Standardization of Diagnostic Immunohistochemistry Coming of Age

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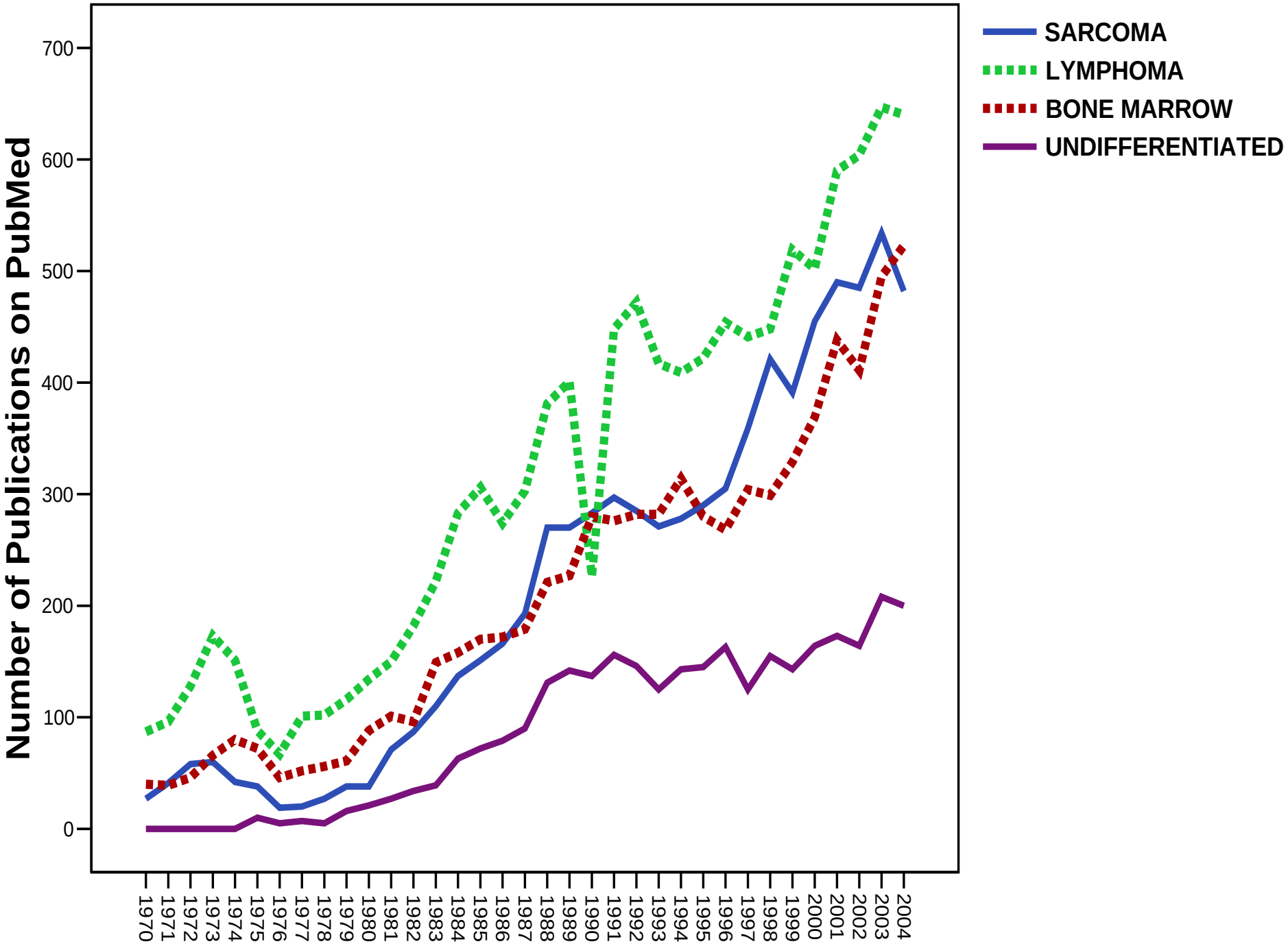
Canada


Number of Publications on PubMed
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BONE MARROW
UNDIFFERENTIATED

Standardization of IHC

- IHC has been in use in diagnostic pathology for about 4 decades
- It is only in the last 10 years that technological advances enabled us to claim that:
- **IHC results are highly reproducible**
- **IHC can be finely tuned/calibrated, and**
- **IHC is amenable to standardization**

"Standardization" is greatly misused term in IHC

- As long as tissue processing cannot be fully standardized, diagnostic IHC can be only optimized.
- Standardization is possible only if there are so-called "gold standards" for reference values.
- Diagnostic IHC has very few "gold standards" at this time.

Standardization vs. Optimization

- **Pre-Analytical variables of IHC tests** – Any and all steps in tissue processing, including intraoperative tissue handling/treatment (prolonged ischemia, delayed fixation, etc.), type and length of fixation, decalcification, and elements of tissue handling. The pre-analytical component is concluded at microtomy and the placement of the tissue section on pre-treated glass slides.

- **Analytical variables of IHC tests** – The analytical variables phase begins with the handling of the cut slides in a clinical IHC laboratory. It is completed with the coverslipping of the stained slides.
  - **Antibodies, controls, automation, reagents**

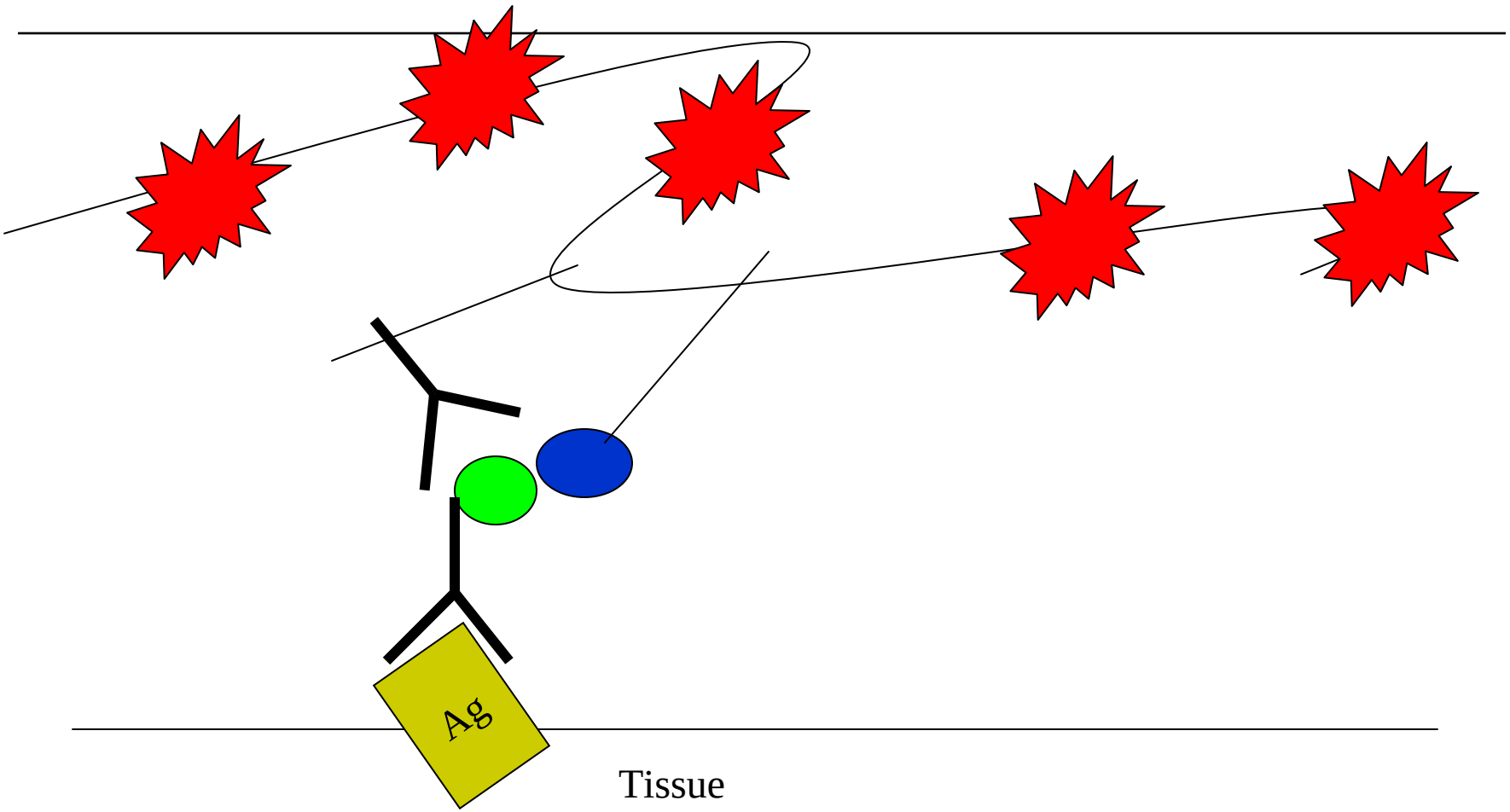
- **Post-Analytical variables of IHC tests** – Interpretation and reporting of the results.

What standards are defined so far?

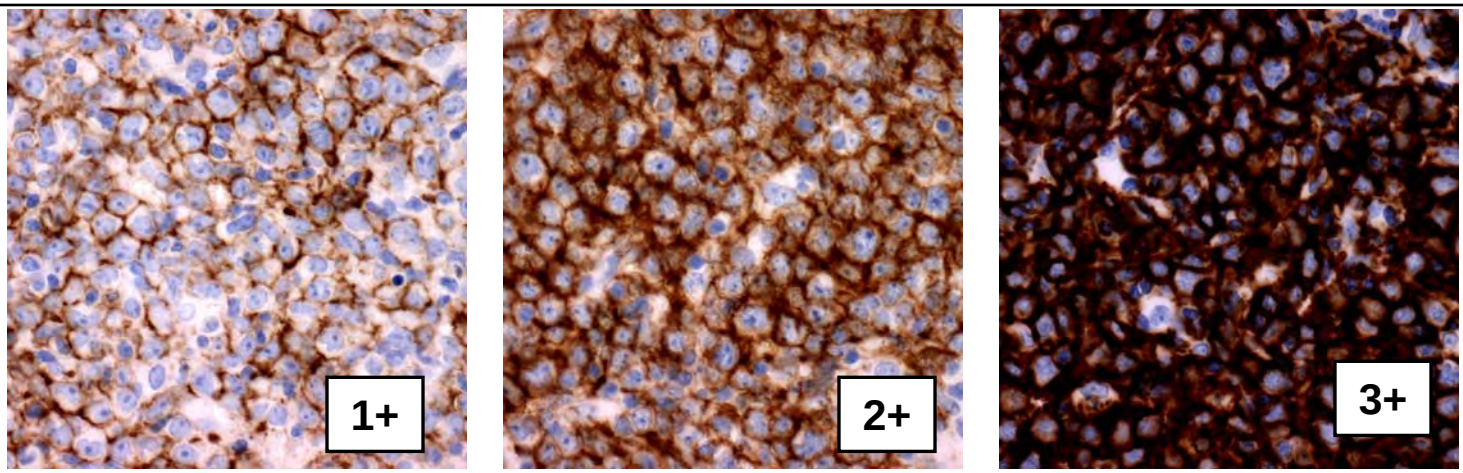
http://www.popsci.com/technology/article/2010-11/celebrating-international-standard-units-meter

- 1983: A meter is the distance light travels in a vacuum in 1/299,792,458th of a second.

Amplification and Detection



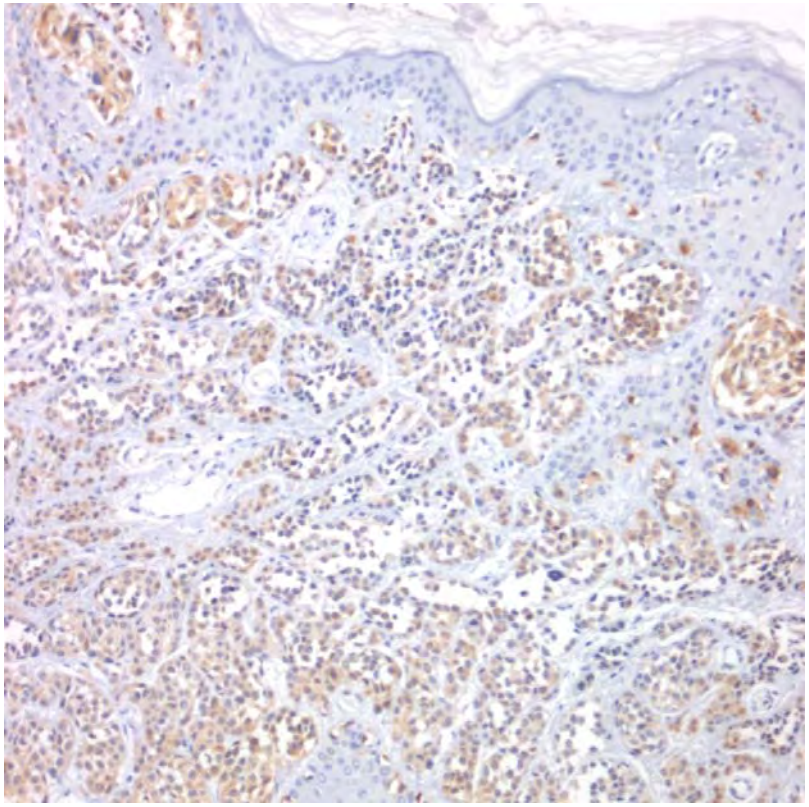
Intensity of Staining



- Most of the time relevant for the interpretation, but not reported.
- Must be stated if class II guidelines are asking for reporting (ER/PR)

MELAN-A

A



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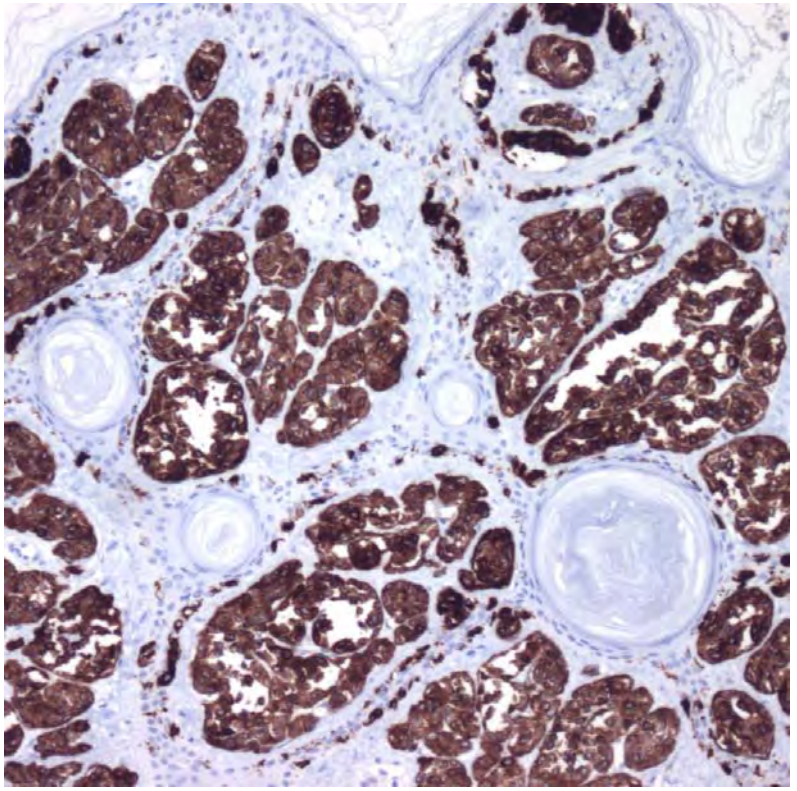


QC/QA for High Complexity Testing

- Compared with other laboratory disciplines, the state of the art in both, quality control (QC) and quality assurance (QA) practices for high complexity testing including IHC and molecular testing has fallen behind.

- IHC and Molecular Testing share similar challenges.

In Common: High Expectations of Accuracy

- Sensitivity and specificity of most test is not defined
- When possible to calculate sensitivity and specificity, standards are not set or not universally agreed upon
- Two types of sensitivity and specificity are applicable and need to be recognized:
  - Clinical (how accurately our test result will detect clinically relevant parameter)
  - Analytical:
    - Expected sensitivity and specificity - design of the test (design of prim. Ab, design of primers)
    - Actual achieved sensitivity and specificity

In Common: New and Rapidly Evolving Technology

- New test targets are described almost daily
- New methodology has a potential to redefine the entire filed and make all published knowledge obsolete

New methodology requires new approach to validation

- FDA-approved tests are considered validated for clinical applications
- Most IHC and molecular and cytogenetic tests are performed as those using ASRs and their validation is in hands of board-certified pathologists (LDT)
- Validation may not be even possible regarding the cost and time required for each new developed test
- Can we put in clinical use tests that were not properly validated?

In Common: Lack of Quality Control Samples and Standardization

- Lack of definitions and/or agreement what samples should be used for either positive or negative controls
- Lack of actual source of QC samples
- Lack of funds for appropriate generation of QC samples
- Lack of standardized calibrators
- Lack of **knowledge dissemination in QC** including both laboratory physicians, technologists, managers, and users (oncologists, other…)

QA in High Complexity Testing

- How to generate data on accuracy and precision?
  - Monitoring outputs in such way to enable application of statistical analysis.

- **Challenge: Is such traditional QC strategy applicable to immunohistochemistry testing and molecular diagnostics?**
  - Produced results of controls can be serially plotted on **Levey-Jennings charts** to monitor the test system for shifts or trends in performance.
  - Produce such results so that **"Westgard Rules"** can be applied o to determine when corrective action should be taken to prevent test failure.
- **Challenge:**
  - **Develop new rules for IHC control monitoring which are more appropriate to data that is generated by IHC?**
  - **Develop new controls that are amenable to be plotted on Levey-Jennings charts?**

Experimental Validation of Peptide Immunohistochemistry Controls

Steven A. Bogen, MD, PhD,† Kodela Vani, MS,* Brian McGraw, BSME,‡ Vin Federico, BSEE,‡ Iqbal Habib, HBSc, MBA,§ Ron Zeheb, PhD,‖ Ed Luther, AB,¶ Colin Tristram, MSc,# and Seshi R. Sompuram, PhD*†*

(*Appl Immunohistochem Mol Morphol* 2009;17:239–246)

ract: Peptide immunohistochemistry (IHC) controls are a

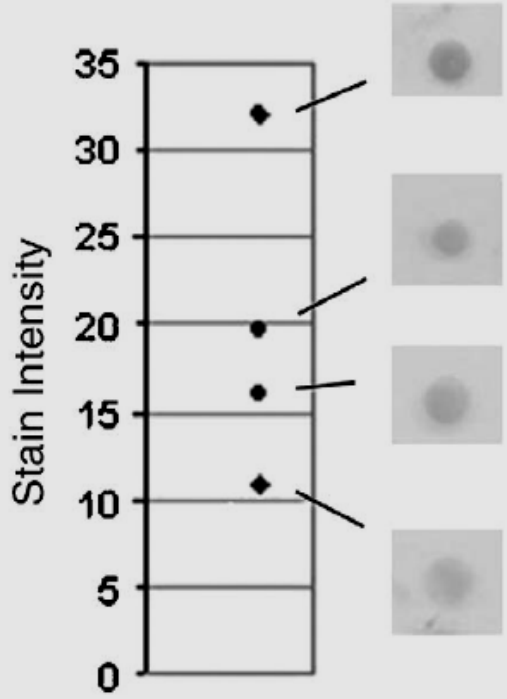


FIGURE 1. Examples of stained peptide control spots and their respective numerical scores. The *y*-axis is expressed as mean pixel intensity, in relative units, and is not calibrated against a color intensity reference standard.

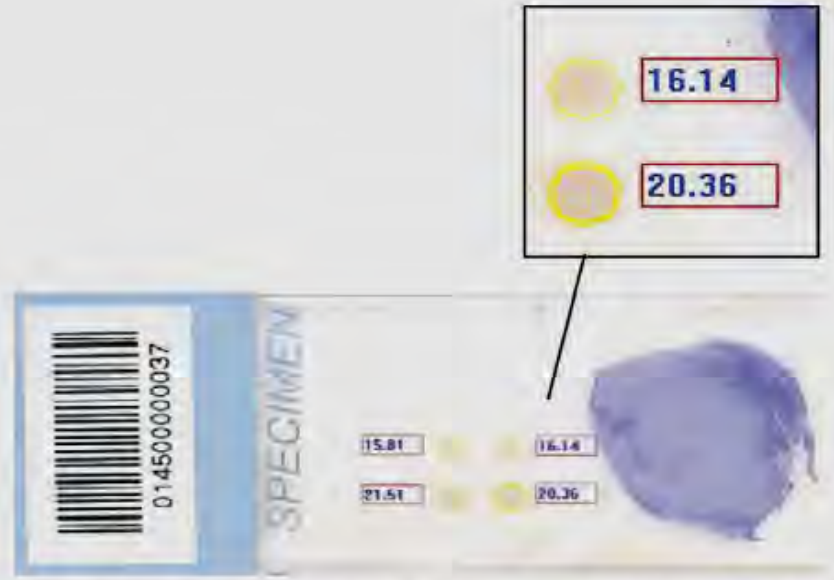


FIGURE 2. Software program screen for measuring stained peptide spot intensity. After subtracting the background level of intensity over irrelevant areas of the slide (not shown), color intensity is measured over the stained peptide spots (contained within the yellow circles). The value, in mean pixel intensity, is depicted in the higher magnification inset. The peptide control spots shown in the inset are not duplicates of each other, but rather serial dilutions. Duplicate peptide controls were printed with a horizontal orientation on the slide.

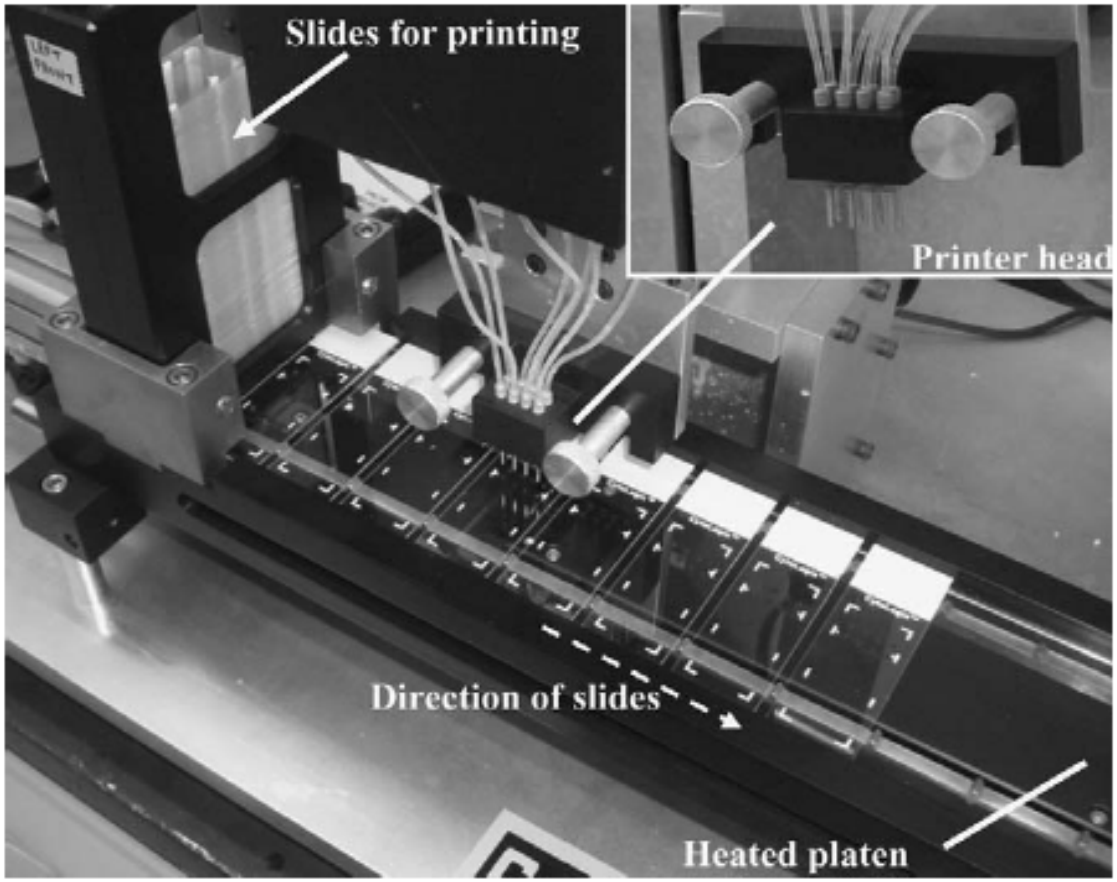


FIGURE 3. Photograph of the prototype slide printer, with a higher magnification of the printer head (inset), showing 8 nozzles, each of which dispense microliter-sized droplets of peptide onto passing slides. As the slides proceed toward the right, they pass onto a heated platen, which accelerates the peptide coupling reaction to the activated glass surface.

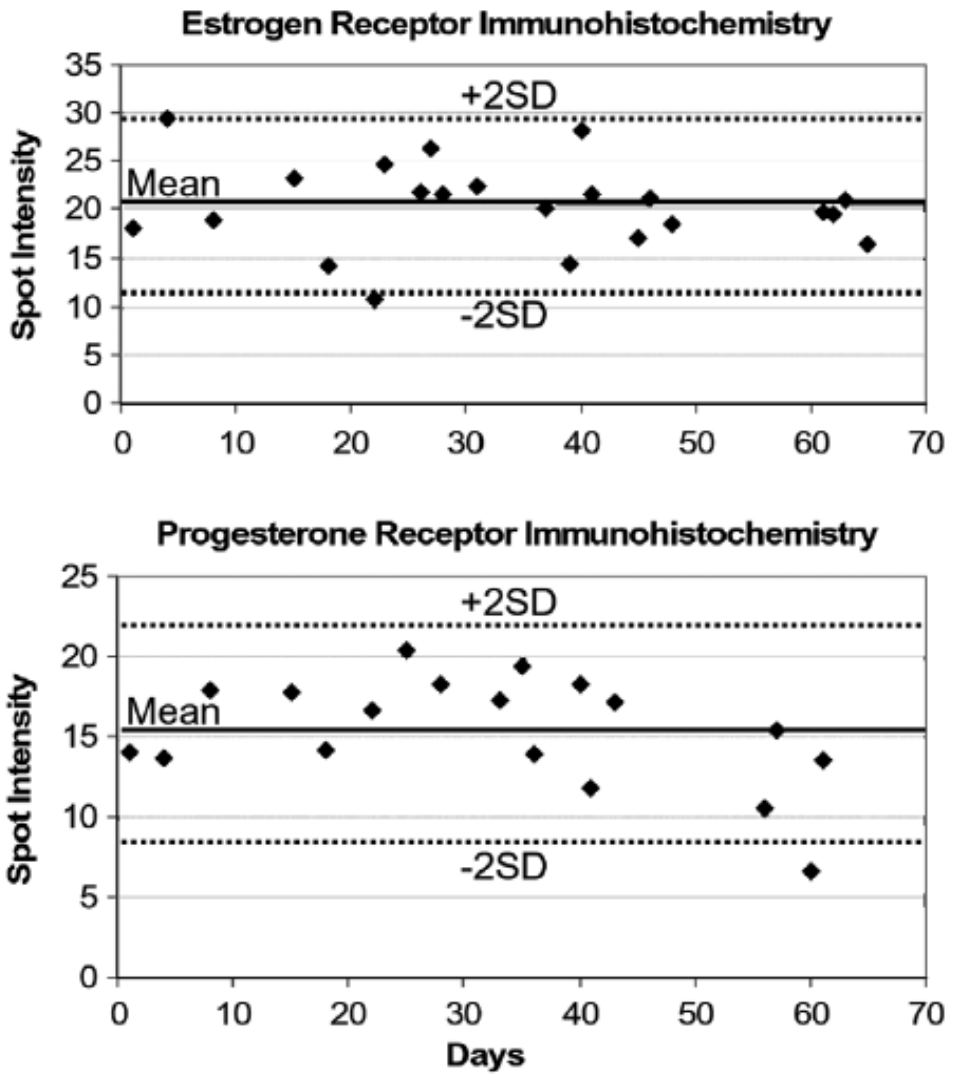


FIGURE 8. Levy-Jennings charting for estrogen and progesterone receptor immunohistochemistry, over a 2-month period. The mean and ±2 SD boundaries are also shown. No staining failures occurred in this time frame.

Alternative: Use of Relative Values Introducing LSRMSR

- Sample for controls is prepared by an inexpensive cell line (cell block).
- One slide is sent to reference laboratory to be stained.
- H-score is determined by image analysis.

Lab H-SCORE / Ref Method or Lab H-SCORE = LSRMSR

- LSRMSR can be plotted on the Levy-Jennings charts.

LDT, “Home Brew Tests”

- All IHC tests except FDA approved kits.
- All molecular tests except FDA approved kits.
- All cytogenetic tests and FISH.

- “CLIA regulated laboratories qualified to perform high complexity testing have demonstrated expertise and ability to use ASRs in test procedures and analyses”.

FDA Enforcement Discretion for LDTs

- Starting in 1992, FDA asserted that all LDTs are devices subject to regulation under the Federal Food, Drug, and Cosmetic Act. Since then, the agency said it was exercising its enforcement discretion and not regulating LDTs.
- Thus, the primary federal regulation of laboratories has been under the Clinical Laboratory Improvement Amendments of 1988 (CLIA).
- LDTs are also regulated by the states (notably New York) and other bodies (notably the College of American Pathologists) ("CAP").
- Until recently, FDA has departed from this position of enforcement discretion in relatively few instances. However, FDA now took a different stand and will address in much more detail LDTs.

FDA: Upcoming New Regulation of LDTs?

- Regulating these tests will raise many policy, regulatory, legal, and public health questions.
- Elements that need to be outlined by the agency include: risk categorization, a phase-in period for premarket review and quality systems requirements for new LDTs; registration and listing; and inspections of laboratories.

Requirements for Test Validation

- The FDA does not specify requirements for test validation, but it provides guidance for commercial manufacturers that intend to submit validation data for FDA approval or clearance.
- When FDA-approved kits are used, laboratory only need to confirm its performance characteristics (verify the test claims).

LDT Validation

- The laboratory must establish test performance specifications:
  - Accuracy
  - Precision
  - Reportable range
  - Reference range
- The laboratory must develop and plan procedures for calibration and control of the test system.
- The laboratory must establish analytical sensitivity and specificity.

Principles of Test Validation

- ISO 9000 – “Confirmation by using objective evidence, that requirements for a specific intended use or application have been fulfilled.”
- Validation – we are doing the *correct* test.
- Verification – we are doing the test *correctly*.
- Validation requires *identification of the needs of the user*.

Analytic Performance Characteristics

- Accuracy: our result – reference value (or conventional true value) = error
- Trueness: systematic error/bias
- Precision (for quantitative tests): measure of random error (SD)
- Reproducibility (precision)
- Repeatability: reproducibility within-run
- Reference range: range of test values for designated population

Analytic Performance Characteristics

- Analytic sensitivity: positive agreement as compared to reference method
- Analytic specificity: negative agreement as compared to reference method
- Clinical sensitivity: proportion of subjects with a disorder with positive test result
- Clinical specificity: proportion of subjects without disorder with negative test result
- Limit of detection: the lowest amount of analyte (Ag) that is statistically distinguishable from background or negative control

Cochrane Collaboration

- 2003 **Standards for the Reporting of Diagnostic Accuracy**
- Diagnostic accuracy: agreement between test results and reference standard.
- Reference standard: the best available method for establishing the presence or absence of the condition of interests.
- Reference standard: single method, combination of methods, imaging, pathology, clinical follow-up, etc.

Clinical and Laboratory Standards Institute (CLSI) Recommendations: Evaluation Protocols

- Generally:
  - 10 to 20 operating days
  - 20 to 40 patient samples
  - 50 positive and 50 negative

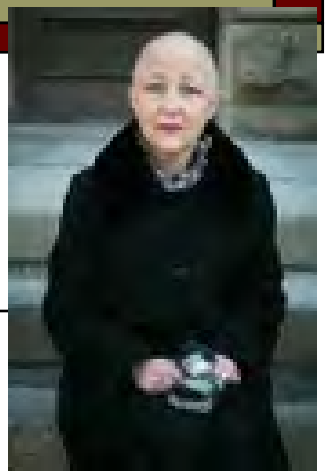
- www.clsi.org/

LDT, ASR, “Home Brew Tests”

- All IHC tests except FDA approved kits.
- All molecular tests except FDA approved kits.
- All cytogenetic tests and FISH.

- “CLIA regulated laboratories qualified to perform high complexity testing have demonstrated expertise and ability to use ASRs in test procedures and analyses”.

CBC News in Depth:



Misdiagnosed - Anatomy of Newfoundland's Cancer-Testing Scandal

- Of the 1,013 breast cancer patients retested (1997-2005), 383 — more than a third — were found to be false negative. That meant 383 patients were denied a fighting chance against cancer. More than 100 of those wrongly tested patients are now dead.
- Not all of those affected were notified that a mistake had been made.

What Went Wrong in Newfoundland? How to Fix it?

- Media responds with various takes on the subject.
  - **“Breast Cancer Testing Scandal Shines Spotlight on Black Box of Clinical Laboratory Testing” JNCI News**

- **What really went wrong? Who was responsible and why?**

Commission of Inquiry on Hormone Receptor Testing

The Honourable Justice Margaret A. Cameron, Commissioner

- The Commission expressed conclusions and recommendations regarding responsibility of various persons or organizations, and delivered its final report and recommendations to the Minister of Health and Community Services on **February 28, 2009**.
- QA
- QA
- QA

http://www.cihrt.nl.ca/about.html

CME/SAM

Canadian Association of Pathologists–Association canadienne des pathologistes National Standards Committee/Immunohistochemistry

Best Practice Recommendations for Standardization of Immunohistochemistry Tests*

Emina Emilia Torlakovic, MD, PhD,[1] Robert Riddell, MD, FRCPath, FRCPC,[2] Diponkar Banerjee, MBChB, FRCPC, PhD,[3] Hala El-Zimaity, MD, MS, FRCPC,[4] Dragana Pilavdzic, MD, FRCPC,[5] Peter Dawe, MS,[6] Anthony Magliocco, MD, FRCPC,[7] Penny Barnes, MD, FRCPC,[8] Richard Berendt, MD, FRCPC,[9] Donald Cook, MD, FRCPC,[10] Blake Gilks, MD, FRCPC,[11] Gaynor Williams, MD, PhD,[12] Bayardo Perez-Ordonez, MD, FRCPC,[13] Bret Wehrli, MD, FRCPC,[14] Paul E. Swanson, MD,[15] Christopher N. Otis, MD,[16] Søren Nielsen, HT, CT,[17] Mogens Vyberg, MD,[17] and Jagdish Butany, MBBS, MS, FRCPC[13]

Key Words: Clinical immunohistochemistry; Standards; Canadian Association of Pathologists

DOI: 10.1309/AJCPDYZ1XMF4HJWK

Guidelines:

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- Principles/Best Practices for Quality Assurance of Clinical IHC Testing
- Class II Immunohistochemistry Tests Principles/Best Practices
- Proficiency testing: Monitoring the quality of laboratory performance
- Education and training standards for laboratory personnel
- References

- CAP-ACP NSC Checklists: Part 1 and Part 2
  http://www.cap-acp.org/publicFiles/CAP%20ACP%20NSC%20IHC%20Checklists%20English.pdf

The Role of Test Classification on Tools for QC/QA in IHC

- Class I – results used by pathologists
- Class II – results used by clinicians
- QC/QA ideally should be the same for both types of tests.
- Class II currently have priority as they are linked to higher risk for patient safety.

Class I IHC Tests (used by pathologists)

- Adjunctive diagnostic information not independently reported by physician
- Used after tumor diagnosed by other methods
- E.g. cytokeratin differentiation markers

Class I

- The results are incorporated into the diagnostic interpretation by the pathologists.
- Results NOT to be listed/described in entirety in the pathology reports?
- Readily available internal and external controls.

How do you know if the IHC test works properly or not?

- Pathologists need to be able to readily identify false-positive and false-negative Class I tests.
- It is assumed that evidence to support the interpretation as false-negative or false-positive test is readily available.
- Use both external and internal positive and negative controls.

IHC Controls

- Internal positive control is most important to exclude false-negative results.
- Internal negative control is most important to exclude false-positive results.

- **External controls monitor system, not individual patient's sample.**

Class II IHC Tests (results used by non-pathologists)

- Stand alone diagnostic
- Predictive or prognostic
- Widely accepted valid scientific claims
- E.g. hormone receptors in breast cancer

Class II

- **Prognostic IHC tests** – The results of these tests independently forecast clinical outcome. They may be either qualitative or quantitative. HER2/neu if used as prognostic marker.

- **Predictive IHC tests** – The results of these tests independently predict response to a particular therapy. They may either be qualitative or quantitative (e.g., ER/PR, HER2/neu in breast carcinoma, CD117 in gastrointestinal stromal tumor).

CAP-ACP IHC Test Classification: Class II Tests

Current	In Consideration	For Discussion
Estrogen Receptor (ER)	C4d	NPM1
Progesterone Receptor (PR)	DOG1	FOXP1
Human Epidermal Growth Factor Receptor 2 (HER2)	MMR	GCET1
Proliferation Marker Ki-67		IgG/IgG4
CD117		c-Myc
CD20		

Interpretation and Test Classification

- Class I IHC tests, which have critical significance for interpretation of overall assessment:

  ALK-1, cyclin D1, CD30, TdT, TTF-1, CDX-2, HMB-45, …

- New IHC test Class may be necessary to rise awareness and prevent wrong diagnoses.
  - Class IA and IB?

FDA and Health Canada are focused on whether this level of regulation is adequate for the protection of public health

- **FDA** is aware that variability in IHC results may be introduced at every step:
- Collection and fixation of the specimen,
- Automated processing,
- Embedding and sectioning,
- Staining of the final slide preparation, and
- Microscopic interpretation by the pathologist.

FDA (also Health Canada) counts on (counted on):

- **Ongoing initiatives by professional organizations and manufacturers** directed at ensuring that pre- and postanalytic, as well as analytic procedures, are properly performed.

Class I and Class II IHC Tests: Incorporating Appropriate Language in Pathology Reports

- For analyte specific reagents (ASR), a U.S. Food and Drug Administration (FDA)-required disclaimer is included in the reports.

- The mandatory language is as follows:

  "These tests were developed and their performance characteristics determined by the name of institution, Pathology Laboratory. They have not been cleared or approved by the FDA. However, the FDA has determined that such clearance or approval is not necessary. These tests are used for clinical purposes. They should not be regarded as investigational or for research. This laboratory is certified under the Clinical Laboratory Improvement Amendments of 1988 (CLIA) as qualified to perform high complexity clinical laboratory testing."

- This does not apply to FDA-approved kits for IHC testing.

Postanalytical: Interpretation of IHC Results

- Are there published guidelines for interpretation?
- Starts with interpretation of results in controls by technologist
- Starts with an agreement on what is considered a desirable result
- Postanalytical component cannot even start without a consensus on positive and negative controls
- **Challenge: Standardization of positive and negative controls**

Postanalytical: Reporting of IHC Results

- **Challenge:** Reporting standardization of Class II tests other than breast cancer
- **Challenge:** Reporting of Class I tests results
- Synoptic reporting for Class II tests

IHC Challenges for Pathology

- Reviewing current QA systems for clinical IHC in each laboratory for their adequacy.
- Proactively building appropriate internal and external QA measures to support development and clinical applications of new IHC tests.
- Reaching agreement/consensus on IHC test classification within our discipline and with our clinical colleagues.
- Identifying key components that are not addressed by laboratory accreditation.
- Changing how we report IHC test results for both Class I and Class II tests.

Challenges

- **There are about 200 IHC tests that are currently in clinical use.**

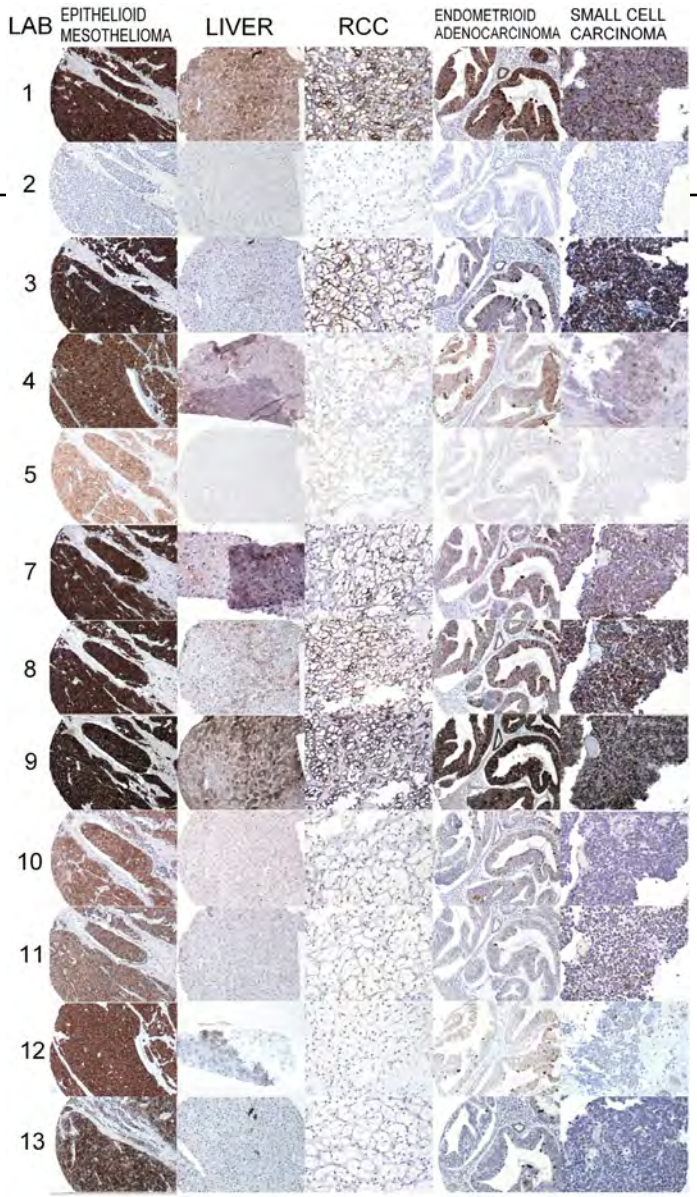
1. Most are not included in proficiency testing
2. Standardized controls are not available
3. National and international agreement on what standardized controls should be does not exist even for Class II markers
4. **Validation** of most IHC assays is not clearly defined:
   - For any calculations of sensitivity, specificity, and agreement power analysis should be considered so that calculations are not misleading (for some tests this may mean close to 100 samples to test).

Lack of Quality Control Samples and Standardization

- Lack of definitions and/or agreement what samples should be used for either positive or negative controls
- Lack of actual source of QC samples
- Lack of funds for appropriate generation of QC samples
- Lack of **knowledge dissemination in QC** including both laboratory physicians, technologists, managers, and users (oncologists, other…)

External Quality Assurance and Proficiency Testing (PT) in IHC

- Does not exist for many tests!
- Various programs that are providing PT do not clearly define their targets:
- **What are gold standards?**
- **What are reference values?**
- **Are assessments quantitative for quantitative IHC tests?**
- **Can participation in EQA be used for test validation?**
- **Are the EQA programs testing analytical or clinical sensitivity and specificity or both (or neither)?**

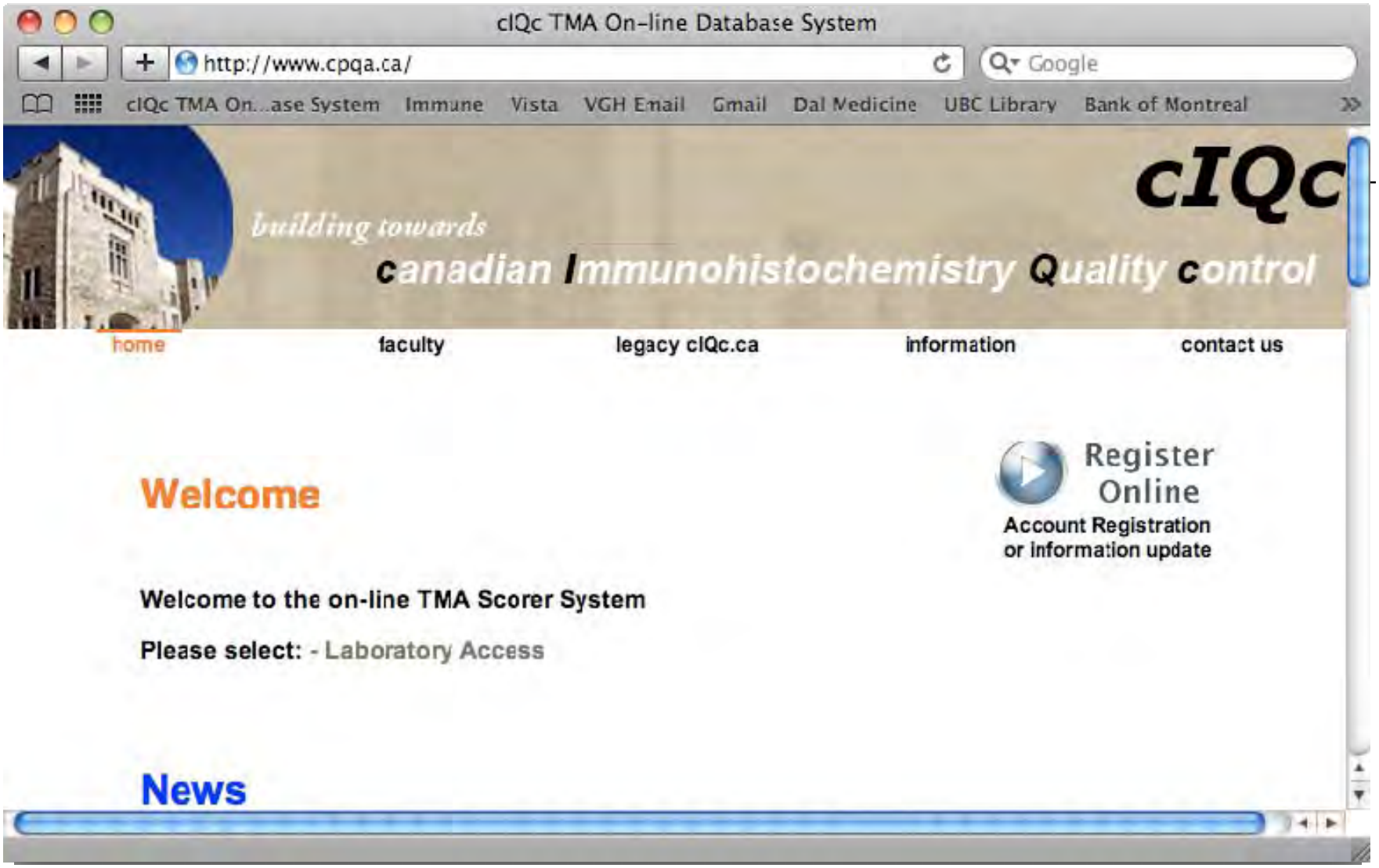


NordiQC and CIQC Experience

33 % optimal

33 % good / suboptimal

33 % poor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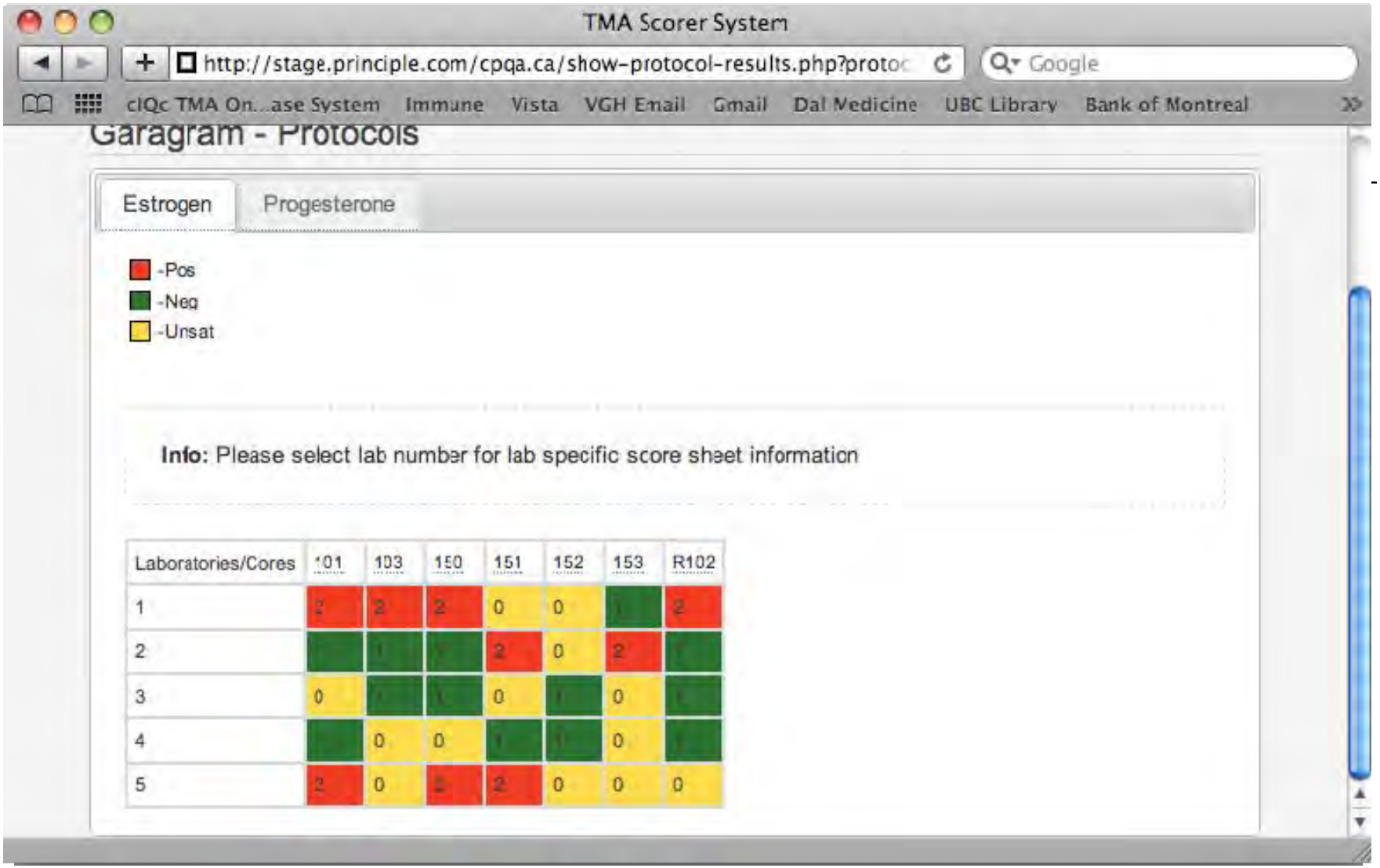
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ESTROGEN RECEPTORS

June/July 2009


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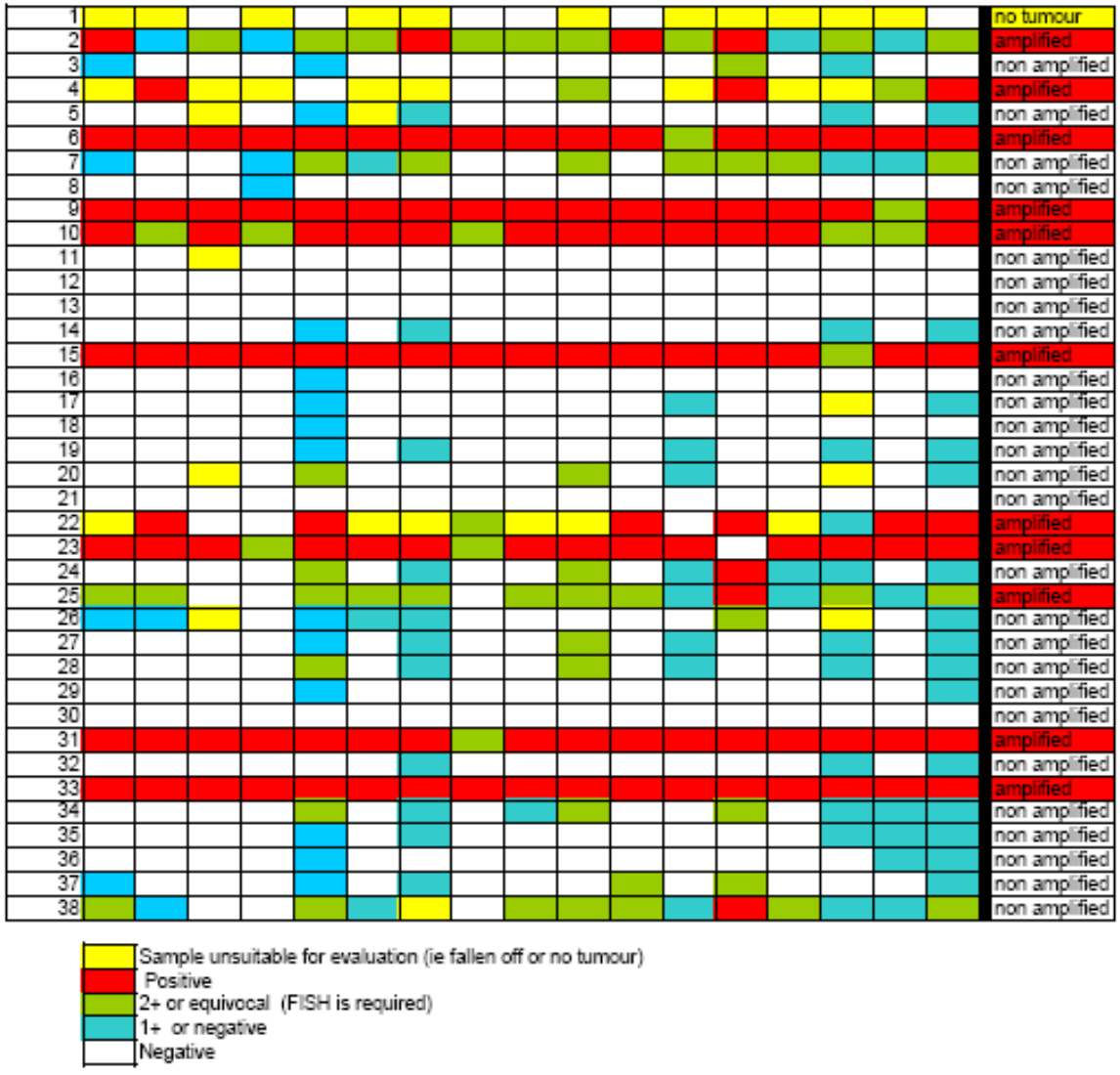
- Positive
- Negative
- Sample unsuitable for evaluation (ie fallen off or no tumour)
- Background staining - unsuitable for evaluation

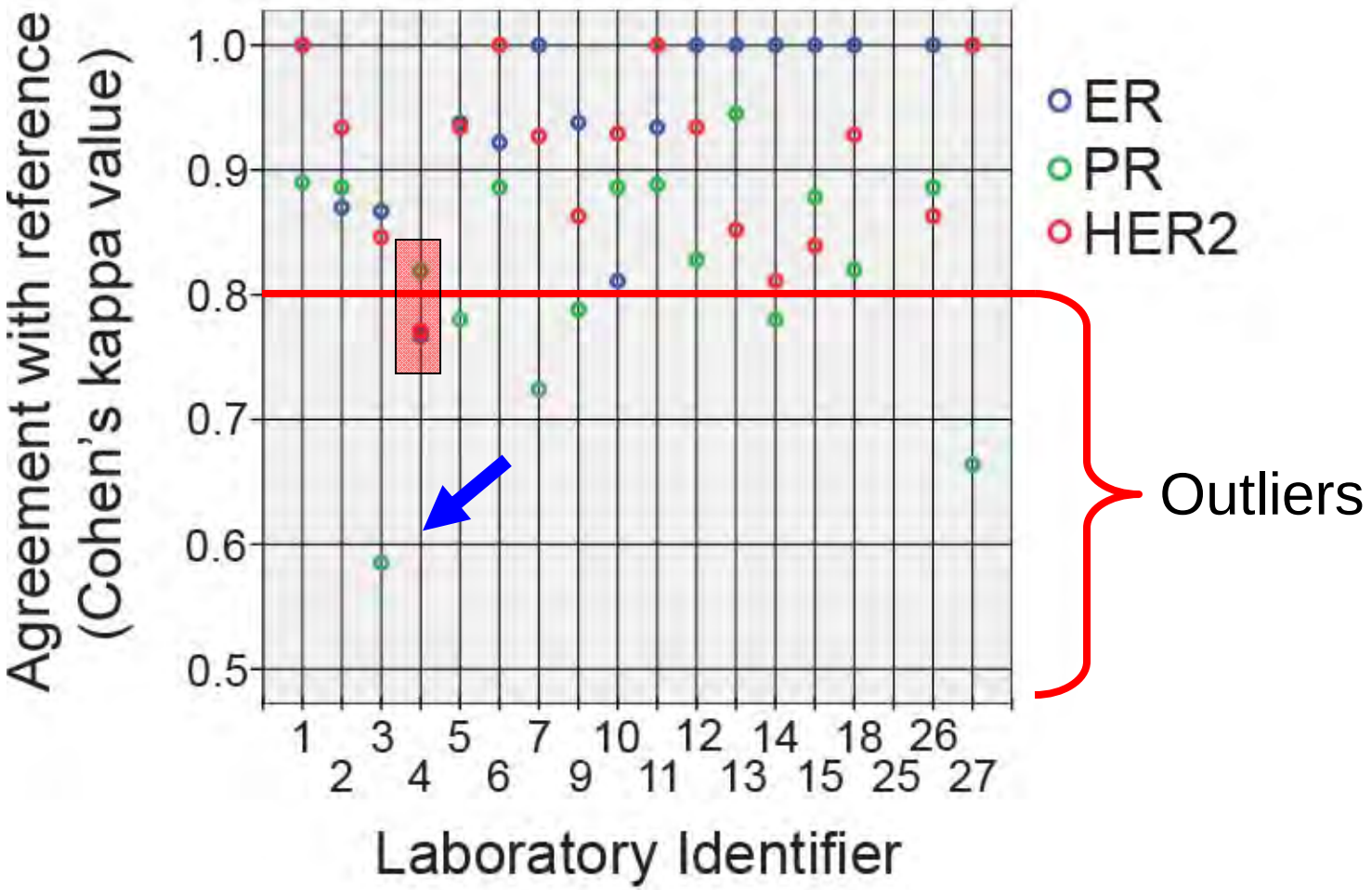
CIQC Run2: HER2

Average sensitivity: 91%, specificity: 98%



Kendall's coefficient of concordance = 0.96

How to Define Discordant Results?



Call for a European programme in external quality assurance for bone marrow immunohistochemistry; report of a European Bone Marrow Working Group pilot study

E E Torlakovic,[1] K Naresh,[2] M Kremer,[3] J van der Walt,[4] E Hyjek,[5] A Porwit[6]

ABSTRACT

Background and Aims: In diagnostic immunohistochemistry (IHC), daily quality control/quality assurance measures (QC/QA) and participation in external quality

in metastatic breast carcinoma in the BMTB varies from one institution to another, and most published literature does not address reporting of these markers in the BMTB.[7 8] Importantly, based on recent guide-

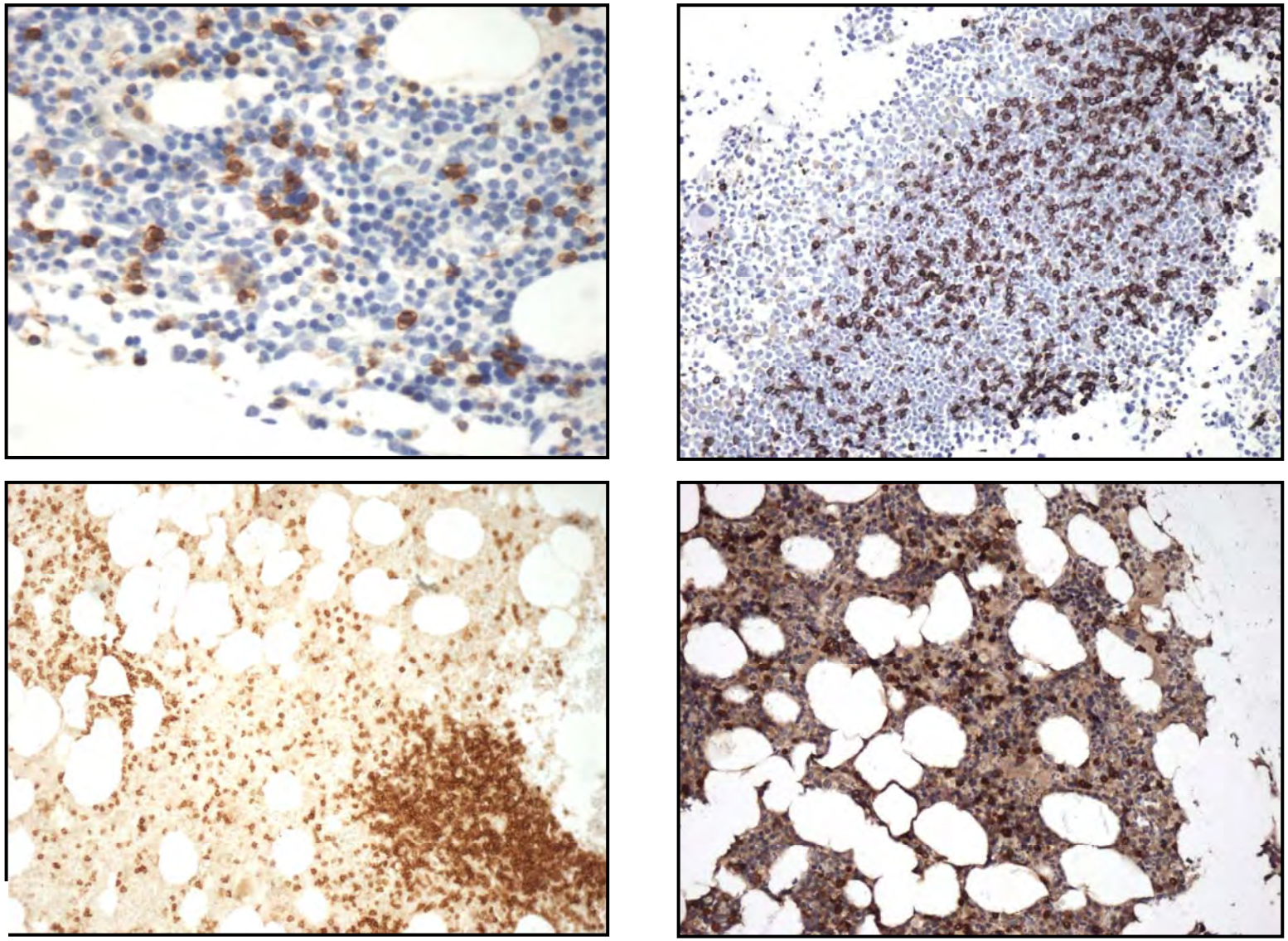
Pilot Project

- Survey posted on www.cIQc.ca

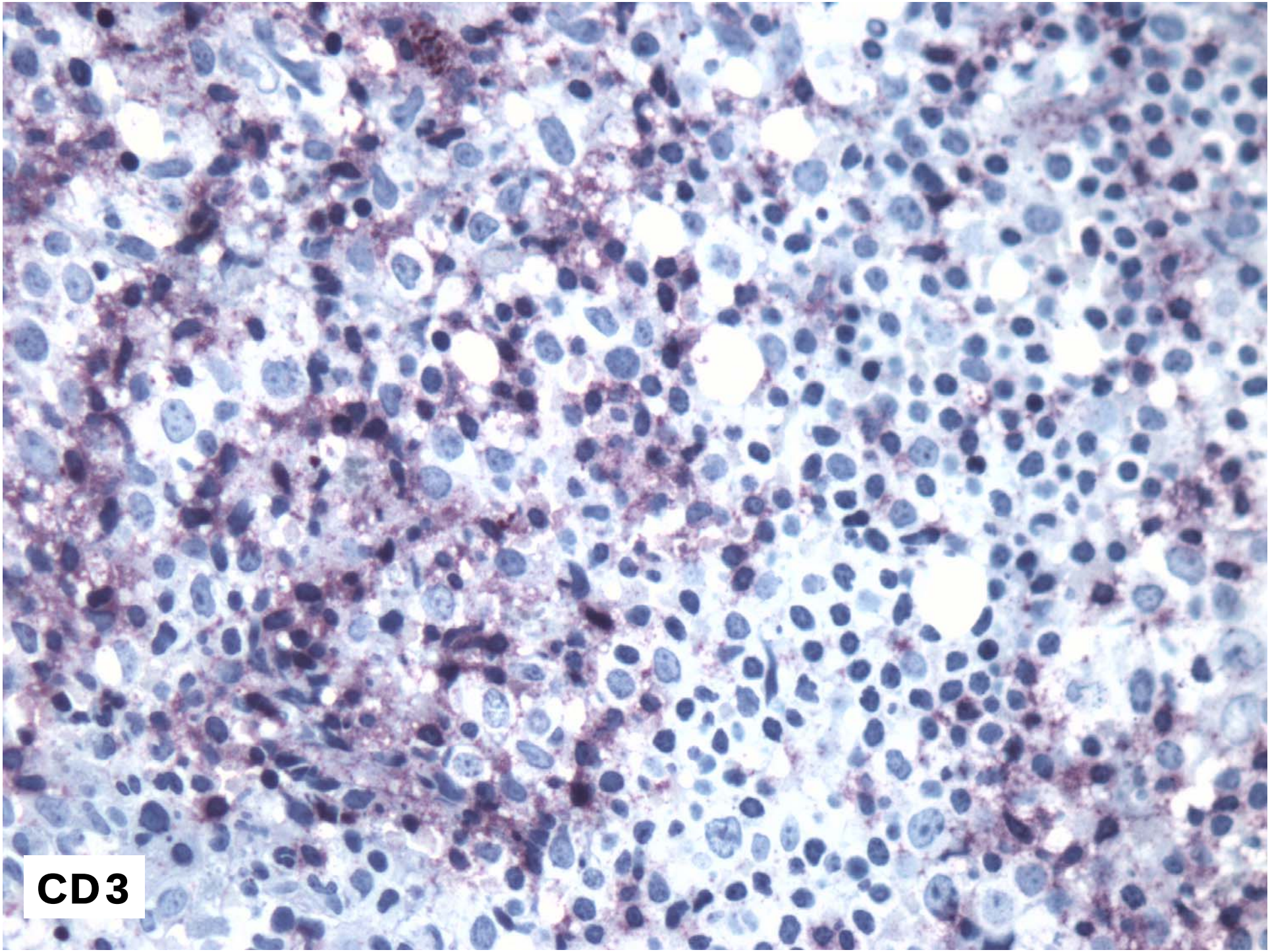
- Evaluation of stained slides:

  - **Ki-67**
  - **CD117**
  - **CD3**
  - **CD20**
  - **CD34**
  - **CD61/F8-ra**

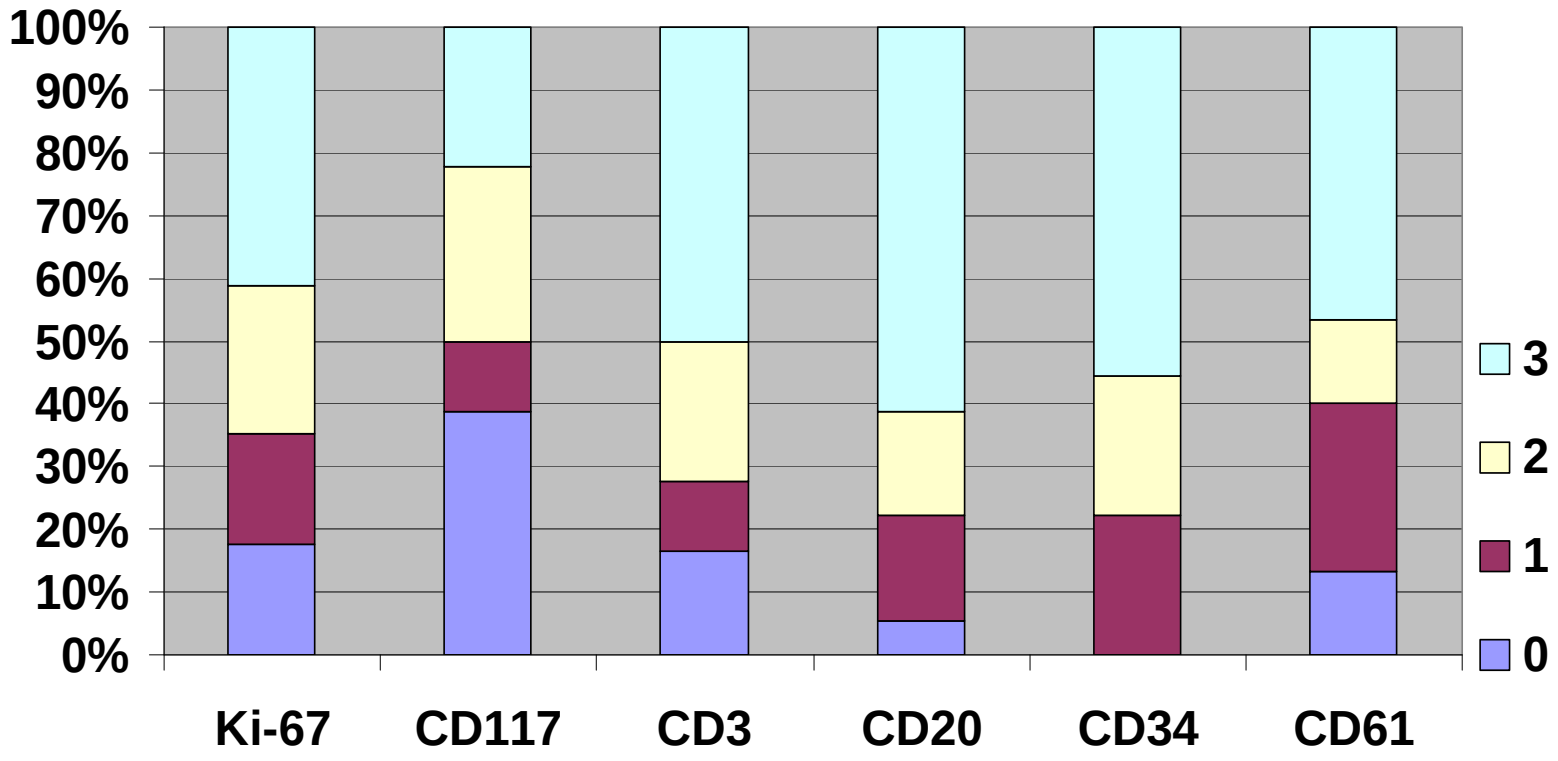
EBMWG



CD3


CD3

SUMMARIZED RESULTS



3= optimal, 2=good, 1=suboptimal, 0=poor

Center	Ki-67	CD117	CD3	CD61	CD34	CD20	Suboptimal/Poor	Total
1	1	1	3	1	1	1	5	6
2	1	0	2	2	1	2	6	6
3	1	3	3	3	3	3	1	6
4	3	1	3	3	2	3	1	6
5	2	2	2	2	3	2	0	6
6	2	3	2	3	3	3	0	6
7	0	0	0	.	3	3	3	5
8	3	2	3	3	3	3	0	6
9	3	0	3	0	2	3	2	6
10	3	2	0	1	3	3	2	6
11	2	2	2	.	3	3	0	5
12	0	0	1	.	2	2	3	5
13	3	0	3	1	1	1	4	6
14	0	3	3	0	3	3	2	6
15	.	0	0	1	1	0	5	5
16	2	2	3	3	3	3	0	6
17	3	3	1	3	3	1	2	6
18	3	0	3	3	2	3	1	6

Total 35.50% **104**

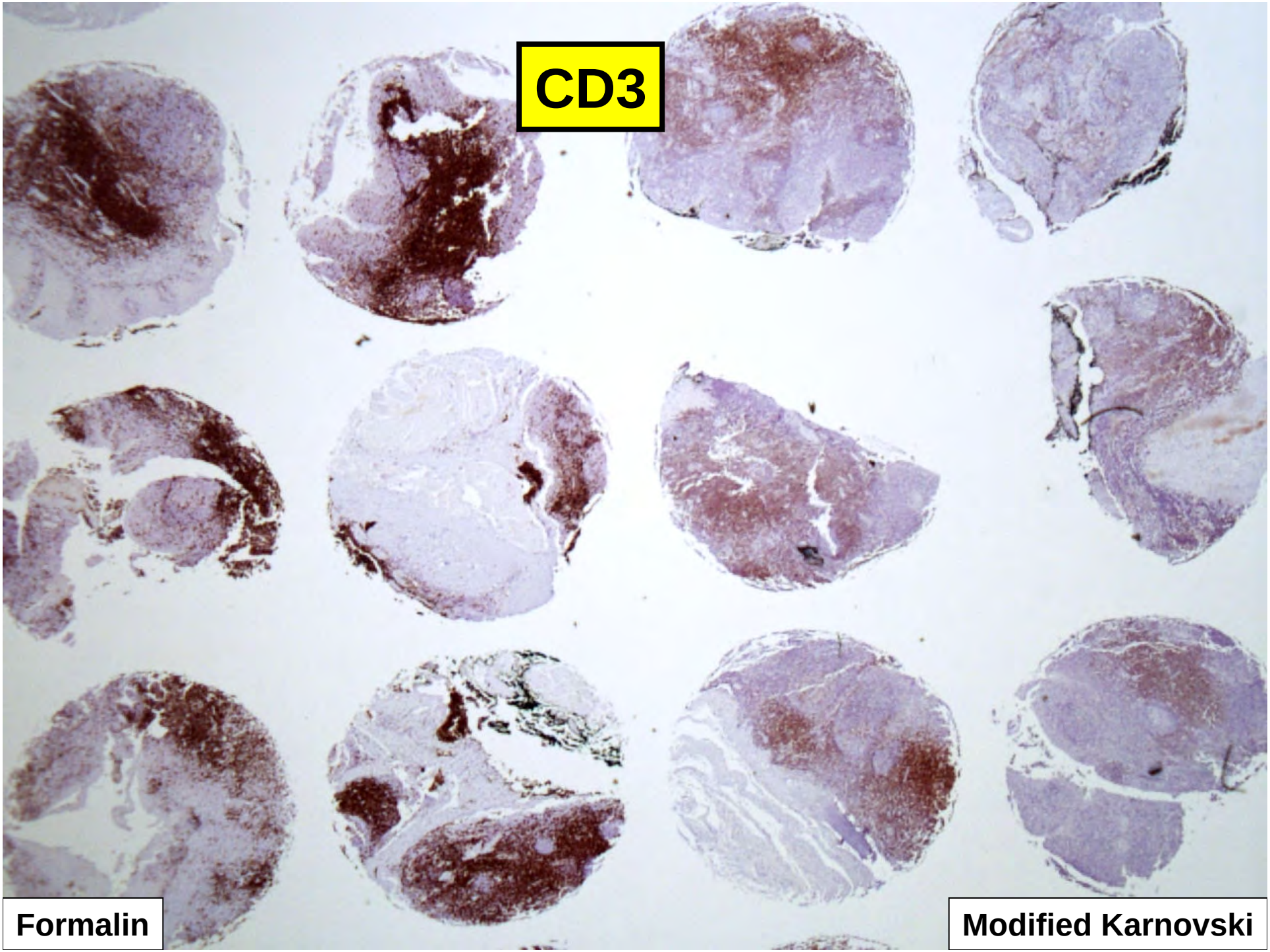
Standardization of Bone Marrow Immunohistochemistry

International Council for Standardization in Haematology (ICSH) Working Party for Standardization of Bone Marrow Immunohistochemistry

Emina Torlakovic, MD, PhD, FCAP – Canada (co-chair)
Anna Porwit, MD, PhD -- Sweden (co-chair)
Szu-Hee Lee, MBBChir, PhD, FRCPEdin, FRCPath, FRCPA -- Australia
Marciano Reis, MD, PhD, FRCPC – Canada
Hans Kreipe, MD, PhD – Germany
Kikkeri N. Naresh, MBBS, CCP, MD, FRCPath – United Kingdom
Alexander Tzankov, MD -- Switzerland
Yoshito Sadahira, MD -- Japan
Elizabeth Hyjek, MD, PhD – USA
Russell K. Brynes, MD – USA
Robert McKenna, MD -- USA

Literature Review: Methods' Description is Insufficient

	Not Described (%)	Significance for IHC Results
Fixation **Fixaton time**	27 75	**Essential**
Decalcification **Decalcification time**	52 72	**Essential**
Positive controls	87	**Very important**
Negative controls	80	**Very important**
Pretreatment methods	48	**Essential**
Pretreatment buffer	61	**Essential**
Pretreatment time	68	**Essential**
Detection system	17	**Essential**


CD3
Formalin
Modified Karnovski

High Expectations of Accuracy: To Treat or Not Treat?

- Sensitivity and specificity of most tests is not defined
- When possible to calculate sensitivity and specificity, standards are not set or not universally agreed upon
- There is no tracking of clinical impact of reported IHC tests
- There is no tracking of clinical impact of proficiency testing (PT) results of various programs that provide PT

Targeted Therapy: Anti-ABC

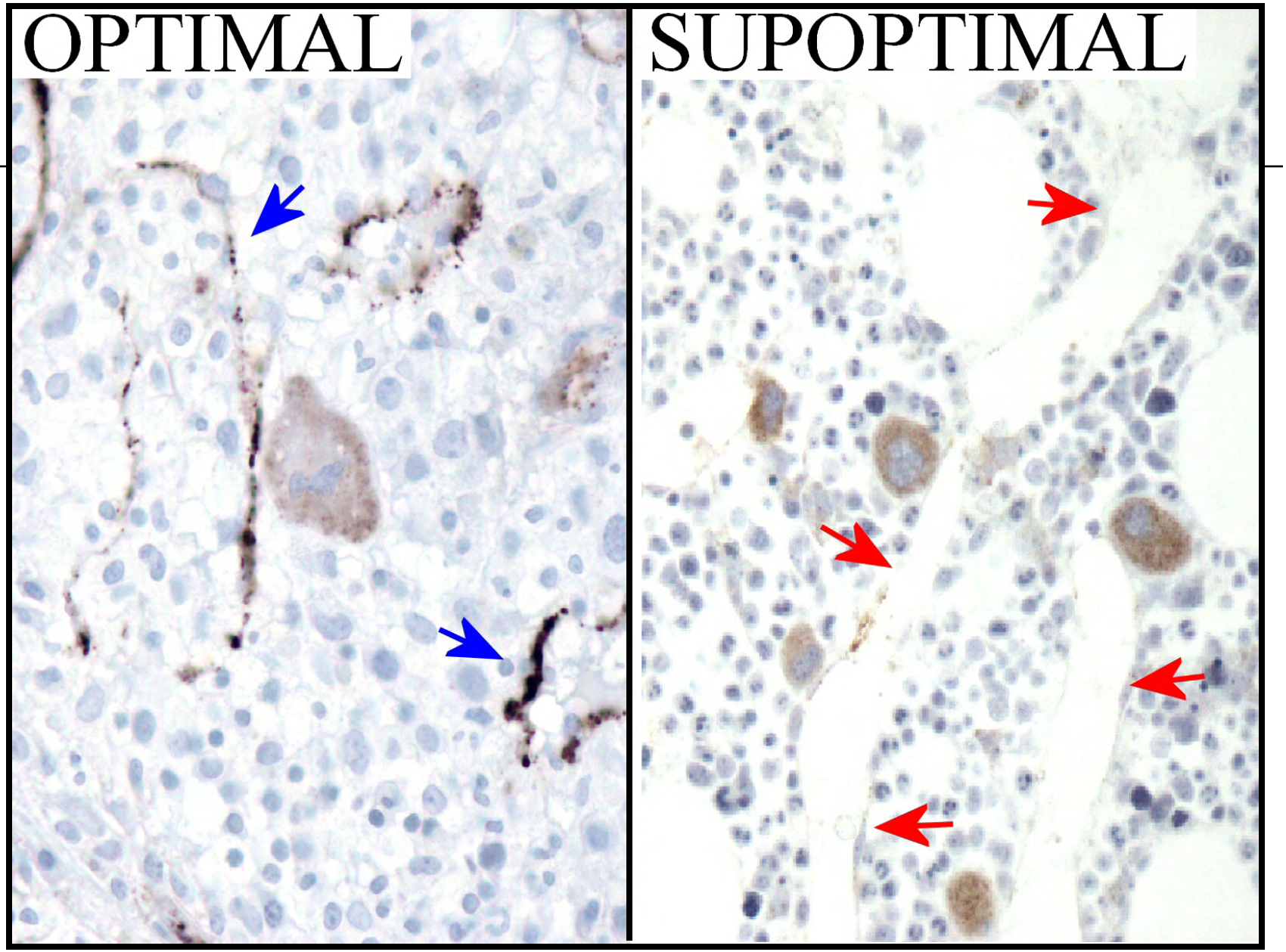
Future

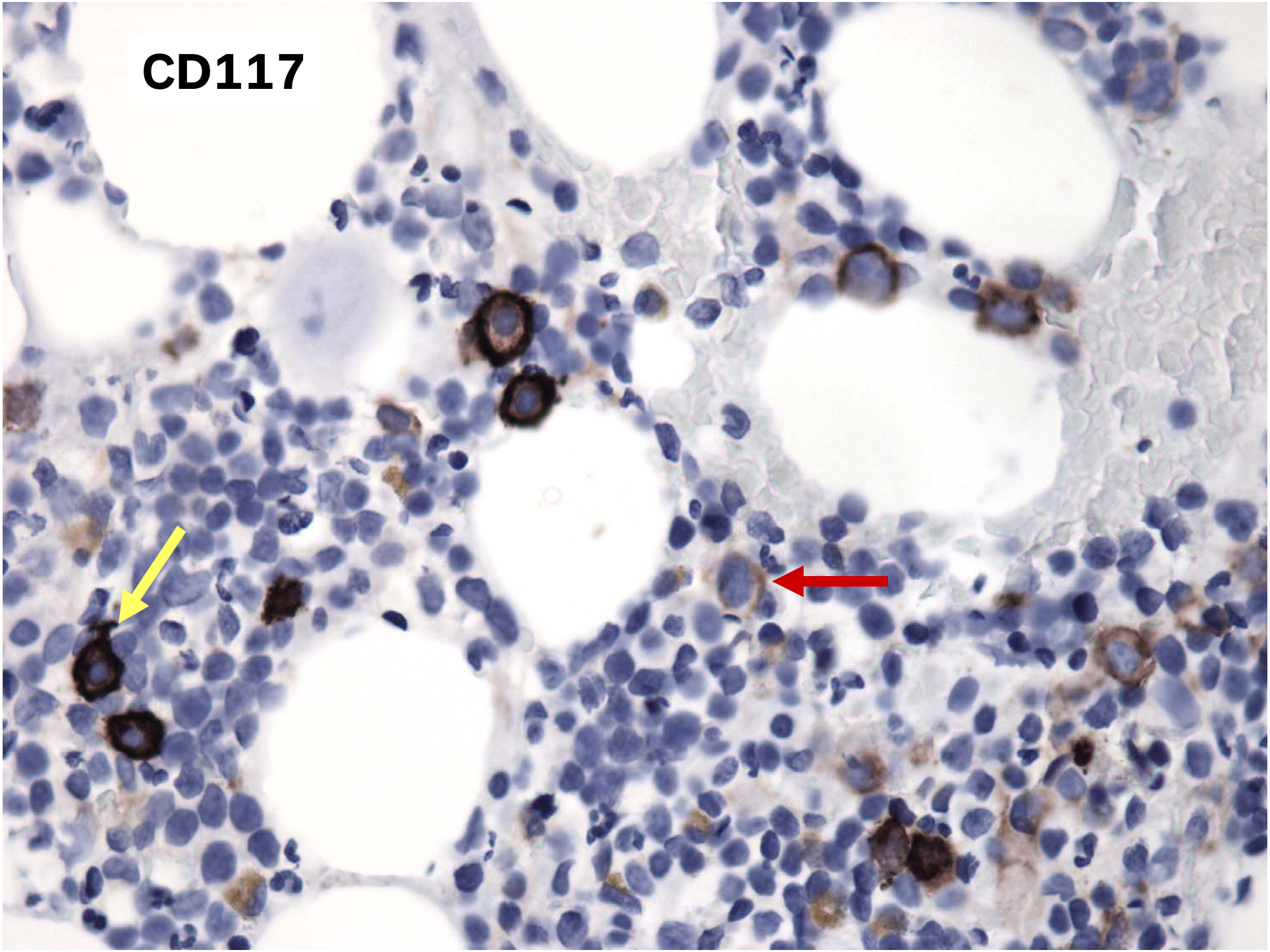
- Patient safety is clearly identified as the no. 1 priority in the design and regulation of laboratory testing by all agencies and organizations
- Standardization needs to address all parameters of the testing (pre-analytical, analytical, and post-analytical)
- QA measures need to be tailored to the test type (Class I vs. Class II) as well as to make biological and statistical sense.

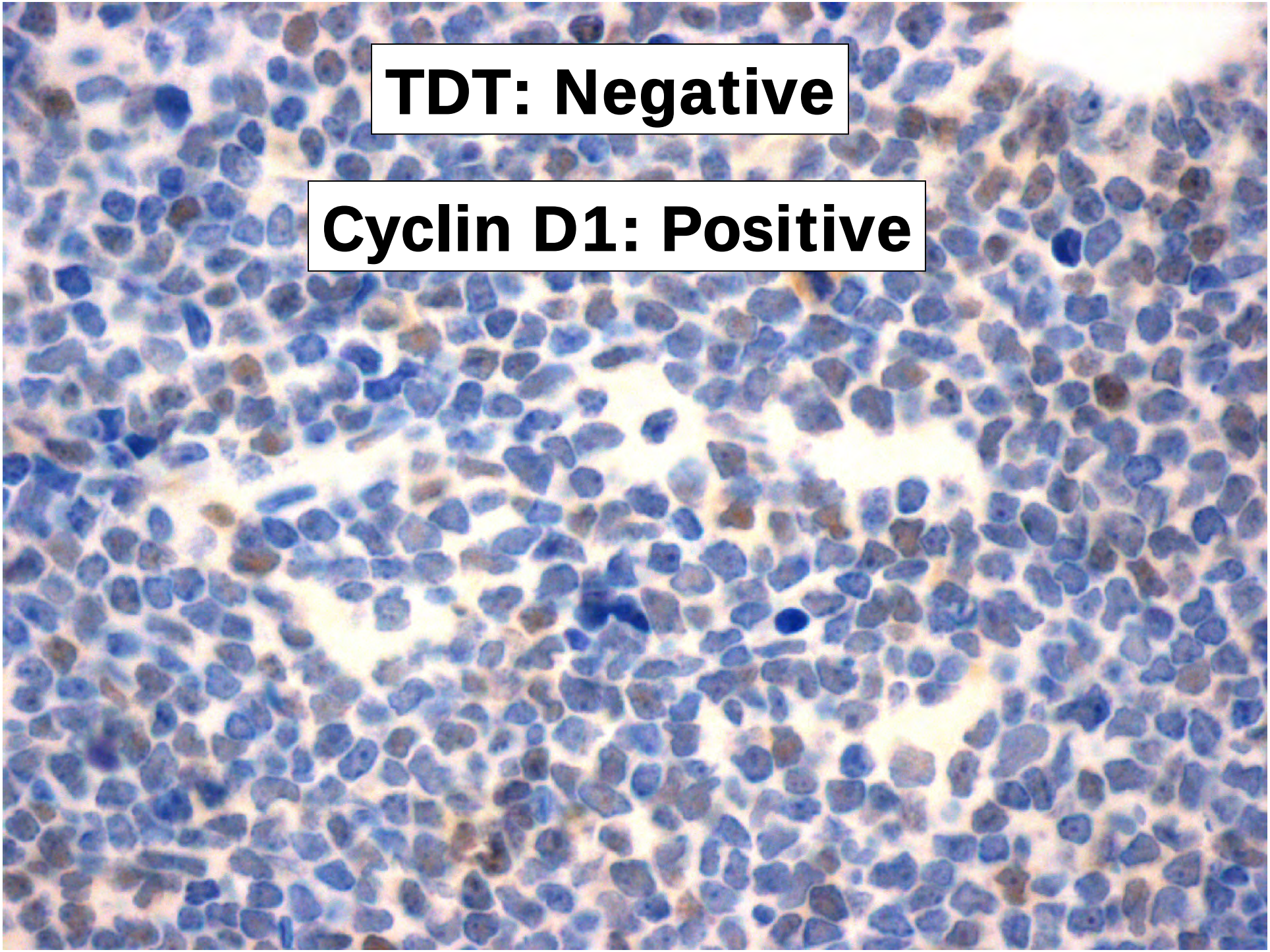
Future: Era of -OMICS

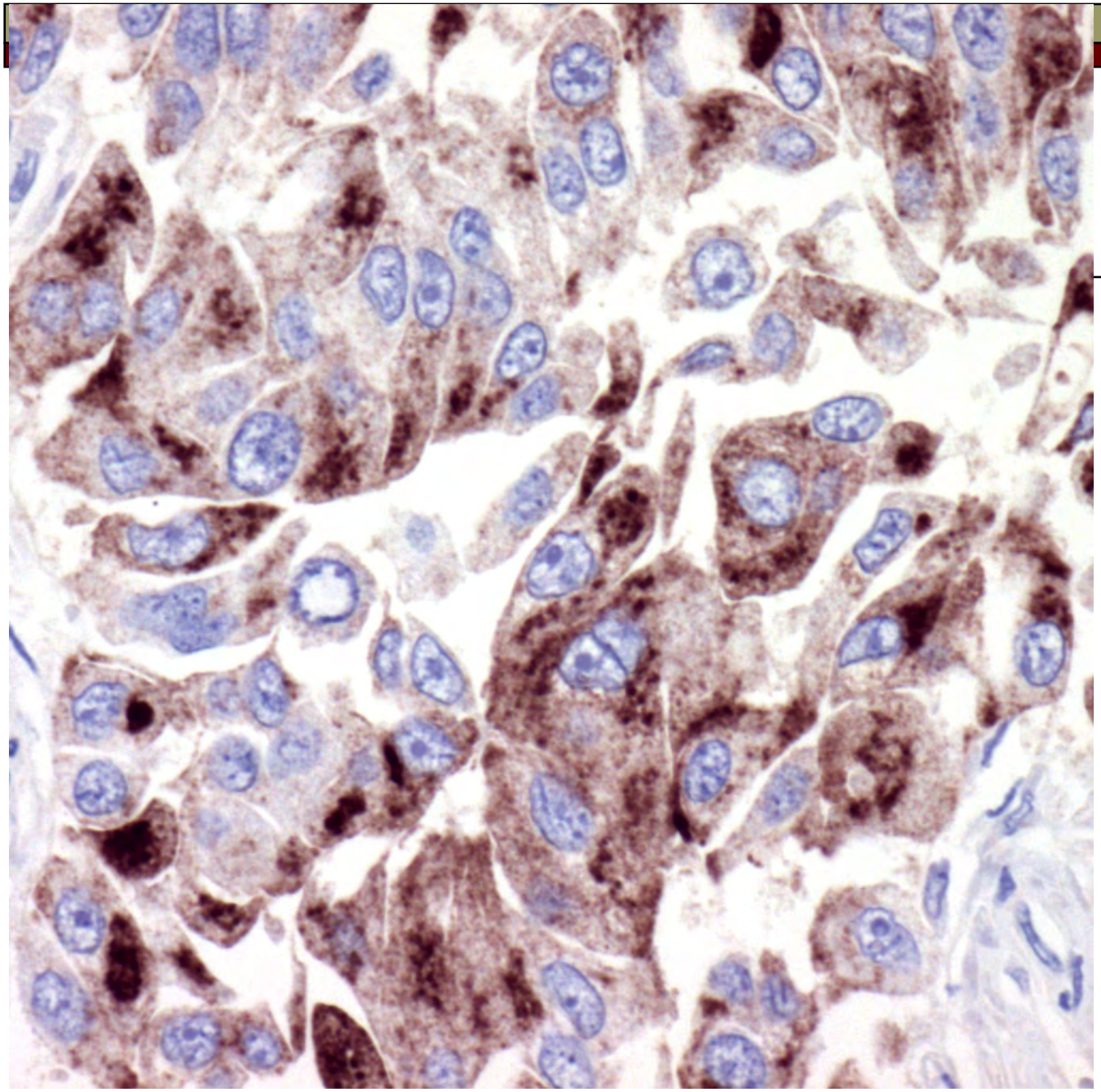
- In situ demonstration of protein expression
- -Omics studies at the moment are relatively expensive and discovery-focused
- IHC required to confirm data obtained by other methods including standard NB, WB, and SB
- Omics studies narrow our focus from large scale to small scale (most important genes)
- Many “most important genes” are being detected by immunohistochemistry

Paying Attention & Common Sense

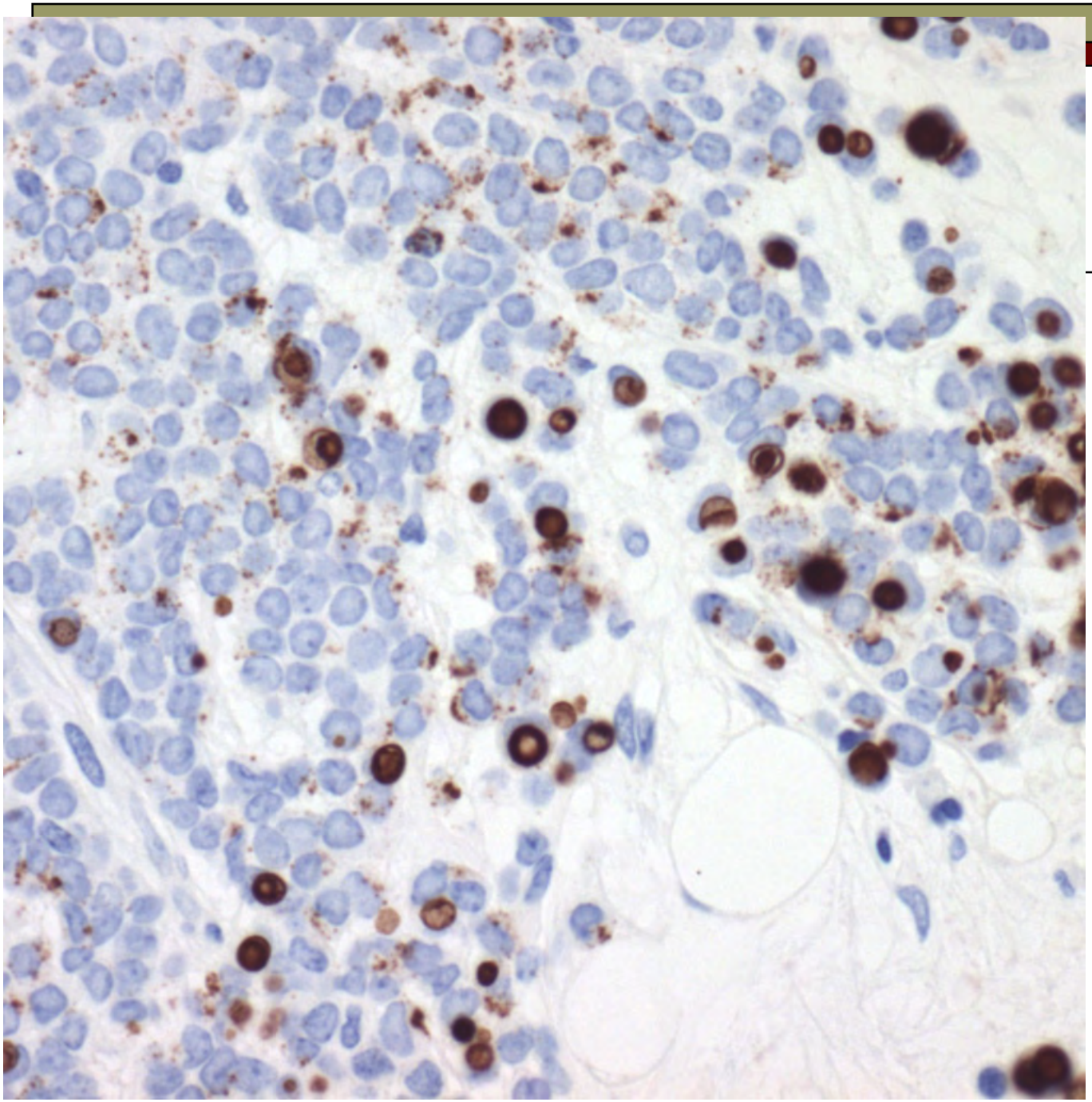

OPTIMAL
SUPOPTIMAL


CD117

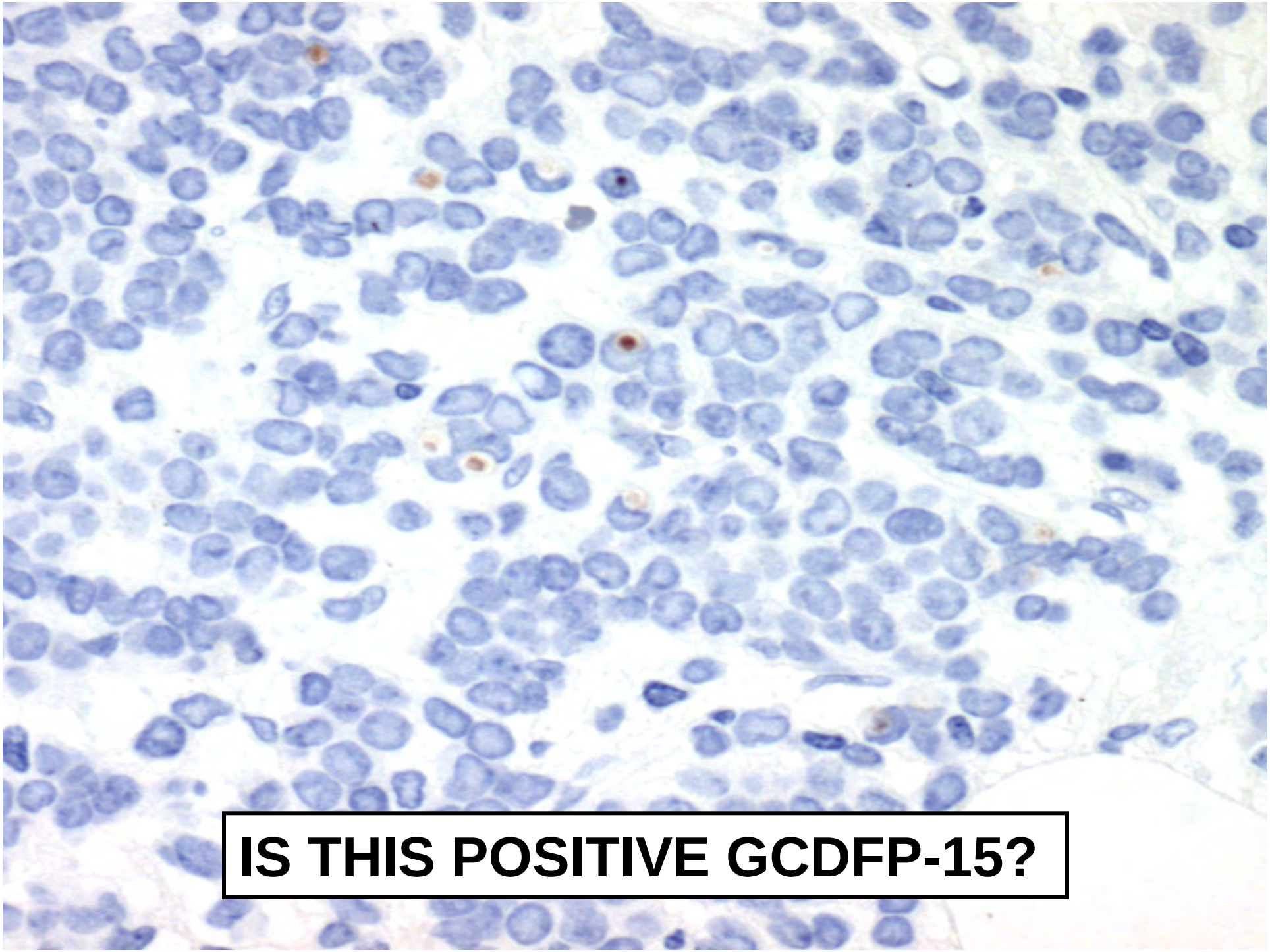

TDT: Negative
Cyclin D1: Positive



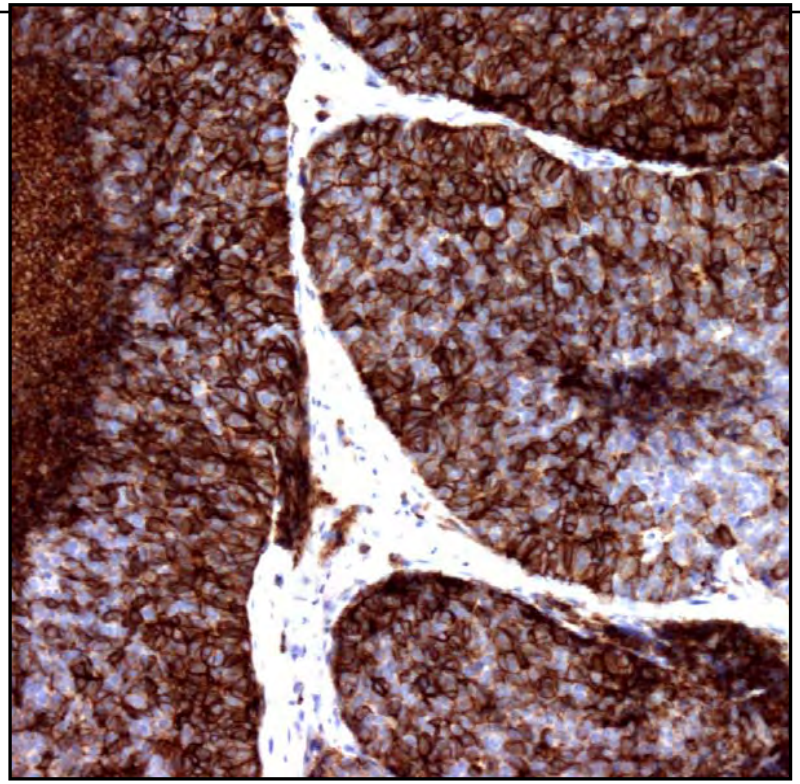
AP-15 in Breast CA: Apocrine Pattern



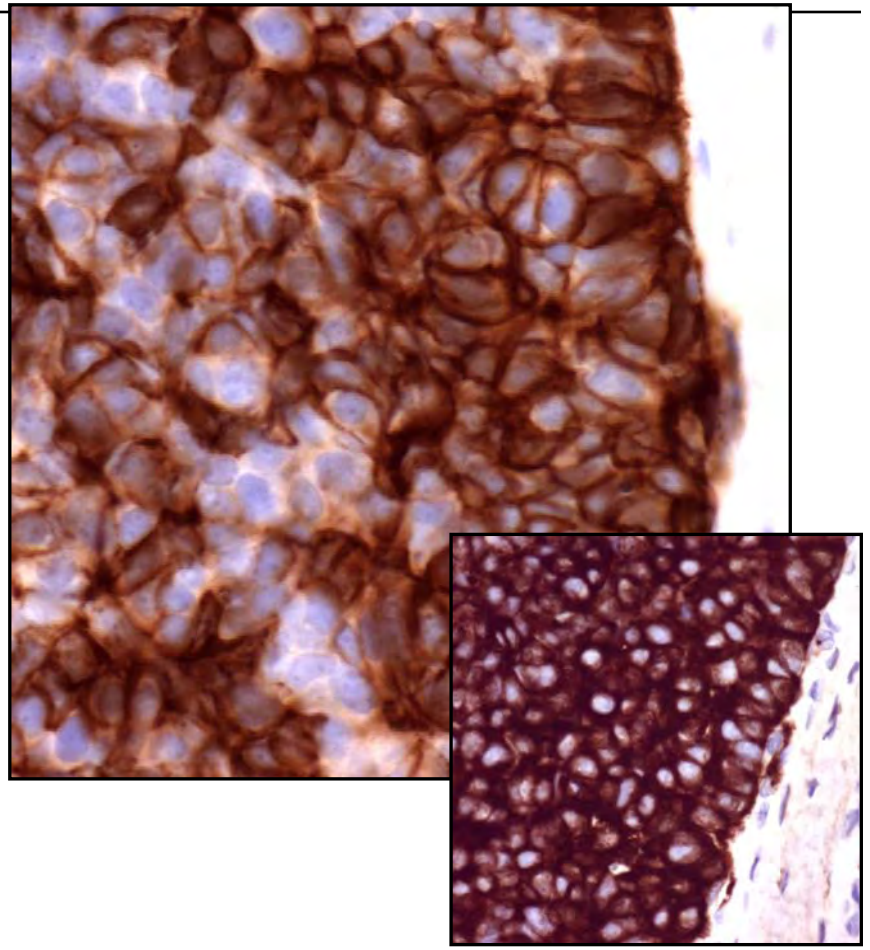
AP-15 in Breast CA: Intracytoplasmic Luminal Pattern


IS THIS POSITIVE GCDFP-15?

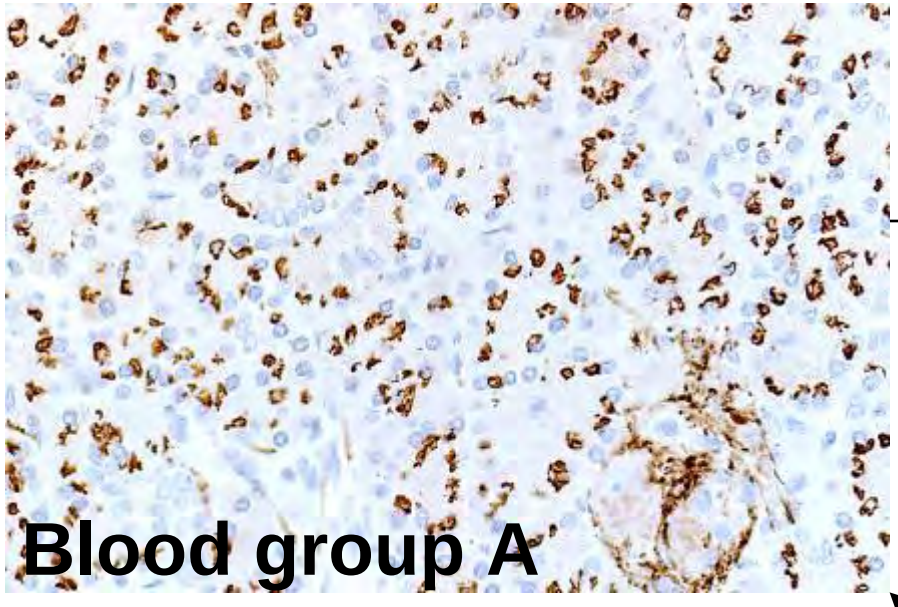
Allow for Biological Variation or Unknown Technical Arterfact



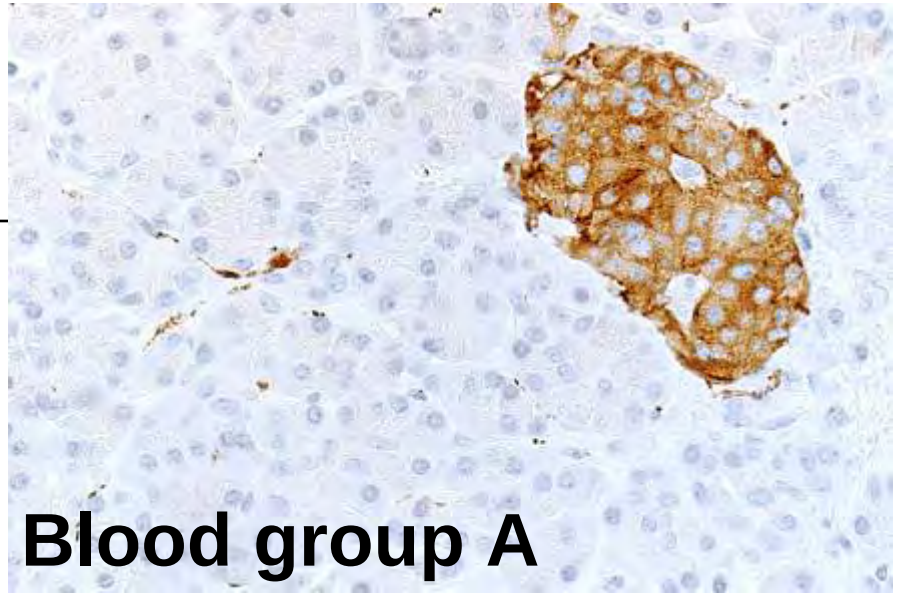
CD45 in SCC



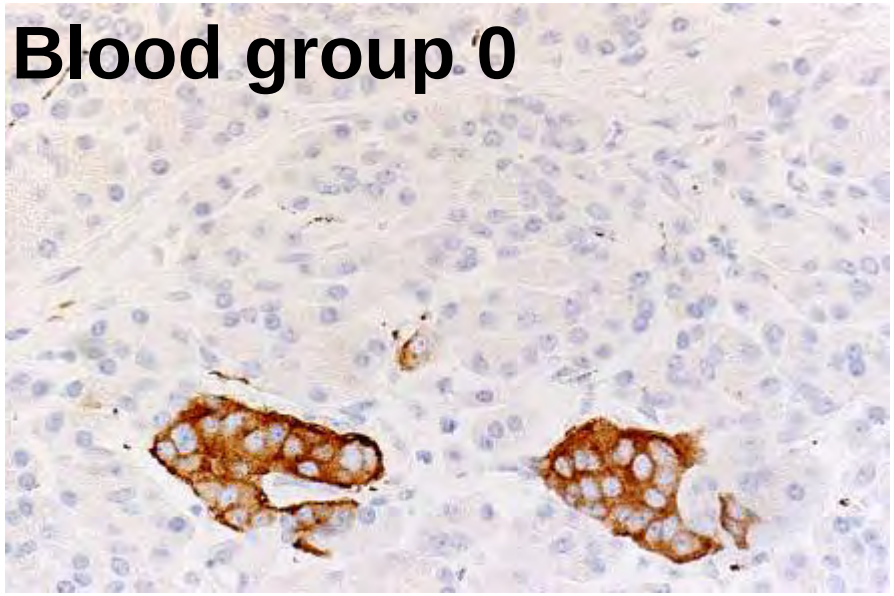
panCK



Snp88



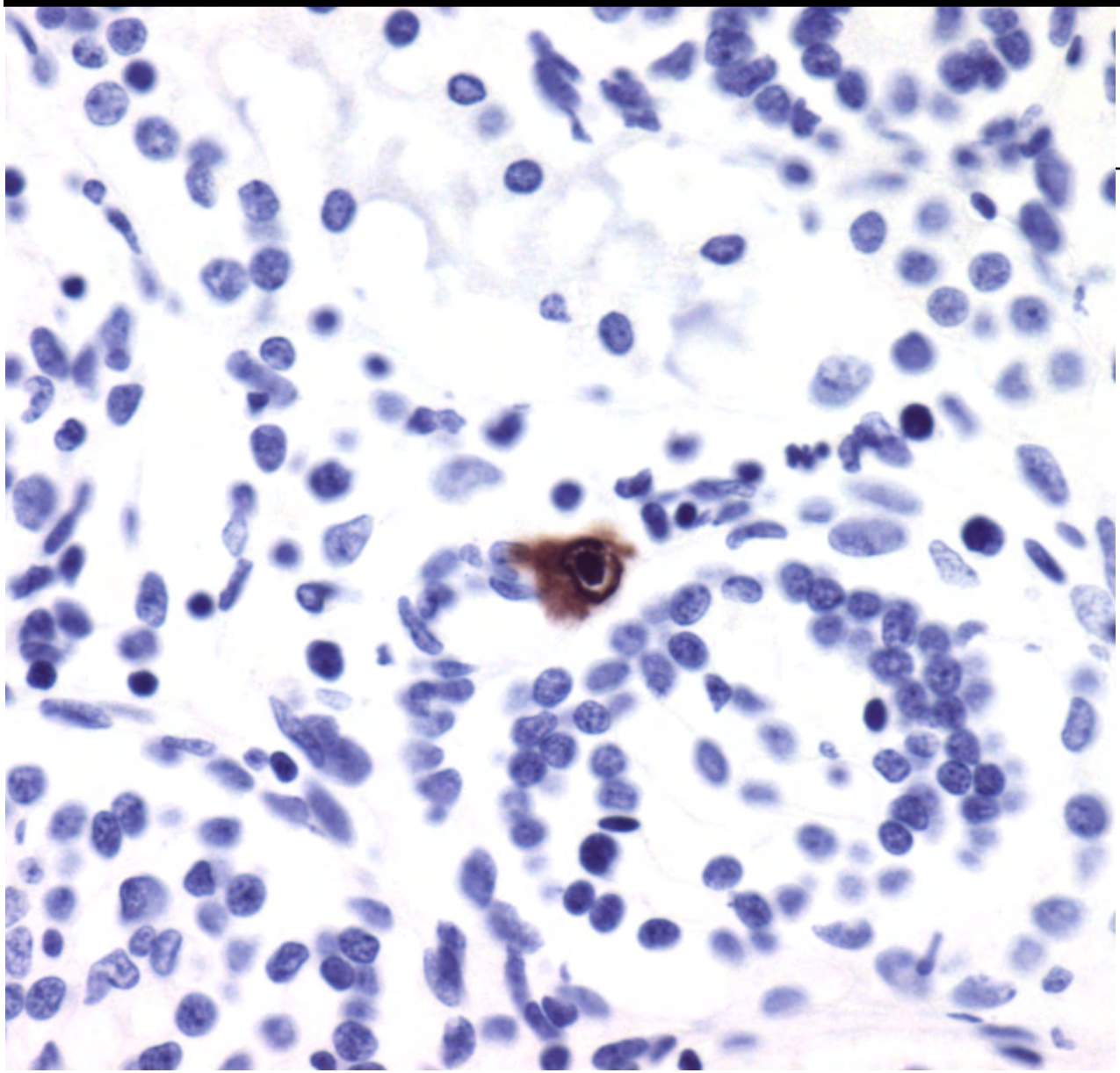
27G12



MAG

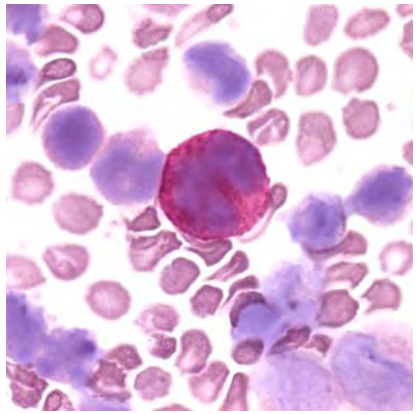
Synaptophysin

Single Cell Positivity: YES and NO

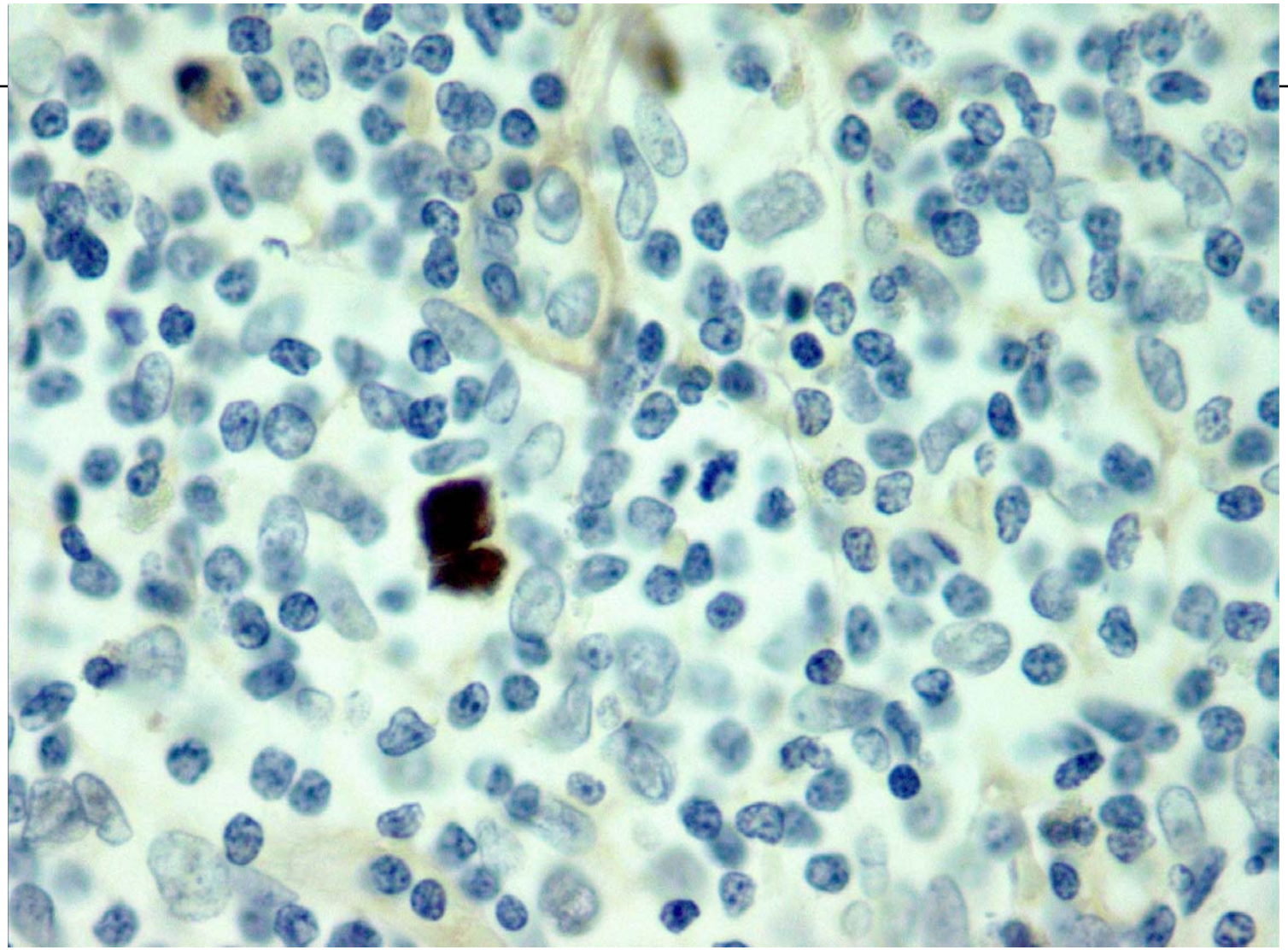


CMV (Kidney Bx): YES

CD30 in BM aspirate: NO



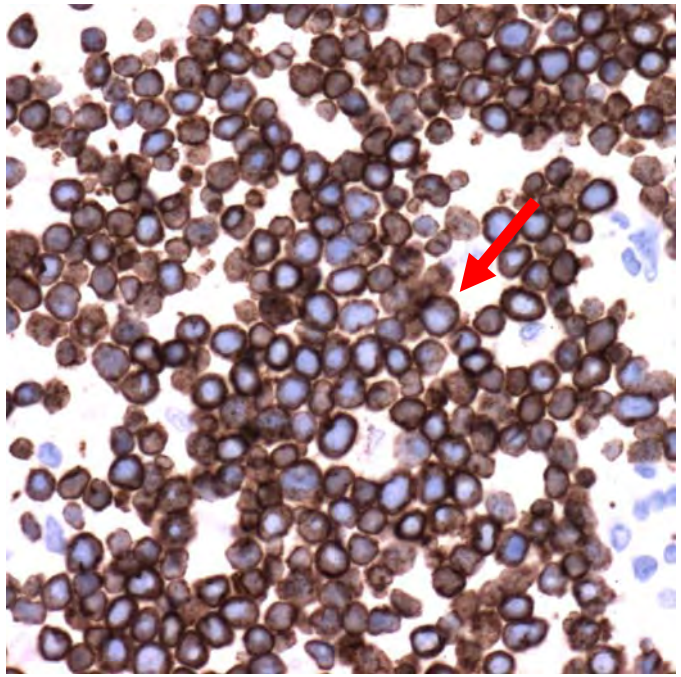
EBER in cHL: YES



Membranous vs. Cytoplasmic vs. Nuclear vs. Extracellular

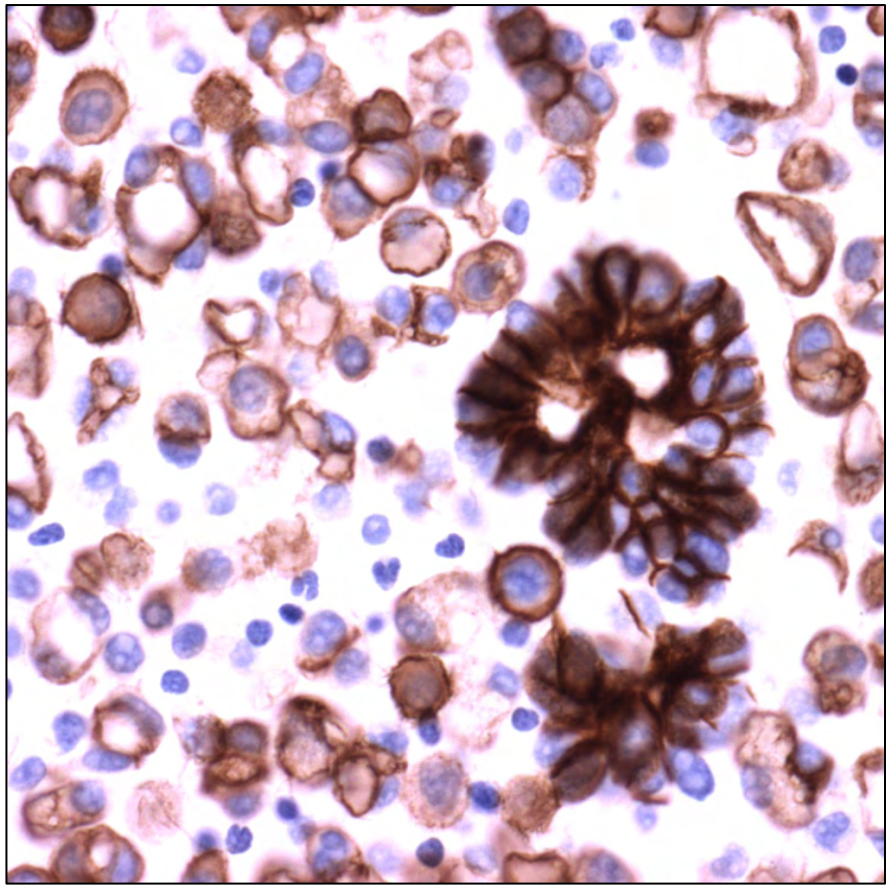
- CD3: could be both, membranous and cytoplasmic
- CK: cytoplasmic only
- CD30: Golgi,cytoplasmic, membranous
- CD20: membranous only
- PSA: any pattern is good

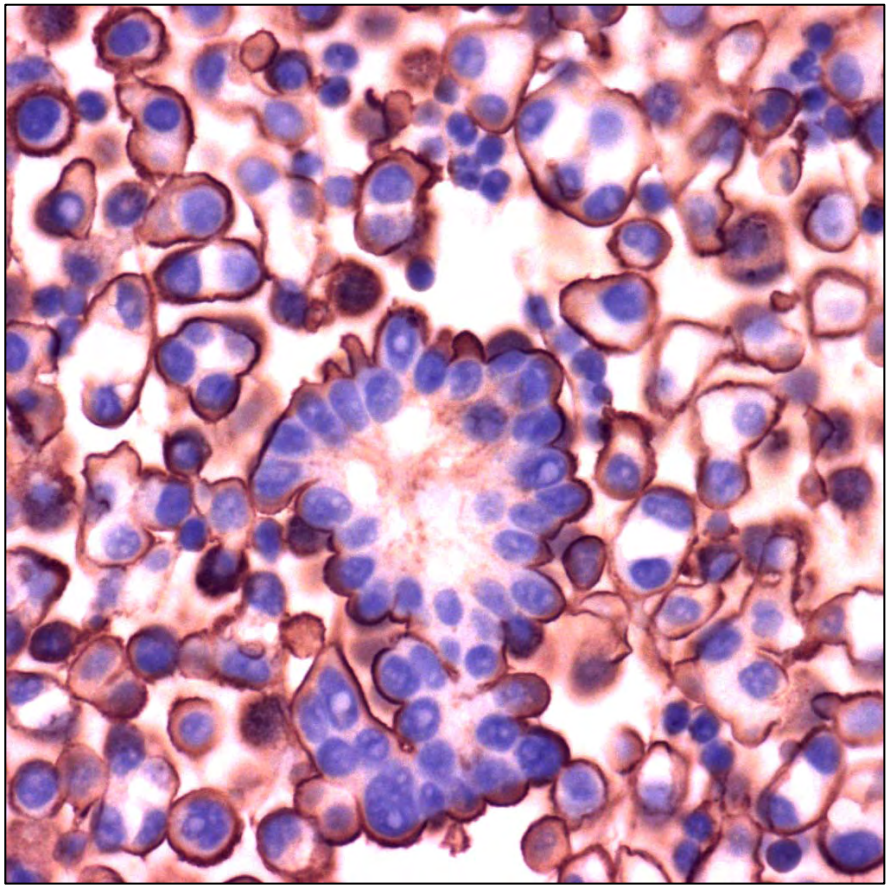
CD3



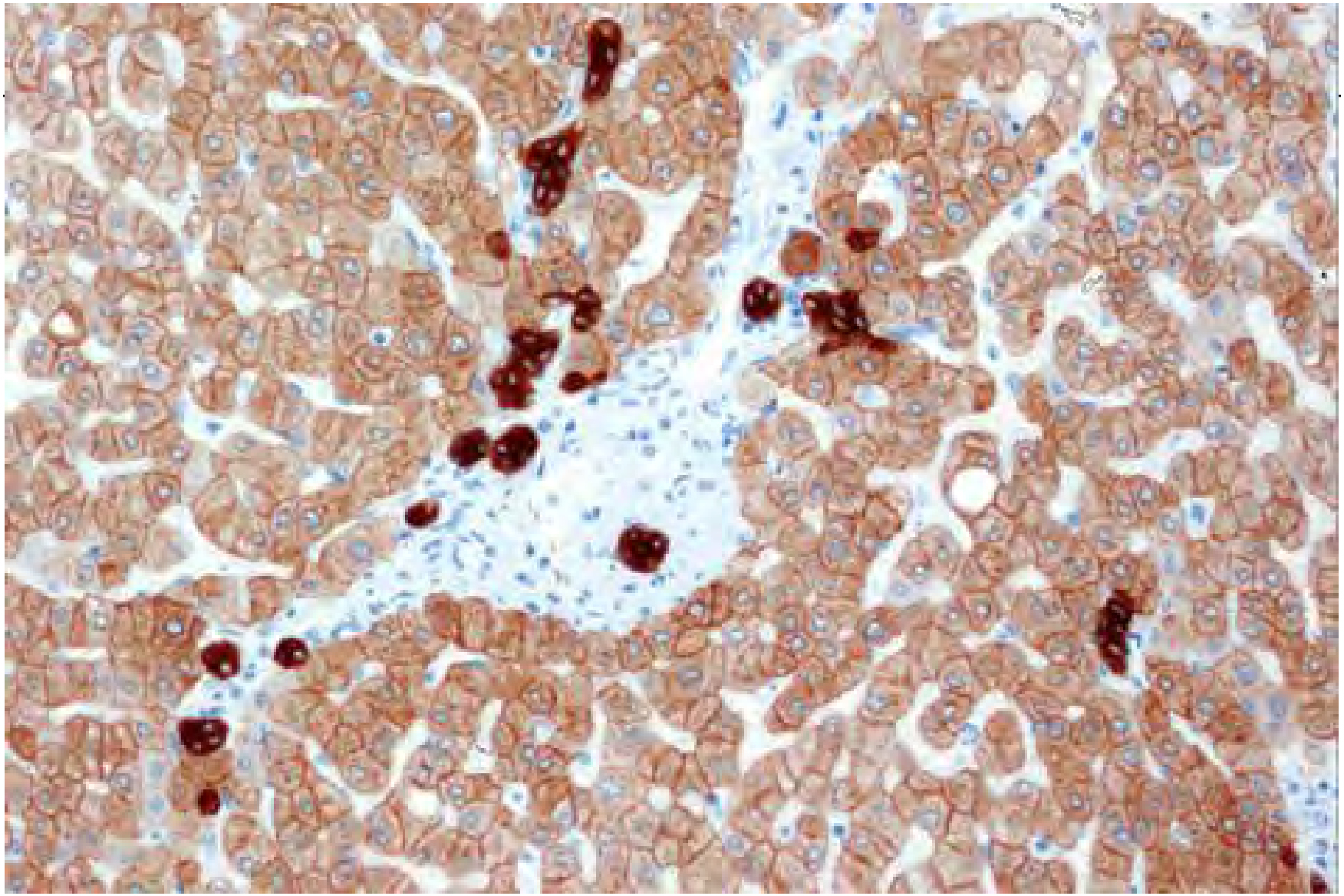
Membranous

Ber-EP4 **CA-125**

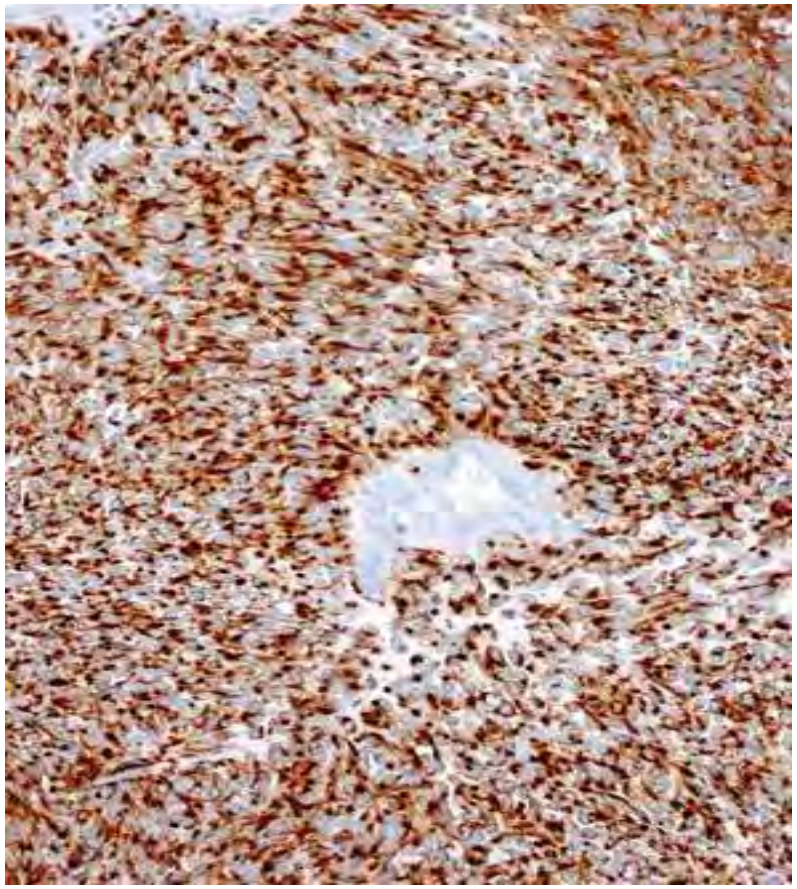


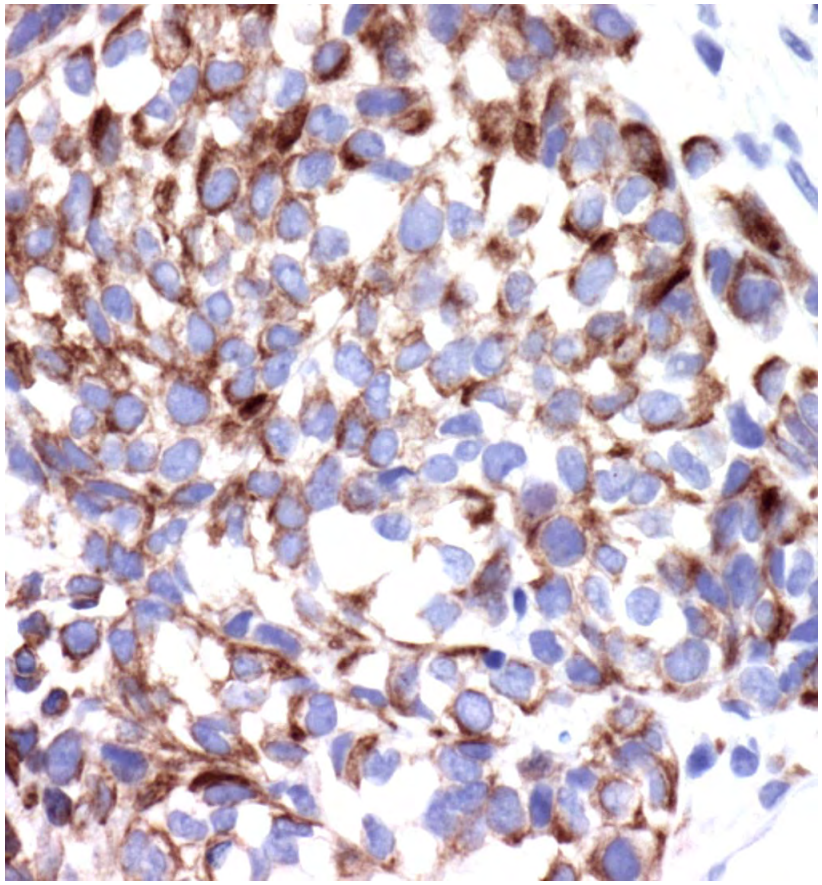


Membranous: Cytokeratins

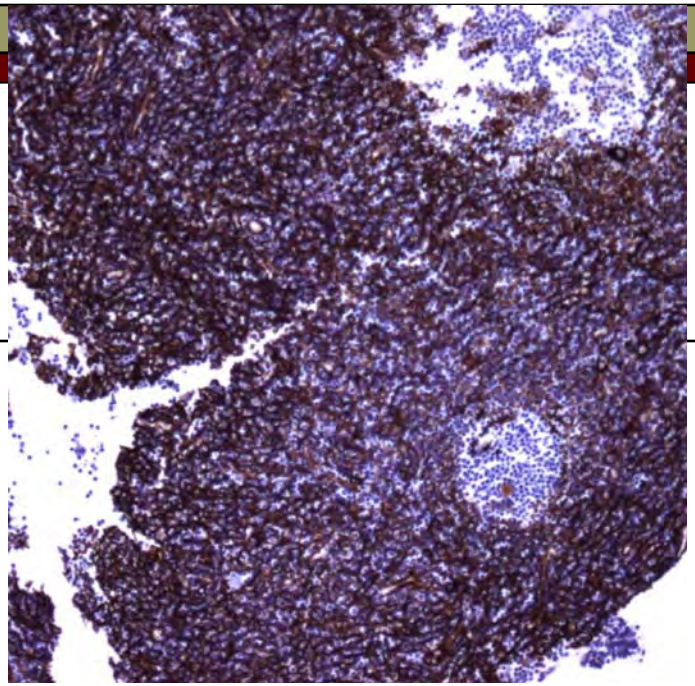


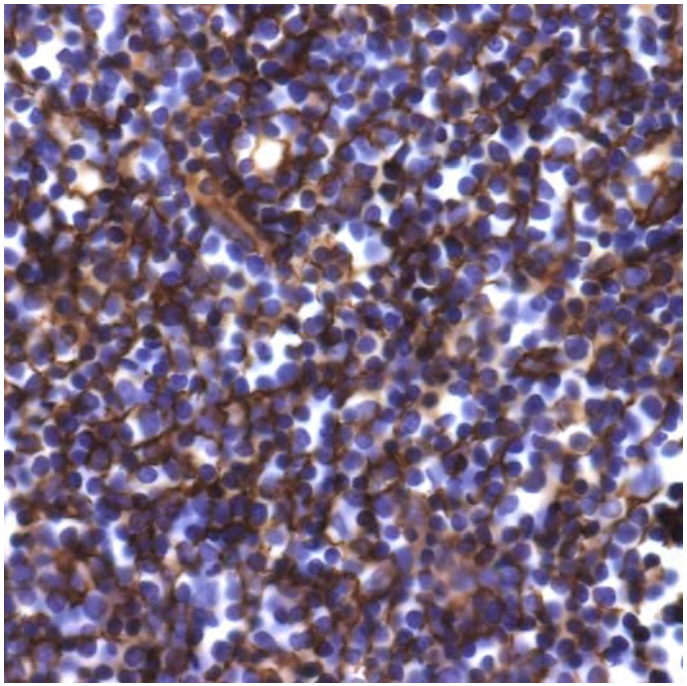
Cytokeratin: Dot-like & Filamentous Cytoplasmic

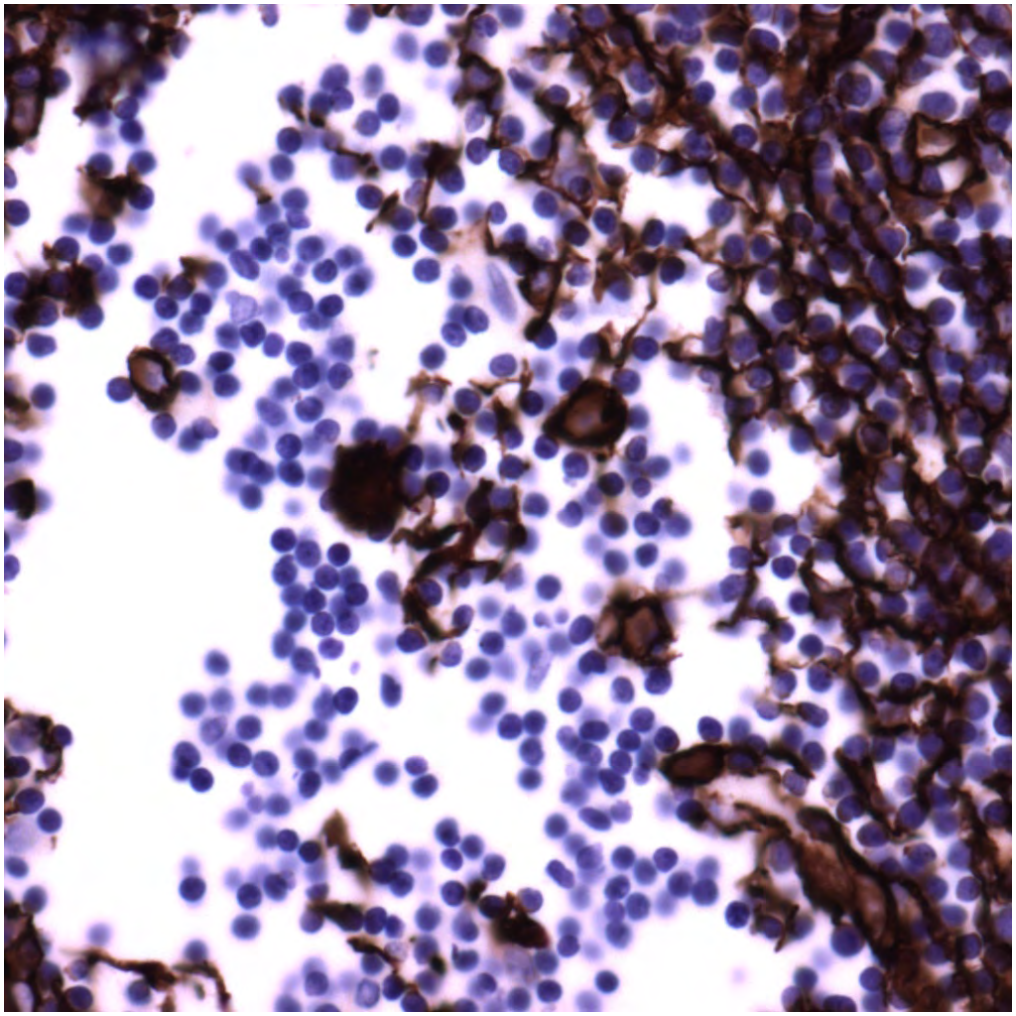




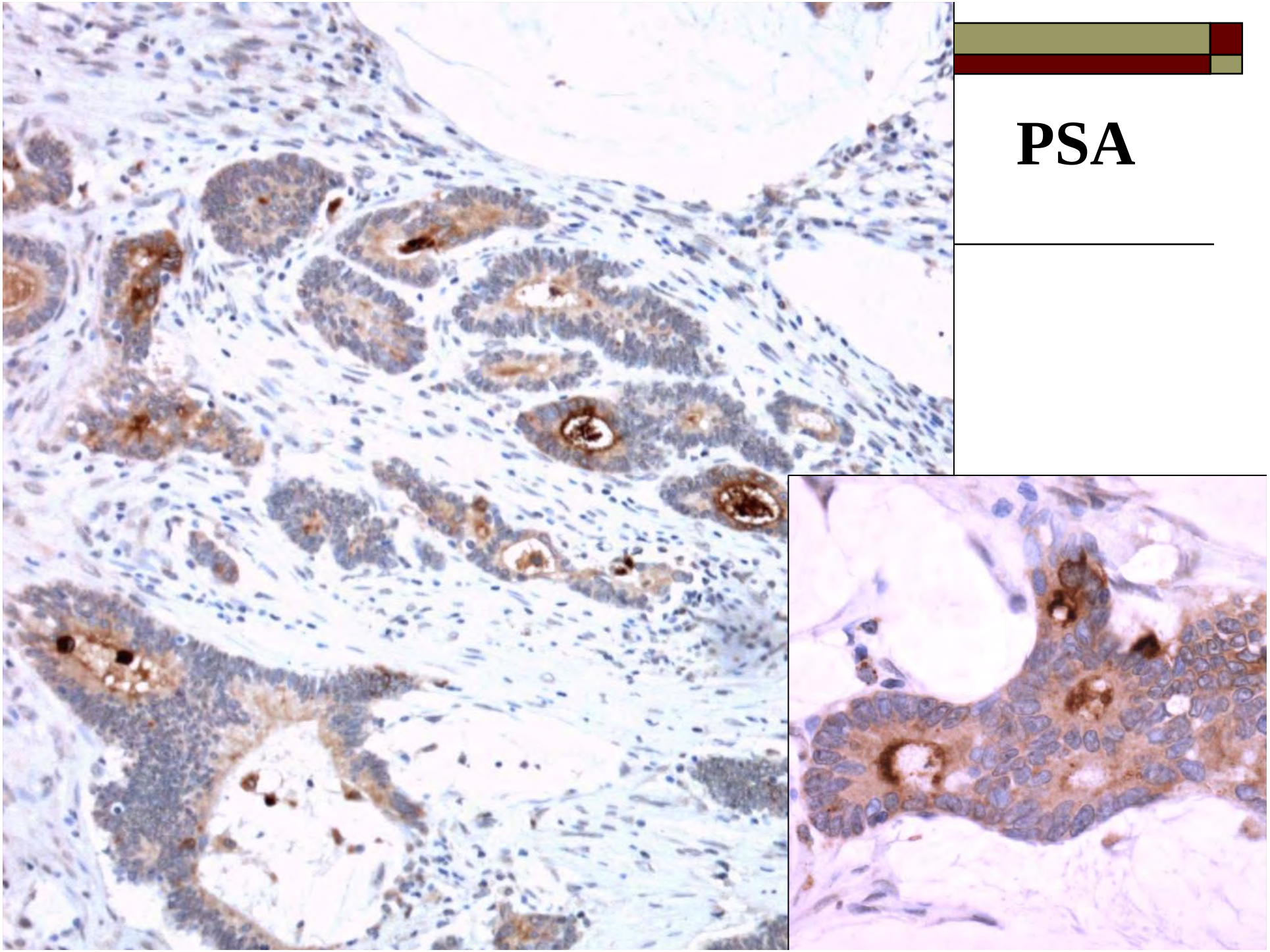
CK in Cortical Type Thymoma: Dendritic

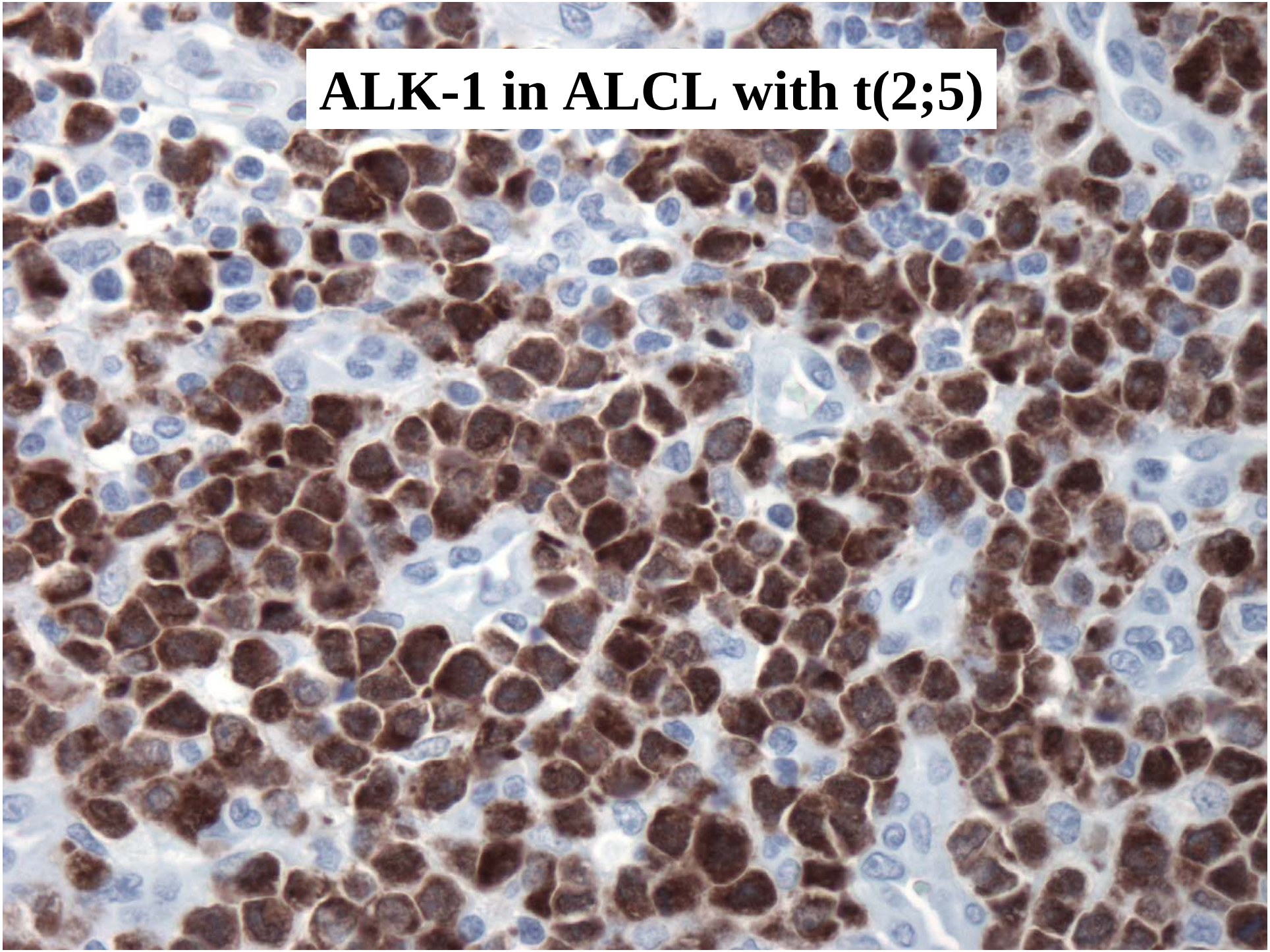






PSA



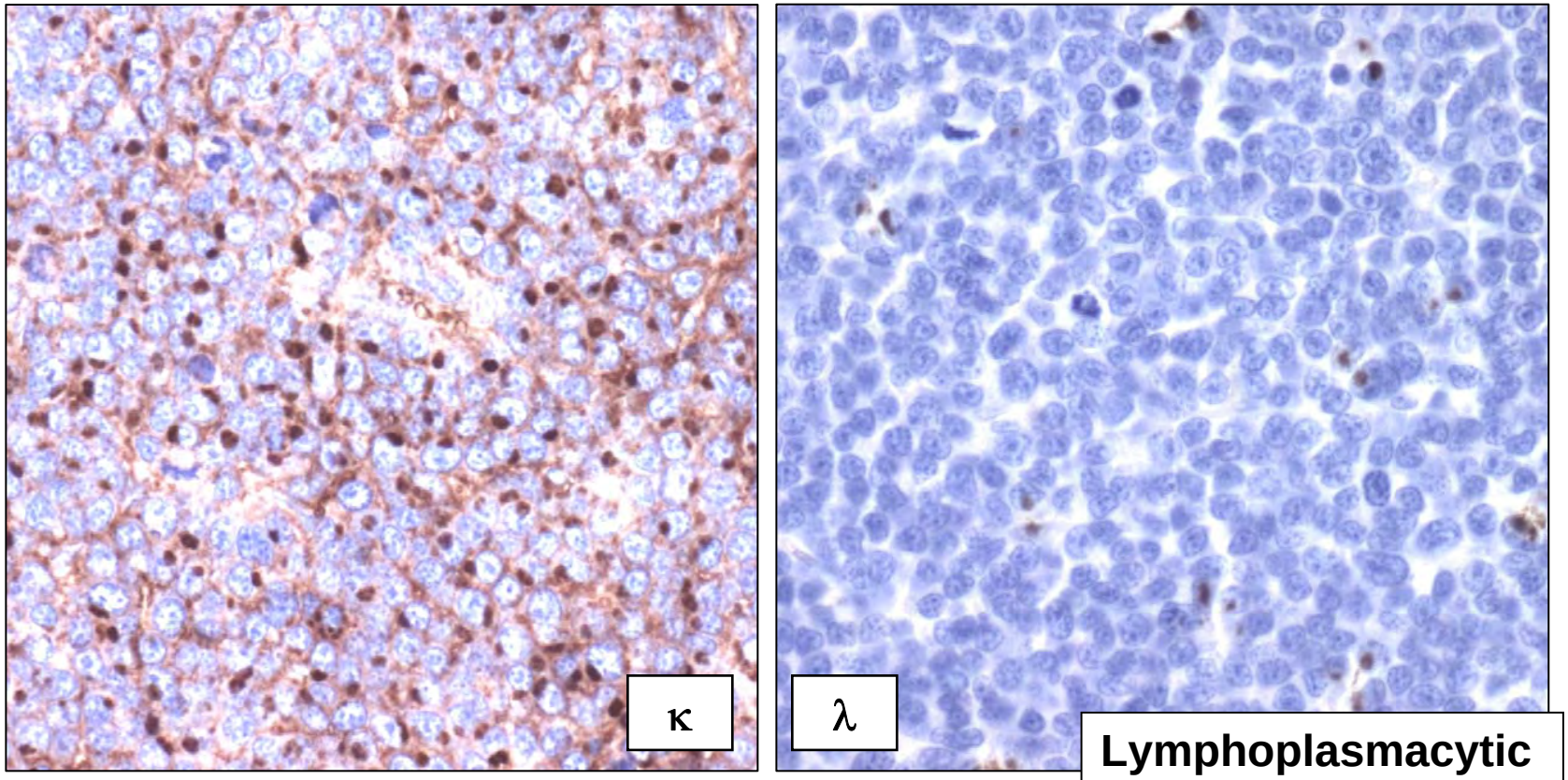

ALK-1 in ALCL with t(2;5)

Other Markers with Cytoplasmic + Nuclear Positivity

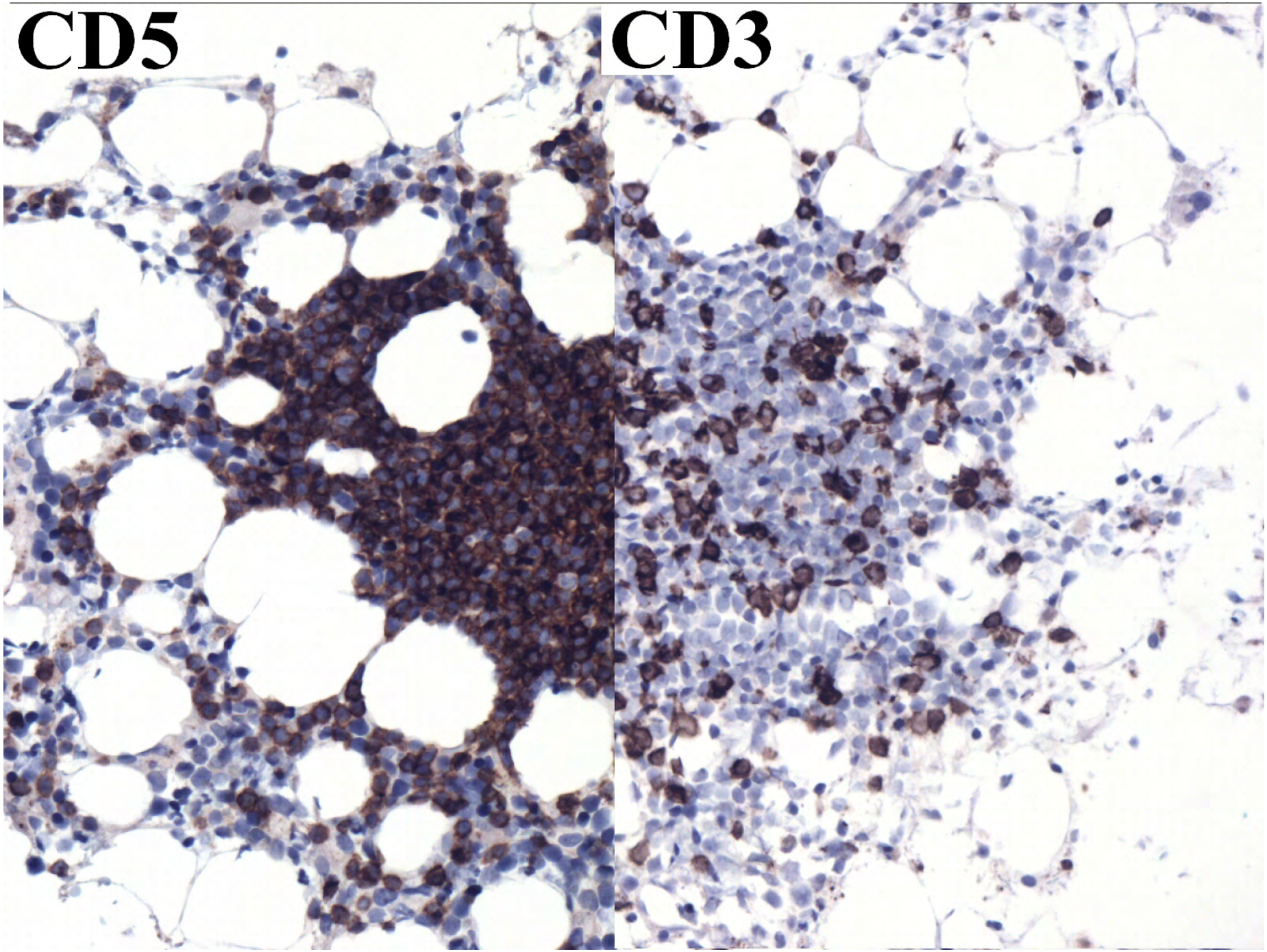
- S-100
- Calretinin
- Mutated NPM-1
- CMV
- Hemoglobin A
- ER/PR and other typically nuclear markers (sometimes)
- Other

Always Interpreted Together

- Kappa and lambda
- CD3, CD20 (and CD5)
- CD4 and CD8…

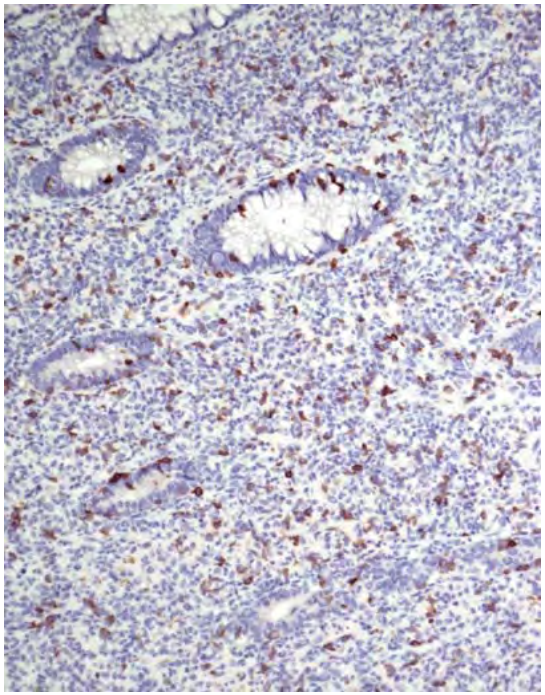


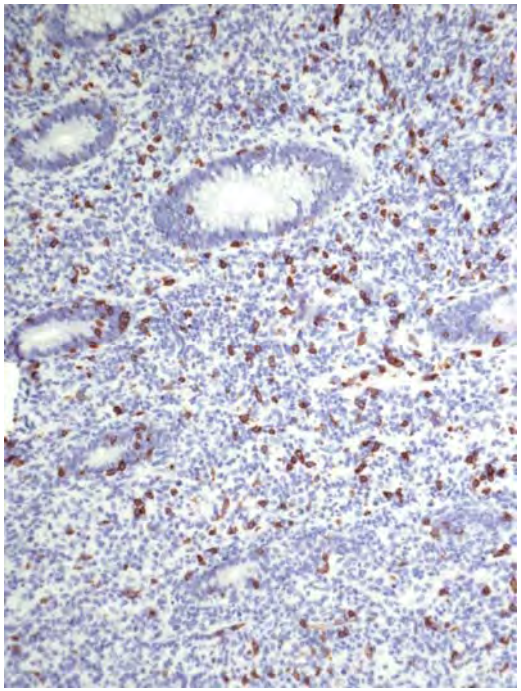
Lymphoplasmacytic lymphoma


CD5
CD3

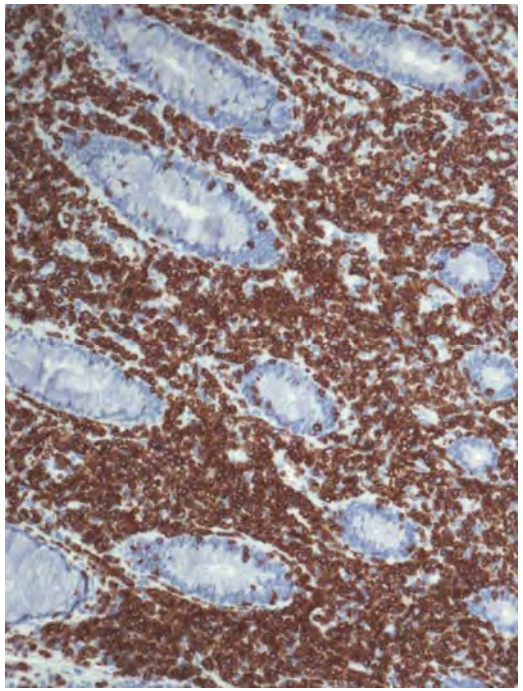
CD3 CD4 CD8

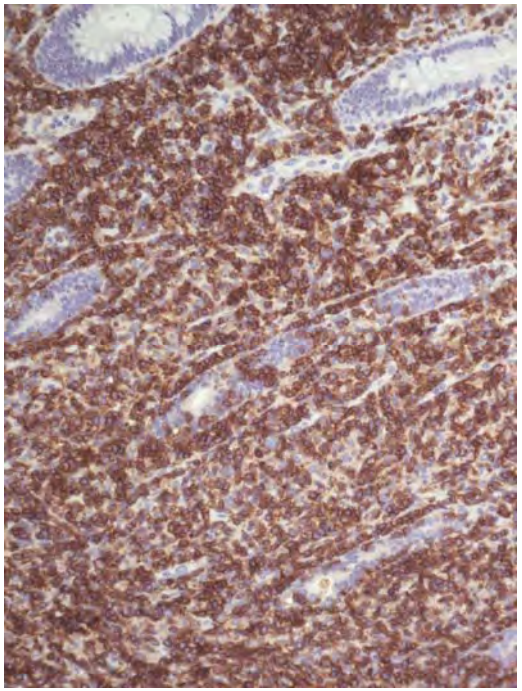
?

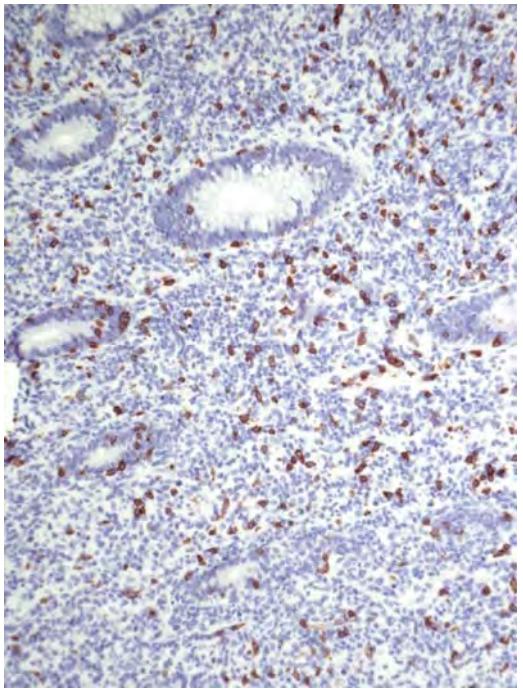




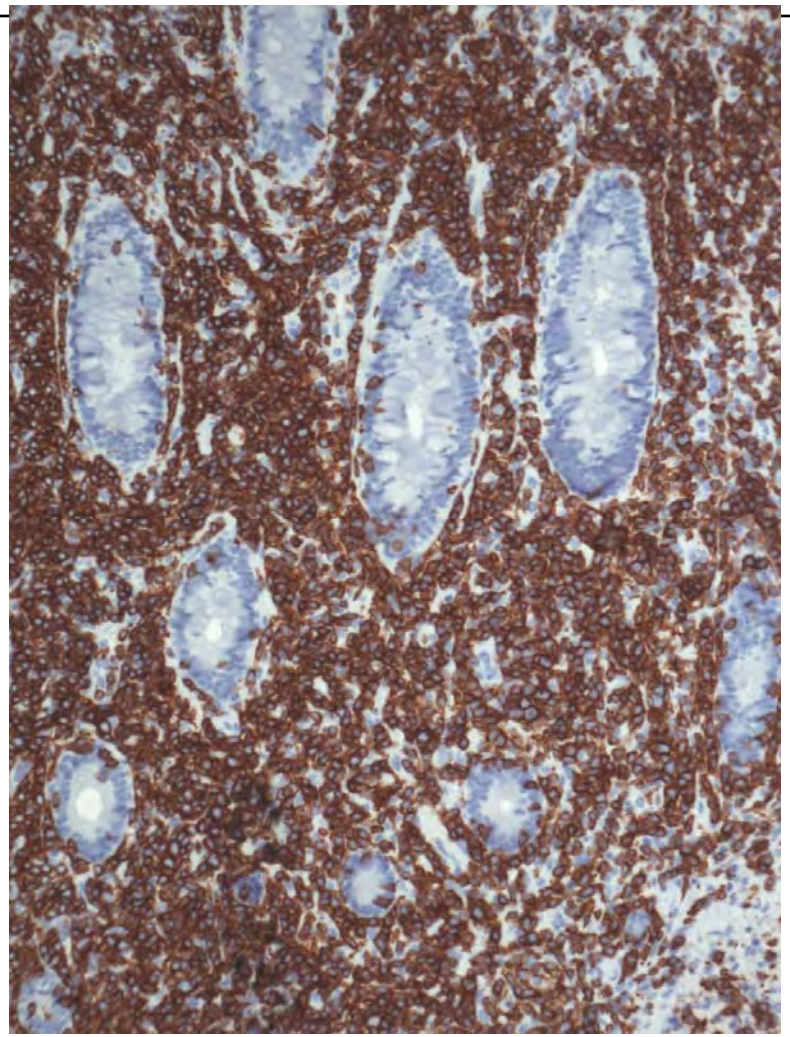
CD3 CD4 CD8



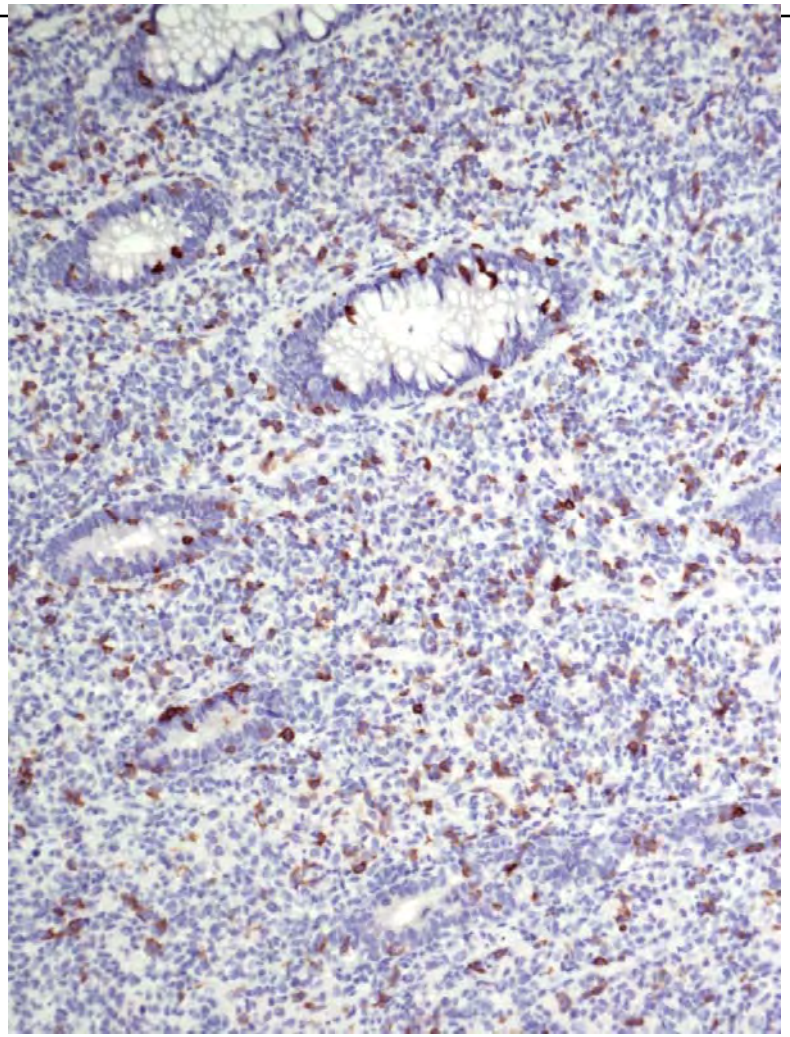




CD5

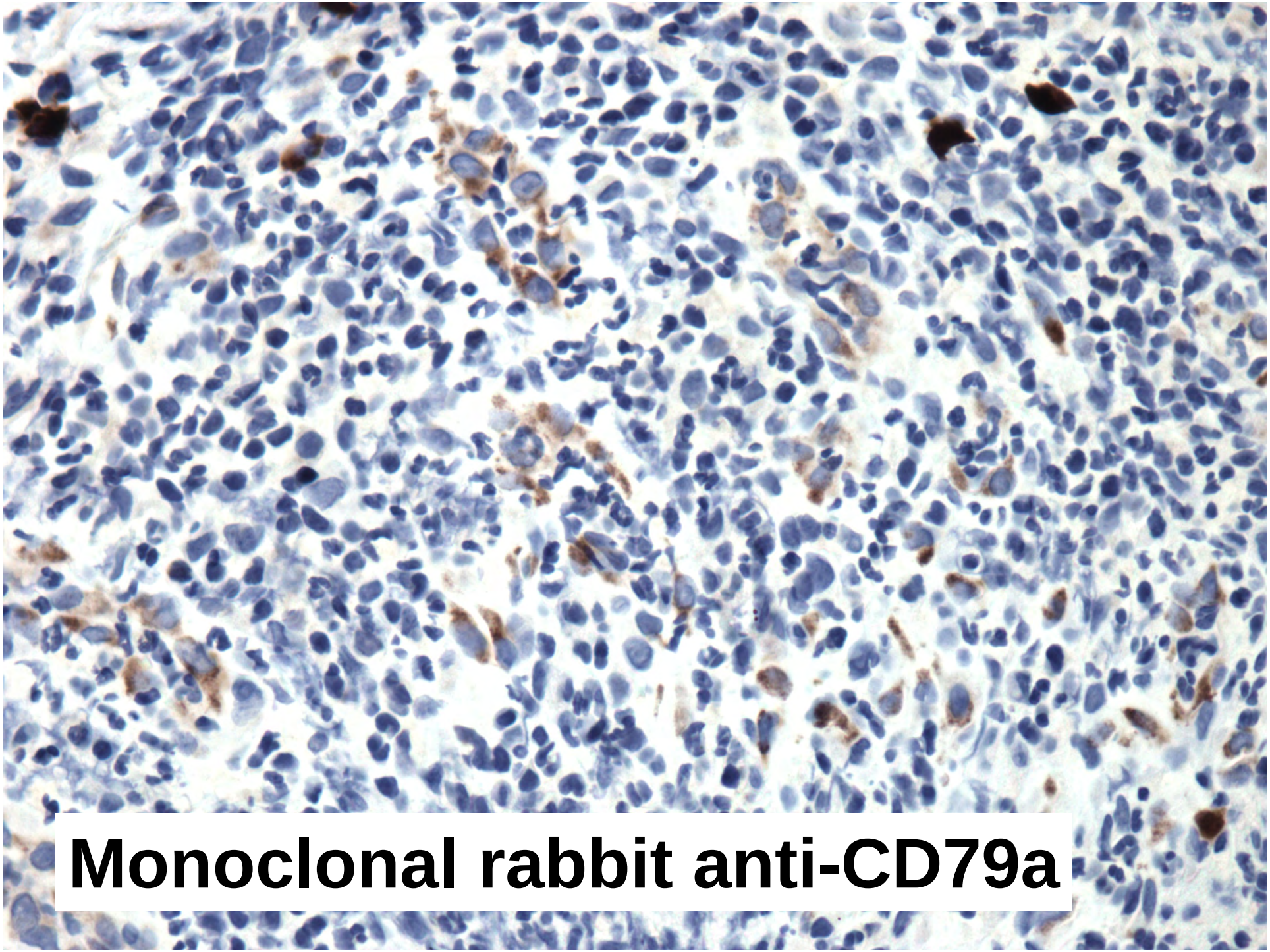


CD7

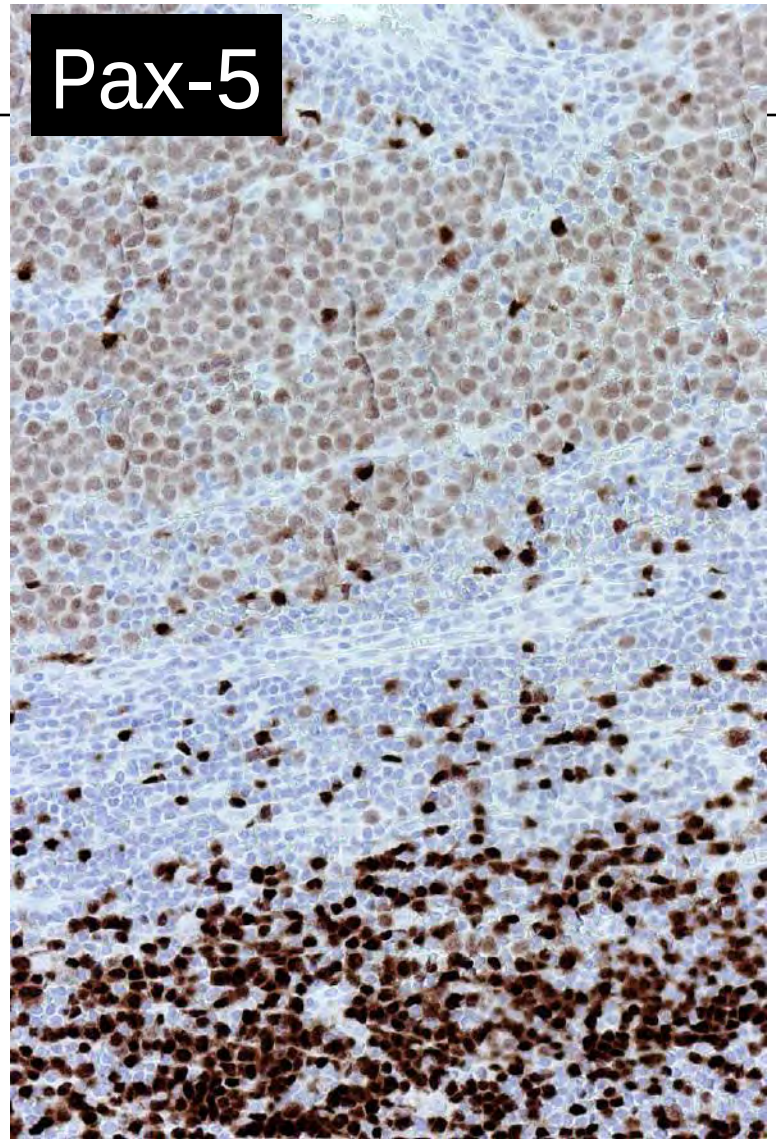


Distribution

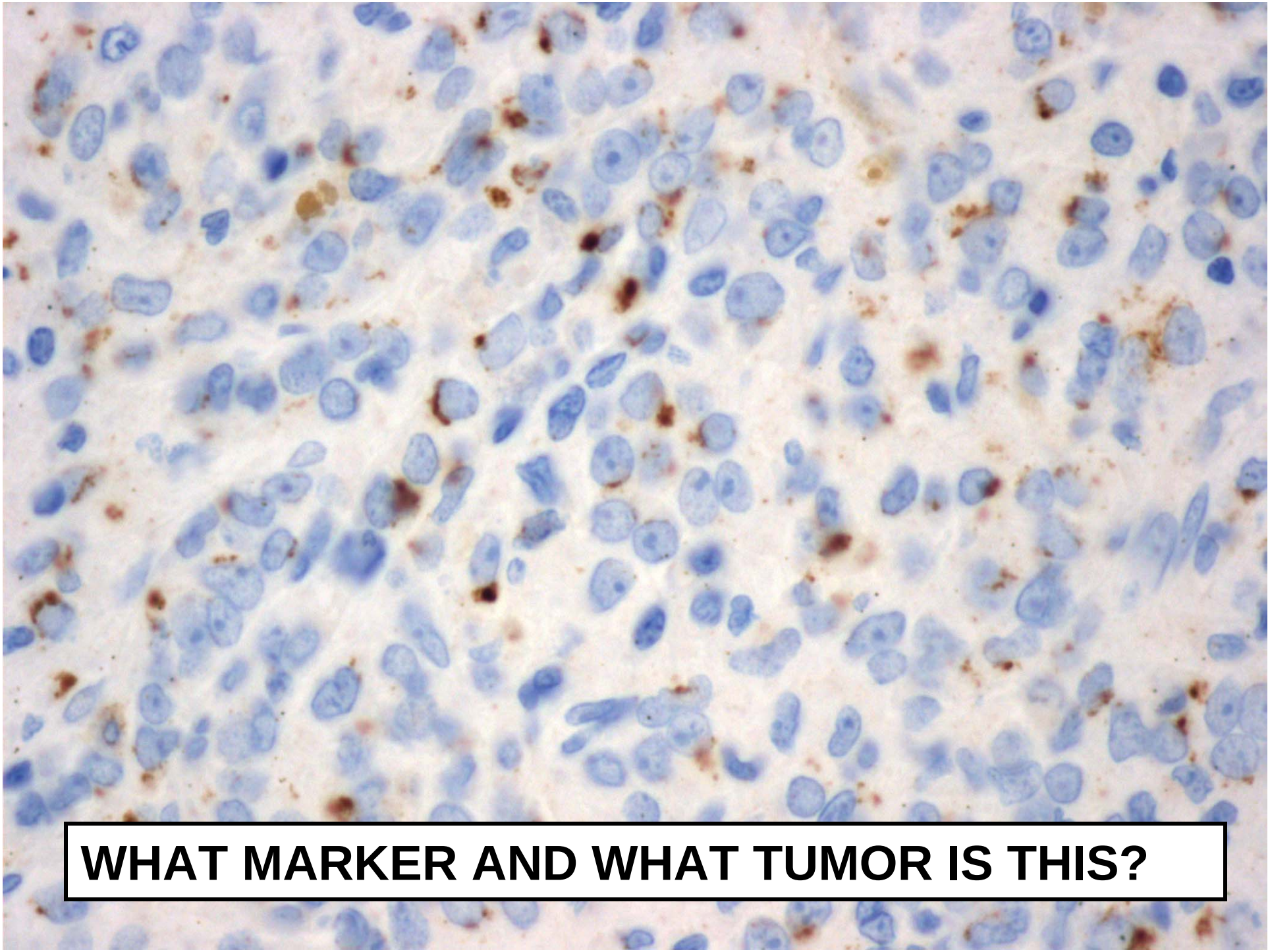
- Variation from cell to cell is more likely to be specific
- Uniform positivity in all present cells should warrant special consideration to rule out false positive result

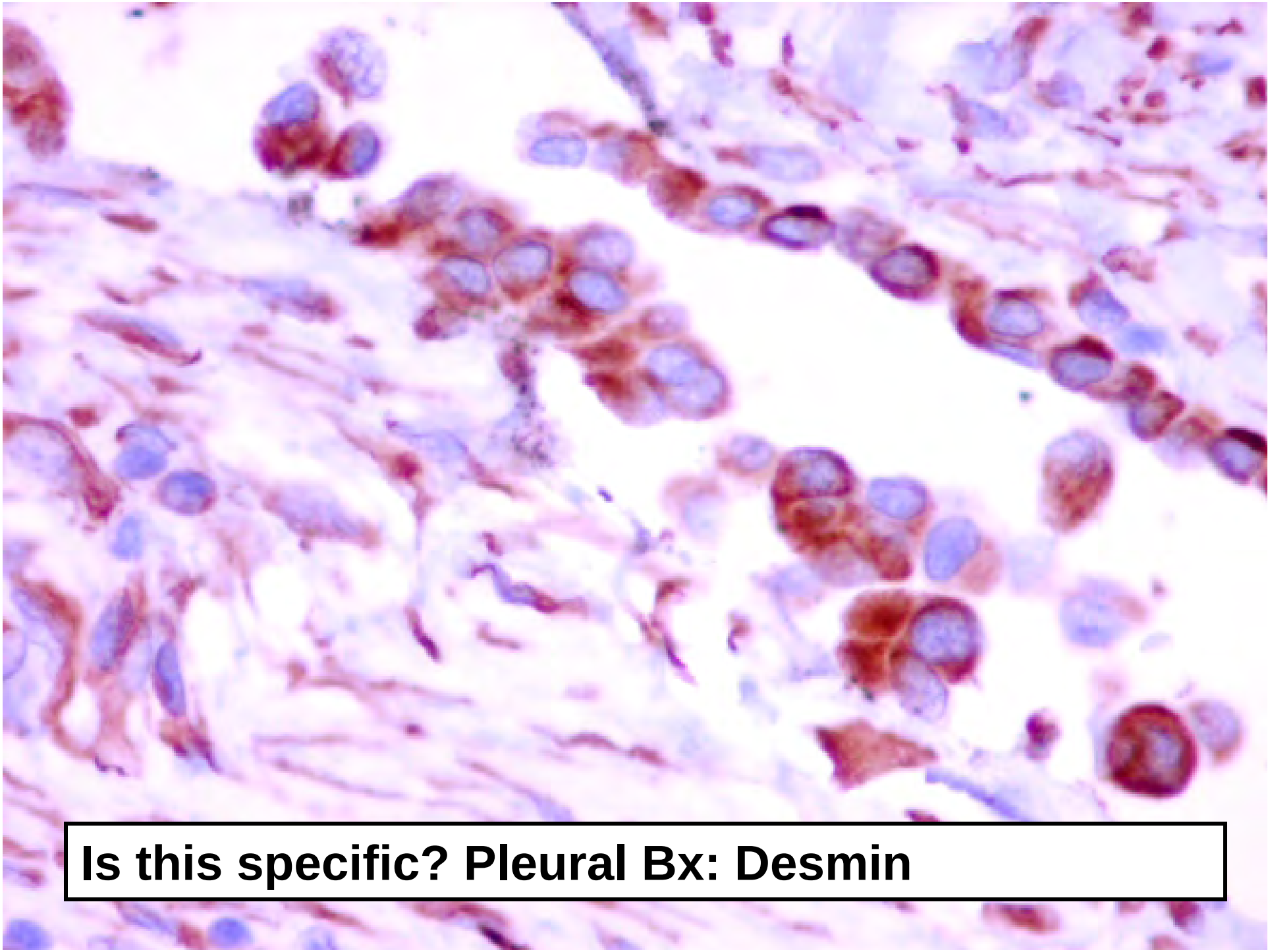

Monoclonal rabbit anti-CD79a

WHAT IS YOUR DIAGNOSIS?



Skin Bx:
Blastic morphology,
TdT+, Pax-5+,
CD45-


WHAT MARKER AND WHAT TUMOR IS THIS?


Is this specific? Pleural Bx: Desmin