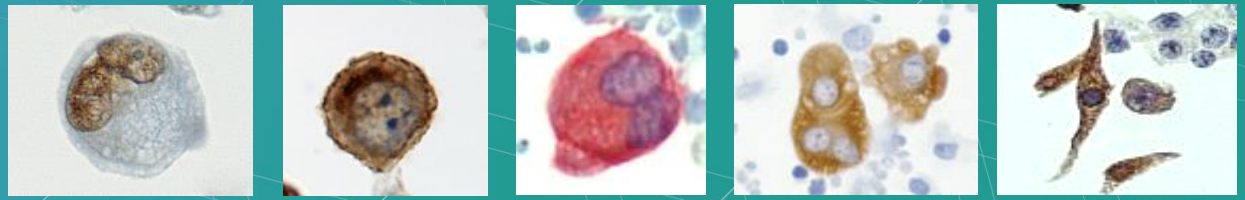
Immunocytochemistry – overview, considerations and applications

Irena Srebotnik Kirbis



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My expertise

- Introducing and development of ICC in cytology
- Development of universal cytology sample processing for ancillary methods
- Optimization and validation of ICC on cytology samples
- Assessor and sample provider for UK NEQAS ICC
- Published studies
  - 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

ICC

- Tumor typization
- Metastasis origin
- Prognostic/predictive

Immunohistochemistry (IHC) = Immunocytochemistry (ICC)

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

Immunohistochemistry (IHC) ≠ Immunocytochemistry (ICC)

Pre-analytic

- Sample management and processing
- Fixation

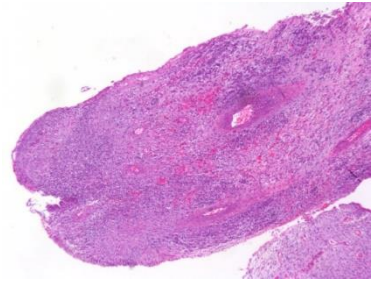
Analytic

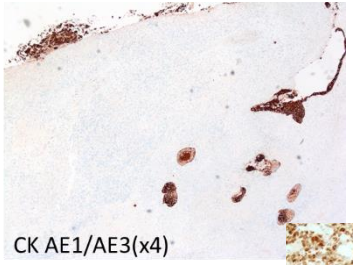
- Pretreatment
- Dilutions
- Detection kits

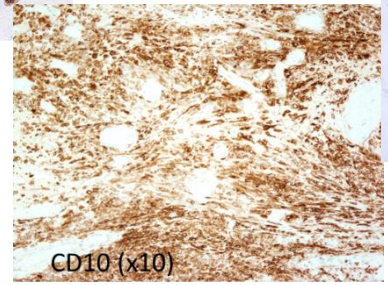
QA/QC

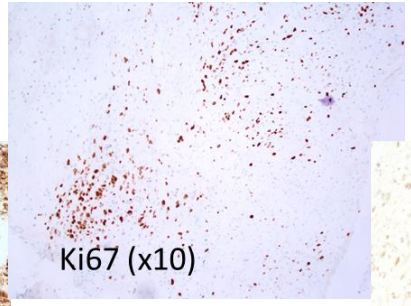
- Control slides
- Optimization
- Validation

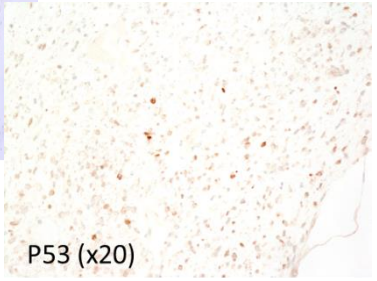
Histology – IHC workflow











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 sample processing – **slide preparation** options

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

Cell blocks

FFPE cell blocks ≈ FFPE tissue samples

Advantages

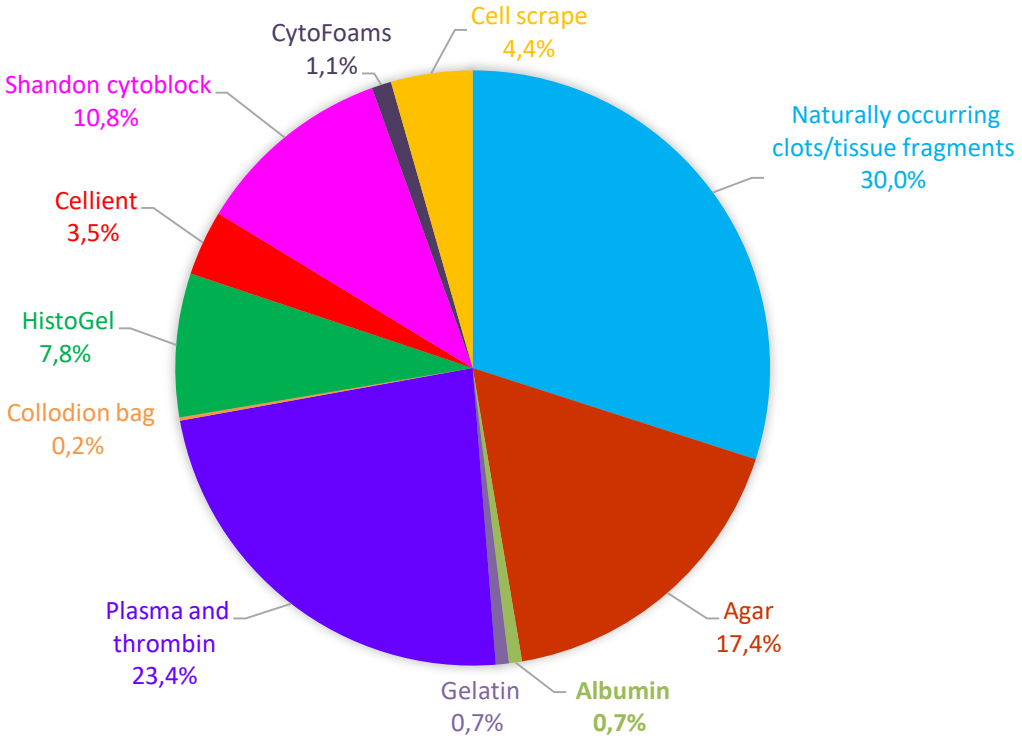
- easy storage
- multiple sections
- same protocols as for FFPE
- same QC/QA
- no additional validation studies

Cell blocks - disadvantages

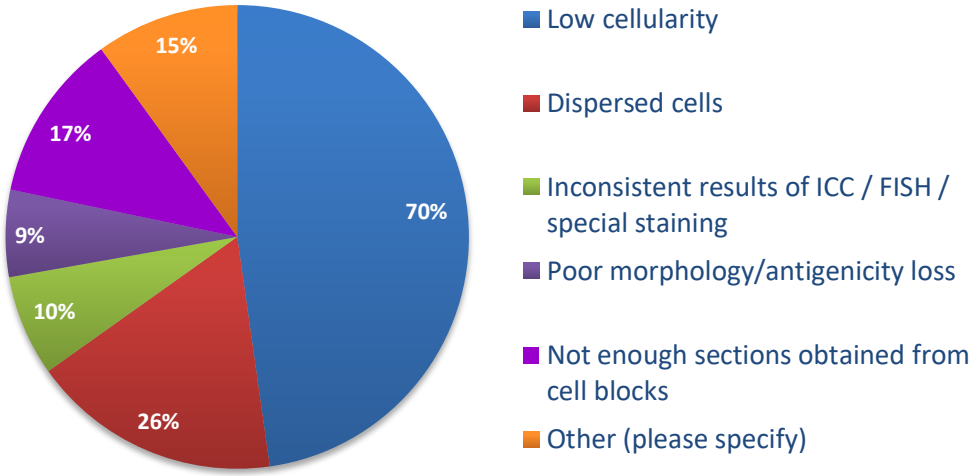
- no standardized protocol*
  - medium for sample collection (fixative, PBS, commercial solutions, RPMI, other)
  - fixation (formalin and non- formalin based)
  - cell pellet preparation (agar, HistoGel, plasma thrombin, Cellient, ...)
- **not suitable for low cellular samples**
- time consuming (↑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

Crapanzano JP et al. The state of cell block variation and satisfaction in the era of molecular diagnostics and personalized medicine. Cytojournal 2014;11:7.

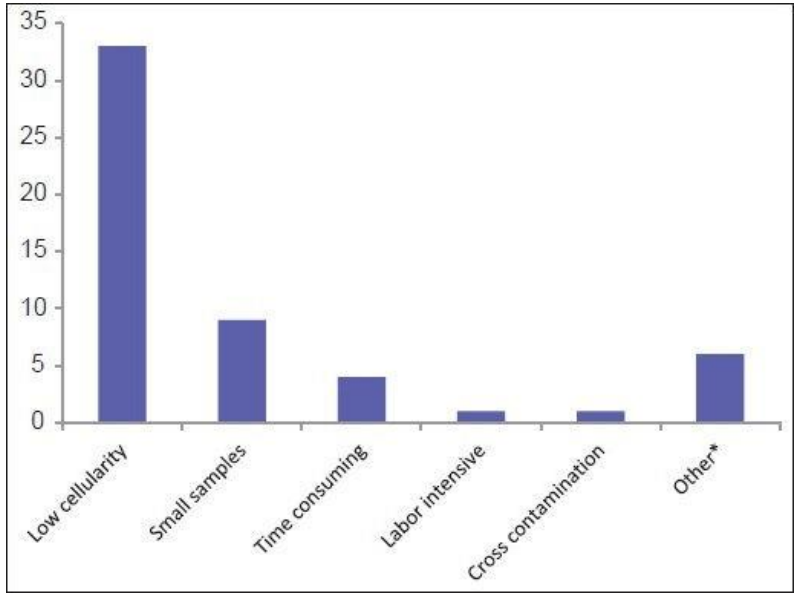
Cell block preparation methods – EFCS survey



Low cellularity is the main issue of CB



CB Survey – EFCS and UK NEQAS CPT project 2022

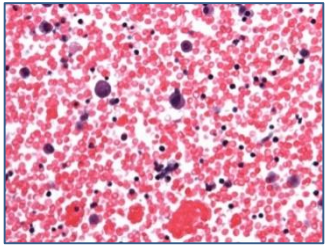


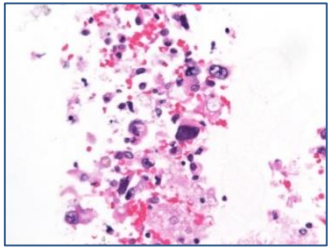
Crapanzano, J. P., Heymann, J. J., Monaco, S., Nassar, A., & Saqi, A. (2014). The state of cell block variation and satisfaction in the era of molecular diagnostics and personalized medicine. CytoJournal, 11, 7. https://doi.org/10.4103/1742-6413.129187

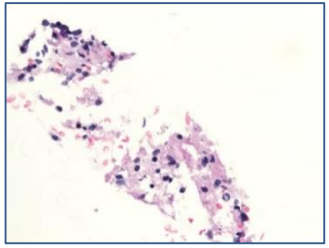
20 x 10^6 cells

1.2 x 10^6 cells

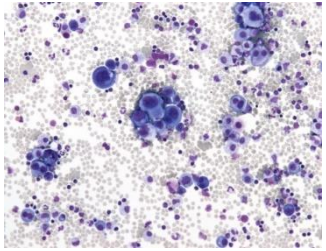
0.1 x 10^6 cells

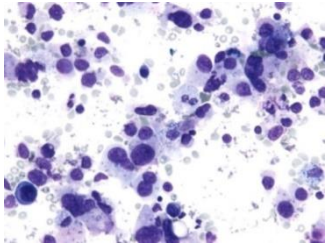


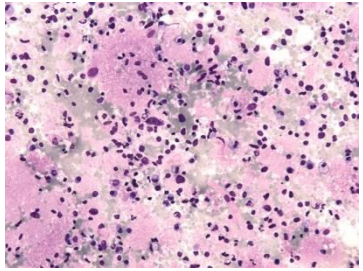




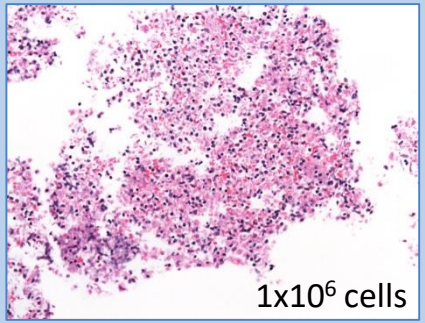
corresponding cytospins

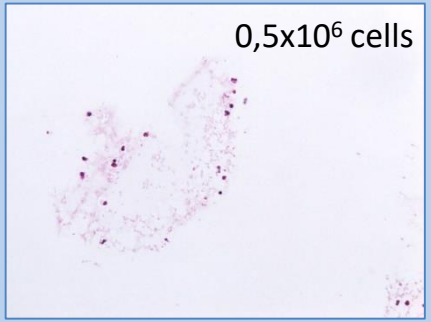


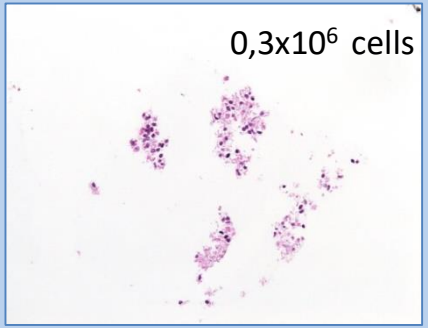


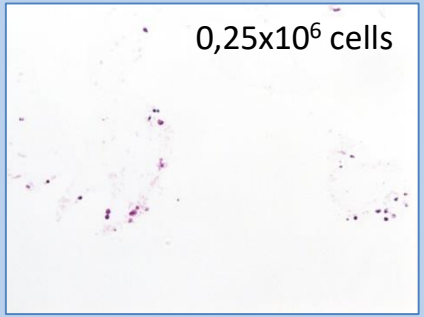


Cellularity of cell blocks





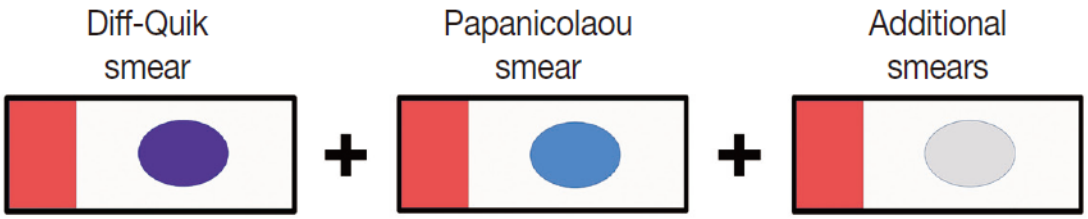




Alternatives to cell blocks?

Smears - advantages

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



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

Cytospins

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

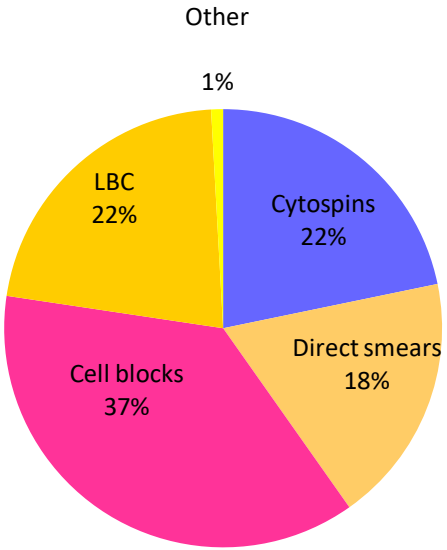
Advantages

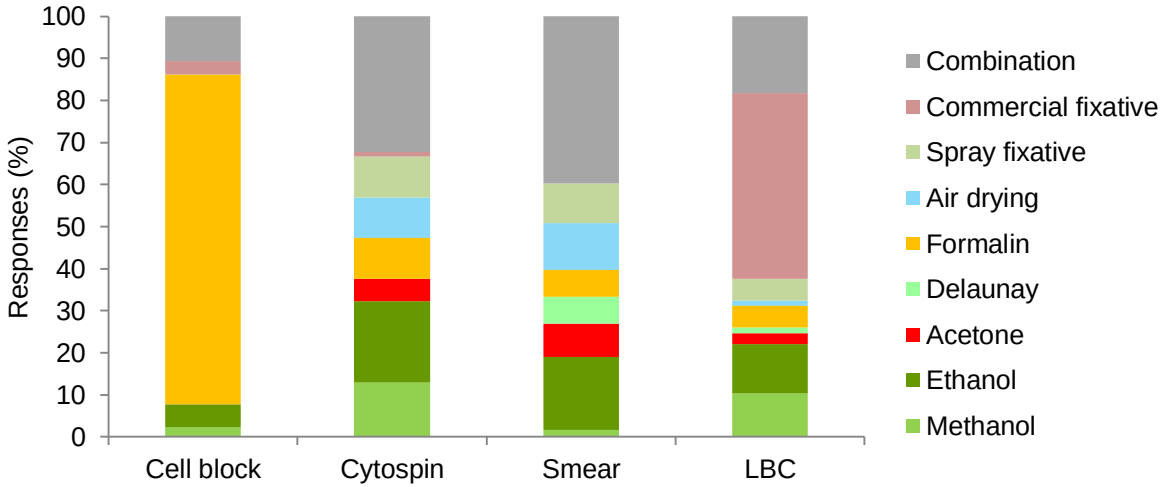
- easy storage of samples
- postpone decision
- monolayer distribution of cells
- multiple slides

Disadvantages

- expensive equipment
- ↑ cost
- Prefixed cells - clumping

Slides used for ICC – European survey



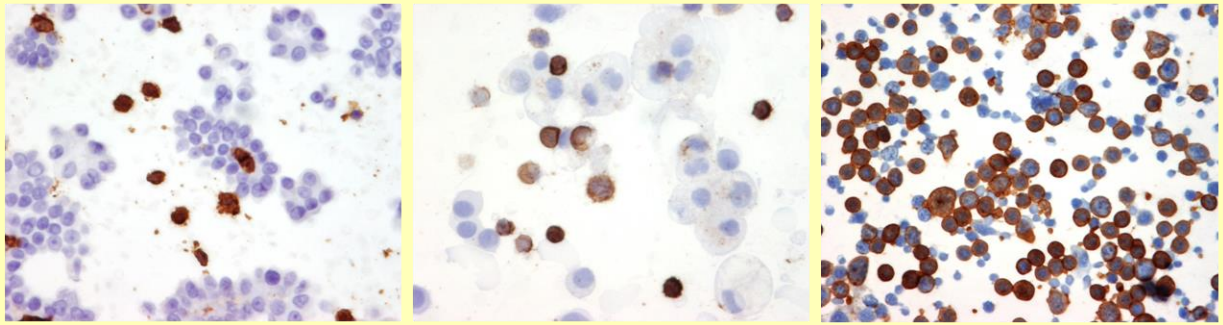


Fixatives used for the fixation of ICC preparations

Schmitt F, Cochand-Priollet B, Toetsch M, et al. Immunocytochemistry in Europe: results of the European Federeation of Cytology Societes (EFCS) inquiry. Cytopathology 2011, 22, 238–242.

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:

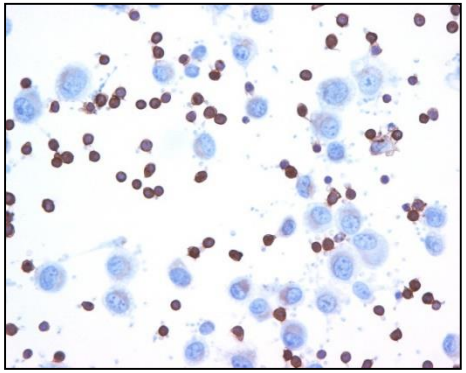
- non-specific staining

Control should be prepared the same as test sample

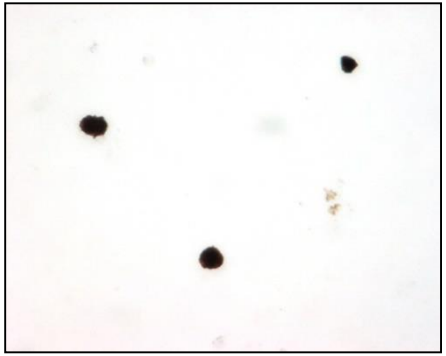
Sample	Control
FFPE tissue	FFPE tissue
Cell blocks -Histogel	Cell blocks -Histogel
Cell blocks - Shandon	Cell blocks - Shandon
Cell block - other	Cell block - other
Cytospins - methanol	Cytospins - methanol
Cytospins - aceton	Cytospins - aceton
LBC - ThinPrep	LBC - ThinPrep
LBC- SurePath	LBC- SurePath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

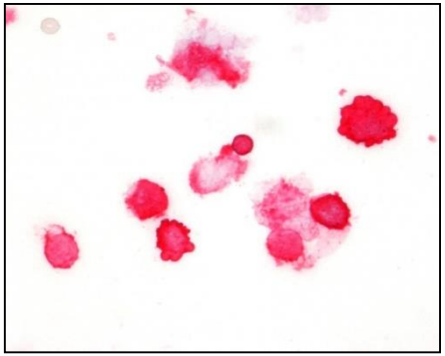
- Each step in sample preparation can affect IR
- ICC procedure for FFPE and cytology slides not identical

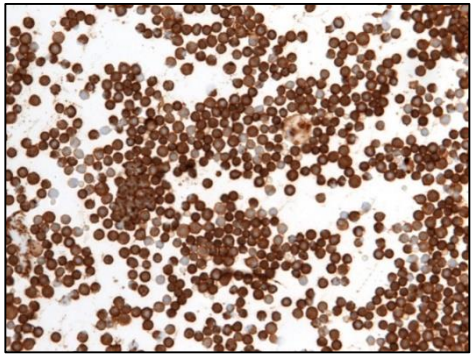
Positive control slides



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







How to prepare enough good control slides from cytology samples?

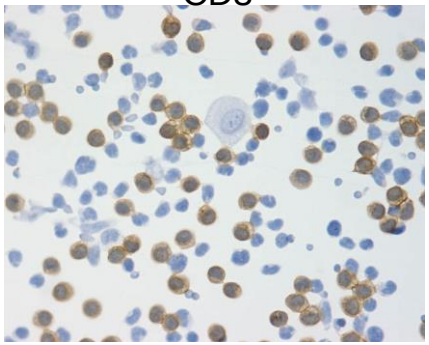
Cytology samples for controls

- leftovers of diagnostic cytology samples
- cytology samples of resected tumours
- cell lines

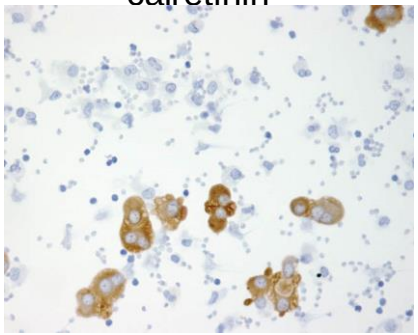
Effusion for controls

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

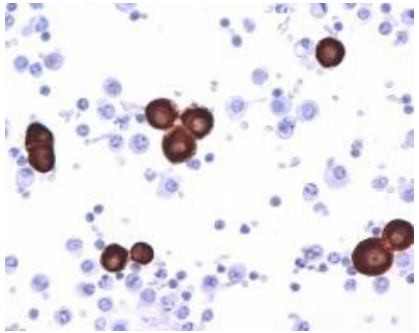
CD3



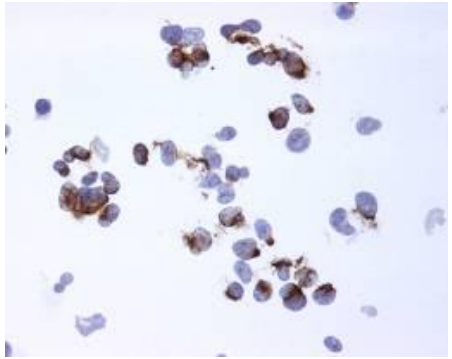
calretinin



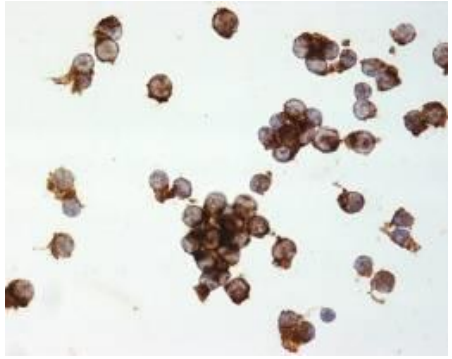
cytokeratin



FNAB 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

FFPE

cell block

methanol fixed cytospins

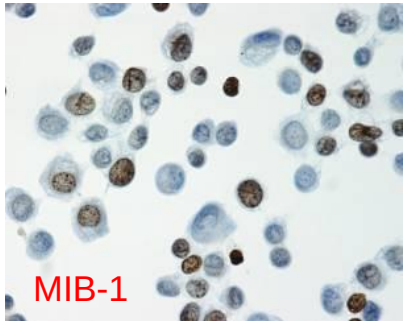
Cell lines for controls

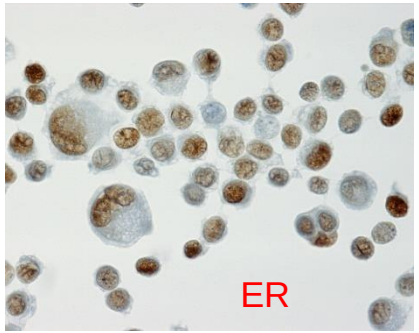
- known antigen expression
- enough cells

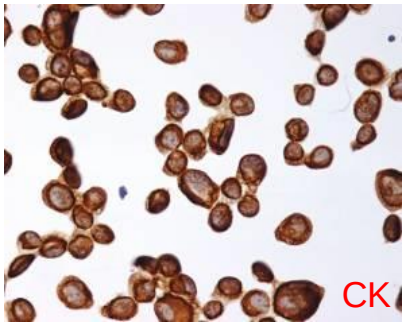
- access to a cell culture facility
- not suitable for all markers
- only positive cells!

Cell lines for controls

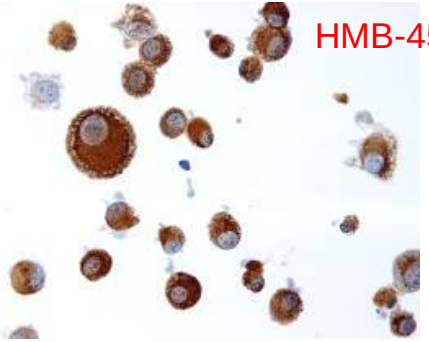
Human breast cancer cell line MCF-7

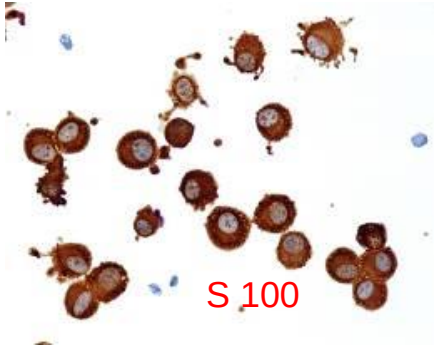






Human melanoma cell line SK-MEL 28





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:

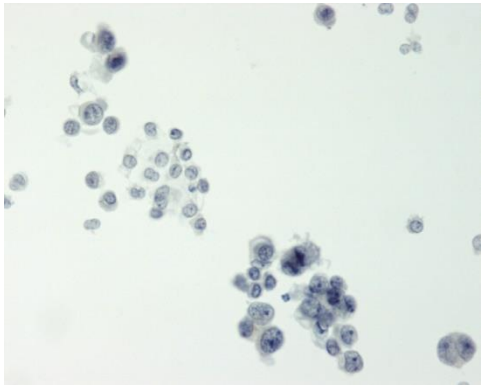
- non-specific staining

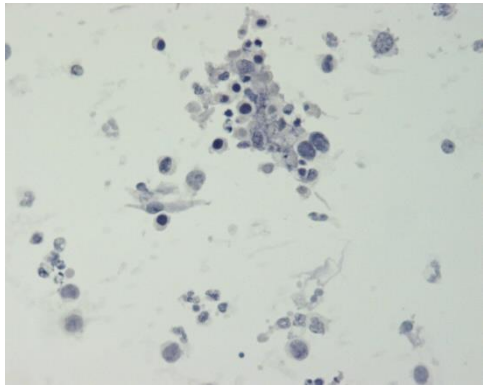
each sample?

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

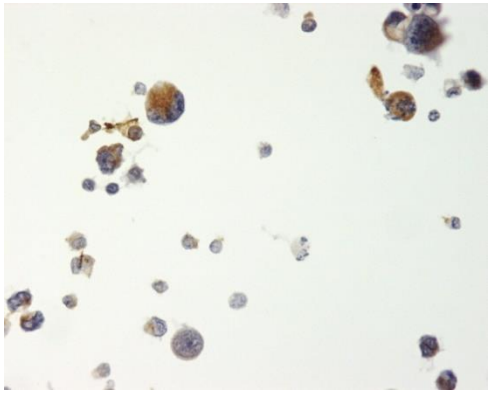
Negative controls - detection kit

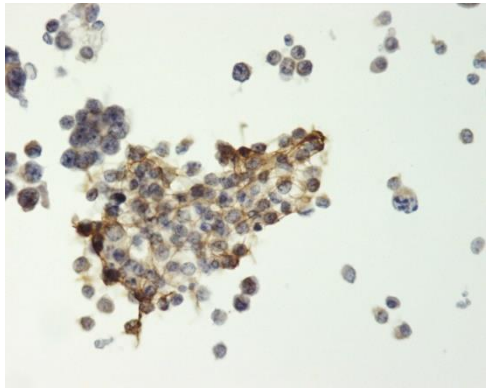
Detection kit 1





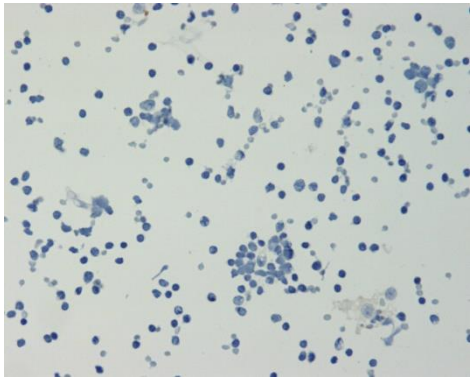
Detection kit 2

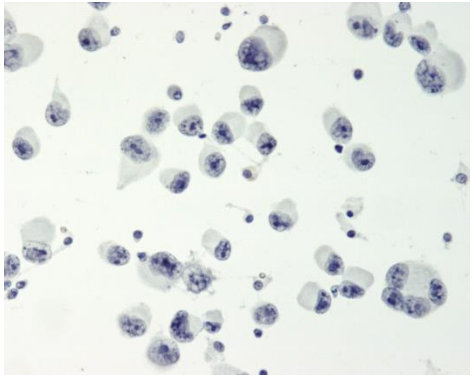




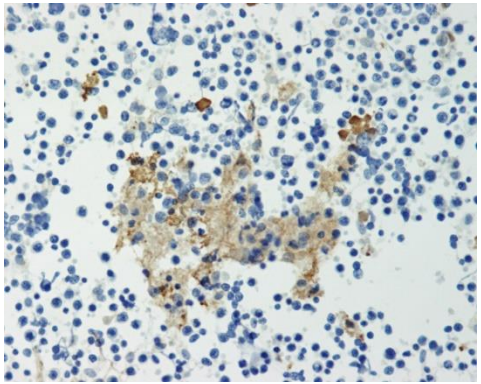
Negative controls - detection kit

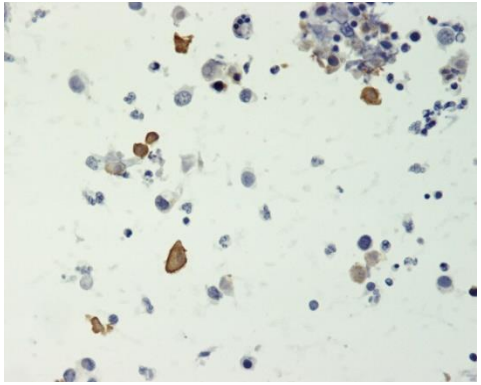
Detection kit 1



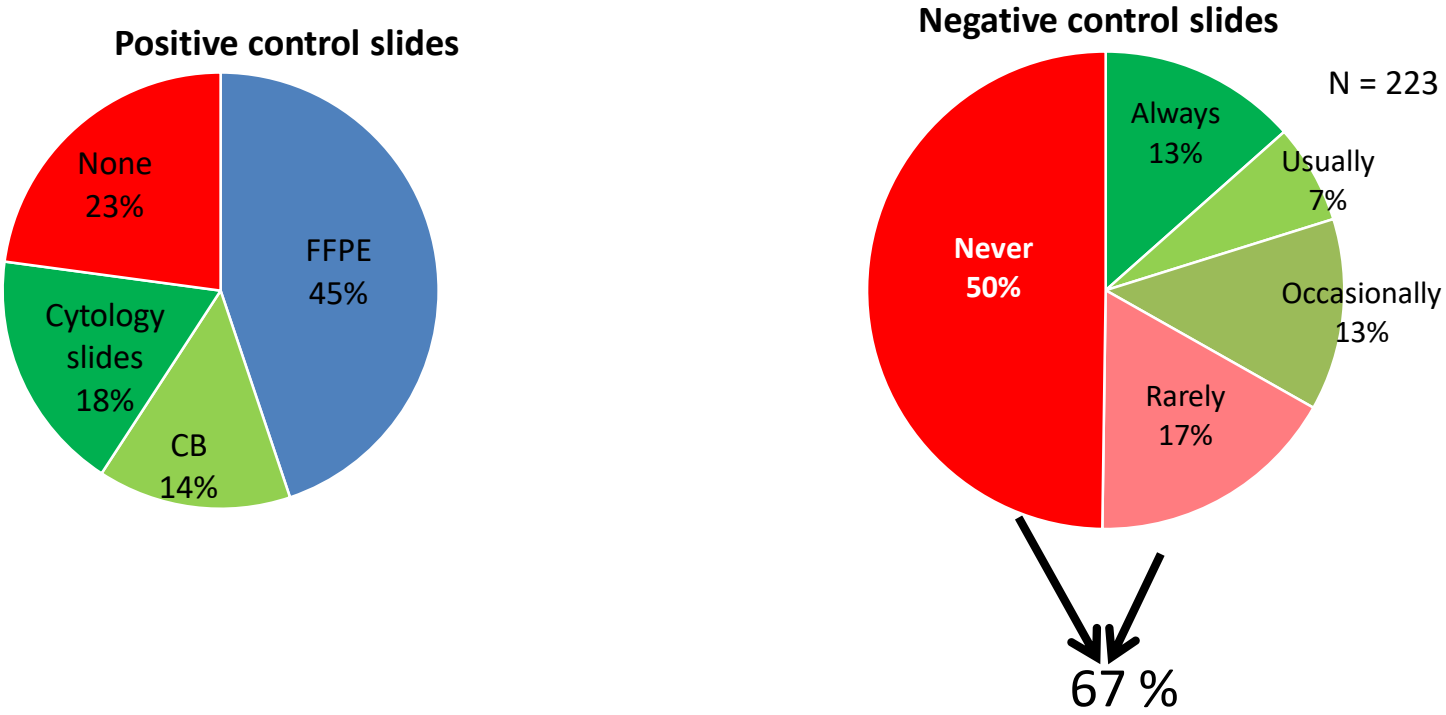


Detection kit 2





ICC Controls - European survey



Schmitt F, Cochand-Priollet B, Toetsch M, et al. Immunocytochemistry in Europe: results of the European Federeation of Cytology Societes (EFCS) inquiry. Cytopathology 2011, 22, 238–242.

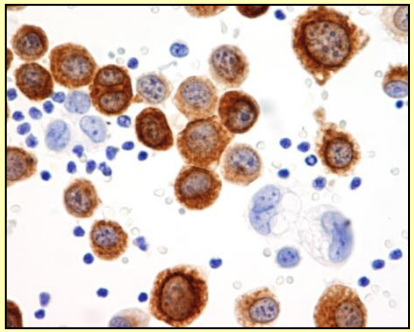
Optimization of IHC/ICC protocols

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

ICC protocols ≠ IHC protocols

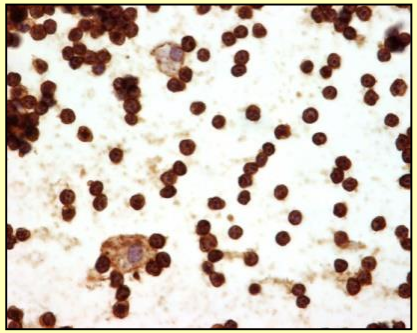
ICC
quality assessment

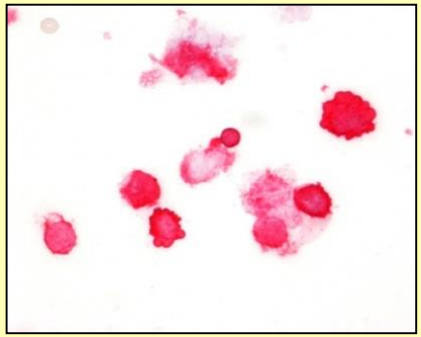
Optimal quality ICC



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

Poor quality ICC



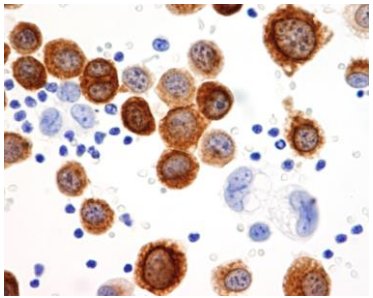


- poor cell morphology
- non specific staining
- background

Discrepancy in perception of imunocytochemical staining quality

HMB-45 on identical UK NEQAS slides

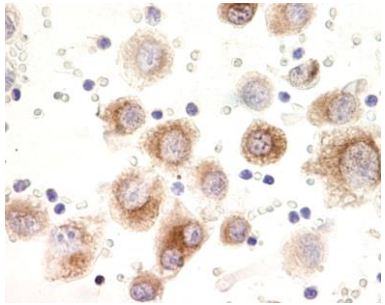
Lab 1



Very good

Very good

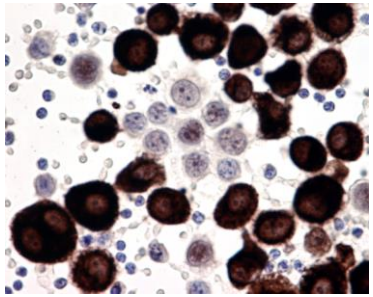
Lab 2



Very good

Borderline

Lab 3



Very good

Borderline

In house assessors

External assessors

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

JL. Sauter et al. Validation and Optimization of Immunohistochemistry Protocols for Use on Cellient Cell Block Specimens. Cancer (Cancer Cytopathol) 2016;124:89-99.

ICC protocols ≠ IHC protocols

- LBC: FFPE from the same sample
- IHC protocols
- 7/71 (10 %) Ab non reactive/inconsistent on LBC

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.

ICC protocols ≠ IHC protocols

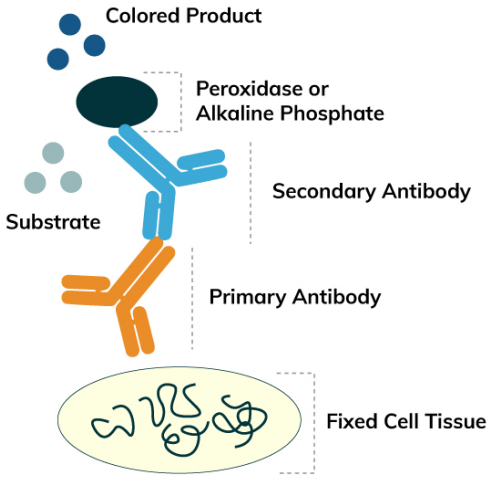
- 70 samples
- Thrombin CB : Cellient CB
- Cellient CB - modified FFPE protocol (43 %)

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

- **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



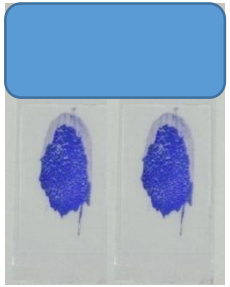
Antibodies for IHC detect epitopes in FFPE!

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

Validation of ICC on cytospins – our approach

FNA,
EUS-FNA
ES-FNA
effusion



+

universal home-made cell medium

cytospins → fixation and storage?

Sample in cell medium

molecular tests

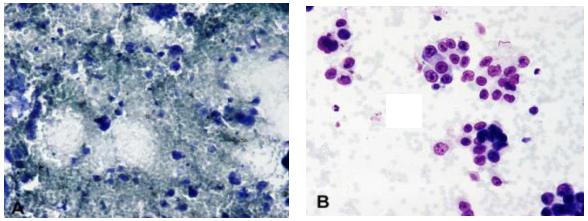
flow cytometry

cytospins

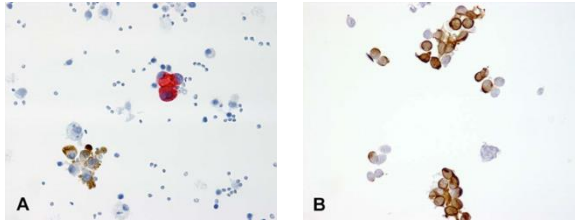
cell block (> 1x10^6 cells)

- 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

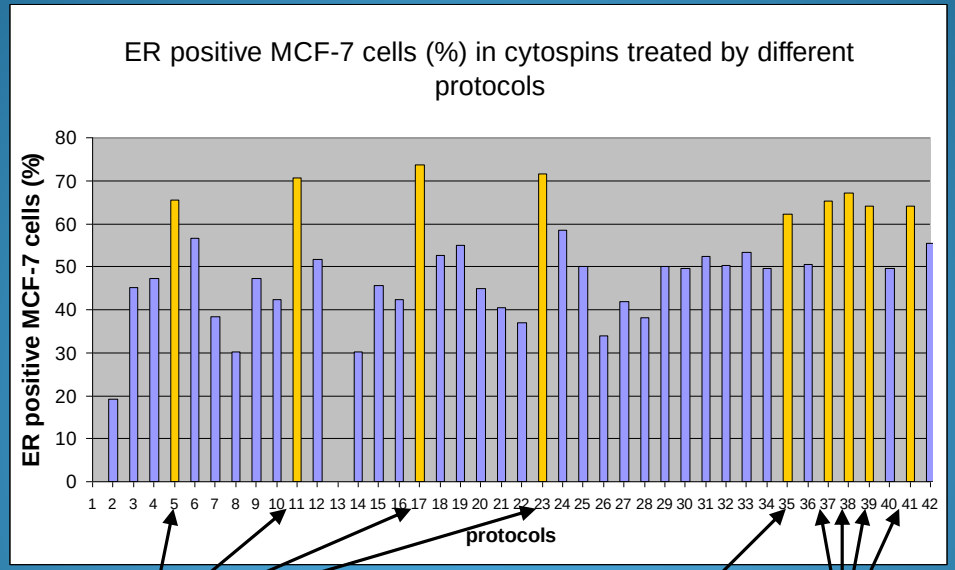
Optimal protocol set-up

MCF-7 cell line: 60-80 % cells ER positive

Influence of fixation and ICK staining procedure

42 protocols:

- 7 fixation
  - Methanol
  - CellFixx
  - Papanicolaou stained slides fixed in
    - Delaunay (1hr, 12 hrs)
    - 96 % ethanol (1hr, 12 hrs)
    - CellFix
- 3 microwave pretreatment: 0,5,10 min
- 2 antibody dilution: 1/100, 1/200



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

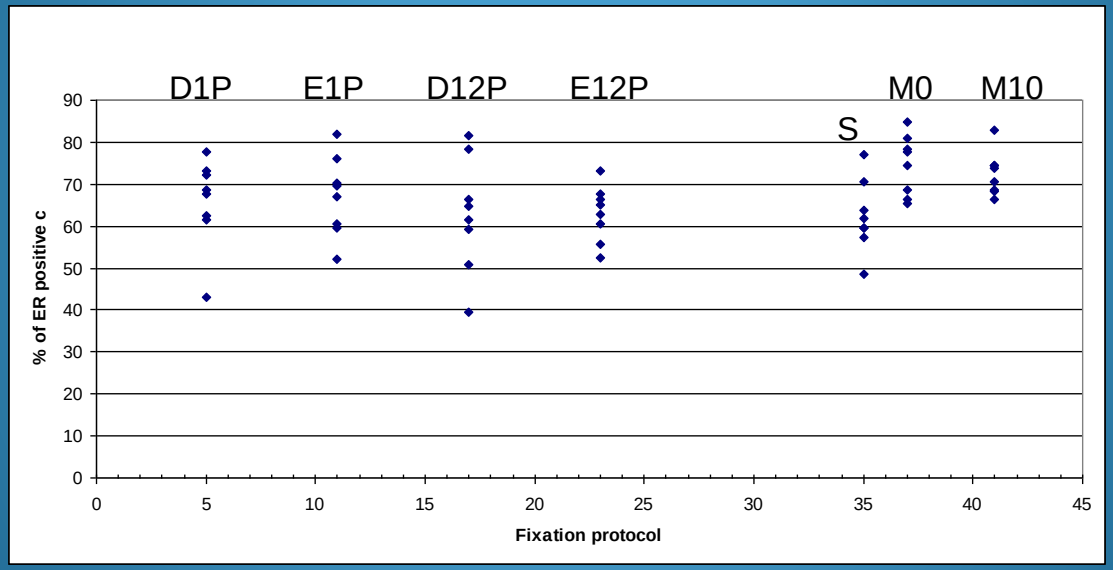
CellFix
Mw10, D100

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

Variability in ICK detection of ER positive cells

- Cytospins prepared from MCF-7 cells
- 7 selected protocols
- 4 independent staining series
- 2 parallel cytospins for each protocol
- Negative control for each protocol

Variability in ICK detection of ER positive MCF-7 cells



Optimal protocol for ICK detection of ER on cytospins prepared from MCF-7 cell line

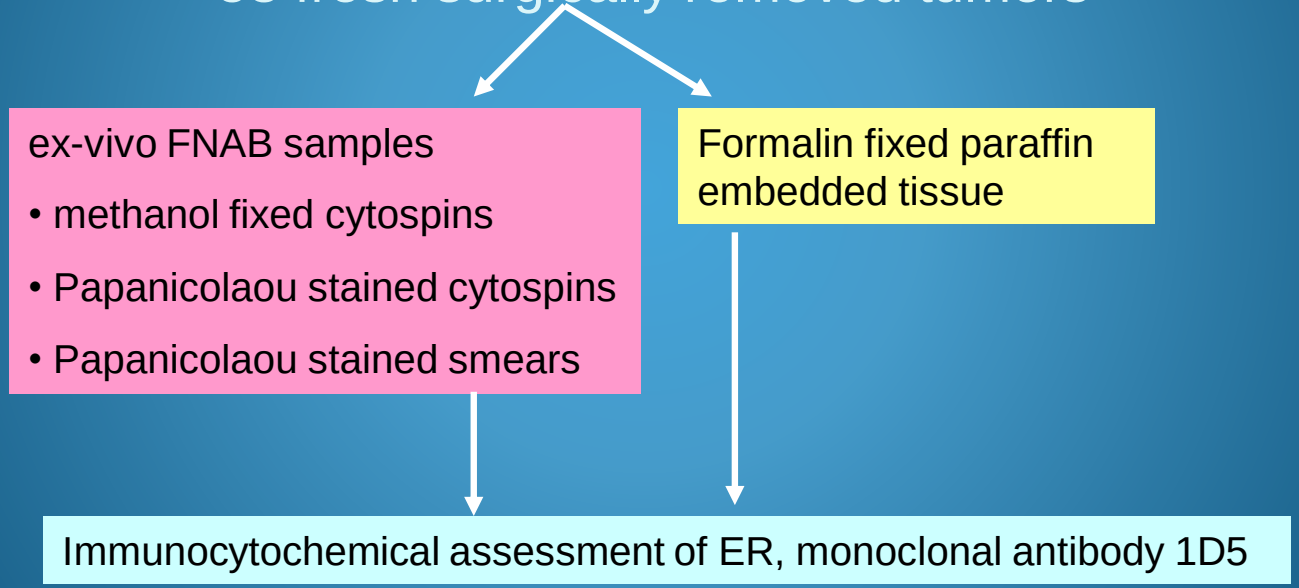
methanol fixed cytospins
no Mw pretreatment

Papanicolaou stained cytospin
10 min Mw pretreatment

manual staining, ABC method,
overnight incubation with monoclonal antibody 1D5

Protocol evaluation on ex-vivo FNAB samples

53 fresh surgically removed tumors

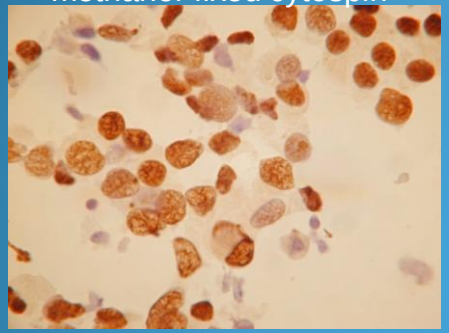


ER on ex-vivo FNAB samples - concordance with corresponding tissue sections

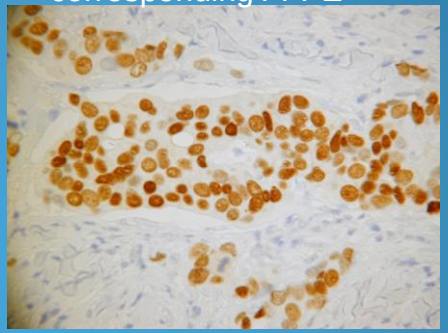
	concordance	kappa
Papanicoalou stained smears	92 %	0.75
Papanicoalou stained cytospins	94 %	0.84
methanol fixed cytospins	**100 %**	**1.00**

ER assessment

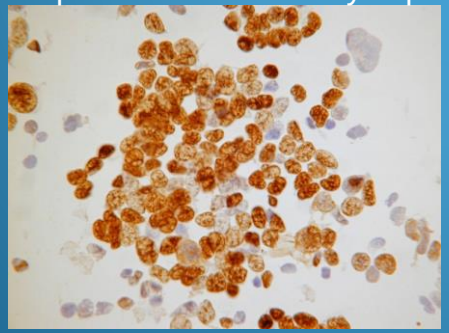
methanol-fixed cytospin



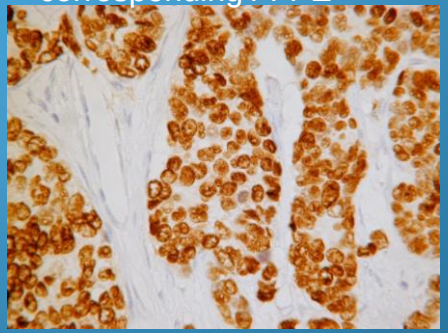
corresponding FFPE



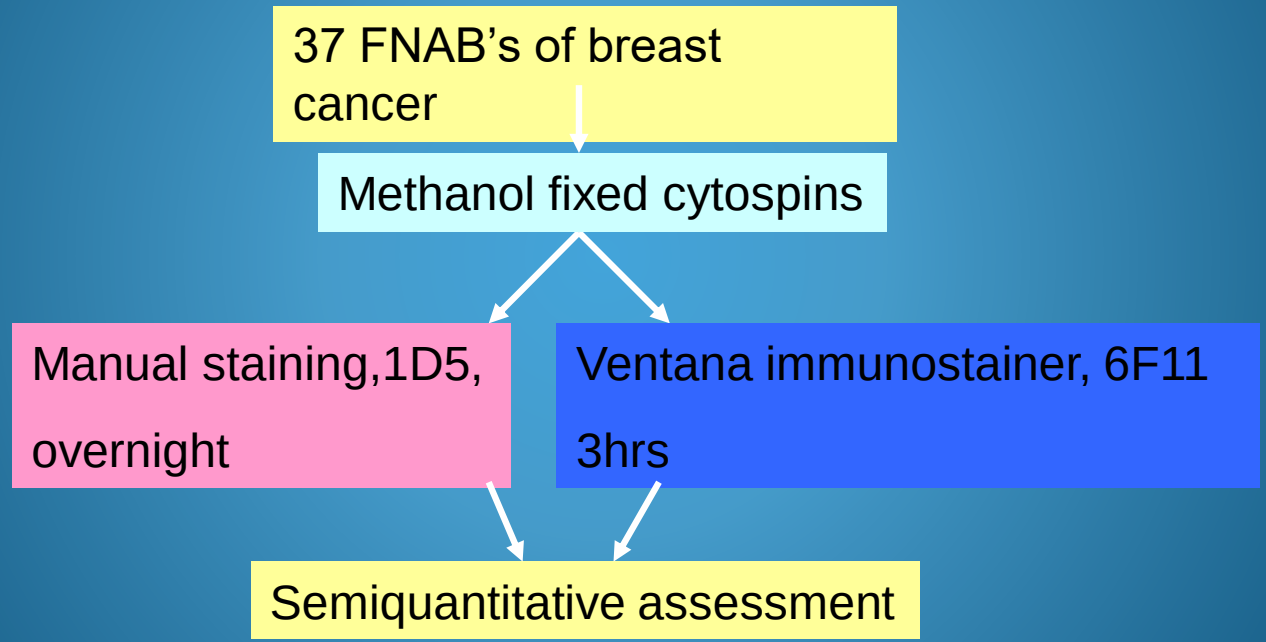
Papanicolaou stained cytospin



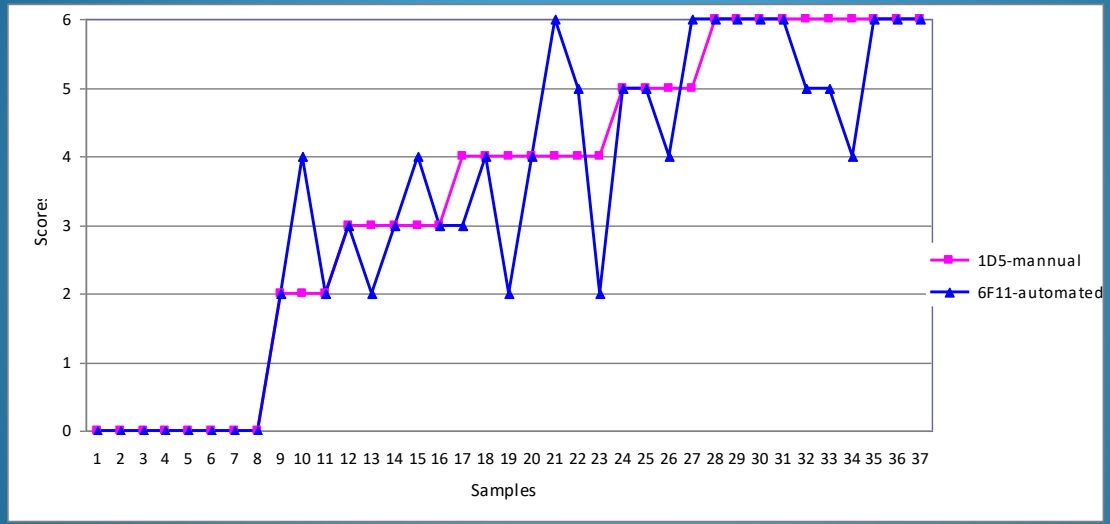
corresponding FFPE



Introduction of automated immunostaining



Manual 1D5: Ventana 6F11



Equal: 24/37

1D5 better : 8/37

6F11 better : 5/37

ER assessed in preoperative FNAB samples - clinical response to hormonal treatment

	localized breast cancer	generalized breast cancer
clinical response	45/49 (92%)	14/22 (64%)

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)

Methanol

Validation of ICC

38 other markers:

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

Validation of ICC

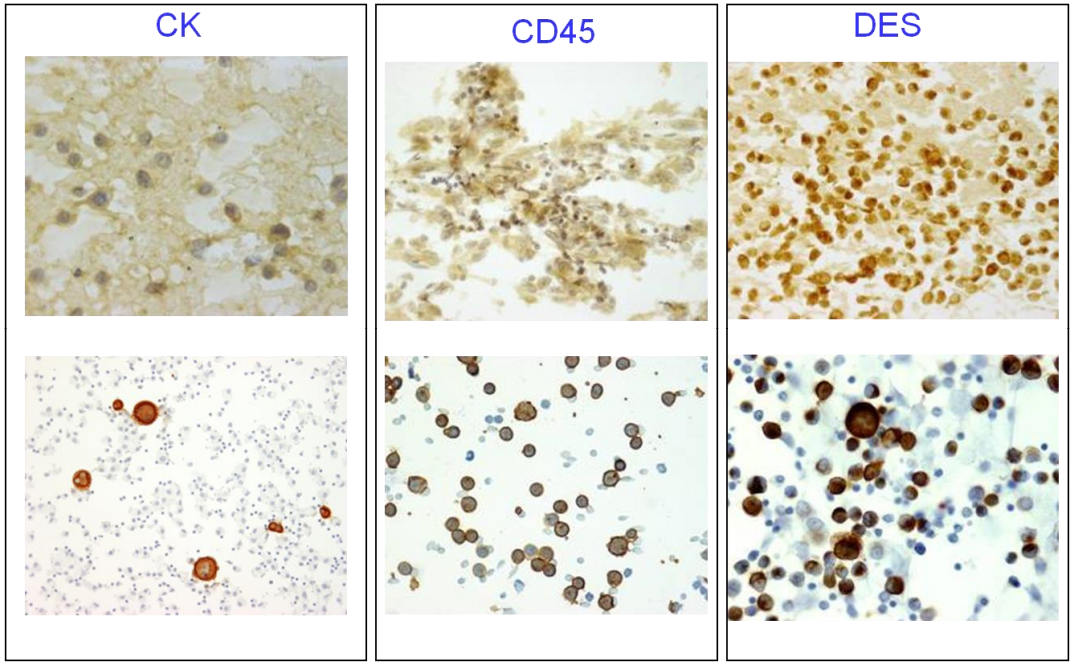
50 diagnostic routine cytology samples
ICC on methanol fixed cytospins : IHC on concordant FFPE

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



Conclusion

Immunocytochemistry

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

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