IMMUNOCYTOCHEMISTRY AS AN ADJUNCT TO DIAGNOSTIC CYTOLOGY

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Specificity of immunocytochemistry

- Various kinds of specimens
  - Air-dried smears
    - Lymph node, thyroid, salivary glands, other organs)
  - Fixed smears (spray, alcohol-based fixative…)
    - Cervical smears
  - Liquid samples
    - Unfixed: serous fluids, cerebrospinal fluid, broncho-alveolar lavage fluid…
    - Fixed: urine
  - Liquid-based cytology
  - Cell blocks

Specimens

Smears

- Air dried
- Often haemorrhagic
- Usually on non-adhesive slides
- May be realised at the laboratory (ex : cell pellet from serous fluid)

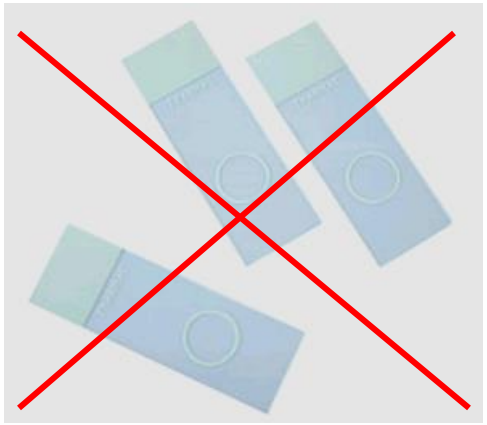
- Ex: cytology from lymph nodes, lung, thyroid, other organs…

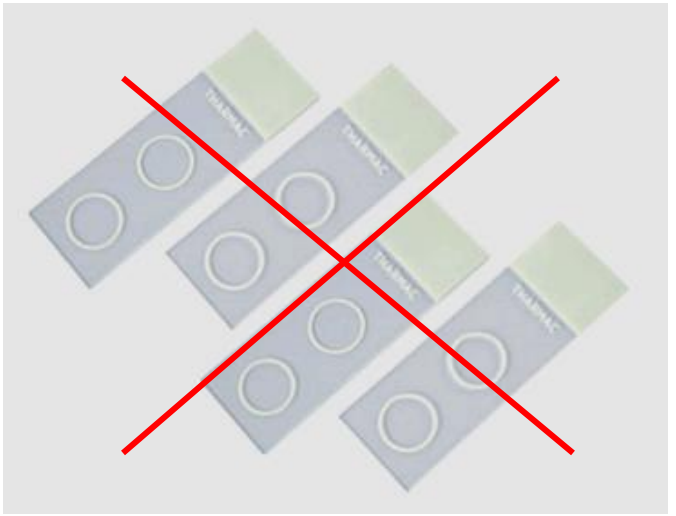
Liquid samples

Cytocentrifugation :

- Serous fluid (cell blocks may also be performed)
- Cerebro-spinal fluid (CSF)
- Broncho-alveolar lavage fluid (BALF)
- Cysts…

Funnel, clamp, filter paper and labelled glass slides ready for cytocentrifugation





Circled slides not suitable for ICC (automate)

Difficulty : adjust cell concentration on the slides

Too much cells → risk of cell detachment
Too few cells → difficult to analyse

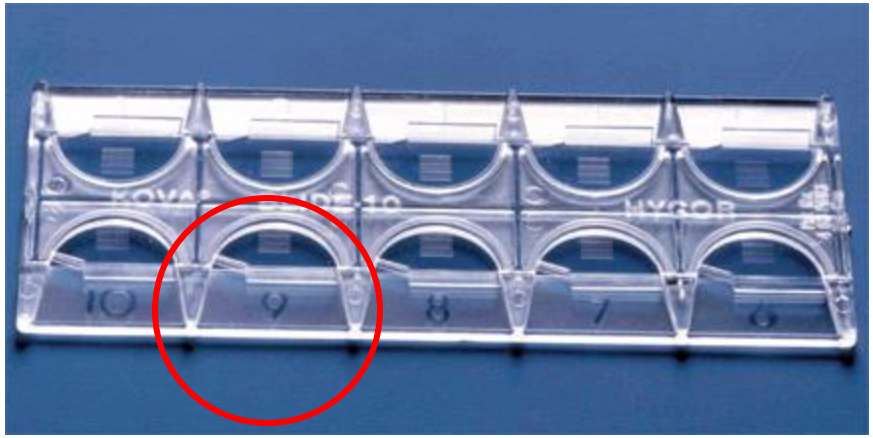
Solution : cell counts

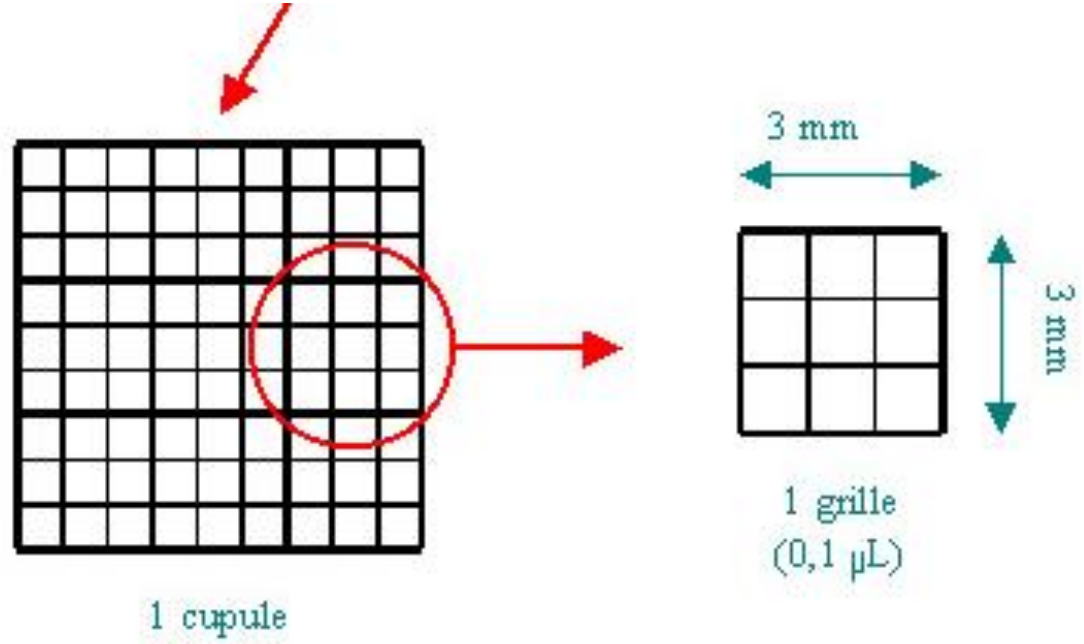
Nageotte slide : liquids with few cells (CSF)

Thomas or Malassez slide : liquids with lots of cells (BALF, serous fluids, other….)

Kovaslide : urine

Kovaslide





Liquid based cytology

Pretreated slides to increase cell adhesion

Cell blocks

If too few liquid remaining :

cytocentrifugation (500µl/slide)

many slides can be performed

Cell blocks

- Many ways to perform cell blocks
  - Thrombin clot +++
  - Agar
  - Histogel*
  - Other
- Fixation: the same as tissue blocks
- Difficulties : too few cells

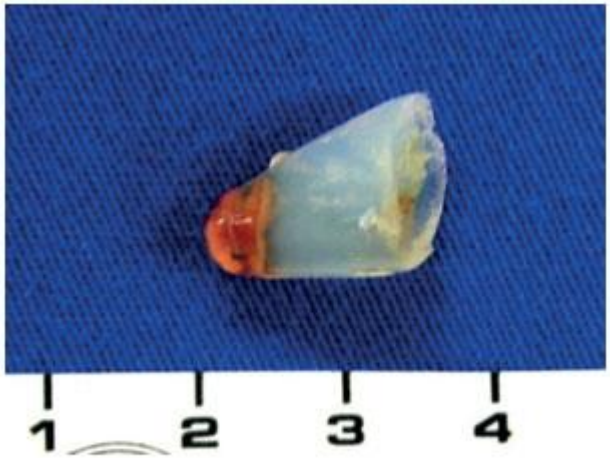


Figure 6 – Paraffin block containing lymph node aspirate. Courtesy of Dr. Cristina Mitteldorf.

Application of Immunohistochemistry to Cytology

Larry J. Fowler, MD; Whitney A. Lachar, MD (*Arch Pathol Lab Med.* 2008;132:373–383)

Table 1. Pros and Cons of Cytology Preparation Methods

Pros	Cons
Direct Smear	
May do when no extra material	Background artifact severe
No wet material needed	Panels unlikely
Can use what available slides were initially obtained (no expense to extra preparations)	Different antibody levels needed
	Prior staining or ethanol may affect results
Cytospins	
Useful with limited material	Background artifact
Panels possible	Different antibody levels needed
	Extra "wet" material needed
Monolayer Preparations	
Possibly decreased background	Different antibody levels needed
Extra material frequently available and easily stored	Extra "wet" material may still be needed
	Ethanol in fixative may interfere with some antigen
Cell Block	
Immunohistochemistry laboratory can handle like routine material with proper controls	Limited cellular specimens cannot be used
Material easily stored	Methodology of cell block preparation must be tested

When no other material

Panels possible

Decreased background

Easy to store IHC techniques

Background artifact

Background artifact +/-

Ethanol in fixative may interfere

Limited cellular specimens

Table 8. Commonly Used Antibodies At Our Cytology Laboratory*

Table 8. Commonly Used Antibodies At Our Cytology Laboratory*

Antibody	IVD/ASR	Vendor	Dilution	Cytology Diagnosis Being Considered
α_1-Antichymotrypsin, polyclonal	IVD	Ventana	PRE	Pancreatic pseudopapillary tumor (SPPT)
Actin muscle, smooth, monoclonal	IVD	Dako	1:200	Skeletal and smooth muscle
α-Fetoprotein (AFP), polyclonal	IVD	Dako	1:4000	Hepatic, yolk sac tumors
ALK-1, monoclonal	IVD	Ventana	PRE	Anaplastic lymphoma
Ber-EP4, monoclonal	IVD	Dako	1:100	Serous tumor
Bcl-2, monoclonal	IVD	Dako	1:50	Lymphoma
CA 19-9, monoclonal	IVD	Ventana	PRE	Pancreas
CA 125, monoclonal	IVD	Ventana	PRE	Ovarian
Calretinin, monoclonal	IVD	Zymed	1:200	Mesothelial
Calcitonin, polyclonal	IVD	Dako	1:200	C cell
c-Kit, CD117, monoclonal	IVD	Dako	1:100	GIST
c-Erb-B2, HER2, monoclonal	IVD	Ventana	PRE	Breast carcinoma
Carcinoembryonic antigen (CEA), monoclonal	IVD	Dako	1:100	Mucinous tumor
CD1a, monoclonal	IVD	Ventana	PRE	Langerhans cell
CD3, monoclonal	RUO	Novo	1:25	T cells
CD5, monoclonal	RUO	Novo	1:50	T cells
CD10, monoclonal	RUO	Novo	1:50	Renal, SPPT
CD15, monoclonal	IVD	Ventana	PRE	Hodgkin
CD20, monoclonal	IVD	Dako	1:2000	B cell
CD30, monoclonal	IVD	Neomark	1:50	Hodgkin and anaplastic
CD31, monoclonal	IVD	Dako	1:20	Vascular
CD34, monoclonal	IVD	Dako	1:40	Solitary tumor
CD45RB, monoclonal	IVD	Dako	1:500	Lymphoid
CD56, monoclonal	IVD	Novo	1:100	Neural
CD68, monoclonal	IVD	Dako	1:2000	Macrophage
CD99, monoclonal	IVD	Neomark	1:50	Ewing tumor
Chromogranin, monoclonal	IVD	Dako	PRE	Neural
Cytomegalovirus (CMV), monoclonal	IVD	Signet	1:10	Viral
Cytokeratin 7, monoclonal	IVD	Dako	1:50	Adenocarcinoma unknown
Cytokeratin 20, monoclonal	IVD	Dako	1:100	Adenocarcinoma unknown
Desmin, monoclonal	IVD	Dako	1:40	Rhabdomyosarcoma
E-cadherin, monoclonal	IVD	Ventana	PRE	Lobular breast carcinoma if negative
Epithelial membrane antigen (EMA), monoclonal	IVD	Dako	1:50	Renal
Estrogen receptor	IVD	Ventana	PRE	Breast and gynecologic
Gastrin, polyclonal	IVD	Ventana	PRE	Neuroendocrine for increased risk
Glucagon, polyclonal	IVD	Dako	PRE	Neuroendocrine
Human chorionic gonadotropin (HCG), polyclonal	IVD	ABCAM	1:50	Choriocarcinoma
Herpes simplex virus type I, polyclonal	IVD	Ventana	PRE	Viral
Herpes simplex virus type II, polyclonal	IVD	Ventana	PRE	Viral
Hepar	IVD	Dako	1:100	Hepatic carcinoma
HMB-45 (melanoma), monoclonal	IVD	Ventana	PRE	Melanoma and angiomyolipoma
Keratin, AE1	IVD	Zymed	1:400	FPS
Keratin, AE3	IVD	Zymed	1:200	FPS
κ Light chains, polyclonal	IVD	Dako	1:20 000	B cell
Ki-67, monoclonal	IVD	Ventana	PRE	Prognosis
λ Light chains, polyclonal	IVD	Dako	1:30 000	B cell
MART1	IVD	Signet	PRE	Melanoma
Myogenin, monoclonal	IVD	Dako	1:500	Rhabdoid
p16	IVD	BCM	1:100	Dysplasia
Pan keratin AE1/AE3	IVD	Ventana	PRE	Epithelial origin
Placental alkaline phosphatase, polyclonal	IVD	Ventana	PRE	Germ cell tumor
Progesterone receptor, monoclonal	IVD	Ventana	PRE	Breast, SPPT
Prostate-specific antigen (PSA), monoclonal	IVD	Dako	1:200	Prostate
S100, monoclonal	IVD	Dako	1:800	Melanoma and neural
Somatostatin, polyclonal	IVD	Ventana	PRE	Neuroendocrine for higher risk
Spirochete	RUO	BCM	1:100	At risk inflammation
Synaptophysin, monoclonal	IVD	Dako	1:10	Neural
Thyroid transcription factor 1 (TTF-1)	IVD	Dako	1:80	Thyroid, lung, and small cell
Thyroglobulin, monoclonal	IVD	Ventana	PRE	Thyroid follicular cell
Vimentin, monoclonal	IVD	Ventana	PRE	Antigenic reactive, stromal

* IVD indicates in vitro diagnostic use; ASR, allied specific reagent; PRE, prediluted; SPPT, solid pseudopapillary pancreatic tumor; GIST, gastrointestinal stromal tumor; RUO, research use only; and FPS, fallopian tube, prostate (skin control). Vendors: Ventana, Tucson, Ariz; Dako, Carpinteria, Calif; Zymed, Carlsbad, Calif; Novo, Novocastra, Burlingame, Calif; Neomark, Fremont, Calif; Signet, Emeryville, Calif; ABCAM, Cambridge, Mass; and BCM, Baylor College of Medicine, Houston, Tex.

Prerequisite to immunocytochemistry

- Slides
- Fixation
- Antigen retrieval

Slides for ICC

Adhesive slides

- Increase cell adhesion
- Especially if heat-induced antigen retrieval
  - Many suppliers for adhesive slides

- Problems with smears:

  non treated slides

  good adhesion if hemorrhagic specimen

Slide fixation

Smears or cytospins : air dried

Fixation:

- Cold acetone (4°C) 10 min
- Ethanol (not suitable for some antigens, ex: ER, pS100)
- Methanol
- Formalin…

Slides may be kept at room temperature for 7 days or -20°C several months

Before use, bring them at room temperature under cover (avoids mist on the slides)

Fixatives

- Acetone, Ethanol, Methanol = coagulating fixatives (precipitate proteins)
  - Remove lipids (permeabilize cell membranes)
  - Dehydrate the cells

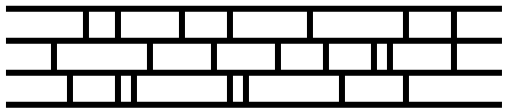
- Formaldehyde (HCHO) : non-coagulant, additive fixative, cross-linking reagent
  - Forms intermolecular bridges, normally through free amino groups, thus creating a network of linked antigens
  - No permeablization

Fixatives

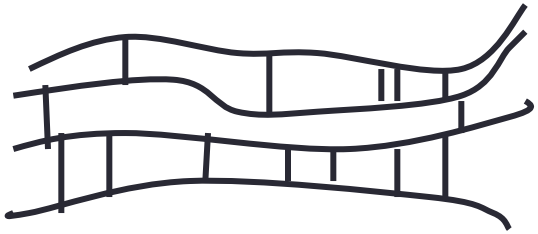
- **Acetone, Ethanol, Methanol :**

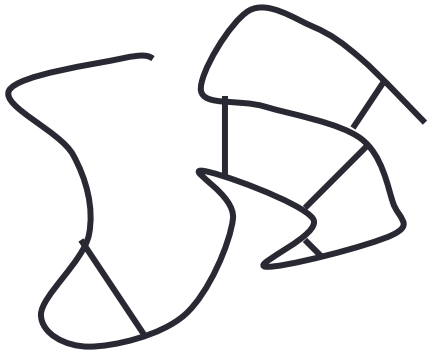
Before fixation

After fixation

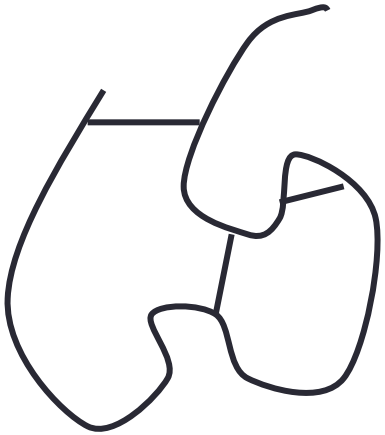


Fibrous protein (shrinkage)





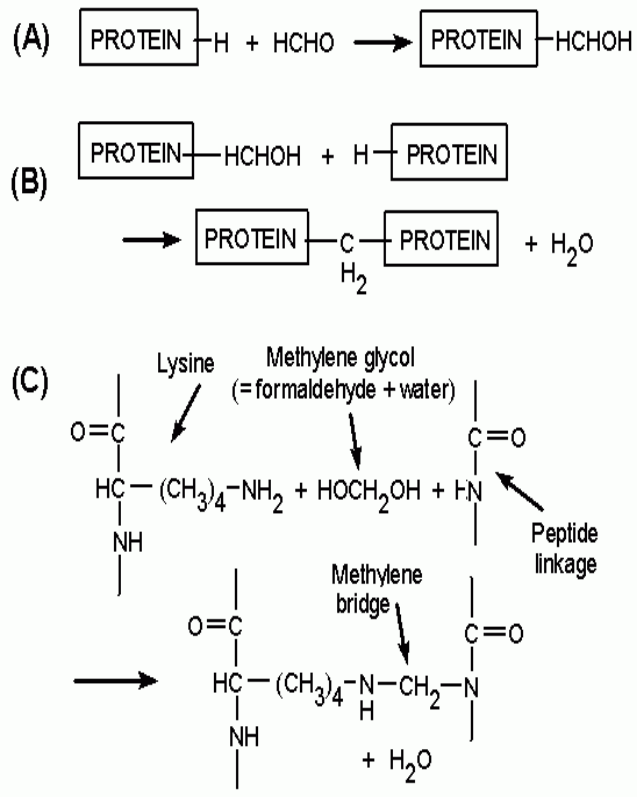
Globulous protein (unfolding)



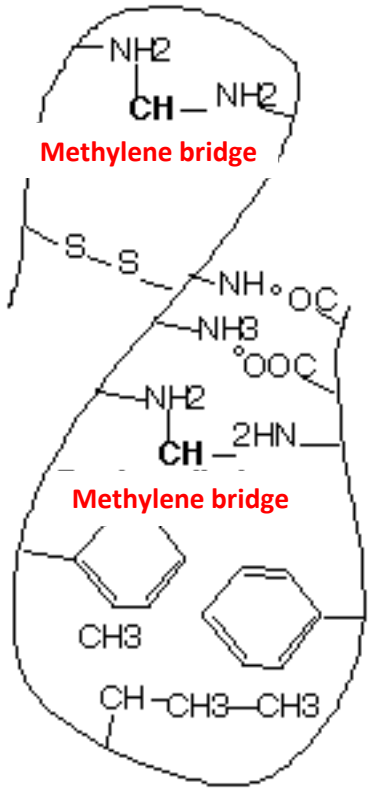
Protein denaturation

Fixatives

- **Formaldehyde (HCHO) : non-coagulant, additive fixative**



After fixation



Antigen retrieval and cytology

- **Not necessary for cells fixed with acetone or alcohol, for membranous or cytoplasmic antigens**

but

- **May reduce background staining and increase some stains**
- **Mandatory with formalin fixation (remove methylene bridges)**
- **Mandatory for nuclear antigens**

Heat-induced antigen retrieval

- **High temperature**
  - **95-100°C 20 min or 90°C 30 min or 80°C 50min**
  - **Water bath +++**
  - **Microwave**

- **Alcaline solution (Tris Buffer pH:8-9) +++ or acidic solution (Citrate buffer pH:6)**

- **Calcium chelating agent (antigens may be masked due to calcium complexes with formalin)**
  - **Ex Tris Buffer pH9 with EDTA**

Antigen retrieval

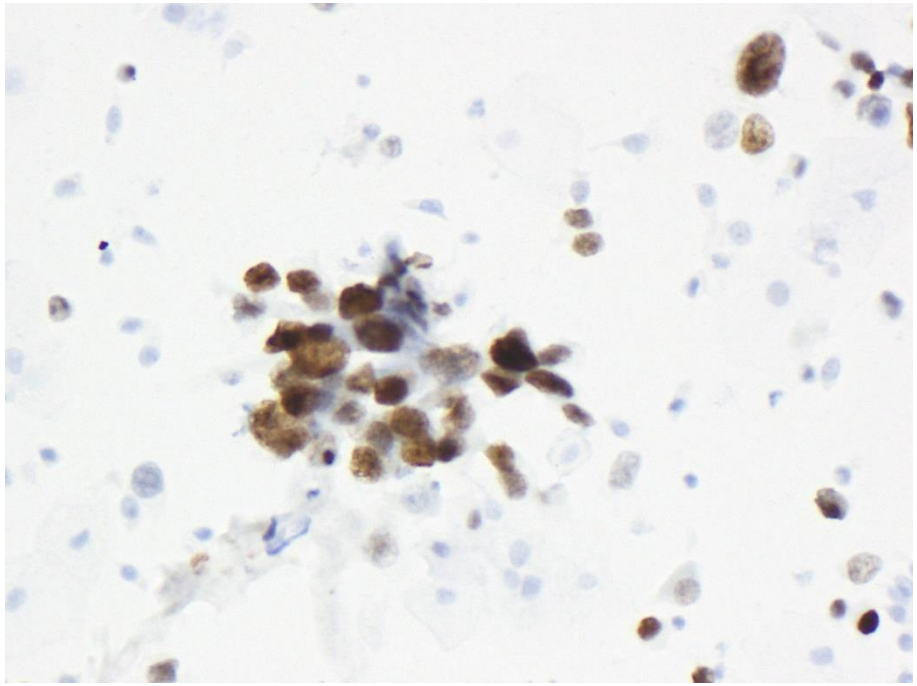
- **Citrate pH6**
  - HBME1
  - BCL2
  - Mib1
  - INI1R
  - CMV
  - MDM2
  - L1
  - TTF1
- **Tris EDTA pH9**
  - ER
  - p63
  - p53
  - p40
  - SV40
  - WT1
  - NF
  - GATA3
  - PAX8
  - PR

Shorter times for cytological samples ++++

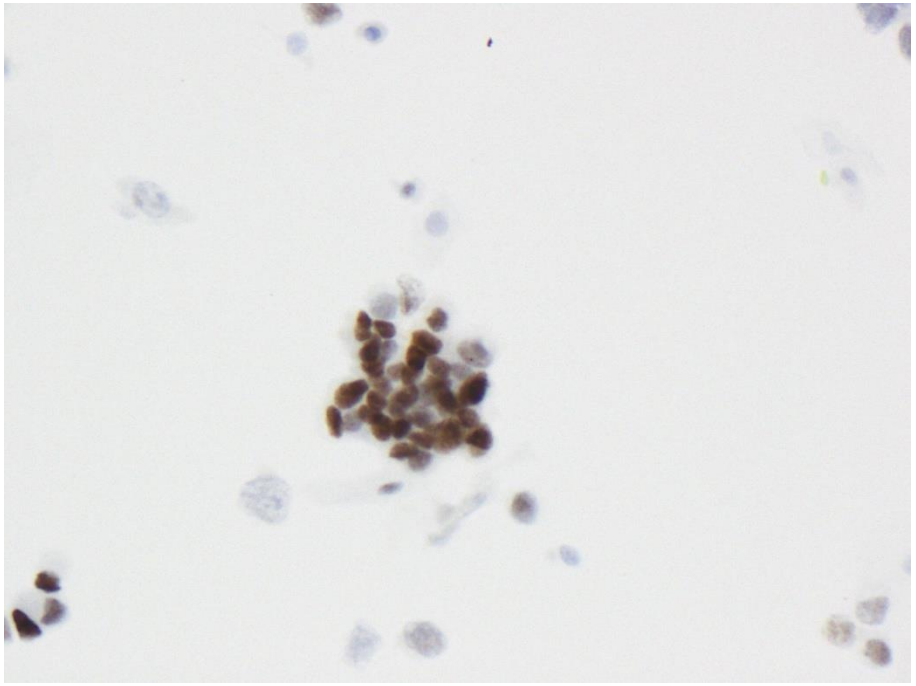
p53 (clone DO7)

- Dilution : 1/30

Citrate buffer alone : no staining



Citrate buffer then EDTA



Flex buffer Tris-EDTA-pH9

Urine : High grade tumor (alcohol fixation)

Revelation systems

- **With Biotin : false positives with endogenous biotin**
  - Liver, kidney, colon, thyroid, breast
  - Removed by albumin
- **Alkaline phosphatase: false positives with endogenous AP**
  - Placental, intestinal, germinal
  - Inhibited by Levamisole
- **Peroxydase : false positives with endogenous peroxydase**
  - Eosinophils, neutrophils, monocytes, erythrocytes, muscle cells
  - Inhibited by hydrogen peroxyde

Some examples

- **Litterature**

Smears

Automated immunostaining of cell smears: an alternative to flow cytometry

J Clin Pathol 2008;**61**:740–743.

L C Happerfield,[1] R Saward,[2] L Grimwade,[2] D Bloxham,[2] W N Erber[2]

Bond-maX (Leica Microsystems)
Peripheral blood and bone marrow

Fixatives

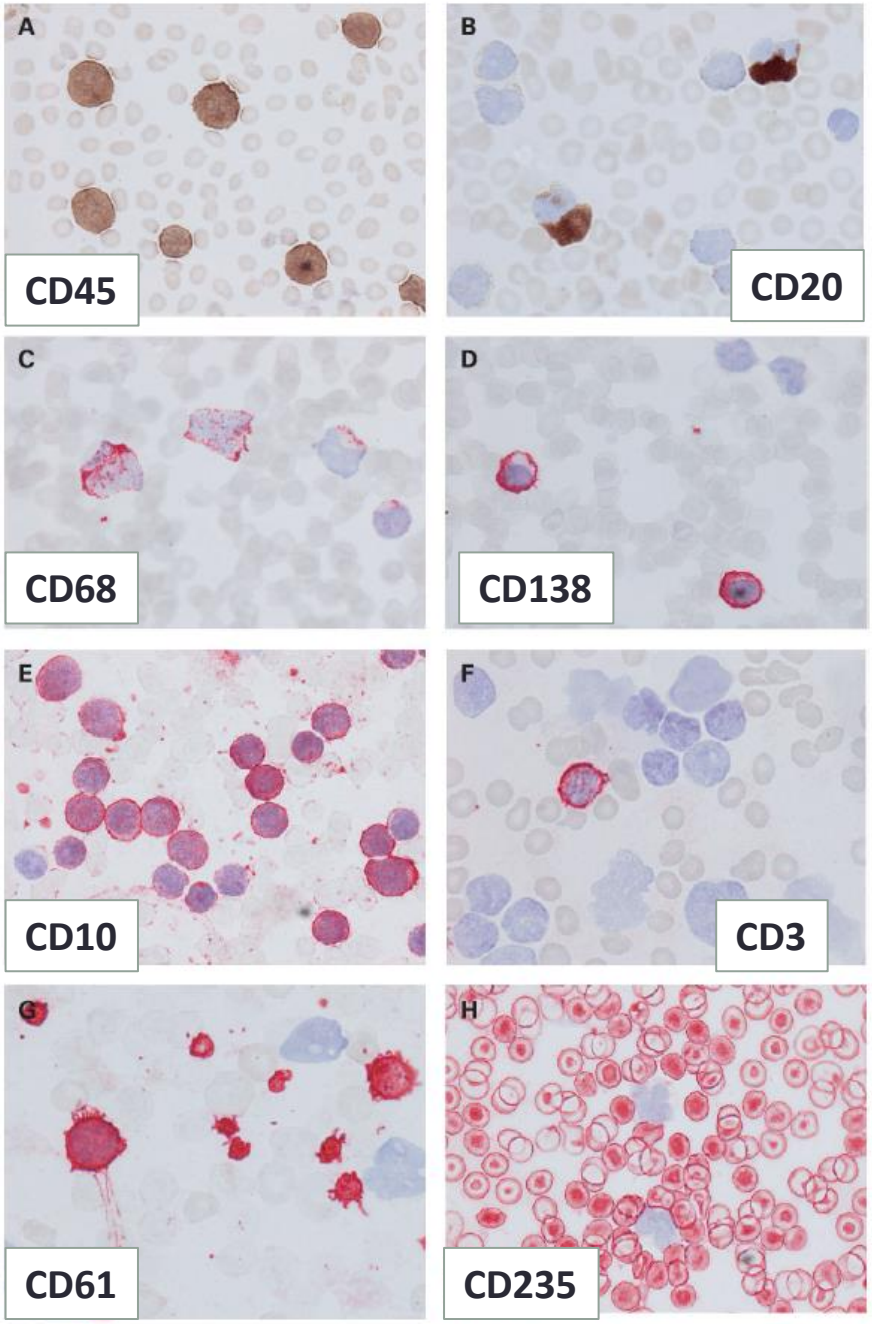
Acetone 100% : 10 min
Acetone/Methanol (vol/vol) : 90 sec

} **Good stain but poor preservation of cells**

Acetone/methanol/formalin (19v/19v/2v) : 90 sec
Buffered formol acetone : 30 sec

} **Best combination**

Figure 1 Examples of immunostaining of blood and bone marrow smears using the Bond-maX automated immunostainer. (A) and (B) Precursor B-lymphoblastic leukaemia stained with CD45 and CD20, respectively, with immunoperoxidase and diaminobenzidine substrate. (C)–(H) Examples of immuno-AP staining and Fast Red detection. (C) CD68 positive myeloblasts in a case of acute myeloid leukaemia. (B) CD138 positive plasma cells in the bone marrow of multiple myeloma. (E) and (F) CD10 and CD3, respectively, in the bone marrow of precursor B-lymphoblastic leukaemia. The blast cells are CD10 positive and CD3 negative. One normal T lymphocyte is CD3 positive. (G) CD61-positive platelets and megakaryoblasts in a case of acute megakaryoblastic leukaemia. (H) Positive erythrocytes with CD235.



Background staining of erythrocytes and polymorphonuclears with peroxydase

No background staining with alkaline phosphatase

Correlation ICC-IHC

Comparative study of cytology and immunocytochemistry with trucut biopsy and immunohistochemistry in diagnosis of localized lung lesions: A prospective study

Sarasvatî Roy, Ayandip Nandi,[1] Indranil Das,[2] and Palash K. Mandal[3]

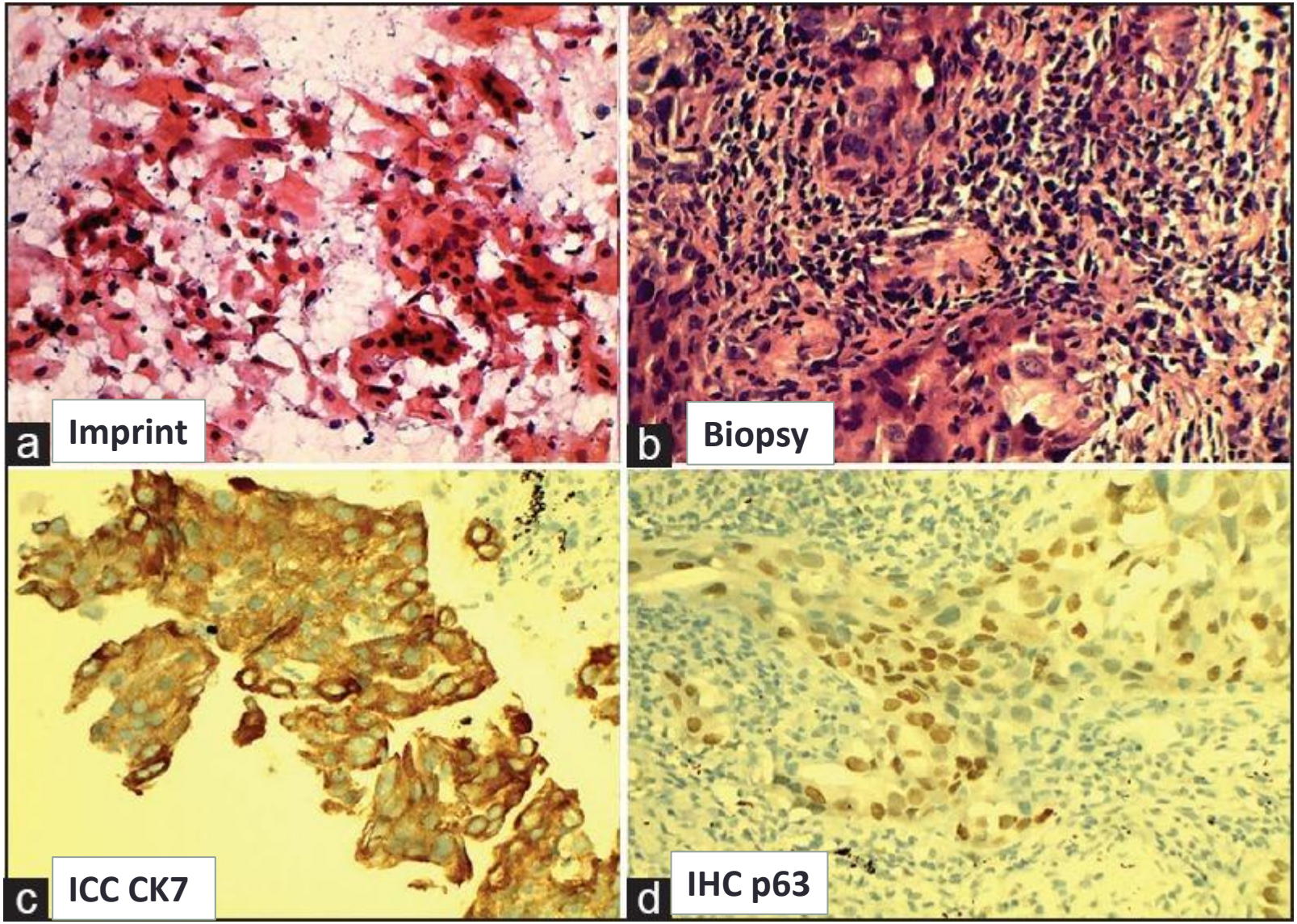
J Cytol. 2015 Apr-Jun; 32(2): 90–95.

Imprints from biopsy, air-dried followed by cold acetone-fixation

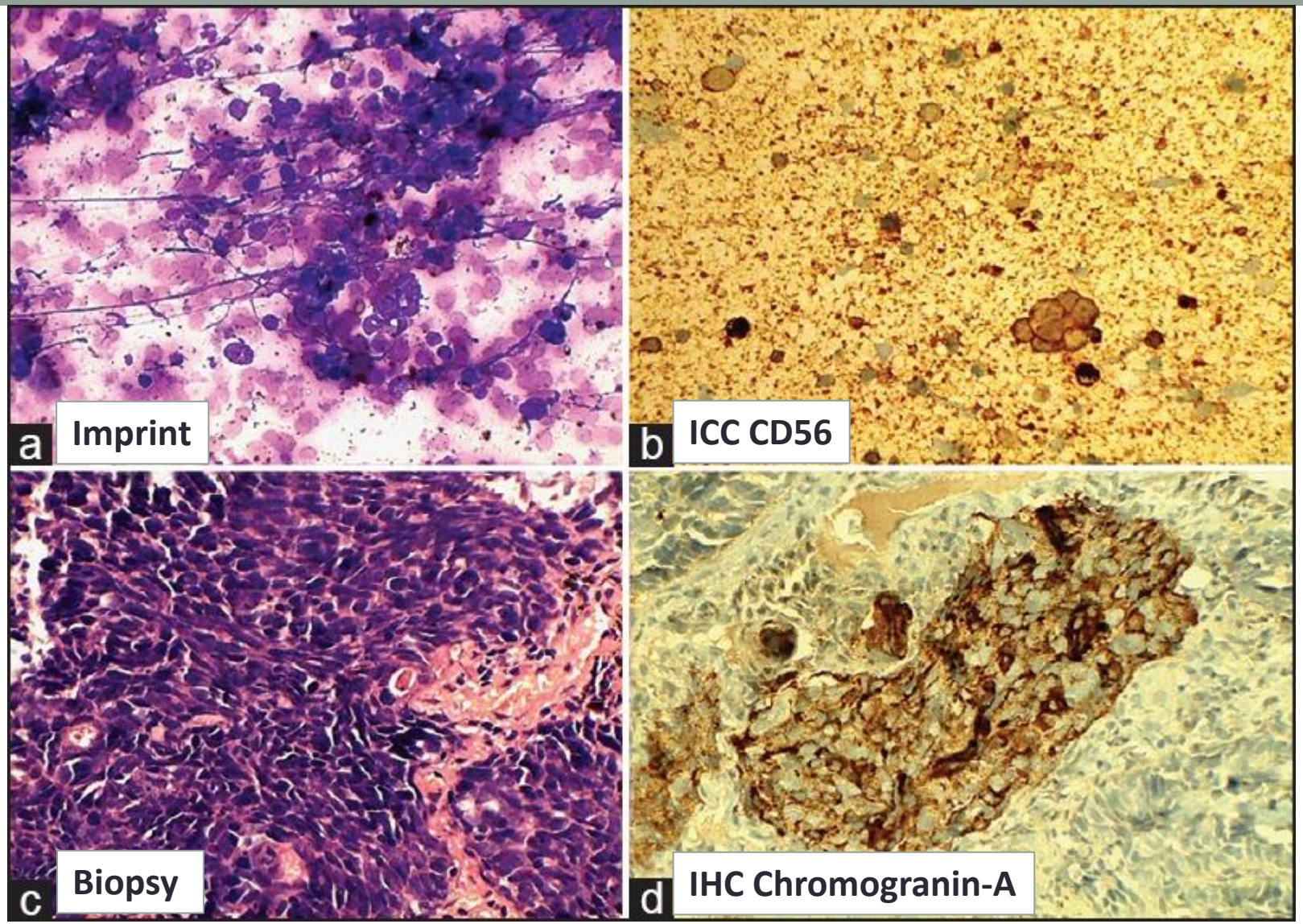
Table 3

Immunocytochemistry — immunohistochemistry correlation (*n* = 34)

Final diagnosis	Cytology	Immunocyto	Histology	Immunohisto	Discordance
AdenoCA (*n*=23)	19	21	23	23	2
Sq cell CA (*n*=4)	3	4	4	4	0
Small cell CA (*n*=4)	3	4	4	4	0
Others (*n*=3)	2	2	3	3	1



(a) Cytology smear demonstrating prominent orangeophilia, diagnosed as squamous cell carcinoma on cytology (Pap, ×400). (b) Trucut biopsy shows squamous areas among cells with glandular differentiation: Adenosquamous carcinoma (H and E, ×400). (c) Immunohistochemistry shows strong CK-7 positivity (IHC, ×400). (d) Patchy p63 positivity in squamoid areas (IHC, ×400)

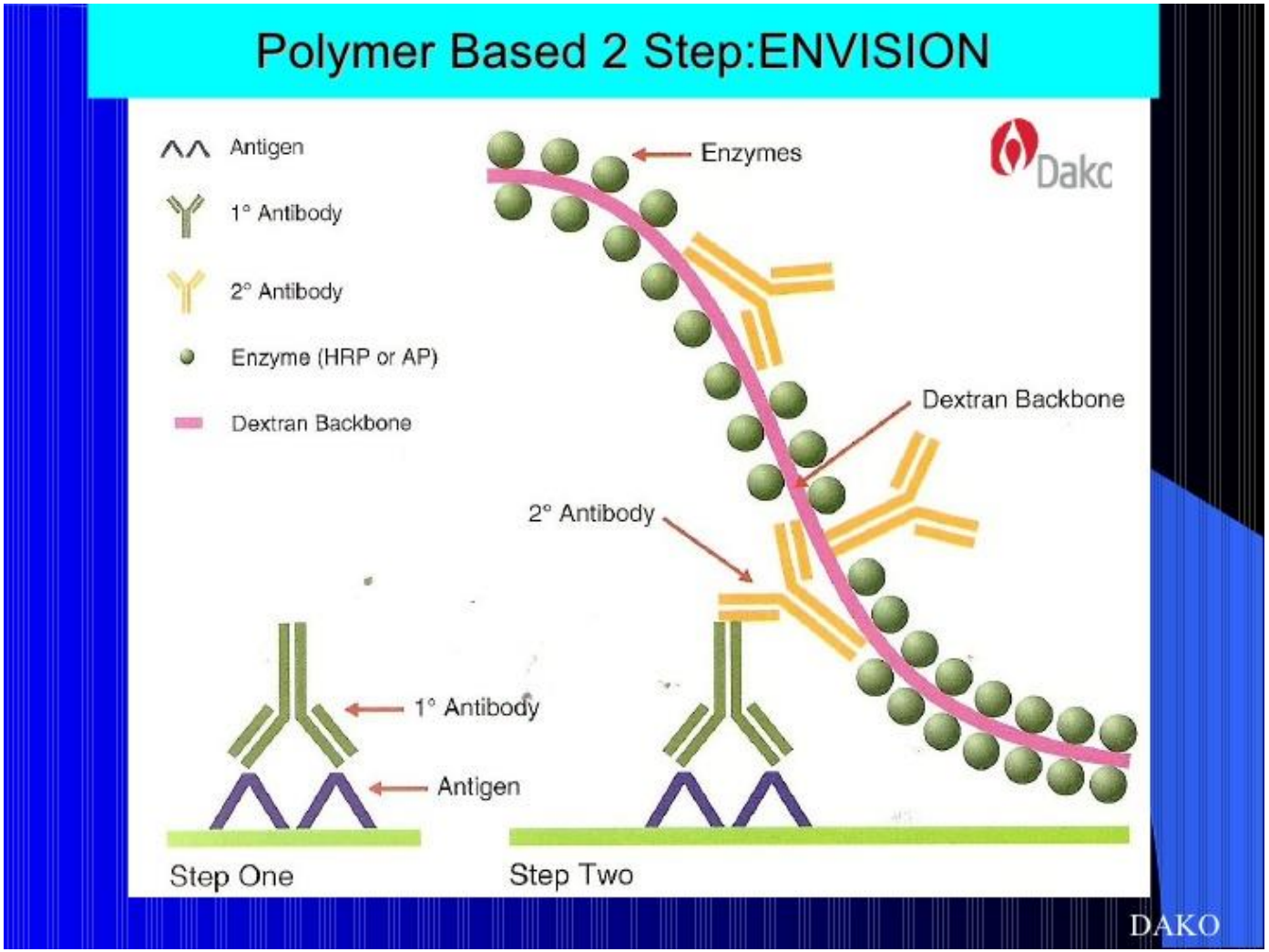


(a) Cytology smear showing clusters of pleomorphic cells with molding and smudging, small cell carcinoma (MGG, ×400). (b) Immunocytochemistry smear showing CD56-positive cells in a necrotic background (IHC, ×400). (c) Trucut biopsy showing solid sheets of cells with smudging (H and E, ×400). (d) Immunohistochemistry showing positivity with chromogranin-A (IHC, ×400)

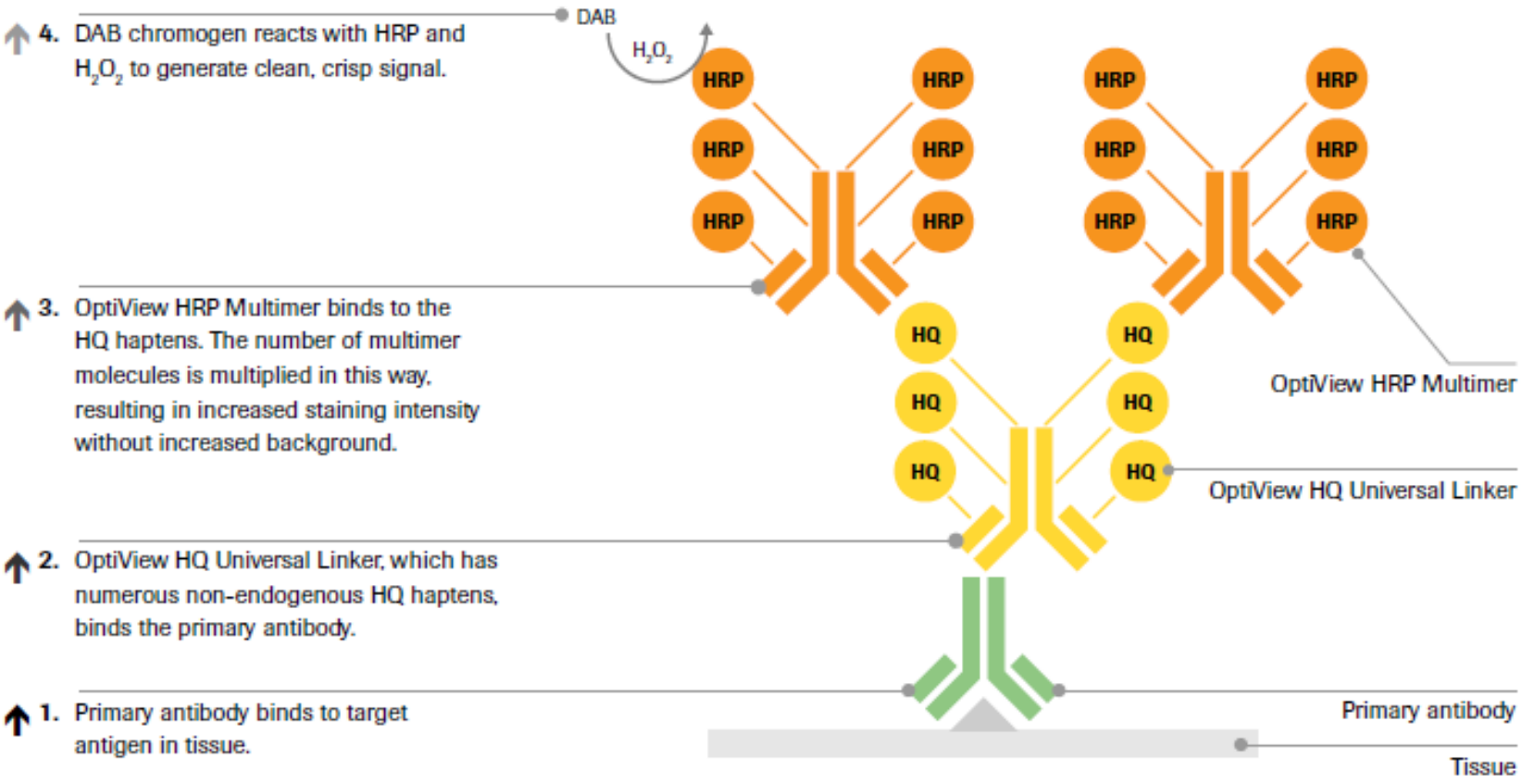
Some examples from our experience

- **Dako Autostainer Link48**
- **Ventana Roche BenchMark ULTRA (leucocyte antigens)**

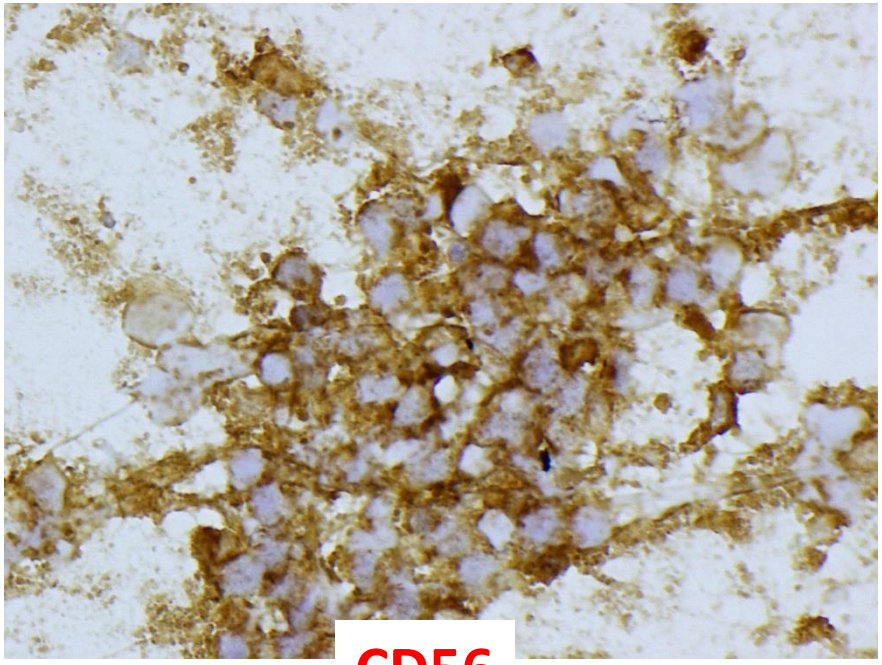
- **Dako**
  - **EnVision FLEX**



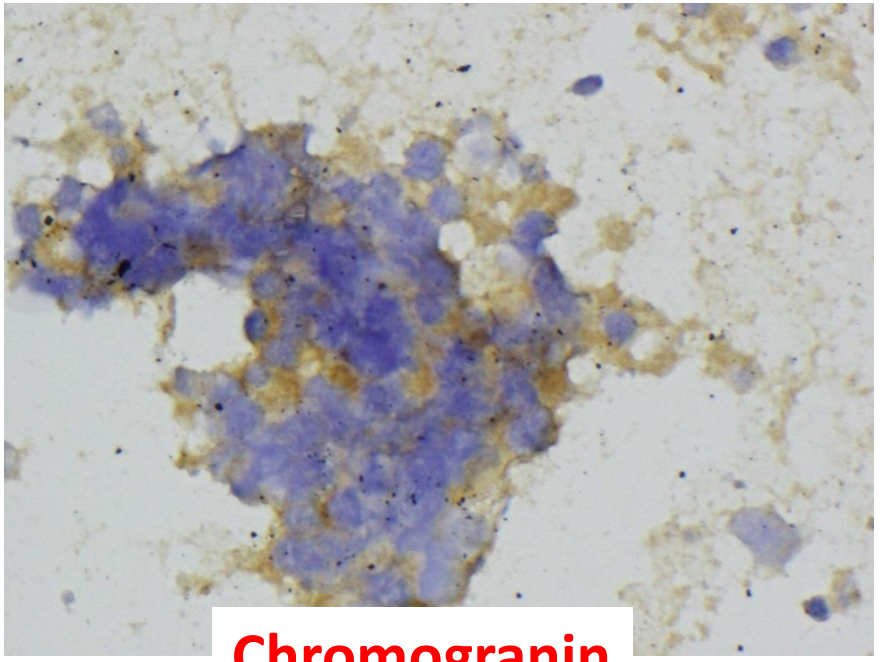
- **Ventana**
  - **OptiView DAB IHC Detection Kit**



Smear

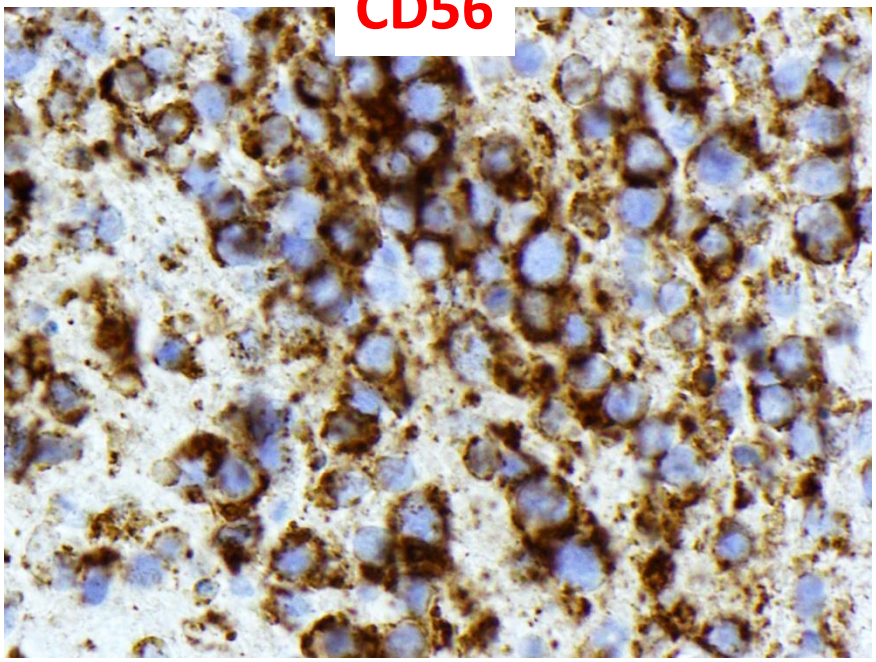


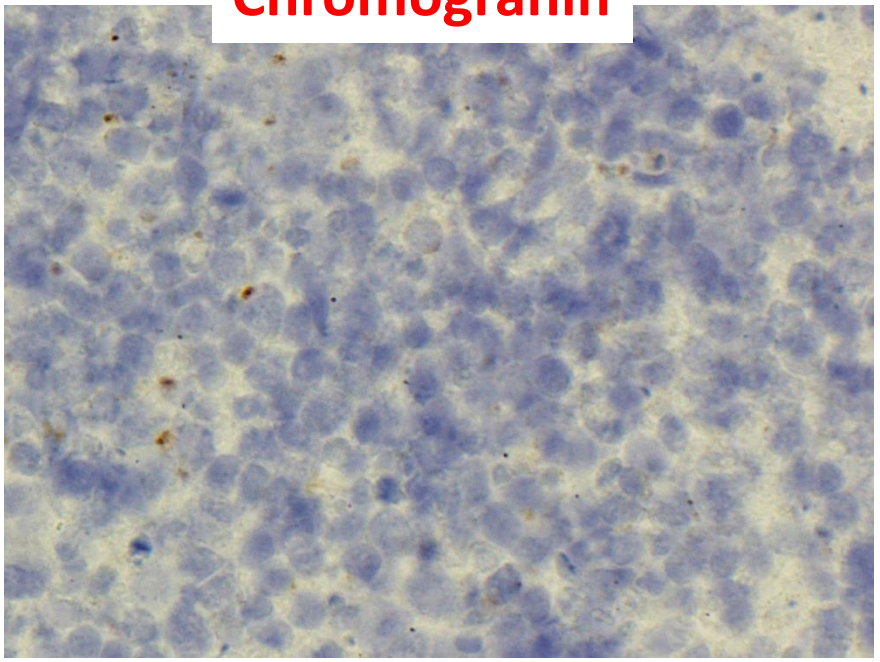
CD56



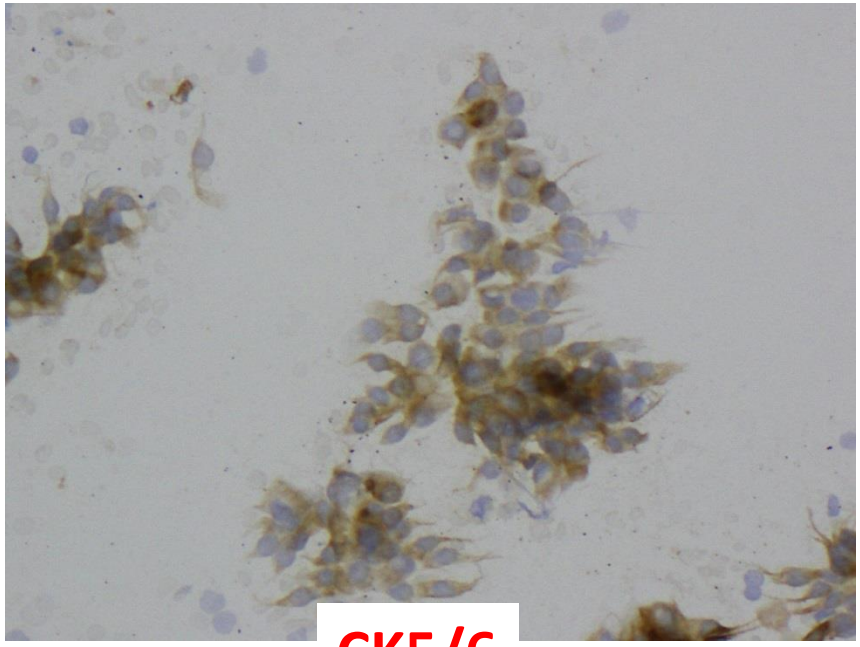
Chromogranin

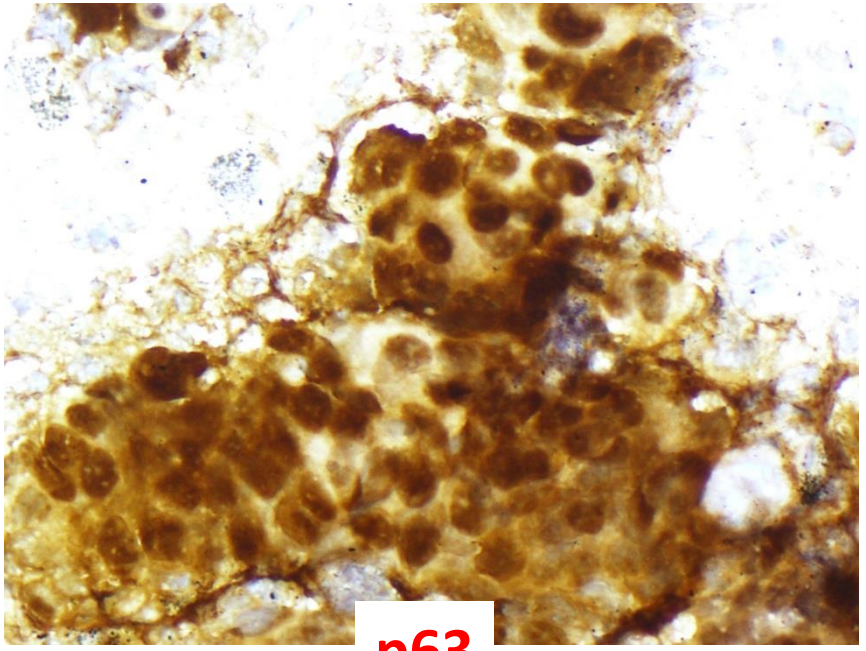
Cellblock





Smear

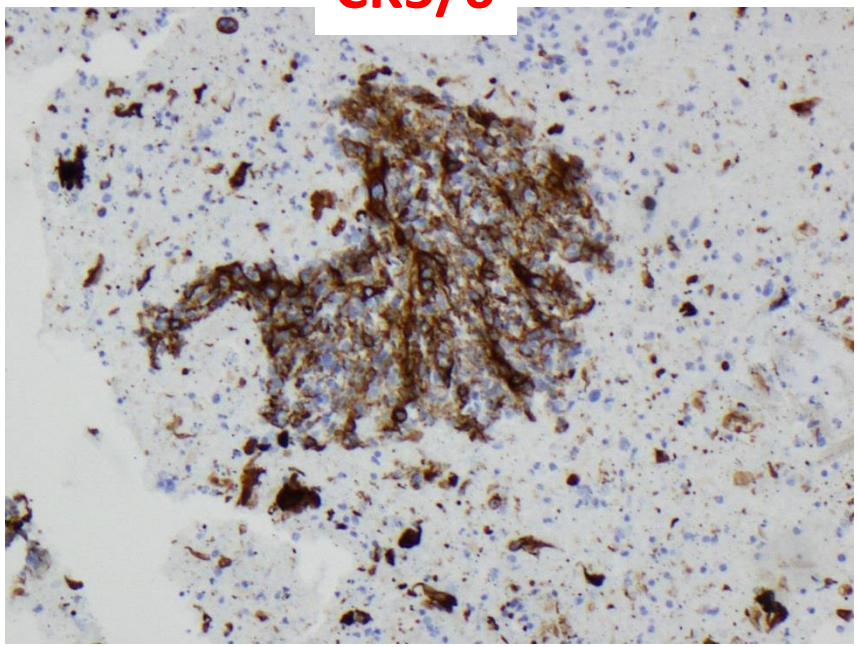


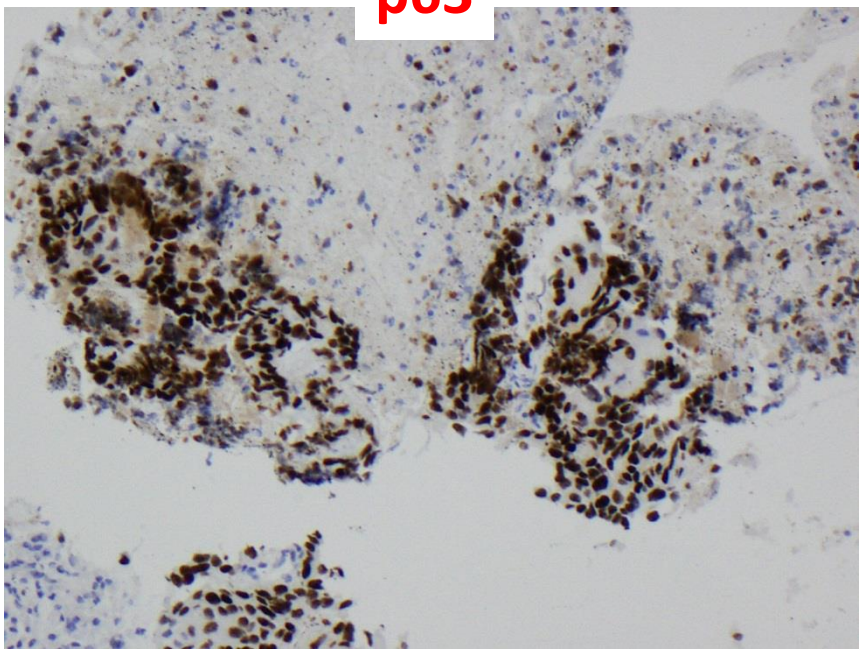


CK5/6

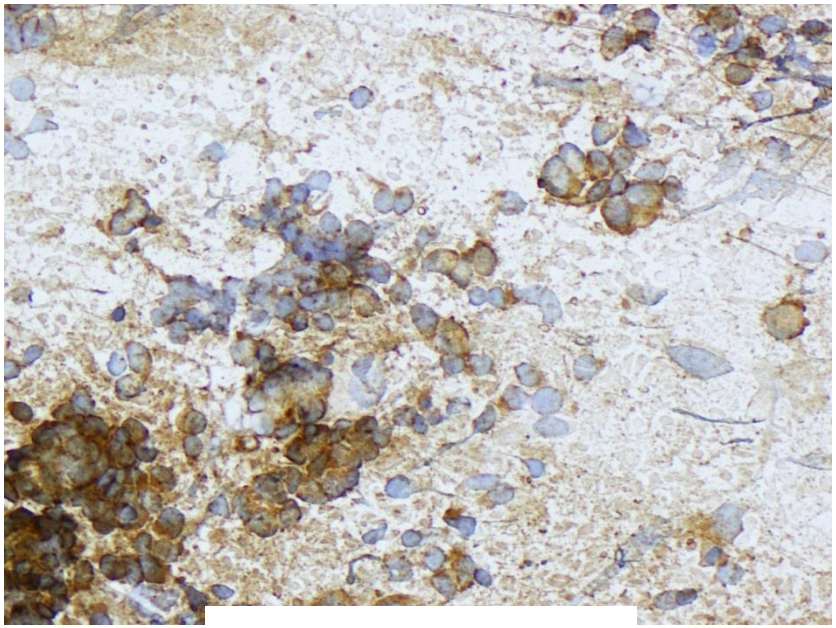
p63

Cellblock

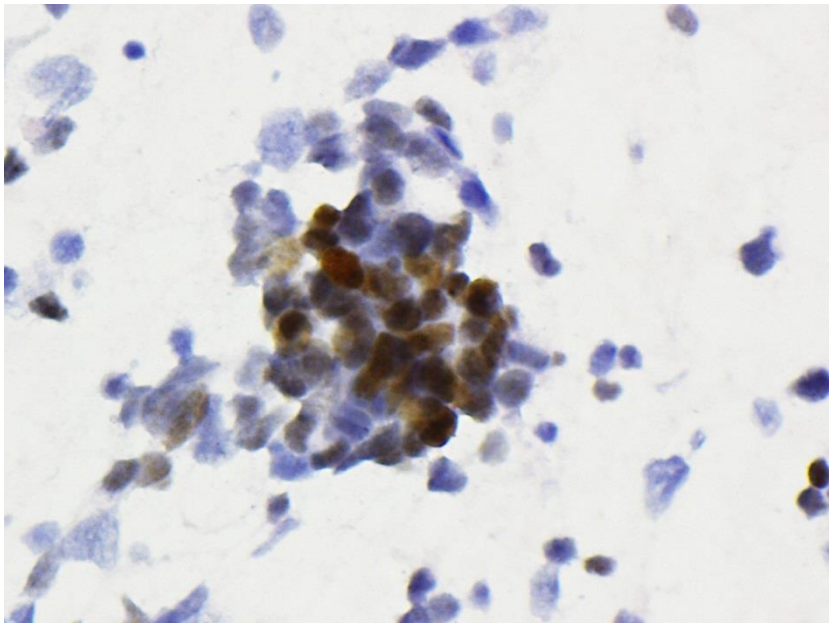




Smear

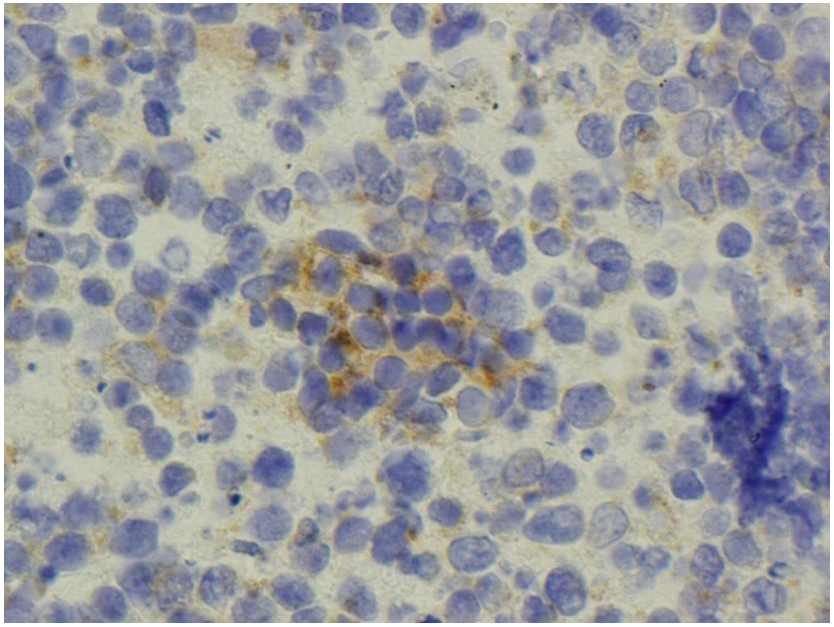


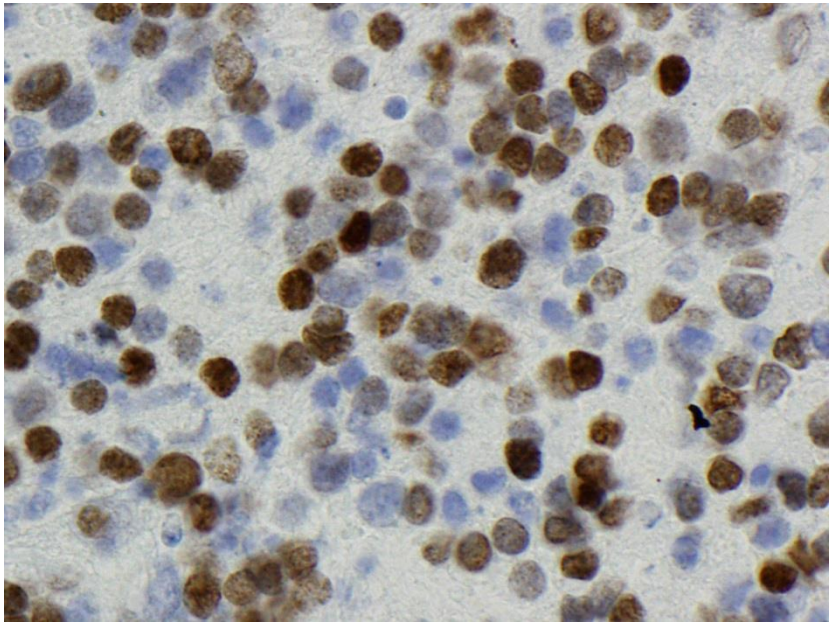
Synaptophysin



TTF1

Cellblock





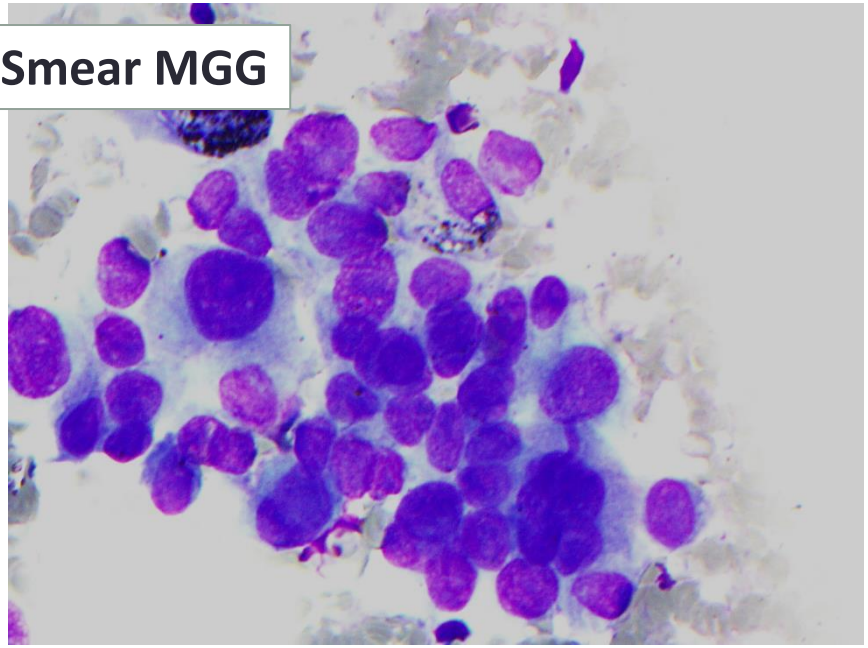
Comparative analysis

- ICC on smears / ICC on cell blocks

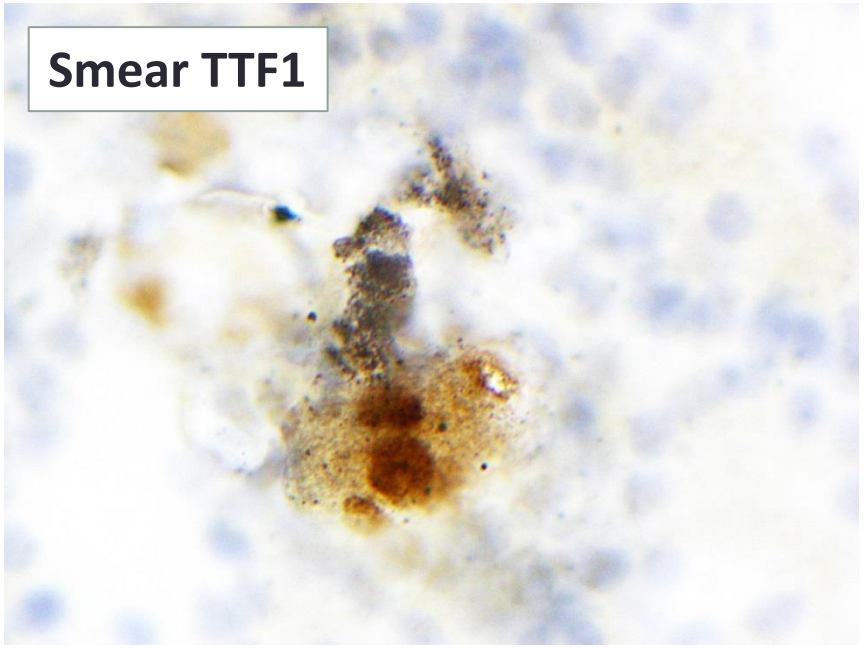
- Metastasis of lung adenocarcinoma in mediastinal lymph node

- Smears
  - Slides MGG stain
  - Slides ICC
- Cytolyt (Hologic) → Preservcyt
  - Slide Pap stain
  - Cellblock

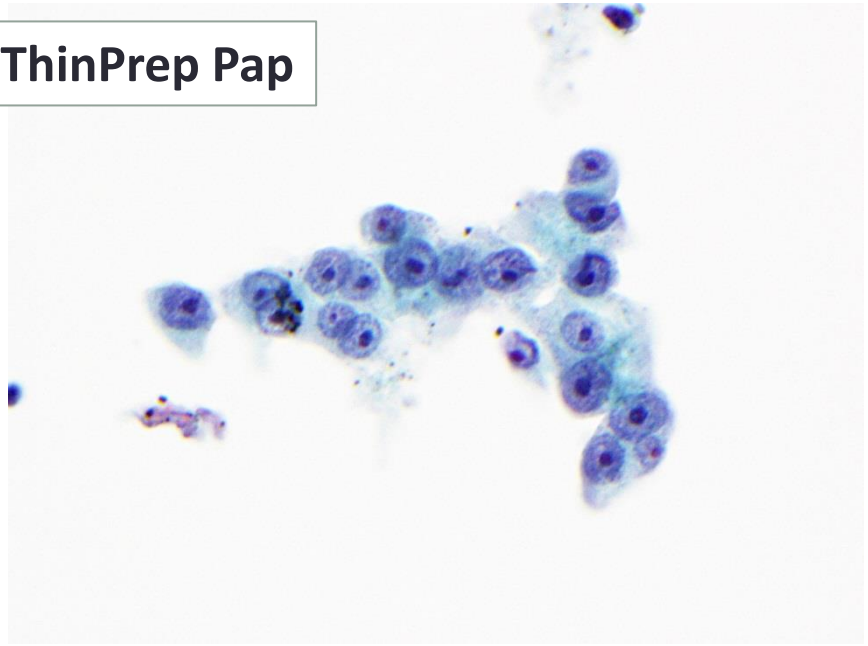
Smear MGG



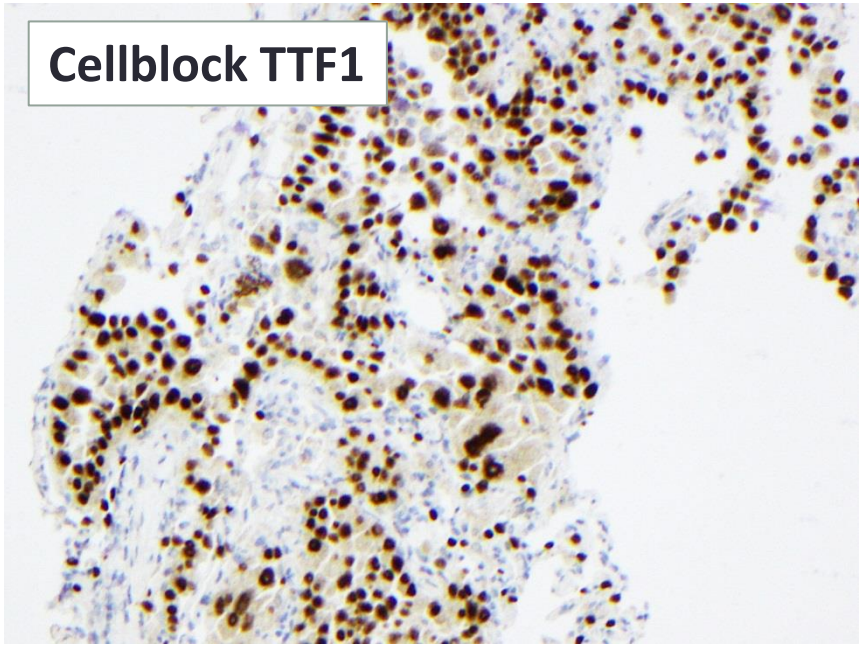
Smear TTF1



ThinPrep Pap



Cellblock TTF1

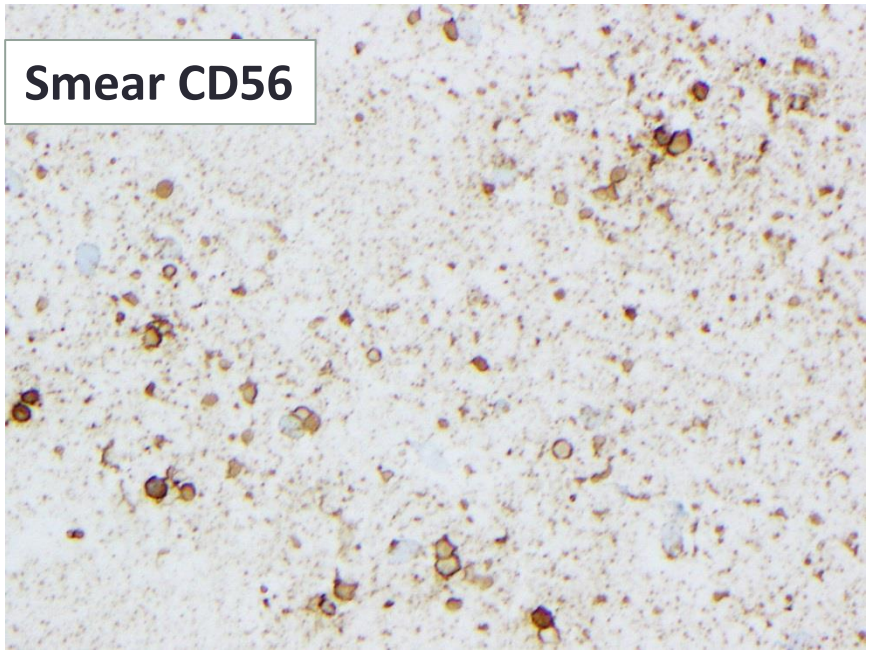


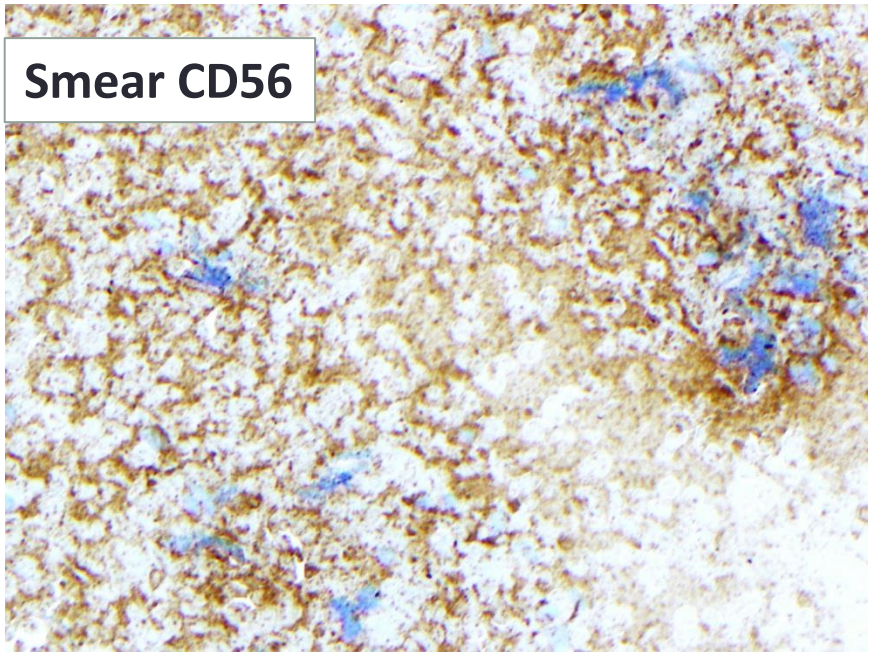
Comparative analysis

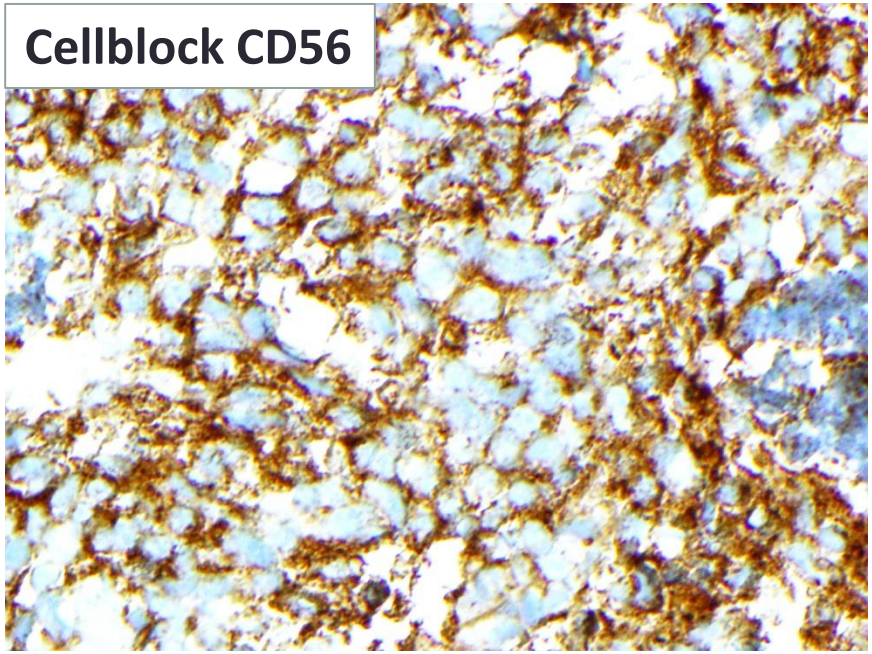
- ICC on smears / ICC on cell blocks

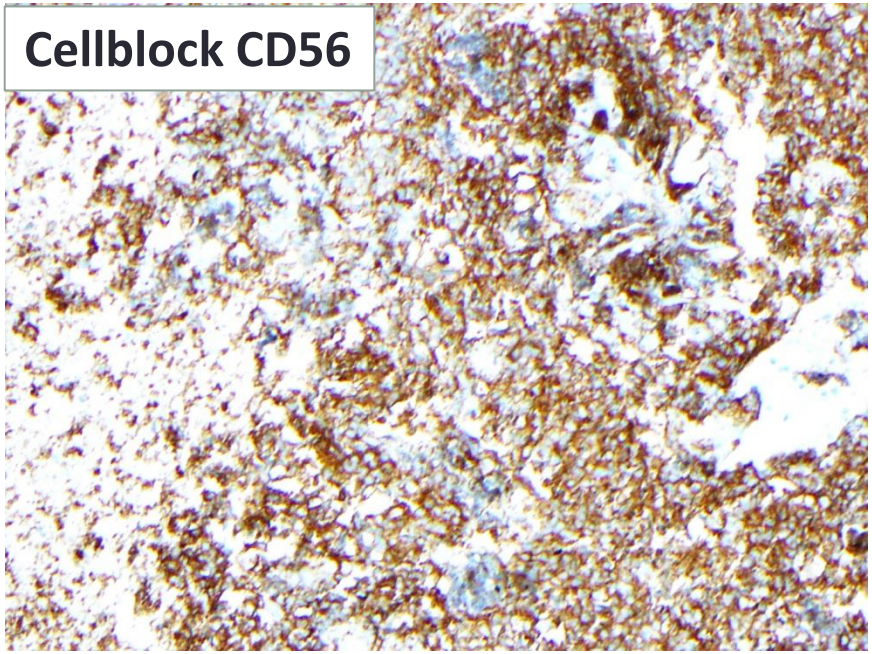
- Metastasis of an atypical carcinoid tumor in a mediastinal lymph node

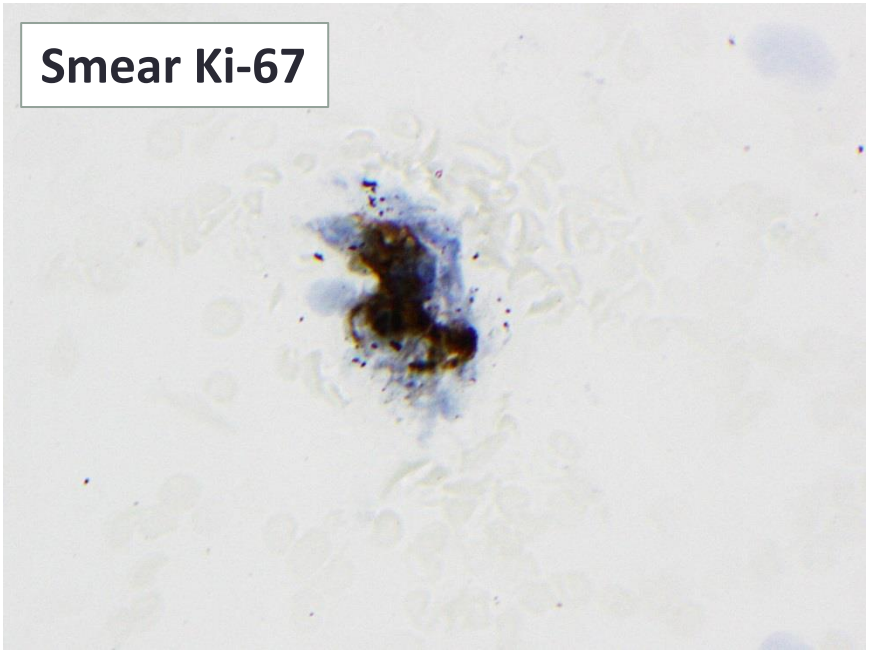
- Smears
  - Slide MGG stain
  - Slides ICC
- Cytolyt (Hologic) → Preservcyt
  - Slide Pap stain
  - Cellblock

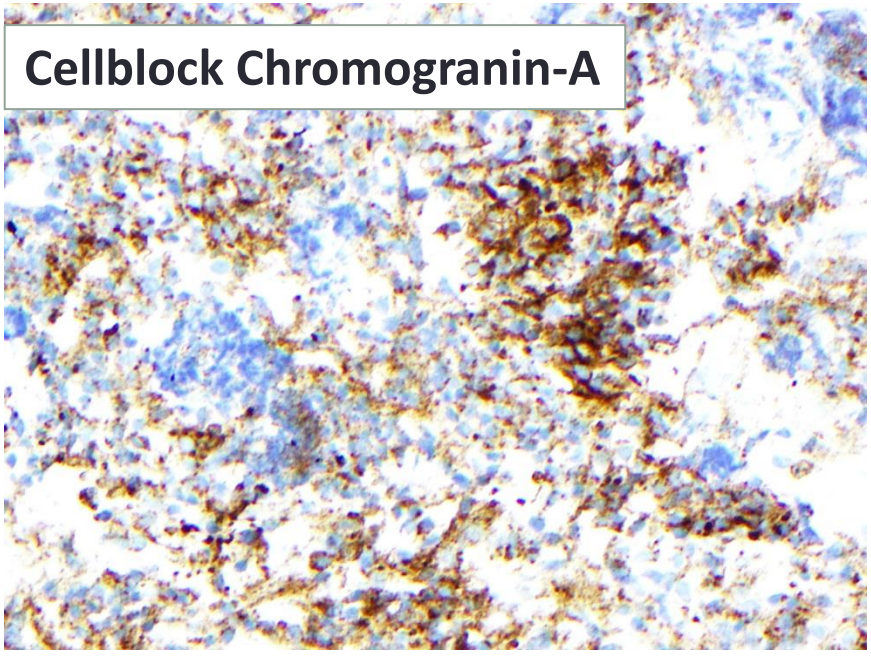

Smear CD56

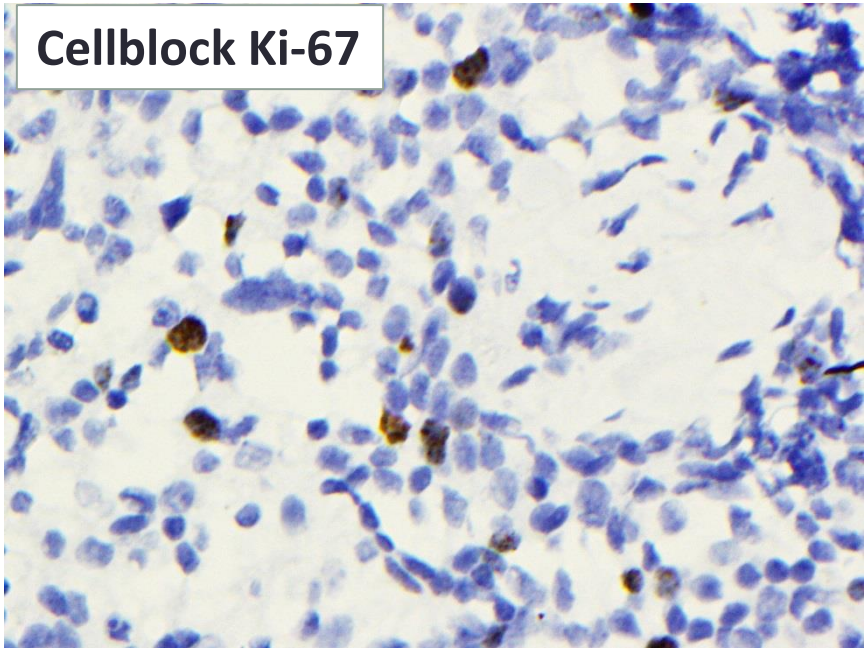

Smear CD56

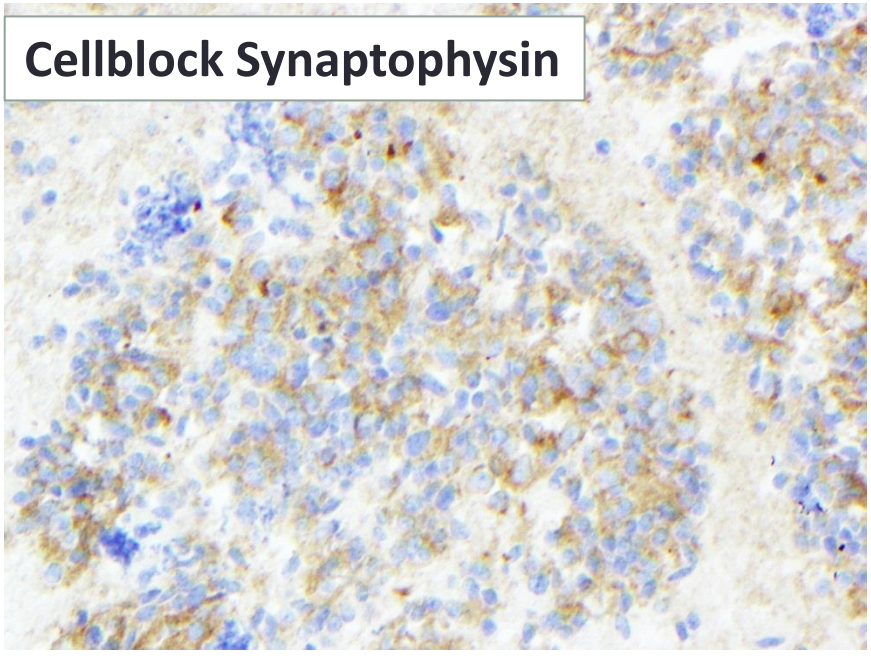

Cellblock CD56


Cellblock CD56


Smear Ki-67


Cellblock Chromogranin-A

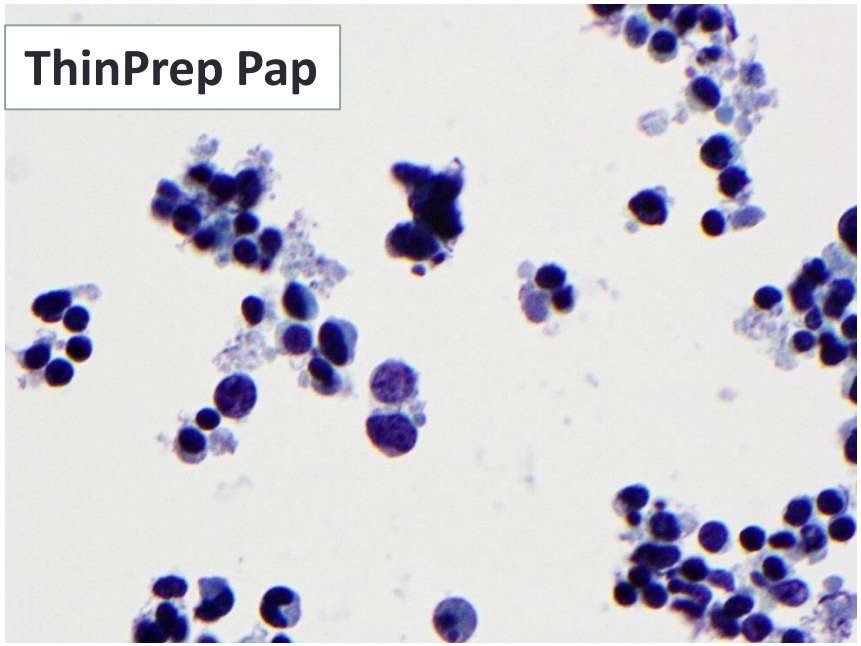

Cellblock Ki-67


Cellblock Synaptophysin

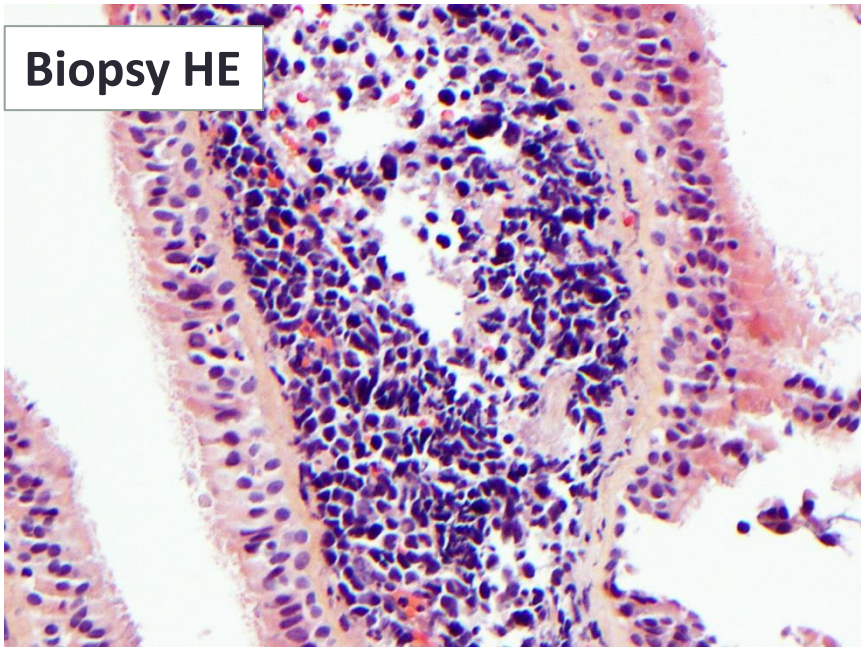
Comparative analysis

- ICC on cytospins from Preservcyt/ IHC on biopsy
- Metastasis of small cell carcinoma in a mediastinal lymph node
- Cytolyt (Hologic) → Preservcyt
  - Slide Pap stain
  - Cellblock
  - Cytospins for ICC
- Bronchial biopsy
  - IHC

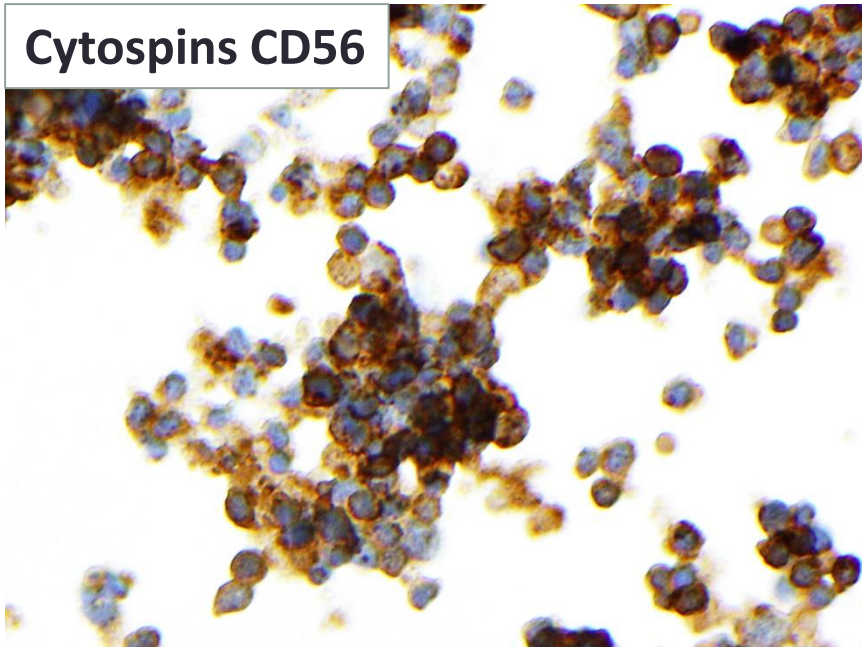
ThinPrep Pap



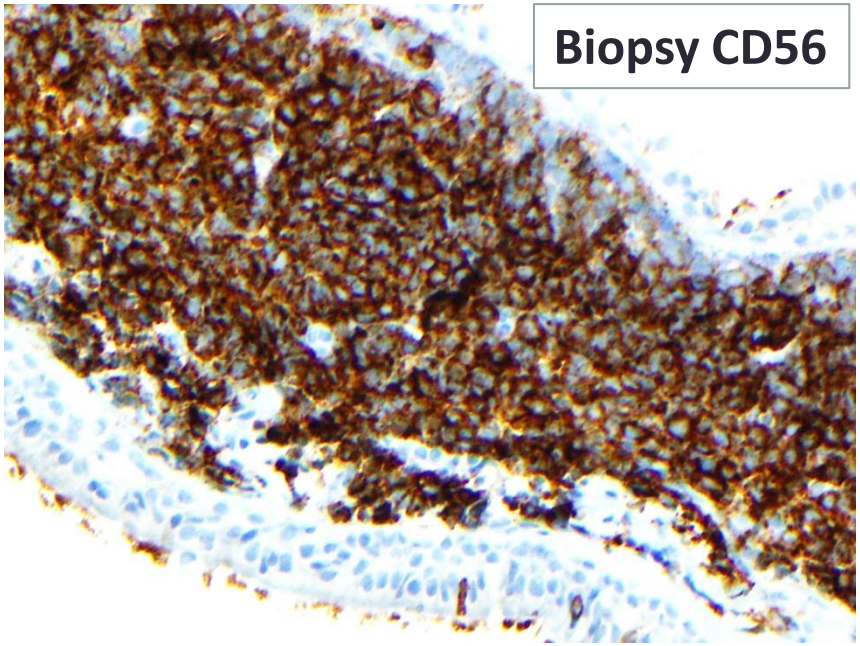
Biopsy HE



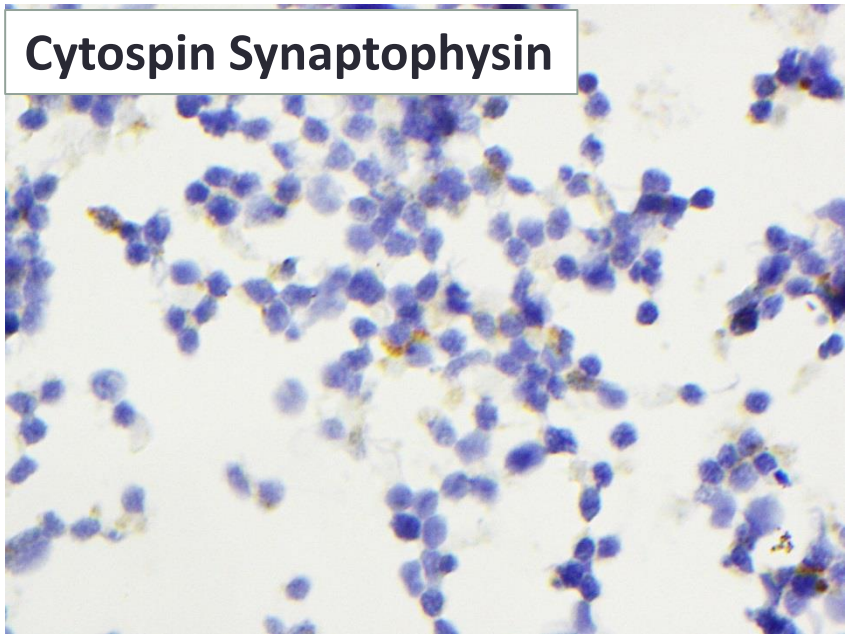
Cytospins CD56



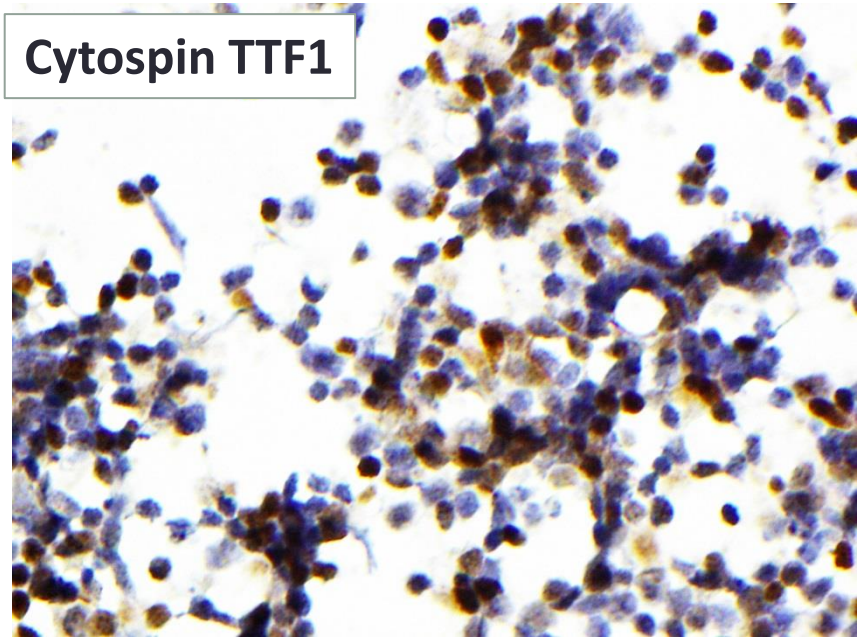
Biopsy CD56



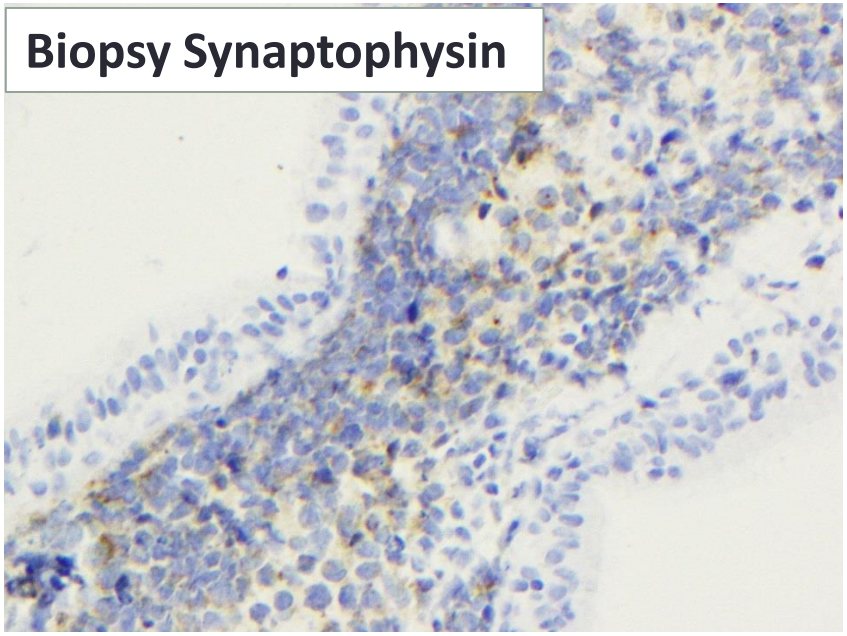
Cytospin Synaptophysin



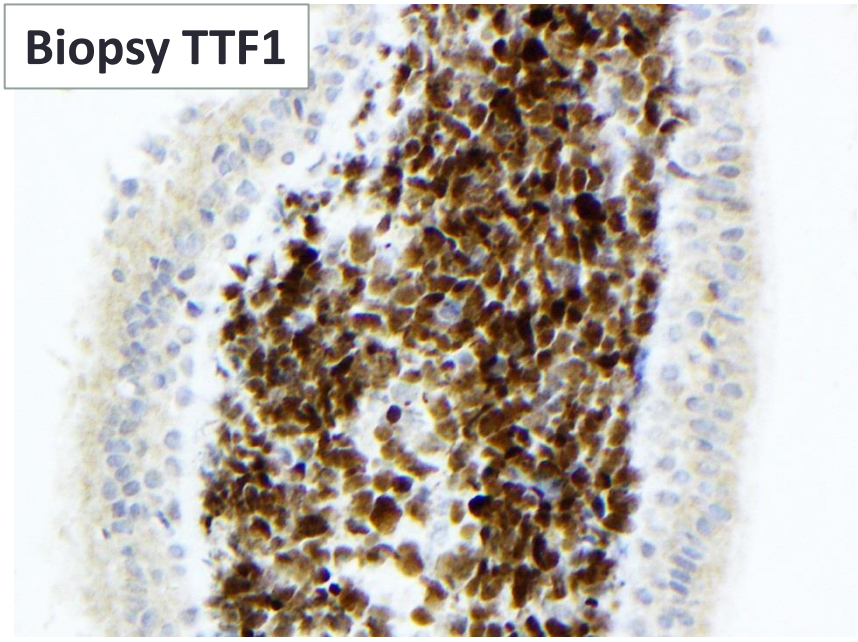
Cytospin TTF1



Biopsy Synaptophysin

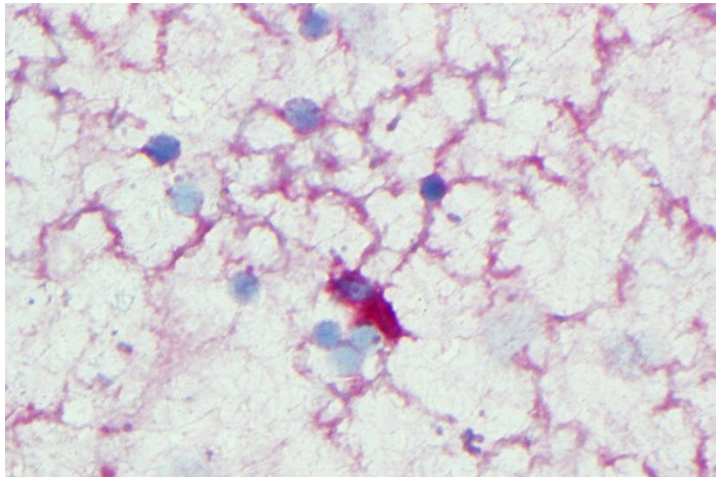


Biopsy TTF1

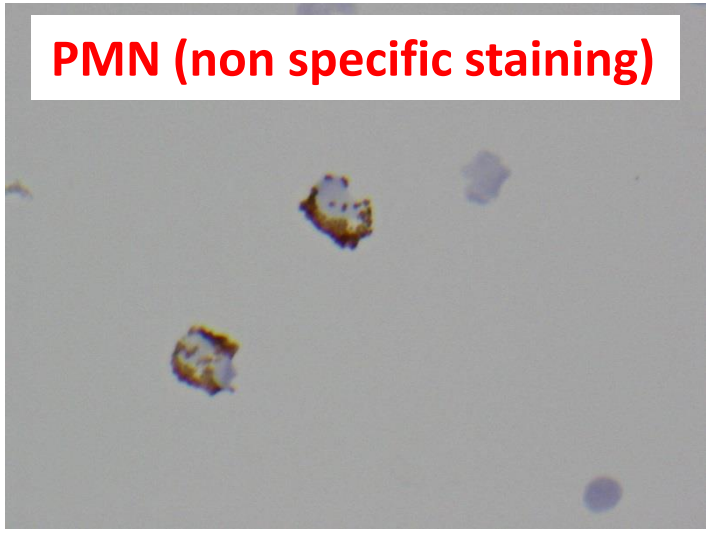


Optimisation and validation

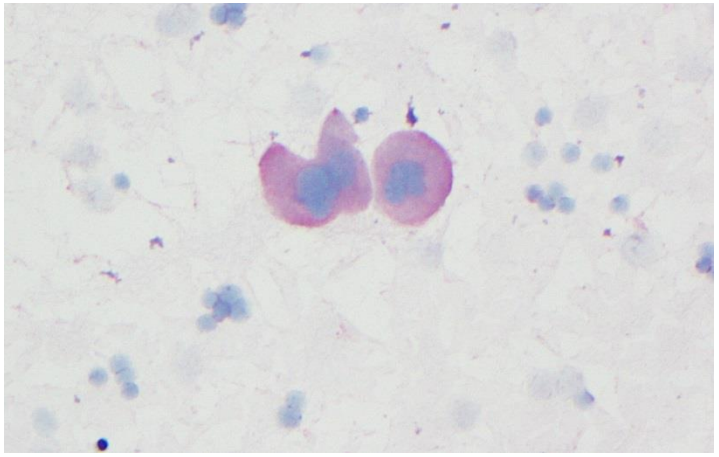
CD25 bone marrow
Background staining



CD117 bone marrow
background and non specific
staining

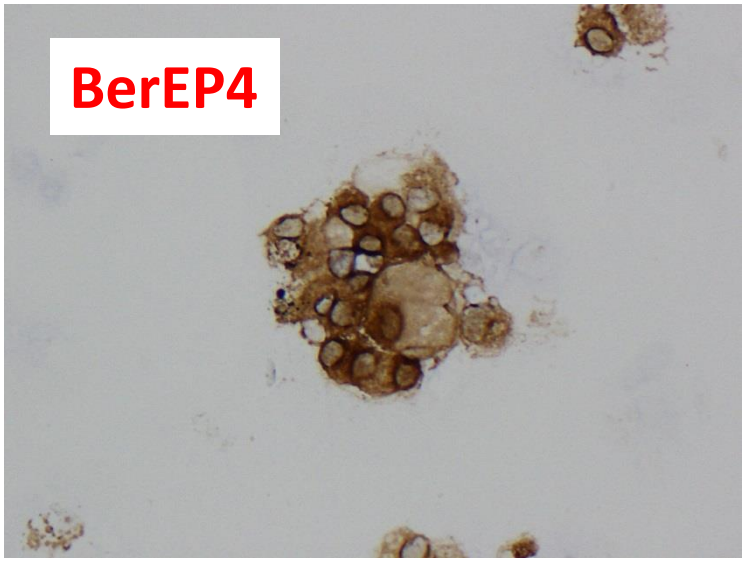


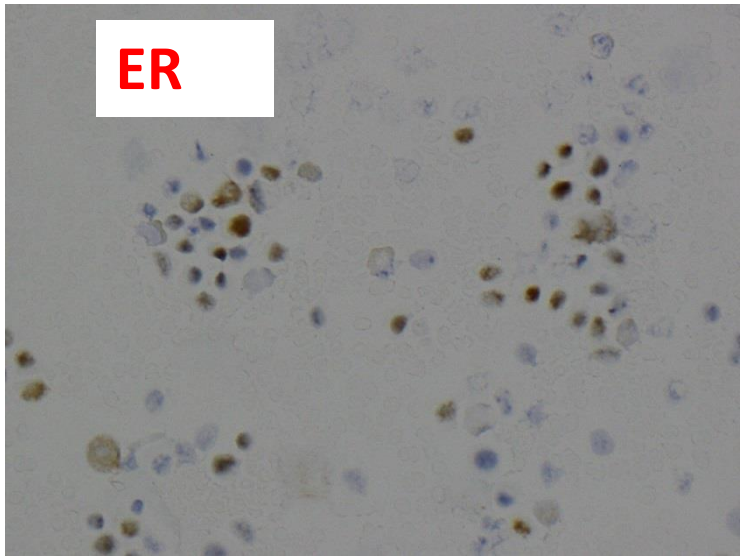
PMN (non specific staining)

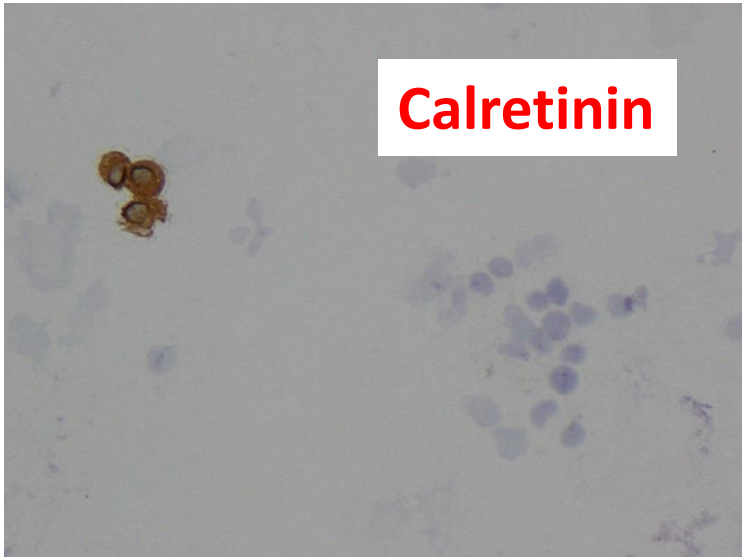


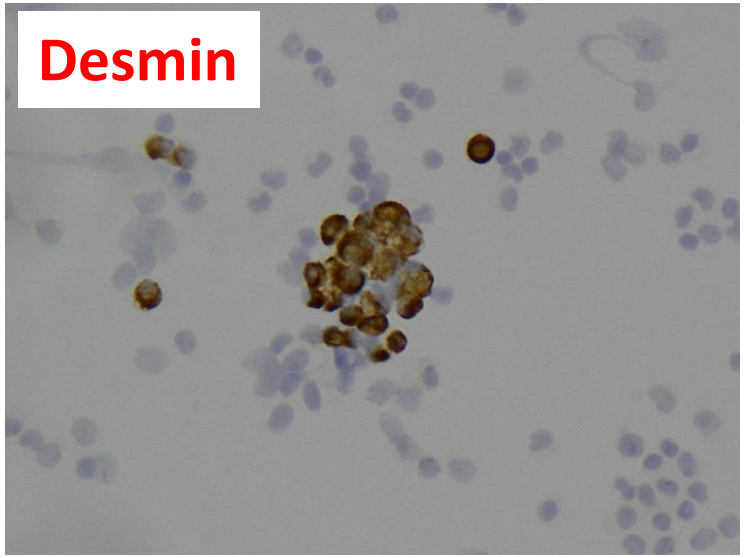
Optimisation and validation

Serous fluid









Use of controls

- Internal controls +++
  - Mesothelial cells in serous fluid
  - Mesothelial or bronchial cells for CK7
  - Small lymphocytes for CD3
- Additional slides
  - Positive slides fixed and stored for 12 months at -20°C
  - Cell lines
- Limited for non usual antibodies

Control specimens for immunocytochemistry in liquid-based cytology

T. Hansen, H. Pedersen, V. Brauner and J. Hariri

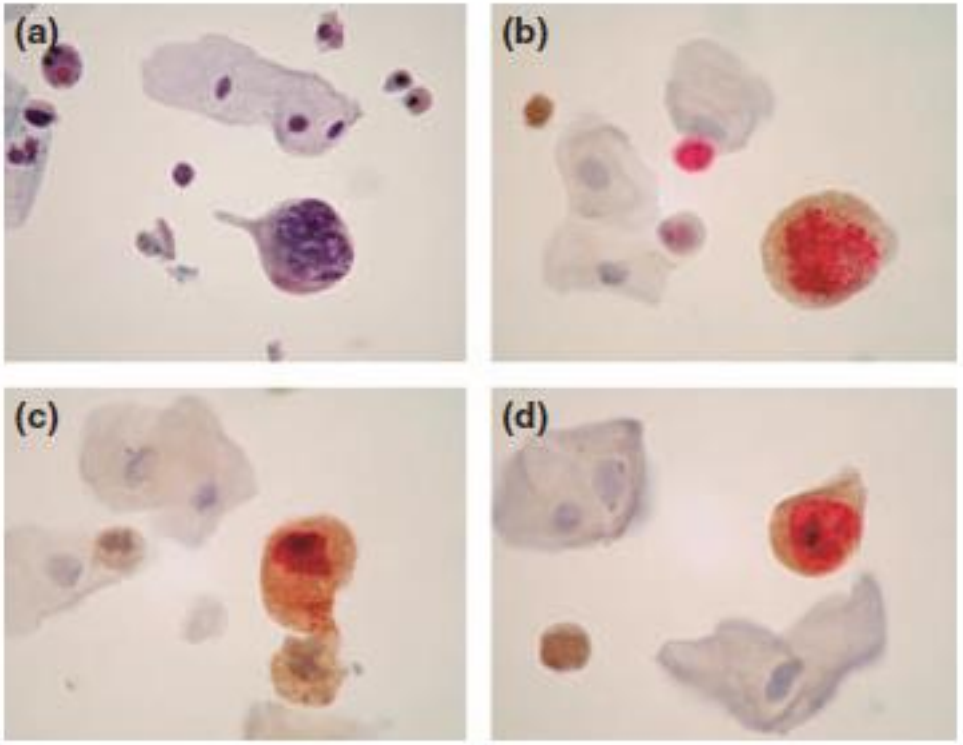
Department of Pathology, Sygehus Sønderjylland, Sønderborg, Denmark

Cytopathology 2011, **22**, 243–246

We do not perform ICC on stained slides

p16^{INK4a}/Ki-67 dual labelling as a marker for the presence of high-grade cancer cells or disease progression in urinary cytopathology

E. Piaton*,†,#, A. S. Advenier‡, C. Carré§, M. Decaussin-Petrucci†,¶, F. Mege-Lechevallier†,** and A. Ruffion†,††



Cytopathology 2013, 24, 327–334

Positive in :

- 87.3% of HG
- 38% of negative or LG
- 94% of CIS

Pitfalls

- **No or insufficient staining:**
  - Antibody sensitivity
  - Verify storage conditions especially temperature (specification sheet). Avoid freezing and thawing (perform aliquots)
  - Antibody concentration too low
  - Demasking protocol inappropriate

- **False positive result**
  - Crushed or degenerated cells or marked necrosis
  - Acute inflammation in background (PMN)
  - Antibody dilution insufficient
  - Antibody specificity

Thank you very much for your attention

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