

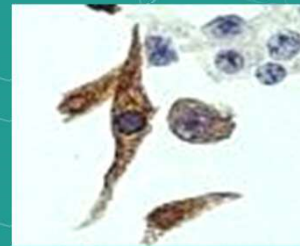
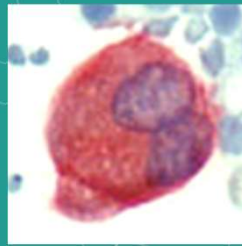
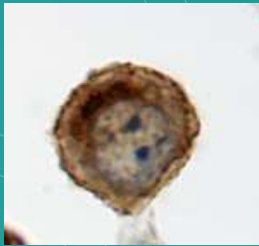
The impact of pre-analytical factors in cytology sample processing on a biomarkers preservation

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INSTITUTE OF PATHOLOGY

UNIVERSITY OF LJUBLJANA ♦ FACULTY OF MEDICINE



Preservation of biomarkers

FFPE

- Ischemic time
- Duration of fixation
- Processing
- Storage of unstained section
- Storage of FFPE blocks

Cytology preparations

????????????????

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

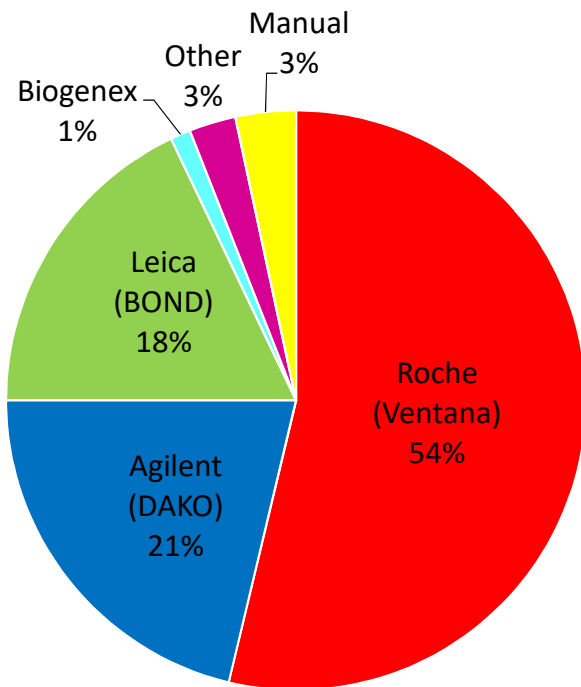
Our experiences

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

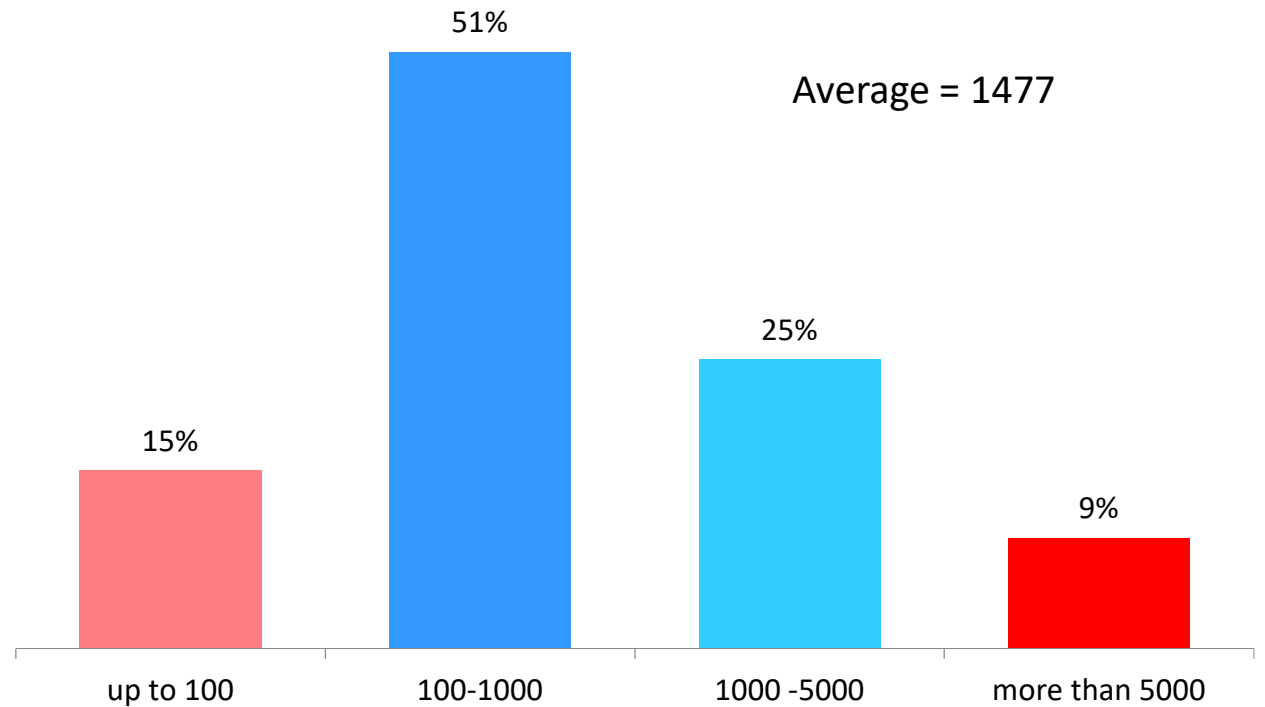
External quality control for immunocytochemistry on cytology samples: a review of UK NEQAS ICC (cytology module) results. Kirbis IS, Maxwell P, Flezar MS, Miller K, Ibrahim M. Cytopathology. 2011;22(4):230-23

ICC Platforms /number of ICC

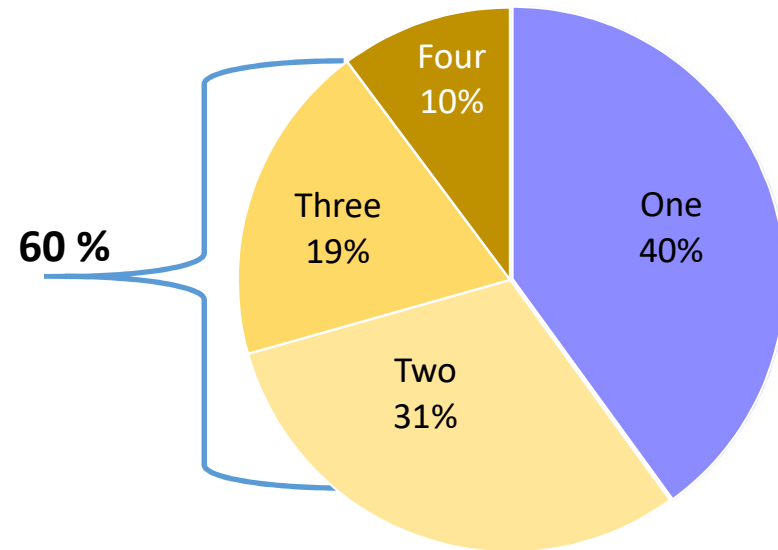
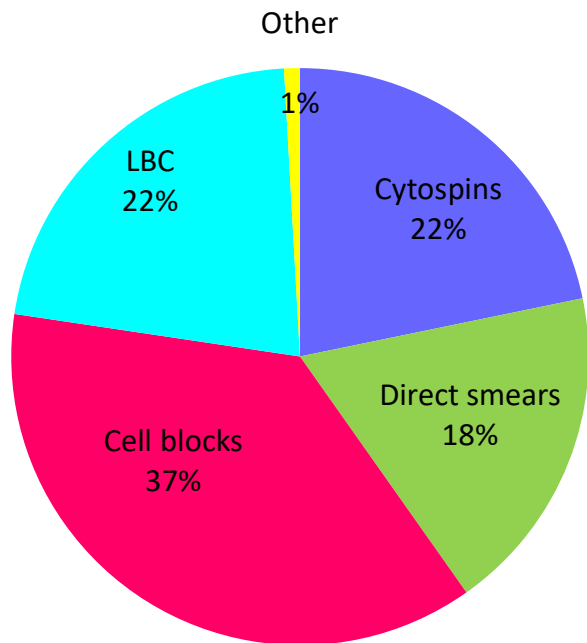
ICC platforms



Number of ICC/year

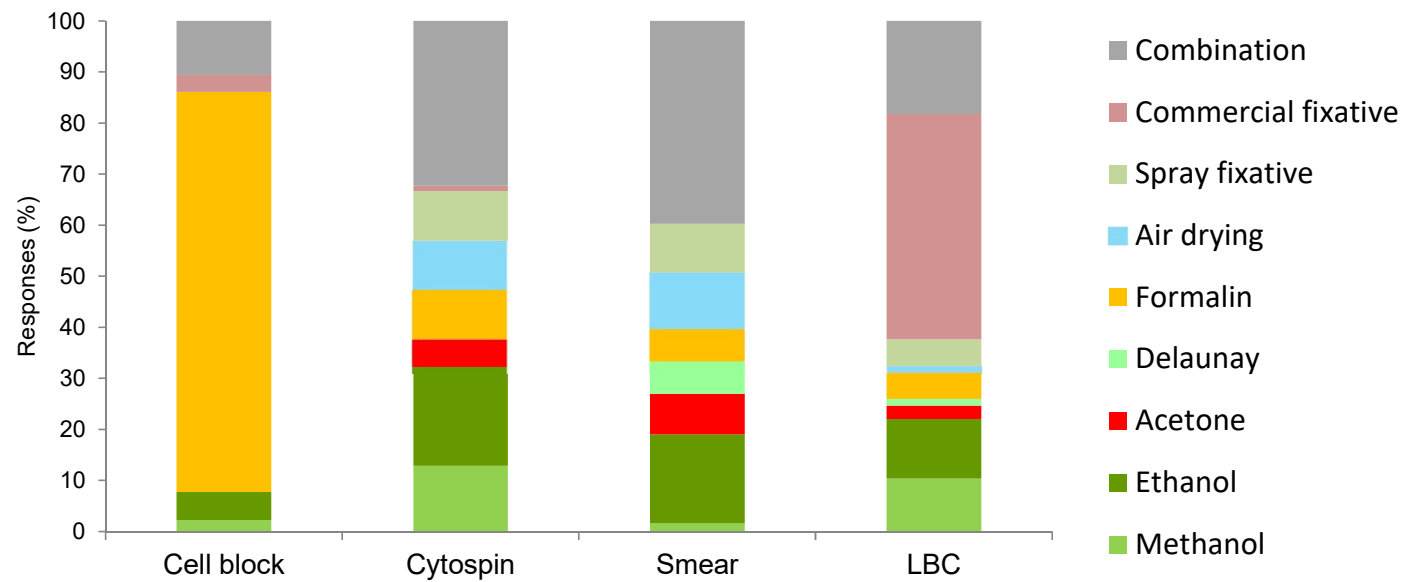


Slides used for ICC



Number of different slides used for ICC

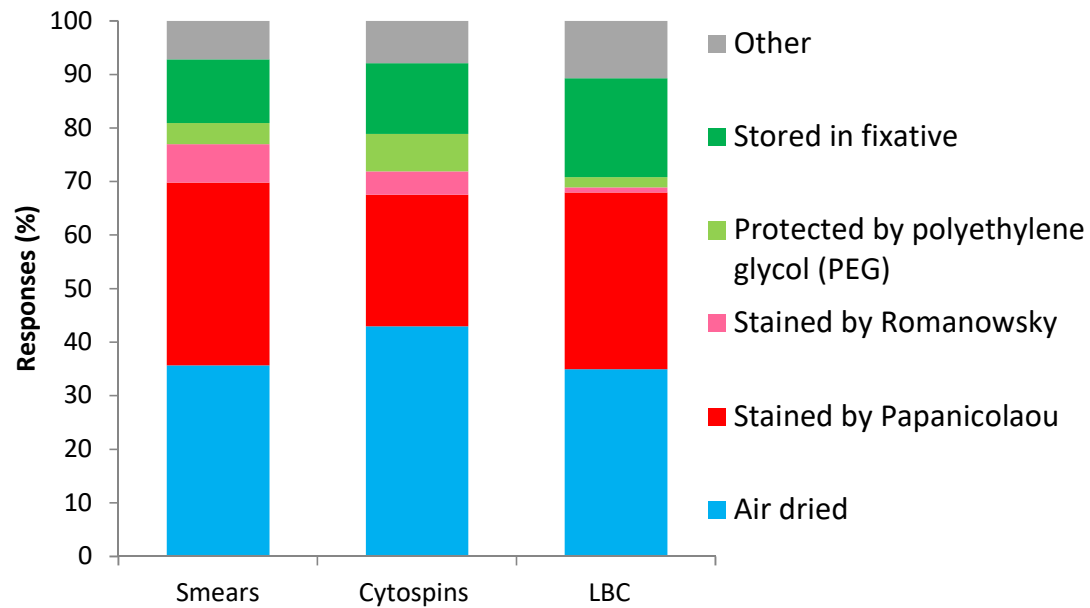
Pre-analytical variability - fixation



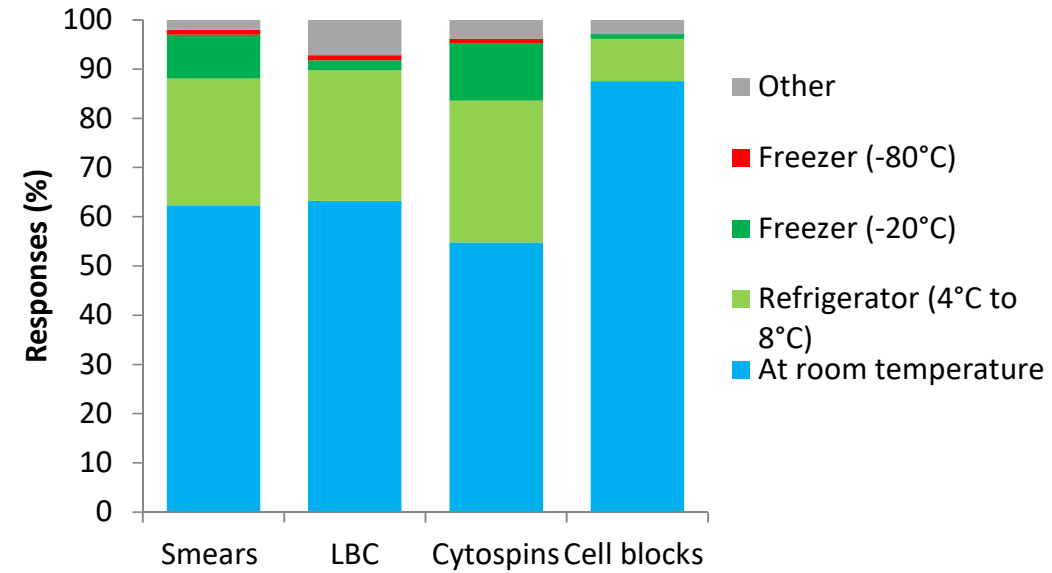
Fixatives used for the fixation of ICC preparations

Pre-analytical variability – post fixation

Post-fixation step for ICC slides



Storage of ICC slides



Optimal slides for ICC?

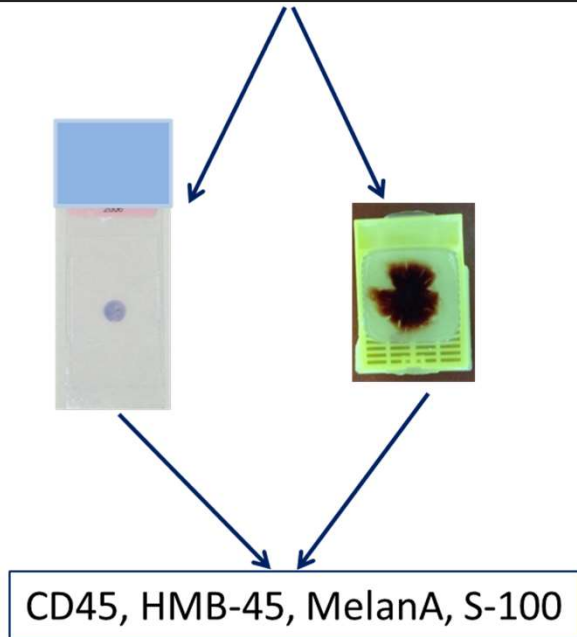
Optimal fixation?

Differences in ICC quality/reliability?

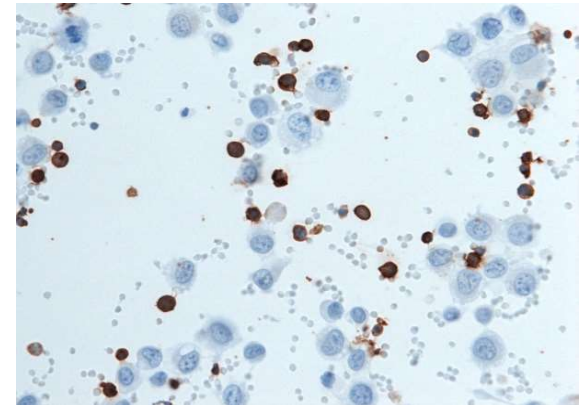
CB the best option?

UK Neqas ICC slides – run 108

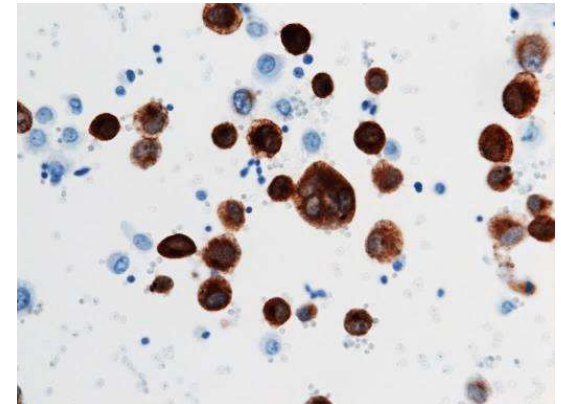
- Human melanoma cell line SK-MEL28
- effusion with carcinoma cells, few mesothelial cells, Erci
- FNAB of lymph node



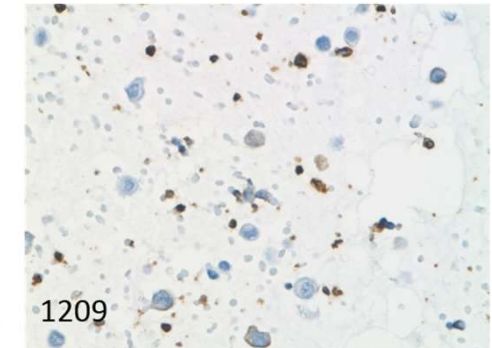
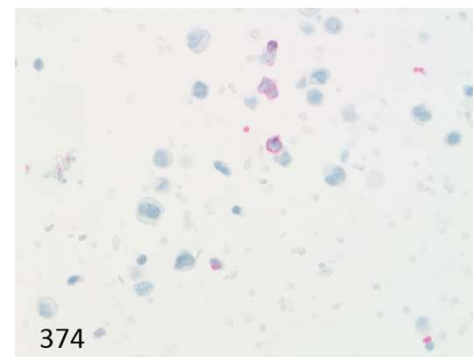
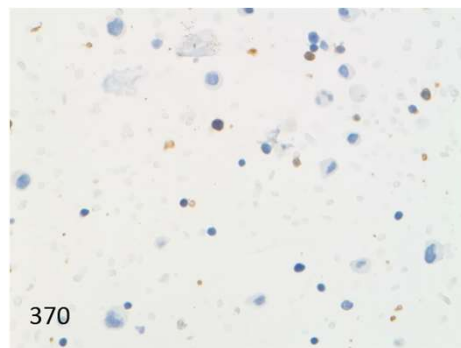
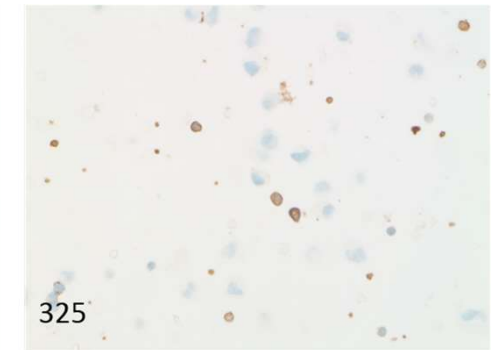
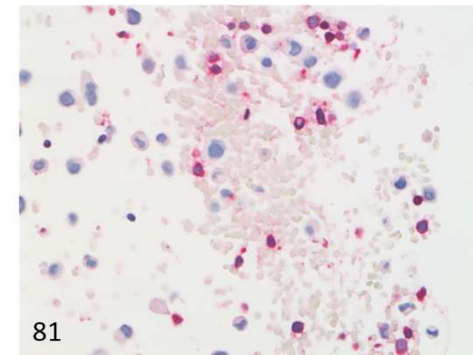
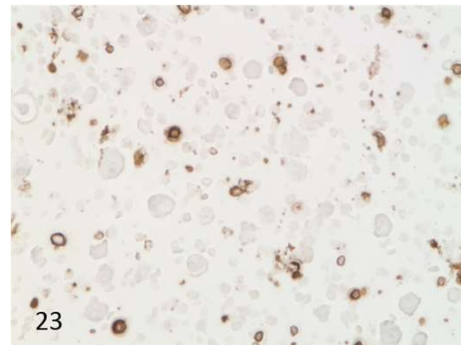
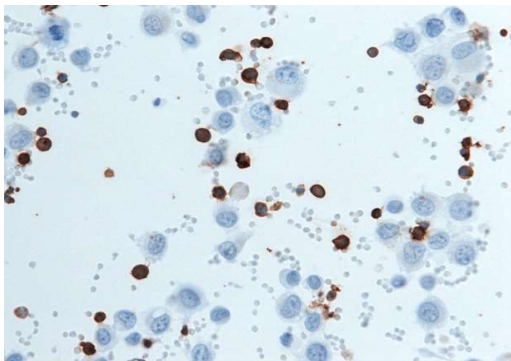
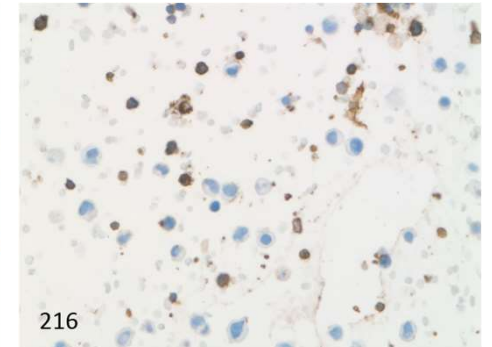
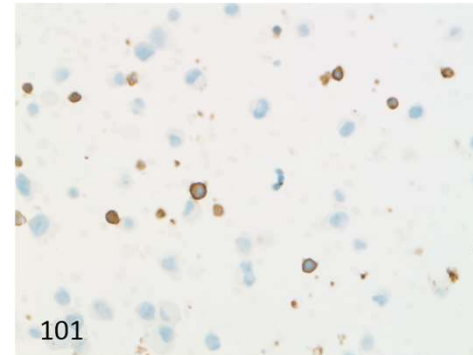
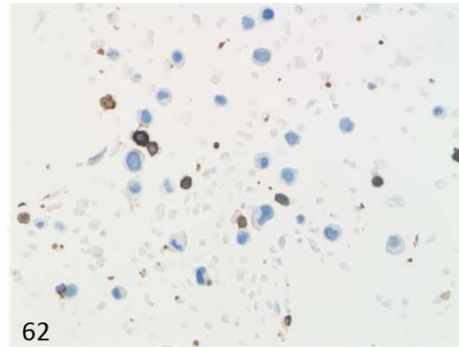
CD45



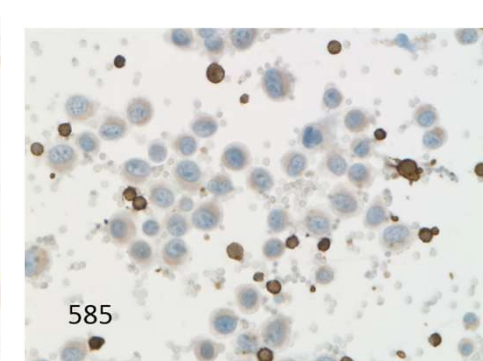
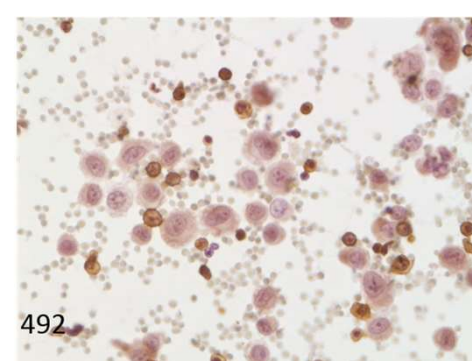
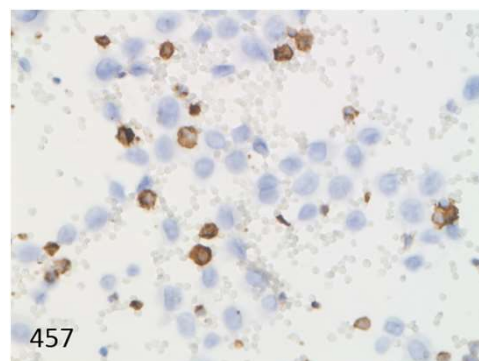
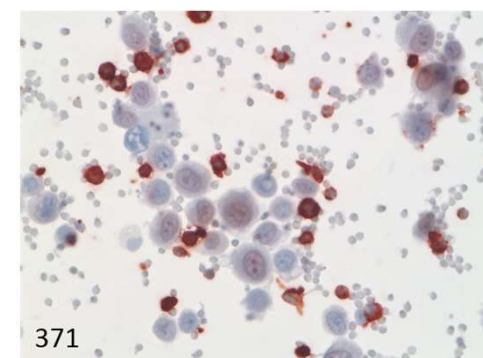
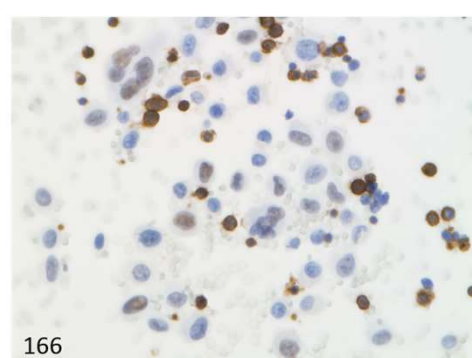
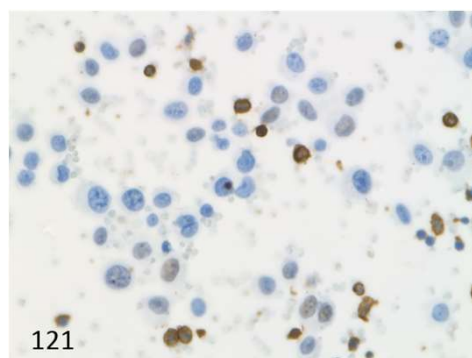
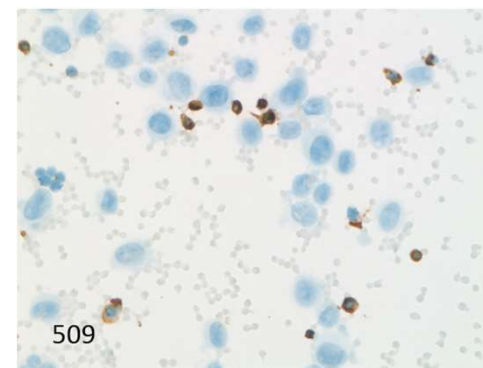
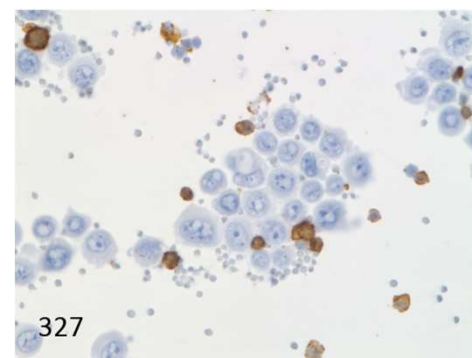
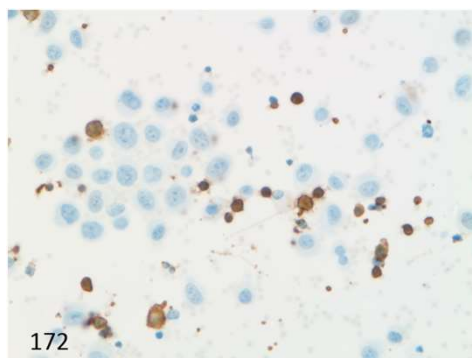
HMB-45



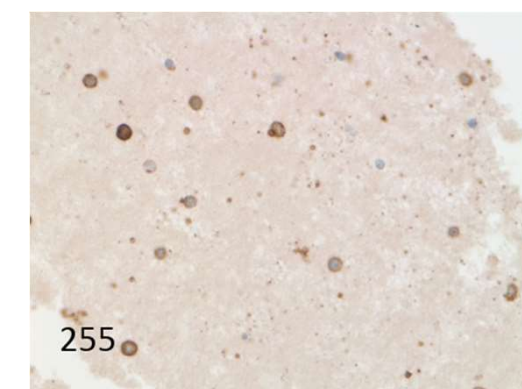
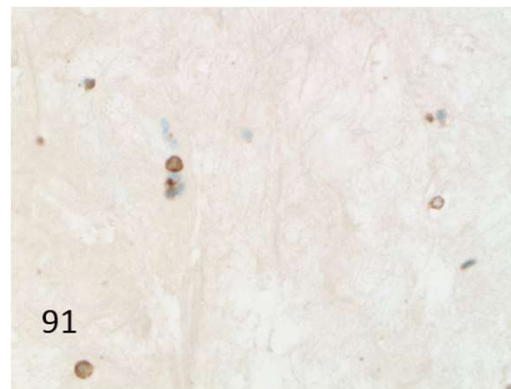
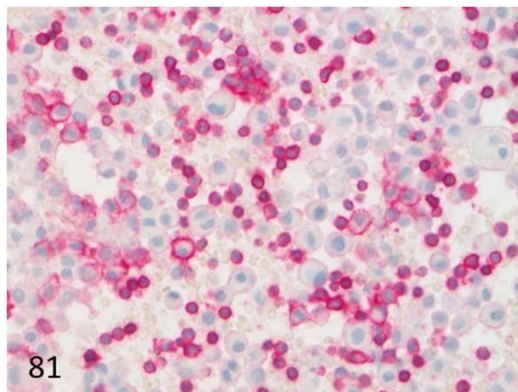
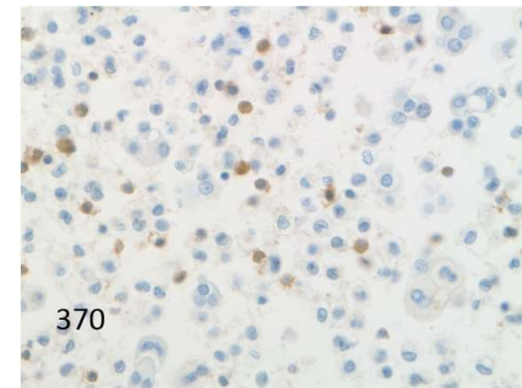
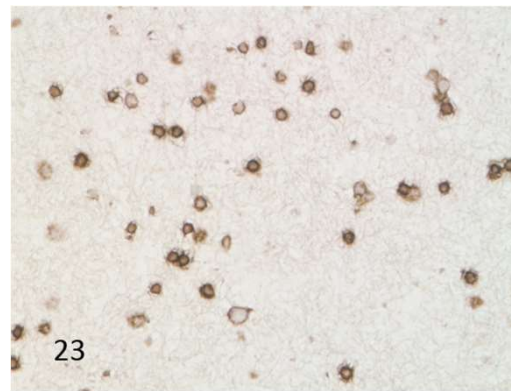
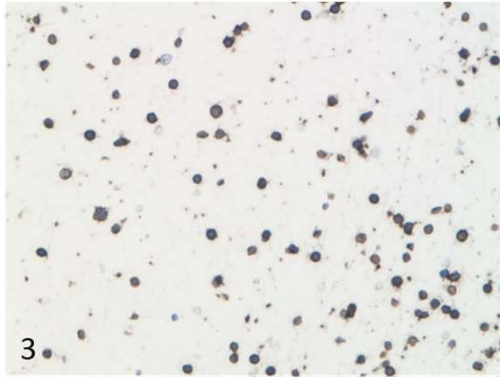
108 R (CD45) - ICC variability on Neqas cell block



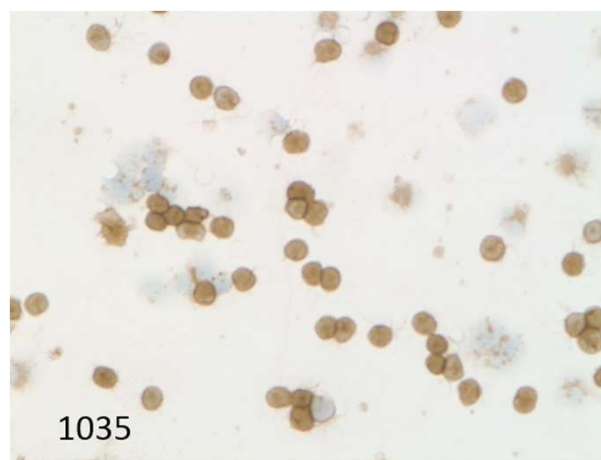
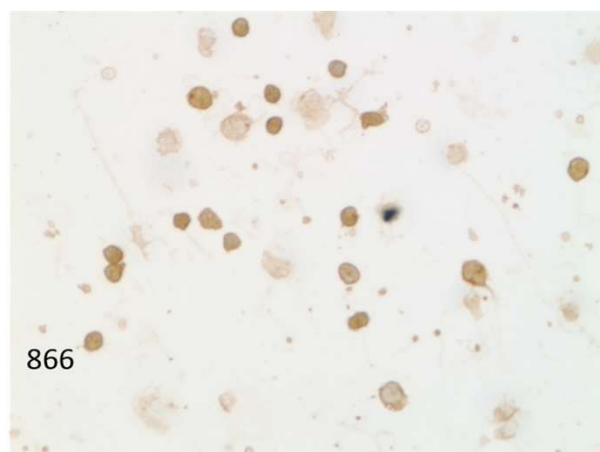
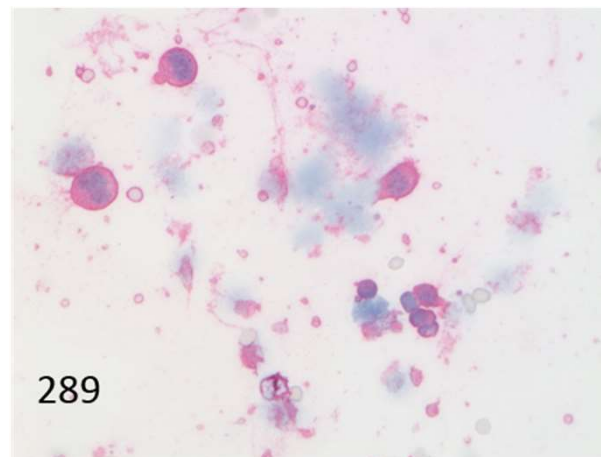
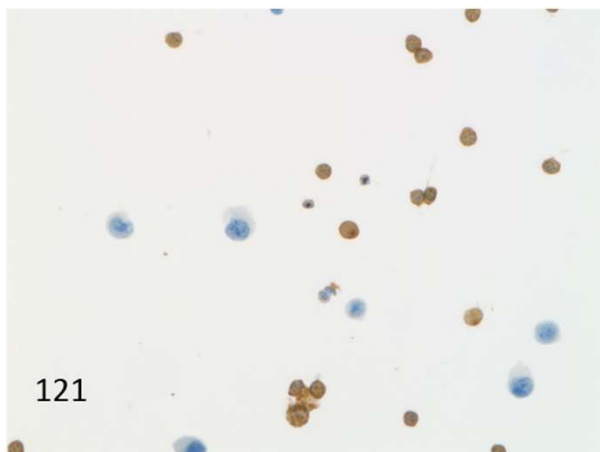
108R (CD45) - ICC variability on Neqas **cytospins**



Run 108, in-house CB, CD45

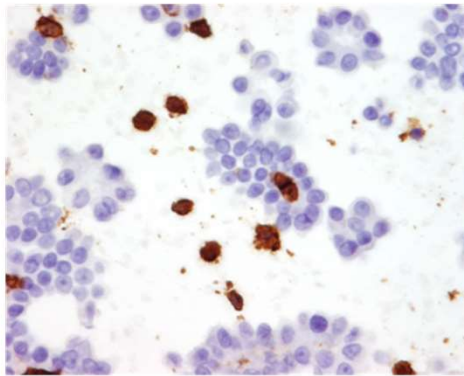


Run 108, in-house cytopsin, CD45

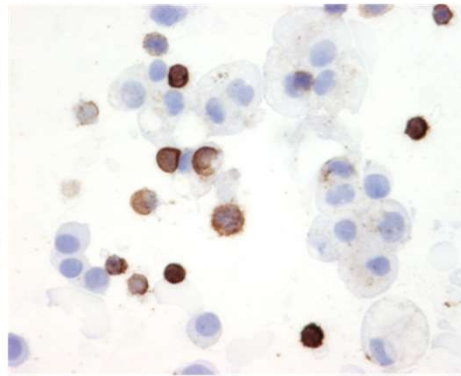


Good ICC quality can be achieved on a differently prepared slides

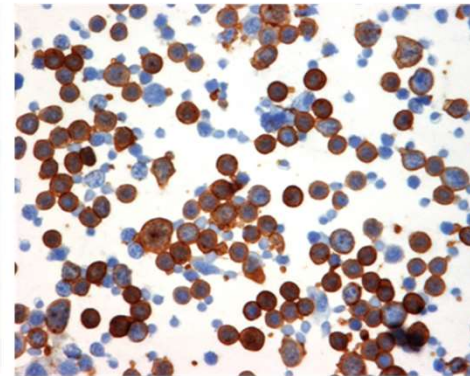
CD 45 (DAKO M701)



Cell block, FFPE



Smear, ethanol

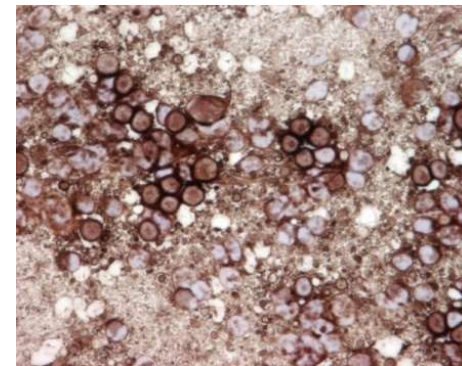
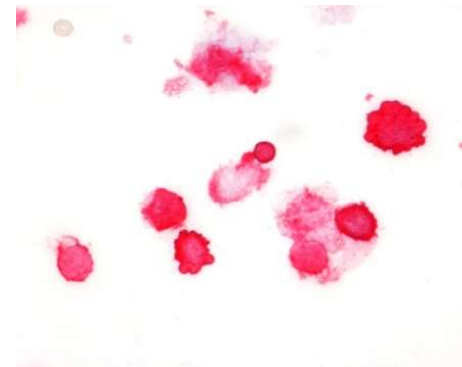


Cytospin, methanol

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.

Acetone is not suitable fixative for ICC

| Fixative | N | UK NEQAS ICC score | |
|--------------------------|-----|--------------------|------|
| | | Mean | SD |
| Delaunay | 70 | 15.40 | 2.61 |
| CytoRich Red | 68 | 15.36 | 2.16 |
| Formalin-based fixative | 76 | 15.21 | 1.90 |
| Methanol-based fixatives | 134 | 15.18 | 3.03 |
| Other | 29 | 14.94 | 2.02 |
| Ethanol-based fixatives | 145 | 14.59 | 2.74 |
| Acetone | 109 | 13.77* | 2.34 |



UK NEQAS ICC samples

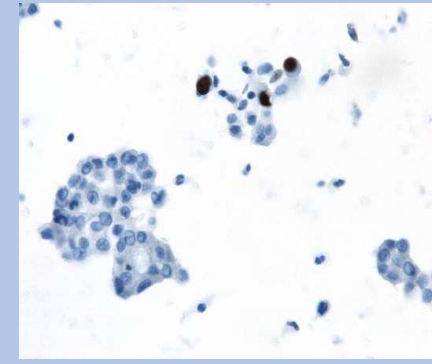
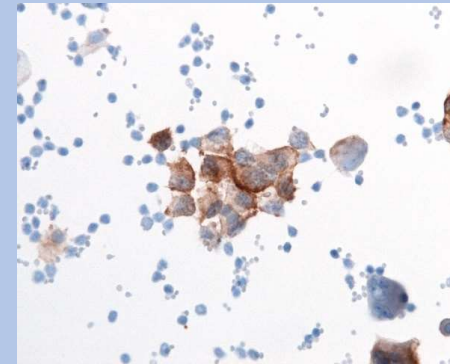
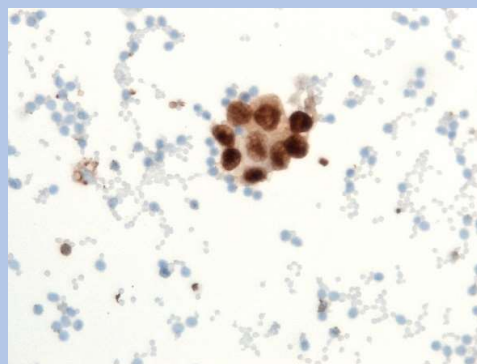
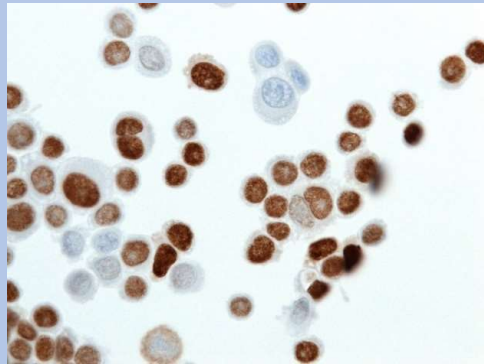
ER

TTF

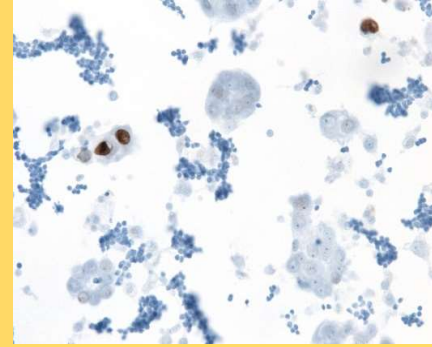
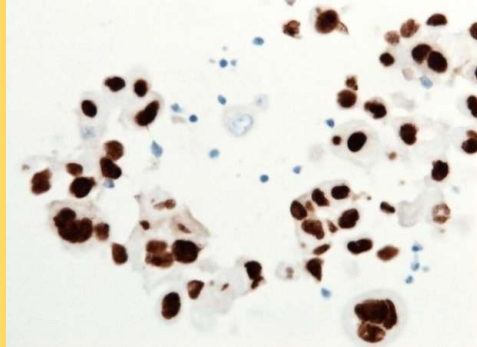
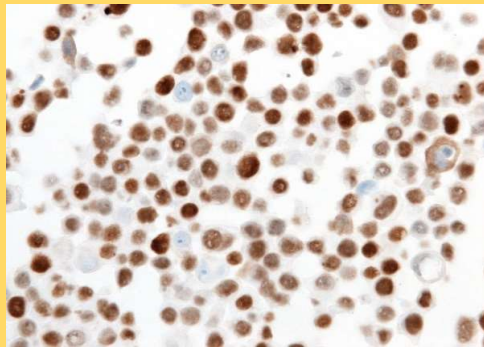
PD-L1

Ki-67

Cytospin



CB

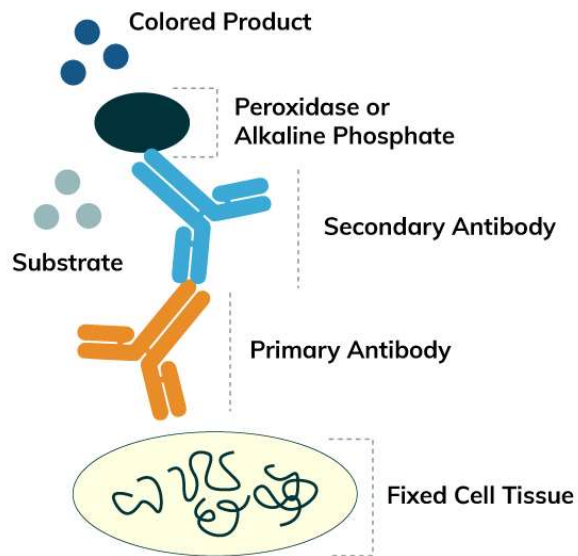


ICC reality

- 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)?

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 of IHC/ICC protocols


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

ICC protocols \neq IHC protocols

ICC protocols \neq 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

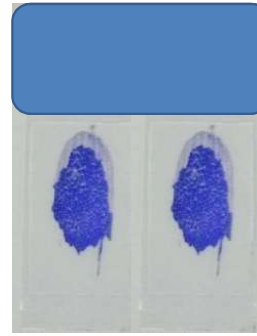
- Validation ensures a test works as intended. Any antibody assay (novel or replacement) must be validated before it is put into use as a diagnostic test.
- Objective evidence that test performs reliable and consistently - accurate, correct, reliable results

ICC: IHC or other method

- **Quality Assurance For Immunocytochemistry: 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

Sample processing – our approach

FNA,
EUS-FNA
US-FNA
effusion



Brushing of fresh
tissue sample





Sample in buffer based cell medium

molecular tests

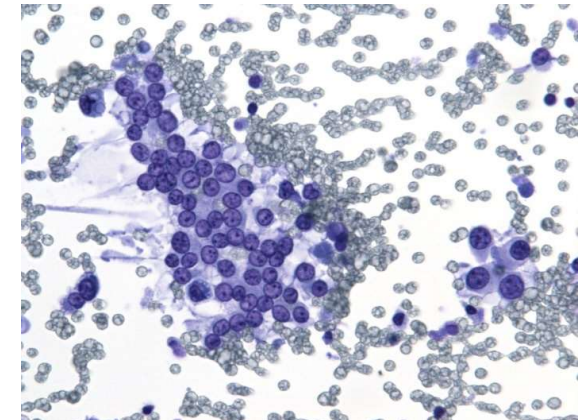
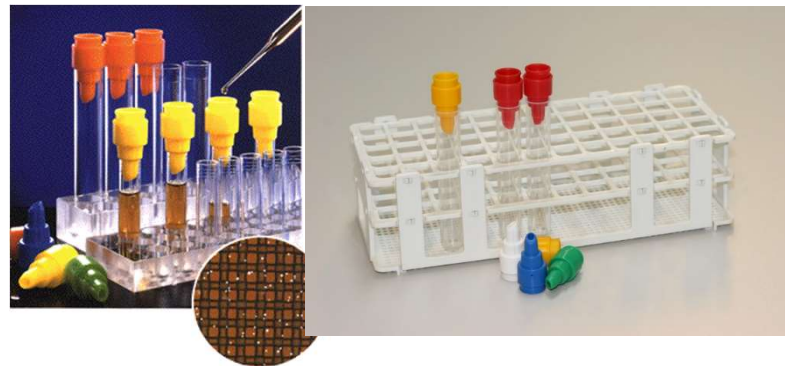
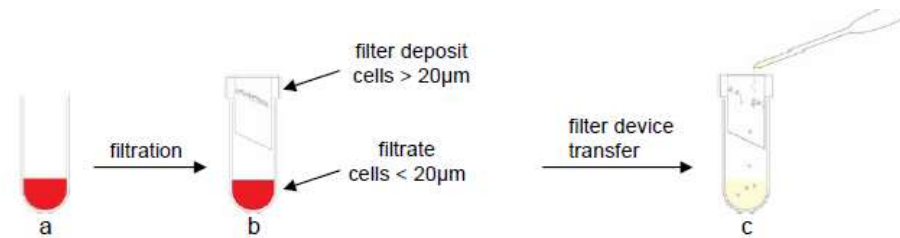
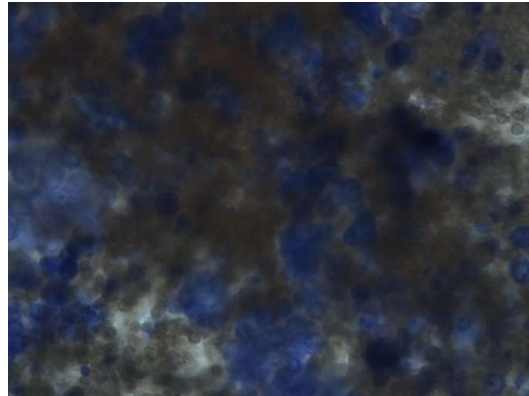
cytospins

cell block ($> 1 \times 10^6$ cells)

flow cytometry

- **immunocytochemistry**
- **special stainings**
- **FISH**
- **control slides**

Hemorrhagic samples - filtration



Validation of ICC on cytopins

- 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 cytopins 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

ICC validation - ensures that test works as intended

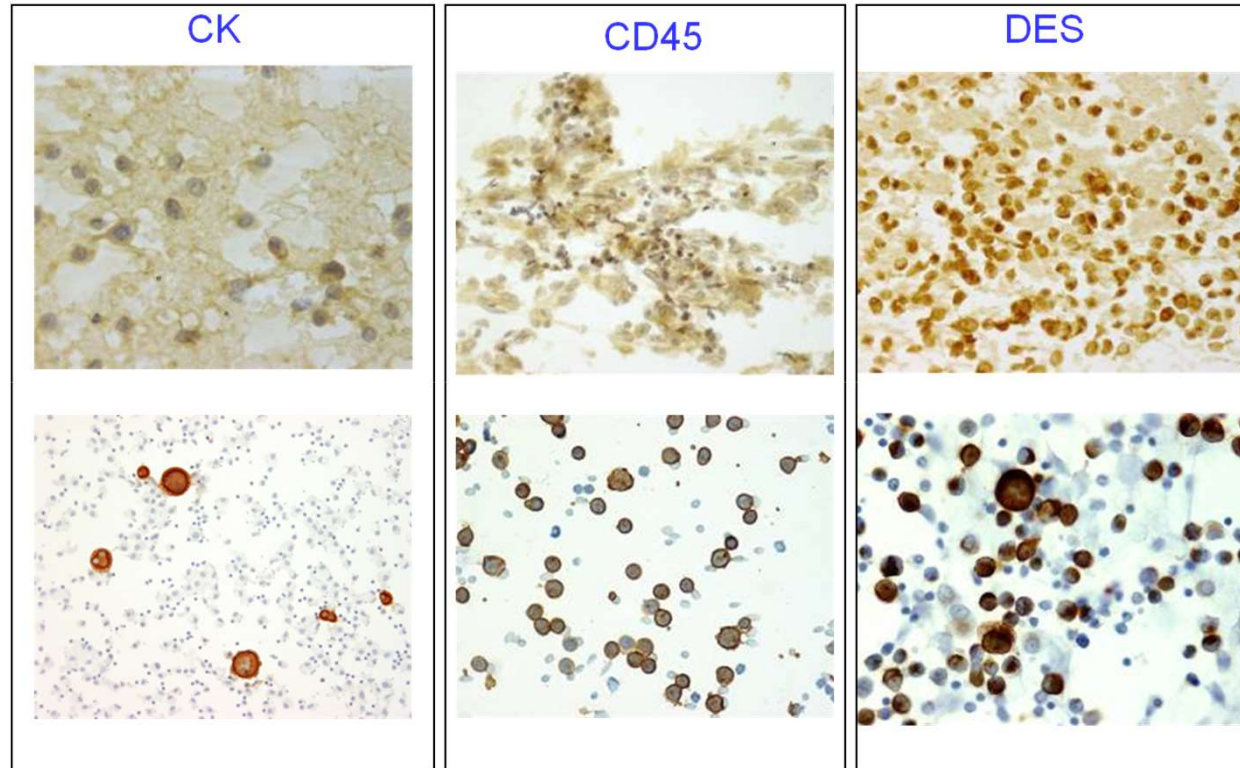
50 diagnostic routine cytology samples

ICC on methanol fixed cytopins : 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 %, $\kappa = 0,93$ | | |

Development of sample processing

1988
Direct smears



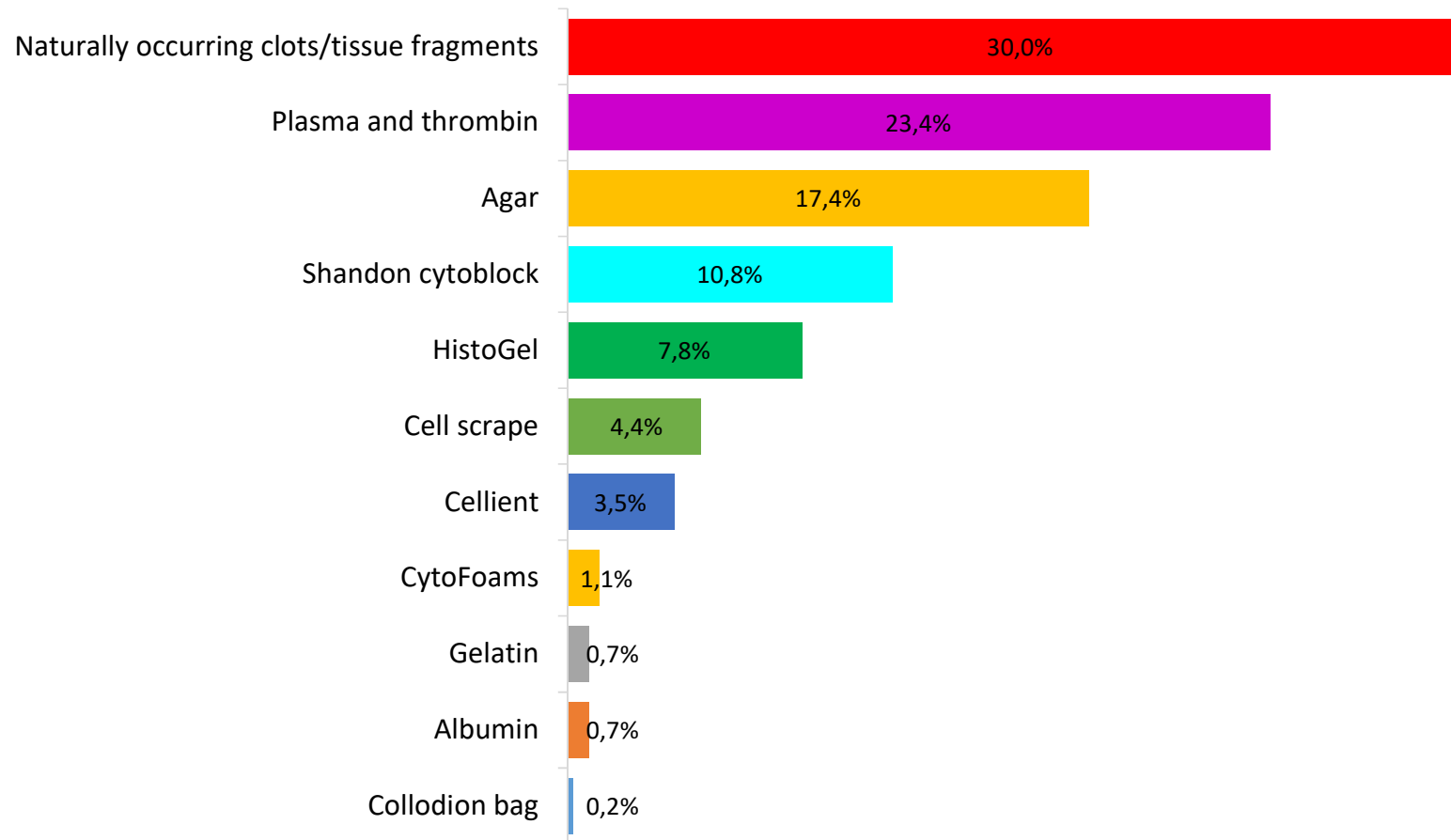
2008
Cytospins

ICC - conclusions

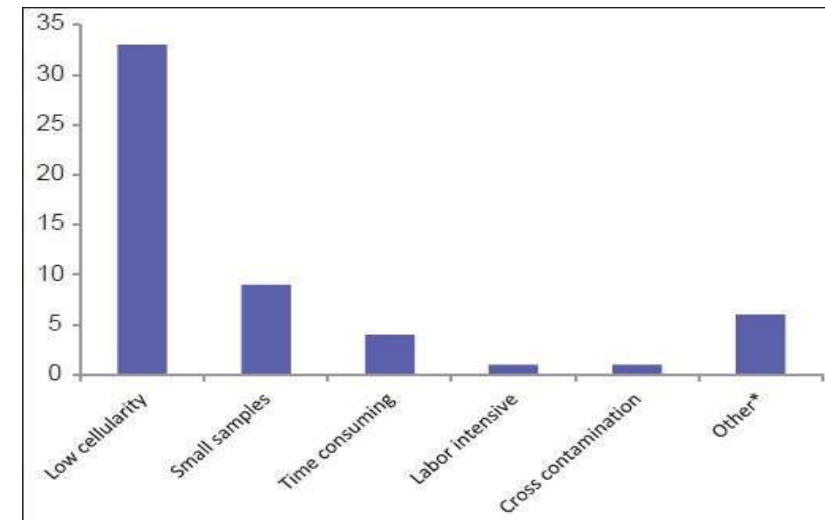
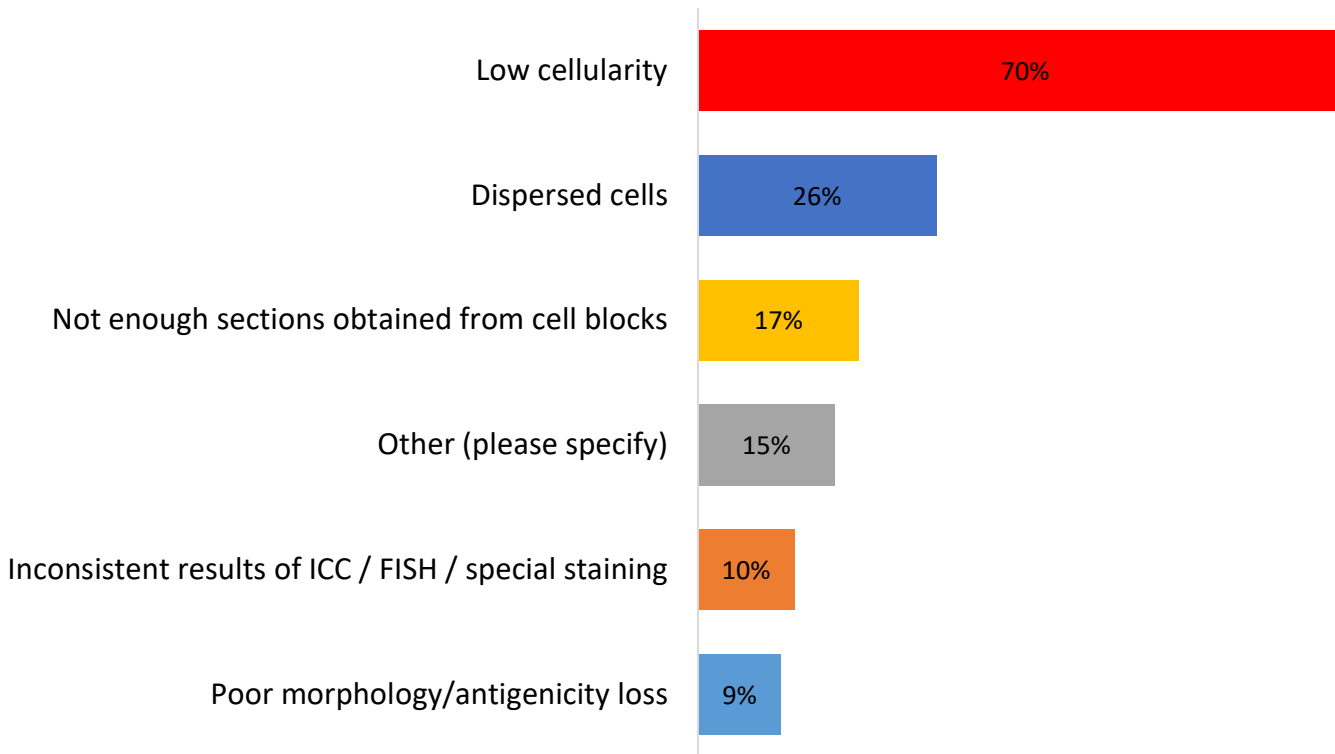
- Great variability in all aspects of ICC
- Good ICC quality can be achieved on a differently prepared slides
- QA/QC
 - Control slides prepared as patient sample
 - Optimization
 - Validation
 - EQA

Are cell blocks the best option for cytology samples?

Methods for CB preparation

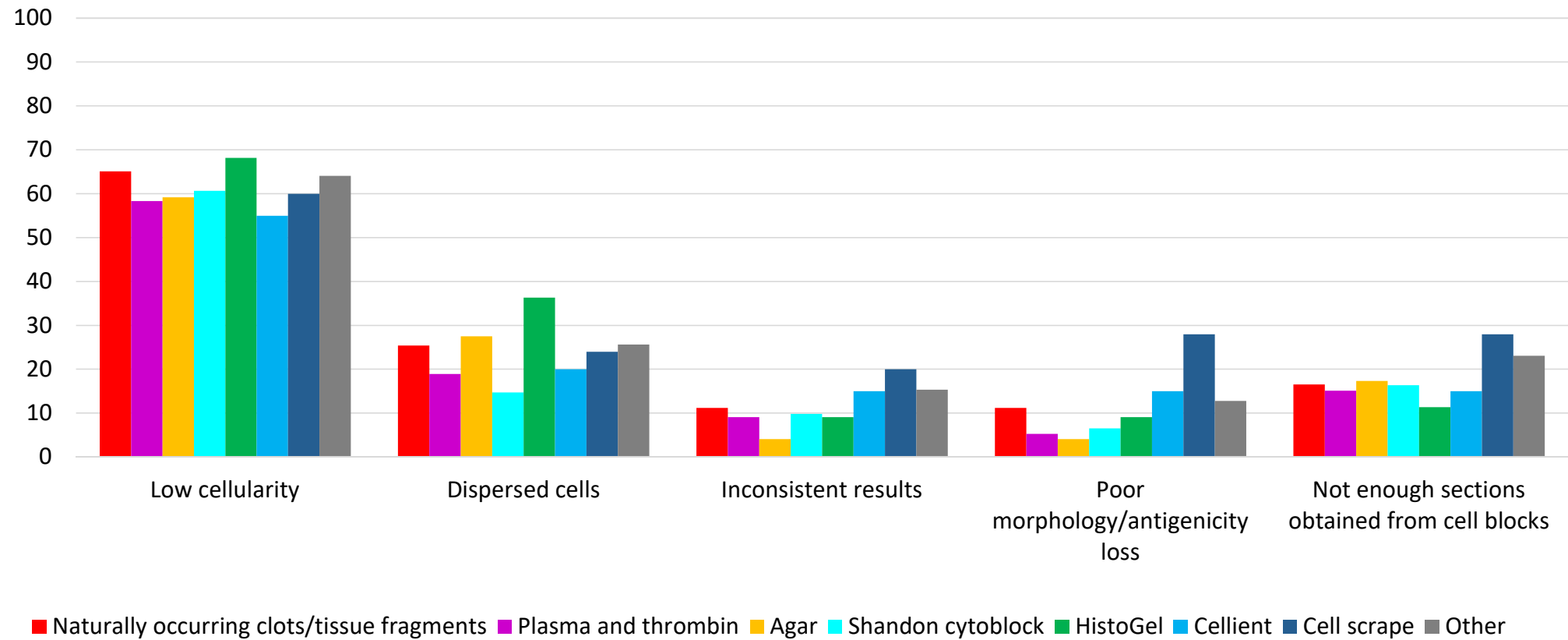


Issues with CB

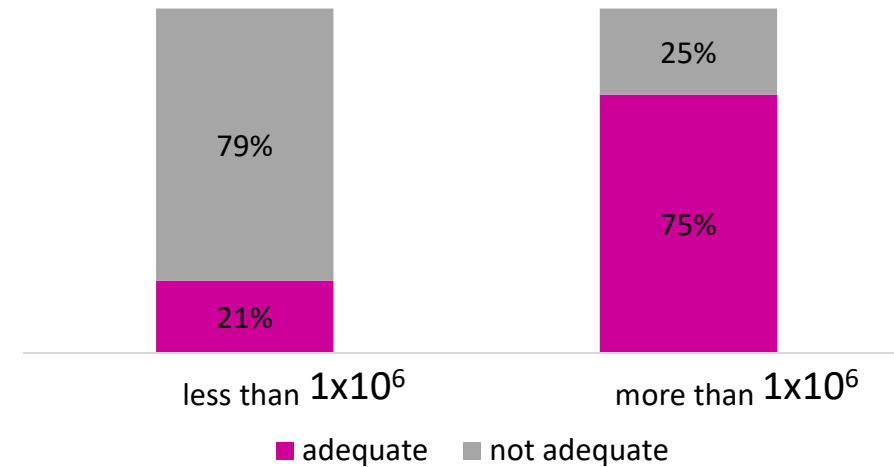
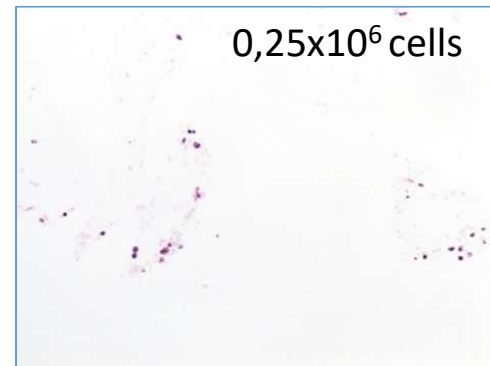
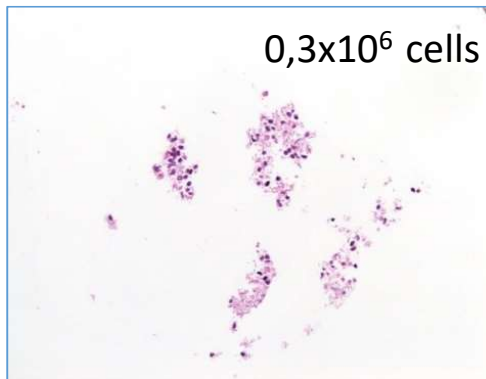
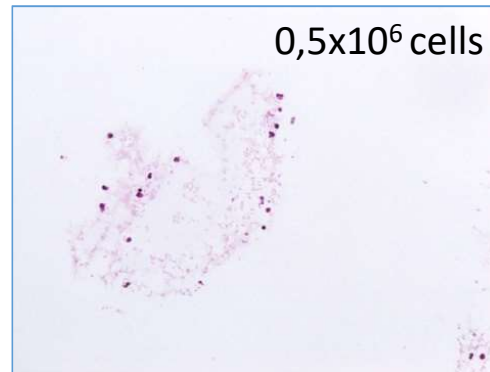
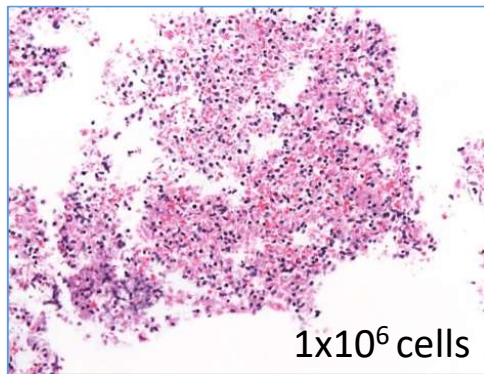


Crapanzano, J. P., Heymann, J. J., Monaco, S., Nassar, A., & Saqi, A. (2014). The state of cell block variation and satisfaction in the era of molecular diagnostics and personalized medicine. *CytoJournal*, 11, 7. <https://doi.org/10.4103/1742-6413.129187>

Issues and CB preparation method



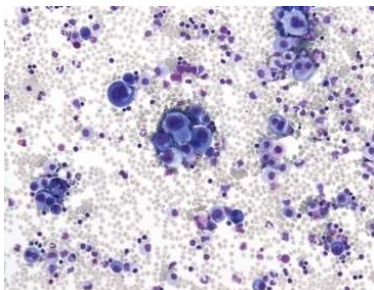
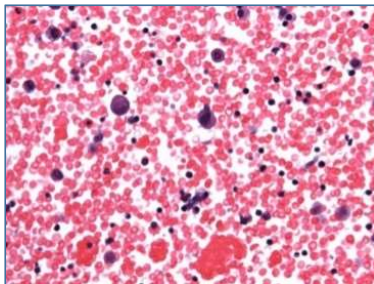
Cell block cellularity



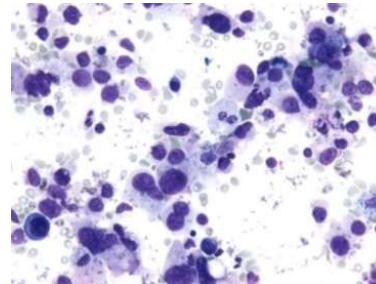
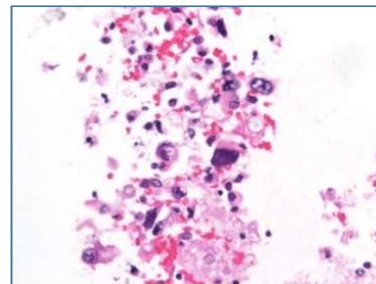
Srebotnik Kirbiš, I. and Strojan Fležar, M. Cell count-based triaging of cytology samples for cell block preparation. Cytopathology 2016, <https://doi.org/10.1111/cyt.12404>

Cell block cellularity

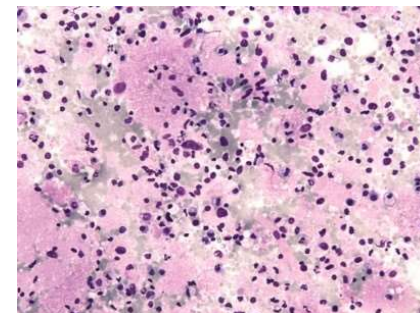
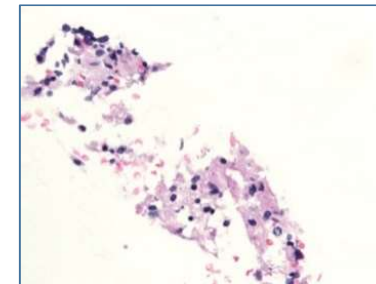
20×10^6 cells



1.2×10^6 cells

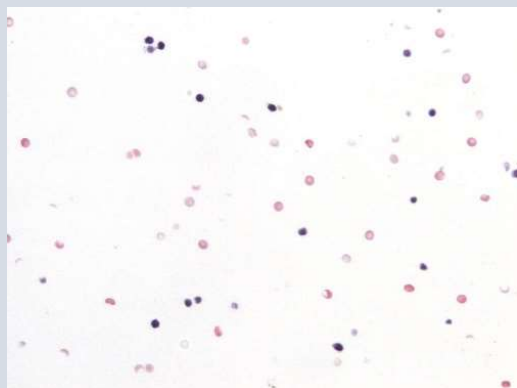


0.1×10^6 cells



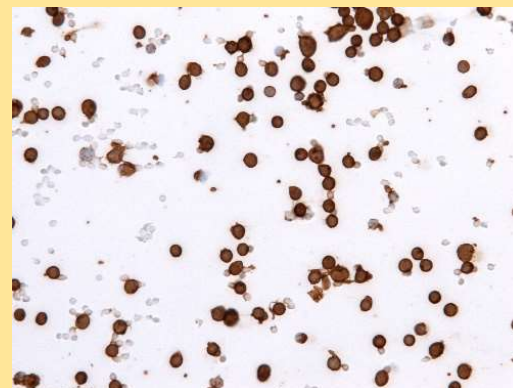
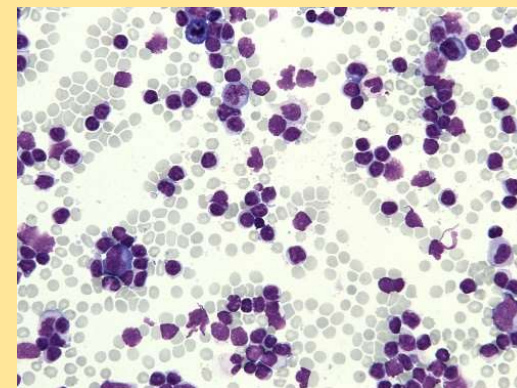
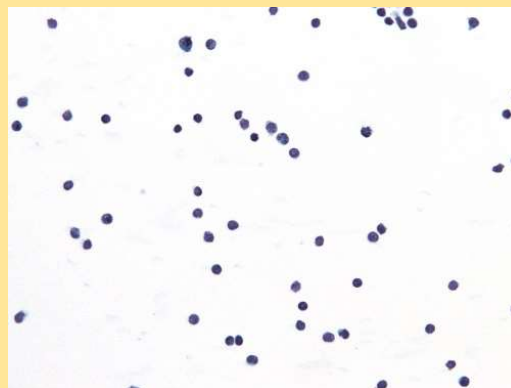
corresponding cytopins

Cell block



← CD45 →

Cytospins



CB - conclusions

- Great variability in CB preparation method
- Low cellularity is the main issue with CB

Recommendations

- Low cellular samples not suitable for CB!
- Sample triage?

Molecular testing on cytology samples

- Cytologic specimens > FFPE tissue (degraded DNA!)
- Various types of cytology preparations > cell blocks – VALIDATION!
- Archived MGG/Papanicolaou stained slides – 100 tumor cells - NGS

Amemiya, K, Hirotsu, Y, Nagakubo, Y, Mochizuki, H, Higuchi, R, Tsutsui, T, Kakizaki, Y, Miyashita, Y, Oyama, T, Omata, M. Actionable driver DNA variants and fusion genes can be detected in archived cytological specimens with the Oncomine Dx Target Test Multi-CDx system in lung cancer. Cancer Cytopathol. 2021. <https://doi.org/10.1002/cncy.22434>

Baltimore, MD
USA

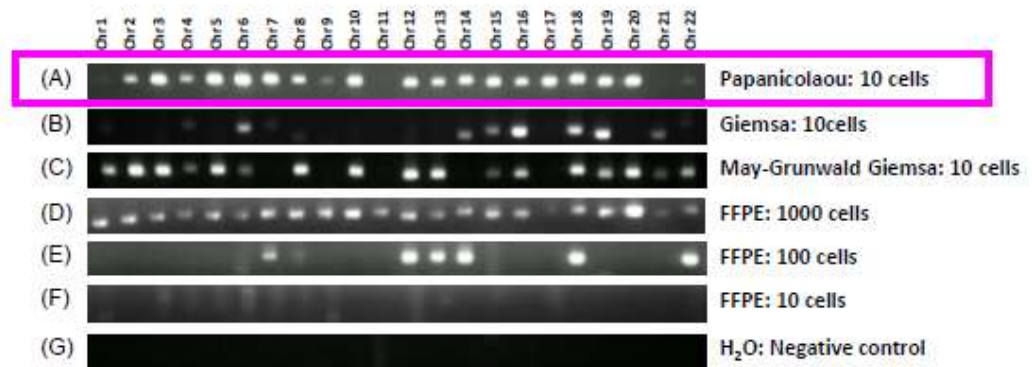
Nov 16, 2022 Platform 1

**Next Generation Sequencing of
Cytological Oligo Tumor Cells
Using Whole Genome Amplification**

Genome Analysis Center, Yamanashi Central Hospital,
Yamanashi, Japan
Kenji Amemiya, MS

ICC 2022
70th

Optimization of WGA using cytology specimens



- Quality of nucleic acid: cytology preparations > FFPE
- Papanicolaou/MGG stained smears yield enough quality DNA/RNA for NGS



Next-Generation Sequencing (NGS) in US-guided Fine Needle Aspirates of (Primary and Metastatic) Malignant Tumors in the Liver

Damjana Cimerman
*Institute of Pathology,
Faculty of Medicine,
University of Ljubljana,
Slovenia*



NGS analysis from US-FNA's of liver tumors

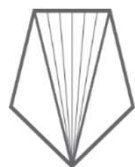
- Successful in 97%
- Fresh cells in cell medium
- Stained smears



➤ *Cancer Med.* 2022 Apr 10. doi: 10.1002/cam4.4728. Online ahead of print.

Optimization of pre-analytical and analytical steps for DNA and RNA analysis of fresh cytology samples

Ana Dolinar ¹, Gašper Grubelnik ¹, Irena Srebotnik-Kirbiš ², Margareta Stojan Fležar ²,
Margareta Žlajpah ¹



dr. Irena Srebotnik Kirbis



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1000**