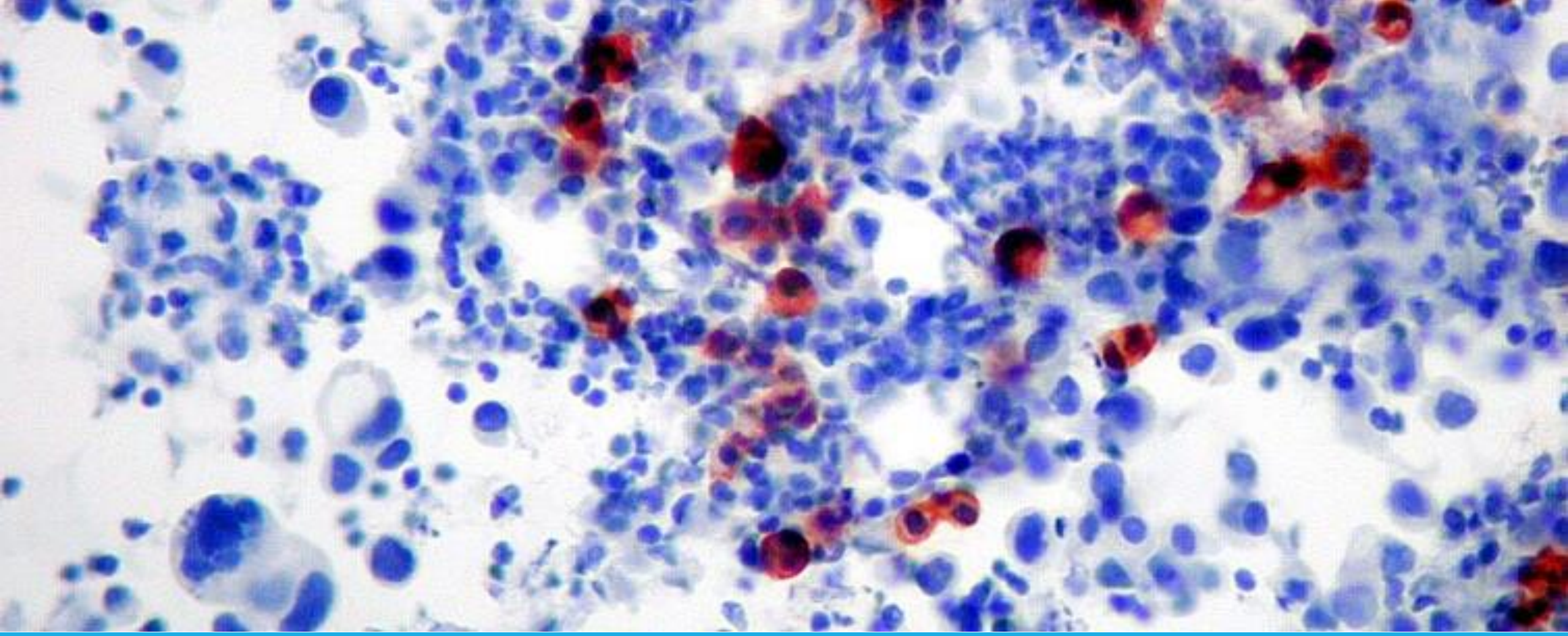


Immunocytochemistry – "What, Why, How"

Søren Nielsen,
Director, NordiQC

Agenda - objectives

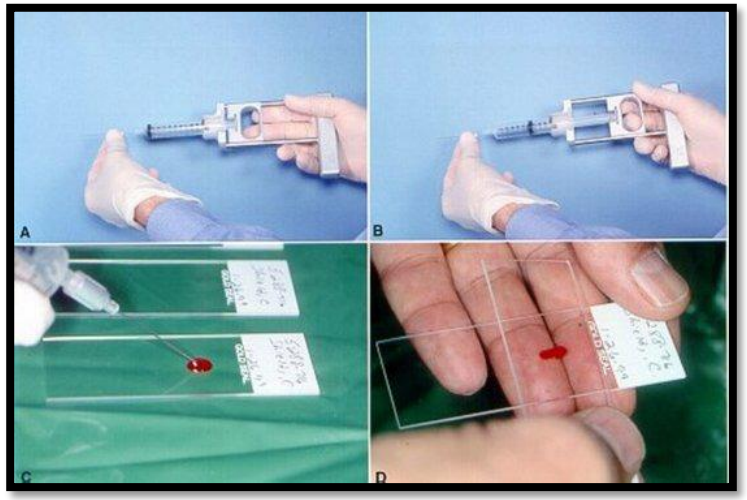
- The potential of Immunocytochemistry (ICC)
- The ”technical challenges” to face and solve
- Input to ”best practice” ICC protocols

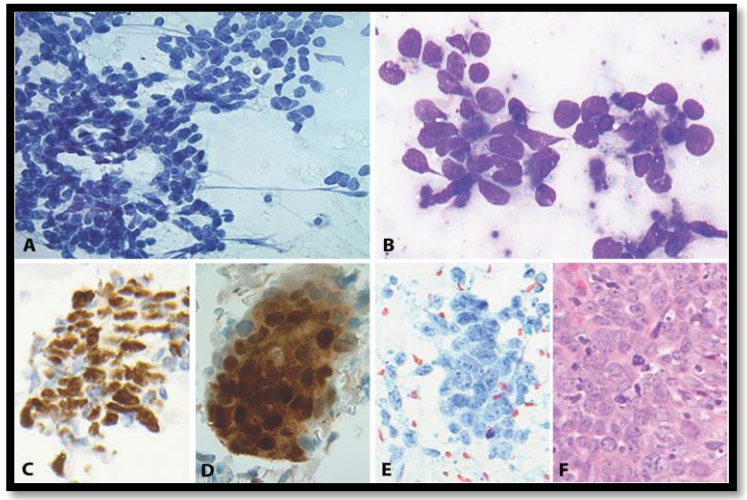
Presentation and data largely generated by Ole Nielsen, Denmark

Definitions

- ICC; Immuno**cyto**chemistry being an ancillary method to be used on cell samples as FNA's, smears and cytospins incl liquid based cytology (LBC)

- IHC; Immuno**histo**chemistry being an ancillary method to be used on formalin fixed tissue samples as biopsies, resection material and cell blocks from cytologic samples





The potential of Immunocytochemistry

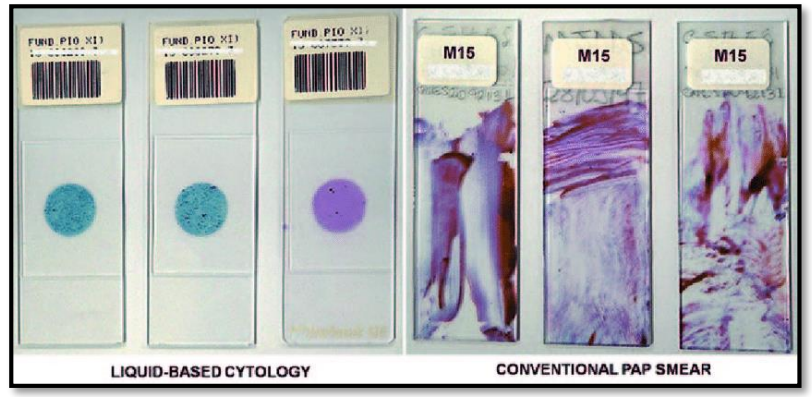
- Essential for the classification of cancer of unknown primary origin (CUP)
  - CDX2, TTF1, GATA3, SOX10, CD45, PCK….
- Subtyping of carcinoma (e.g. squamous cell vs adeno type in NSCLC)
  - P40, CK5, Napsin A, TTF1..
- Predictive information (e.g. breast, lung cancer)
  - ER, PD-L1, ALK….

- No access to histologic sample (non-operable e.g. in NSCLC)

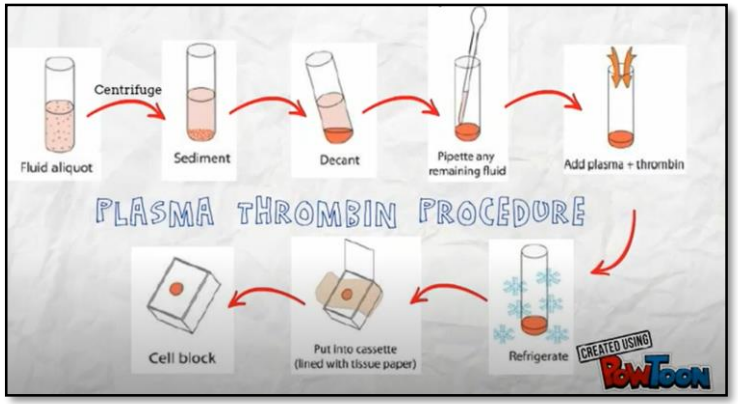
- Primary sample type due to fast TAT, cost effective and minimal invasiveness

Immunocytochemistry – subtype diversity

- The ”classical” cytology samples
  - FNAs / smears
  - Imprints
  - Cytospins / Liquid based cytology (LBC)

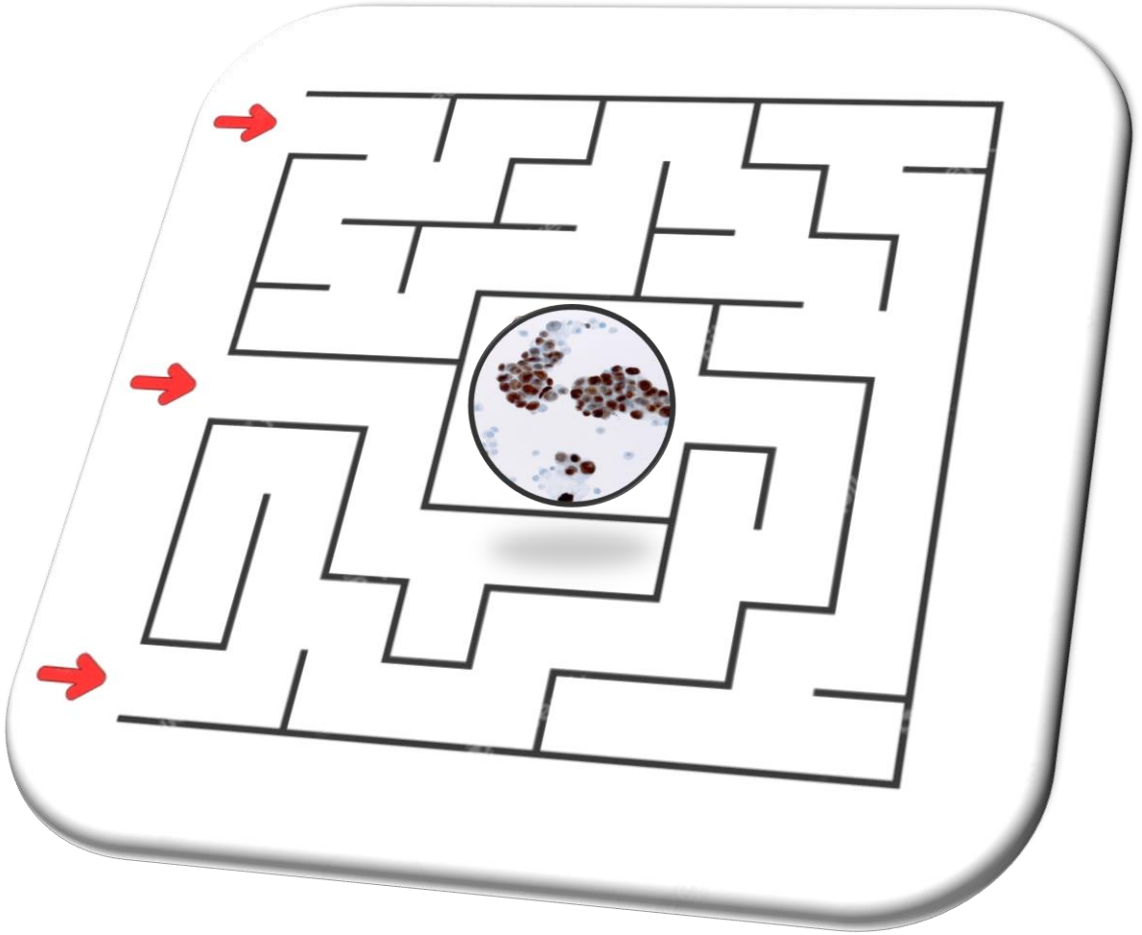


- The histology converted cytology samples
  - Cell blocks



Immunocytochemistry – where to start and where to end….??

- Different sample types & fixatives,
- Variable cellular content
- Limited access to test material
- Limited access to validated ICC methods



Immunocytochemistry – Common practices and challenges

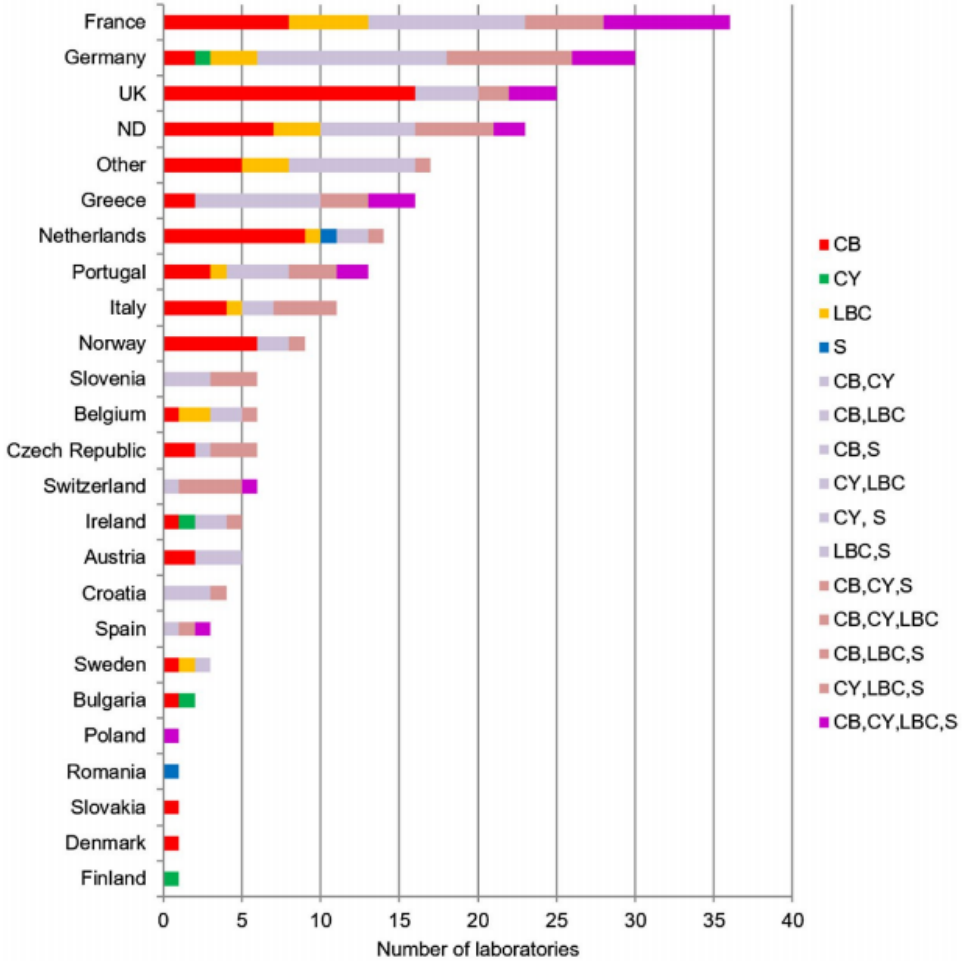


FIGURE 1. This chart illustrates cytology preparations and their combinations used for immunocytochemistry in participating laboratories (n = 245). CB indicates cell block; CY, cytospin; LBC, liquid-based cytology, ND, no data about location; S, smear.

TABLE 2. Cytology Preparations Used for Immunocytochemistry in European Laboratories, n = 245

	No. of Laboratories (%)
Combination	147 (60)
2 Types: CB/CY, CB/LBC, CB/S, CY/S, CY/LBC, LBC/S	75 (31)
3 Types: CB/CY/LBC, CB/CY/S, CY/LBC/S, CB/LBC/S	47 (19)
4 Types: CB/CY/LBC/S	25 (10)
One Type	98 (40)
CB	72 (29)
LBC slides	20 (8)
CY	4 (2)
S	2 (1)

Abbreviations: CB, cell block; CY, cytospin; LBC, liquid-based cytology; S, smear.

Immunocytochemistry Practices in European Cytopathology Laboratories—Review of European Federation of Cytology Societies (EFCS) Online Survey Results With Best Practice Recommendations, Srebotnik Kirbiš et al. Cancer Cytopathology October 2020, 757-766.

Immunocytochemistry – Common practices and challenges

TABLE 5. Troubles With Immunocytochemistry Quality on Different Cytology Preparations and for Different Immunocytochemistry Protocols

		Percentage of ICC Protocols		Percentage of Preparations			
	Quality Issue	Same as FFPE	Optimized and Validated	Cell Block	Cytospin	LBC	Smear
Cell access	Low cellular slides	61[a]	47	62	58	66	55
	Not enough slides	70	61	59	69	66	69
ICC method	Inconsistent staining	50[a]	31	37	32	40	37
	Background staining	44	54	48	51	52	62
	Nonspecific staining	42	38	43	45	45	48
	Weak staining	40	31	37	35	36	40
	Poor cell morphology	32	38	34	38	30	41
Read-out	Difficult interpretation	57[a]	34	50	45	40	48

Abbreviations: FFPE, formalin-fixed, paraffin-embedded; ICC, immunocytochemistry; LBC, liquid-based cytology.
[a]$P < .05$.

Immunocytochemistry Practices in European Cytopathology Laboratories—Review of European Federation of Cytology Societies (EFCS) Online Survey Results With Best Practice Recommendations, Srebotnik Kirbiš et al. Cancer Cytopathology October 2020, 757-766.

Immunocytochemistry – Common practices and challenges

Development, optimization and validation of ICC methods

- Usage of same IHC method as for FFPE material
- Usage of method developed, optimized and validated for cytological preparation

"However, our survey showed that optimization and validation of ICC protocols for cytology preparations are not yet common practice. Staining procedures validated for FFPE tissue sections in participating laboratories are used not only for cell blocks but also for variously fixed and prepared cytology preparations (31%), but only a few laboratories optimized or validated their ICC protocols (26% and 29%, respectively)."

TABLE 3. Immunocytochemistry Protocols Used for Different Cytology Preparations

Protocols: Multiple Responses Allowed, n = 527	No. of ICC protocols (%)				
	Cell Blocks	Cytospins	LBC Slides	Smears	Total
Same as for FFPE sections	165 (69)	22 (9)	28 (12)	23 (10)	238 (45)
Optimized	10 (7)	43 (31)	44 (32)	41 (30)	138 (26)
Validated	14 (9)	49 (32)	39 (26)	49 (32)	151 (29)

Abbreviations: FFPE, formalin-fixed, paraffin-embedded; ICC, immunocytochemistry; LBC, liquid-based cytology.

Immunocytochemistry Practices in European Cytopathology Laboratories—Review of European Federation of Cytology Societies (EFCS) Online Survey Results With Best Practice Recommendations, Srebotnik Kirbiš et al. Cancer Cytopathology October 2020, 757-766.

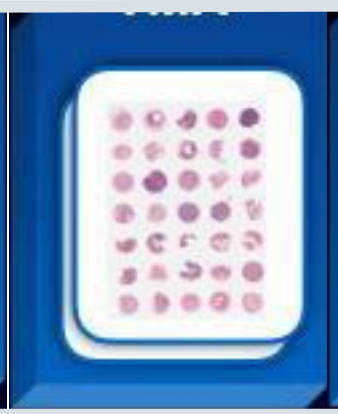
Immunohistochemistry – Requirements for optimal performance

- Appropriate tissue fixation and processing
- Appropriate calibration and validation
  - Appropriate choice of antibody clone and titre
  - Appropriate epitope retrieval
  - Robust and sensitive detection system
- Appropriate choice of tissue samples and controls
  - Clinically relevant range of expression levels

A systematic test battery approach

Immunohistochemistry – The tissue tool box

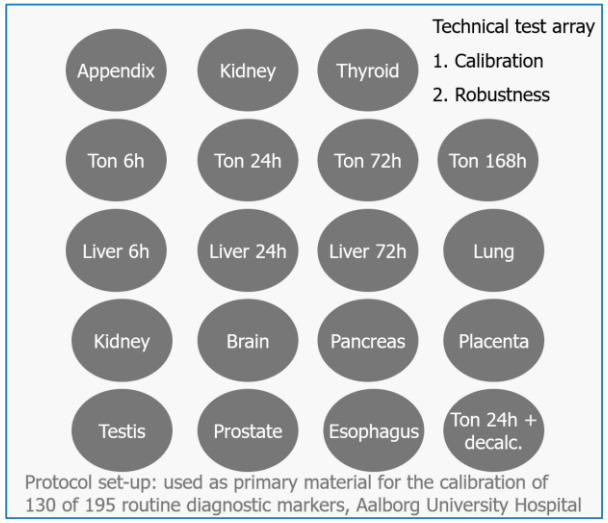
E Torlakovic et al. AIMM, 2017; 25:227-230
Evolution of Quality Assurance for Clinical Immunohistochemistry in the Era of Precision Medicine: Part 4

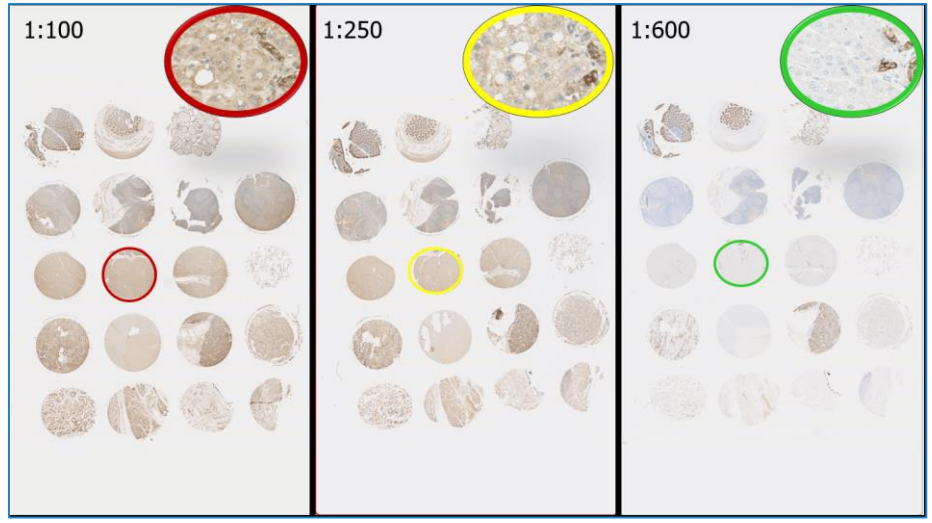
Calibration TMA's			Analytical "Validation" TMA's		Lab QC TMA
iCAPC* TMA	Specificity TMA	Pre-analyt. TMA	Accuracy TMA	Index TMA	"Daily QC" TMA
					
"Gold standard" tissue controls	"Normal" tissues	iCAPCs processed as lab procedures	"Lesional" tissues	"Lesional" tissues	iCAPCs + selected tissues
IHC 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
High expression Low expression No expression	With expression No expression		With expression No expression	High expression Low expression No expression	Method of transfer proof
			20/40 of each Type I/II IHC	+ relevant cut-off	

*Immunohistochemical critical assay performance controls

Immunohistochemistry – Requirements for optimal performance

	No retrieval	HIER pH Hi	HIER pH Lo	Protease	HIER + Prot.	.....
1:100	Slide 1	Slide 2	Slide 3	Slide 4	Slide 5	
1:250	Slide 6	Slide 7	Slide 8	Slide 9	Slide 10	
1:600	Slide 11	Slide 12	Slide 14	Slide 15	Slide 16	.....
	OptiView DAB + BenchMark Ultra or EnVision FLEX+ Dako Omnis					





A systematic test battery approach

Immunocytochemistry – Requirements for optimal performance

- Appropriate choice of fixative and fixation process
- Appropriate calibration and validation
  - Appropriate choice of antibody clone and titre
  - Appropriate epitope retrieval
  - Robust and sensitive detection system
- Appropriate choice of cell controls
  - Clinically relevant range of expression levels

A systematic test battery approach

Immunocytochemistry – subtypes & fixatives

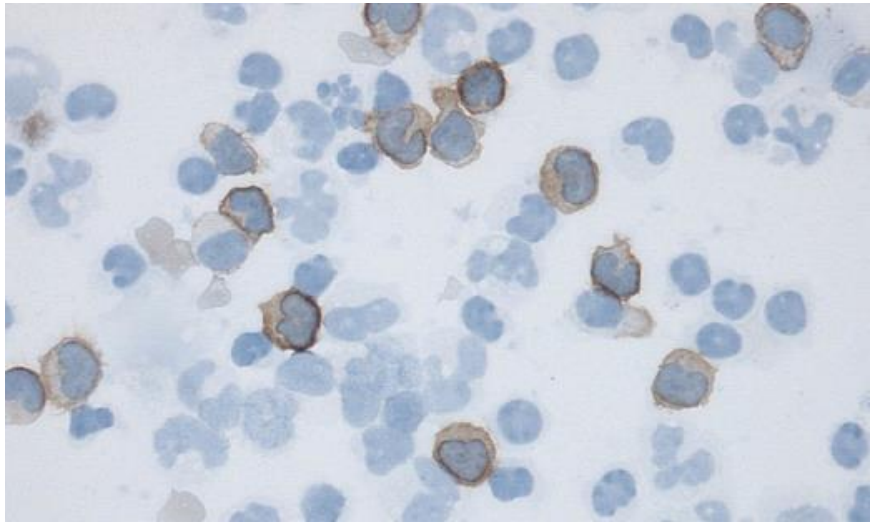
- The ”classical” cytology samples
  - FNAs / smears; Air-dried, etanol, metanol..
  - Imprints; Air-dried
  - Cytospins / LBC; SurePath, PreservCyt (alcohol+NBF)

- The histology converted cytology samples
  - Cell blocks; +/- prefix in e.g alcohol, SurePath and postfix in 10% NBF

Immunocytochemistry – fixative and antigenesity

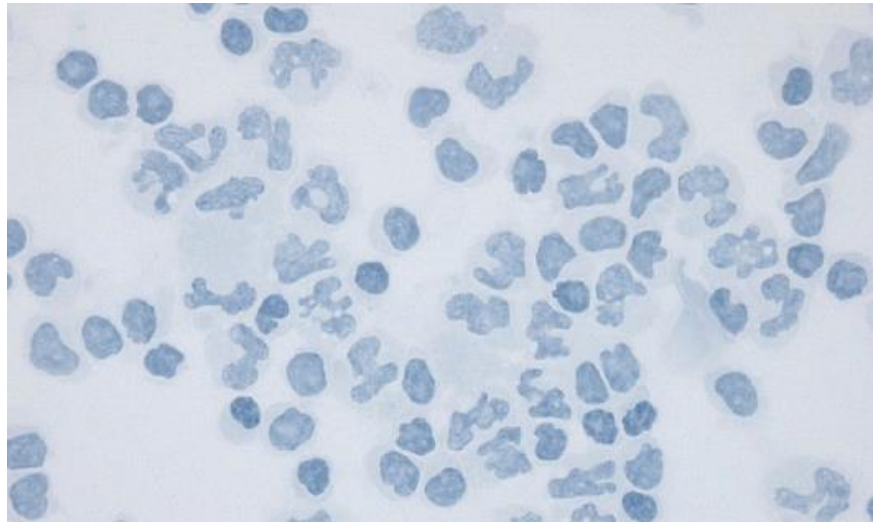
CD8, DK25

Acetone 10 min.



CD8, DK25

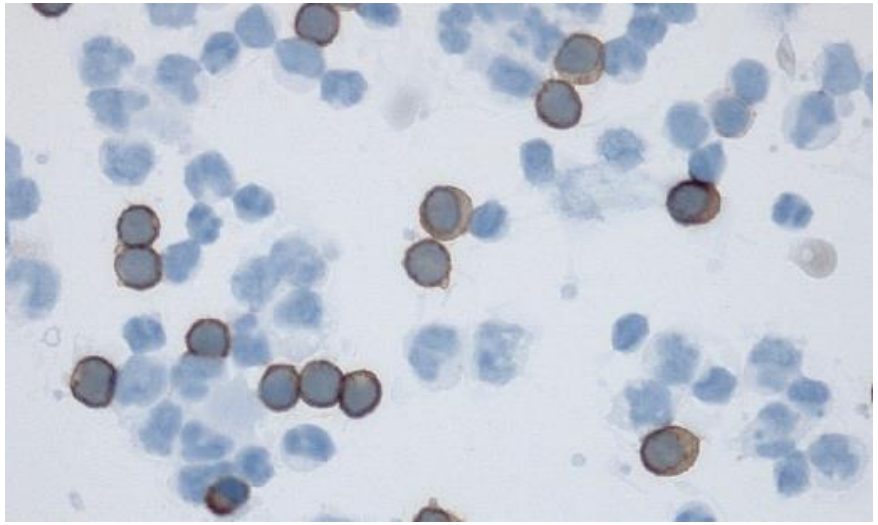
NBF 2 min.



CD8, C8/144

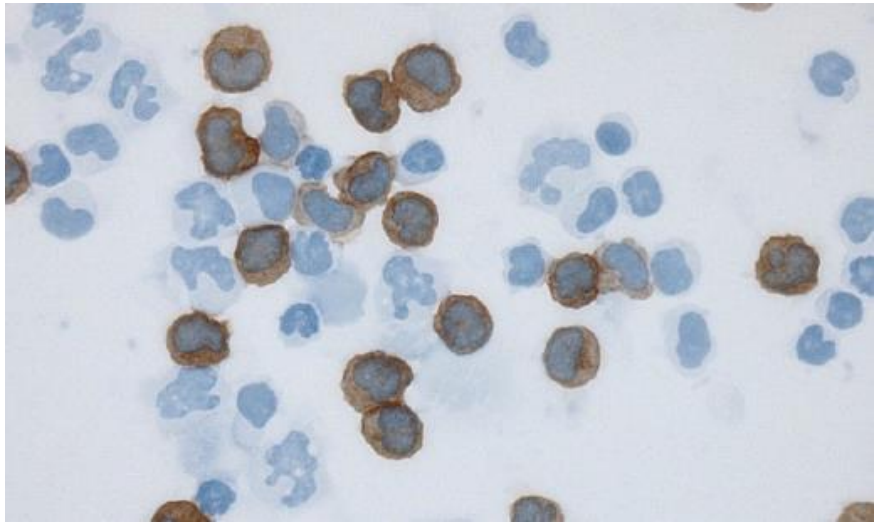
Acetone 10 min.

Blood smear

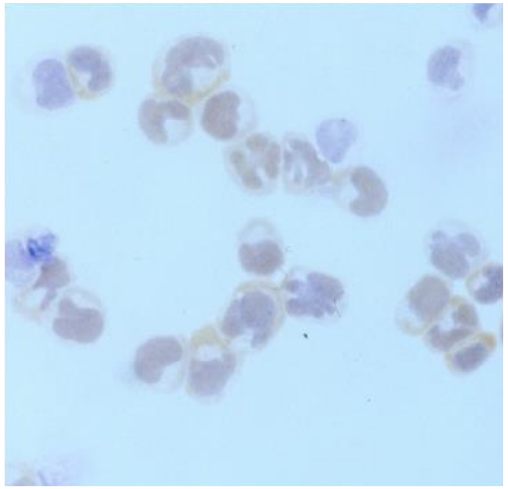


CD8, C8/144

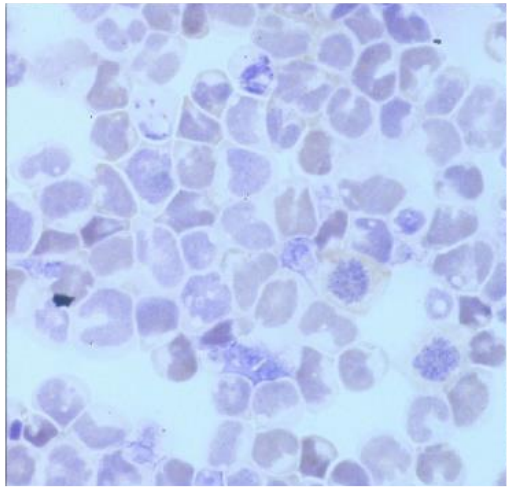
NBF 2 min.



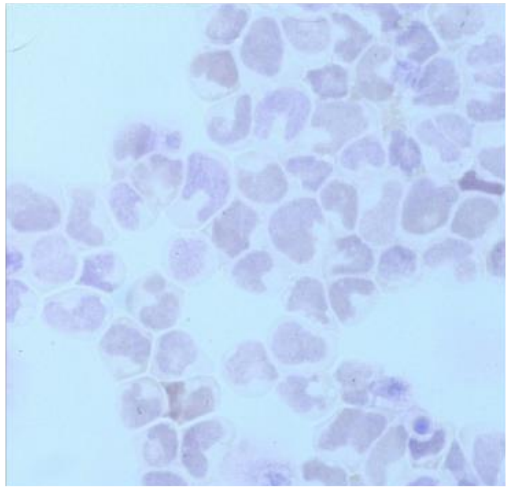
Immunocytochemistry – fixative and antigenesity



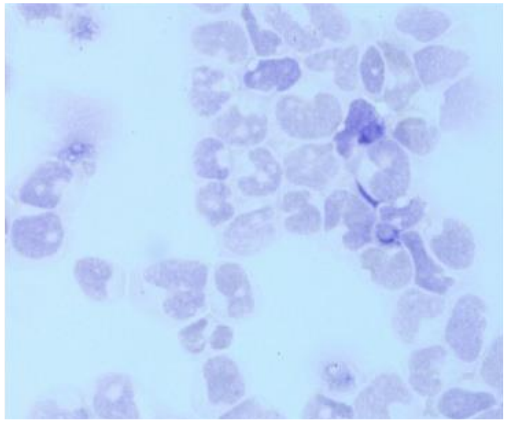
Acetone 5’ min.



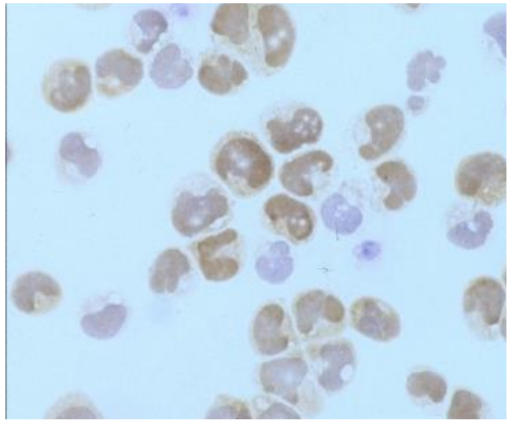
Acetone/Methanol 40’ sec



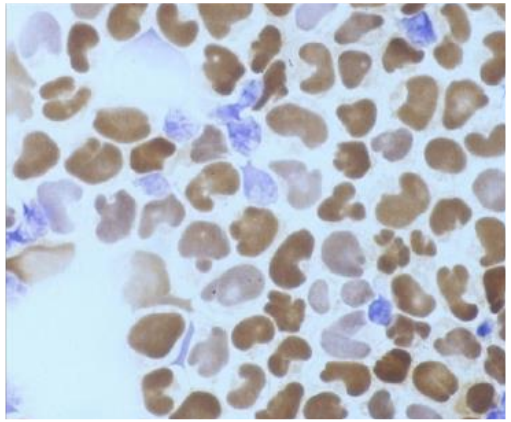
Methanol 5’ min.



Ethanol 5’ min.



NBF 5’ min.



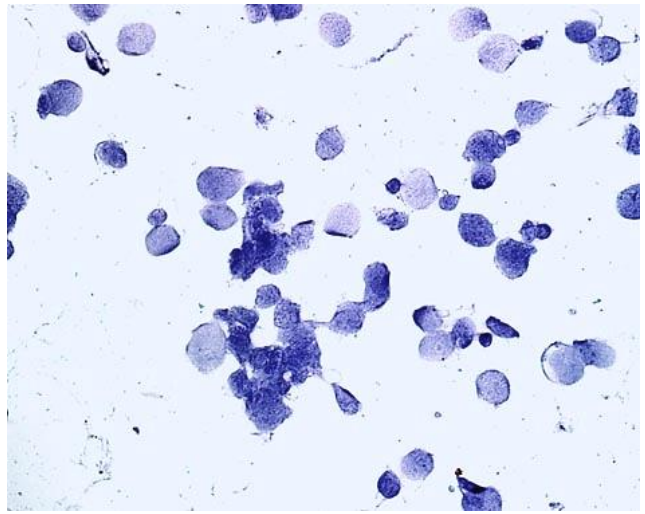
NBF 15’ min.+Triton-X 5’ min.

Cytospin;

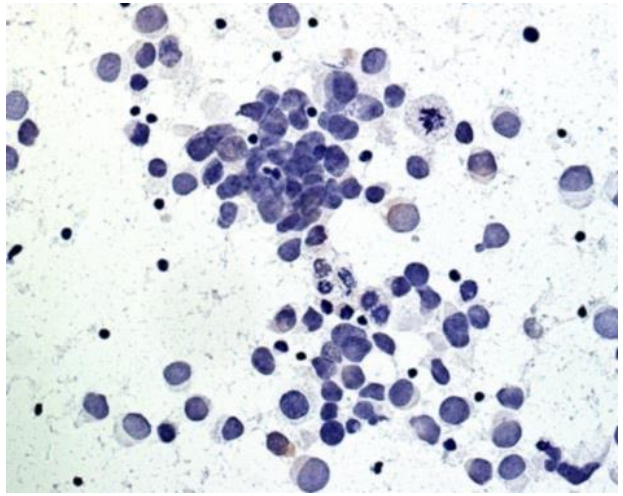
MOLT4 cell line

TdT polyclonal Ab

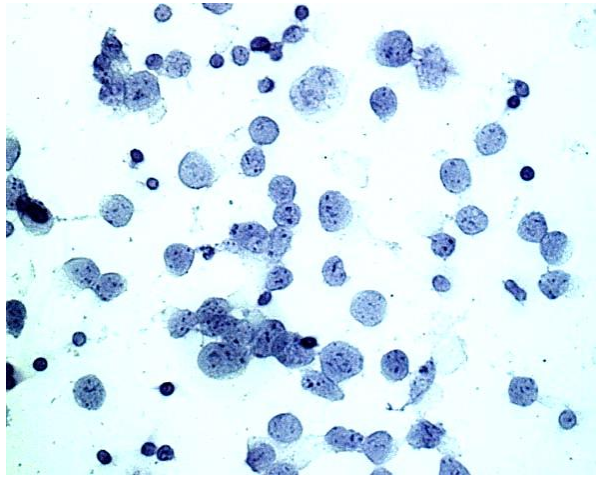
Immunocytochemistry – fixative and antigenesity – Histological…



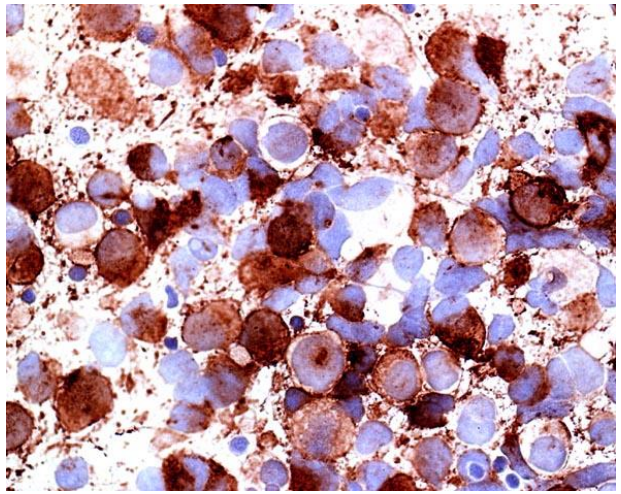
No fixation



NBF 15’ min.



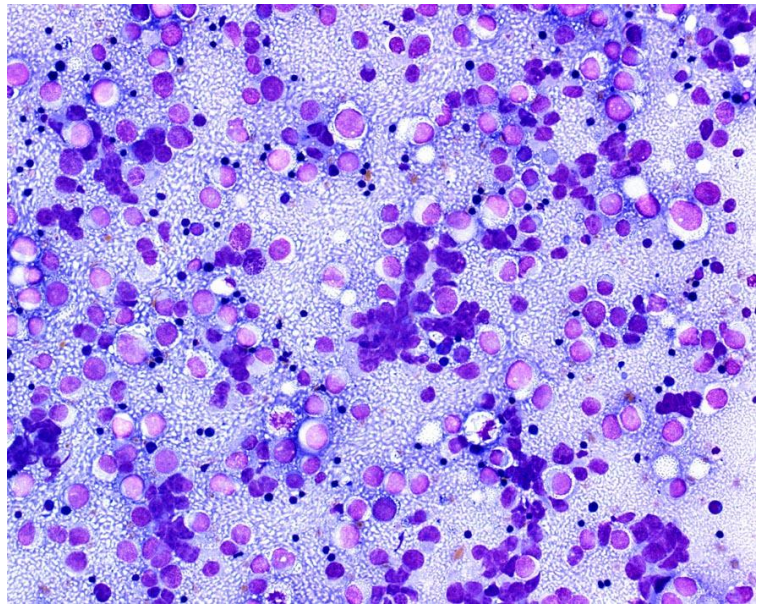
Ethanol 5’ min.



NBF 15’ min. + HIER*

Seminoma imprints

PLAP mAb clone 8A9



* Heat Induced Epitope Retrieval

HIER – concept and intention

Optimized temperature-time-pH-buffer system

‘Heating condition’ = temperature × time:

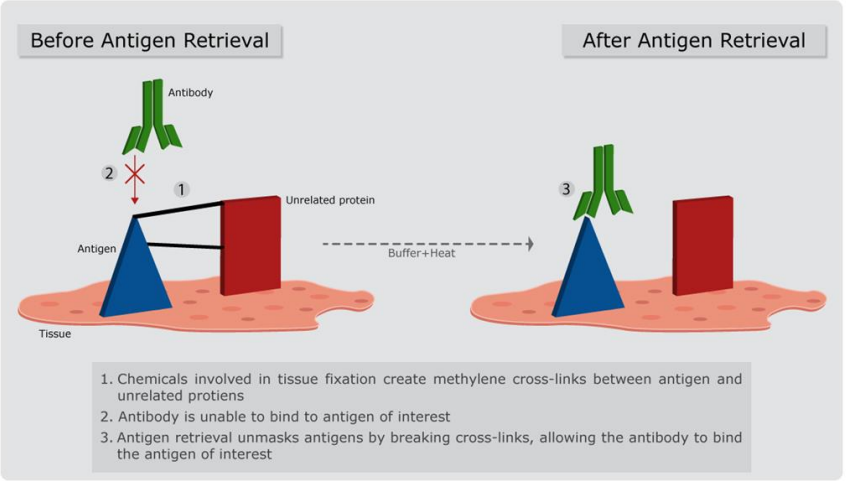
121ºC/1 min 100ºC/20 min 95ºC/40 min 60ºC/24 h.

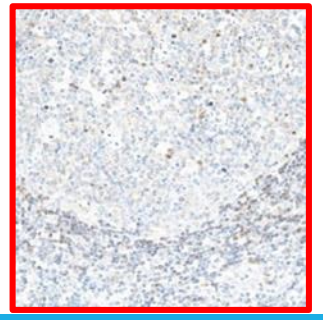
Device:
IHC stainer integrated
Water bath
MWO
Pressure cooker

Considerations:
Efficiency / sensitivity
Standardization
Morphology
Performance

Essential to restore / retrieve

Antigens being blocked by fixation in formalin





CD79a
JCB117

Tonsil

Immunocytochemistry – fixation and HIER test battery

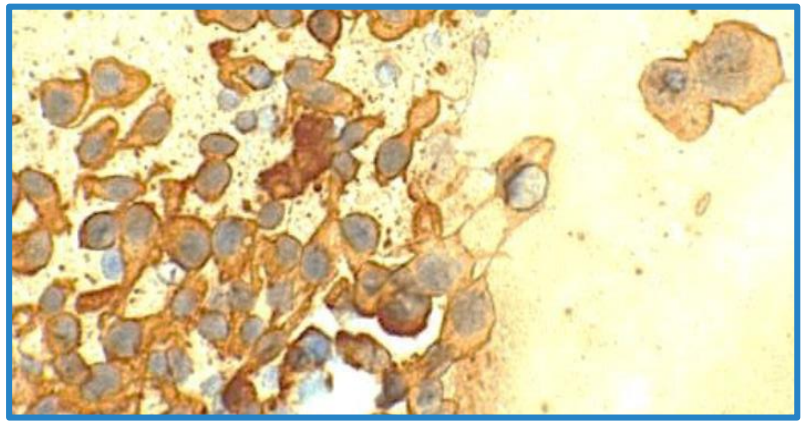
”Classical cytology samples” – direct smears, FNA’s (non-LBC)

	Fixation	HIER	Detection system	IHC stainer
Slide 1	None (drying)	-	OptiView	BenchMark Ultra
Slide 2	Acetone 10 min.	-	OptiView	BenchMark Ultra
Slide 3	10% NBF 5 min.	-	OptiView	BenchMark Ultra
Slide 4	10% NBF 30 min.	CC1 pH 8,5, 8 min.	OptiView	BenchMark Ultra
Slide 5	10% NBF 30 min.	CC1 pH 8,5, 32 min.	OptiView	BenchMark Ultra
Slide 6	Vendor recommendations			

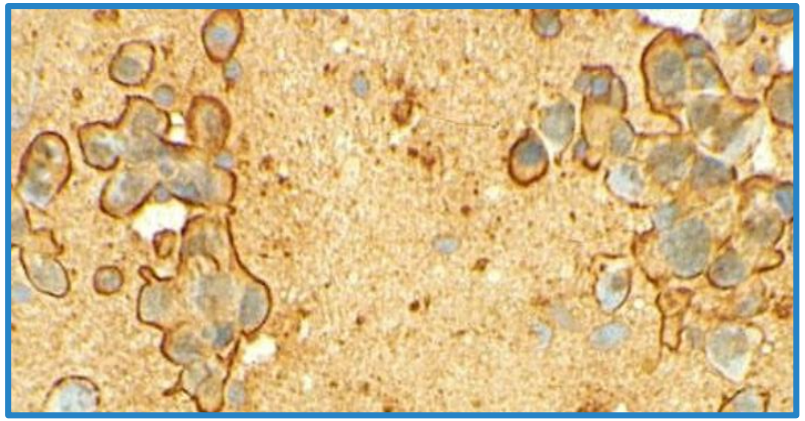
A systematic test battery approach

Immunocytochemistry – fixation and HIER test battery

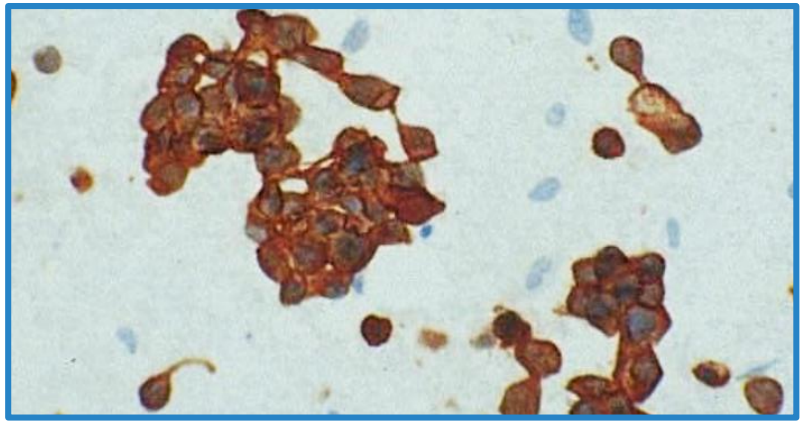
”Classical cytology samples” – direct smears, FNA’s (non-LBC)



Acetone 10 min.



10% NBF 5 min.



10% NBF + HIER.

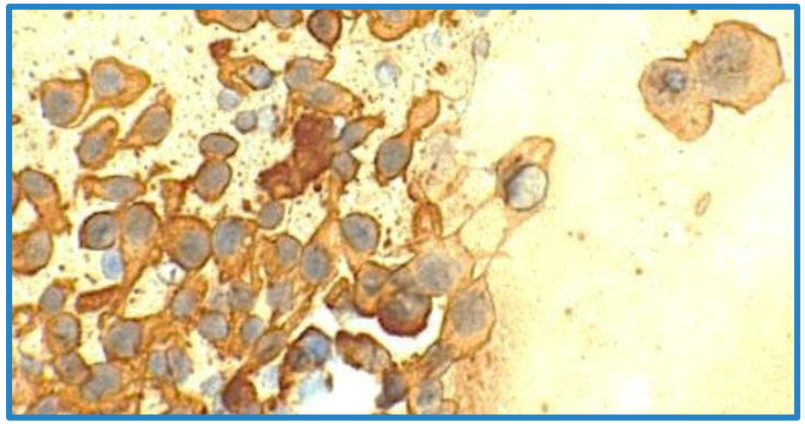
CK7 mAb clone OV-TL 12/30

Imprint of tumour - airdried

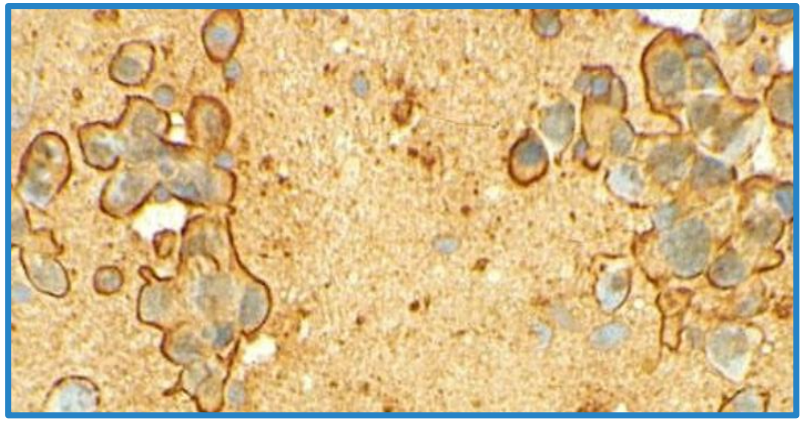
A systematic test battery approach

Immunocytochemistry – fixation and HIER test battery

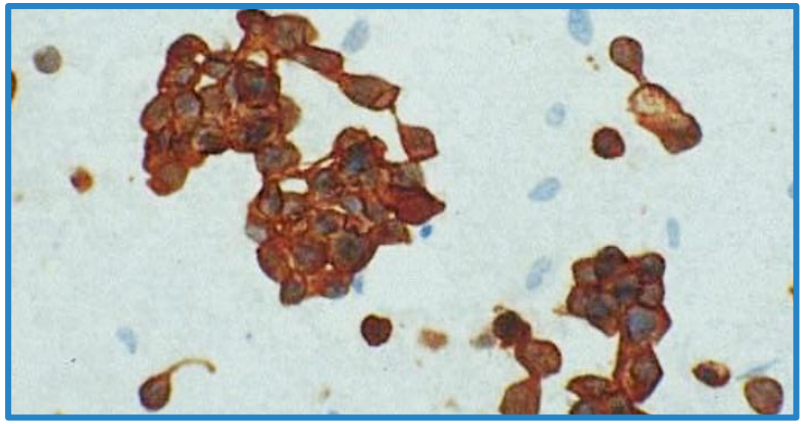
”Classical cytology samples” – direct smears, FNA’s (non-LBC)



Acetone 10 min.



10% NBF 5 min.



10% NBF + HIER.

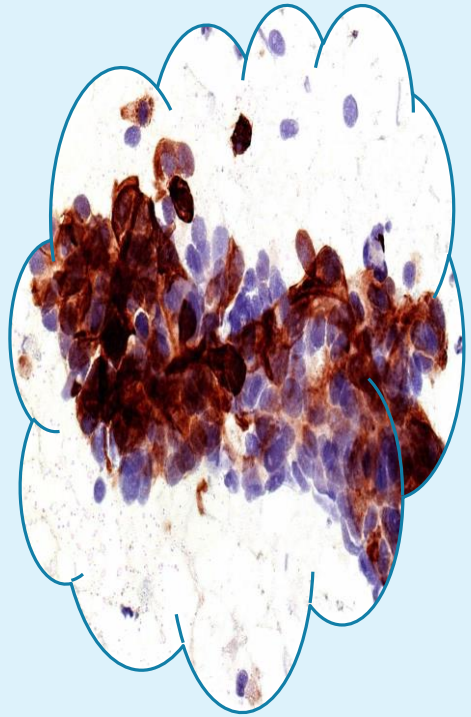
The concept and mechanisms	
Airdrying	A coagulant fixation of the proteins/antigens
+/- Postfixation in NBF	Additional fixation and stabilization
HIER	Restore blocked antigens Allow penetration of abs by reducing cellular surface tensions Block endogenous enzymes used for ICC labelling (peroxidase/phosphase)

Immunocytochemistry – Suggested protocols

”Classical cytology samples” – direct smears, FNA’s (non-LBC)

Marker	Clone	Fixation	Retrieval	Platform
CD1a	EP3622	10% NBF, 30 min.	CC1 8 min.	BenchMark Ultra
CD3	2GV6	10% NBF, 30 min.	CC1 8 min.	BenchMark Ultra
CD10	SP19	Acetone	None	BenchMark Ultra
CD20	L26	10% NBF, 30 min.	CC1 8 min.	BenchMark Ultra
CD30	Ber-H2	10% NBF, 5 min.	None	BenchMark Ultra
CD56	MRQ-42	10% NBF, 30 min.	CC1 8 min.	BenchMark Ultra
CDX2	EPR2764Y	10% NBF, 30 min.	CC1 8 min.	BenchMark Ultra
CK7	OV-TL 12/30	10% NBF, 5 min.	None	BenchMark Ultra
CK20	SP33	10% NBF, 30 min.	CC1 8 min.	BenchMark Ultra
GATA3	L50-823	10% NBF, 5 min.	None	BenchMark Ultra
HEPA	OCH1E5	10% NBF, 30 min.	CC1 8 min.	BenchMark Ultra
Napsin A	IP64	10% NBF, 5 min.	None	BenchMark Ultra
PAX8	ZR1	10% NBF, 30 min.	CC1 8 min.	BenchMark Ultra
p40	BC28	10% NBF, 30 min.	CC1 8 min.	BenchMark Ultra
p63	4A4	10% NBF, 30 min.	CC1 8 min.	BenchMark Ultra
TTF1	SPT24	10% NBF, 30 min.	CC1 8 min.	BenchMark Ultra
WT1	6F-H2	10% NBF, 30 min.	CC1 8 min.	BenchMark Ultra

CK7
FNA



Immunocytochemistry – fixation and HIER test battery

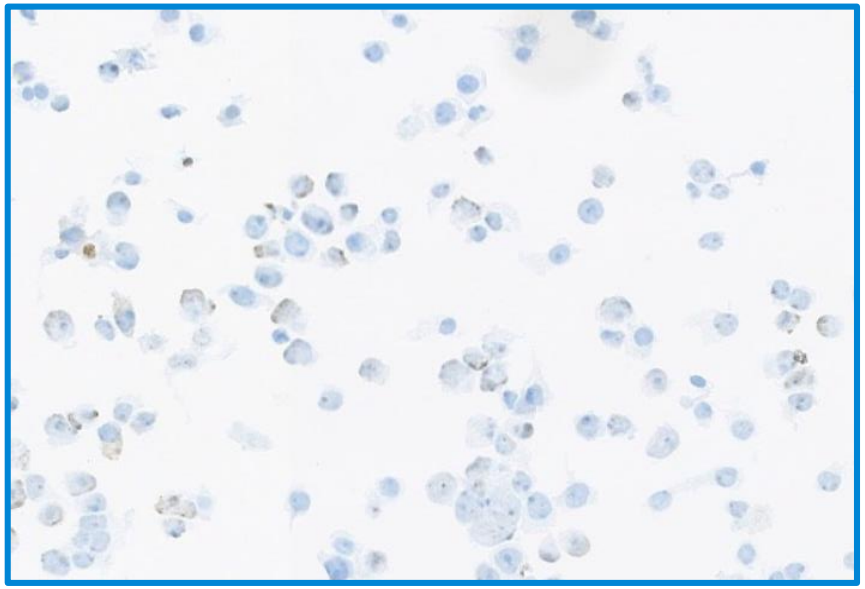
Liquid based cytology – prefixed in preservation medium as SurePath and PreservCyt

	Fixation	HIER	Detection system	IHC stainer
Slide 1	None (drying)	-	OptiView	BenchMark Ultra
Slide 2	None (drying)	CC1 pH 8,5, 8 min.	OptiView	BenchMark Ultra
Slide 3	None (drying)	CC1 pH 8,5, 32 min.	OptiView	BenchMark Ultra
Slide 4	10% NBF 5 min.	-	OptiView	BenchMark Ultra
Slide 5	10% NBF 30 min.	CC1 pH 8,5, 8 min.	OptiView	BenchMark Ultra
Slide 6	10% NBF 30 min.	CC1 pH 8,5, 32 min.	OptiView	BenchMark Ultra
Slide 7	Vendor recommendations			

A systematic test battery approach

Immunocytochemistry – fixation and HIER test battery

Liquid based cytology - SurePath



10% NBF 5 min.

10% NBF + HIER.

p40 mAb clone BC28

Cell line A431

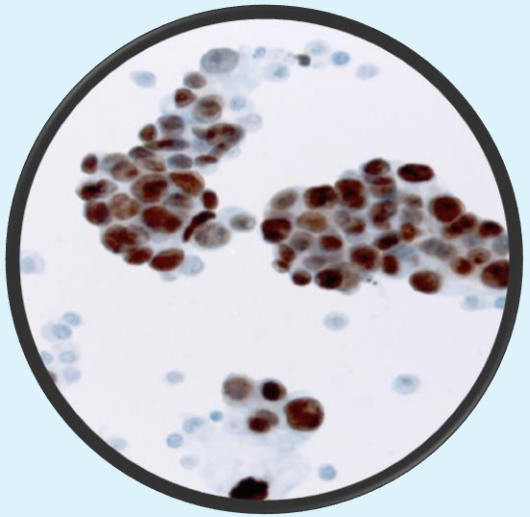
A systematic test battery approach

Immunocytochemistry – Suggested protocols

Liquid based cytology (ThinPrep) on BenchMark Ultra

Marker	Clone	Fixation	Retrieval	Platform
CD1a	EP3622	10% NBF, 30 min.	CC1 32 min.	BenchMark Ultra
CD3	2GV6	10% NBF, 30 min.	CC1 32 min.	BenchMark Ultra
CD5	SP19	10% NBF, 30 min.	CC1 32 min.	BenchMark Ultra
CD7	CBC.37	None	None	BenchMark Ultra
CD56	MRQ-42	10% NBF, 30 min.	CC1 32 min.	BenchMark Ultra
CDX2	EPR2764Y	10% NBF, 5 min.	CC1 32 min.	BenchMark Ultra
CK-LMW	CAM5.2	10% NBF, 30 min.	CC1 32 min.	BenchMark Ultra
CK-Pan	KL1	10% NBF, 30 min.	CC1 32 min.	BenchMark Ultra
CK7	SP52	10% NBF, 30 min.	CC1 32 min.	BenchMark Ultra
CK19	A-53-B/A2.26	10% NBF, 30 min.	CC1 32 min.	BenchMark Ultra
E-Cad	36	10% NBF, 30 min.	CC1 32 min.	BenchMark Ultra
Ki67	30-9	10% NBF, 30 min.	CC1 32 min.	BenchMark Ultra
Melan A	A103	None	CC1 32 min.	BenchMark Ultra
Napsin A	IP64	10% NBF, 30 min.	CC1 32 min.	BenchMark Ultra
p40	BC28	10% NBF, 5 min.	CC1 32 min.	BenchMark Ultra
PSA	pAb	None	CC1 32 min.	BenchMark Ultra
TTF-1	SPT-24	10% NBF, 30 min.	CC1 32 min.	BenchMark Ultra

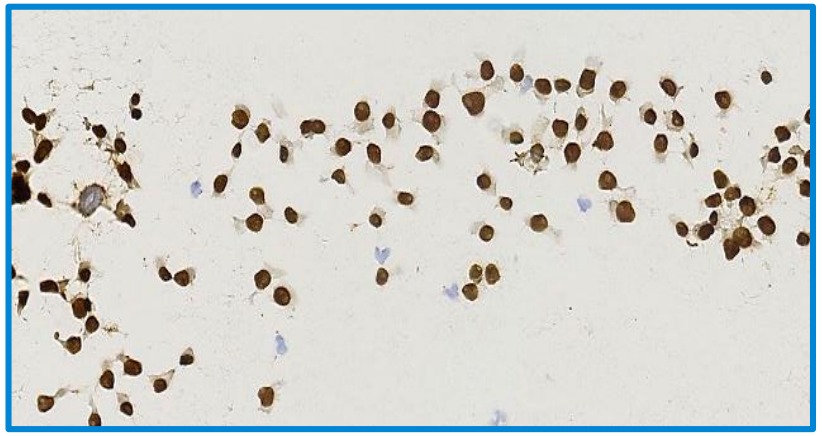
TTF1
LBC



Immunocytochemistry – fixation and HIER test battery

”Classical cytology samples” – direct smears, FNA’s (non-LBC)

10% NBF 30 min. HIER TRS-L-10 min.



10% NBF 30 min. HIER TRS-H-10 min.

10% NBF 30 min. HIER TRS-H-5 min.

TTF1 mAb clone SPT24

Imprint of thyroid - airdried

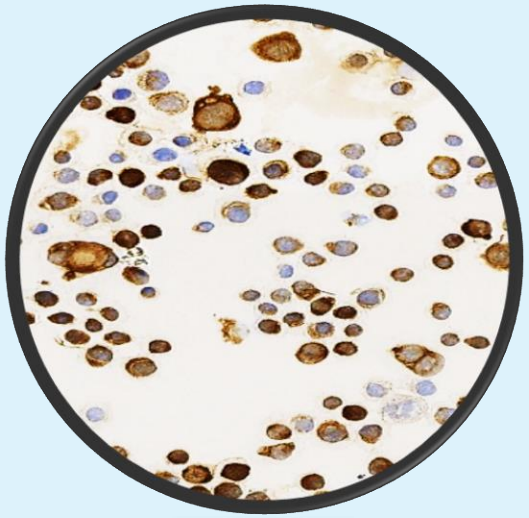
A systematic test battery approach

Immunocytochemistry – Suggested protocols

Liquid based cytology (ThinPrep) on Dako Omnis

Marker	Clone	Fixation	Retrieval	Platform
CD34	EP88	10% NBF, 30 min.	TRS-Low 10 min.	Omnis
CDX2	EPR2764Y	10% NBF, 30 min.	TRS-Low 10 min.	Omnis
CEA-M	COL-1	10% NBF, 30 min.	TRS-Low 10 min.	Omnis
CK5	XM26	10% NBF, 30 min.	TRS-Hi 10 min.	Omnis
EMA	E29	10% NBF, 30 min.	TRS-Low 10 min.	Omnis
EP-CAM	BS14	10% NBF, 5 min.	TRS-Low 10 min.	Omnis
GATA3	EP368	10% NBF, 30 min.	TRS-Low 10 min.	Omnis
p40	BC28	10% NBF, 30 min.	TRS-Low 10 min.	Omnis
PAX8	SP348	10% NBF, 30 min.	TRS-Hi 10 min.	Omnis
TTF1	SPT24	10% NBF, 30 min.	TRS-Low 10 min.	Omnis
WT1	EP122	10% NBF, 30 min.	TRS-Low 10 min.	Omnis

CK5
LBC



Immunocytochemistry – Requirements for optimal performance

- Appropriate choice of fixative and fixation process
- Appropriate calibration and validation
  - Appropriate choice of antibody clone and titre
  - Appropriate epitope retrieval
  - Robust and sensitive detection system
- Appropriate choice of cell controls
  - Clinically relevant range of expression levels

A systematic test battery approach

ImmunoHistochemistry – Requirements for optimal performance

iCAPCs - Immunohistochemical critical assay performance controls

High expression Low expression No expression

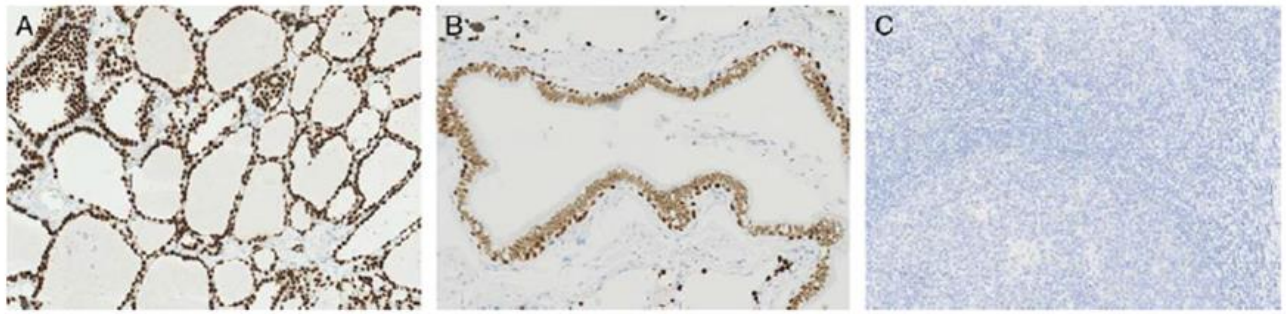


FIGURE 7. TTF-1 iCAPC. A, Thyroid: virtually all epithelial cells must show a strong nuclear staining reaction. B, Lung: virtually all all pneumocytes and basal cells of terminal bronchi must show a moderate to strong nuclear staining reaction. Columnar epithelial ial cells of terminal bronchi must show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

Right Ab Right Sensitivity Right 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‡§

- 2-3 Normal tissues
- Well characterized
- Relevant expression levels
- Processed as clinical samples

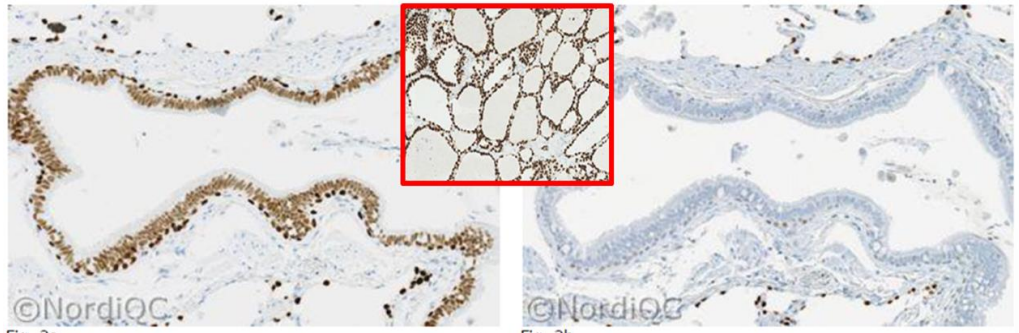


Fig. 2a
Optimal TTF1 staining of the lung using same protocol as in Fig. 1a. The type II pneumocytes and the basal epithelial cells lining the terminal bronchioles show a strong distinct nuclear staining reaction, whereas the columnar epithelial cells show a moderate nuclear staining reaction. No background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

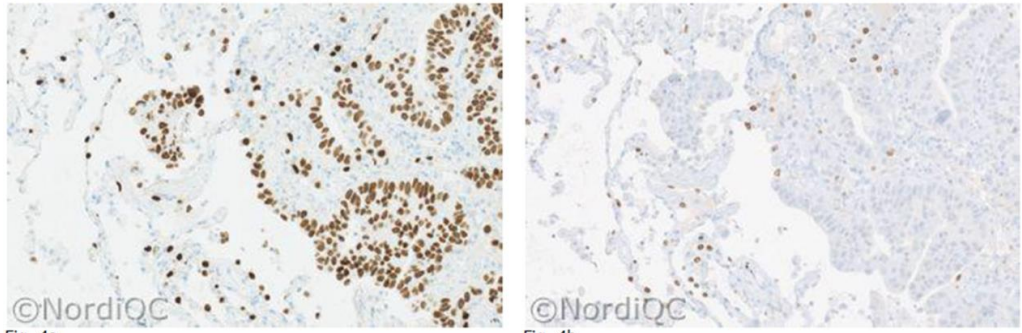


Fig. 4a
Optimal TTF1 staining of the lung adenocarcinoma no. 4 using same protocol as in Figs. 1a, 2a & 3a. Tumour (right side) with adjacent normal lung tissue. Virtually all the neoplastic cells

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

Immuno**Histo**chemistry – Requirements for optimal performance

	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	Tonsil	Tonsil	Tonsil	Different comp.

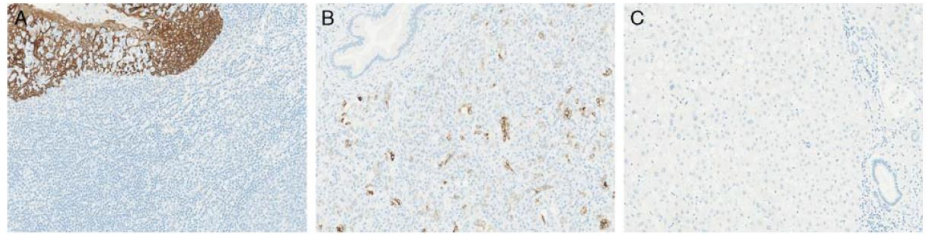


FIGURE 3. CK5 and/or CK14 (high–molecular weight cytokeratin) iCAPC. (A) Tonsil: Virtually all squamous epithelial cells throughout all cell layers must show a moderate to strong cytoplasmic staining reaction. (B) Pancreas: Scattered columnar epithelial cells of intercalated ducts must show a weak to moderate predominantly membranous staining reaction (LLOD). (C) Liver: No staining reaction must be seen. iCAPC indicates immunohistochemistry critical assay performance controls; LLOD, low limit of detection.

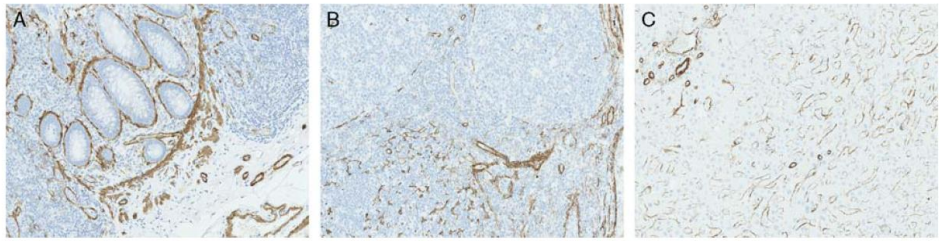


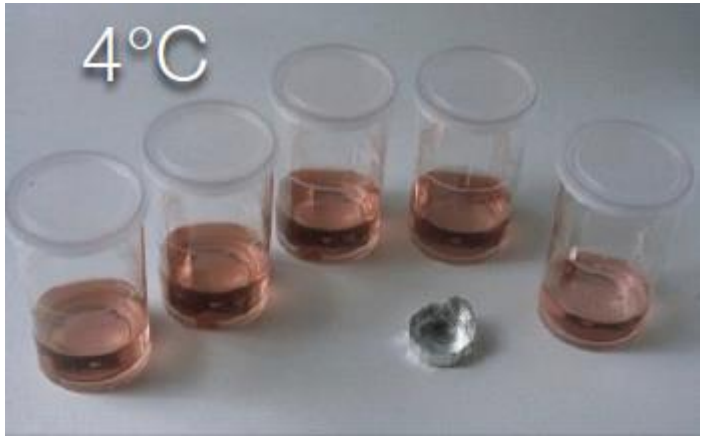
FIGURE 11. α-smooth muscle actin (α-SMA) iCAPC. A, Appendix: all smooth muscle cells in vessels, muscularis mucosae, and muscularis propria of the appendix must show a moderate to strong, distinct cytoplasmic staining reaction. B, Tonsil: virtually all smooth muscle cells in vessels must show a moderate to strong, distinct cytoplasmic staining reaction. C, Liver: the majority of the perisinusoidal cells must show an at least weak to moderate, distinct cytoplasmic staining reaction (LLOD). iCAPC indicates immunohistochemistry critical assay performance controls; LLOD, low limit of detection.

Immunocytochemistry – Requirements for optimal performance

Collection of pre-identified fresh tissues for imprints

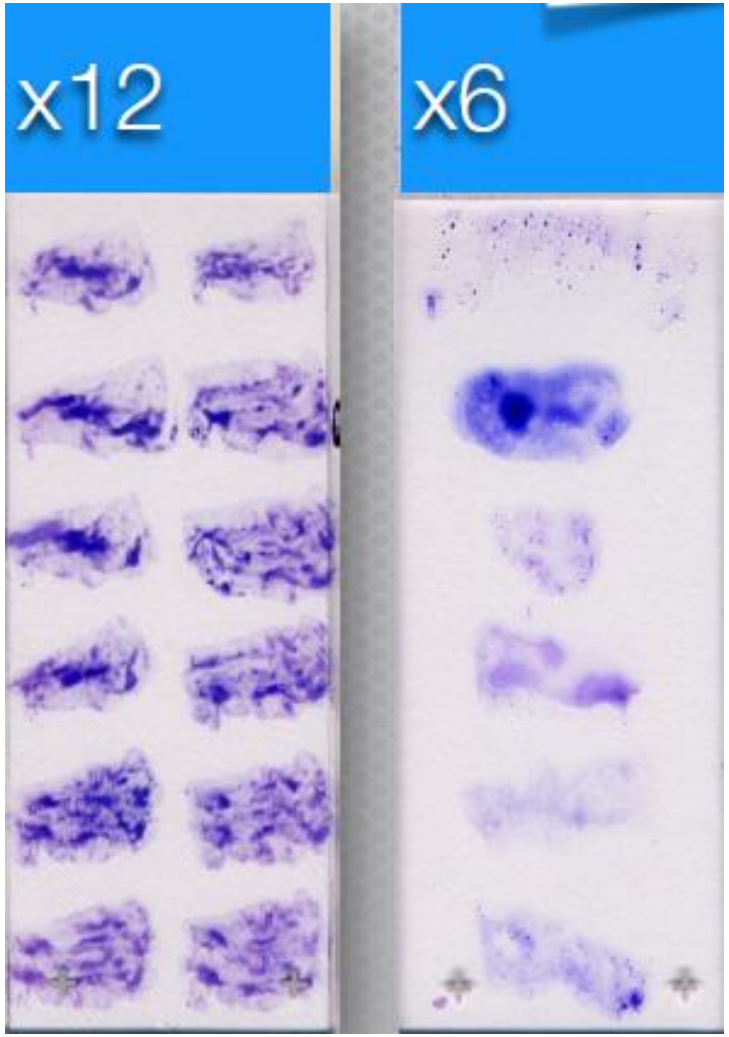
Tissues with High expression
Tissues with Low expression
Tissues with No expression

Either as single imprints or ”TMA” imprints



Tissue storage 1-3 days at 4°C in e.g. DMEM or similar

Faes, Katrien, and Ellen Goossens. "Short-term hypothermic preservation of human testicular tissue: the effect of storage medium and storage period. " *Fertility and sterility* 105.5 (2016): 1162-1169.



Kidney

Colon

Lung

Liver

Pancreas

Breast

……

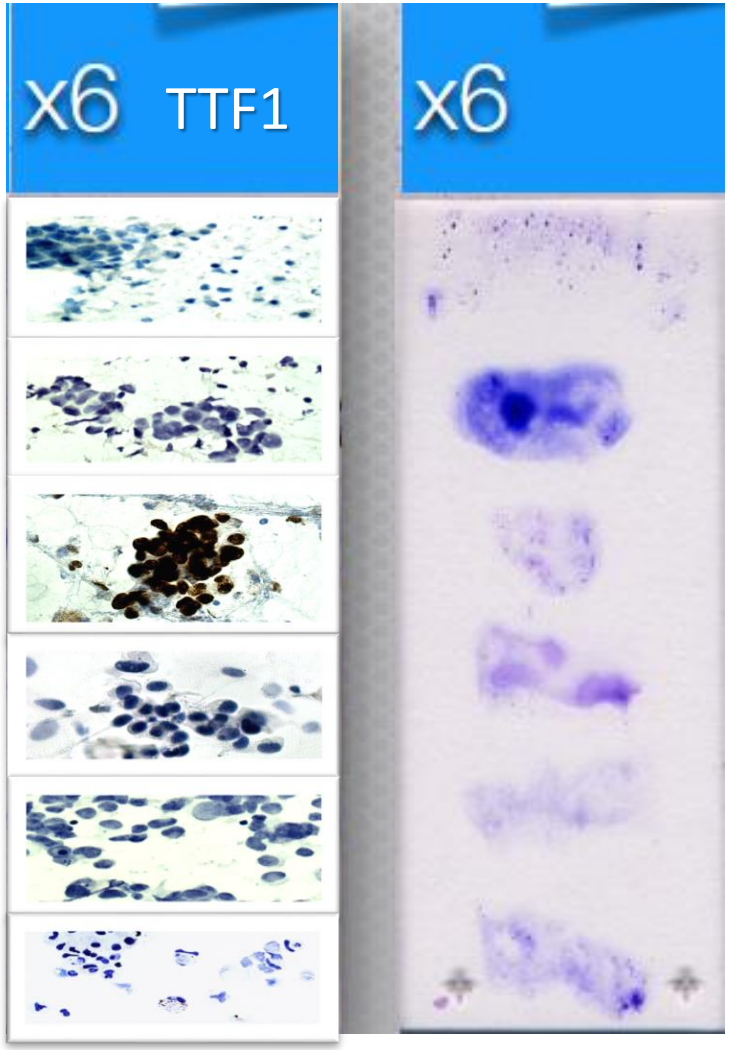
Immunocytochemistry – Requirements for optimal performance

Collection of pre-identified fresh tissues for imprints

Tissues with High expression
Tissues with Low expression
Tissues with No expression

Either as single imprints or ”TMA” imprints

Tissue storage 1-3 days at 4°C in e.g. DMEM or similar



Kidney

Colon

Lung

Liver

Pancreas

Breast

……

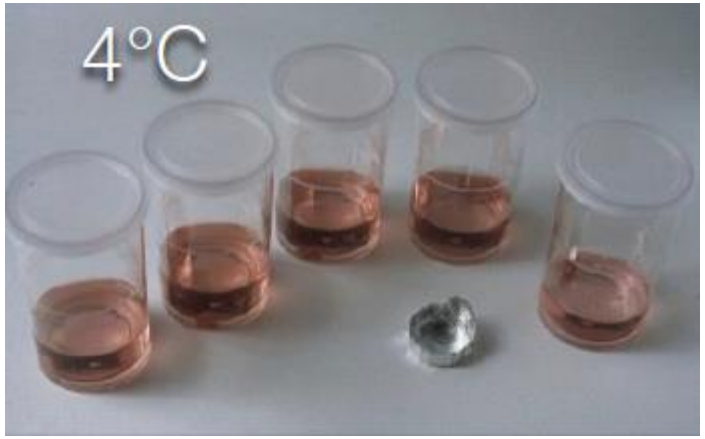
Faes, Katrien, and Ellen Goossens. "Short-term hypothermic preservation of human testicular tissue: the effect of storage medium and storage period. " *Fertility and sterility* 105.5 (2016): 1162-1169.

Immunocytochemistry – Requirements for optimal performance

Collection of pre-identified fresh tissues for imprints

Tissues with High expression
Tissues with Low expression
Tissues with No expression

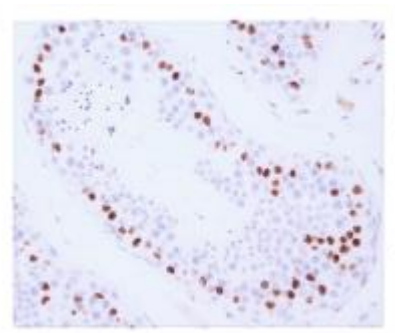
Either as single imprints or ”TMA” imprints

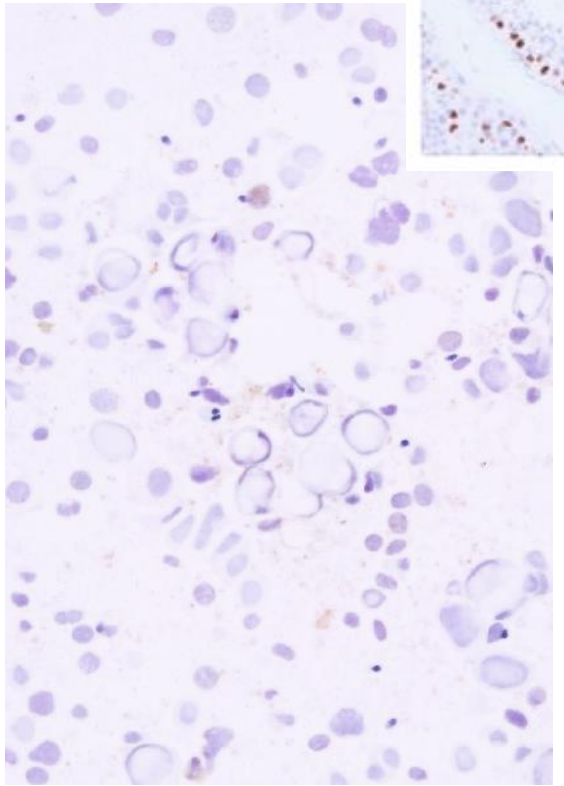


Tissue storage 1-3 days at 4°C in e.g. DMEM or similar

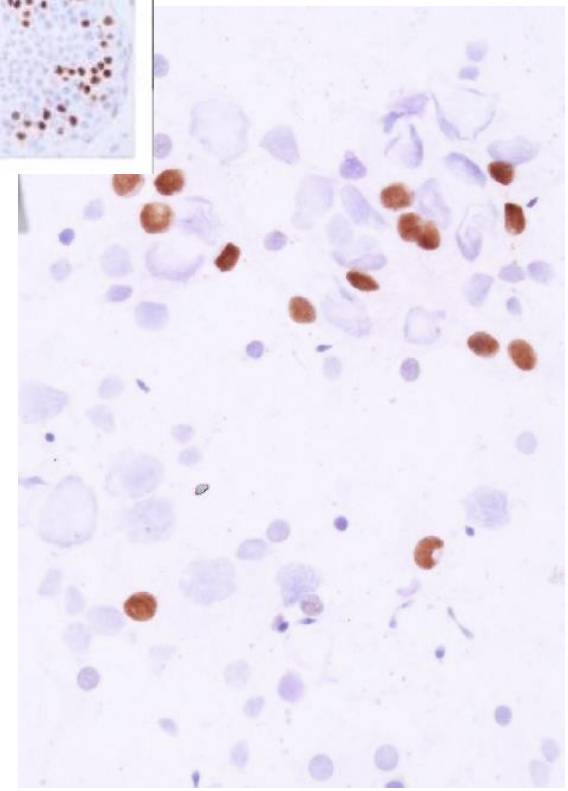
Faes, Katrien, and Ellen Goossens. "Short-term hypothermic preservation of human testicular tissue: the effect of storage medium and storage period. " *Fertility and sterility* 105.5 (2016): 1162-1169.

Ref; IHC on FFPE donor imprint mat.





10% NBF 5 min.



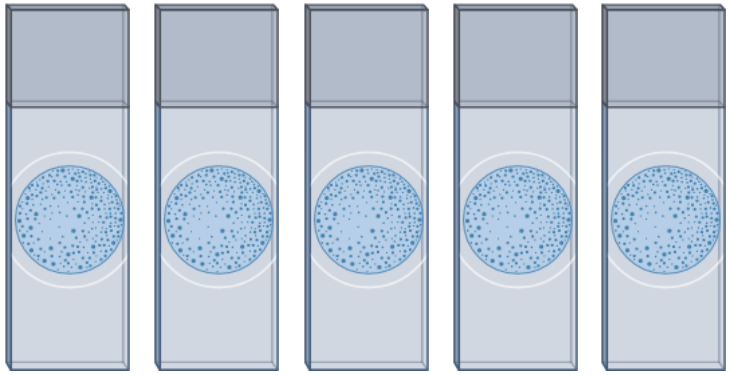
10% NBF + HIER

Imprint of Testis
WT1; EP122

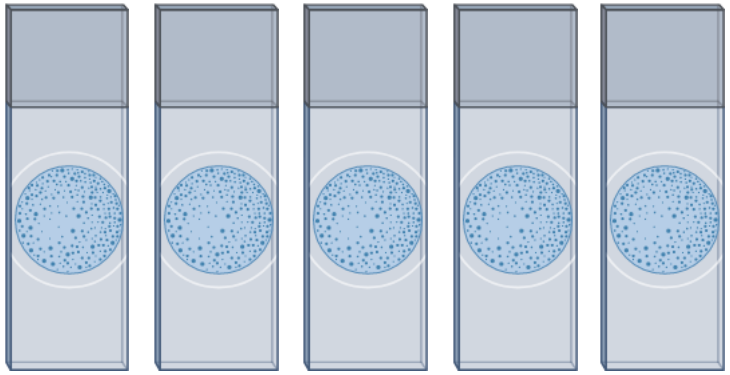
Immunocytochemistry – Requirements for optimal performance

Collection of different pt samples for LBC slides

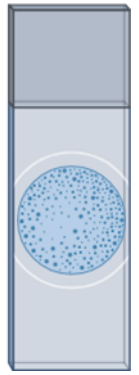
Samples / cells with High expression
Samples / cells with Low expression
Samples / cells with No expression



Ab method development
Titres, fixation, retrieval



Ab method validation
Pos. and neg. samples



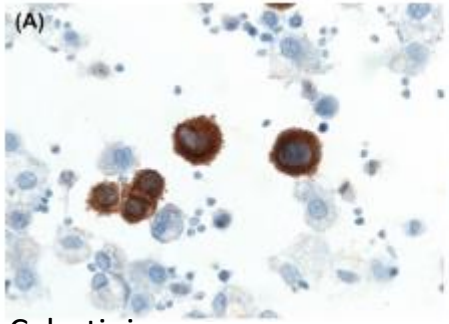
Ab method QC
Pos. sample

Immunocytochemistry – Requirements for optimal performance - Storage

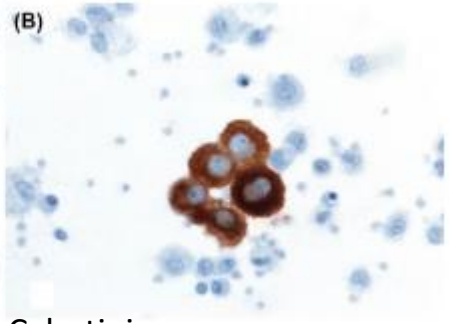
Antigens in airdried smears, imprints and cytospin/LBC deterioate over time.

Storage optimally in -80°C (years), alternatively -20°C (months)*

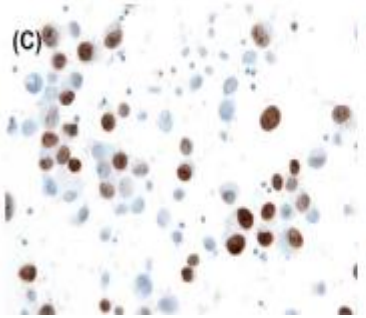
Storage at room temp. by Polyethylene glycol (PEG) coatning (months)**



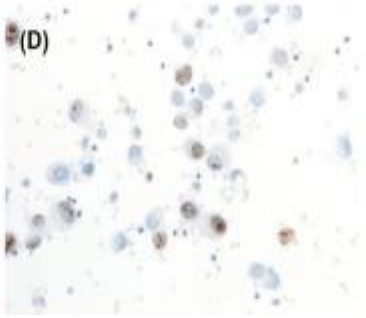
Calretinin
Baseline



Calretinin
PEG 15 months, RT



Ki67
Baseline



Ki67
PEG 4 months, RT

Storage essential for ICC control material identical to cytological samples and alternative to FFPE controls

*Skoog L, Tani E. Immunocytochemistry: an indispensable technique in routine cytology. Cytopathology. 2011 Aug;22(4):215-29.

**Srebotnik Kirbis I et al. Preservation of biomarkers immunoreactivity on cytospins protected with polyethylene glycol. Cytopathology. 2021 Jan;32(1):84-91.

Cytology sample preparations – pros and cons….

	FNA – smear	FNA – liquid based cytology	FNA – cell block
+	1. Excellent cytomorphology 2. Extra smears easily made during aspiration 3. Applicable even for smears with few tumor cells	1. Uniform cell spread in single layers facilitating IHC and interpretation 2. Multiple slides with same cell content 3. Standardarized assay supporting QC	1. Morhology comparable to histology 2. Multiple slides with same cell content 3. IHC protocol similar to conventional FFPE material
-	1. Variable content of tumor cells. 2. Require special ICC protocol and validation **3. Airdried / different fix.**	1. Altered cytomorphology. 2. Require special IHC protocol and validation **3. Different fixatives**	1. Inconsistent cellularity 2. Different methods to generate cell blocks 3. Time consuming ***4. Different fixatives +/-***

Cell block procedure – pros and cons

Table 1. Advantages and limitations of different cell block methods

Cell block technique	Clot and scrape method	Formalin or alcohol vapor method	BBC fixative method	Cell block pellet alcohol fixation	Cell block pellet formalin fixation	Plasma thrombin method	Collodion method	Cellient automated system	HistoGel method
Advantages	Inexpensive, no additional equipment required	Inexpensive	Fast, no additional equipment, low cost, excellent results	Inexpensive, easy and rapid method, good for FNAs of any type and fluids	Inexpensive, easy and rapid method, good for FNAs of any type and fluids, optimal results for IHC and molecular studies	Simple, low cost, clean background for ancillary studies, suitable for FNAs of any type and fluids	Good cellular yield, great for samples with scant cellularity	Great for small/ scanty samples, crisp architecture, fully automated processing, consistent results, no cross-contamination	Concentration of cells in HistoGel for an adequate sample, good cellular preservation
Disadvantages	Does not work well with small samples, crush artifact is common, variable cellularity	Time-intensive, variable quality	None	Cellular yield variable, limited data on IHC due to alcohol fixation	Cellular yield variable	Cross-contamination from plasma and thrombin, uneven concentration of cells	Time-consuming method of preparation, toxic ether fumes for storage	Time-consuming method of prep—45 mins. for each cell block prep, expensive, training of histology for cutting thin blocks, limited data on IHC due to alcohol fixation*	Can be a tedious process—HistoGel has to be converted to liquid state

Modified from Jain D, et al. *Cytopathology*. 2014;25(6)356–371.

*Formalin protocol available

Cell block procedure – pros and cons

Received: August 20, 2020; **Revised:** September 28, 2020; **Accepted:** September 30, 2020

Formalin Fixation for Optimal Concordance of Programmed Death-Ligand 1 Immunostaining Between Cytologic and Histologic Specimens From Patients With Non–small Cell Lung Cancer

Bregje M. Koomen, MD [1]; Jose van der Starre-Gaal, MD, PhD[2]; Judith M. Vonk, PhD[3]; Jan H. von der Thüsen, MD, PhD[4]; Jacqueline J. C. van der Meij, MD[5]; Kim Monkhorst, MD, PhD[6]; Stefan M. Willems, MD, PhD[1,7]; Wim Timens, MD, PhD[7]; and Nils A. 't Hart, MD, PhD[2,7]

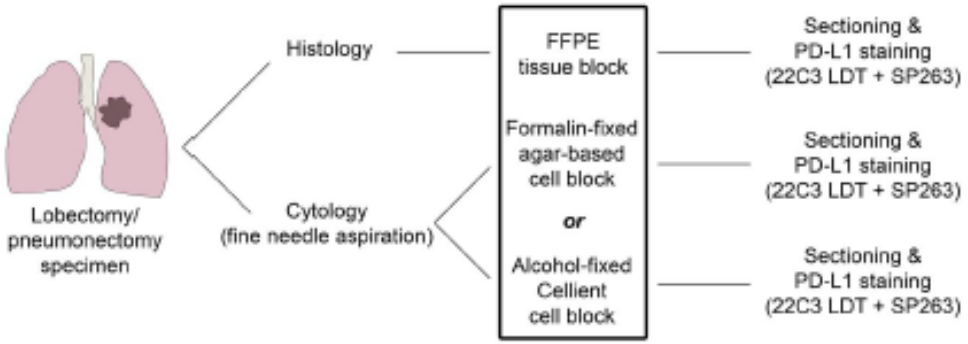


Figure 1. This is a schematic representation of the study design. FFPE indicates formalin-fixed, paraffin-embedded; LDT, laboratory-developed test (using the 22C3 antibody); PD-L1, programmed death-ligand 1; SP263, antibody used in the standardized assay.

TABLE 1. Concordance of Programmed Death-Ligand 1 (PD-L1) Expression (Tumor Proportion Score) Between Histologic and Cytologic Specimens When the 22C3 Laboratory-Developed Test Was Used to Stain for PD-L1[a]

	Agreement of TPS in 3 Categories: <1%, 1%-49%, and ≥50%		Agreement When Dichotomizing TPS at 1% Cutoff				Agreement When Dichotomizing TPS at 50% Cutoff			
Cytologic Specimen	OPA, %	Weighted κ (95% CI)	OPA, %	PPA, %	NPA, %	Cohen κ (95% CI)	OPA, %	PPA, %	NPA, %	Cohen κ (95% CI)
Agar, formalin-fixed	73	0.70 (0.51-0.88)	88	90	83	0.74 (0.49-0.99)	85	67	95	0.65 (0.37-0.94)
Cellient, alcohol-fixed	44	0.28 (0.06-0.49)	62	48	85	0.28 (0.00-0.57)	76	20	100	0.26 (−0.05, 0.57)

Abbreviations: LDT, laboratory-developed test; NPA, negative percent agreement; OPA, overall percent agreement; PPA, positive percent agreement; TPS, tumor proportion score.
[a]Analyses were performed separately for formalin-fixed, agar-based cell blocks and for alcohol-fixed Cellient cell blocks.

Cell block procedure – pros and cons

Received: August 20, 2020; **Revised:** September 28, 2020; **Accepted:** September 30, 2020

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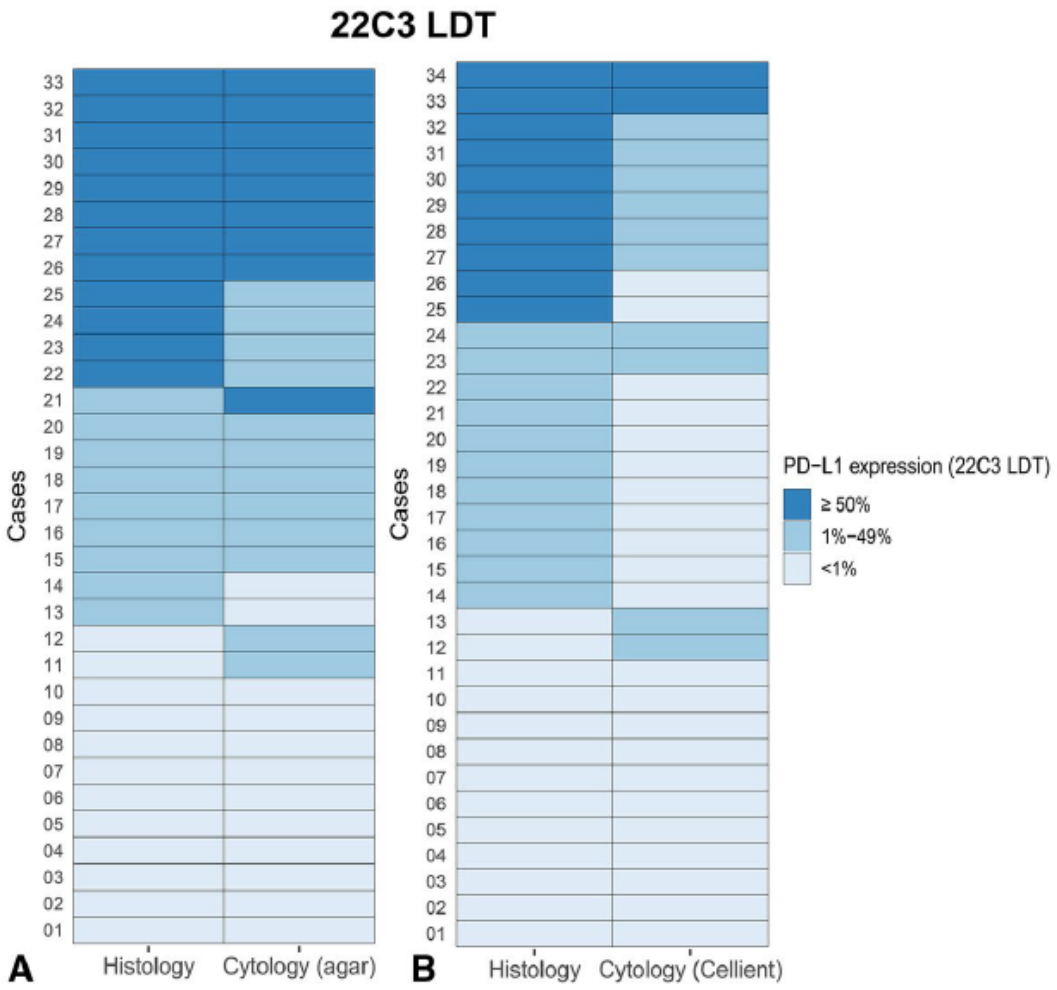
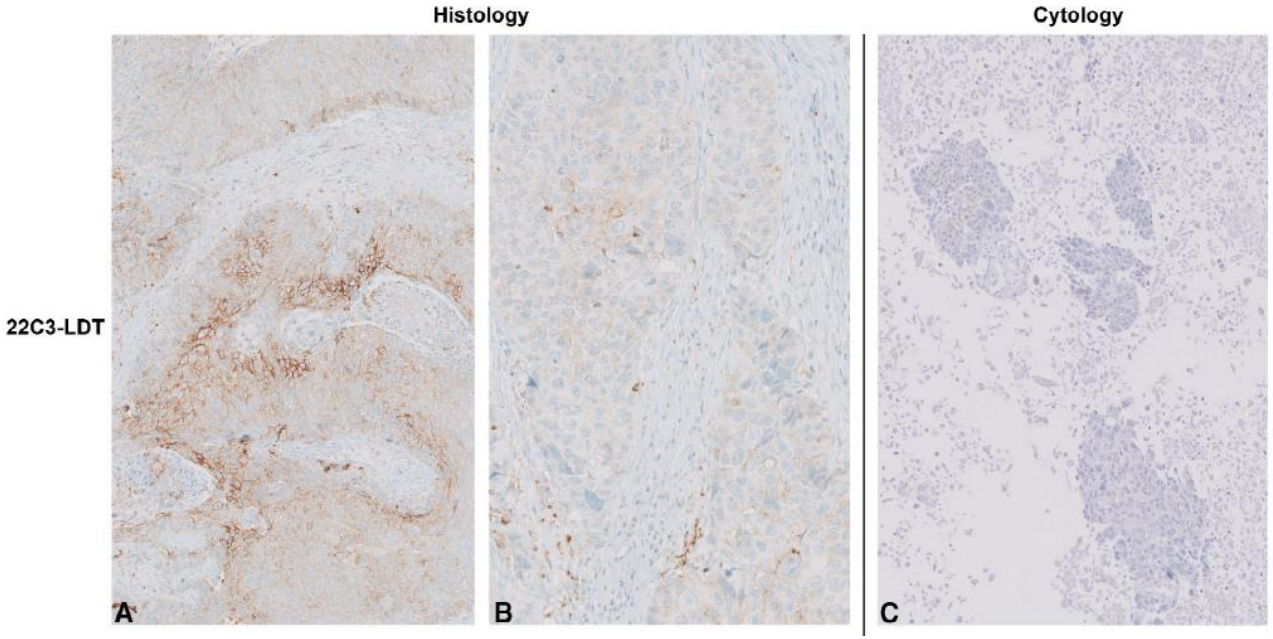
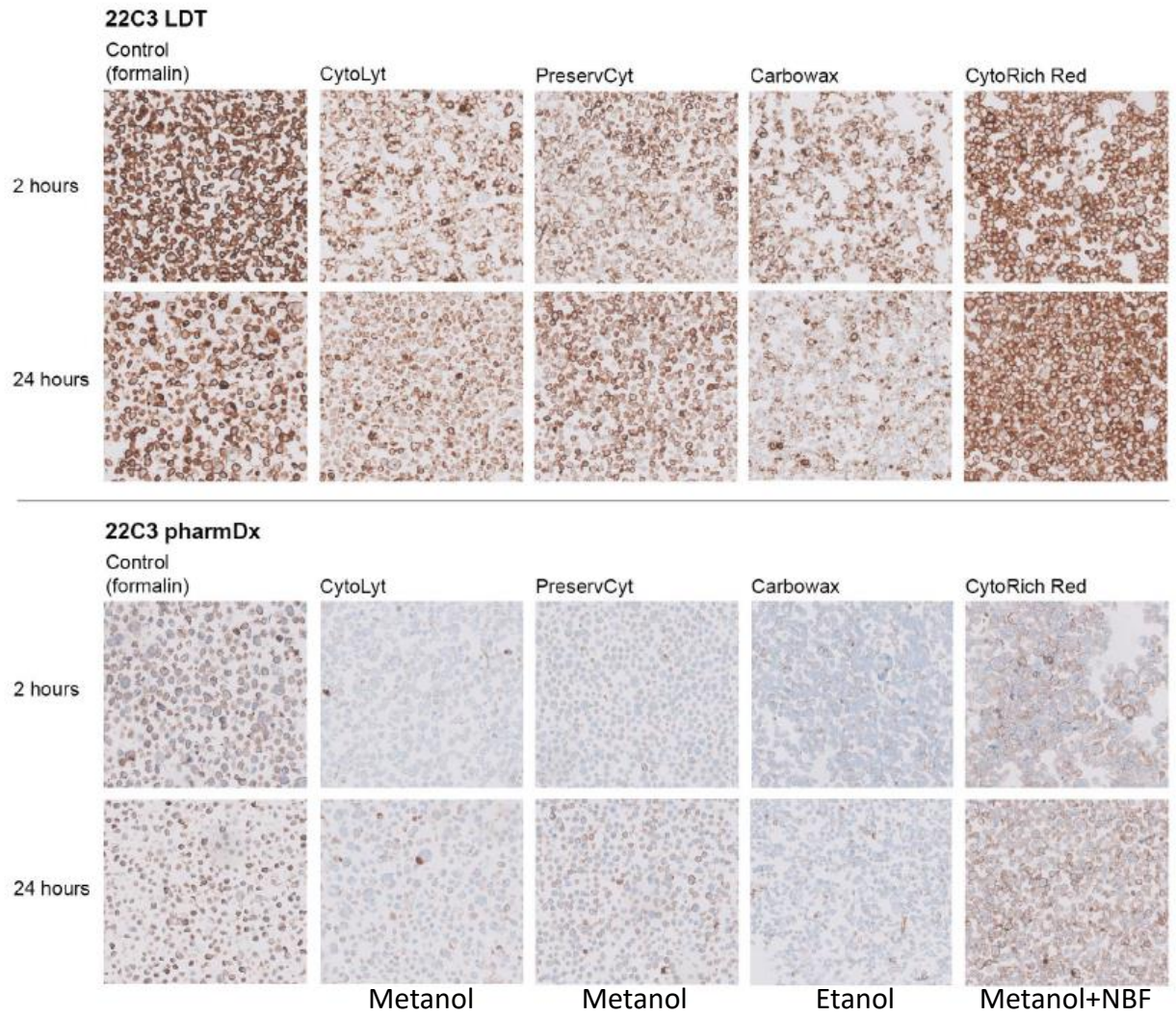




22C3 LDT
A&B; Histology of NSCLC 1; TPS low
C; Cytology of NSCLC1 ; TPS neg (Cellient)

Cell block procedure – pros and cons



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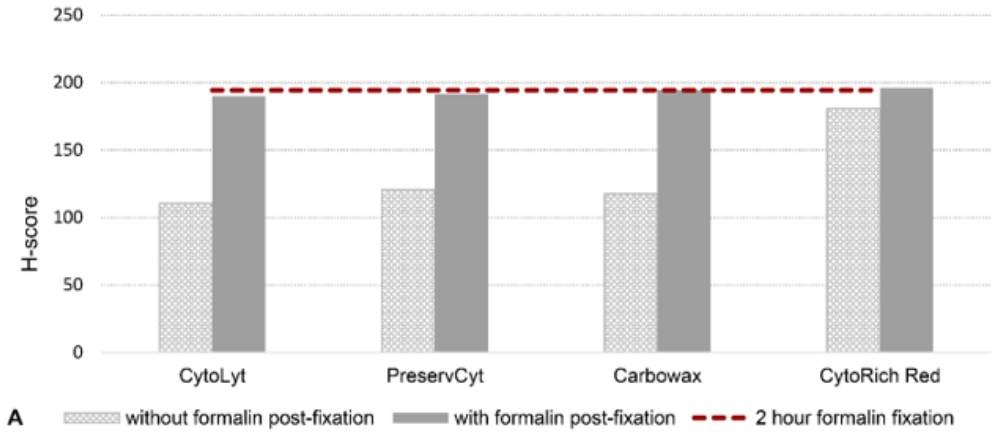
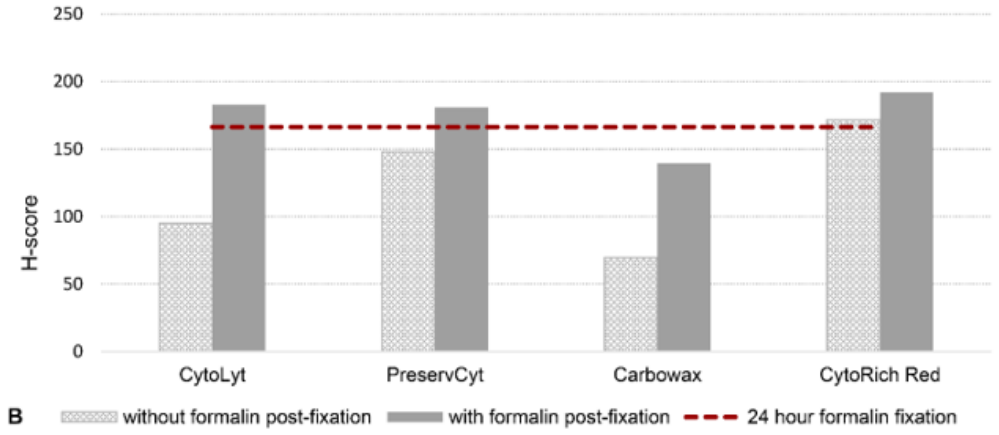
Cell block procedure – pros and cons

Supporting Figure 2. Programmed death-ligand 1 (PD-L1) expression (expressed in H-scores) in cell lines fixed in alcohol-based fixatives with and without formalin post-fixation, stained with 22C3 LDT. **A:** PD-L1 H-scores for a total fixation duration of 2 hours. The dotted line represents the H-score of a cell line fixed in formalin only. **B:** PD-L1 H-scores for a total fixation duration of 24 hours. The dotted line represents the H-score of a cell line fixed in formalin only.





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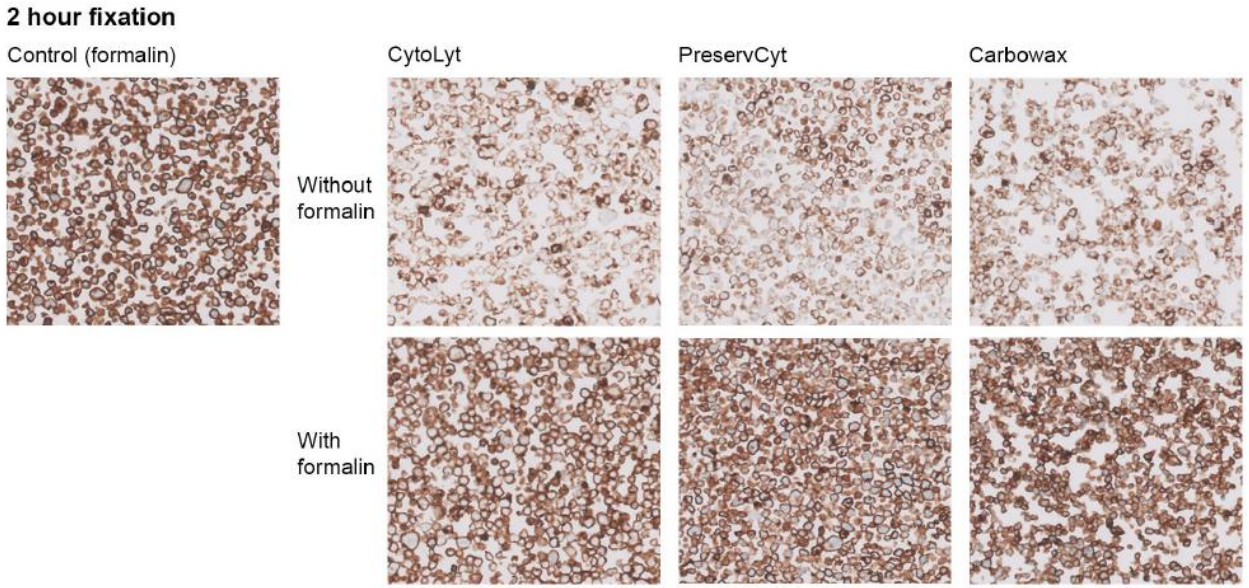

The reduced PD-L1 expression in Cellient processed samples was in additional studies on cell lines to some degree restored by post-fixation in 10% NBF for 30 min.

Cell blocks – Best practices

- Time to formalin max 30 min.
- Time in formalin 6-72 hours
- Cell blocks optimally made from unfixed cytologic material alternatively e.g. alcohol + post fixation in formalin. Plasma thrombin preffered method – Cellient also adequate.
- 4 um sections
- Sections dried at 55-60°C 30-60 min.
- IHC performed asap within 1-2 weeks.

Immunocytochemistry – Best practices, conclusions

- ✓ Develope, optimize and validate ICC methods with focus on the sample types relevant
- ✓ Ideally use serial imprints of relevant tissue types and/or different LBC samples in the process
- ✓ Use the test battery strategy with focus on fixatives, +/- HIER and a highly sensitive IHC/ICC method
- ✓ Methanol and ethanol based fixatives can cause reduced expression – caveat for LBC and CB
- ✓ The Histological concept based on postfixation in NBF and HIER seems to favour ICC methods
- ✓ Caution on storage conditions of slides for ICC

Thank You for the attendance.

Questions ??