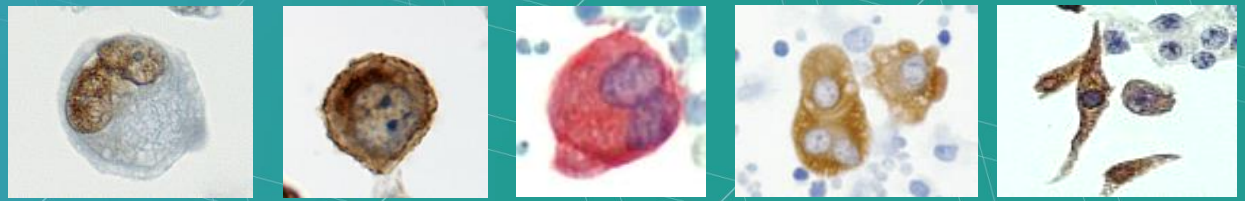
Immunocytochemistry – overview, considerations and applications

Irena Srebotnik Kirbis



INSTITUTE OF PATHOLOGY
UNIVERSITY OF LJUBLJANA ◊ FACULTY OF MEDICINE

Slovenia


POLAND
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SLOVAKIA
FRANCE
AUSTRIA
HUNGARY
SWITZERLAND
CROATIA
ITALY
BOSNIA & HERZEGOVINA
SPAIN

Sources

EFCS surveys

- Immunocytochemistry
  - 245 participants; 94% from 26 European countries, 6% from 5 non-European countries
  - Cancer Cytopathol. 2020;128(10):757-766. doi:10.1002/cncy.22311
- Cell blocks
  - 402 participants; 97% from 27 European countries, 3% from 10 non-European countries

UK NEQAS ICC results

Our studies and experiences

- Preservation of biomarkers immunoreactivity on cytospins protected with polyethylene glycol. Cytopathology. 2021; 32: 84–91.
- Time-related changes in cell morphology and biomarker immunoreactivity for cells stored in a buffer-based cell medium. Cytopathology. 2021;32(4):513-518.
- Immunocytochemistry practices in European cytopathology laboratories - review of European Federation of Cytology Societies (EFCS) online survey results with best practice recommendations, Cancer cytopathology 128 (10): 757-766, 2020.
- Cell count-based triaging of cytology samples for cell block preparation, Cytopathology.2016; 28(3): 216-220.
- Optimization and validation of immunocytochemical detection of oestrogen receptors on cytospins prepared from fine needle aspiration (FNA) samples of breast cancer, Cytopathology. 2015;26(2): 88-98.
- External quality control for immunocytochemistry on cytology samples : a review of UK NEQAS ICC (cytology module) results, Cytopathology.2011; 22(4): 230-237.
- Haemorrhagic cytology samples: how to get the best diagnostic results, Cytopathology.2007; 18(3):175-179.
- MIB-1 immunostaining on cytological samples: a protocol without antigen retrieval, Cytopathology.2004; 15(3):154-159.

Immunocytochemistry (ICC) = IHC on cytology samples

Cytology

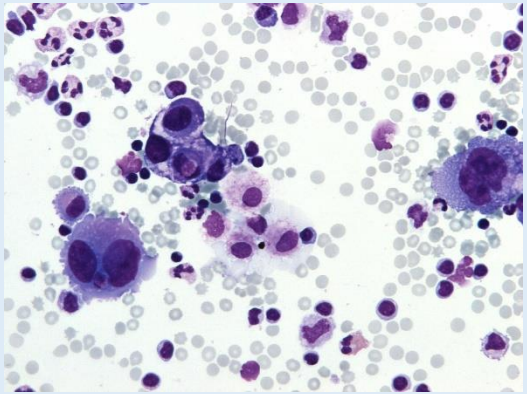
- Minimally invasive diagnostic method
- First line, sometimes ONLY available
- US-FNA, EUS-FNA

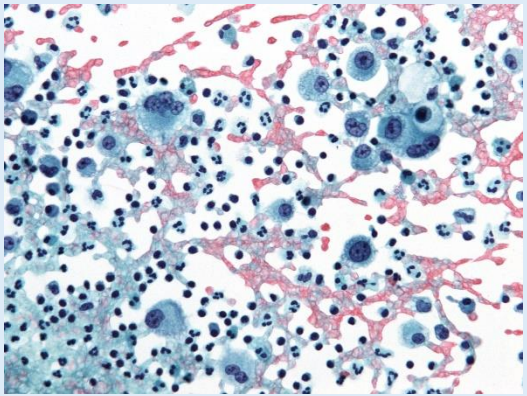
ICC

- Tumor typization
- Metastasis origin
- Prognostic/predictive

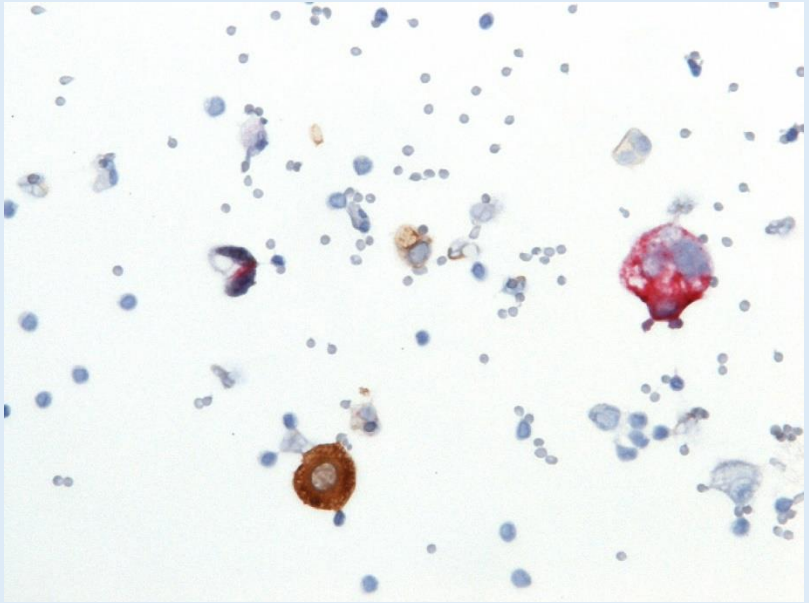
Value of ICC in a modern cytopathology- effusions

Pleural effusion, M, 80 yrs, ca pancreas



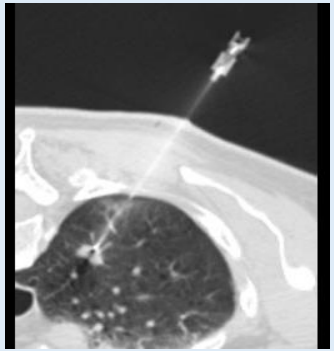


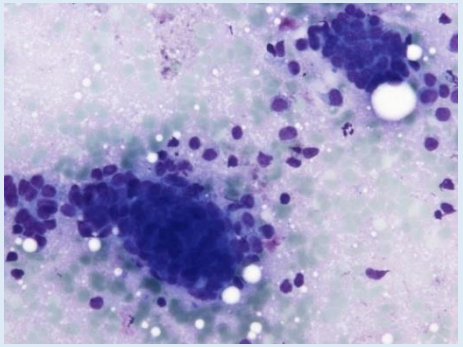
Double ICC – Calretinin/MOC31

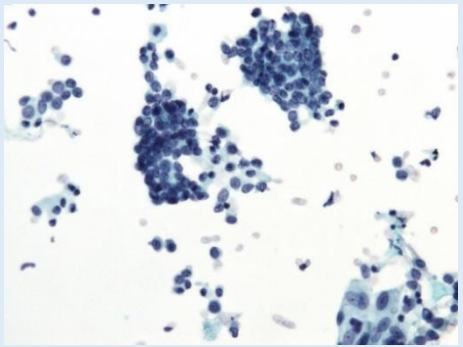


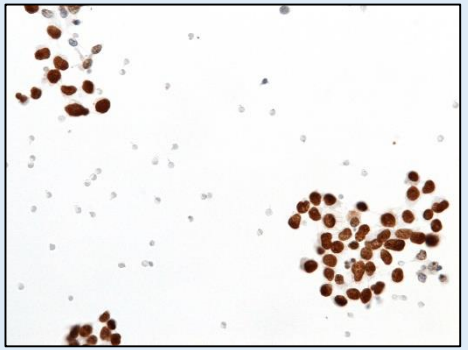
Value of ICC in a modern cytopathology - breast cancer- hormone receptor status

F, 87 yrs, tumour in right breast 4 x 3,5 cm

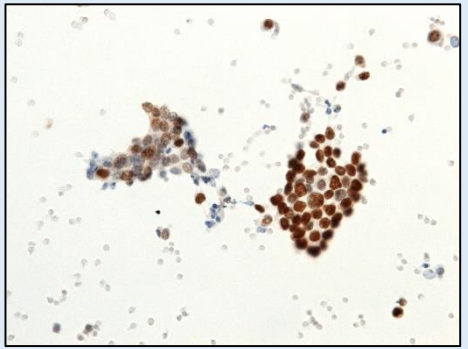






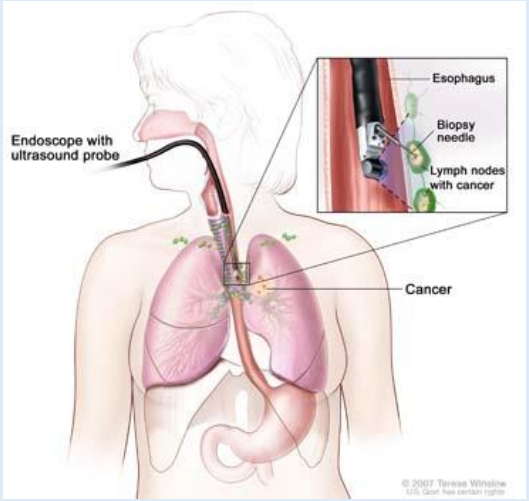


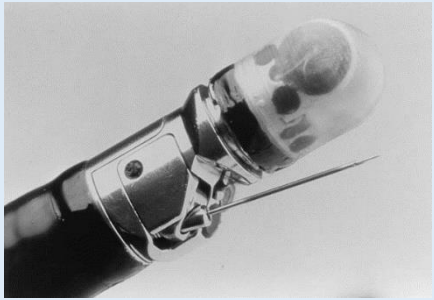
ER

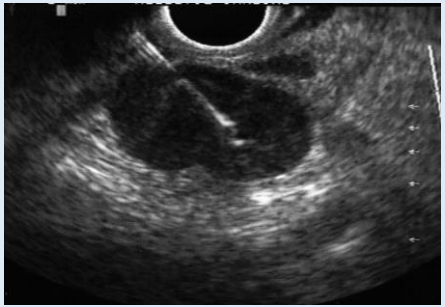


PR

Endoscopic ultrasound guided FNAs (EUS-FNAs)







Immunohistochemistry (IHC) = Immunocytochemistry (ICC)

- Principles
- Basic steps
- Antibodies
- Reagents
- Platforms
- QA/QC measures

Immunohistochemistry (IHC) ≠ Immunocytochemistry (ICC)

Pre-analytical

- Sample management and processing
- Fixation

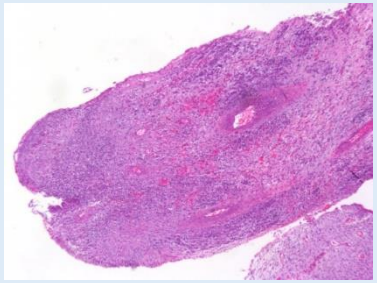
Analytical

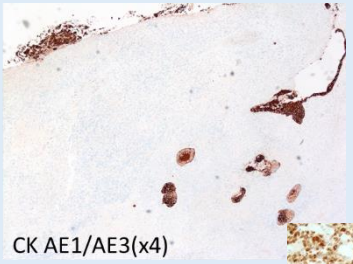
- Pretreatment
- Dilutions
- Detection kits

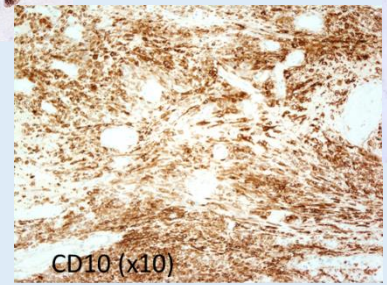
QA/QC

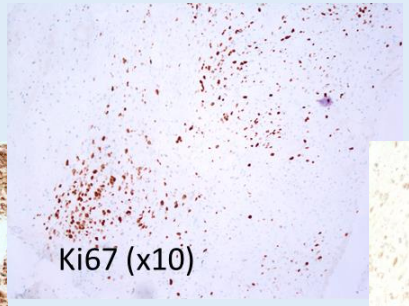
- Control slides
- Optimization
- Validation

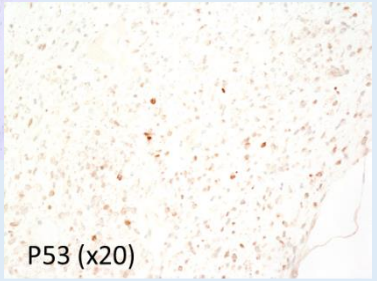
Histology – IHC workflow











Cytology –ICC workflow

On site immediately

Diagnostic smears

Smear for **R**apid **O**n **S**ite **E**valuation (ROSE)
sample adequacy ? ancillary test ?

Sample for ICC, special stain, flow cytometry, FISH, ISH, molecular test **?**

Cytology – **ICC workflow**

- Low and unknown sample volume/cellularity
- Sample adequacy
- Immediate decision for ancillary methods

Cytology sample processing – **slide preparation** options

- Cell blocks
- Direct smears
- Cytospins
- Liquid based cytology – LBC

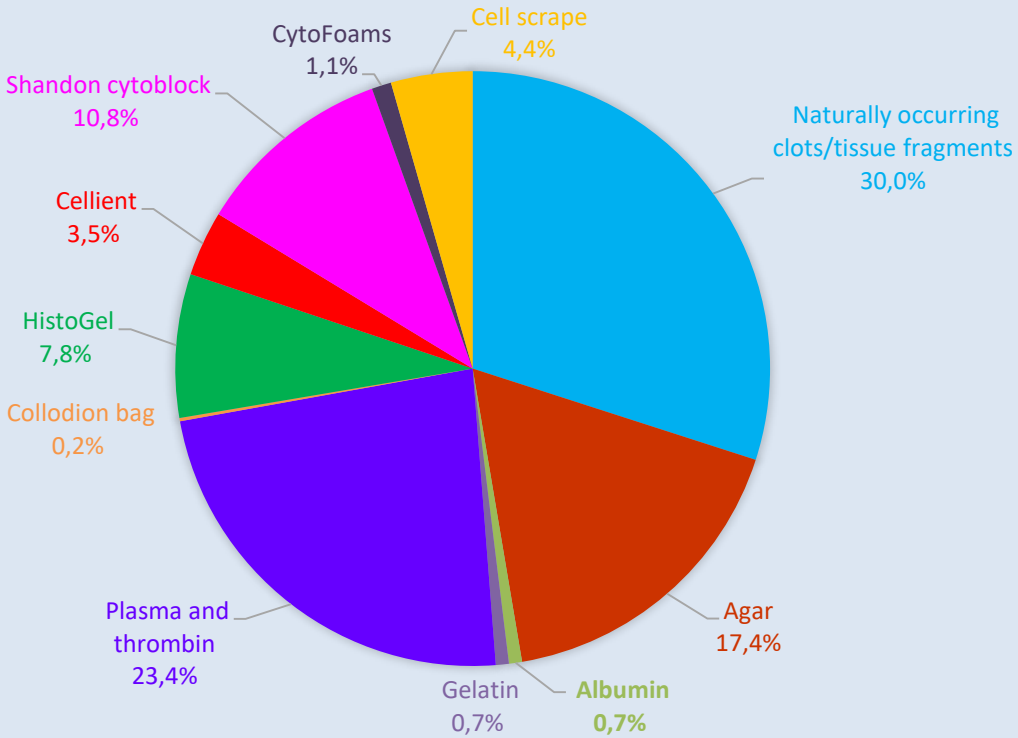
Cell blocks

FFPE cell blocks ≈ FFPE tissue samples

Advantages

- easy storage
- multiple sections
- same protocols as for FFPE
- same QC/QA
- no additional validation studies

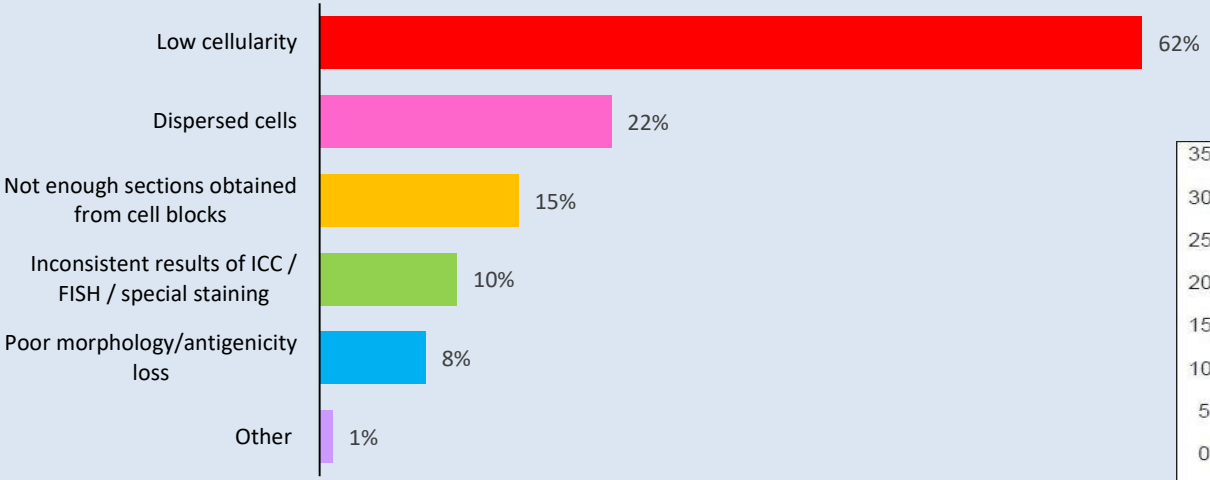
Cell block preparation methods – EFCS survey

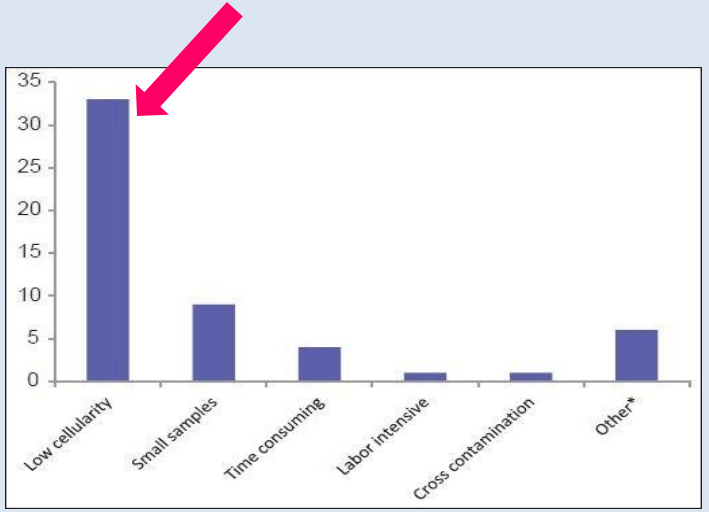


Cell blocks - disadvantages

- no standardized protocol
  - medium for sample collection (fixative, PBS, commercial solutions, RPMI, other)
  - fixation (formalin and non- formalin based)
  - cell pellet preparation (agar, HistoGel, plasma thrombin, Cellient, ...)
- **not suitable for low cellular samples**
- time consuming (↑ TAT)
- ↑ price
- sample triaging

Issues with CB



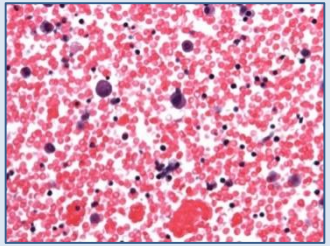


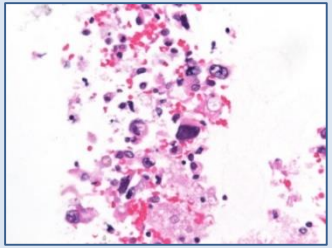
Crapanzano, J. P., Heymann, J. J., Monaco, S., Nassar, A., & Saqi, A. (2014). The state of cell block variation and satisfaction in the era of molecular diagnostics and personalized medicine. CytoJournal, 11, 7. https://doi.org/10.4103/1742-6413.129187

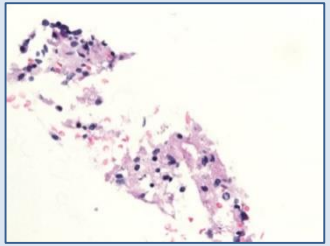
20 x 10^6 cells

1.2 x 10^6 cells

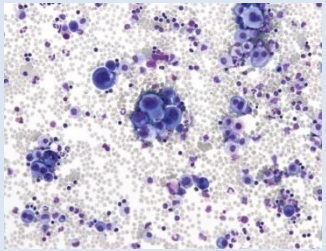
0.1 x 10^6 cells

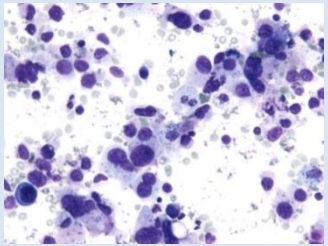


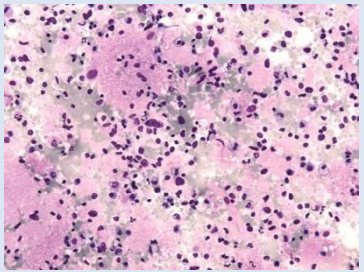




corresponding cytospins

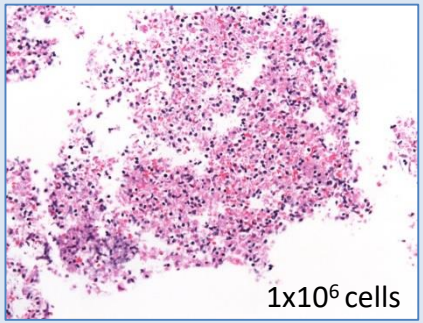


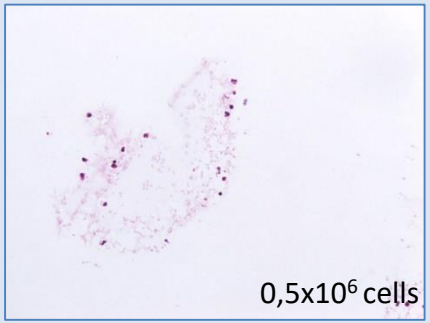


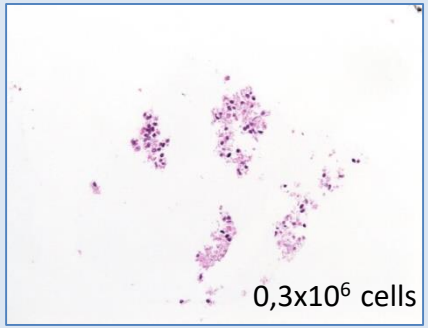


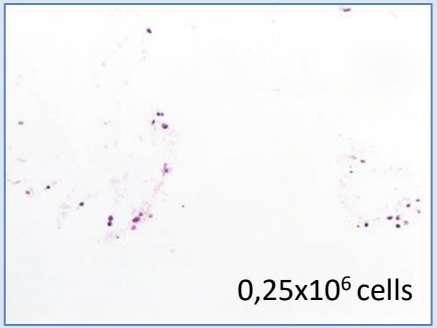
CB cellularity – number of cells embbedded

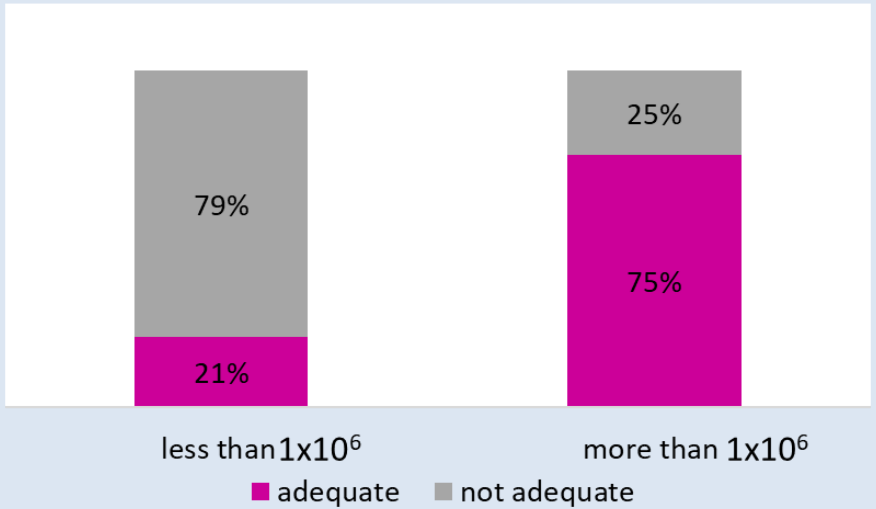
CB from aliquotes of the same sample with different cellularity





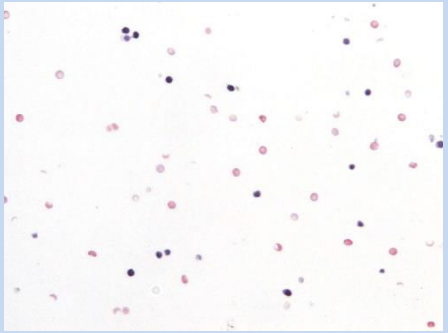


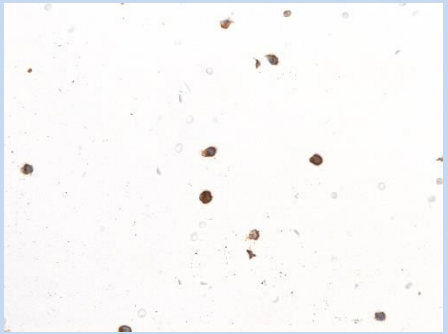




Srebotnik Kirbiš I, Strojan Fležar M. Cell count-based triaging of cytology samples for cell block preparation. Cytopathology. 2017;28(3):216-220. doi:10.1111/cyt.12404

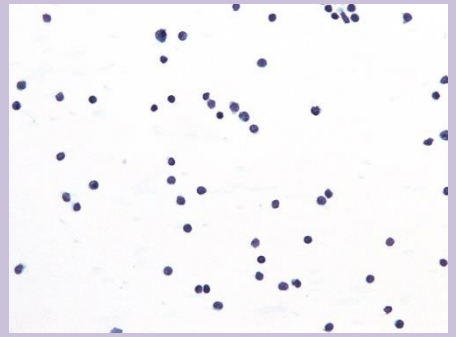
Cell block

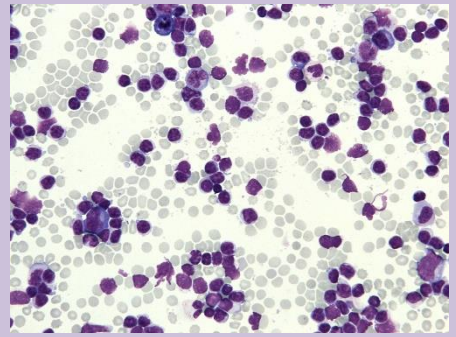


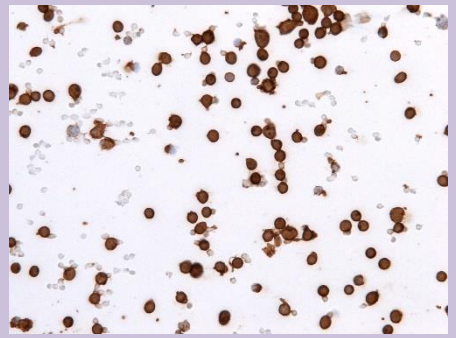


CD45

Cytospins







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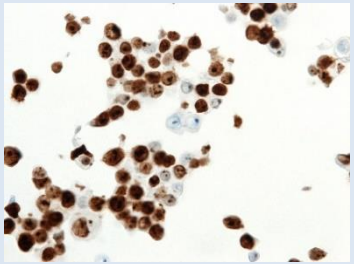
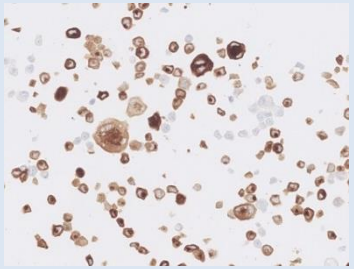
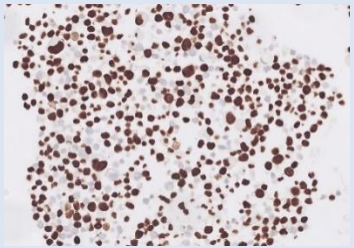
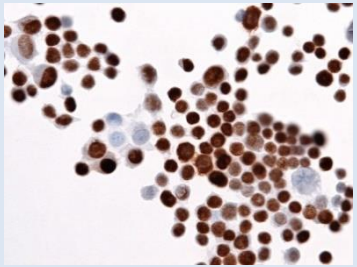
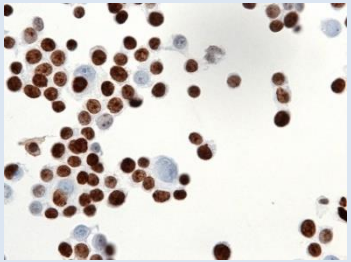
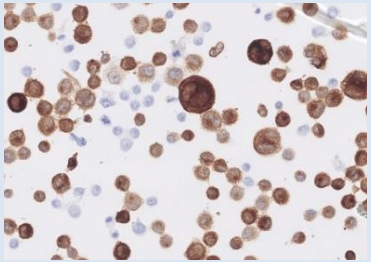
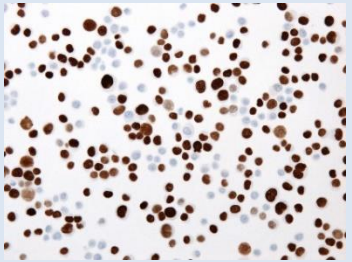
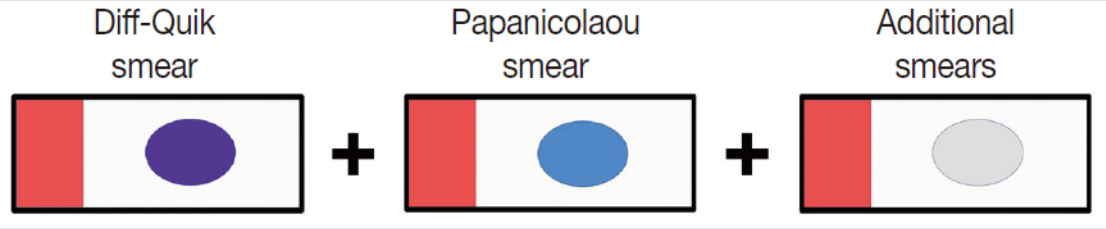
CB				
Cytospin				

Smears - advantages

- always available
- quick, simple, inexpensive
- morphological evaluation before ICC



Alternatives to cell blocks

- Establishing a protocol for ICC staining and CISH of Giemsa and Diff-Quick **prestained cytological smears** (E. Beraki, TK Olsen, T Sauer, CytoJournal **2012**)
- The application of ICC to **direct smears** of metastatic Merkel cell carcinoma (SM Knoepp et al. Diagn Cytopathol **2013**)
- ER, PR, and Her2 immunocytochemistry on cell-transferred **cytologic smears** of primary and metastatic breast carcinomas: a comparison study with formalin-fixed cell blocks and surgical biopsies (Ferguson J et al. Diagn Cytopathol. **2013** Jul;41(7):575-81. doi: 10.1002/dc.22897. Epub 2012 Jul 16.

Smears - disadvantages

- sample triaging: which case/ how many smears
- uneven and uncontrolled distribution of the cells
- background ICC staining
- unstandardized:
  - unstained, Papanicolaou stained, MGG, Diff-Quick
  - fixation: drying before or after, acetone, ethanol based, formalin based, combination of fixatives, one step, multi steps
  - storage: freezer, refrigerator, RT, dried, in a fixative, PEG

Cytospins

- slides prepared by cytocentrifuge from cell suspension
- Cell suspension:
  - PBS, RPMI, ...
  - methanol and ethanol based solutions
- Fixation:
  - before or after drying
  - methanol/ethanol/formalin based fixative
- Storage:
  - fixed or unfixed slides
  - freezer, refrigerator, RT

Stokes B.O. Principles of cytocentrifugation. *Lab. Med.* 2004;35:434–437.

Cytospin

Advantages

- multiple slides
- monolayer, controlled distribution of the cells
- short or long term storage of cell suspension/slides
- postponed decision for ancillary tests

Disadvantages

- cytocentrifuge
- non standardized procedure
- knowledge, experience, cooperation

Liquid based cytology (LBC)

- sample suspended in commercial transport medium
- automated slide preparation (ThinPrep, SurePath, CellPrep....)
  - membrane filtration
  - gradient centrifugation

LBC

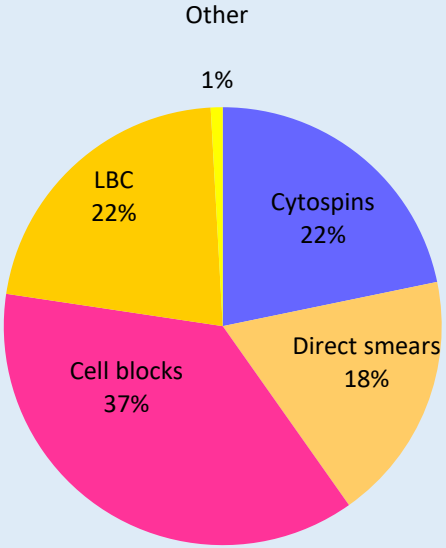
Advantages

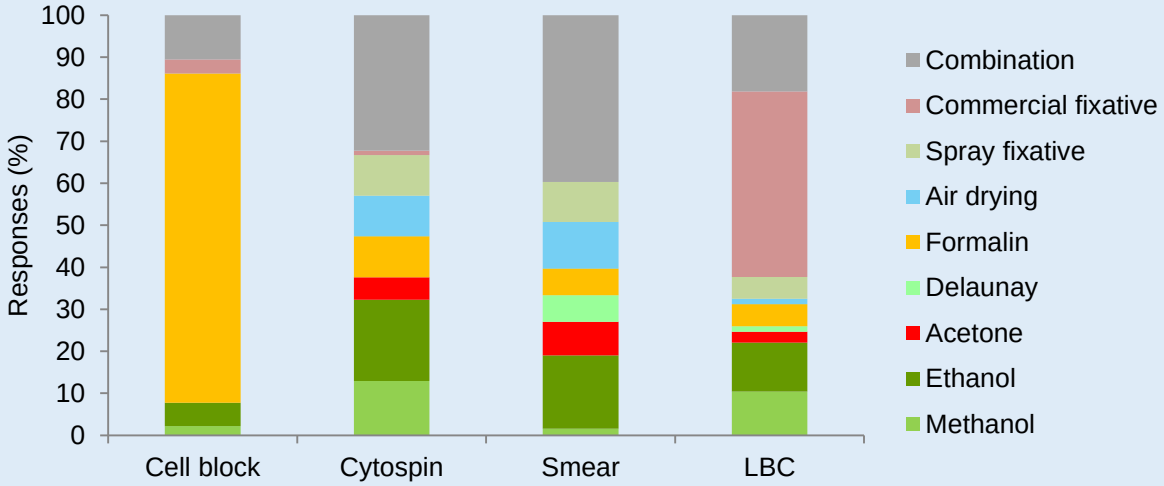
- easy storage of samples
- postpone decision
- monolayer distribution of cells
- multiple slides

Disadvantages

- expensive equipment
- ↑ cost
- Prefixed cells - clumping

Slides used for ICC – European survey



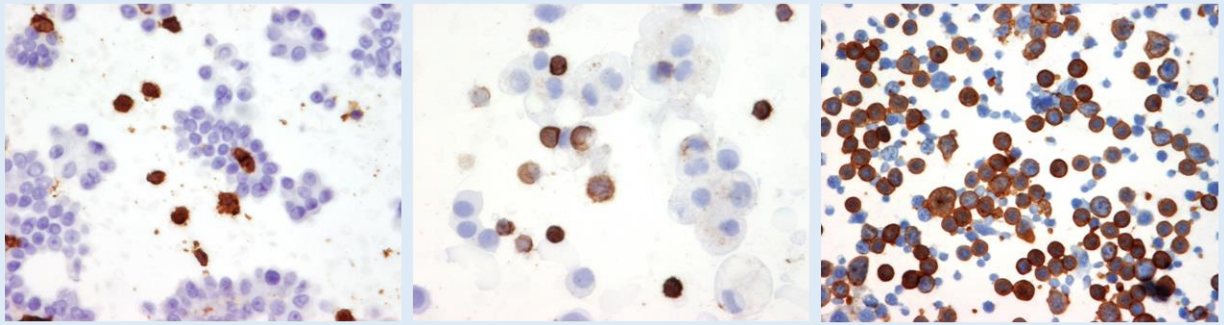


Fixatives used for the fixation of ICC preparations

Srebotnik Kirbiš I, Rodrigues Roque R, Bongiovanni M, Strojan Fležar M, Cochand-Priollet B. Immunocytochemistry practices in European cytopathology laboratories-Review of European Federation of Cytology Societies (EFCS) online survey results with best practice recommendations. Cancer Cytopathol. 2020;128(10):757-766.

Good ICC quality can be achieved on a differently prepared slides

CD 45 (DAKO M701)



Cell block, FFPE Smear, ethanol Cytospin, methanol

Kirbis IS, Maxwell P, Flezar MS, Miller K and Ibrahim M. External quality control for immunocytochemistry on cytology samples: a review of UK NEQAS ICC (cytology module) results. Cytopathology 2011, 22, 230–237.

ICC reality

- Processing of cytology samples for ICC is not standardized
- Great variability in all aspects of ICC on cytology samples
- Good ICC quality can be achieved on a differently prepared slides
- Reliability of ICC (correct, accurate, repeatable)?

Quality assurance/quality control (QA/QC)

Why?

- Reliable ICC results (correct, accurate, repetable)
- Accreditation

How?

- Control slides
- ICC optimization and validation
- External quality control (EQA)

- Institute CLS. Quality assurance for design control and implementation of immunohistochemistry assays: approved guideline, second edition. CLSI Document I/LA28-A2: Clinical and Laboratory Standards Institute; 2011.
- Hardy LB, Fitzgibbons PL, Goldsmith JD, Eisen RN, Beasley MB, Souers RJ, et al. Immunohistochemistry validation procedures and practices: a College of American Pathologists survey of 727 laboratories. Arch Pathol Lab Med. 2013;137(1):19-25.
- Torlakovic EE, Riddell R, Banerjee D, El-Zimaity H, Pilavdzic D, et al. Canadian Association of Pathologists-Association canadienne des pathologistes National Standards Committee/Immunohistochemistry: best practice recommendations for standardization of immunohistochemistry tests. Am J Clin Pathol. 2010;133(3):354-65.

Control slides

Positive control slides

- Sample with known expression of antigen
- Prepared as patients sample

Check:

- staining procedure
- antibody reactivity

Negative control slides

- Additional slide from diagnostic sample
- Replacing primary antibody with diluent buffer

Check:

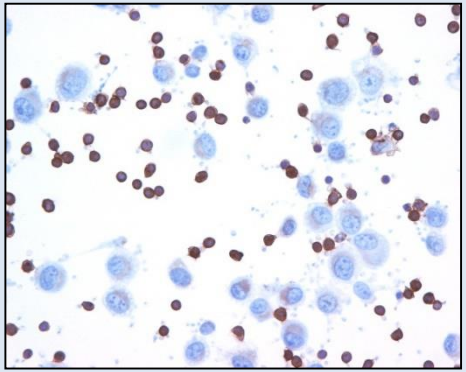
- non-specific staining

Control should be prepared the same as test sample

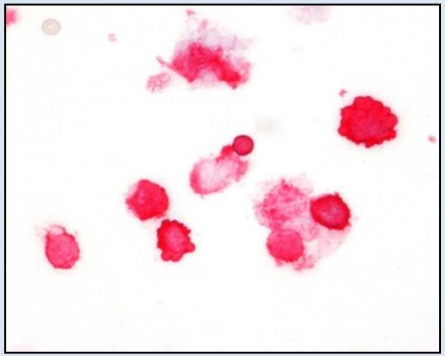
Sample	Control
FFPE tissue	FFPE tissue
Cell blocks -Histogel	Cell blocks -Histogel
Cell blocks - Shandon	Cell blocks - Shandon
Cell block - other	Cell block - other
Cytospins - methanol	Cytospins - methanol
Cytospins - aceton	Cytospins - aceton
LBC - ThinPrep	LBC - ThinPrep
LBC- SurePath	LBC- SurePath
Smear - air dried	Smear - air dried
Smear - formalin	Smear - formalin

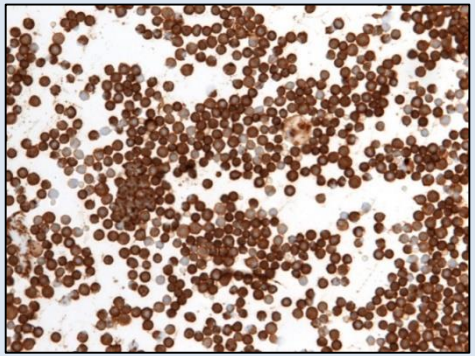
- Each step in sample preparation can affect IR
- ICC procedure for FFPE and cytology slides not identical

Positive control slides

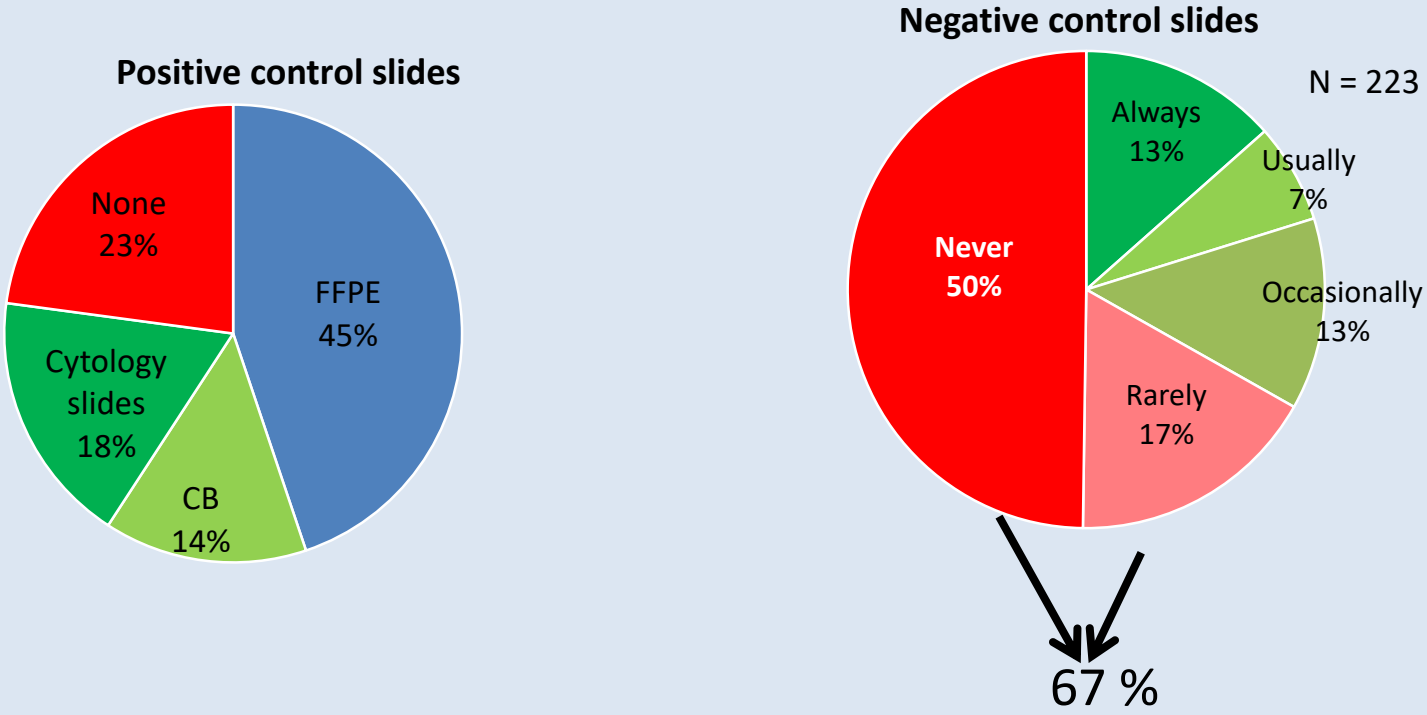


- enough well distributed cells in monolayer
- positive and negative cell population
- good cell morphology





ICC Controls - European survey

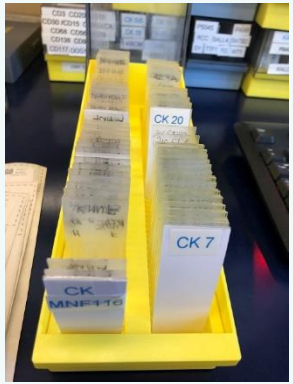


Srebotnik Kirbiš I, Rodrigues Roque R, Bongiovanni M, Strojan Fležar M, Cochand-Priollet B. Immunocytochemistry practices in European cytopathology laboratories-Review of European Federation of Cytology Societies (EFCS) online survey results with best practice recommendations. Cancer Cytopathol. 2020;128(10):757-766.

How to prepare enough good control slides from cytology samples?

Cytology samples for controls

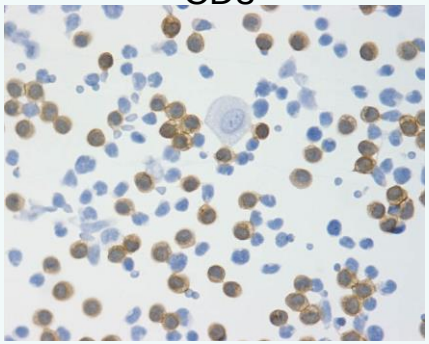
- leftovers of diagnostic cytology samples
- effusions
- cytology samples (FNA‘s, brushings) of fresh resected tumours
- human cell lines



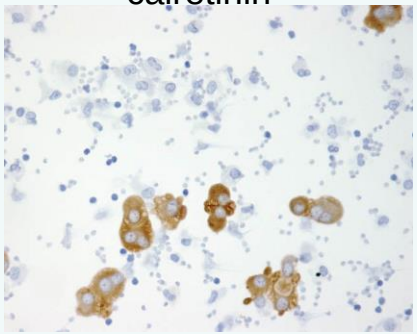
Effusion for controls

- lymphoid cells (CD3,CD20,CD45)
- mesothelial cells (calretinin, HBME, CK5/6)
- carcinoma cells (cytokeratins, MOC-31)

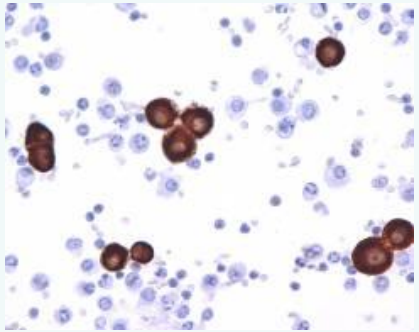
CD3



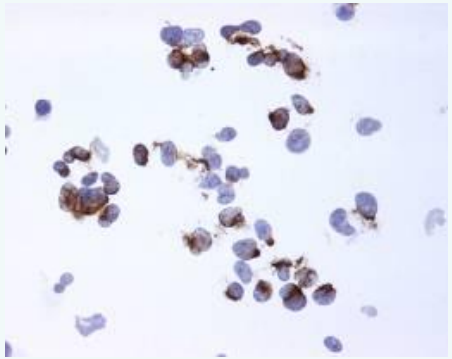
calretinin



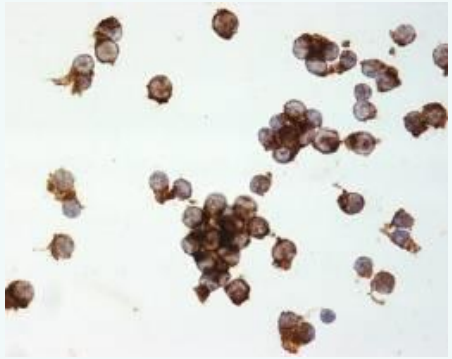
cytokeratin



FNA‘s of resected tumors



ex-vivo FNAB sample of intra-abdominal desmoplastic small cell tumour; desmin on Papanicolaou stained cytospin



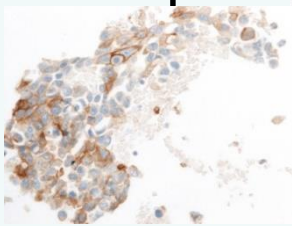
ex-vivo FNAB sample of thyroid carcinoma; thyroglobulin on Papanicolaou stained cytospin

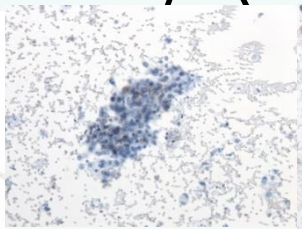
Brushing of resected tumors- PDL1 study

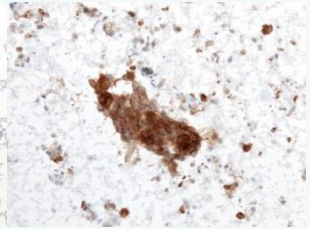
FFPE

cell block

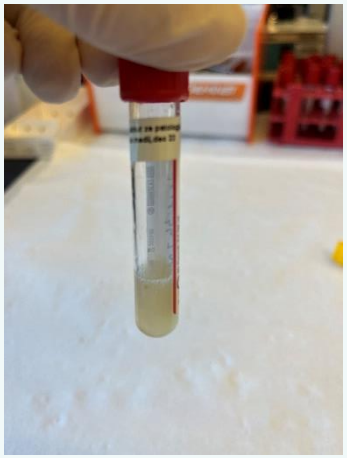
methanol fixed cytospins



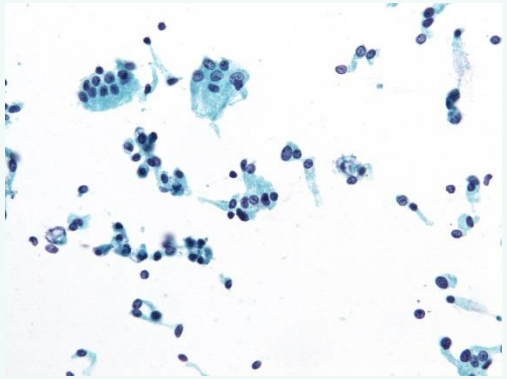


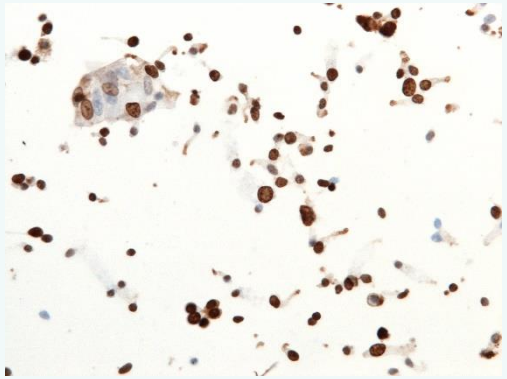


Brushing of fresh tissue – controls SATB2



→ 30 cytospins

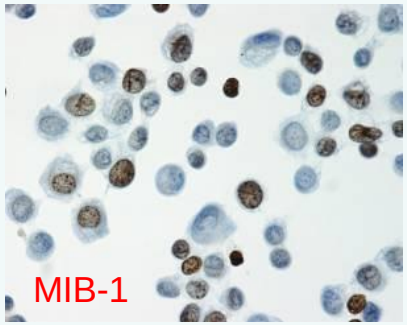


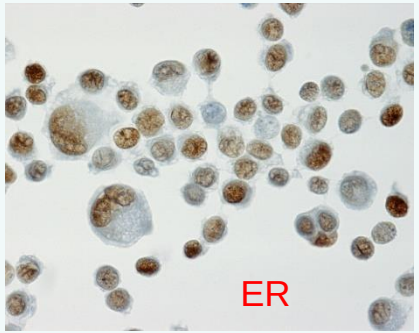


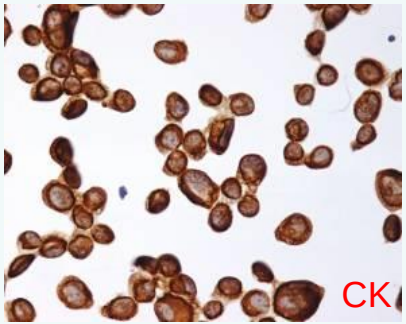
SATB2 on cytospin

Cell lines for controls

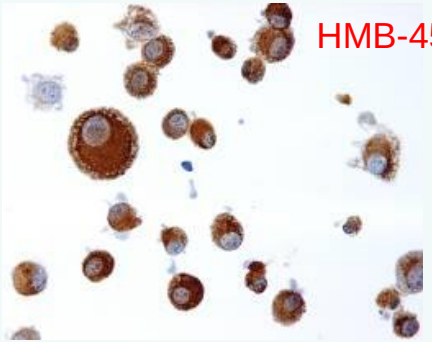
Human breast cancer cell line MCF-7

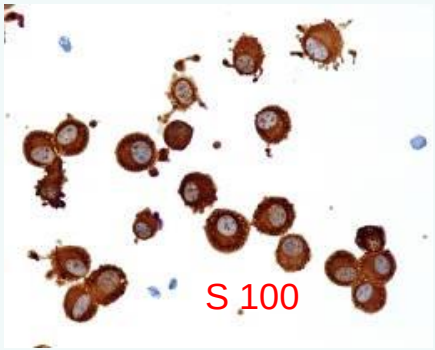






Human melanoma cell line SK-MEL 28





Good control slides from cytology samples

TEAM work:
- hunt suitable sample
- testing

TIME:
- slide preparation
- analysis (evaluation, comparison)
- documentation

Negative controls

Negative control slides

- Additional slide from diagnostic sample
- Replacing primary antibody with diluent buffer

Check:

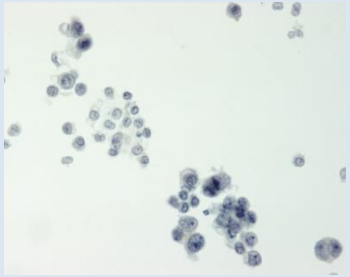
- non-specific staining

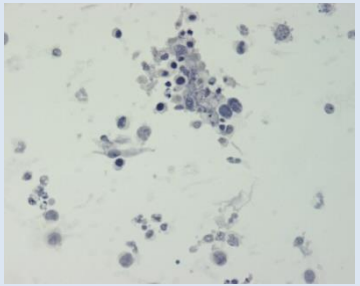
Each sample?

- according to lab experiencies
- any change in slide preparation technique
- any change in immunostaining protocol

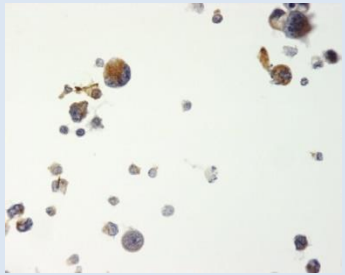
Negative controls – new detection kit

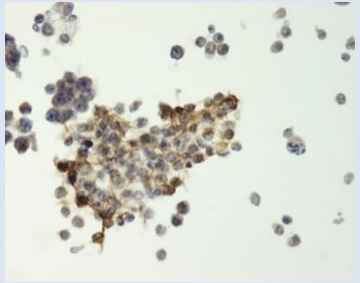
Detection kit 1



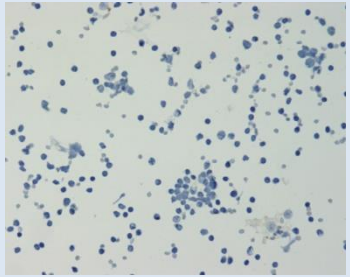


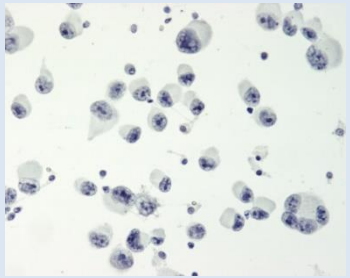
Detection kit 2



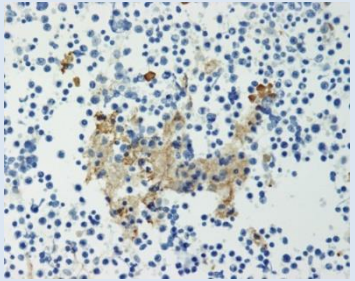


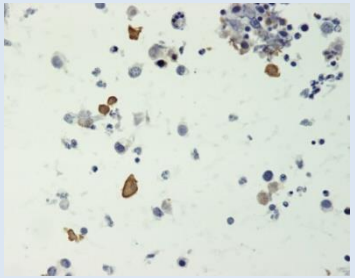
Detection kit 1



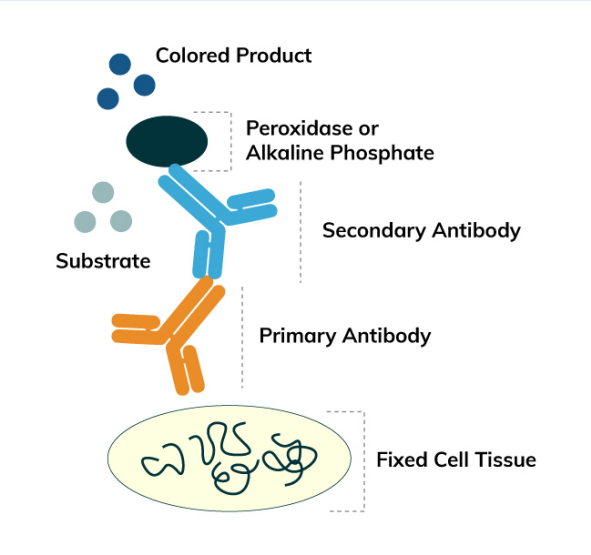


Detection kit 2





Optimization and validation



Antibodies for IHC detect epitopes in FFPE!

Each modification/variation from standard FFPE should be validated

Quality Assurance For Immuncytochemistry: Approved Guideline, Clinical Laboratory Standards Institute (formerly NCCLS), Wayne PA, USA, publication MM4-A, Vol. 19, No. 26, 1999. www.clsi.org

Optimization and validation

Optimization – adjustment of steps in ICC procedure

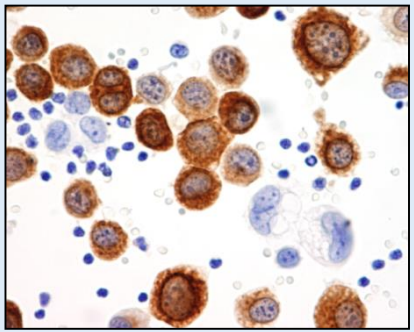
Validation – reliable, correct, results

Basic requirements

- Adequate positive controls
- Assessment of ICC quality!

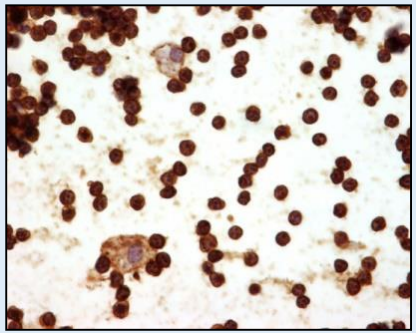
Quality of ICC

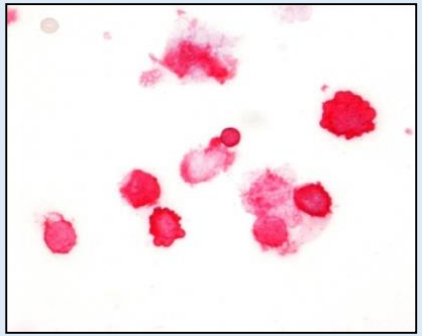
Optimal quality ICC



- properly localized
- clearly visible
- specific
- well preserved cell morphology
- no background

Poor quality ICC



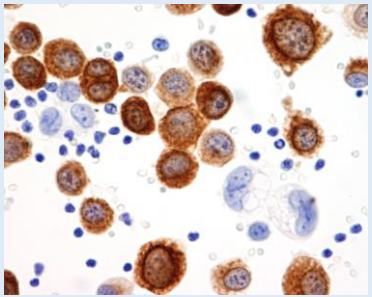


- poor cell morphology
- non specific staining
- background

Discrepancy in perception of imunocytochemical staining quality

HMB-45 on identical UK NEQAS slides

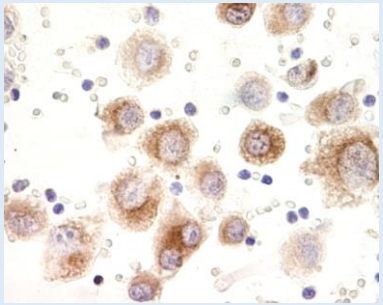
Lab 1



Very good

Very good

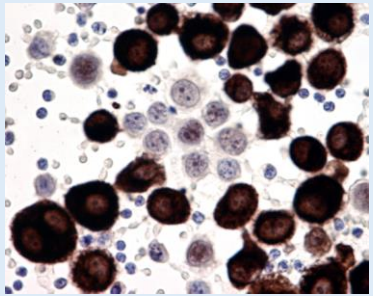
Lab 2



Very good

Borderline

Lab 3



Very good

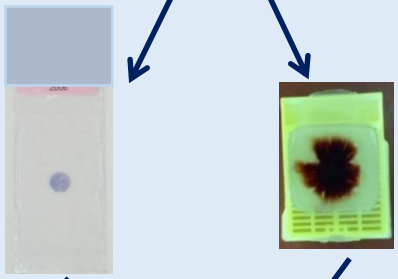
Borderline

In house assessors

External assessors

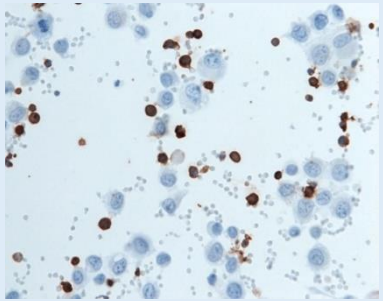
Run 108 – CD45, melanoma

- Human melanoma cell line SK-MEL28
- Effusion with carcinoma cells, few mesothelial cells, Erci
- FNAB of lymph node

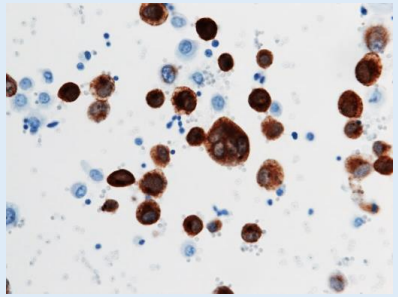


CD45, HMB-45, MelanA, S-100

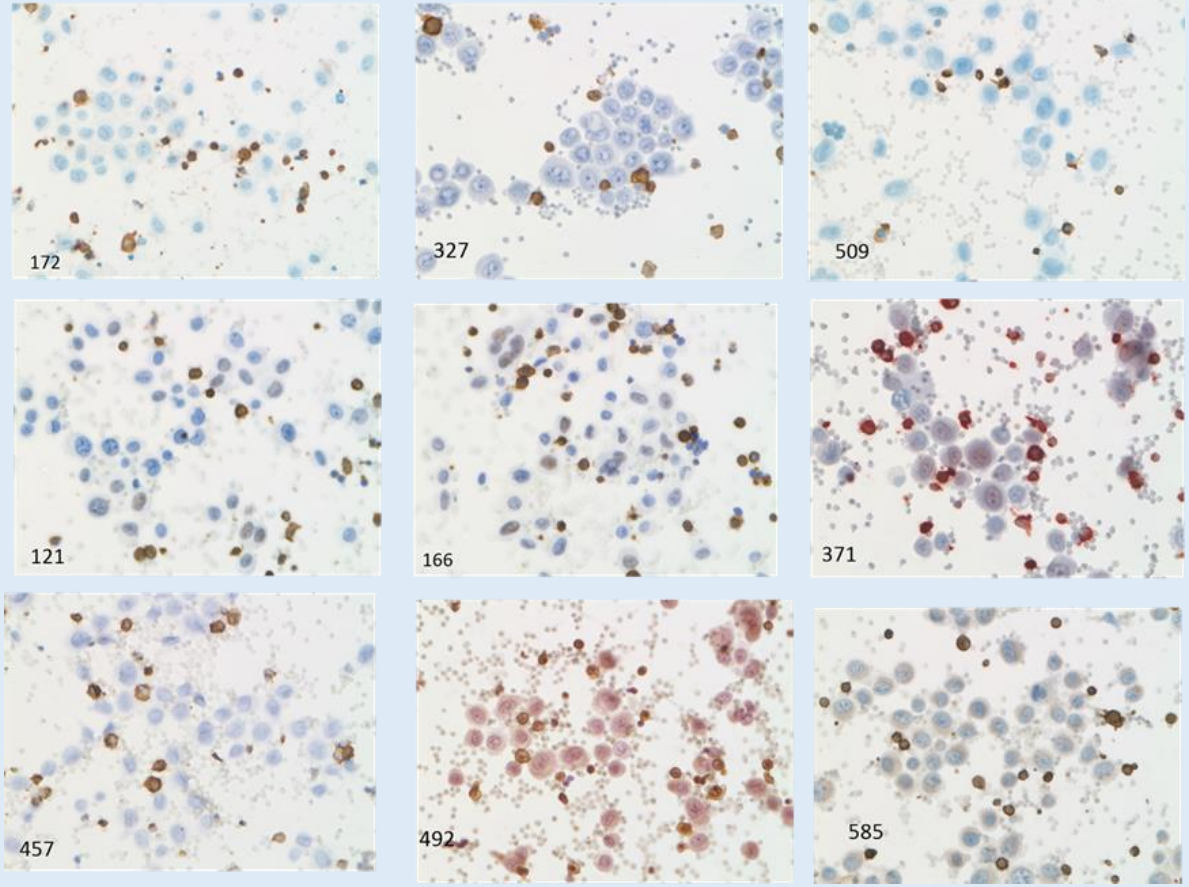
CD45



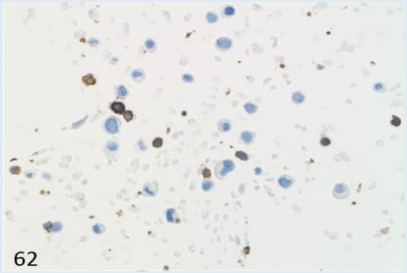
HMB-45

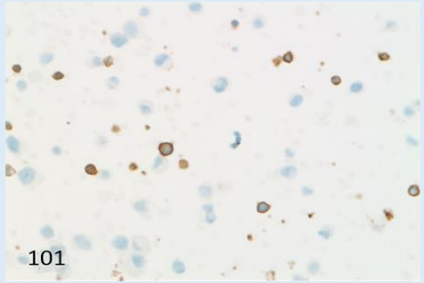


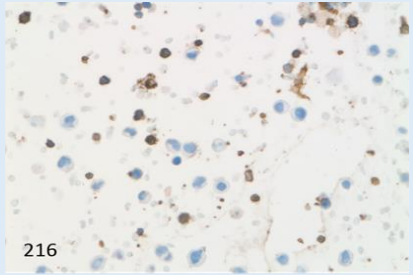
108R (CD45) - ICC variability on cytospins

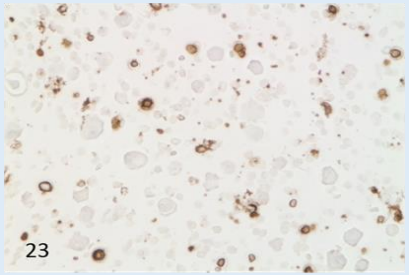


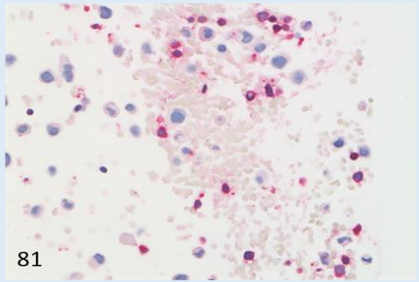
108 R (CD45) - ICC variability on CB

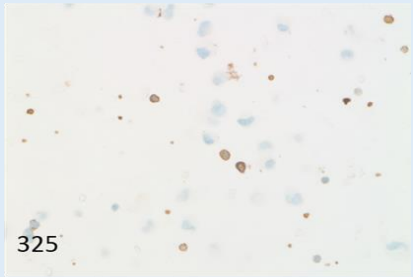


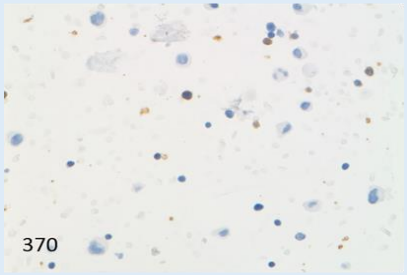


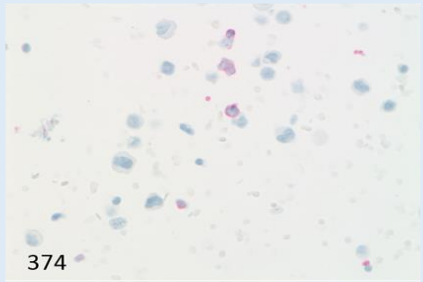


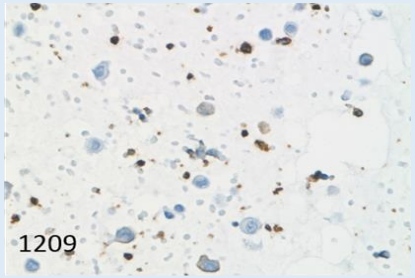












Optimization of IHC/ICC protocols

Optimization – adjusting steps in IHC/ICC staining procedure yielding the best ratio between specific/nonspecific staining

ICC protocols ≠ IHC protocols

ICC protocols ≠ IHC protocols

Our optimization

- Cytospins fixed in methanol
- 39 antibodies

Step	ICC	IHC
Deparaffination	no	yes
H2O2/methanol	yes	no
Antigen retrieval	1/39 (2 %)	38/39 (97 %)
iView	34/39 (87 %)	2/39 (5 %)
ultraView	4/39 (10 %)	32/39 (82 %)
optiView	0	4/39 (10 %)
Antibody dilutions ICC : IHC	⇧ 27/39 (69 %) = 12/39 (31 %)	

ICC protocols ≠ IHC protocols

- Cellient cell blocks - adapted IHC protocol for 15/30 antibodies
- LBC: FFPE from the same sample - 10 % Ab non reactive/inconsistent on LBC using IHC protocols
- Thrombin CB : Cellient CB (70 samples)- Cellient CB - modified FFPE protocol (43 %)

- Sauter et al. Validation and Optimization of Immunohistochemistry Protocols for Use on Cellient Cell Block Specimens. Cancer (Cancer Cytopathol) 2016;124:89-99.
- Sauter JL, Ambaye AB, Mount SL. Increased utilization, verification, and clinical implications of immunocytochemistry: Experience in a northern New England hospital. Diagn Cytopathol 2015;43(9):688-95.
- Sauter JL, Grogg KL, Vrana JA, Law ME, Halvorson JL, Henry MR. Young investigator challenge: Validation and optimization of immunohistochemistry protocols for use on cellient cell block specimens. Cancer Cytopathol. 2016;124(2):89-100.

Validation

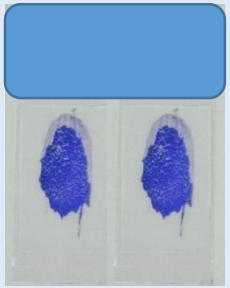
- Validation ensures a test works as intended. Any antibody assay (novel or replacement) must be validated before it is put into use as a diagnostic test.
- Objective evidence that test performs reliable and consistently - accurate, correct, reliable results

ICC : IHC/flow cytometry immunophenotyping/….

- **Quality Assurance For Immuncytochemistry: Approved Guideline**, Clinical Laboratory Standards Institute (formerly NCCLS), Wayne PA, USA, publication MM4-A, Vol. 19, No. 26, 1999. www.clsi.org
- College of American Pathologists

Validation of ICC on cytospins – our approach

FNA,
EUS-FNA
ES-FNA
effusion



+

universal home-made
buffer-based cell medium

cytospins → fixation and storage?

Sample in cell medium

molecular tests

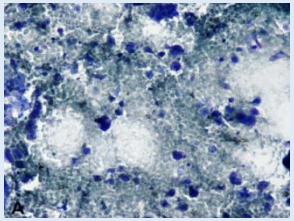
flow cytometry

cytospins

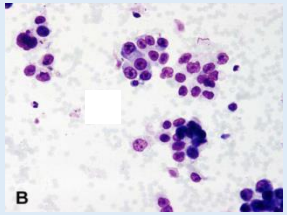
cell block (> 1x10^6 cells)

- immunocytochemistry
- special stainings
- FISH
- control slides

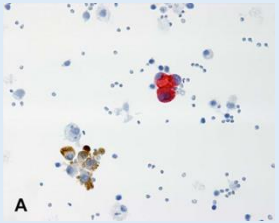
Hemorrhagic sample

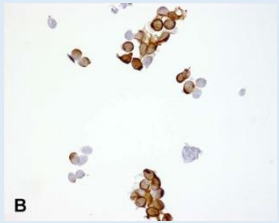


After filtration



ICC





Validation of ICC

- Optimal fixation for CD markers (ICC : IHC: flow cytometry)
- Optimal fixation for Ki67 (ICC: S-phase)
- Optimal fixation for ER (MCF-7 cell line, ICC:IHC)

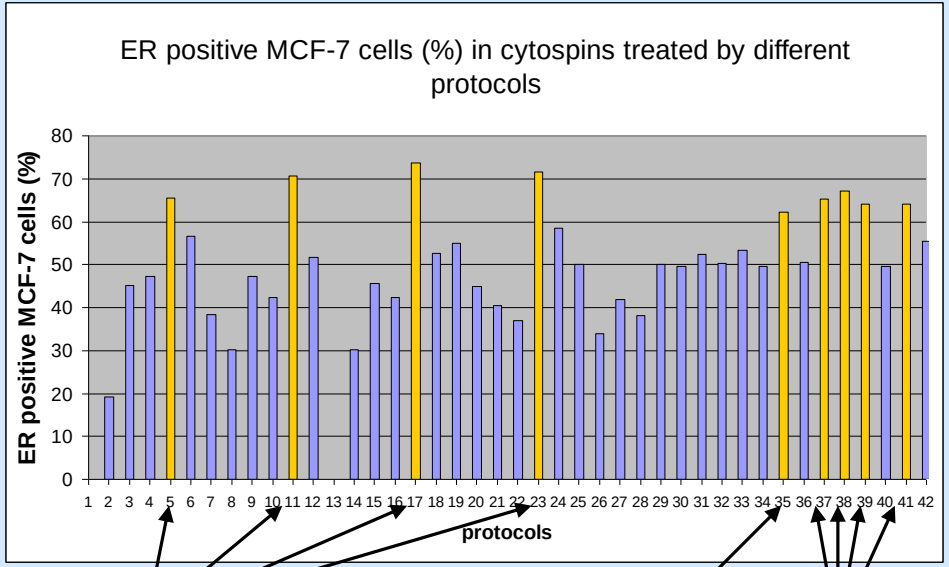
ER optimization and validation

Optimal protocol set-up on MCF-7 cell line

Evaluation of protocols on ex-vivo FNAB samples

Introduction of automated immunostaining

Follow up - response to hormonal treatment

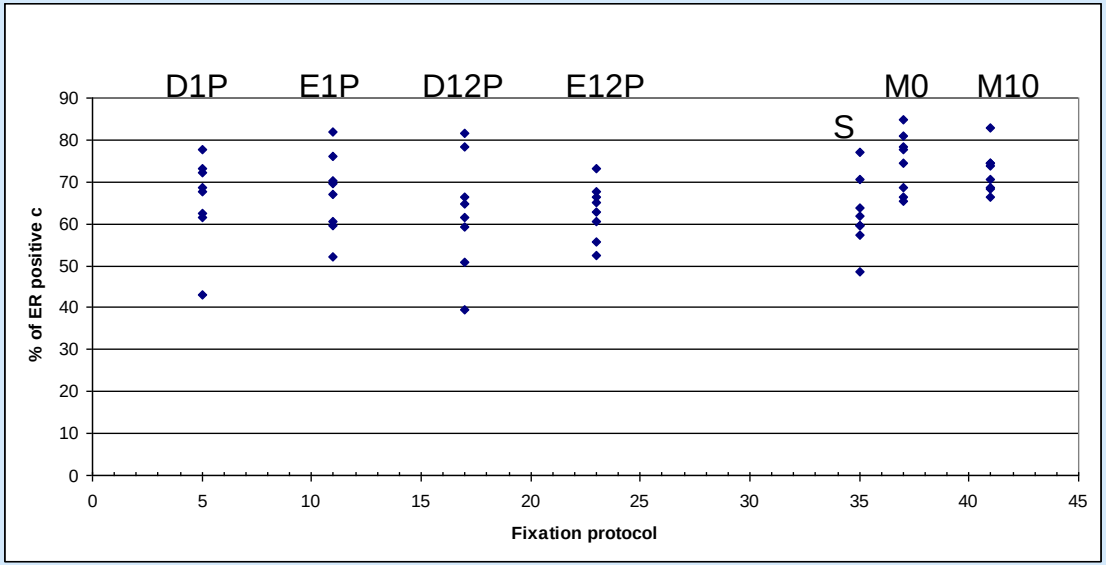


Papanicolaou stained, **Mw10, D100**
5: Delaunay 1hr
11: 96 % ethanol 1hr
17: Delaunay 12 hrs
23: 96 % ethanol 12 hrs

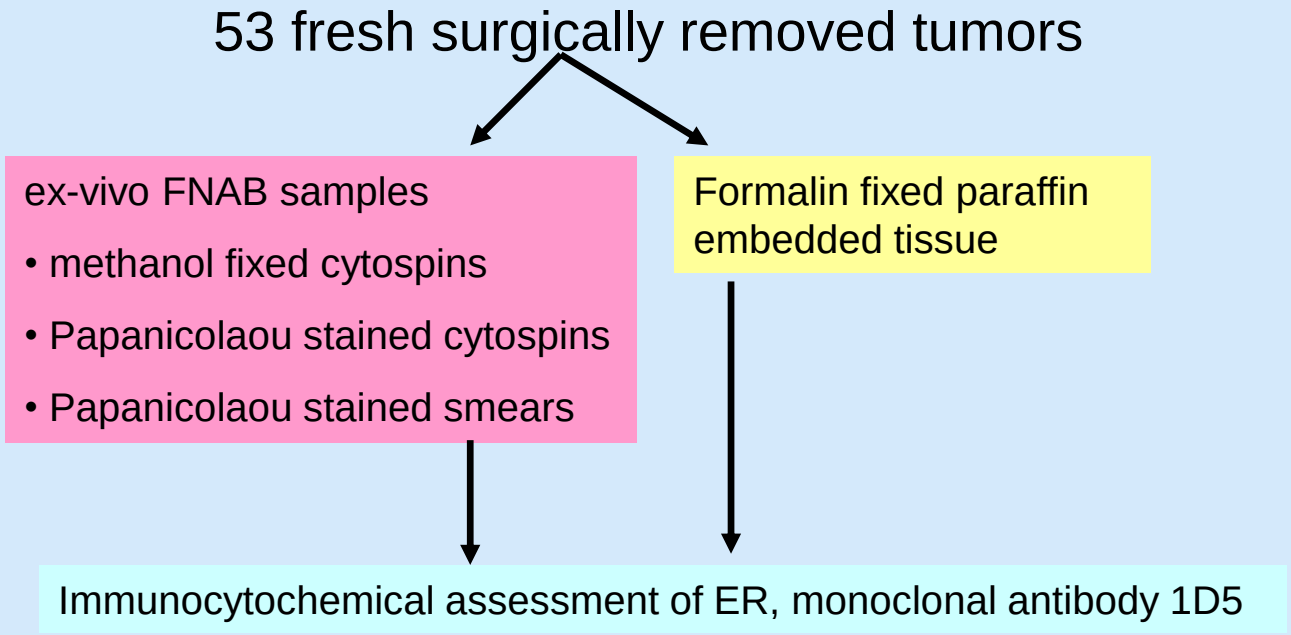
CellFix
Mw10, D100

Methanol
37: Mw 0, D100
38: Mw 0, D200
39: Mw 5, D100
41: Mw 10, D100

Variability in ICK detection of ER positive MCF-7 cells



Protocol evaluation on ex-vivo FNAB samples

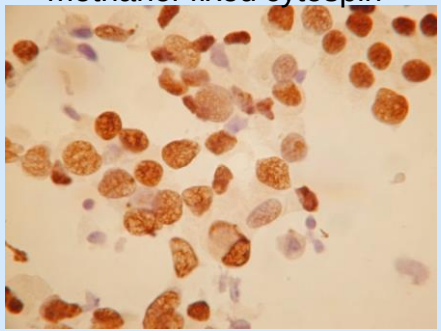


ER on ex-vivo FNAB samples - concordance with corresponding tissue sections

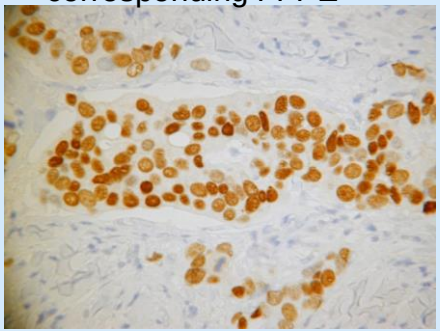
	concordance	kappa
Papanicoalou stained smears	92 %	0.75
Papanicoalou stained cytospins	94 %	0.84
methanol fixed cytospins	**100 %**	**1.00**

ER assessment

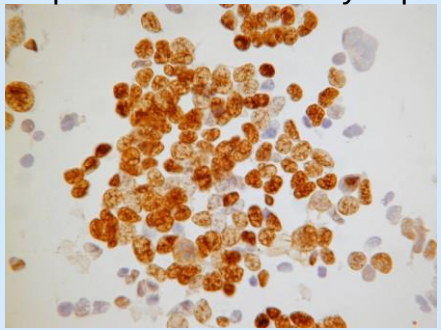
methanol-fixed cytospin



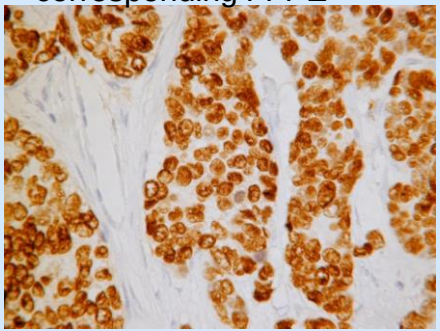
corresponding FFPE



Papanicolaou stained cytospin



corresponding FFPE



Validation of ICC on cytospins

- Optimal fixation for CD markers (ICC : IHC: flow cytometry)
- Optimal fixation for Ki67 (ICC: S-phase)
- Optimal fixation for ER (MCF-7 cell line, ICC:IHC)

} Methanol

Kirbis IS, Flezar MS, Krasovec MU. MIB-1 immunostaining on cytological samples: a protocol without antigen retrieval. Cytopathology. 2004;15(3):154-159. doi:10.1111/j.1365-2303.2004.00146.x

Srebotnik Kirbiš I, Us Krašovec M, Pogačnik A, Strojan Fležar M. Optimization and validation of immunocytochemical detection of oestrogen receptors on cytospins prepared from fine needle aspiration (FNA) samples of breast cancer. Cytopathology. 2015;26(2):88-98. doi:10.1111/cyt.12143

Srebotnik Kirbis I, Prosen L, Strojan Flezar M. Time-related changes in cell morphology and biomarker immunoreactivity for cells stored in a buffer-based cell medium. Cytopathology. 2021;32(4):513-518. doi:10.1111/cyt.12980

Validation of ICC

38 other markers:

- positive controls with known/expected expression
- methanol preserve all tested antigens

Validation of ICC

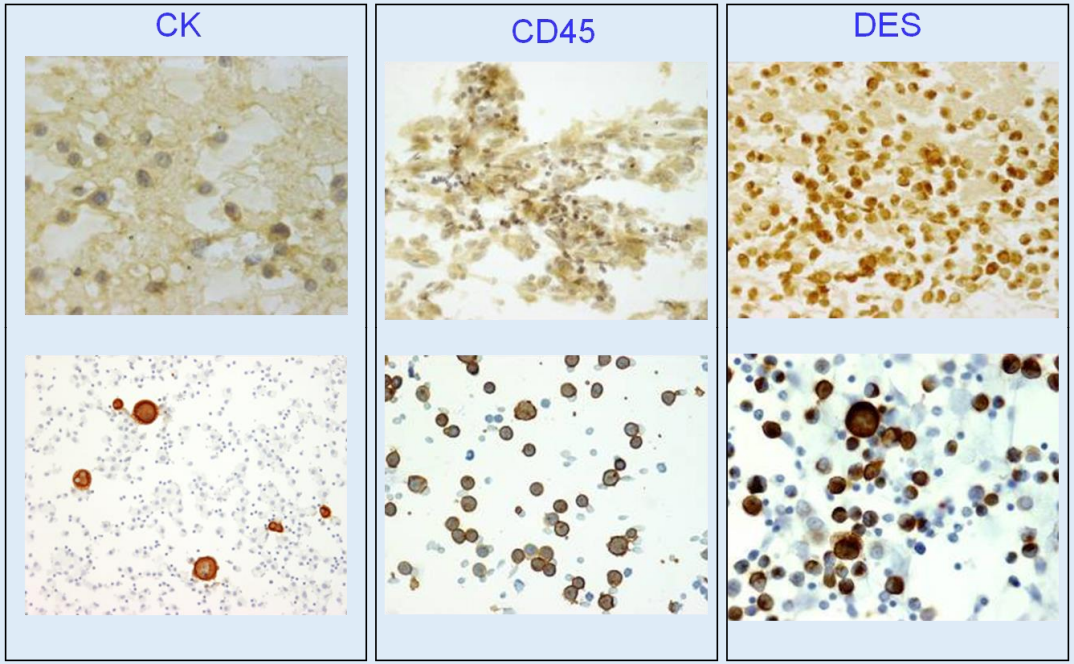
50 diagnostic routine cytology samples
ICC on methanol fixed cytospins : IHC on concordant FFPE

IHC	ICC		
	Neg	Poz	Together
Neg	67	0	67
Poz	5	74	79
Together	72	74	146
Concordance	141/146, 97 %, κ = 0,93		

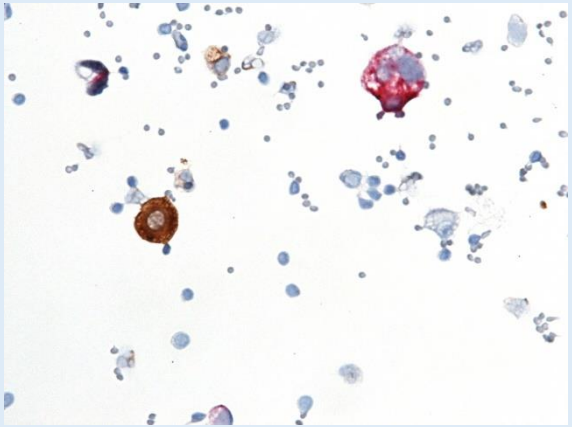
Development of sample processing

1988
Direct smears

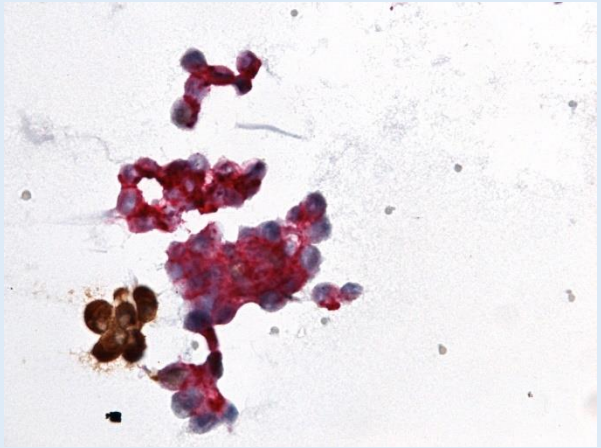
2008
Cytospins



Patient sample

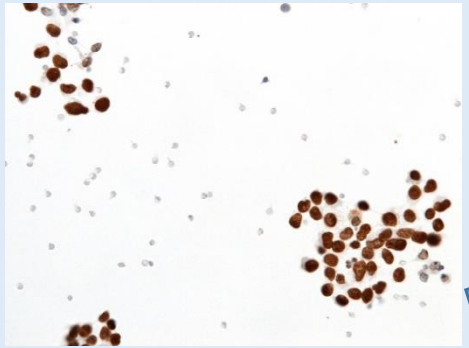


Integrated control

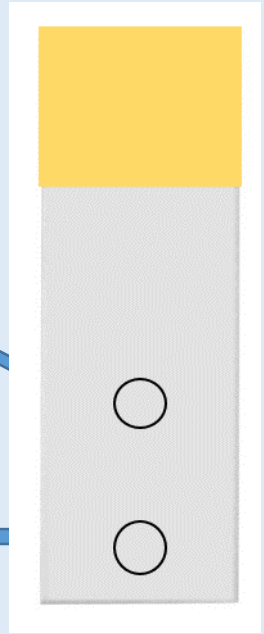


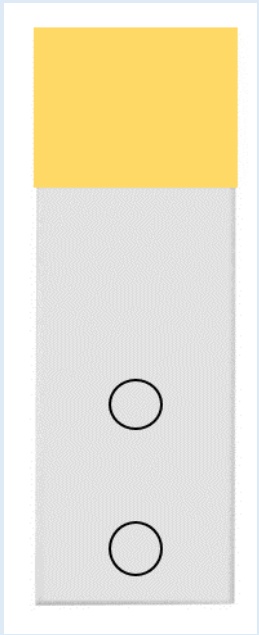
ER

PR

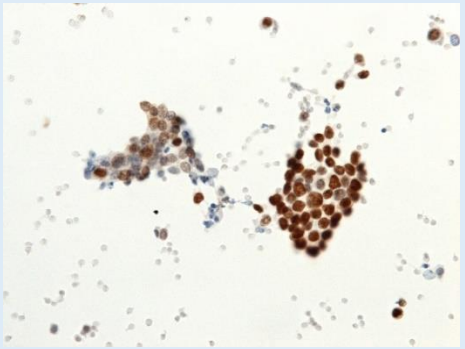


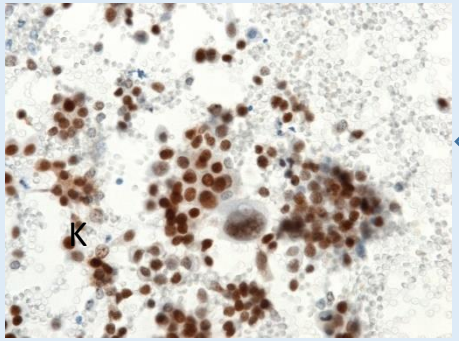
Patient sample



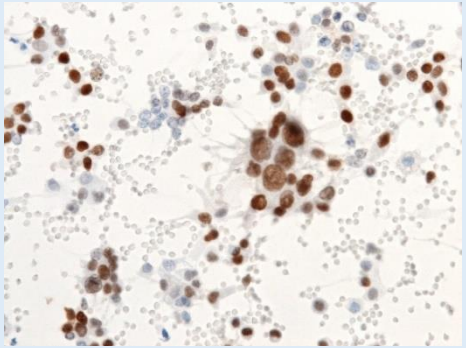


Patient sample





K



Integrated positive control

Conclusion

Immunocytochemistry

- Essential in modern cytopathology
- Proper QA/QC mandatory for reliable, consistent, correct results
- Demanding but feasible

Thank you for your attention

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