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

The Tissue Tool Box

-

IHC Critical Assay Performance Controls

Søren Nielsen,
Director
NordiQC

Agenda and focus areas

- What is recommended and best practice for IHC controls in diagnostic IHC?
- What are the potentials and limitations for the use of IHC controls ?
- How can IHC controls be used by laboratories and IHC stakeholders?
  - How to use IHC controls to implement new markers.
  - How to use IHC controls to monitor assay consistency.
  - How to use IHC controls to adress inter and intra test accuracy (e.g. EQA).

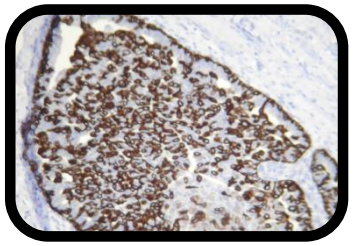
The role and concept behind ICAPCs -

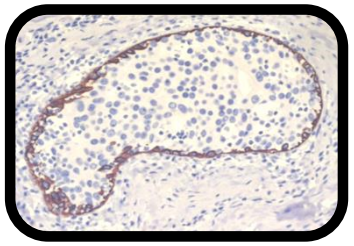
IHC Critical Assay Performance Controls

Power of IHC

Hyperplasia or In-situ

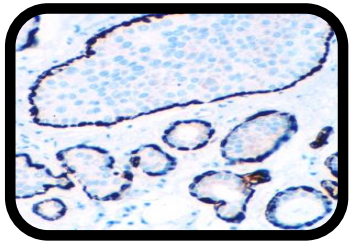
CK5, CK14, Heavy chain myosin, p63

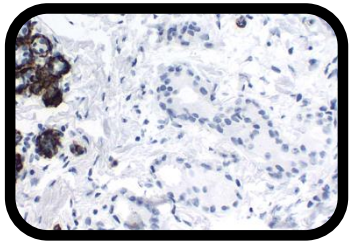




In-situ or invasive

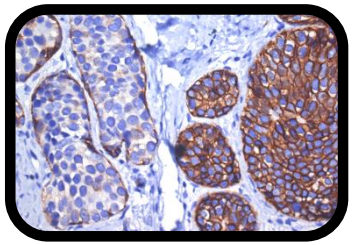
CK5, CK14, Heavy chain myosin, p63

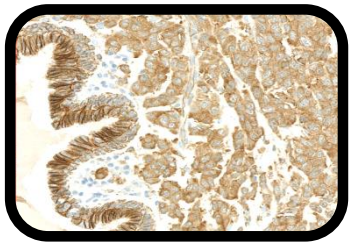




Lobular or ductal lesion

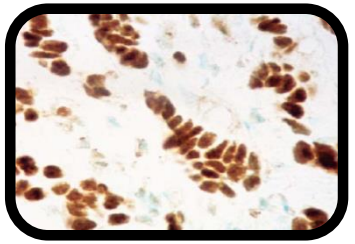
E-cadherin, p120

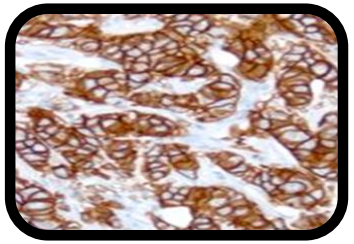




Predictive - Prognostic

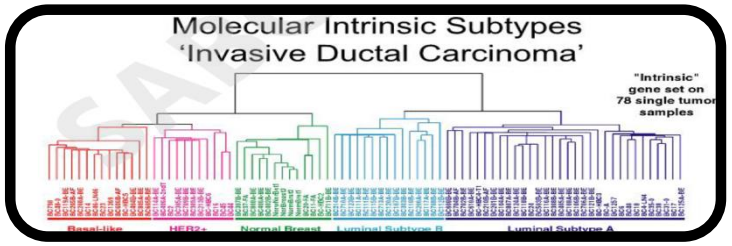
ER, PR, HER2, Ki67





”Molecular risk signature”

IHC4 – *ER, PR, HER2, Ki67 (CK5)*



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

Decalcification
Preparation

Fixation
Time, Type, Volume

Tissue
Type, Dimension,
Laser resection,
De-differentiation

Pre-analytic

Section
Thickness
Storage
Drying

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

Epitope retrieval
HIER conditions
Proteolysis

Primary antibody
Clone, Dilution
Buffer, Time, Temp

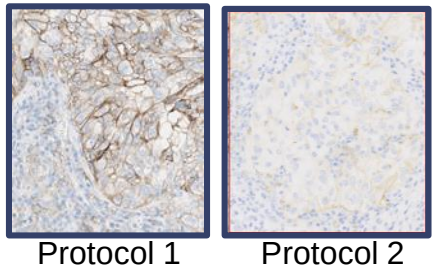
Manual
Stainer

Analytic

Visualization
Sensitivity, Specificity

Development
Sensitivity,
Localization

PD-L1 - NSCLC



Protocol 1 Protocol 2

Controlment

Quantification
Reporting

Post-analytic

Read-out / Interpretation
Positive/Negative - cut-off level

The right control material will expose right or wrong choices

Importance of IHC controls have been neglected….

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

Diagnostic Cytopathology, Vol 39, No 4 2011

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 Gladv

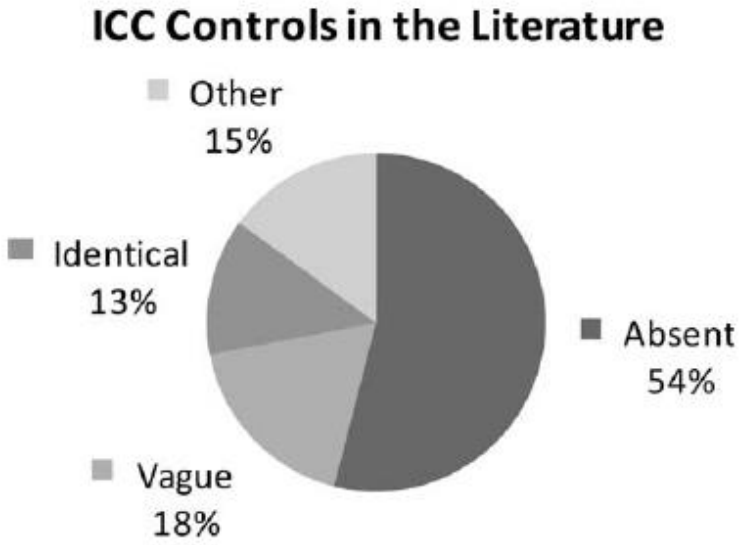


Fig. 1. Description of immunocytochemistry controls in articles reviewed.

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 controls to guide reliability of data…

PAX8 expression in breast cancer – true of false…?

Comparison of PAX8 Expression in Breast Carcinoma Using MRQ50 and BC12 Monoclonal Antibodies

Kamaljeet Singh, MD, Linda C. Hanley, MD, C. James Sung, MD, and M. Ruhul Quddus, MD, MPhil (Path)

41% MRQ-50
0% BC12

Unexpected PAX8 Immunoreactivity in Metastatic High-grade Breast Cancer

Mark R. Kilgore, MD, Dustin E. Bosch, MD, PhD, Kathi H. Adamson, MD, Paul E. Swanson, MD, Suzanne M. Dintzis, MD, PhD, and Mara H. Rendi, MD PhD

31% MRQ-50
11% pAb CM

Metastatic Carcinoma of Unknown Primary: Diagnostic Approach Using Immunohistochemistry

James R. Conner, MD, PhD and Jason L. Hornick, MD, PhD

< 5%

Right choice, use and results reported in positive and negative IHC control tissues needed to verify data

IHC controls to guide reliability of data…

NordiQC Assessments of PAX8 Immunoassays

Rasmus Roge, MD,† Ole Nielsen, HT,‡ Michael Bzorek, HT,§ Søren Nielsen, HT,* and Mogens Vyberg, MD*†*

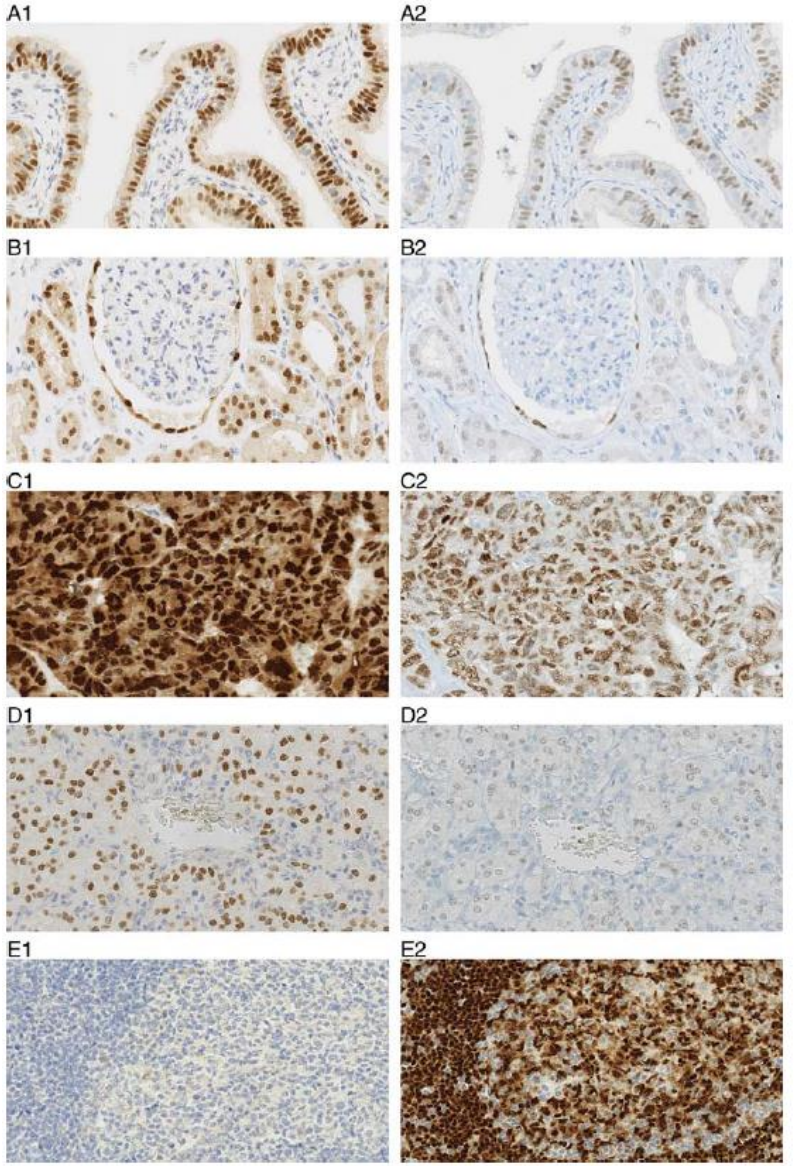
Positive tissue control 1

Positive tissue control 2

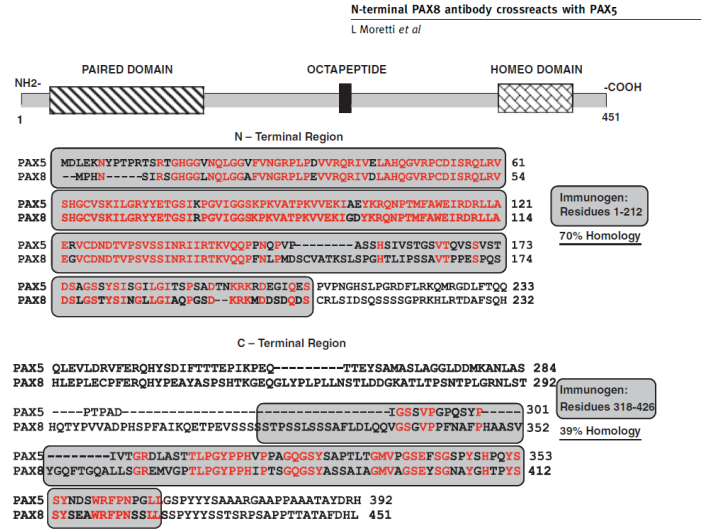
Tumour type 1

Tumour type 2

Negative tissue control 1



Level of analytical sensitivity



Level of analytical specificity

Protocol 1 Protocol 2

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Right choice, use and results reported in positive and negative IHC control tissues needed to verify data

References central for the area of IHC controls

The ”Kick-off” phase for

”Standardization of IHC controls”

Definitions and requirements
Usage
Potentials / Limitations
Perspectives

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,†††
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Paul E. Swanson, MD,¶¶## Xiaoge Zhou, MD,***††† Clive R. Taylor, MD, PhD,‡‡‡
and Mogens Vyberg, MD‡§*

References central for the area of IHC controls

The 4-paper evolutions series

Recommendations and road-map for IHC QA provided by

International Society For Immuno-Histochemistry and Molecular Morphology (ISIMM)

International Quality Network for Pathology (IQN-PATH)

Published AIMM 2017 (Jan-April)

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

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

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

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

Main elements to develop & validate IHC assays

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

The journey from an antibody to a diagnostic IHC assay with a specific purpose
Based on external tissue control.

Tissue controls

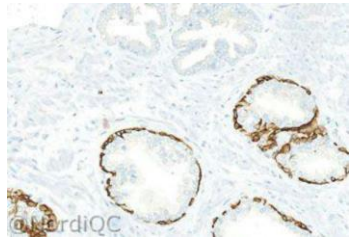
- Reagent and **tissue** controls are necessary for the validation of immunohistochemical staining results.

- 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

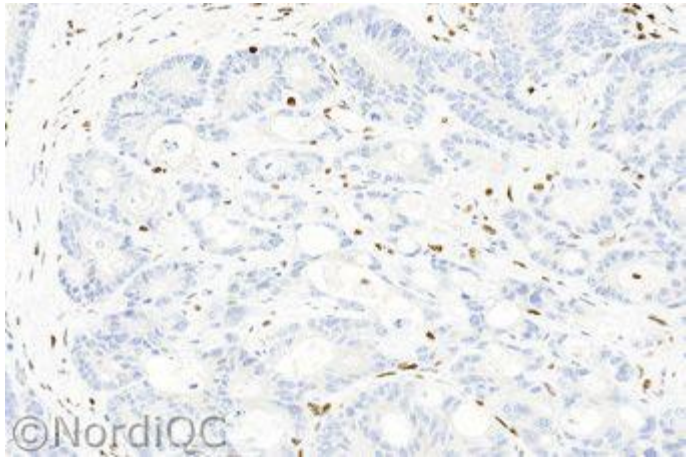
How to use internal tissue controls

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 / target evaluated



Limitations of internal tissue controls

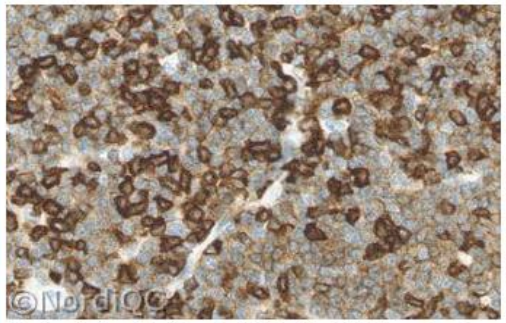


Fig. 4a. Optimal staining for CD5 of the B-CLL no. 5 using same protocol as in Figs. 1a - 4a.
The majority of the neoplastic cells show a moderate and distinct staining reaction, while the infiltrating normal T-cells show a strong staining reaction.

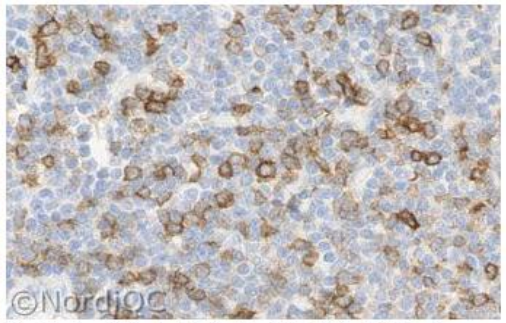


Fig. 4b. Insufficient staining for CD5 of the B-CLL using same protocol as in Figs. 1b - 3b – same field as in Fig. 4a.
The neoplastic cells are virtually negative and only the normal T-cells are clearly demonstrated.

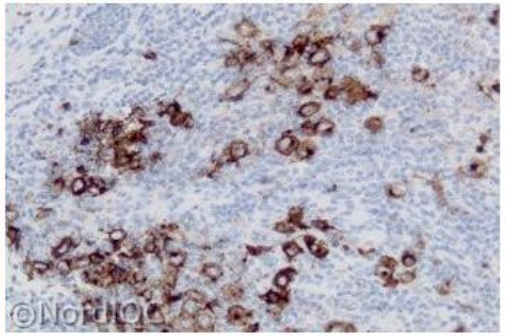


Fig. 2a. Optimal CD15 staining of the Hodgkin lymphoma no 2 (NS) using same protocol as in Fig. 1a. The Reed-Sternberg and Hodgkin cells show a strong membranous staining and a dot-like positivity.

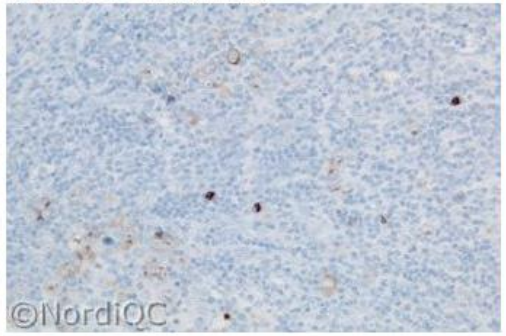


Fig. 2b. CD15 staining of the Hodgkin lymphoma no 2 (NS) using same protocol as in Fig. 1b. Only few Reed-Sternberg and Hodgkin cells show a weak staining - same field as in Fig. 2a.

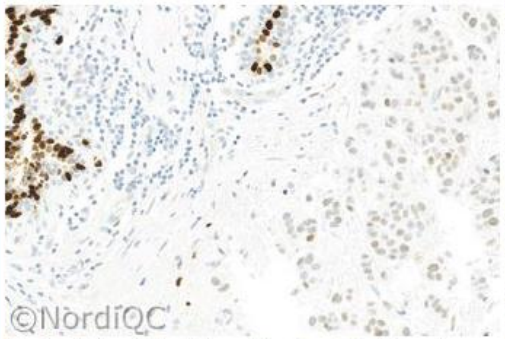


Fig. 3a. Optimal ER staining of the breast ductal carcinoma no. 3 with 60 – 80 % cells positive. A weak but distinct nuclear staining is seen in the appropriate proportion of the neoplastic cells. Same protocol as in Figs. 1a and 2a.

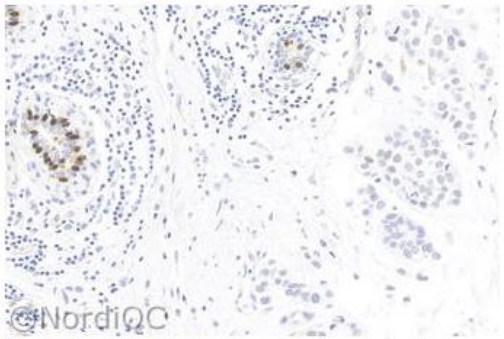
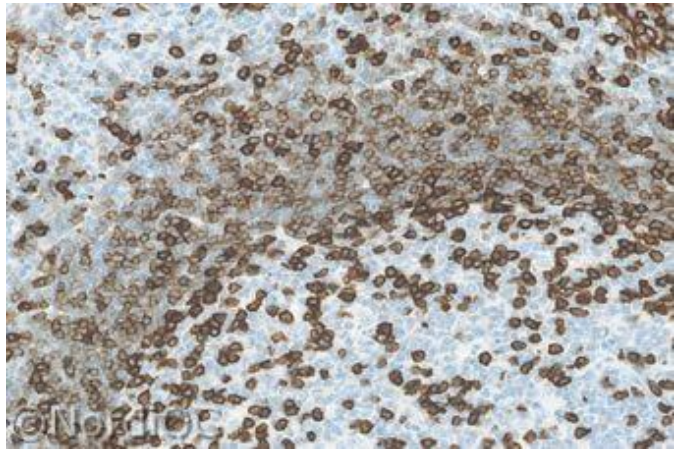


Fig. 3b. Insufficient ER staining of the breast ductal carcinoma no. 3 with 60 – 80 % cells positive using same protocol as in Figs. 1b and 2b – same field as in Fig. 3a.
Only dispersed neoplastic cells show an equivocal staining

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

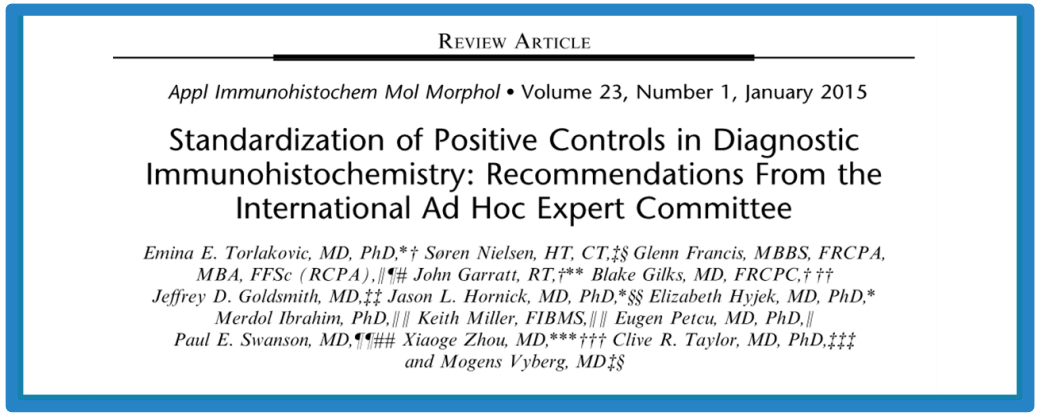


Critical tissue controls = ICAPCs

IHC Critical Assay Performance Controls (ICAPCs)

are basically human positive control tissues with

- clinical relevant range of target analyte (antigen) – especially with low limit detection
- well characterized expression pattern – preferable normal tissues
- predictable levels and specified cellular and architectural localization



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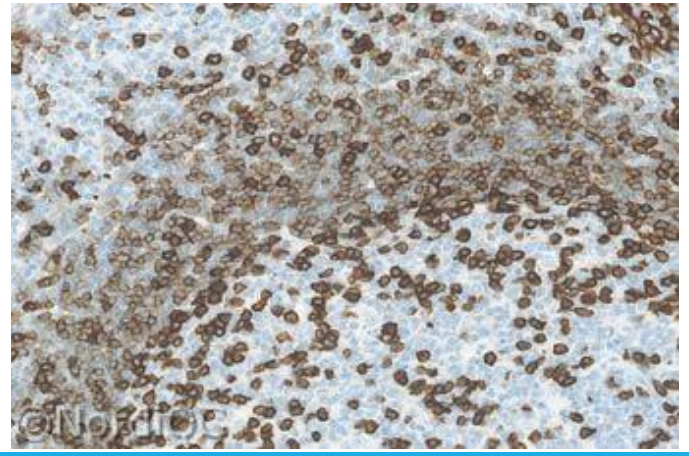

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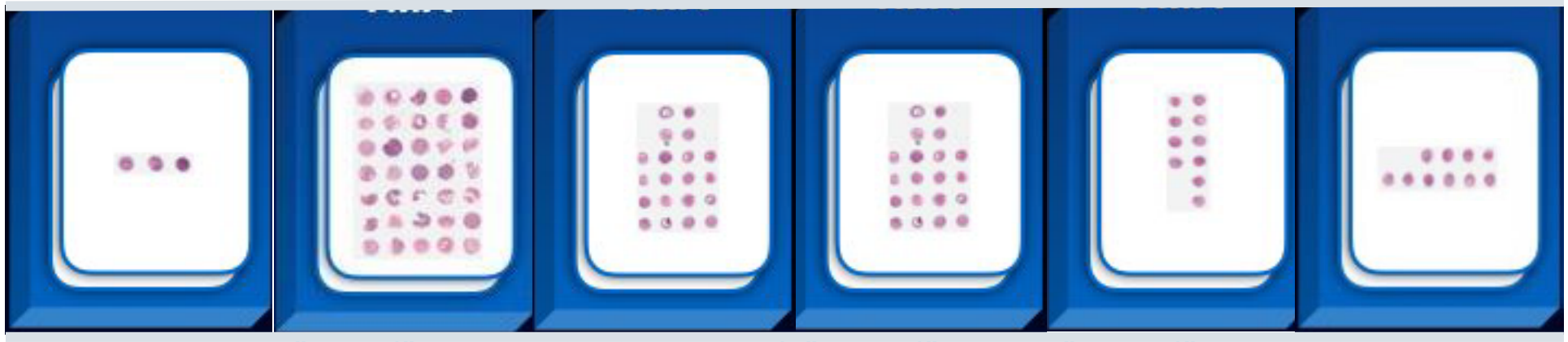
3. Evaluation of analytical sensitivity / specificity
4. Identification of IHC performance controls providing information that the established level of detection is obtained in each test performed in daily practice.

Based on selection and use of appropriate external tissue controls

External tissue control tool box

E Torlakovic et al. AIMM, 2017; 25:227-230
Evolution of Quality Assurance for Clinical Immunohistochemistry in the Era of Precision Medicine: Part 4

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 Test performance characteristics	Fixation time Fixative(s) Decalcification	Range of relevant expression levels	Range of relevant expression levels	Reproducibility
			With expression	High expression Low expression	Method of transfer proof
High expression Low expression No expression	With expression		No expression	No expression	
	No expression		20/40 of each Type I/II IHC	+ relevant cut-off	

*Immunohistochemical critical assay performance controls

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	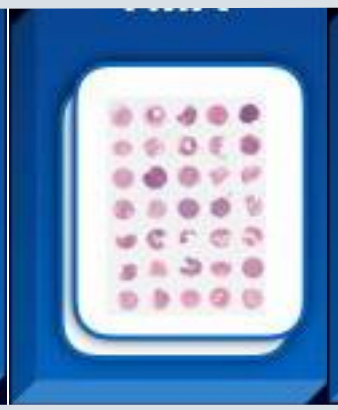		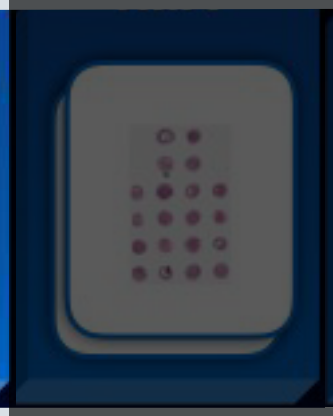		
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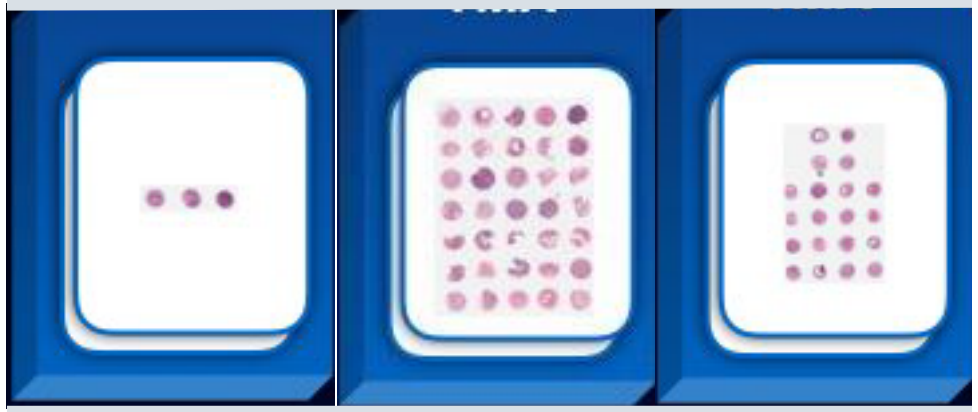		
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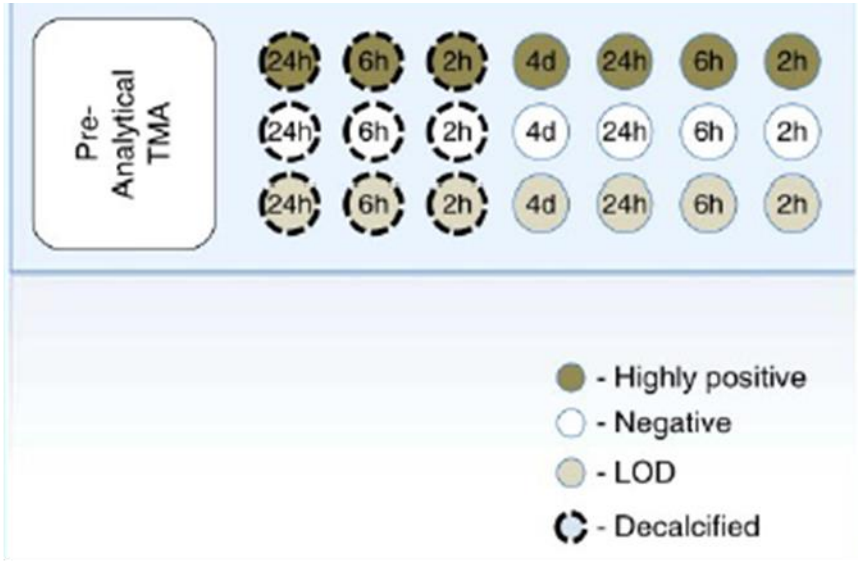
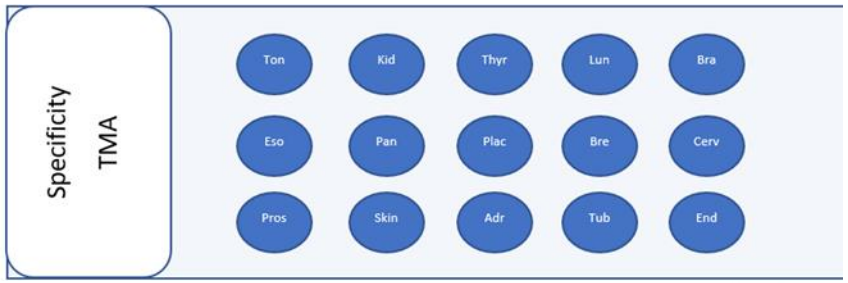
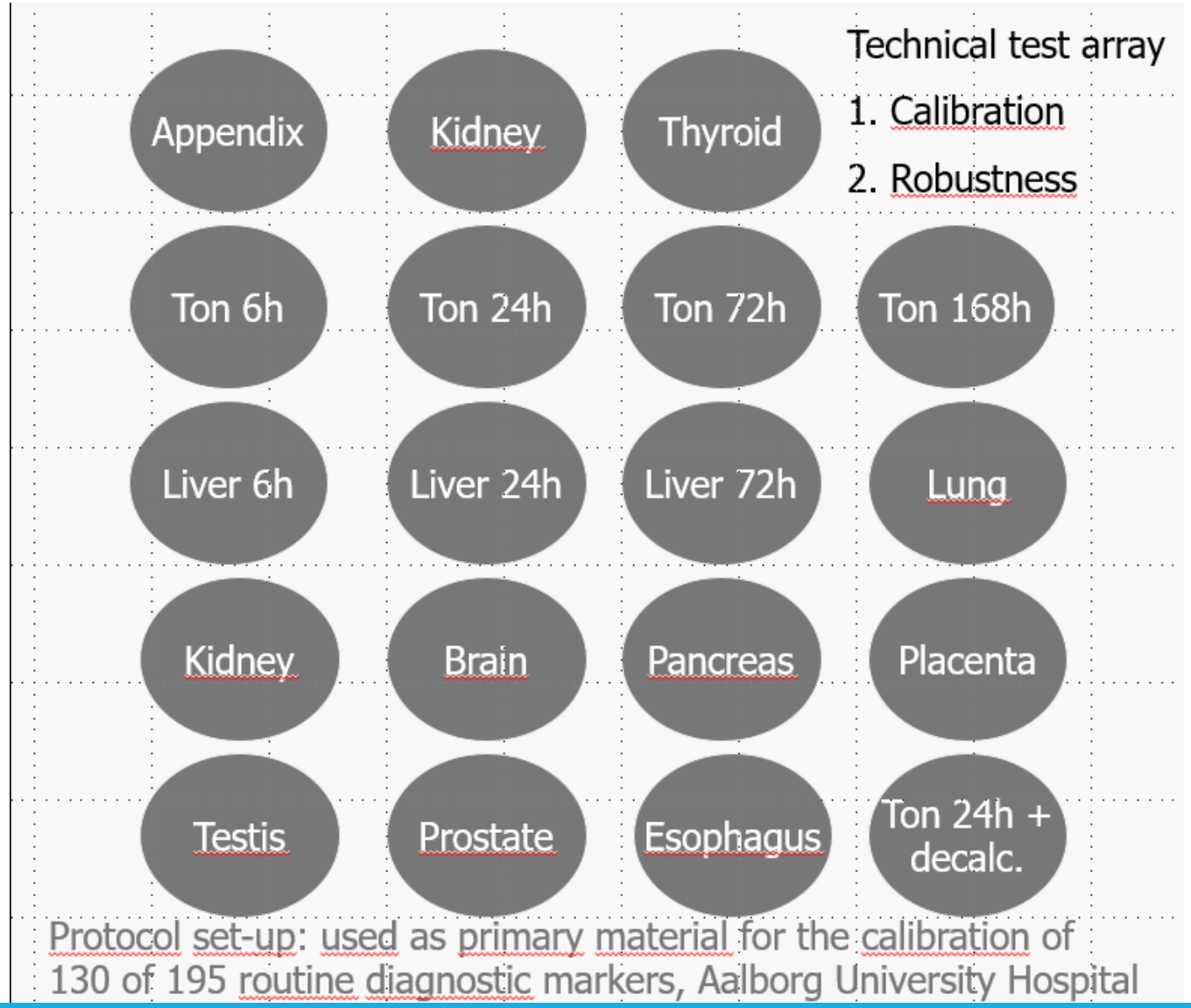




*Immunohistochemical critical assay performance controls

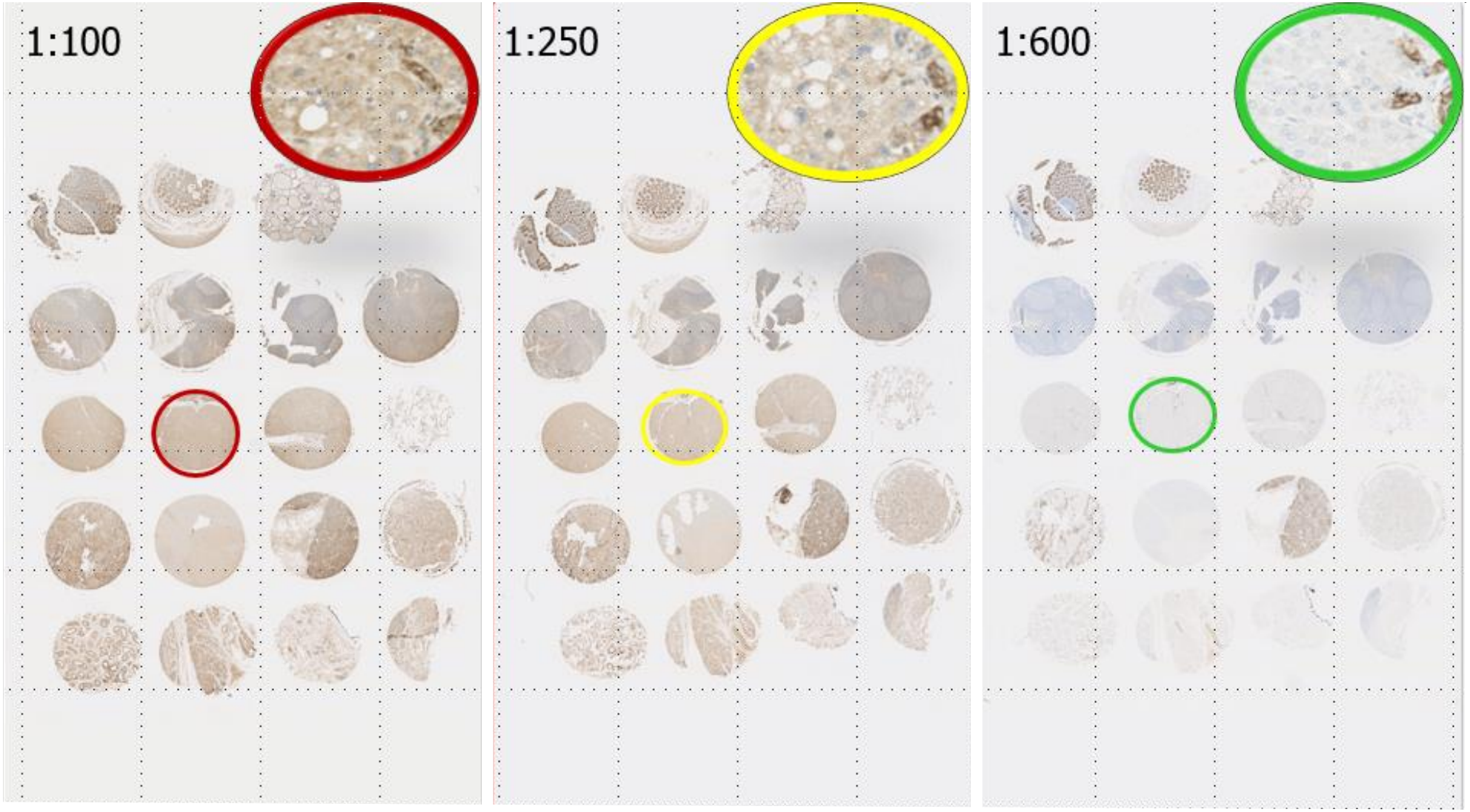
”Poor mans” specificity and pre-analytical TMAs



Inspirational set-up to address issue of specificity and impact on pre-analytics

Source:
NordiQC and Aalborg University Hospital

”Poor mans” specificity and pre-analytical TMAs

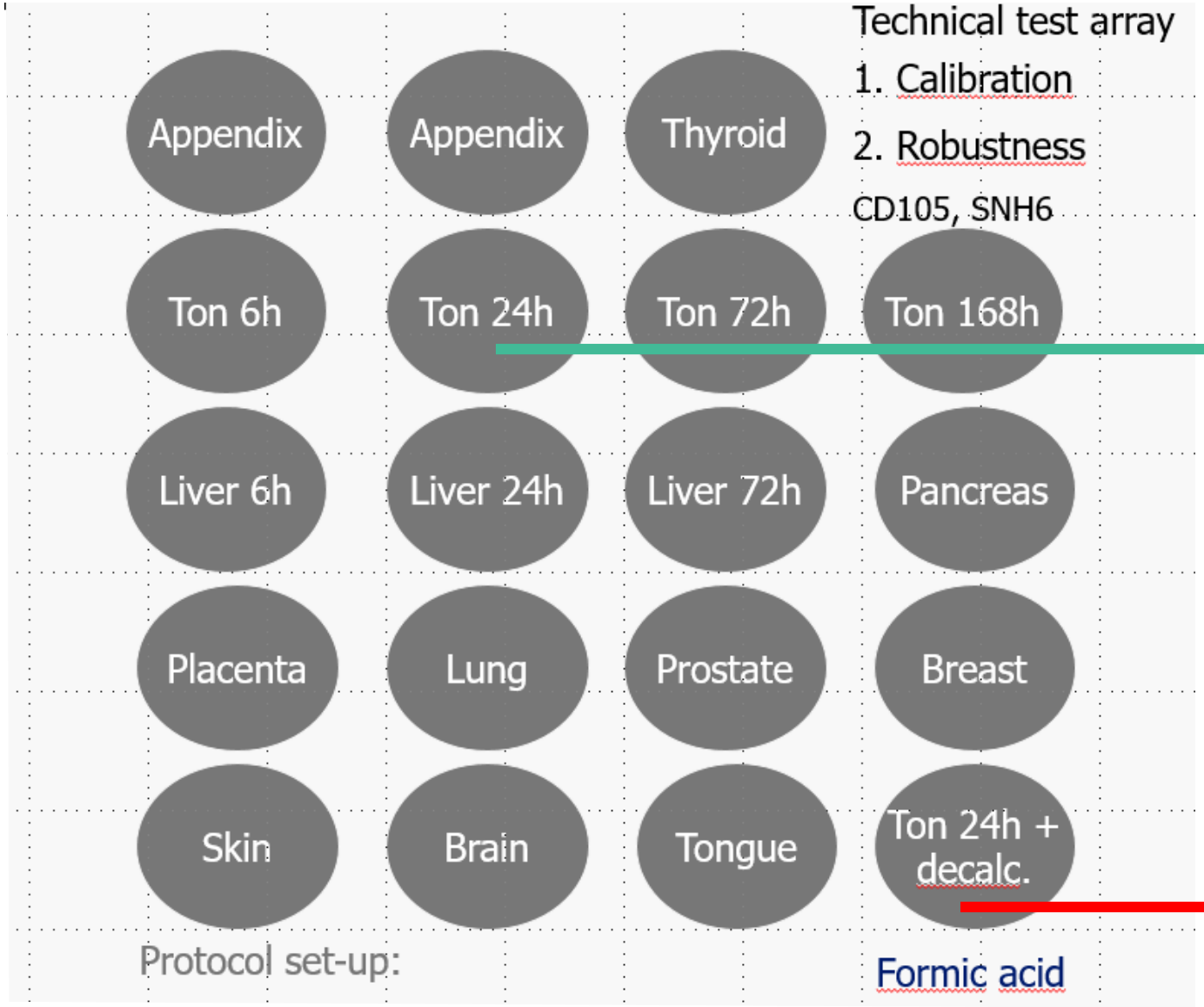


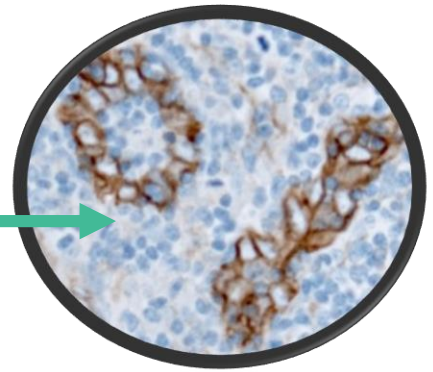
EPCAM calibration

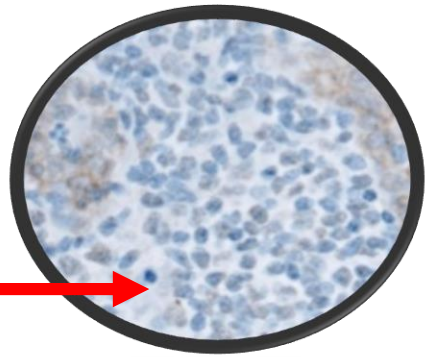
Tissue cores are used to identify best practice protocol providing highest signal-to-noise ratio for qualitative IHC markers

Source:
NordiQC and Aalborg University Hospital

”Poor mans” specificity and pre-analytical TMAs





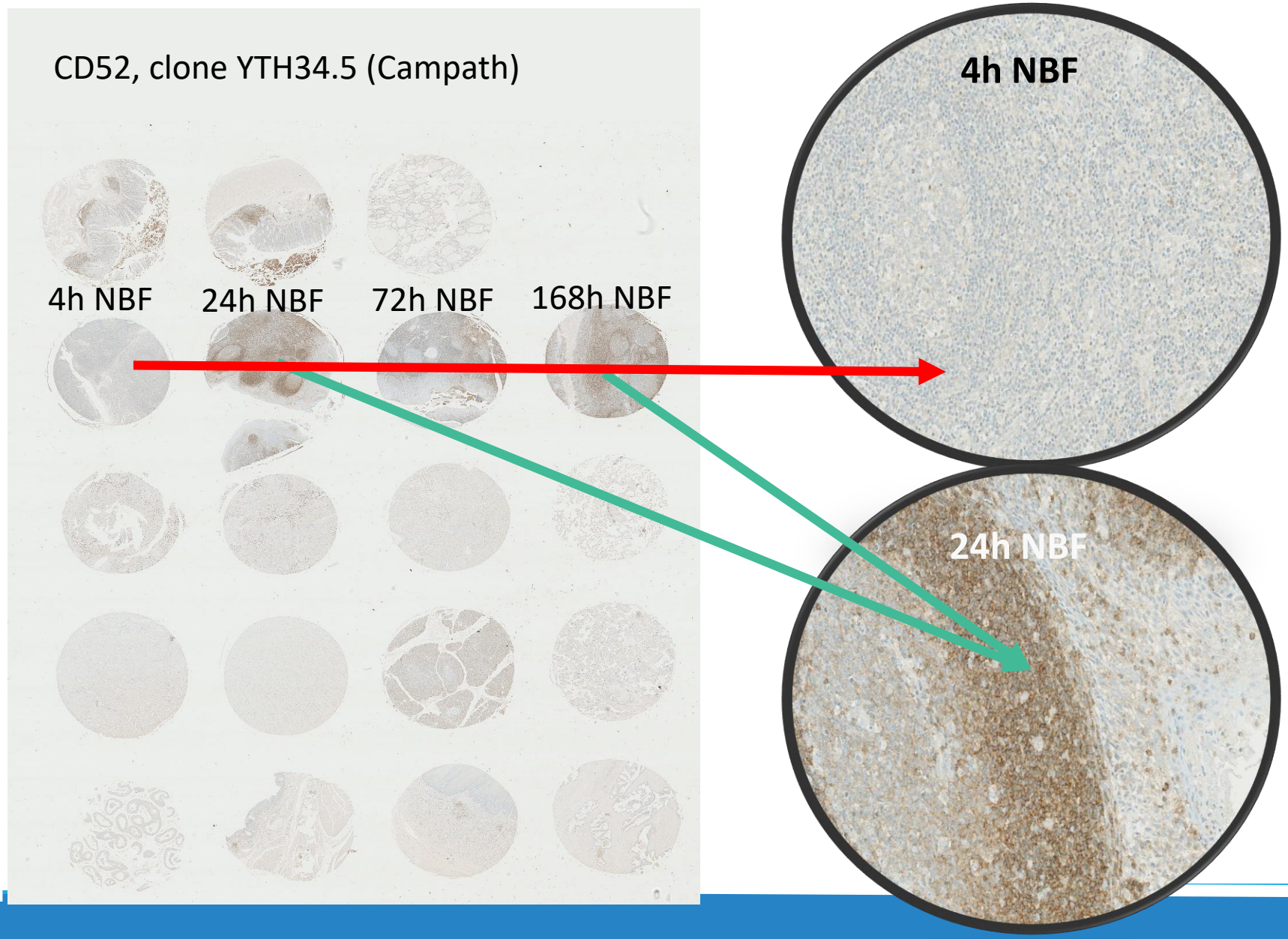


CD105 calibration

Tissue cores are used to identify best practice protocol providing impact on on pre-analytics

Source:
NordiQC and Aalborg University Hospital

”Poor mans” specificity and pre-analytical TMAs

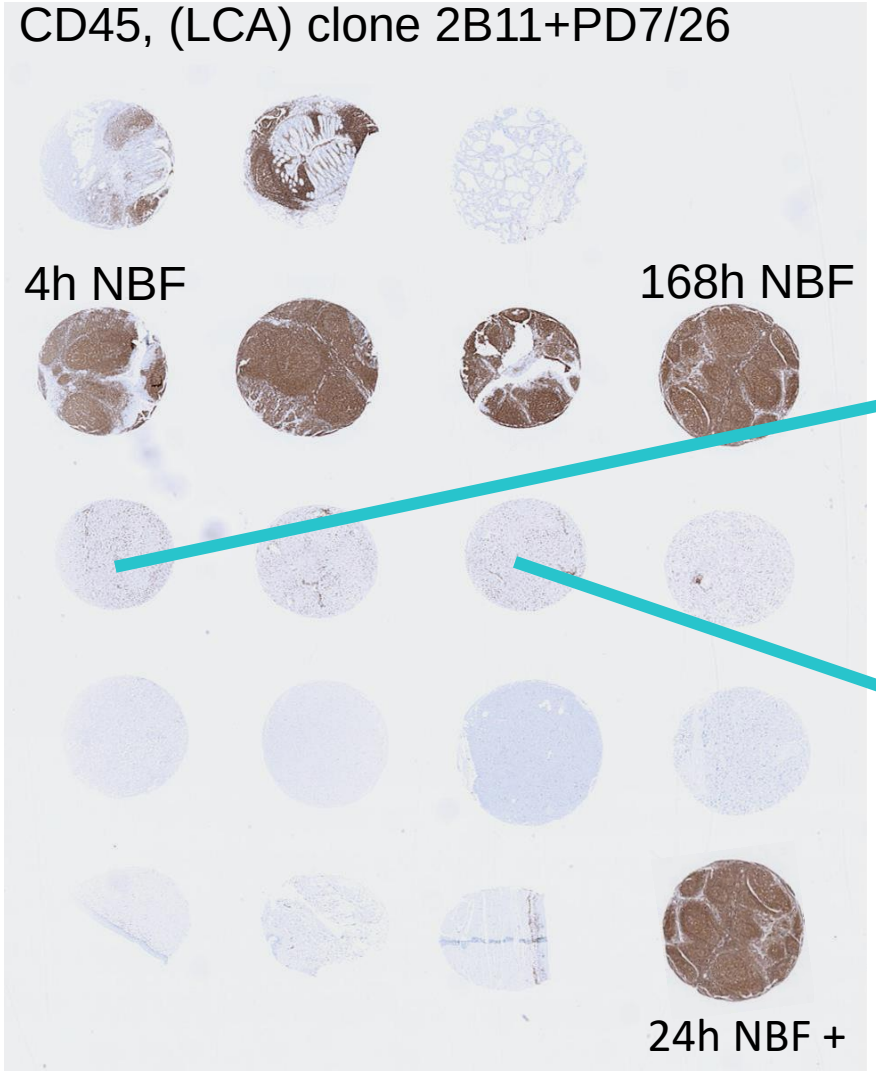


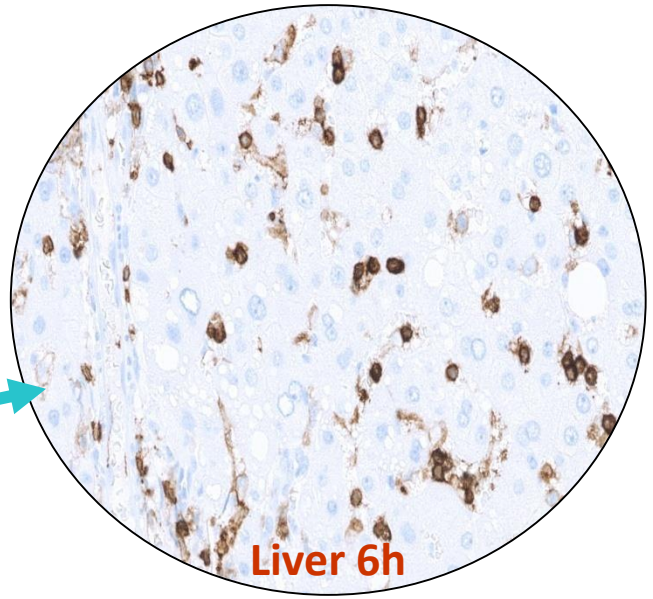
CD52 calibration

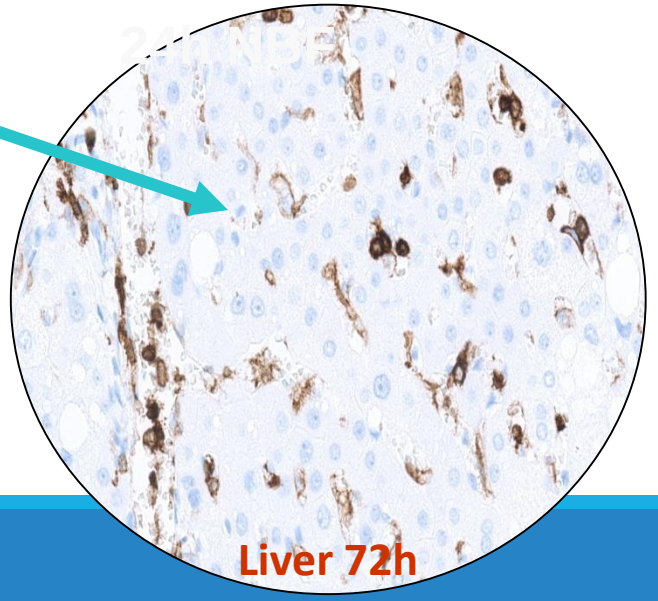
Tissue cores are used to identify best practice protocol providing impact on on pre-analytics

Source:
NordiQC and Aalborg University Hospital

”Poor mans” specificity and pre-analytical TMAs







CD45 calibration

Tissue cores are used to identify best practice protocol providing impact on on pre-analytics

1. Not affected by pre-analytics
2. IHC protocol found
3. *Liver and tonsil as Controls.....???*

Which reaction pattern indicates optimal result?

Source:
NordiQC and Aalborg University Hospital

Test Performance Characteristics - TPCs

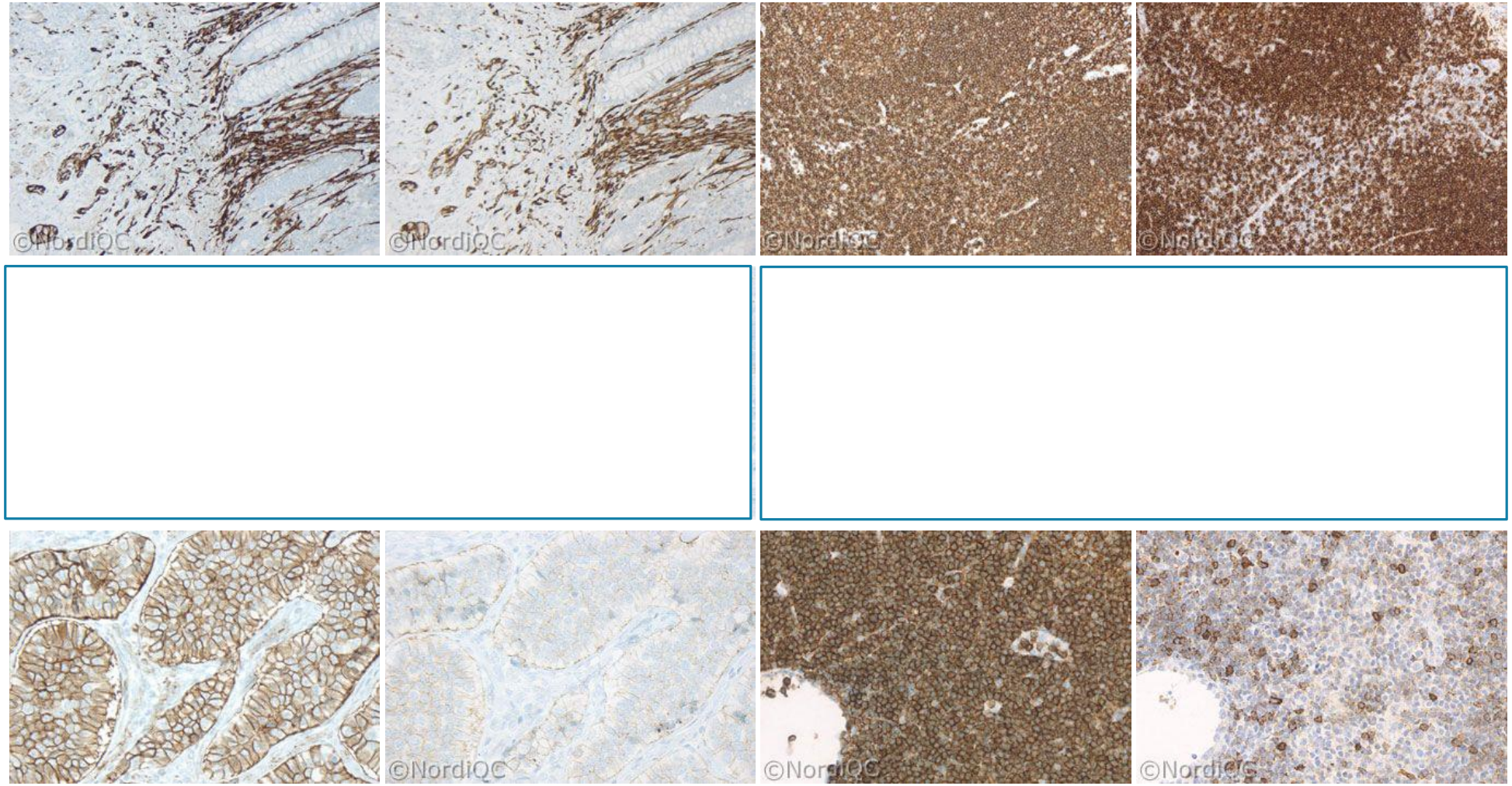
Test performance characteristics;

Which staining pattern characterizes an optimally calibrated IHC assay for a specific purpose?

- Analytical sensitivity
- Analytical specificity
- Precision / reproducibility of IHC assay

Which tissues / cellular structures show the clinical relevant range of the target analyte with focus on required low level of demonstration – **CRITICAL CONTROLS - ICAPCs**?

Fit For Purpose; relevant range of target analyte



CD56

App – Tonsil – Neuroendocrine carc.

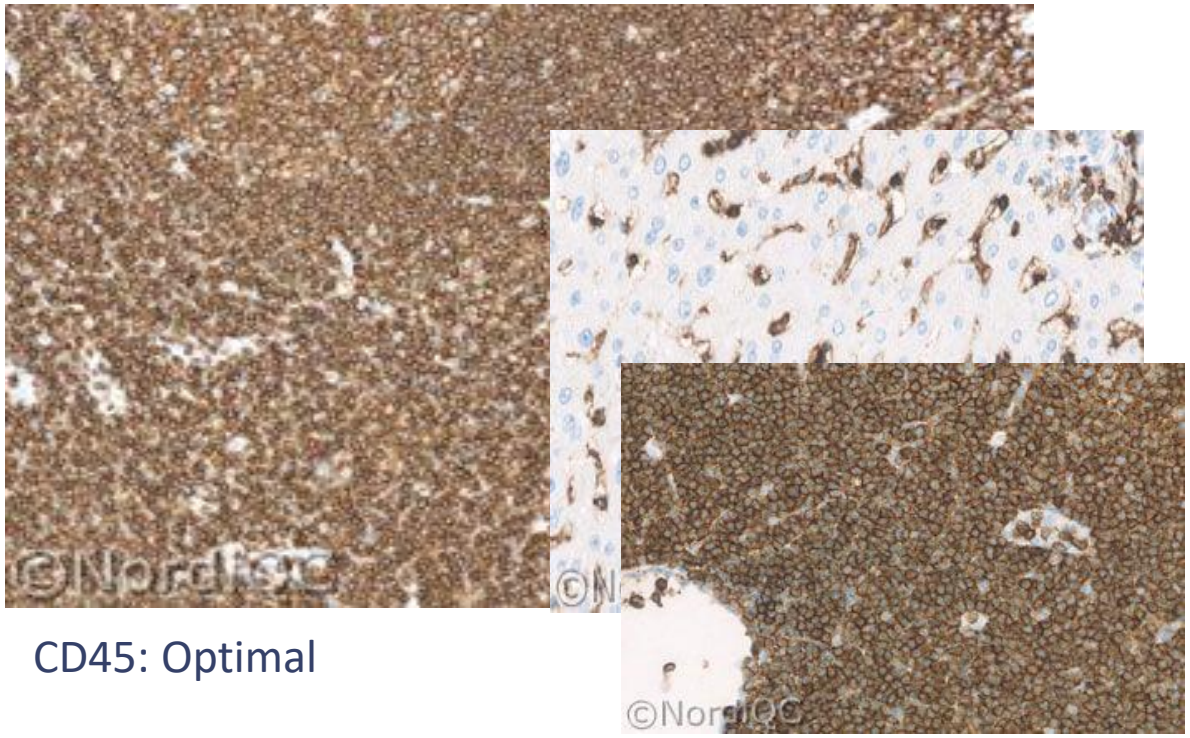
Protocol A Protocol B

CD45

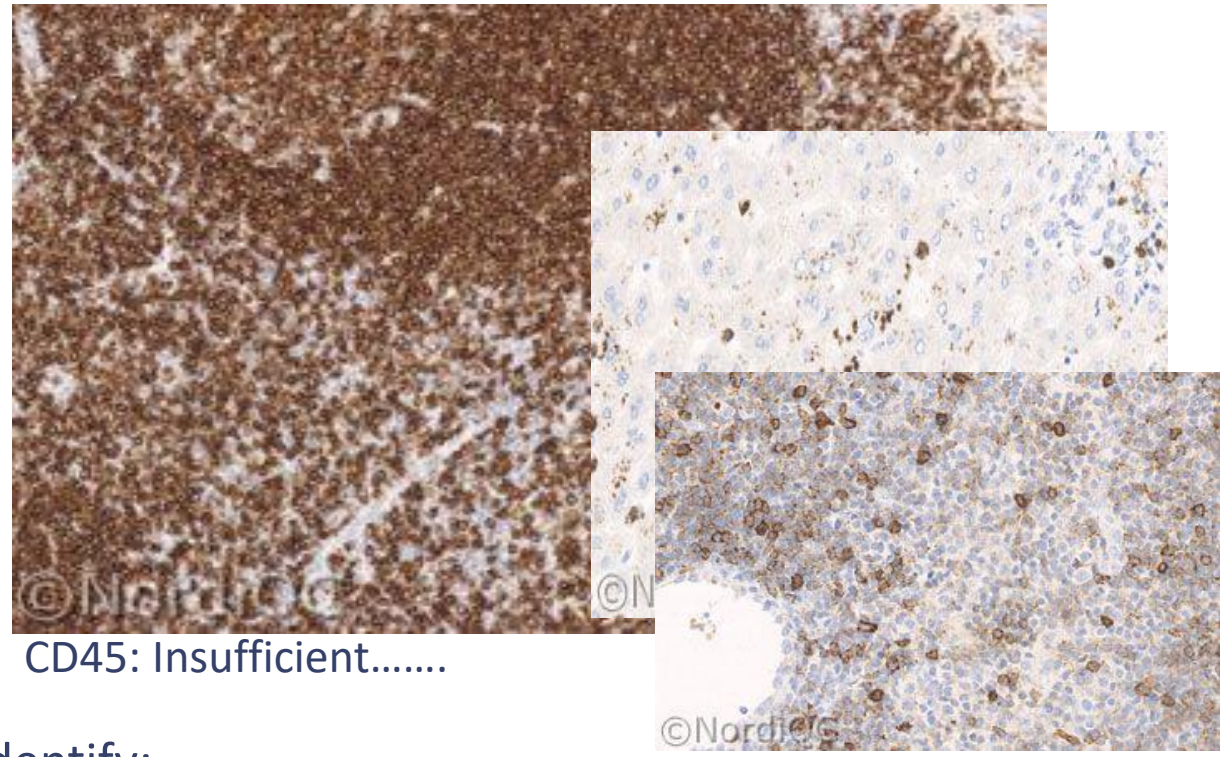
Tonsil – Liver – B-CLL.

Protocol A Protocol B

Fit For Purpose; relevant range of target analyte



CD45: Optimal



CD45: Insufficient…….

Tissues/cells with only high level 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 !

iCAPCs - concept

IHC Critical Assay Performance Controls (iCAPCs)

Which tissues are recommended ?

What is the expected staining pattern ?

Which tissues / cells are critical ?

Right antibody
Appropriate level of sensitivity
Guidance level of specificity

REVIEW ARTICLE

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

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

iCAPCs – potential and use

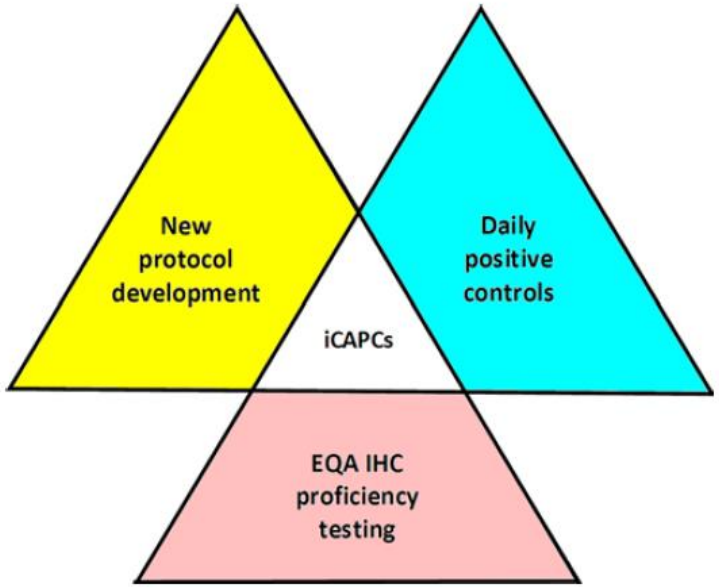


FIGURE 19. The roles of iCAPCs in clinical immunohistochemistry (IHC) laboratories. iCAPCs are an essential part of new protocol development, daily quality controls, and proficiency testing. EQA indicates External Quality Assurance; iCAPC, immunohistochemistry critical assay performance controls.

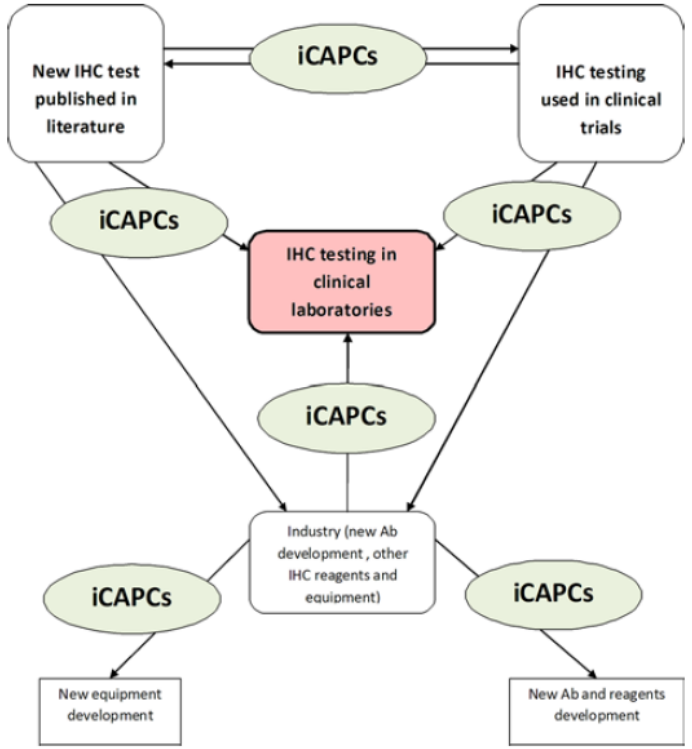


FIGURE 20. iCAPCs and Methodology Transfer. iCAPCs are proposed as important elements for harmonization of immunohistochemistry (IHC) testing between clinical research, product development, and clinical IHC testing. iCAPCs enable IHC harmonization of protocol transfer between research, industry, and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

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

Expected level – calibration
Analytical sensitivity and specificity

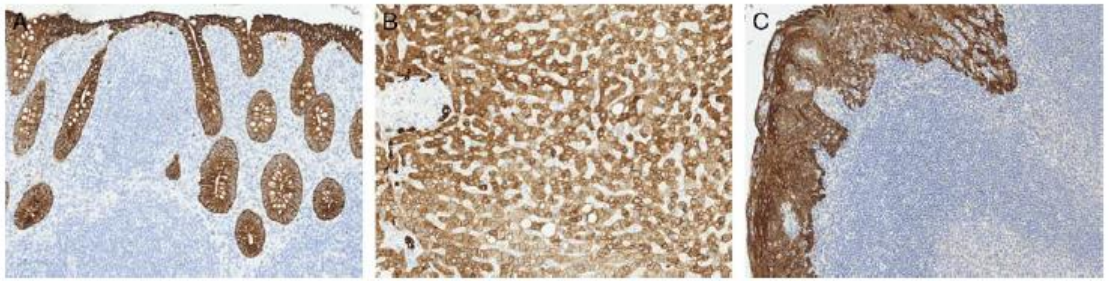


FIGURE 1. Pan-keratin iCAPC. A, Appendix: virtually all columnar epithelial cells must show a moderate to strong predominantly cytoplasmic staining reaction (a membranous accentuation will typically be seen). B, Liver: the vast majority of hepatocytes must show at least weak to moderate cytoplasmic staining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

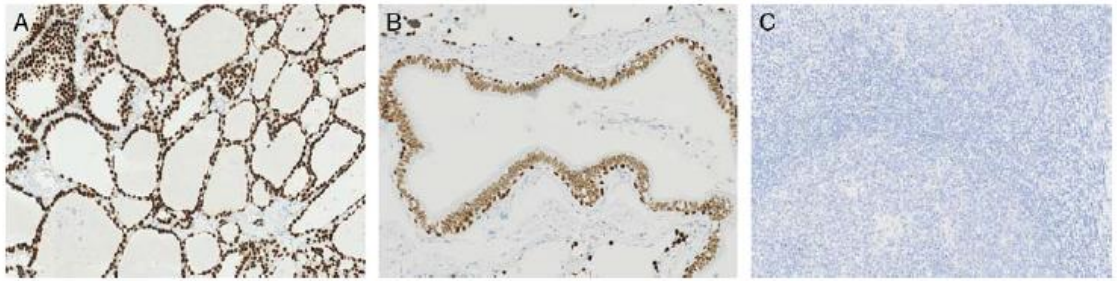


FIGURE 7. TTF-1 iCAPC. A, Thyroid: virtually all epithelial cells must show a strong nuclear staining reaction. B, Lung: virtually all pneumocytes and basal cells of terminal bronchi must show a moderate to strong nuclear staining reaction. Columnar epithelial cells of terminal bronchi must show an at 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.

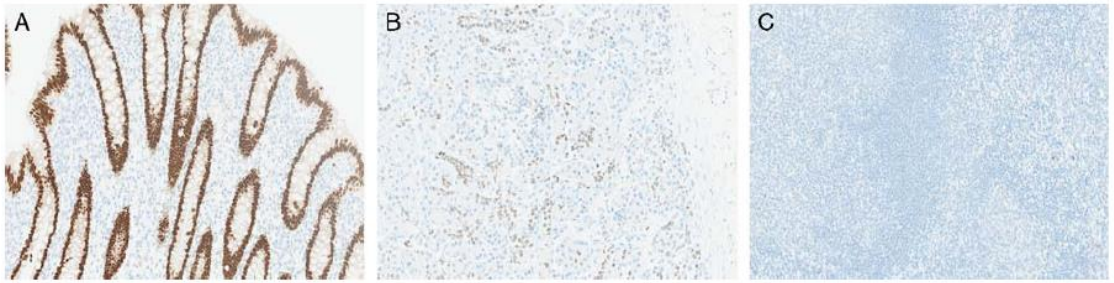


FIGURE 8. CDX-2 iCAPC. A, Appendix: virtually all epithelial cells must show a strong nuclear staining reaction. A weak cytoplasmic staining reaction in addition to strong nuclear staining is often present. B, Pancreas: the majority of epithelial cells of intercalated ducts must show a weak to moderate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

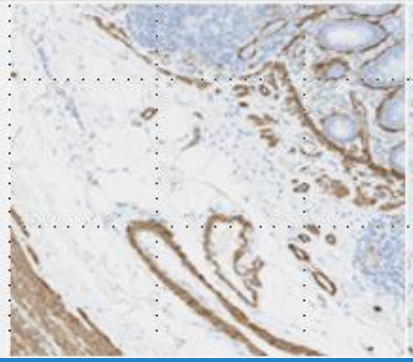
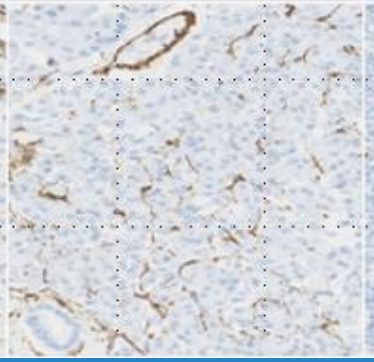
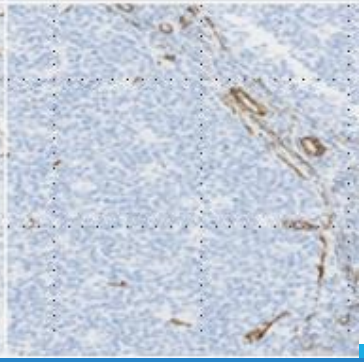
High expression
(Right antibody)

Low expression
(Appropriate sensitivity)

No expression
(Appropriate specificity)

Which tissue
Which cells
Which extension
Which intensity

	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.

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
				

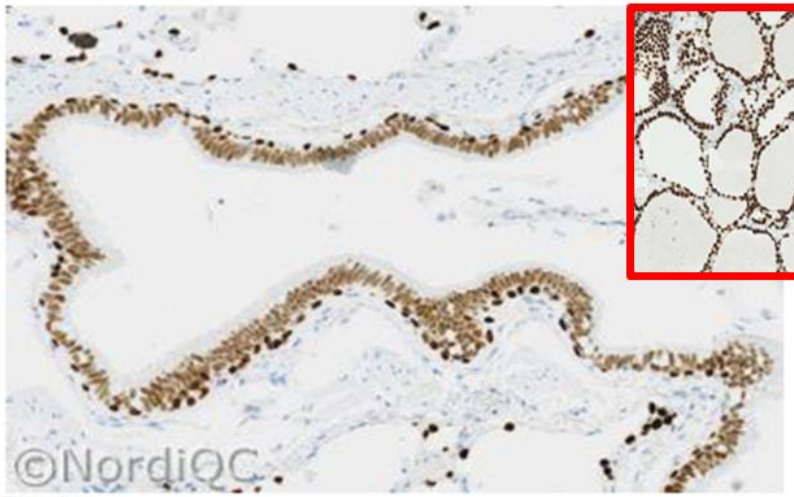


Fig. 2a
Optimal TTF1 staining of the lung using same protocol as in Fig. 1a. The type II pneumocytes and the basal epithelial cells lining the terminal bronchioles show a strong distinct nuclear staining reaction, whereas the columnar epithelial cells show a moderate nuclear staining reaction. No background staining is

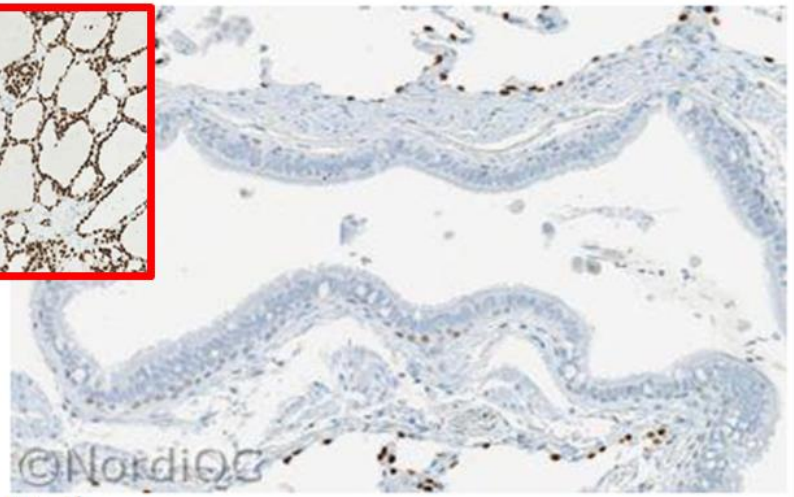


Fig. 2b.
Insufficient TTF1 staining of the lung using same protocol as in Fig. 1b. The type II pneumocytes and the basal epithelial cells lining the terminal bronchioles show only a weak to moderate positive nuclear staining reaction and no reaction is seen in the columnar epithelial cells - same field as in Fig. 2a.

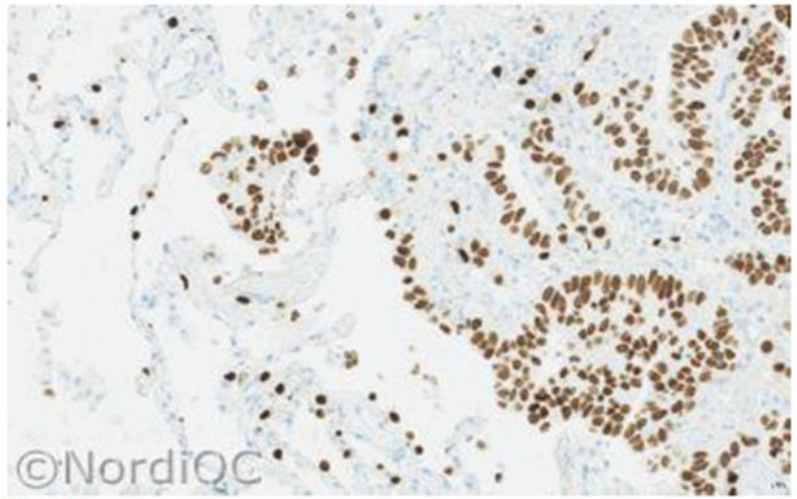


Fig. 4a
Optimal TTF1 staining of the lung adenocarcinoma no. 4 using same protocol as in Figs. 1a, 2a & 3a. Tumour (right side) with adjacent normal lung tissue. Virtually all the neoplastic cells

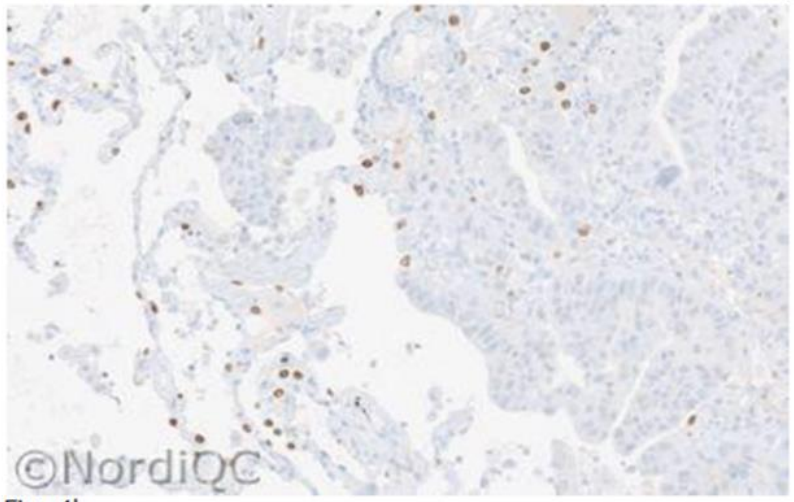


Fig. 4b
Insufficient TTF1 staining of the lung adenocarcinoma no. 4 using same protocol as in Figs. 1b, 2b & 3b. Despite a moderate positive staining reaction in the majority of type II

TTF1

iCAPCs:

Thyroid + lung

Epithelial cells lining terminal bronchi

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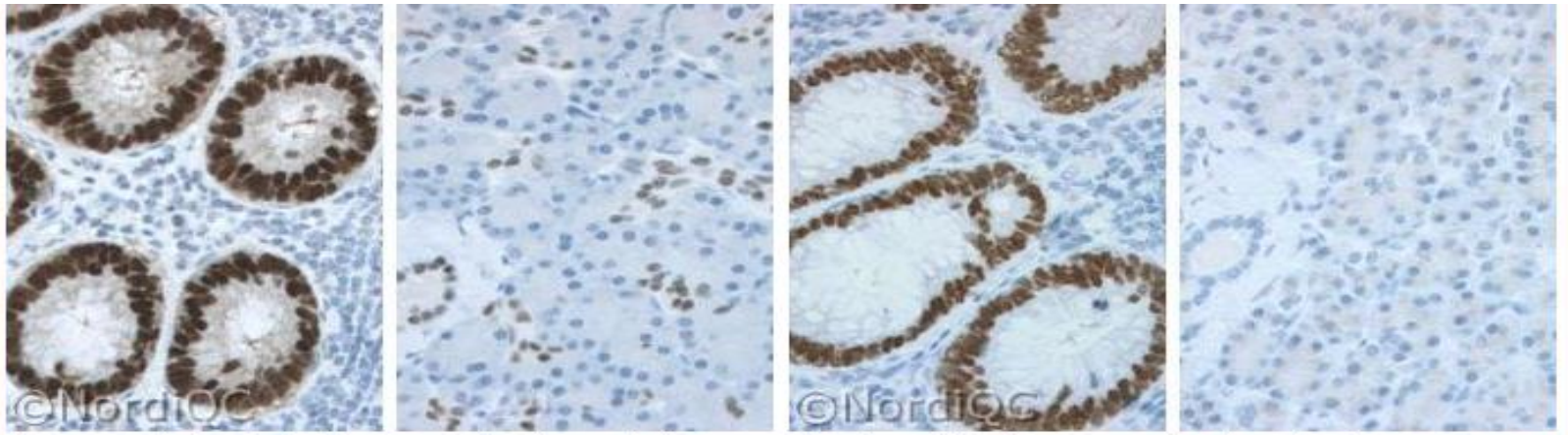

Fig. 1a. Optimal staining for CDX2 using the mAb clone CDX2-88.
Left, colon: A strong nuclear staining is seen in all the enterocytes with a minimal cytoplasmic reaction.
Right, pancreas: A weak to moderate staining is seen in the majority of the ductal epithelial cells.

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.

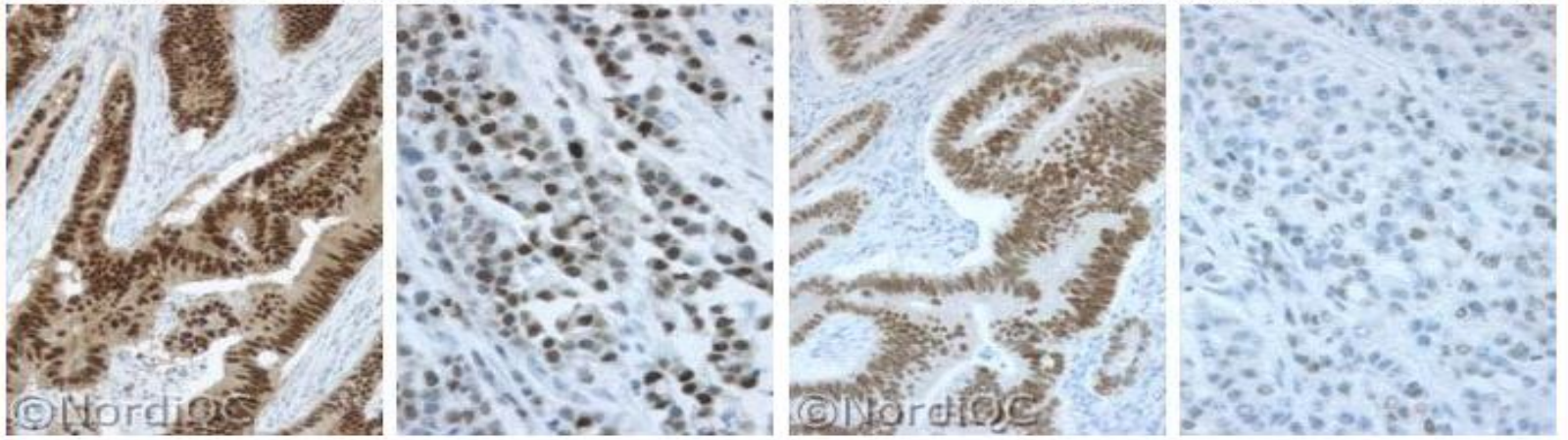

Fig. 2a. Optimal staining for CDX2 using same protocol as in Fig. 1a.
Left: Colon adenocarcinoma with high expression of CDX2: The nuclei of the neoplastic cells show an intense staining while the cytoplasmic compartment is weakly stained.
Right: Colon adenocarcinoma with low expression of CDX2: The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

Colon + pancreas

Pancreatic duct ep. cells

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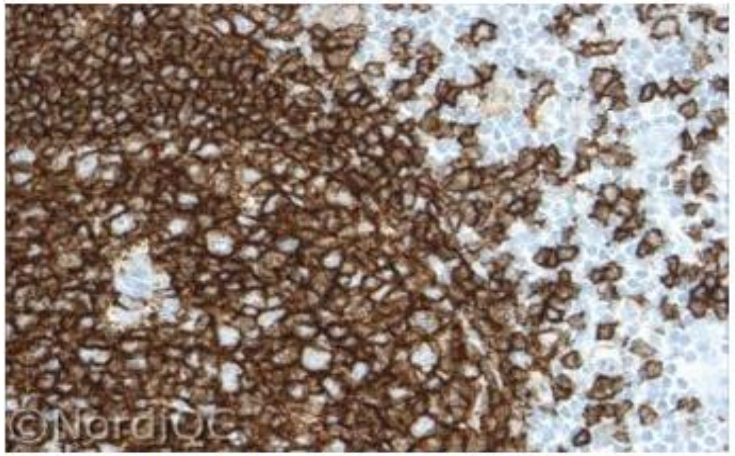

Fig. 1a. Lymphatic tissue in the appendix showing an optimal staining reaction for CD20 using the mAb clone L26 in a RTU format on the BenchMark platform. HIER was performed using Cell Conditioning 1. A very strong membranous staining reaction is seen in virtually all the B-cells.

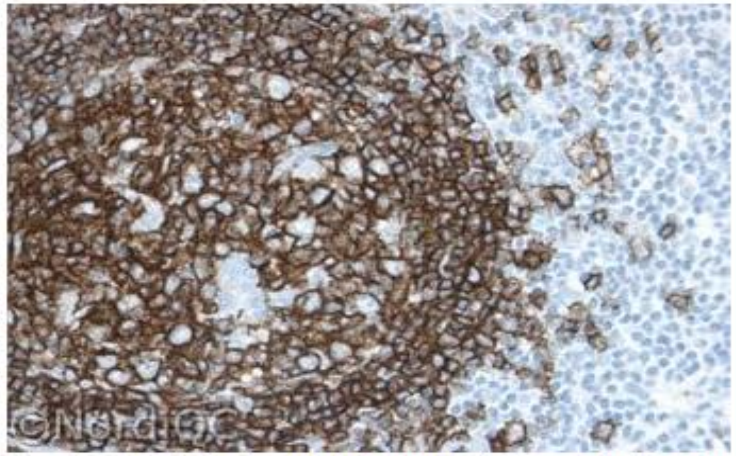

Fig. 1b. Lymphatic tissue in the appendix. Same field as in Fig. 1a. Insufficient staining for CD20 using the mAb clone L26 in a RTU format at the BenchMark platform. No HIER was performed. A moderate to strong staining reaction is seen in virtually all the B-cells. The normal B-cells are high expressors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

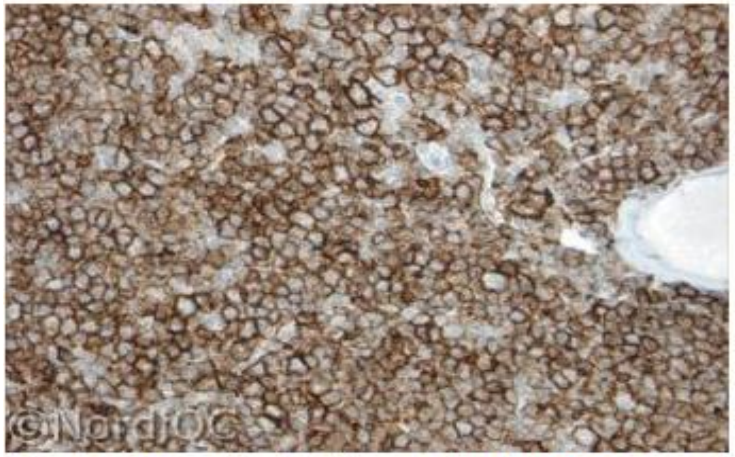

Fig. 2a. B-CLL. Optimal staining reaction for CD20. Same protocol as in Fig. 1a. A moderate to strong membranous staining is seen in virtually all the neoplastic cells.

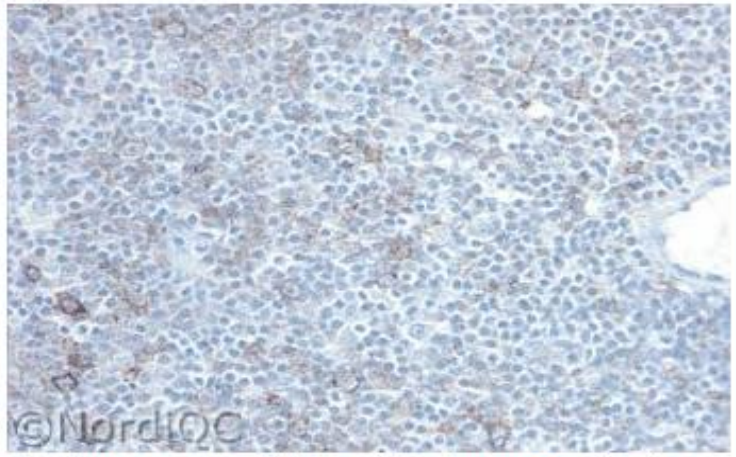

Fig. 2b. B-CLL. Insufficient staining for CD20 using the same protocol as in Fig. 1b. Omitting HIER, only scattered cells are positive. The majority of the neoplastic cells are negative. Compare with the optimal result in Fig. 2a, same field.

CD20:

iCAPCs: ????

Tonsil;

B-cells to be ASAP….

As strong as possible…

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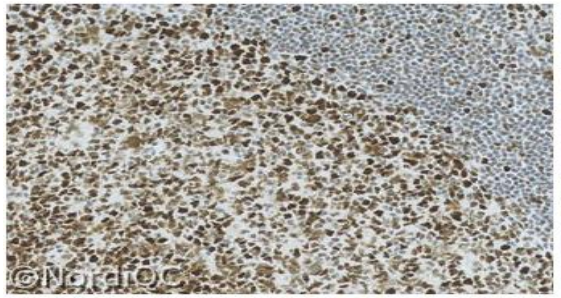


Fig. 1a. Optimal staining for MSH6 of the tonsil using the rmAb clone EP49 optimally calibrated, HIER in an alkaline buffer and a 3-step polymer based detection system.
Virtually all the mantle zone B-cells show a distinct, moderate to strong nuclear staining, while the germinal centre B-cells show a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

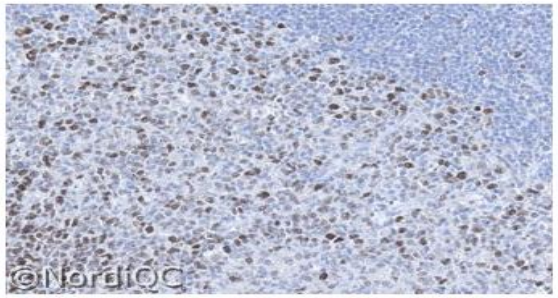


Fig. 1b. Insufficient staining for MSH6 of the tonsil using the mAb clone 44. by a protocol with a too low sensitivity (2-step polymer and too low. conc. of the primary Ab), same field as in Fig. 1a.
Only the germinal centre B-cells are demonstrated, while the mantle zone B-cells expressing limited MSH6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

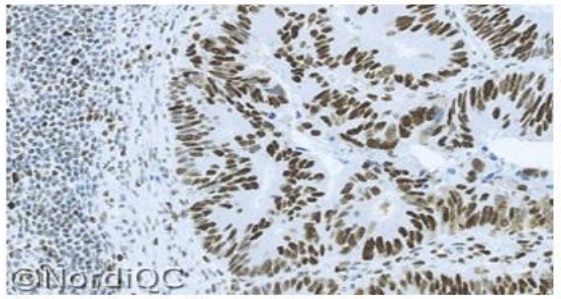


Fig. 2a. Optimal staining for MSH6 of the colon adenocarcinoma no. 3 with intact MSH6 protein using same protocol as in Fig. 1a.
The majority of the epithelial and the stromal cells show a moderate to strong nuclear staining. No background staining is seen.

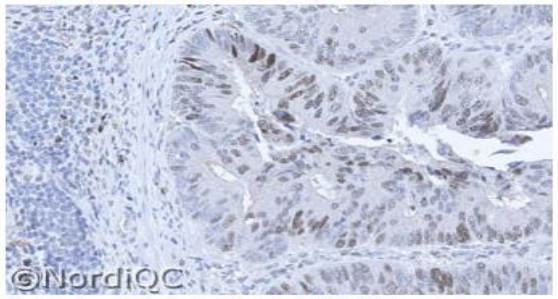


Fig. 2b. Insufficient staining for MSH6 of the colon adenocarcinoma no. 3 with intact MSH6 protein using same protocol as in Fig. 1b., same field as in Fig. 2a.
The proportion of positive cells and the intensity of the staining reaction is significantly reduced compared to the result in Fig. 2a.
Also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

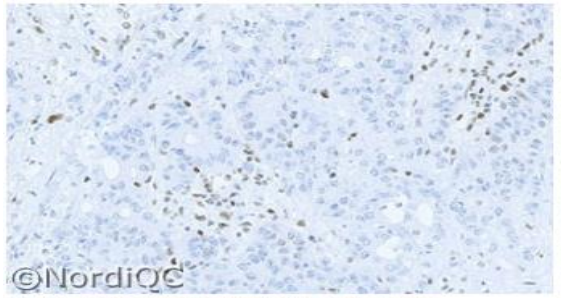


Fig. 3a. Optimal staining for MSH6 of the colon adenocarcinoma no. 5 with loss of MSH6 protein using same protocol as in Figs. 1a. & 2a.
The neoplastic cells are negative, while the remnants of entrapped lymphocytes and stromal cells show a distinct nuclear staining, serving as internal positive control.

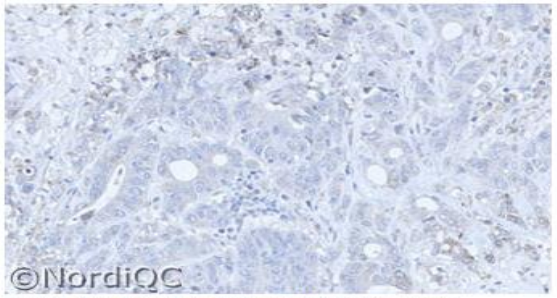


Fig. 3b. Insufficient staining for MSH6 of the colon adenocarcinoma no. 5 with loss of MSH6 protein using same protocol as in Figs. 1b. & 2b., same field as in Fig. 3a.
No nuclear staining reaction is seen in the neoplastic cells, but as virtually no nuclear staining reaction is seen in the normal cells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

MMR:

iCAPCs:

External tissue control

Mantle zone B-cells in tonsil

Assay run-to-run consistency

Internal tissue control

Stromal cells!!

www.nordiqc.org

”Poor mans” specificity and pre-analytical TMAs

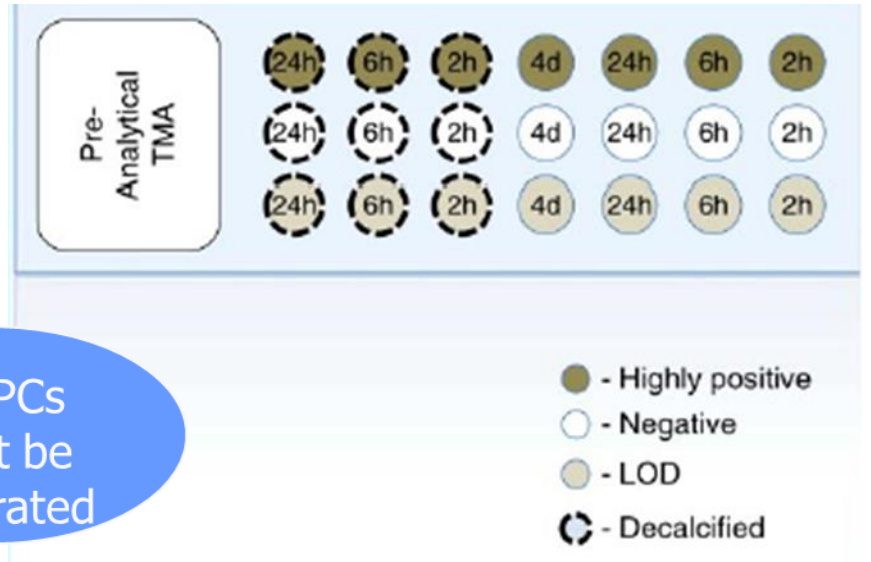
Technical test array

1. Calibration
2. Robustness

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

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

ICAPCs must be integrated

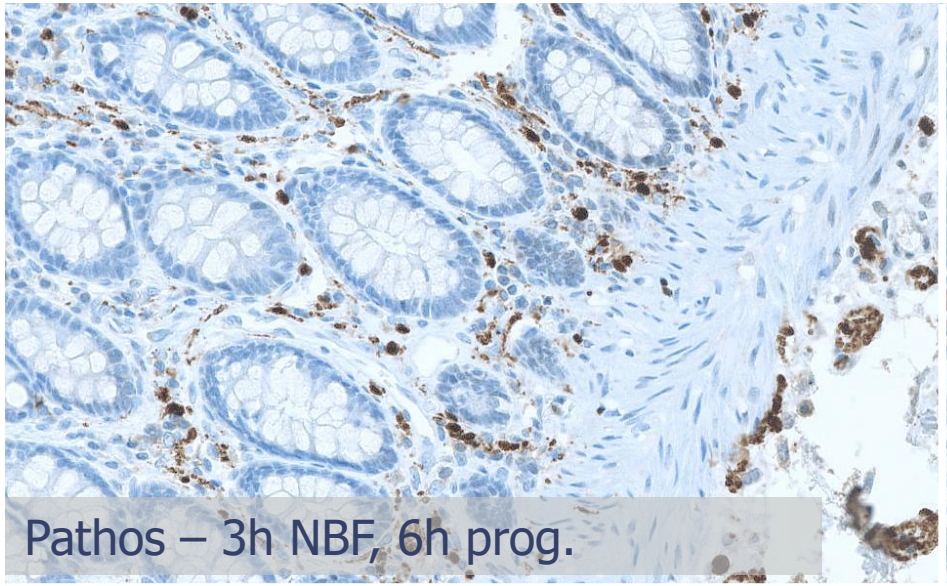


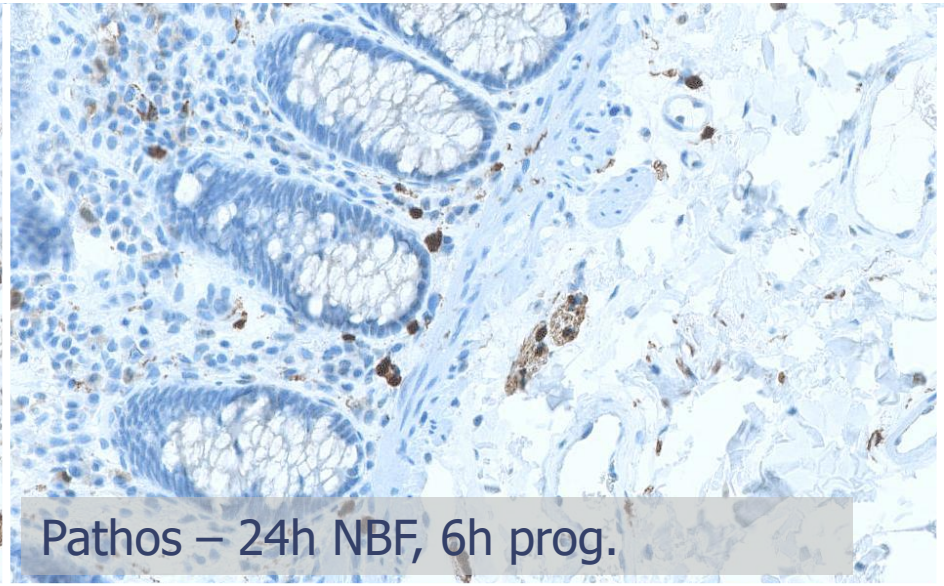
ICAPCs must be integrated

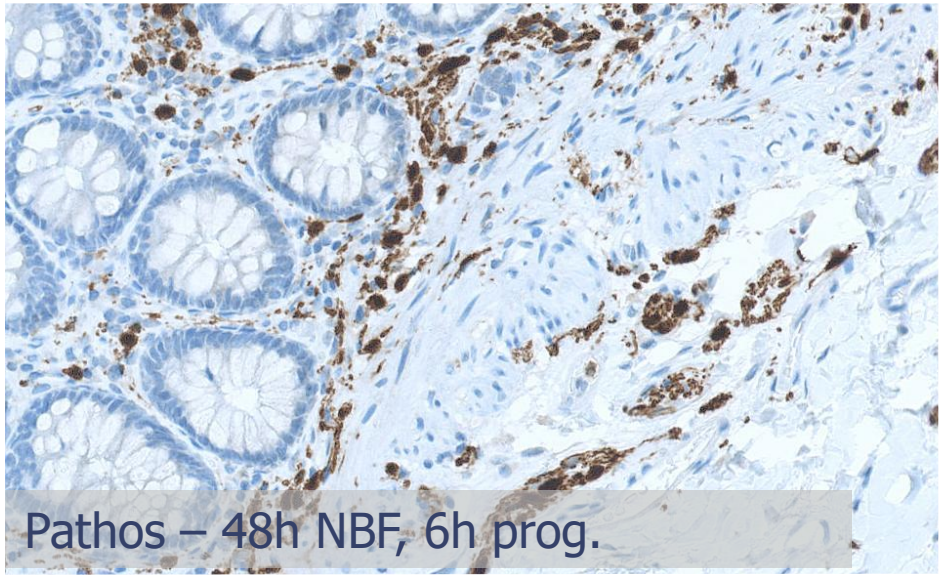
”Poor mans” specificity and pre-analytical TMAs

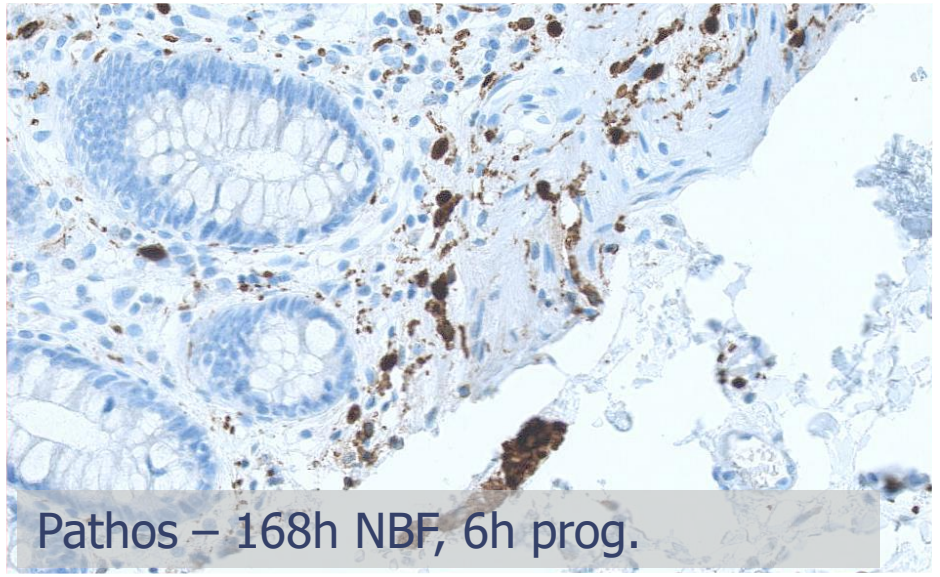
Colon:

S100, polyclonal


Pathos – 3h NBF, 6h prog.


Pathos – 24h NBF, 6h prog.


Pathos – 48h NBF, 6h prog.

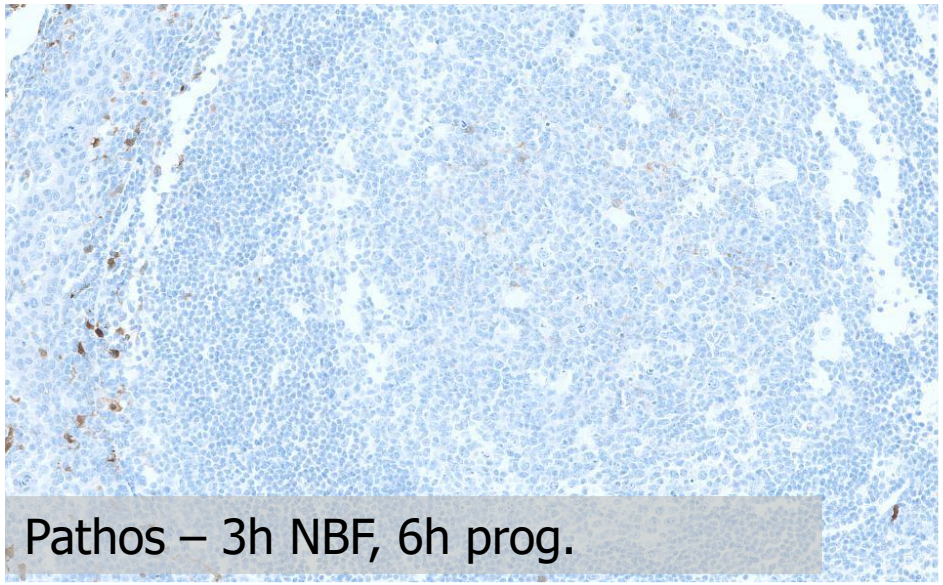

Pathos – 168h NBF, 6h prog.

”Poor mans” specificity and pre-analytical TMAs

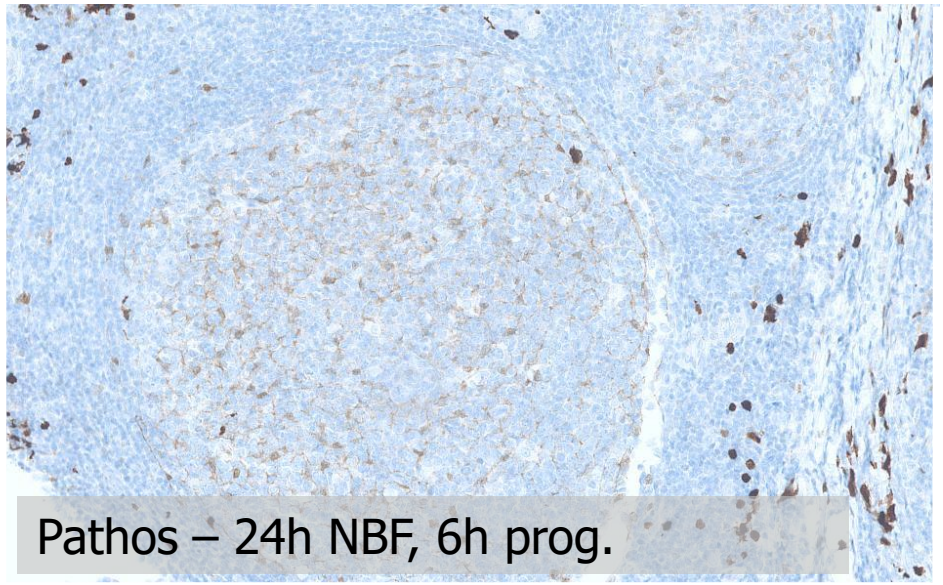
ICAPCs

Tonsil:

S100, polyclonal



Pathos – 3h NBF, 6h prog.



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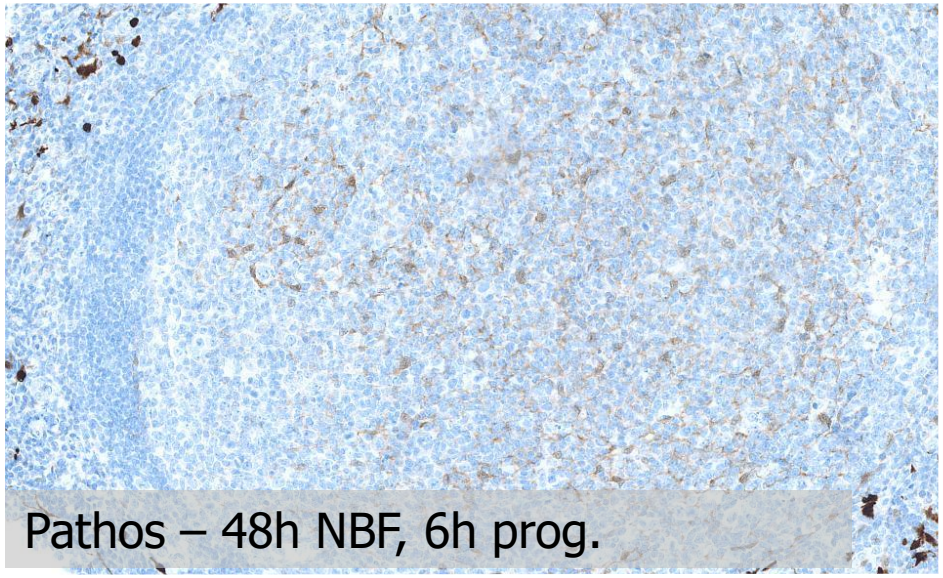

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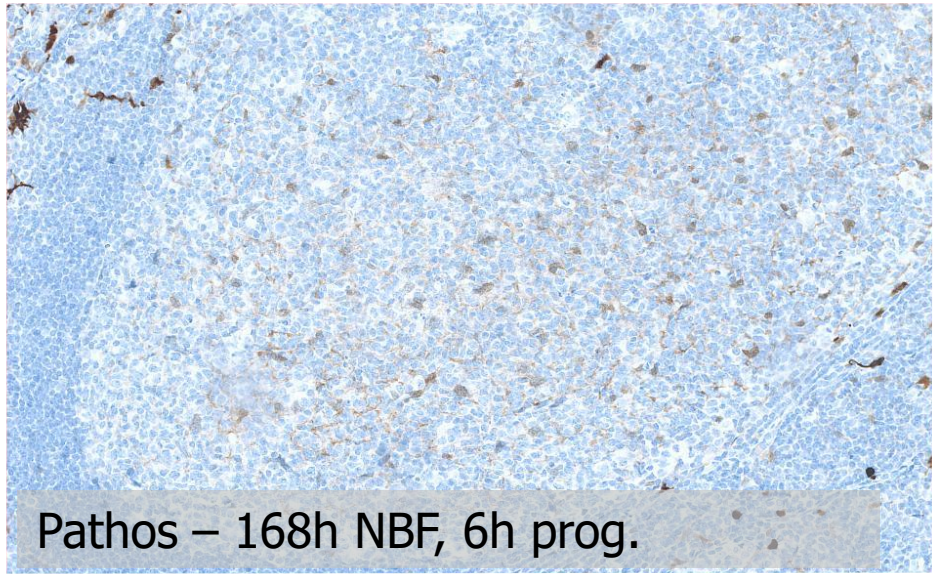


Pathos – 168h NBF, 6h prog.

Main elements to develop & validate IHC assays

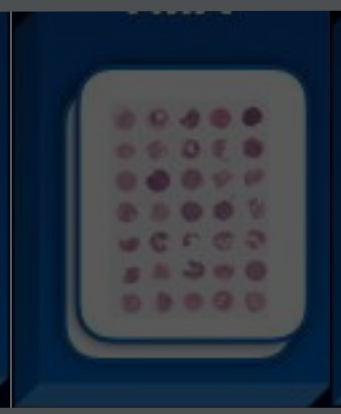
The journey from an antibody to a diagnostic IHC assay with a specific purpose

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

Based on selection and use of appropriate external tissue controls

External tissue control tool box

E Torlakovic et al. AIMM, 2017; 25:227-230
Evolution of Quality Assurance for Clinical Immunohistochemistry in the Era of Precision Medicine: Part 4

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 Test performance characteristics	Fixation time Fixative(s) Decalcification	Range of relevant expression levels	Range of relevant expression levels	Reproducibility
			With expression	High expression Low expression	Method of transfer proof
High expression Low expression No expression	With expression No expression		No expression	No expression	
			20/40 of each Type I/II IHC	+ relevant cut-off	

*Immunohistochemical critical assay performance controls

Sample sets for technical / analytical validation of IHC

- Technical / 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 perhaps less important compared to use of tissue with full range of expression patterns reflecting the diagnostic use and purpose of test

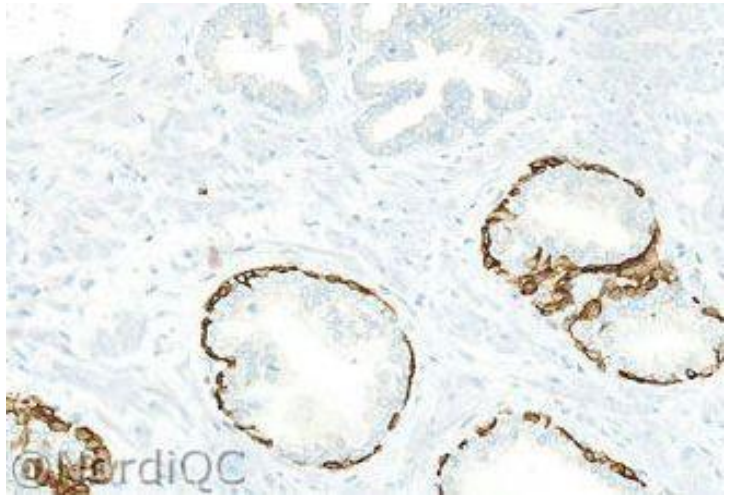
Identification of purpose of the test

E Torlakovic et al. AIMM 2017;25:4-11
Evolution of Quality Assurance for Clinical Immunohistochemistry in the Era of Precision Medicine: Part 1

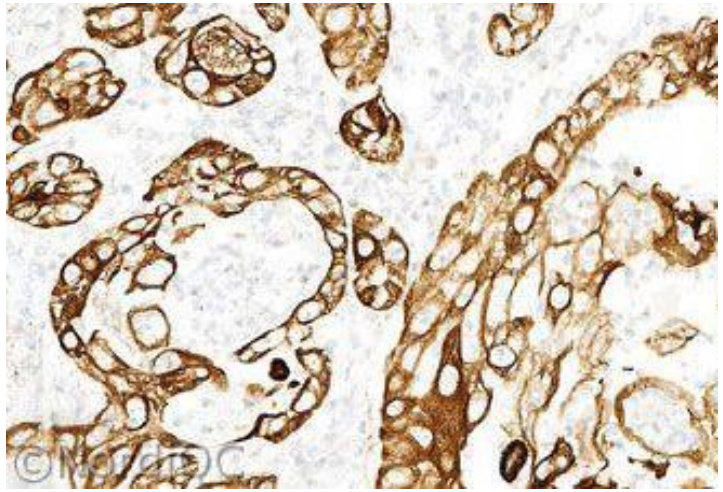
An IHC assay can have one or more purposes and it is crucial to secure the need is fulfilled

IHC for CK5

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



Prostate sample



Lung sample

Same protocol applied for different purposes and meeting the requirements

(source; www.nordiqc.org)

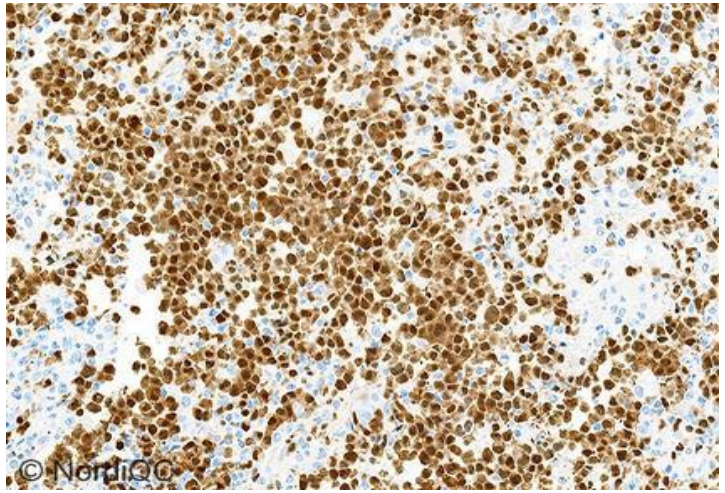
Identification of purpose of the test

E Torlakovic et al. AIMM 2017;25:4-11
Evolution of Quality Assurance for Clinical Immunohistochemistry in the Era of Precision Medicine: Part 1

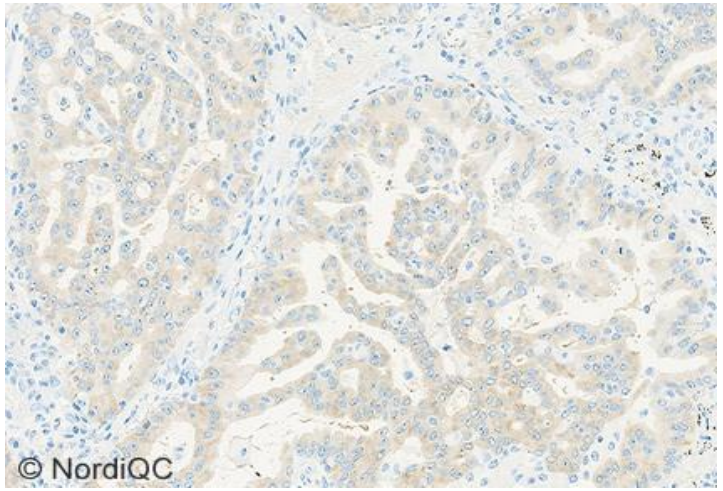
An IHC assay can have one or more purposes and it is crucial to secure the need is fulfilled

IHC for ALK

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



ALCL



Lung ad. carc + EML-ALK mutation

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

(source; www.nordiqc.org)

Identification of purpose of the test

E Torlakovic et al. AIMM 2017;25:4-11
Evolution of Quality Assurance for Clinical Immunohistochemistry in the Era of Precision Medicine: Part 1

CK5 for prostate Dx

Purpose 1 / Test 1

Protocol 1

CK5 for carcinoma subtyping

Purpose 2 / Test 2

Protocol 1

Match of
1 Protocol for 2 Purposes

ALK for ALCL

Purpose 1 / Test 1

Protocol 1

ALK for Critonib/RX in lung DX

Purpose 2 / Test 2

Protocol 1

Mis-match of
1 Protocol for 2 Purposes

Purpose 1 / Test 1

Protocol 1

Purpose 2 / Test 2

Protocol 2

Match of
2 Protocols for 2 Purposes

Identification of purpose of the test

E Torlakovic et al. AIMM 2017;25:4-11
Evolution of Quality Assurance for Clinical Immunohistochemistry in the Era of Precision Medicine: Part 1

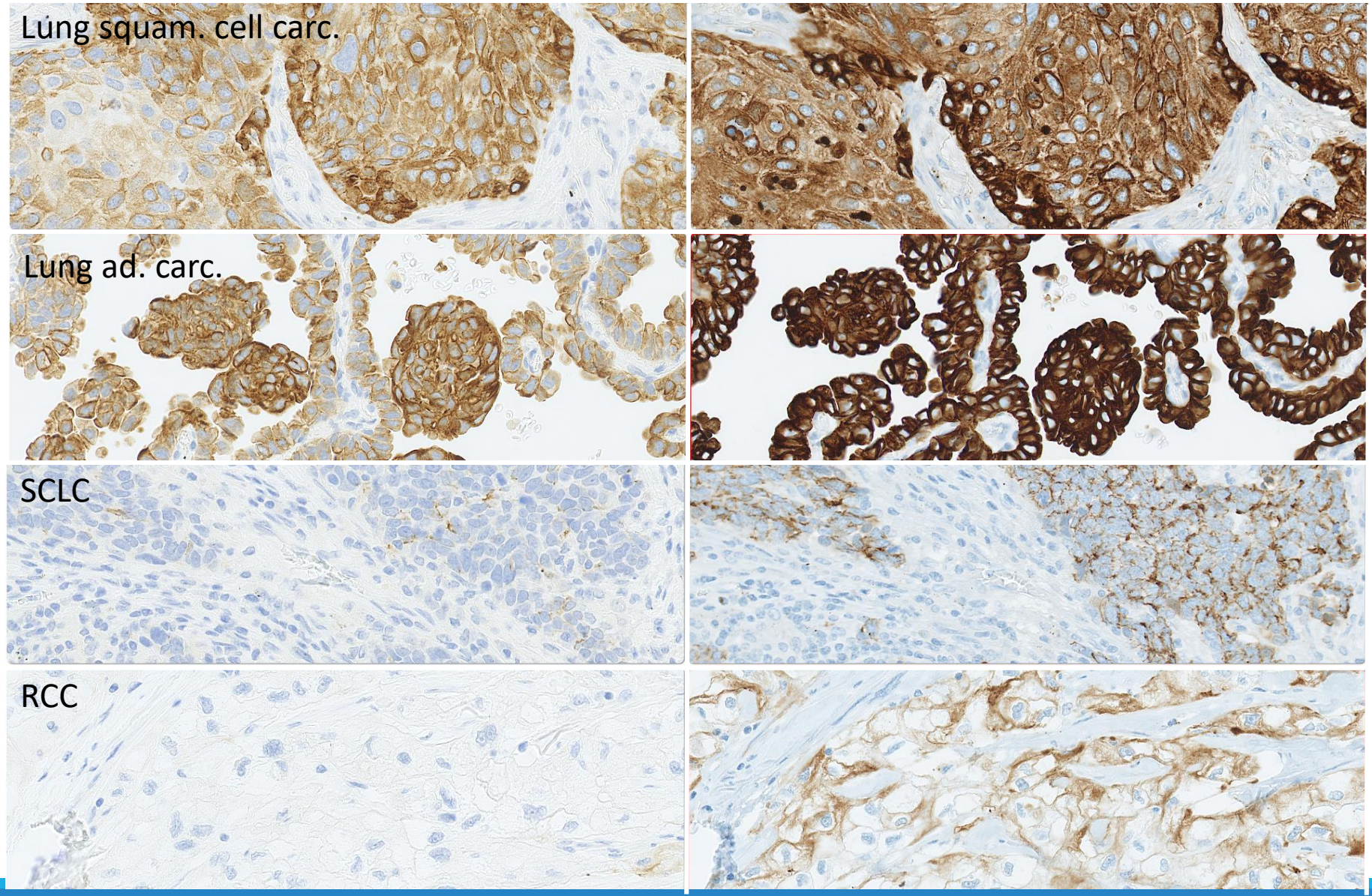
An IHC assay can have one or more purposes and it is crucial to secure the need is fulfilled

	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
GATA3	Breast carcinoma – CUP*	Urothelial carcinoma - CUP	
IgK / IgL	Clonality myeloma (Cytopl)	Clonality lymphoma (Membrane)	
Melan A	Melanoma	Sex cord tumours	(mAb A103)
PAX5	B-cell lineage marker (Lymphoma)	Hodgkin	
.....			

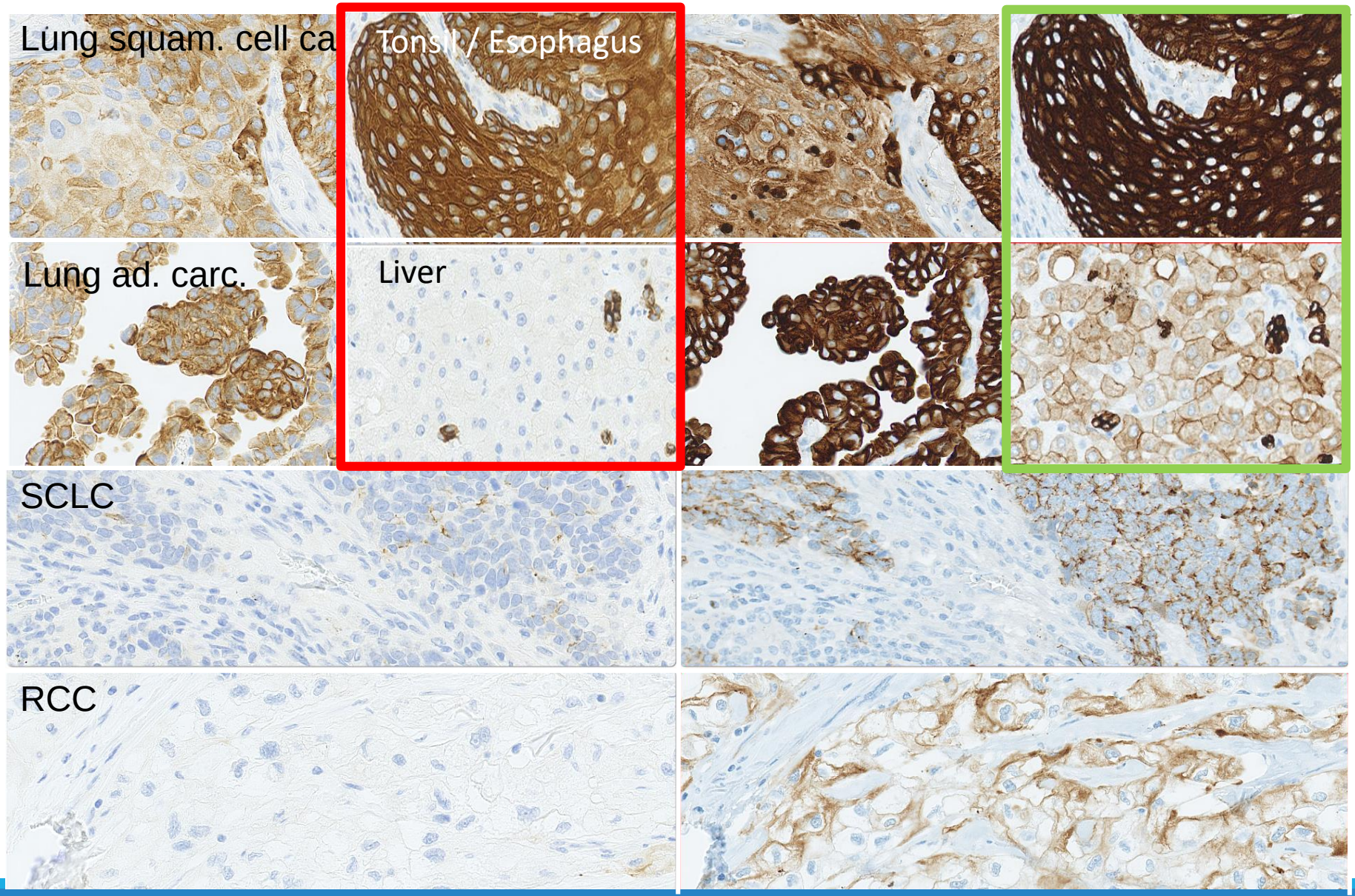
In addition an extensive range within same purpose can be seen....
E.g. Pan-CK for carcinoma identification (primary panel)

* CUP= Cancer Unknown Primary

Use of samples for technical / analytical validation of IHC



Use of samples for technical / analytical validation of IHC



Identification of purpose of the test

E Torlakovic et al. AIMM 2017;25:4-11
Evolution of Quality Assurance for Clinical Immunohistochemistry in the Era of Precision Medicine: Part 1

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

	Purpose I	Purpose II	Influenc. factors
CK-Pan	CUP - carcinoma lineage	Sentinel node – carcinoma metastatis	Clone, titer, retrival
CK 19	Sentinel node – carcinoma metastatis	Thyroid adenoma vs carcinoma	Titer, retrieval
EPCAM	CUP - carcinoma lineage	Lung carcinoma vs mesothelioma	Titer, retrieval
TTF1	CUP - lung adenocarcinoma	Lung adenocarcinoma vs squam.	Clone, titer
…….			

High analytical sensitivity can compromise clinical utility…..

Protocol developed, optimized and validated for purpose I will most likely compromise use for purpose II due to reduced analytical selectivity and specificity

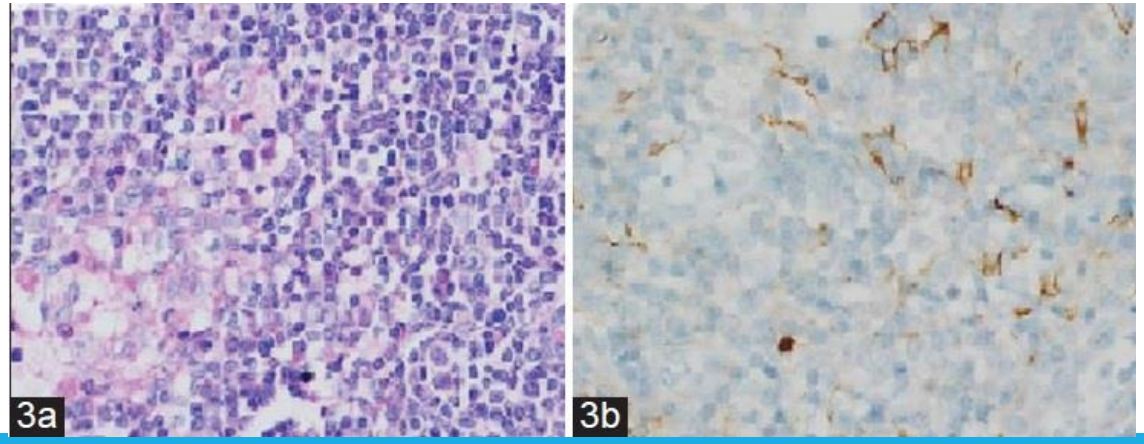
Protocol developed, optimized and validated for purpose II will most likely compromise use for purpose I due to a reduced level of analytical sensitivity

Identification of purpose of the test

Sensitivity , specificity – what to choose…?

	Purpose I	Purpose II	Influenc. factors
CK-Pan	CUP - carcinoma lineage	Sentinel node – carcinoma metastatis	Clone, titer, retrival
CK 19	Sentinel node – carcinoma metastatis	Thyroid adenoma vs carcinoma	Titer, retrieval

Jacob PM, Nair RA, Nair SP, Jayasudha A V.
Cytokeratin-positive interstitial reticulum cells in the lymph node:
A potential pitfall. Indian J Pathol Microbiol 2016;59:128-9



CK-Pan e.g. Clone AE1/AE3 with HIER

Can and will provide interpretational challenges in SN due to labelling of specialized macrophages with CK8/18

CK19 more selective
(CK19 mRNA applied for OSNA technique)

Conclusions for technical / analytical validation of IHC

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.

Challenges for technical / analytical validation of IHC

1. Limited access to relevant tissues – rare incidences
   - ALK (lung), ROS1

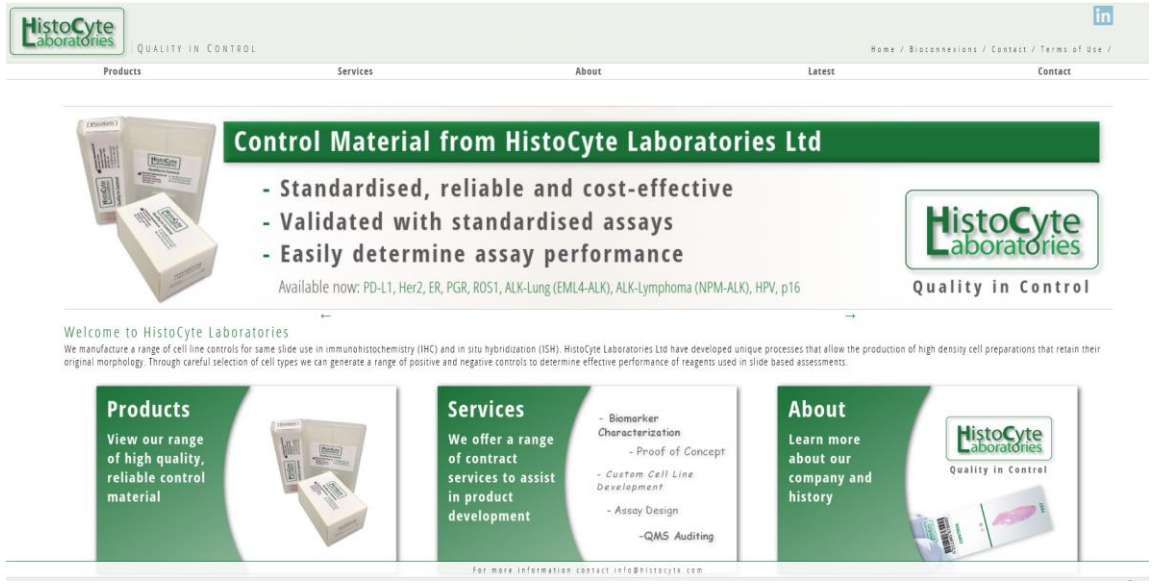
2. New markers not described in details – no data on test performance characteristics
   - SATB2, Claudin-4

3. Limited access to reference material and/or non-IHC method to monitor quality
   - PD-L1 IHC

Role of cell lines & histoids for IHC test development

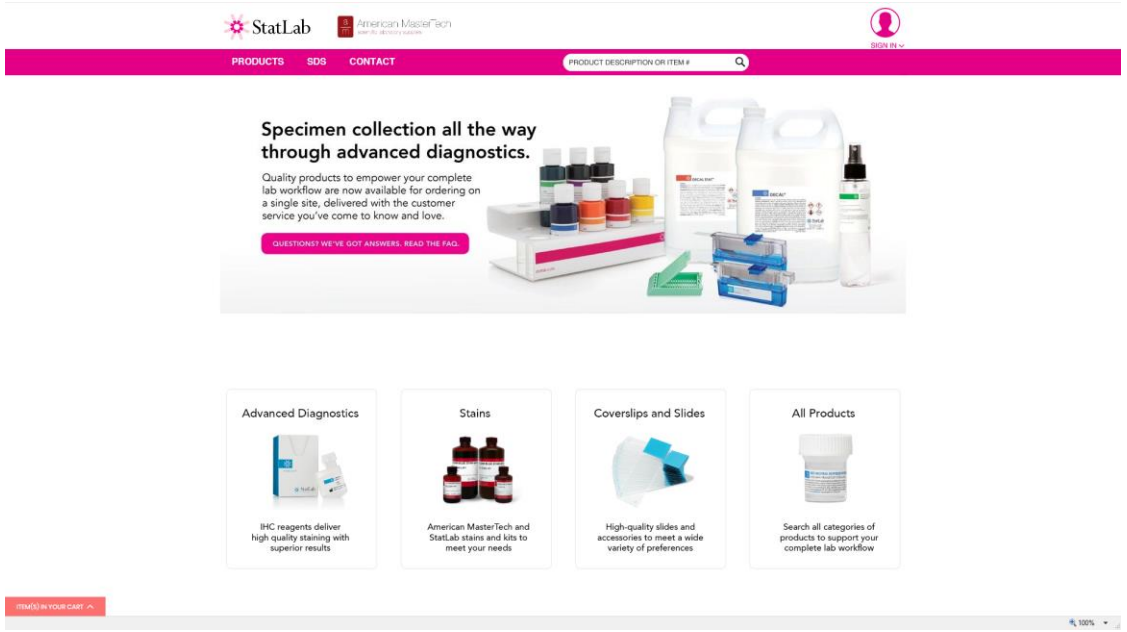
Starting help to guide development – validation still required….

1. Limited access to relevant tissues – rare incidences
   - ALK (lung), ROS1



www.histocyte.com

Cell lines
ALK and ROS1 being +/-
HER2, PD-L1 with dynamic range

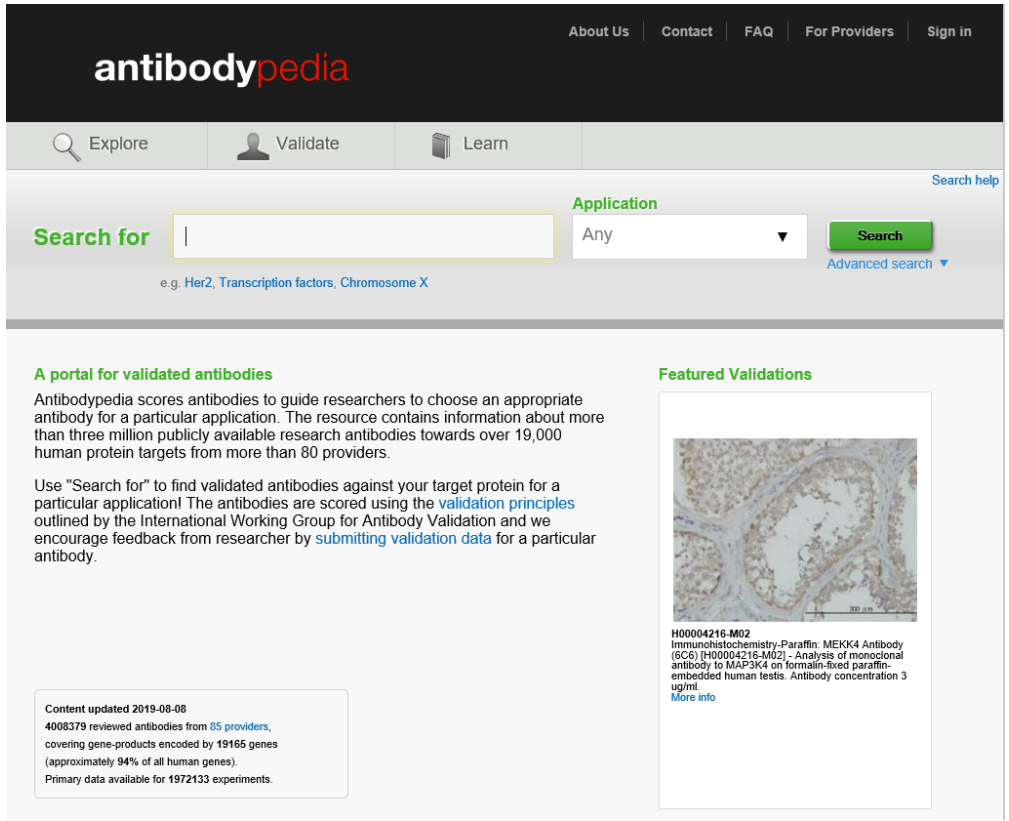


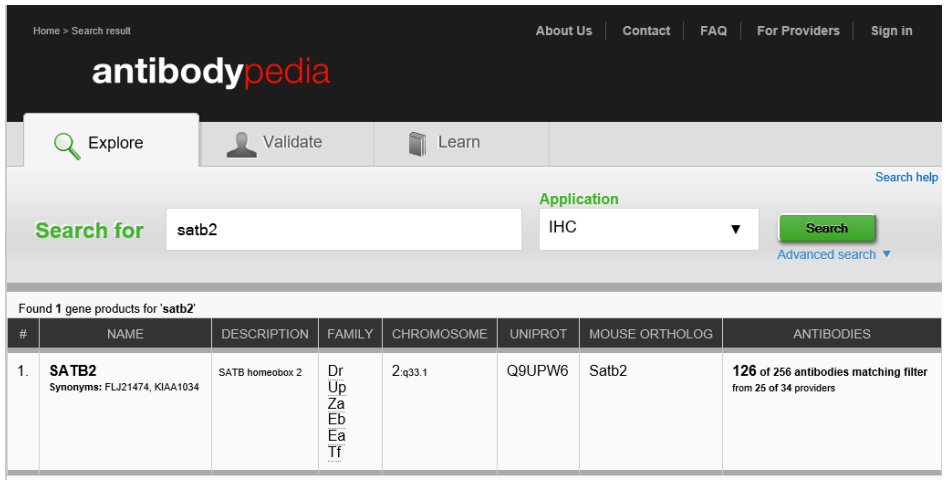
www.statlab.com

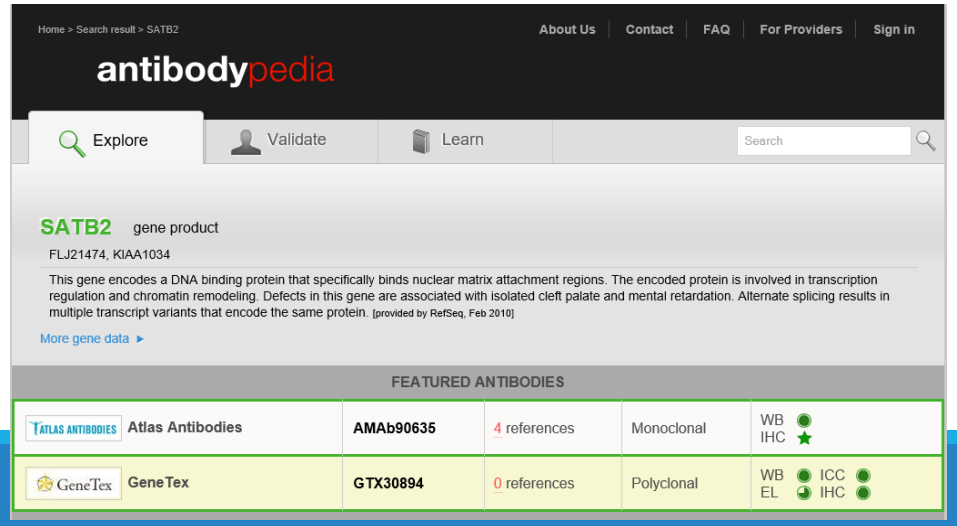
Histoids / Faux tissue
ALK +/-
HER2, PD-L1 with dynamic range

Online ressources – “www.antibodypedia.com”

2. New markers not described in details – no data on test performance characteristics
   - SATB2, Claudin-4







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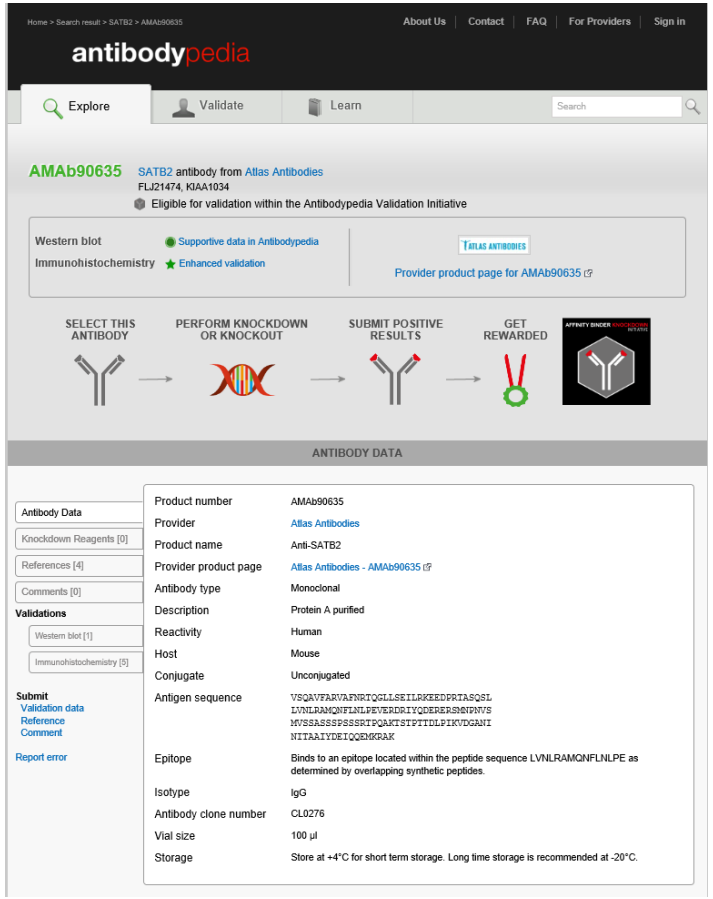
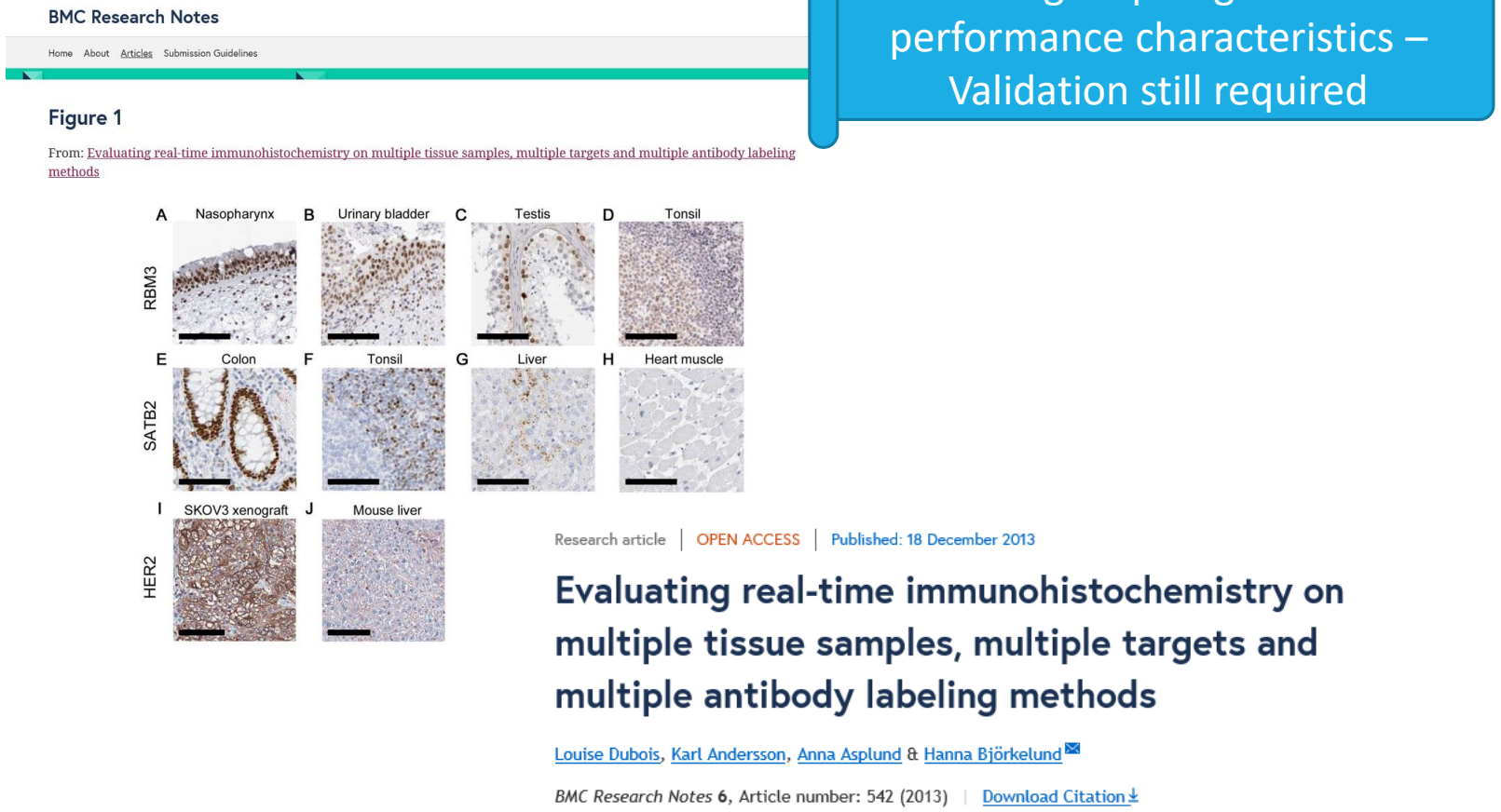




Starting help to guide test performance characteristics – Validation still required

Role of non-IHC methods to guide quality / accuracy

Target	Treatment	Indication	Test methods
HER2	Herceptin	Breast C. GI cancer	IHC ISH
ALK ROS1	Crizotinib	NSCLC	IHC ISH NGS
PD-1 CD274	Keytruda	NSCLC Urothelial Head & Neck	IHC; PD-L1

Considerations

- Predictable value - responders
- Clinical accuracy – confidence
- Test commercially available
- Test complexity – to perform
- Test complexity – to analyze
- Test turn-around-time
- Tissue sample size / type
- Number of relevant targets
- .....

*Only indicative overview and does not reflect any approved regulatory status or guidelines

The challenge to validate a PD-L1 assay

E Torlakovic et al. AIMM 2017;25:151-159
Evolution of Quality Assurance for Clinical Immunohistochemistry in the Era of Precision Medicine: Part 3

44 - 62% of the participants in NordiQC PD-L1 IHC runs C1 – C4 used LDT's

How is correct accuracy identified? – do I identify right proportion of pos / neg tumours?

How is correct index identified? - do I identify tumours with the clinical relevant range from weak to strong?

By access to reference material (e.g. slides / tumours) tested with validated IHC assay

By access to second line non-IHC test as ISH to confirm accuracy of LDT

The central challenges to meet

*E Thunissen et al.
Lung cancer 113 (2017) 102-105
PD-L1 IHC in NSCLC with a global and methodological perspective

*E Thunissen et al.
Arch Pathol Lab Med. 2017-0106-SA
doi: 10.5858/arpa.

**E Torlakovic et al. AIMM 2017;25:151-159
Evolution of Quality Assurance for Clinical Immunohistochemistry in the Era of Precision Medicine: Part 3

As outlined in the references:

1. Identify the purpose of the test – E.g. detection of PD-L1 in NSCLC for KEYTRUDA® decision
2. Develop in-house IHC (LDT) – E.g. using tissue tool-box
3. Perform IHC with reference test – 40* – 100** NSCLCs tested with validated PD-L1 test
4. Perform IHC with developed LDT – Same 40* – 100** NSCLCs
5. Use relevant cut-off's for treatment – 0%, ≥1%, ≥50%
6. Analyze concordance – ≥90% then LDT is validated (if <90% LDT must be recalibrated)
7. Assay reproducibility – Secure stability of reagents, monitor lot-to-lot variations etc etc

Main elements to develop & validate IHC assays

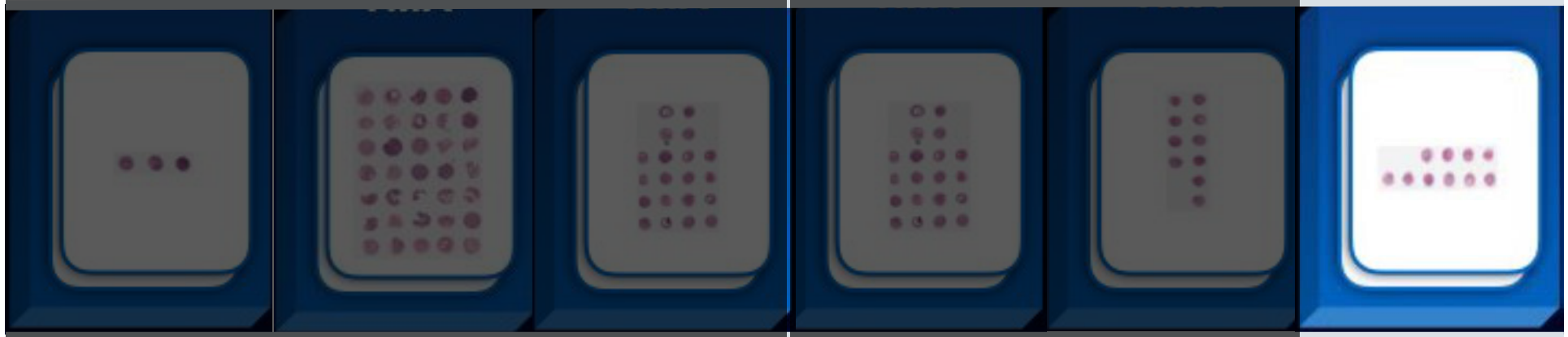
The journey from an antibody to a diagnostic IHC assay with a specific purpose

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

Based on selection and use of appropriate external tissue controls

External tissue control tool box

E Torlakovic et al. AIMM, 2017; 25:227-230
Evolution of Quality Assurance for Clinical Immunohistochemistry in the Era of Precision Medicine: Part 4

Calibration TMA's			Analytical "Validation" TMA's		Lab QC TMA
iCAPC* TMA	Specificity TMA	Pre-analyt. TMA	Accuracy TMA	Index TMA	"Daily QC" TMA
					
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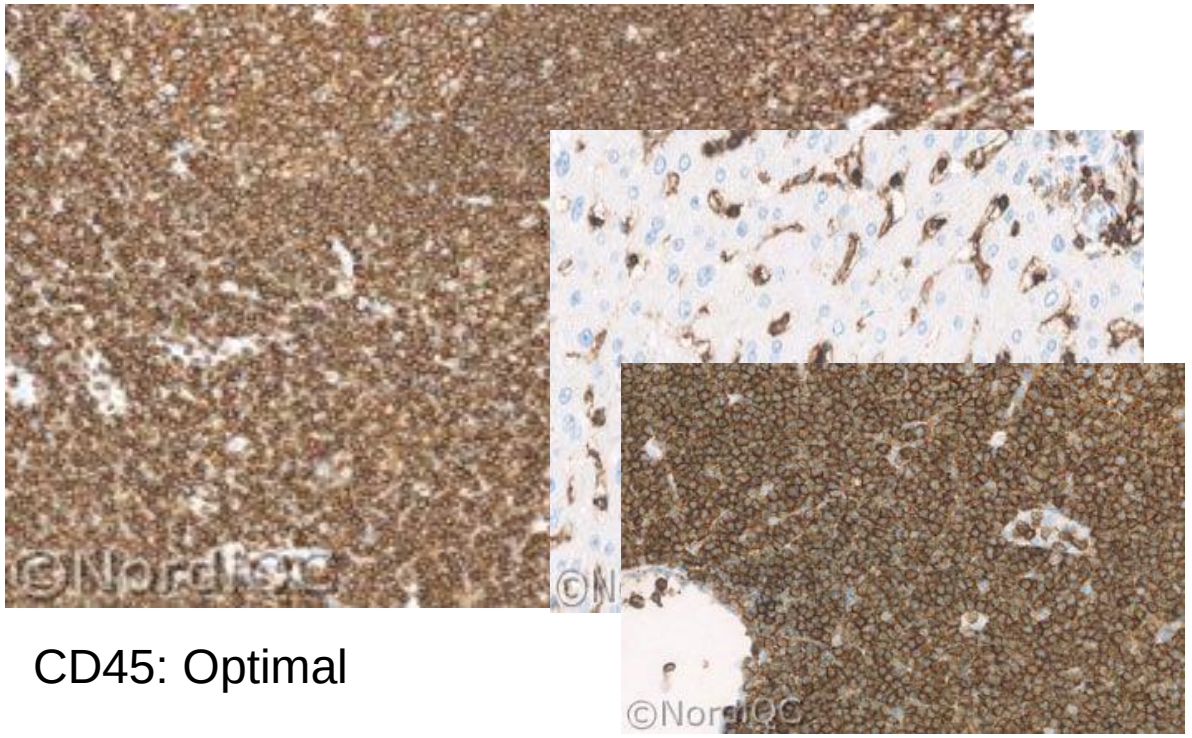
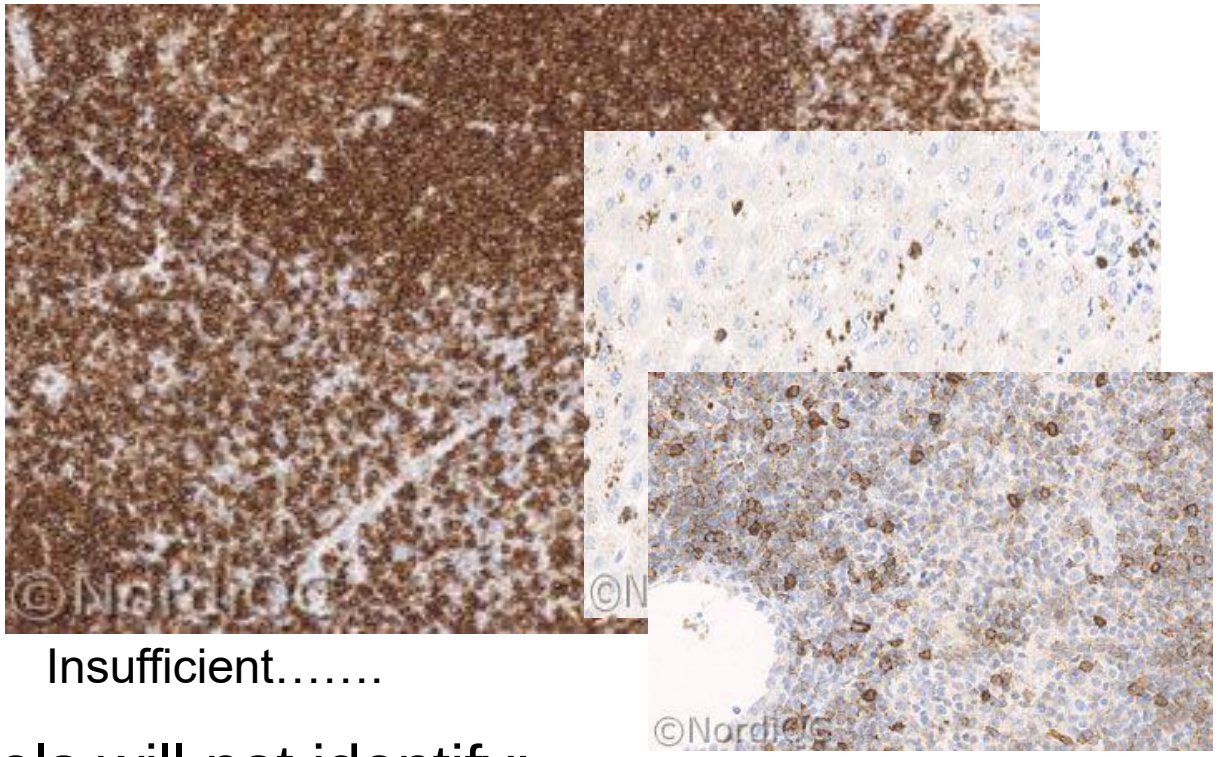
IHC critical assay performance controls	Maps Ab reaction pattern Test performance characteristics	Fixation time Fixative(s) Decalcification	Range of relevant expression levels	Range of relevant expression levels	Reproducibility
			With expression	High expression Low expression	Method of transfer proof
High expression Low expression	With expression		No expression	No expression	
No expression	No expression		20/40 of each Type I/II IHC	+ relevant cut-off	

*Immunohistochemical critical assay performance controls

Tissue controls; Fit for purpose - relevant range of analyte



CD45: Optimal



Insufficient…….

Tissues/cells with only high expression levels will not identify:

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

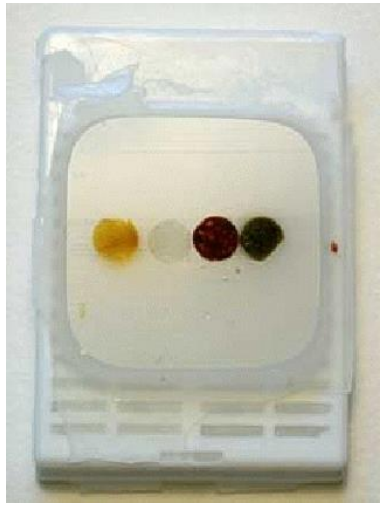
If an IHC test is used to demonstrate the target antigen being expressed at different levels, the controls must reflect this !

Composition of TMA for QC of diagnostic IHC

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

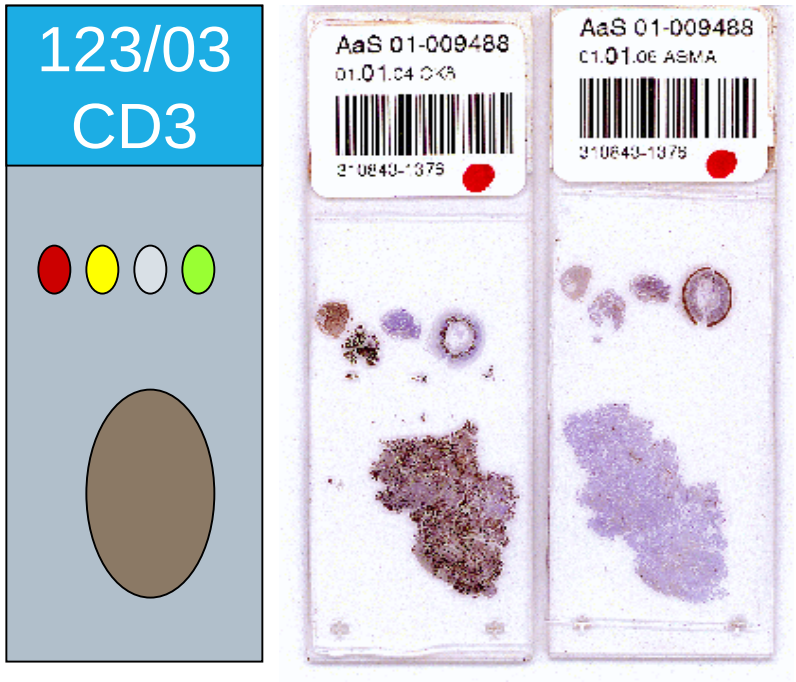


www.nordiqc.org

Application of TMA for QC of diagnostic IHC

Daily IHC 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

Application of TMA for QC of diagnostic IHC

	TMA On-slide control	TMA Run / 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		

Application of TMA for QC of diagnostic IHC

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

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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".

Application of TMA for QC of diagnostic IHC

2% error rate;

Class I 0,8%
Class II 9,0%

(452/22.234 slides)

RESEARCH ARTICLE

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

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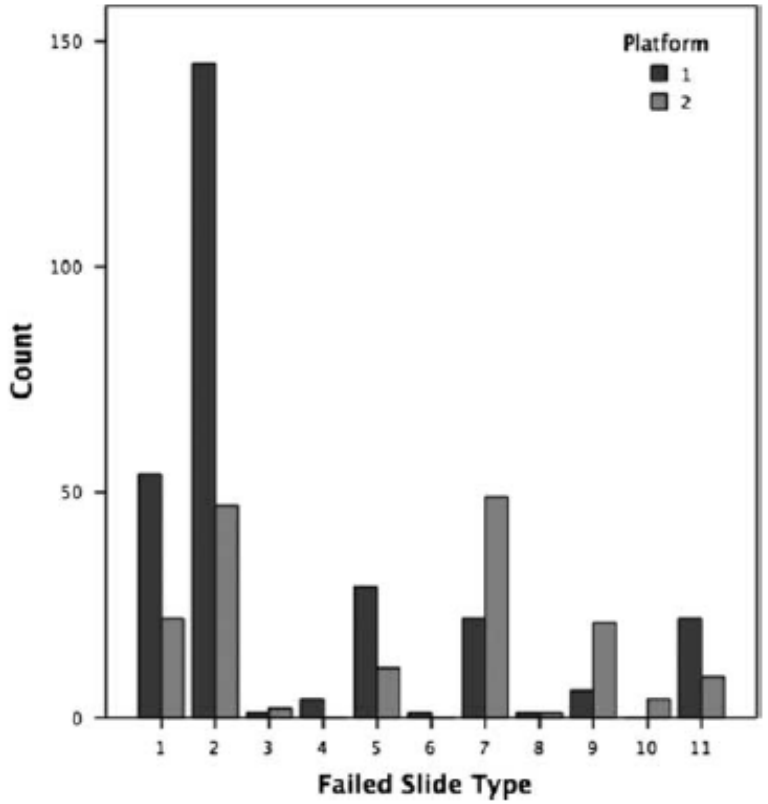


FIGURE 1. Frequency of failed immunohistochemistry slides by category and platform.

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.

Application of TMA for QC of diagnostic IHC

A: On-slide controls

IHC slides stained for ALK (Class II),
same run, same instrument, same protocol
14/19 passed
5/19 failed (5 x 150 USD)

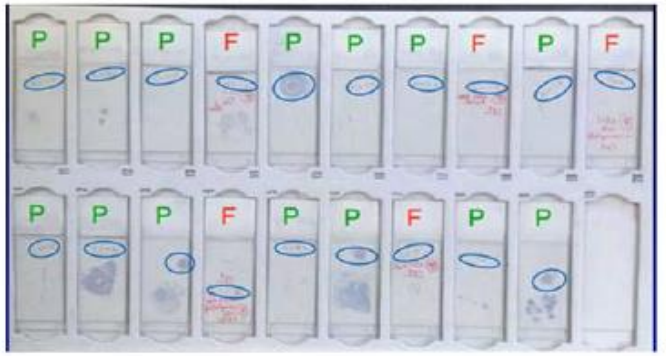
B: Batch-control - Theoretically:

Batch control **failed** by same conditions as above
0/19 passed
19/19 failed (no consistent internal control…) (20 x 150 USD)

C: Batch-control - Theoretically:

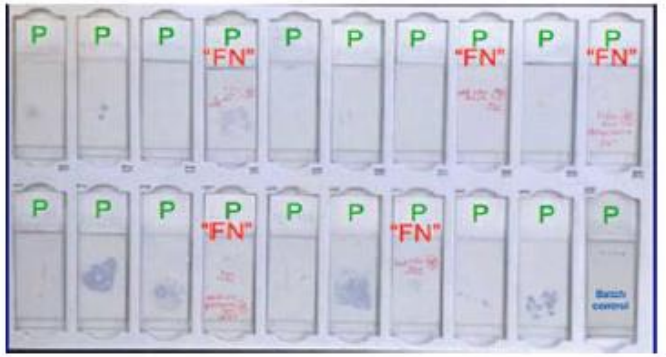
Batch control **passed** by same conditions as above
19/19 passed
0/19 failed (the 5 failed slides not identified….) (Cost…???)

A



B

C



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 and value
- Tissue controls to monitor consistency of IHC assay
- Use of critical tissue controls / ICAPCs with relevant range of target analyte is crucial

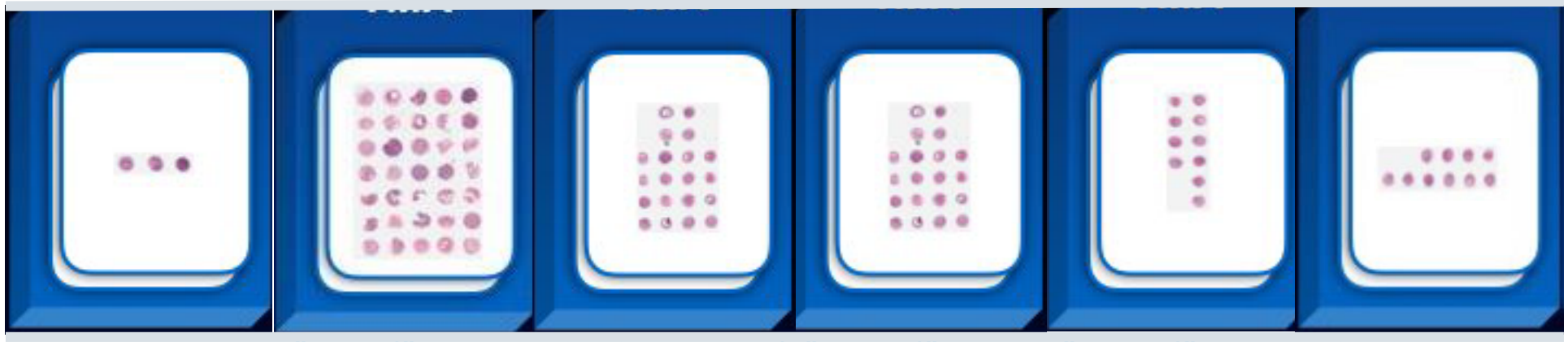
Conclusions

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
- Need to generate consensus guidelines on ICAPCs for all IHC tests – which tissues, which staining pattern. Interaction of industry, EQA and pathology organisations and societies required.

External tissue control tool box

E Torlakovic et al. AIMM, 2017; 25:227-230
Evolution of Quality Assurance for Clinical Immunohistochemistry in the Era of Precision Medicine: Part 4

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 Test performance characteristics	Fixation time Fixative(s) Decalcification	Range of relevant expression levels	Range of relevant expression levels	Reproducibility
			With expression	High expression Low expression	Method of transfer proof
High expression Low expression	With expression		No expression	No expression	
No expression	No expression		20/40 of each Type I/II IHC	+ relevant cut-off	

*Immunohistochemical critical assay performance controls

Questions and/or comments

Thank You for the attention and…..