IMMUNOCYTOCHEMISTRY AS AN ADJUNCT TO DIAGNOSTIC CYTOLOGY

M Courtade-Saïdi
Department of Pathology and Cytology
Toulouse Cancer Institute, France
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 $\rightarrow$ risk of cell detachment
Too few cells $\rightarrow$ 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

1 cupule

1 grille (0.1 μL)

3 mm
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
# Application of Immunohistochemistry to Cytology

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

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

- **When no other material**
- **Panels possible**
- **Decreased background**
- **Easy to store**
- **IHC techniques**
<p>| Commonly Used Antibodies At Our Cytology Laboratory |</p>
<table>
<thead>
<tr>
<th>Antibody</th>
<th>IVD/ASR</th>
<th>Vendor</th>
<th>Dilution</th>
<th>CytoLOGY Diagnosis Being Considered</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Antichymotrypsin, polyclonal</td>
<td>IVD</td>
<td>Ventana</td>
<td>PRE</td>
<td>1:200</td>
</tr>
<tr>
<td>Actin muscle, smooth, monoclonal</td>
<td>IVD</td>
<td>Dako</td>
<td>PRE</td>
<td>1:400</td>
</tr>
<tr>
<td>α-Fetoprotein (AFP), polyclonal</td>
<td>IVD</td>
<td>Ventana</td>
<td>PRE</td>
<td></td>
</tr>
<tr>
<td>AUK-1, monoclonal</td>
<td>IVD</td>
<td>Ventana</td>
<td>PRE</td>
<td>1:200</td>
</tr>
<tr>
<td>Ber-EP4, monoclonal</td>
<td>IVD</td>
<td>Ventana</td>
<td>PRE</td>
<td>1:50</td>
</tr>
<tr>
<td>Bcl-2, monoclonal</td>
<td>IVD</td>
<td>Dako</td>
<td>PRE</td>
<td>1:100</td>
</tr>
<tr>
<td>CA 19-9, monoclonal</td>
<td>IVD</td>
<td>Ventana</td>
<td>PRE</td>
<td></td>
</tr>
<tr>
<td>CA 125, monoclonal</td>
<td>IVD</td>
<td>Ventana</td>
<td>PRE</td>
<td>1:100</td>
</tr>
<tr>
<td>Cytokeratin, monoclonal</td>
<td>IVD</td>
<td>Zymed</td>
<td>PRE</td>
<td>1:200</td>
</tr>
<tr>
<td>Calcitonin, polyclonal</td>
<td>IVD</td>
<td>Dako</td>
<td>PRE</td>
<td>1:100</td>
</tr>
<tr>
<td>c-Kit, CD117, monoclonal</td>
<td>IVD</td>
<td>Dako</td>
<td>PRE</td>
<td>1:100</td>
</tr>
<tr>
<td>c-Erb-B2, HER2, monoclonal</td>
<td>IVD</td>
<td>Ventana</td>
<td>PRE</td>
<td>1:100</td>
</tr>
<tr>
<td>Carcinoma embryonic antigen (CEA), monoclonal</td>
<td>IVD</td>
<td>Dako</td>
<td>PRE</td>
<td>1:100</td>
</tr>
<tr>
<td>CD2a, monoclonal</td>
<td>IVD</td>
<td>Ventana</td>
<td>PRE</td>
<td>1:100</td>
</tr>
<tr>
<td>CD3, monoclonal</td>
<td>IVD</td>
<td>Novo</td>
<td>PRE</td>
<td>1:25</td>
</tr>
<tr>
<td>CD5, monoclonal</td>
<td>IVD</td>
<td>Novo</td>
<td>PRE</td>
<td>1:50</td>
</tr>
<tr>
<td>CD10, monoclonal</td>
<td>IVD</td>
<td>Novo</td>
<td>PRE</td>
<td>1:50</td>
</tr>
<tr>
<td>CD15, monoclonal</td>
<td>IVD</td>
<td>Ventana</td>
<td>PRE</td>
<td></td>
</tr>
<tr>
<td>CD20, monoclonal</td>
<td>IVD</td>
<td>Dako</td>
<td>PRE</td>
<td>1:200</td>
</tr>
<tr>
<td>CD30, monoclonal</td>
<td>IVD</td>
<td>Neomark</td>
<td>PRE</td>
<td>1:50</td>
</tr>
<tr>
<td>CD31, monoclonal</td>
<td>IVD</td>
<td>Dako</td>
<td>PRE</td>
<td>1:20</td>
</tr>
<tr>
<td>CD34, monoclonal</td>
<td>IVD</td>
<td>Dako</td>
<td>PRE</td>
<td>1:40</td>
</tr>
<tr>
<td>CD45, monoclonal</td>
<td>IVD</td>
<td>Dako</td>
<td>PRE</td>
<td>1:500</td>
</tr>
<tr>
<td>CD56, monoclonal</td>
<td>IVD</td>
<td>Novo</td>
<td>PRE</td>
<td>1:100</td>
</tr>
<tr>
<td>CD68, monoclonal</td>
<td>IVD</td>
<td>Dako</td>
<td>PRE</td>
<td>1:200</td>
</tr>
<tr>
<td>CD99, monoclonal</td>
<td>IVD</td>
<td>Neomark</td>
<td>PRE</td>
<td>1:50</td>
</tr>
<tr>
<td>Chromogranin, monoclonal</td>
<td>IVD</td>
<td>Dako</td>
<td>PRE</td>
<td></td>
</tr>
<tr>
<td>Cytomegalovirus (CMV), monoclonal</td>
<td>IVD</td>
<td>Signet</td>
<td>PRE</td>
<td>1:10</td>
</tr>
<tr>
<td>Cytokeratin 7, monoclonal</td>
<td>IVD</td>
<td>Dako</td>
<td>PRE</td>
<td>1:50</td>
</tr>
<tr>
<td>Cytokeratin 20, monoclonal</td>
<td>IVD</td>
<td>Dako</td>
<td>PRE</td>
<td>1:100</td>
</tr>
<tr>
<td>Desmin, monoclonal</td>
<td>IVD</td>
<td>Ventana</td>
<td>PRE</td>
<td></td>
</tr>
<tr>
<td>E-cadherin, monoclonal</td>
<td>IVD</td>
<td>Dako</td>
<td>PRE</td>
<td>1:40</td>
</tr>
<tr>
<td>Epithelial membrane antigen (EMA), monoclonal</td>
<td>IVD</td>
<td>Ventana</td>
<td>PRE</td>
<td></td>
</tr>
<tr>
<td>Estrogen receptor</td>
<td>IVD</td>
<td>Ventana</td>
<td>PRE</td>
<td></td>
</tr>
<tr>
<td>Gastrin, polyclonal</td>
<td>IVD</td>
<td>Ventana</td>
<td>PRE</td>
<td></td>
</tr>
<tr>
<td>Glucagon, polyclonal</td>
<td>IVD</td>
<td>Dako</td>
<td>PRE</td>
<td></td>
</tr>
<tr>
<td>Human chorionic gonadotropin (HCG), polyclonal</td>
<td>IVD</td>
<td>ABCAM</td>
<td>PRE</td>
<td>1:50</td>
</tr>
<tr>
<td>Herpes simplex virus type I, polyclonal</td>
<td>IVD</td>
<td>Ventana</td>
<td>PRE</td>
<td></td>
</tr>
<tr>
<td>Herpes simplex virus type II, polyclonal</td>
<td>IVD</td>
<td>Ventana</td>
<td>PRE</td>
<td></td>
</tr>
<tr>
<td>Hepar</td>
<td>IVD</td>
<td>Dako</td>
<td>PRE</td>
<td>1:100</td>
</tr>
<tr>
<td>HMBA-45 (melanoma), monoclonal</td>
<td>IVD</td>
<td>Ventana</td>
<td>PRE</td>
<td></td>
</tr>
<tr>
<td>Keratin, AE1</td>
<td>IVD</td>
<td>Zymed</td>
<td>PRE</td>
<td>1:400</td>
</tr>
<tr>
<td>Keratin, AE3</td>
<td>IVD</td>
<td>Zymed</td>
<td>PRE</td>
<td>1:200</td>
</tr>
<tr>
<td>α-Light chains, polyclonal</td>
<td>IVD</td>
<td>Dako</td>
<td>PRE</td>
<td>1:20000</td>
</tr>
<tr>
<td>Ki-67, monoclonal</td>
<td>IVD</td>
<td>Ventana</td>
<td>PRE</td>
<td></td>
</tr>
<tr>
<td>λ-Light chains, polyclonal</td>
<td>IVD</td>
<td>Dako</td>
<td>PRE</td>
<td>1:30000</td>
</tr>
<tr>
<td>MART1</td>
<td>IVD</td>
<td>Signet</td>
<td>PRE</td>
<td></td>
</tr>
<tr>
<td>Myogenin, monoclonal</td>
<td>IVD</td>
<td>Dako</td>
<td>PRE</td>
<td>1:500</td>
</tr>
<tr>
<td>p16</td>
<td>IVD</td>
<td>Dako</td>
<td>PRE</td>
<td>1:100</td>
</tr>
<tr>
<td>Pan keratin AE1/3</td>
<td>IVD</td>
<td>Ventana</td>
<td>PRE</td>
<td></td>
</tr>
<tr>
<td>Phosphatidylcholine, polyclonal</td>
<td>IVD</td>
<td>Ventana</td>
<td>PRE</td>
<td></td>
</tr>
<tr>
<td>Progesterone receptor, monoclonal</td>
<td>IVD</td>
<td>Dako</td>
<td>PRE</td>
<td></td>
</tr>
<tr>
<td>Prostate-specific antigen (PSA), monoclonal</td>
<td>IVD</td>
<td>Dako</td>
<td>PRE</td>
<td>1:200</td>
</tr>
<tr>
<td>S100, monoclonal</td>
<td>IVD</td>
<td>Dako</td>
<td>PRE</td>
<td>1:800</td>
</tr>
<tr>
<td>Somatostatin, polyclonal</td>
<td>IVD</td>
<td>Ventana</td>
<td>PRE</td>
<td></td>
</tr>
<tr>
<td>Spirochete</td>
<td>IVD</td>
<td>Dako</td>
<td>PRE</td>
<td>1:100</td>
</tr>
<tr>
<td>Synaptophysin, monoclonal</td>
<td>IVD</td>
<td>Dako</td>
<td>PRE</td>
<td>1:10</td>
</tr>
<tr>
<td>Thyroid transcription factor 1 (TTF-1)</td>
<td>IVD</td>
<td>BCM</td>
<td>PRE</td>
<td>1:80</td>
</tr>
<tr>
<td>Thyroglobulin, monoclonal</td>
<td>IVD</td>
<td>Ventana</td>
<td>PRE</td>
<td></td>
</tr>
<tr>
<td>Vimentin, monoclonal</td>
<td>IVD</td>
<td>Ventana</td>
<td>PRE</td>
<td></td>
</tr>
</tbody>
</table>

* IVD indicates in vitro diagnostic use; ASR, allied specific reagent; PRE, preformed; PST, solid pseudopapillary pancreatic tumor; GIST, gastrointestinal stromal tumor; RUO, research use only; and FFS, falsidrome, prostate (skin control). Vendors: Ventana, Tucson, Ariz.; Dako, Carpinteria, Calif.; Zymed, Berkeley, Calif.; Novo, Novocastor, Burlington, 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 permeabilization
Fixatives

• Acetone, Ethanol, Methanol:

Before fixation

- Fibrous protein (shrinkage)
- Globulous protein (unfolding)

After fixation

Protein denaturation
Fixatives

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

(A) PROTEIN - H + HCHO → PROTEIN - HCHOH

(B) PROTEIN - HCHOH + H → PROTEIN

(C) Lysine + Methylene glycol (HCHO + water) → Peptide linkage + Methylene bridge

After fixation

Methylene bridge
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 50 min
  - 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 peroxide
Some examples

- Litterature
Smears

Automated immunostaining of cell smears: an alternative to flow cytometry

L C Happerfield,¹ R Saward,² L Grimwade,² D Bloxham,² W N Erber²

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

Fixatives
Acetone 100% : 10 min  Good stain but poor preservation of cells
Acetone/Methanol (vol/vol) : 90 sec

Acetone/methanol/formalin (19v/19v/2v) : 90 sec  Best combination
Buffered formol acetone : 30 sec
Examples of immunostaining of blood and bone marrow smears using the Bond-mX 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 peroxidase

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


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

Table 3

Immunocytochemistry — immunohistochemistry correlation (n = 34)

<table>
<thead>
<tr>
<th>Final diagnosis</th>
<th>Cytology</th>
<th>Immunocyto</th>
<th>Histology</th>
<th>Immunohisto</th>
<th>Discordance</th>
</tr>
</thead>
<tbody>
<tr>
<td>AdenoCA (n=23)</td>
<td>19</td>
<td>21</td>
<td>23</td>
<td>23</td>
<td>2</td>
</tr>
<tr>
<td>Sq cell CA (n=4)</td>
<td>3</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Small cell CA (n=4)</td>
<td>3</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Others (n=3)</td>
<td>2</td>
<td>2</td>
<td>3</td>
<td>3</td>
<td>1</td>
</tr>
</tbody>
</table>
(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

1. Primary antibody binds to target antigen in tissue.
2. OptiView HQ Universal Linker, which has numerous non-endogenous HQ haptens, binds the primary antibody.
3. OptiView HRP Multimer binds to the HQ haptens. The number of multimer molecules is multiplied in this way, resulting in increased staining intensity without increased background.
4. DAB chromogen reacts with HRP and \( \text{H}_2\text{O}_2 \) to generate clean, crisp signal.
Smear

Synaptophysin

Cellblock

TTF1
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
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
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
Optimisation and validation

- CD25 bone marrow background staining

- PMN (non specific staining)

- CD117 bone marrow background and non specific staining
Optimisation and validation

Serous fluid

BerEP4

Calretinin

ER

Desmin
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

p16INK4a/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†,††

Positive in:
- 87.3% of HG
- 38% of negative or LG
- 94% of CIS

Cytopathology 2013, 24, 327–334
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