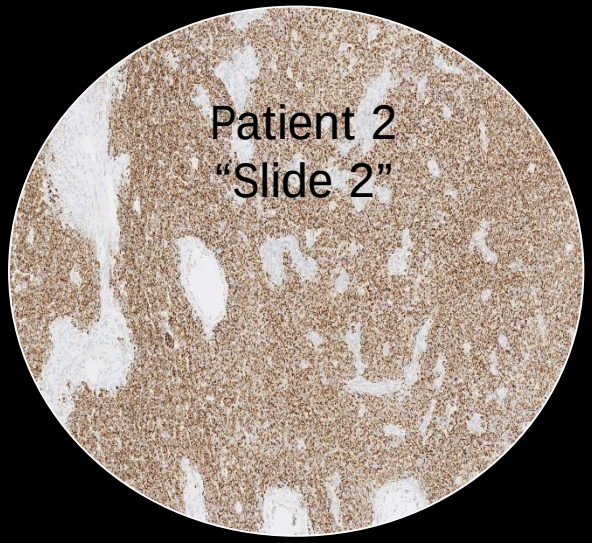


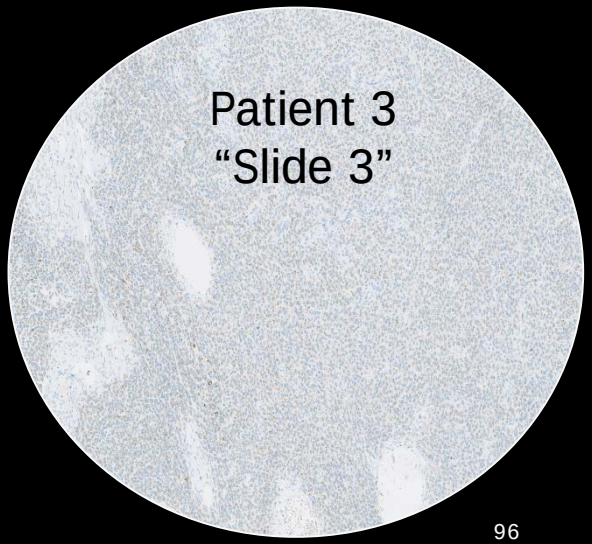
IHC – Biomarker controls

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

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







IHC – Biomarker controls

REVIEW ARTICLE

(*Appl Immunohistochem Mol Morphol* 2015;23:1–18)

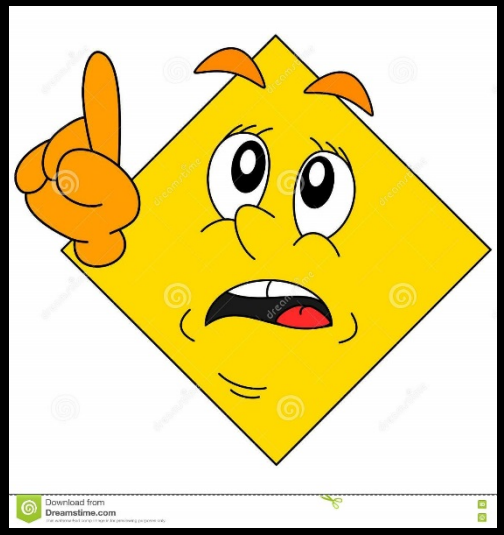
Standardization of Positive Controls in Diagnostic Immunohistochemistry: Recommendations From the International Ad Hoc Expert Committee

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‡§*

TABLE 3. (*continued*)

	Special Considerations
Cut and submit "own on-slide control" if sending patients' unstained slides to another laboratory for IHC testing	The positive controls should match patients' sample tissue processing so far as is possible This is difficult if the sender does not know which IHC assays will be performed or if the sender does not have dIHC laboratory and has no positive controls
Use on-slide positive controls	"Run" or "batch" positive controls are not recommended
Date unstained slides with on-slide controls	Without the date when the slides are prepared, it will be impossible to determine if a unexpected weak result is due to variation in protocol or to an "expired" positive control

dIHC indicates diagnostic immunohistochemistry; iCAPCs, immunohistochemistry critical assay performance controls; SOP, standard operating procedure.



”even for automated stainers, where it cannot be guaranteed that every slide in fact receives identical treatment”.

IHC – Biomarker controls

RESEARCH ARTICLE

(*Appl Immunohistochem Mol Morphol* 2017;25:308–312)

An Audit of Failed Immunohistochemical Slides in a Clinical Laboratory: The Role of On-Slide Controls

Carol C. Cheung, MD, PhD, JD,† Clive R. Taylor, MD, DPhil,‡ and Emina E. Torlakovic, MD, PhD†*

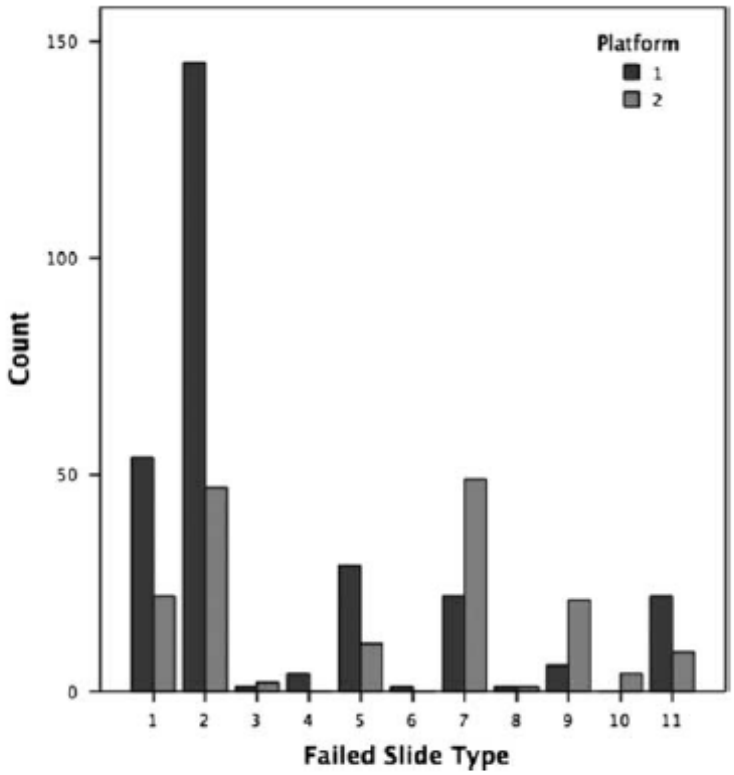


FIGURE 1. Frequency of failed immunohistochemistry slides by category and platform.

TABLE 1. Categories of Failed IHC Slides

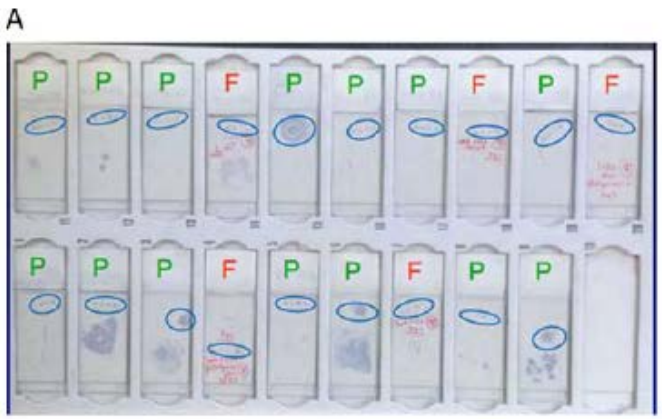
Failed IHC Slide Category	Description	Comments
1	On-slide control too weak, patient tissue negative	Correct primary Ab was applied, but test sensitivity is possibly too low
2	On-slide control negative, patient tissue negative	Total slide failure; the result of the test does not suggest possible cause of the failure
3	On-slide control too weak, patient tissue weakly positive but no internal control	May indicate decreased technical sensitivity
4	On-slide control negative, patient tissue weakly positive but no internal control	There is uncertainty whether the correct primary Ab was applied or if there was significantly decreased sensitivity
5	No on-slide control, patient tissue negative	Uncertain results; cannot distinguish if the staining was optimal, suboptimal, or total failure
6	No on-slide control, patient tissue positive	No internal control present; lesion positive; failed only if there is uncertainty over whether the proper primary Ab was applied
7	Failed signal-to-noise ratio	Usually too high background; potential false positive, involving both patient sample and on-slide external control
8	Counter staining problem	If severe, may render result uninterpretable
9	Wrong protocol	Wrong protocol selected when >1 protocol for the given primary Ab exists in the system
10	Uneven staining	Large or critical areas of the patient tissue or controls were missed by uneven staining
11	Wrong control	Either wrong tissue control or areas relevant to the test were missing (detached during staining or paraffin block with control tissue cut through)

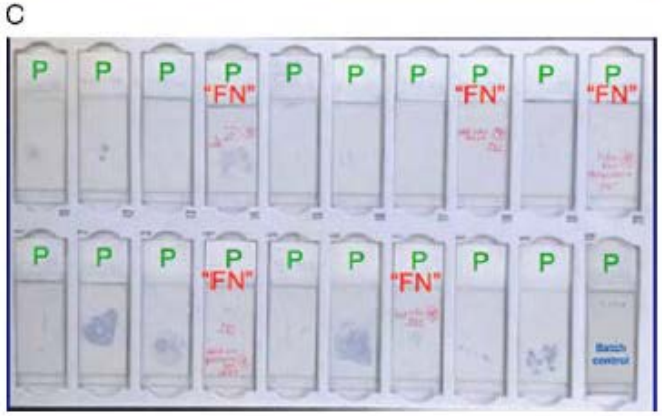
IHC indicates immunohistochemistry.

2% error rate (452/22.234 slides) - Class I 0,8% - Class II 9,0%

Assay / Instrument related; 78%

IHC – Biomarker controls





On-slide controls
IHC slides stained for ALK (Class II),
same run, same instrument, same protocol
14/19 passed
5/19 failed
Cost; (5 x 150 USD)

Batch-control - Theoretically:
Batch control failed by same conditions as above
0/19 passed
19/19 failed (no consistent internal control...)
Cost; (20 x 150 USD)

Batch-control - Theoretically:
Batch control passed by same conditions as above
19/19 passed
0/19 failed (the 5 failed slides not identified....)
Cost; ???

IHC – Biomarker controls

Standardisation of external tissue controls enables a more objective evaluation of IHC assay consistency and potential trouble shooting.

The area still needs to be improved an requires surveillance and registration of IHC results of the controls

E.g. registration of aberrant staining results in controls

Ab	Slide	Weak	FN	PS	FP	Accept	Retest
CK5	144001	+				+	
CD10	144780		+				+
MLH1	144899			+			

Or scoring of all controls

Ab	Slide	0 negative	1 weaker	2 standard	3 stronger
CK5	144001			+	
CK5	144210		+		

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

External tissue control tools:

Calibration TMA's			Analytical "Validation" TMA's		Lab QC TMA
iCAPC TMA	Specificity TMA	Pre-analyt. TMA	Accuracy TMA	Index TMA	"Daily QC" TMA
					
"Gold standard" tissue controls	"Normal" tissues	iCAPCs processed as lab procedures	"Lesional" tissues	"Lesional" tissues	iCAPCs + selected tissues
IHC critical assay performance controls (iCAPCs)	Maps Ab reaction pattern	Fixation time Fixative(s) Decalcification	Range of relevant expression levels	Range of relevant expression levels	Reproducibility Method of transfer proof
High expression Low expression No expression	With expression No expression	High expression Low expression No expression	With expression No expression 20/40 of each Type I/II IHC	High expression Low expression No expression + relevant cut-off	

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

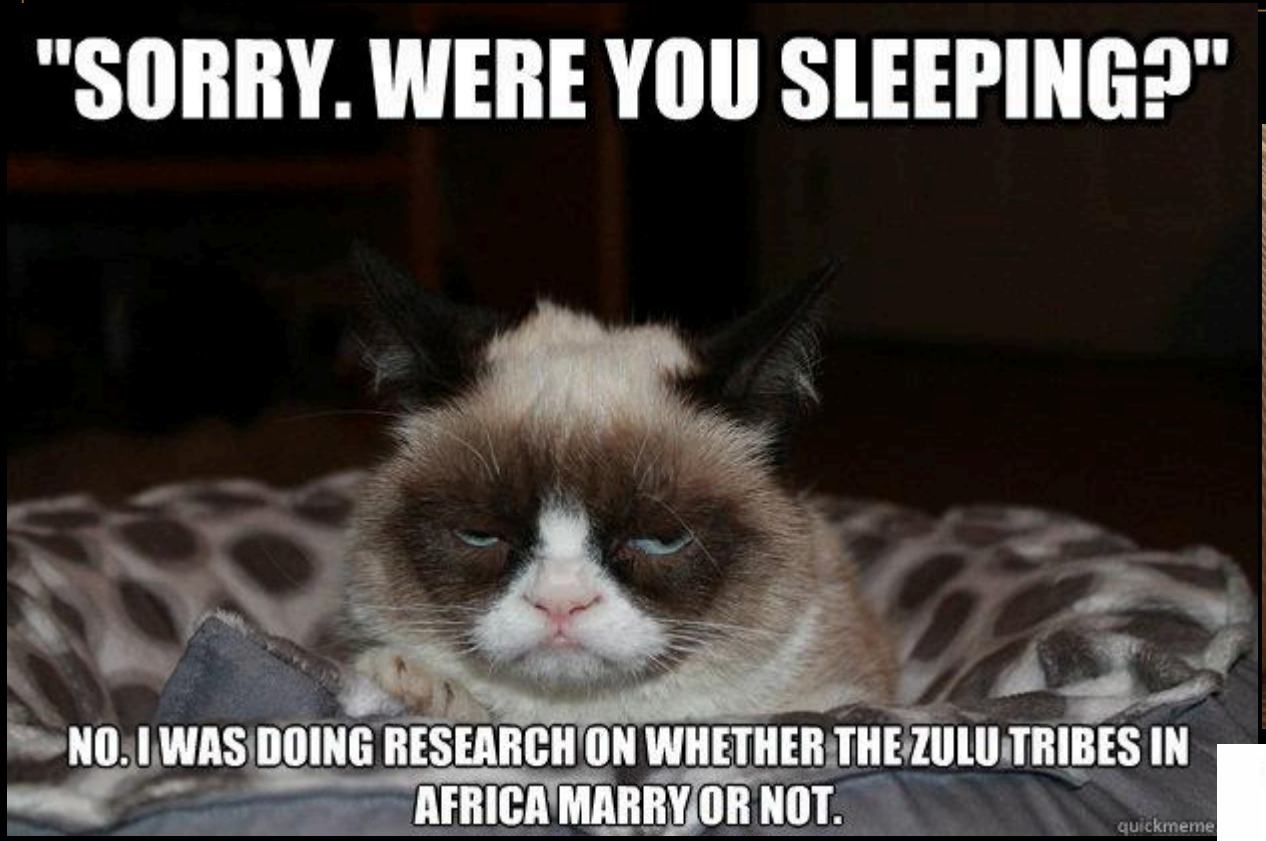
IHC – Biomarker controls

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



Thank You for the attention and.....