

## Immunohistochemistry: Interpretation of Results

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## Analytic Interpretation vs. Clinical Interpretation

- Developing guidelines for interpretation is a part of validation process.
- Cytokeratin expression:
  - In positive controls (analytical)
  - Undifferentiated tumor panel vs. ALCL and plasmablastic lymphoma (clinical).

## Interpretation of IHC Results

- Postanalytical component
- Class I and Class II require different approach
- Biology + published guidelines + experience

## Important articles

1. **Mighell AJ, Hume WJ, Robinson PA.** An overview of the complexities and subtleties of immunohistochemistry. *Oral Dis*, September 1, 1998; 4(3): 217-23
2. **Seidal T, Balaton AJ, Battifora H.** Interpretation and quantification of immunostains. *Am J Surg Pathol*. 2001 Sep;25(9):1204-7. Review.
3. **Cheuk W, Chan JK.** Subcellular localization of immunohistochemical signals: knowledge of the ultrastructural or biologic features of the antigens helps predict the signal localization and proper interpretation of immunostains. *Int J Surg Pathol*. 2004 Jul;12(3):185-206. Review.

## Basic Approach to Interpretation

- Positive vs. negative (qualitative assessment)
- Quantitative assessment (estimate or exact %)
- Specific vs. non-specific

## Positive vs. Negative Results

- Are there published guidelines?
- What is a cut off point?
- Are there any degrees of freedom? Can I have my own opinion?
- How can I detect false positive and false negative results?

## Are there published guidelines?

### Class II




- **HER2** – yes
- **ER/PR** – yes
- **Ki-67** in mantle cell lymphoma and in neuroendocrine tumor

### Class I

- TTF-1, Fli-1, p53, TdT, Pax-5: nuclear
- Pan CK, other cytokeratins: cytoplasmic filaments
- Melan-A & HMB-45: cytoplasmic granular
- Calretinin and S-100: cytoplasmic& nuclear

## HER2 Guidelines: Canadian Consensus for HER2/neu Testing Guidelines in Breast Cancer

### POST-ANALYTIC Interpretation Criteria – IHC

Result Category	IHC Score HER2 Protein Expression	Testing Method	Example
		Interpretation Criteria	
Positive	3+	Strong complete homogeneous membrane staining (chicken wire pattern) in >30% of cells	
Equivocal*	2+	Strong complete membrane staining (chicken wire pattern) in ≤30% of cells Weak/moderate heterogeneous complete membrane staining in at least 10% of cells	
Negative	0 – 1+	No staining (0) or weak, incomplete membrane staining (1+) in any % of cells	

\*Confirm by: FISH analysis of original sample

## Ki-67

- **PROSTATE** >10% favors prostate carcinoma over simple atrophy (around 3%) and postatrophic hyperplasia (around 4%), or high grade PIN (around 6%).
- **SARCOMA** >30% predicts worse disease specific survival in patients with high-risk (>10 cm, high-grade, deep) completely resected primary extremity soft tissue sarcomas.
- **LUNG CA** >30% predicts worse outcome in non-small cell lung carcinoma.
- **PNET** Hot spot (areas with the highest proliferation rate) proliferation index as measured by Ki-67 is a significant and independent prognostic factor.

## Ki-67

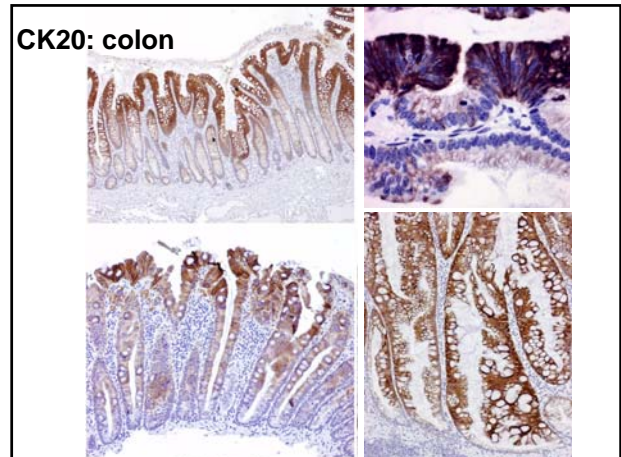
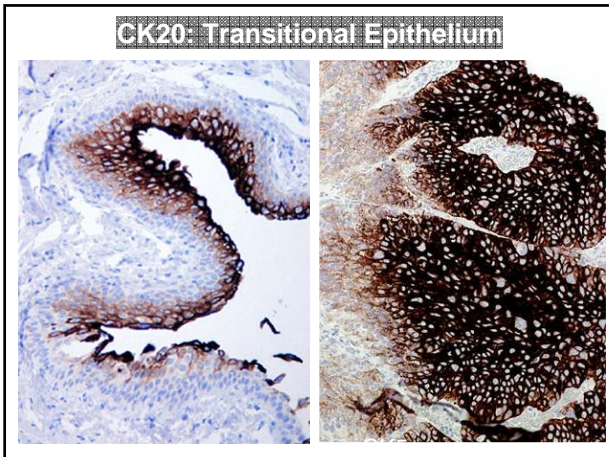
- **PHYLLOIDES TUMORS** generally benign has < 10% labeling index, low grade has between 10 and 20% and malignant has >20%.
- **ENDOCERVIX** Normal endocervix shows Ki-67 positivity in <10% of the cells, but usually in >20% of cells in cervical adenocarcinoma. Because of the significant overlap, it cannot be used alone for grading squamous intraepithelial lesions in the cervix.
- **ORAL LEUKOPLAKIA** Ki-67 is a reliable marker able to differentiate normal (<10%) from both low-grade dysplasia and high-grade dysplasia (both > 30%), when the labeling index of the basal layer was used, whereas this same marker was able to differentiate between low-grade dysplasia (<10%) and high-grade dysplasia (>10%), when the superficial layer was considered.
- **UTERINE SMOOTH MUSCLE TUMORS**: <5% in cellular leiomyomas, <10% in smooth muscle tumors of uncertain malignant potential (STUMP), and >10% in leiomyosarcomas.
- **COLON SERRATED POLYPS**: increased and regular distribution in HP, but irregular and often decreased in SSA and TSA

## Ki-67 in Hematopathology

- Burkitt lymphoma: “nearly 100%” (**class I**)
- Mantle cell lymphoma: > 40% adverse prognostic indicator (**class II**)
- ALL (low – better prognosis): higher in adults than in children and higher in T-ALL than in B-ALL; good prognosis ALL <25% (**class II**)
- Very high in ALPS (**class I**)
- Low in most low-grade LPD (**class I**)
- Plasmablastic lymphoma: minimum 75%, usually >90% (**class I**)
- Other...

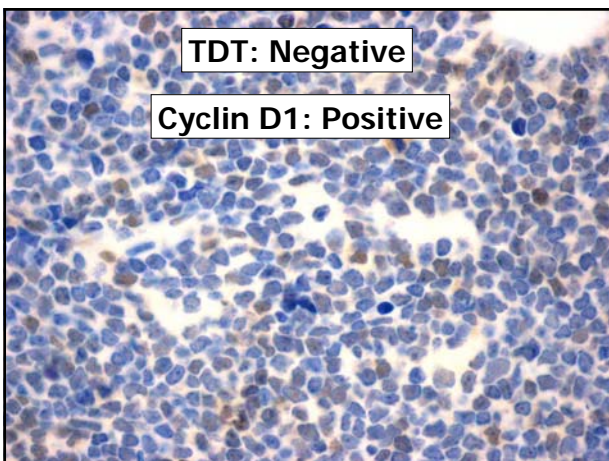
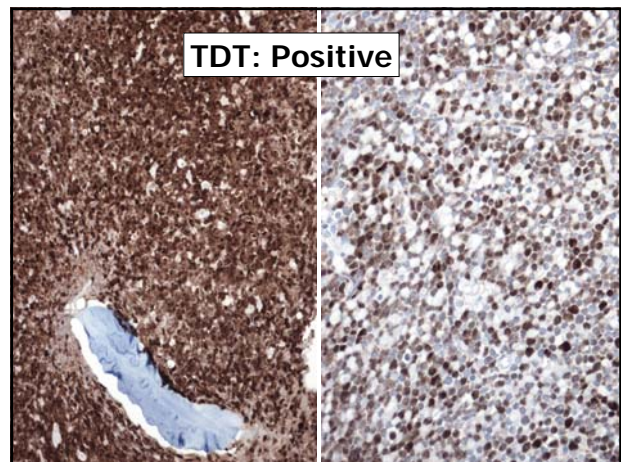
## CK20

- Dot-like cytoplasmic in Merkel cell carcinoma
- Transitional epithelium
  - Normal: Surface only
  - Dysplastic: Whole thickness
- Colon epithelium
  - Normal: Mainly surface of the crypts
  - Hyperplasia: Extends towards the base
  - Neoplasia: Irregular and/or whole thickness



### What is a cut off point?

- **Traditional: 10%**
- **Not to be applied** for those tests that have published guidelines.
  - Breast Ca markers
  - CK typing (10% vs. 20% vs. 30%)
- **Not to be applied** for those tests that biology strongly suggests that even minor population of positive cells is significant.
  - This mostly applies for results with very specific patterns of expression.
- **To be dogmatically applied** for any other tests.
  - MPO staining in BM trephine bx with leukemia (compare with the general guideline for histochemical results at 3%)



### CORRESPONDENCE

#### Limitations of transferability of absolute cut-points in non-standardised assays

We read the paper of Matull *et al*<sup>1</sup> on the biochemical markers of acute pancreatitis with much interest. We would, however, like to comment on some aspects that could be misleading.

First, absolute cut-off points as part of diagnosis or risk prediction need to be employed with great care.<sup>2</sup> This is especially true for enzyme assays such as alanine aminotransferase, aspartate aminotransferase, lactate dehydrogenase and amylase. For example, more than 200 methods of activity determination have been described for amylase.<sup>3</sup> Amylase analytical methods vary with respect to the type of substrate and substrate concentration, use of auxiliary enzymes as well as assay conditions such as pH, temperature, incubation period and protein concentration.<sup>4</sup> Accordingly, reference intervals are method dependent, and it could be confusing to imply that values such as 600 or 1000 U/L have certain clinical sensitivities and specificities<sup>5</sup> without mentioning which analytical methods were employed, what the reference intervals were or that the figures will vary between laboratories owing to a lack in assay standardisation.<sup>6</sup> Reference intervals for amylase range from 90–300 U/L (Phadebas, MagJe AB, Lund, Sweden) through 25–115 U/L (Hexamon, Refl, Dade Behring, Milton Keynes, UK) to 22–69 U/L (Olympus, Clare, Ireland). Not surprisingly, there are large differences between different assays when results from external quality assessment schemes are examined. The difference between the intermethod reference ranges is so significant that they have been flagged up as a clinical performance issue in some hospitals when changing routine clinical chemistry analysers.

The second, unrelated, aspect on which we wish to comment relates to interferences. Significant increases in serum triglyceride concentration is a recognised interferent in some assays; however, it is unlikely to be an interferent or a competitive interferent in all 220+ amylase assays<sup>7,8</sup> due to the inherent differences in assay formulation. Most modern amylase assays are not subject to such problems when the triglyceride concentration is <10 mmol/L.

It also needs to be noted that prognostic criteria such as Ranson and Glasgow are also affected by the above transferability limitations. Direct transfer without validation, especially when assays have not been standardised will lead to avoidable errors in diagnosis and assessing prognosis.

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### Are there any degrees of freedom? Can I have my own opinion?

- Yes, but this only applies if:
- You can scientifically validate your opinion.

**OR**

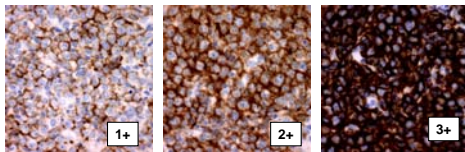
- **There is no good scientific answer how to interpret results of a specific test.**
  - Do not forget to declare that your report is based on your personal expertise and experience.



### Quantitative Assessment (Estimate or Exact %)

- **Estimate only:** Works for great majority of tests, even for ER/PR and HER2
- **Exact count:** Rarely needed
  - Ki-67 in hematopathology (Burkitt, MCL, Plasmablastic Lymphoma, neuroendocrine tumor)
  - CD34 (MDS classification and MDS vs. AML)
  - Other...

### 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)

### How can I detect false positive and false negative results?

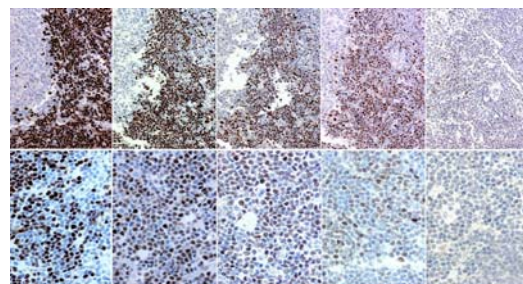
- Daily QC in IHC laboratory
- Positive and negative controls
- Internal controls
- External controls



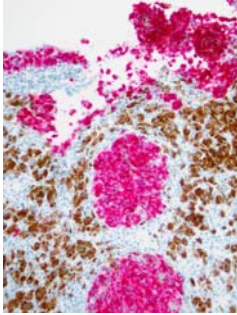
### Control Interpretation

- All types of controls need to be carefully selected and evaluated.
- **Internal controls are critically important if present.**
- **Add some normal breast tissue with the tumor tissue in the same block.**
- There are no positive internal controls for ALK-1, mutated NPM-1, and many other tests. In most samples, even "simple" markers are not represented adequately (Ki-67).
- Negative internal controls are present most of the time.

### Cyclin D1 in Mantle Cell Lymphoma



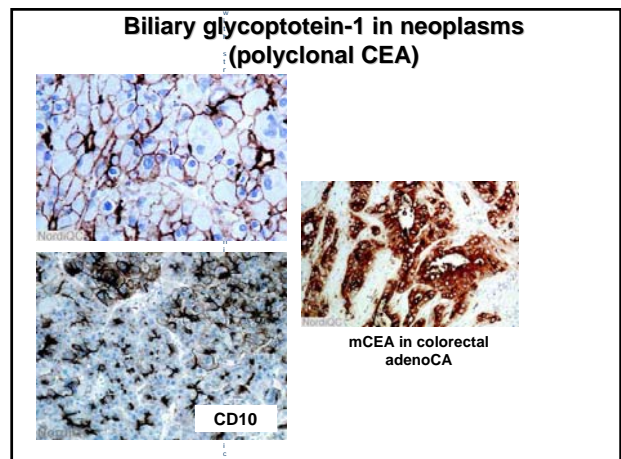
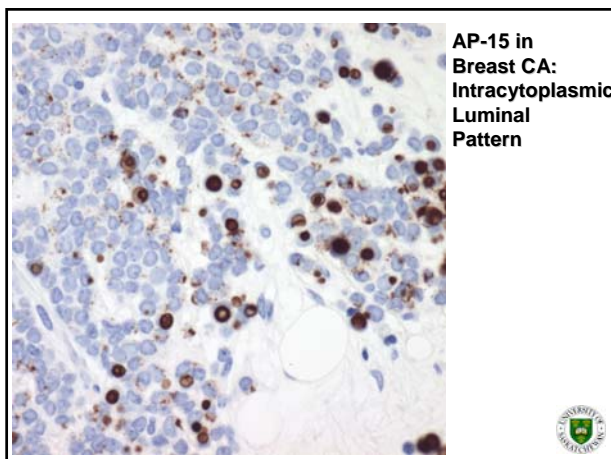
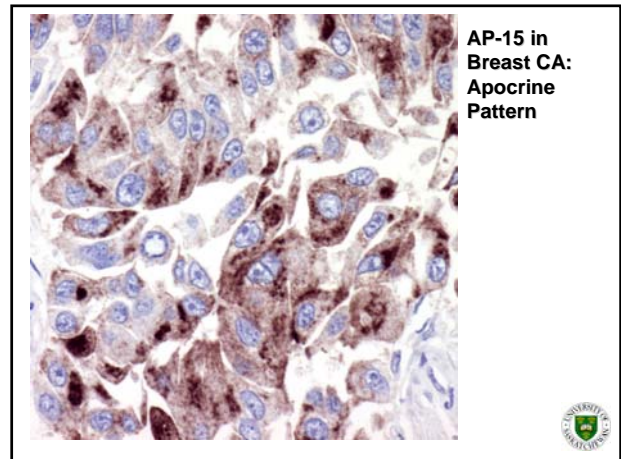
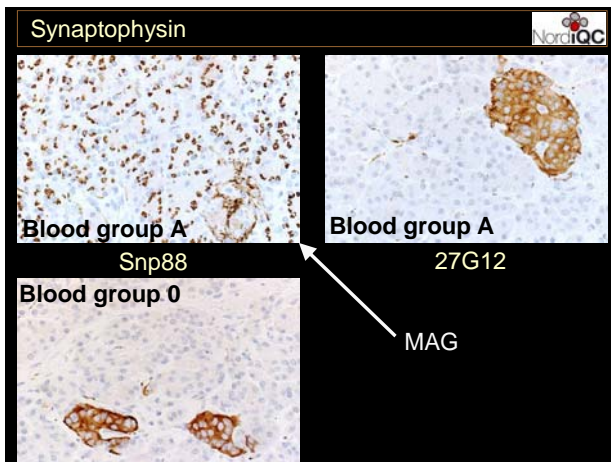
## Primary Melanoma (Skin/Mucosa)



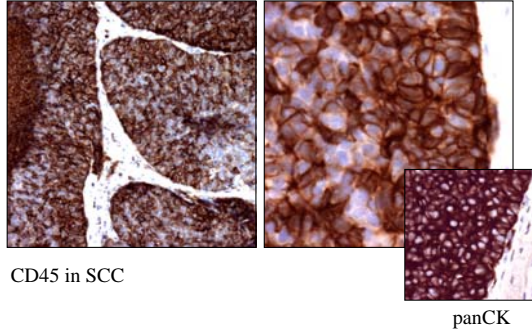
- Endogenous pigments including iron or melanin may interfere with interpretation.
- Red color development in IHC is highly recommended for this application.

## Specific vs. Non-Specific

- Biology/Published Guidelines:
- Cellular localization: nucleus, cytoplasm, Golgi, membrane, secreted
- Tumor biology (genotype vs. phenotype)
- Internal and external controls
- Special consideration: Ag leakage, mouse anti-Golgi (MAG) reaction



Allow for Biological Variation or  
Unknown Technical Artifact



CD45 in SCC

panCK

Published Guidelines:

- Always abnormal: ALK-1, viral epitopes (CMV, HHV-8, LMP-1, HVS)
- Quantification deems results abnormal:
  - Diffuse strong expression of p53
  - Ki-67
- HER2: Membranous overexpression
- ER/PR: Any nuclear
- Abnormal localization: NPM1 in AML: Cytoplasmic (fine granular)
- ALK-1 in t(2;5): Cytoplasmic and nuclear

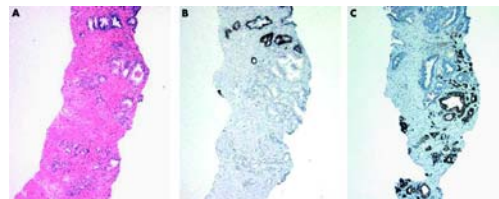
CRITERIA for immunohistochemical staining with anti- $\alpha$ -methylacyl CoA racemase (AMACR/P504S) that would support a diagnosis of malignancy on prostate needle biopsies

1. Intense staining easily visible on low power examination
2. Circumferential, granular, luminal (apical) to diffuse cytoplasmic staining of acini with malignant features on haematoxylin and eosin sections
3. Negative staining or weak, non-circumferential staining in adjacent benign glands

**AMACR/P504S**

**Small focus of prostatic adenocarcinoma on a needle biopsy.**

- (A) Haematoxylin and eosin.  
(B) Immunostaining for 34(beta)E12; note the absence of staining for basal cells within the malignant glands as compared with the adjacent benign glands.  
(C) Malignant glands showing intensely positive, circumferential staining for AMACR/P504S compared with negative staining in the benign glands.

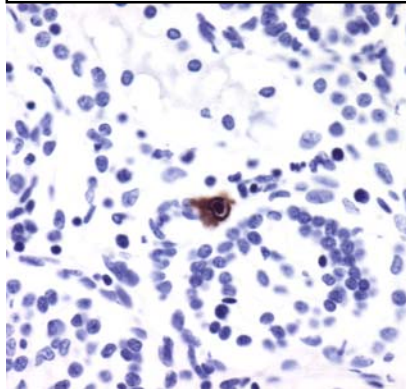


Evans, A J J Clin Pathol 2003;56:892-897

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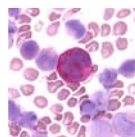
JCP  
ONLINE

Single Cell Positivity: YES and NO

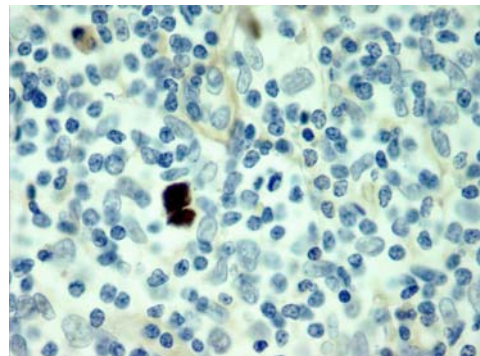


CMV  
(Kidney Bx):  
YES

CD30 in BM  
aspirate: NO

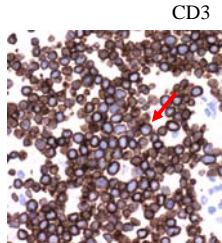


EBER in cHL: YES



### Membranous vs. Cytoplasmic vs. Nuclear vs. Extracellular

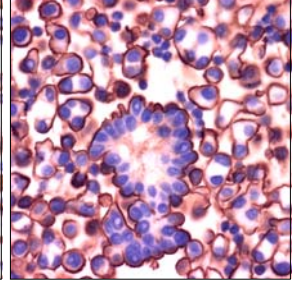
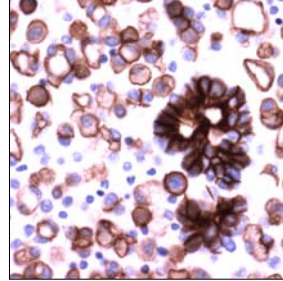
- CD3: could be both, membranous and cytoplasmic
- CK: cytoplasmic only
- CD30: Golgi, cytoplasmic, membranous
- CD20: membranous only
- PSA: any pattern is good



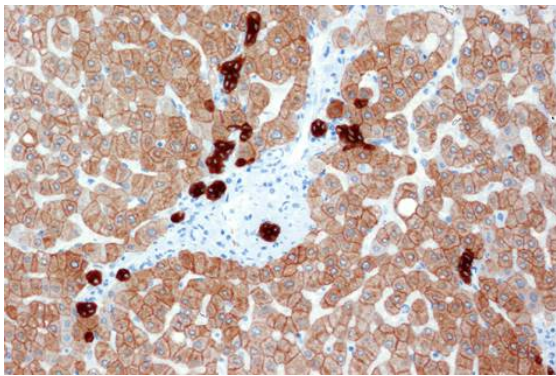
### Membranous

Ber-EP4

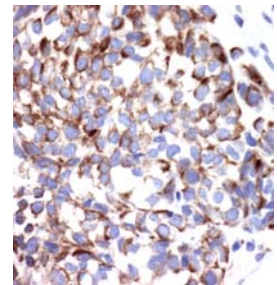
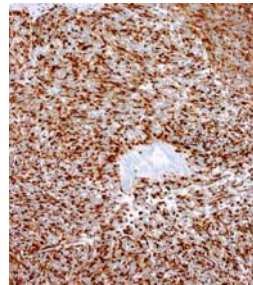
CA-125



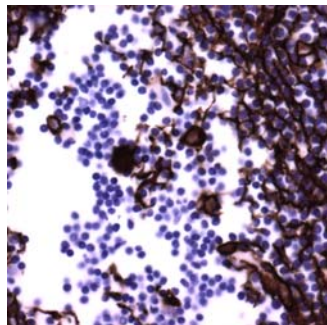
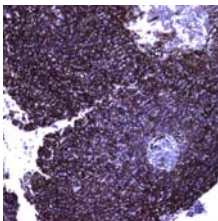
### Membranous: Cytokeratins



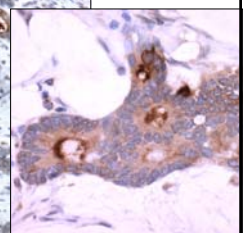
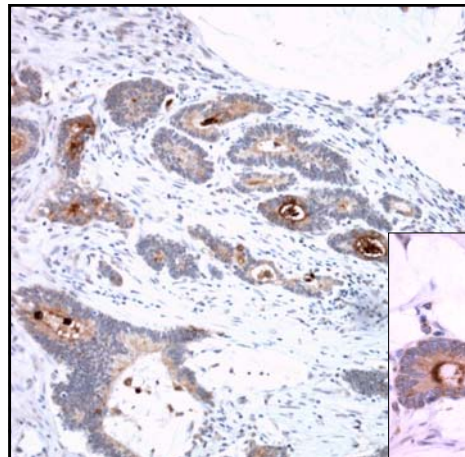
### Cytokeratin: Dot-like & Filamentous Cytoplasmic

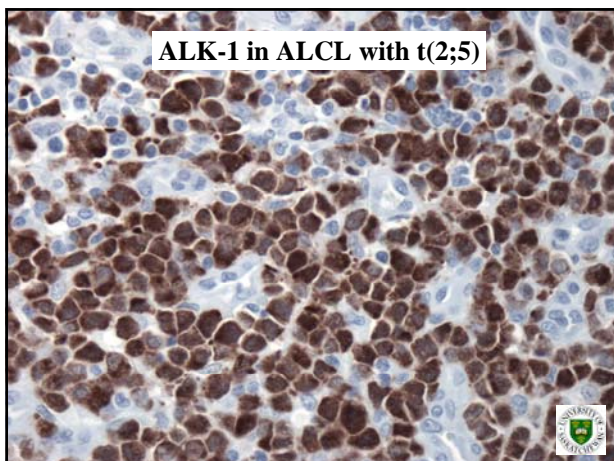


### CK in Cortical Type Thymoma: Dendritic



### PSA



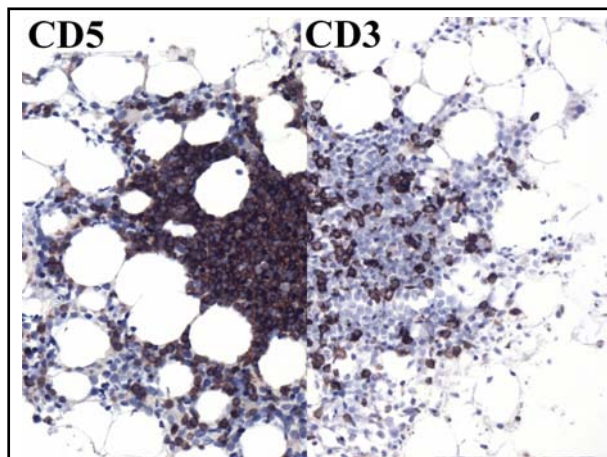
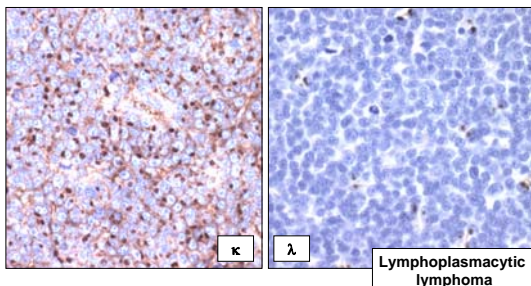


### Other Markers with Cytoplasmic + Nuclear Positivity

- S-100
- Calretinin
- Mutated NPM-1
- CMV
- CD10 in neutrophils?
- Hemoglobin A
- ER/PR and other typically nuclear markers (sometimes)
- Other

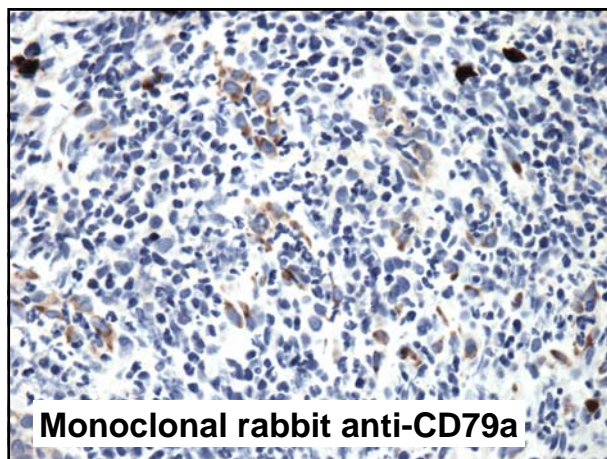
### Always Interpreted Together

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



### Distribution

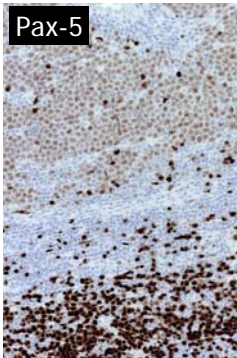
- Variation from cell to cell is more likely to be specific
- Uniform positivity in all present cells should warrant special consideration to rule out false positive result



### Ab Cocktails vs. Double Staining

- **Single staining color is sufficient** if cellular localization is specific enough or when it does not matter which marker is positive or negative:
  - AE1/AE3 or melanoma cocktail
  - Prostate Ca markers: **HMWCK+p63**
  - Plasma cell differentiation: **CD138+MUM1**
  - KI-67 + any surface or cytoplasmic marker (e.g. KI-67+CD3)

### WHAT IS YOUR DIAGNOSIS?



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

