Critical Technical Aspects of Diagnostic Immunohistochemistry

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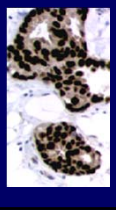
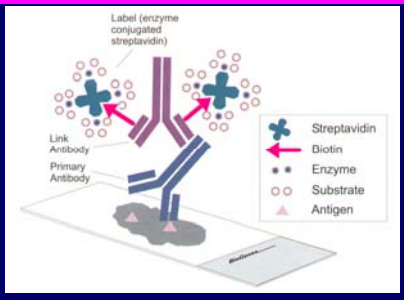


Technical Aspects of IHC: Overview

1. Immunostain methods (and the endogenous biotin problem)
2. Interpretation of stains
3. Controls and Quality Control
4. Special situations (i.e., no block)
5. Artifacts in IHC
6. Oops…….
7. Miller's Rules of IHC

IHC: Avidin-Biotin Method

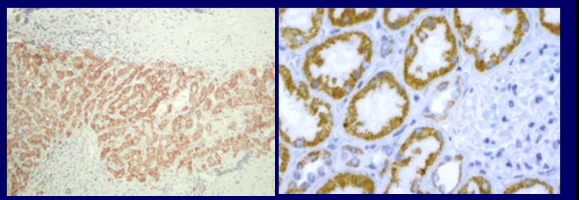


1 Avidin binds 4 Biotin molecules (irreversible)

The Endogenous Biotin Problem

- Biotin: vitamin - for pyruvate carboxylase (mitochondrial enzyme in Krebs Cycle)
- Liver, kidney, oncocytomas
- Nonspecific binding of avidin or streptavidin detection complex (false pos)
- Artifact enhanced by epitope retrieval (especially with pressure cooker method)
- Varies with antigen retrieval solution used

Endogenous Biotin: Normal Tissues

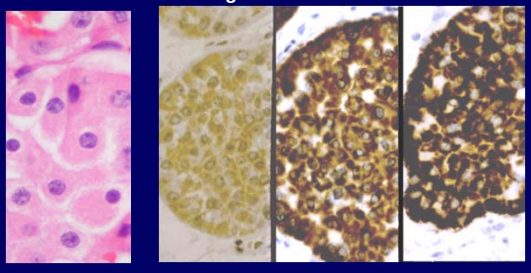


Normal Liver (low) Normal Kidney (high)

Negative Control Immunostains

Oncocytoma: Endogenous Biotin

H&E Negative Control Immunostains



Citrate Buffer Tris Buffer Dilute Urea

The Endogenous Biotin Problem

Blocking of Endogenous Biotin

- After epitope retrieval step, incubate with dilute avidin solution (binds to all endogenous biotin), then incubate with dilute biotin solution (to saturate all the biotin binding sites on the avidin)
- Then add primary antibody

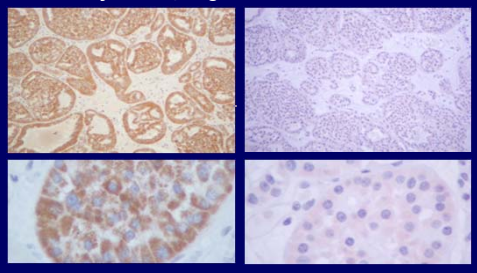
AVIDIN IS EXPENSIVE!! ($3400/gm)

Blocking of Endogenous Biotin (on the cheap)

- After Antigen Retrieval step, immerse sections in dilute egg white solution for 15 min at RT (2 egg whites in 200 ml DW). Rinse with tap water from a squirt bottle.
- Immerse sections in 0.2% biotin in PBS for 15 min at RT (5% reconstituted non-fat dried milk also works well, since milk is a rich source of biotin).

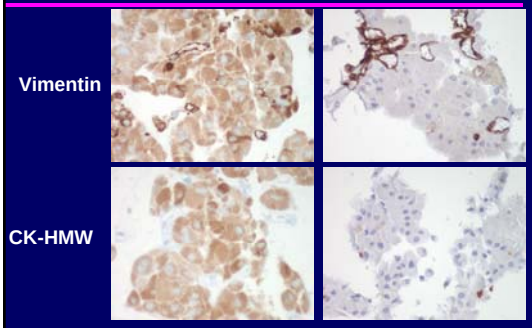
Egg whites as source of avidin

Kidney tissue, negative control sections



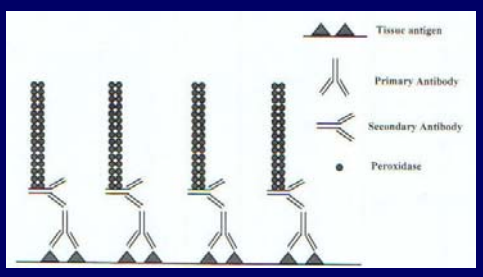
No endogenous biotin block | Endogenous biotin block, egg whites

Endogenous Biotin: It's still out there!



Easier solution to the biotin problem

- Use a polymer-based method



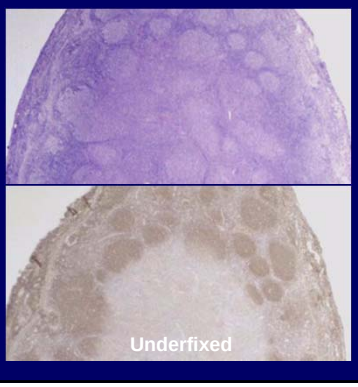
IHC: FIXATION

- GARBAGE IN - GARBAGE OUT
- Cut sections thinly (2 mm)
- If H&E's don't look good, the IHC won't look good either

GOOD

BAD

Lymph Node - Poor Fixation



H&E Stain

CD20 Immunostain

IHC: Fixatives

- Neutral Buffered formalin is the best all-around fixative
- Do not let tissues languish in formalin
- For prolonged storage: 70% ethanol works well

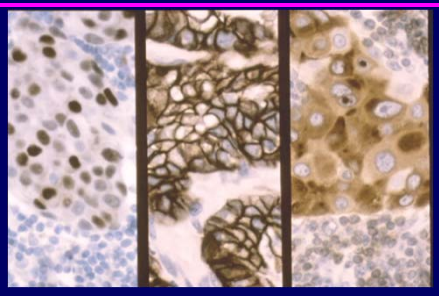
IHC: Interpretation of Stains

TRUE POSITIVE STAINING

- Important to know expected pattern of immunoreactivity (membranous vs. cytoplasmic vs. nuclear)

- Cell to cell heterogeneity critical

IHC: True-Positive Staining



ER nuclear | HER-2 membranous | GCDFP-15 cytoplasmic

IHC: Interpretation of Stains

FALSE POSITIVE STAINING

- Uniform cytoplasmic "blush" that lacks cell-to-cell heterogeneity ("spray-painted")

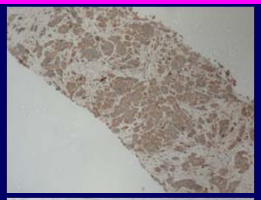
- Can be very intense, and localized to tumor cells with clean background

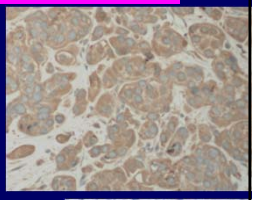
- Often due to inappropriate primary Ab titer (or use of "predilute ready-to-use" Ab)

False Positive Immunostaining

Secondary to inappropriate primary Ab titer

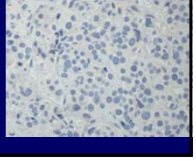
69M LN Bx CD56 automated predilute





Pos Control Original Lab Carcinoid

Rpt CD56



IHC: Controls

Negative Reagent Control Slide

- A slide where primary Ab is omitted and replaced with something else
- As of July 2012, no longer required by CAP if using polymer-based method
- Required if Avidin-Biotin method used – best to have 1 NRC slide for each different type of antigen retrieval

IHC: Tissue Controls

Positive Tissue Controls (PTC)

Tissues expected to be positive

Negative Tissue Controls (NTC)

Tissues expected to be negative

Internal PTC and NTC: Within patient tissue

External PTC and NTC: Non-patient tissue – should be mounted on same slide as patient

Close attention must be paid to BOTH External AND Internal PTC and NTC !

IHC: Controls

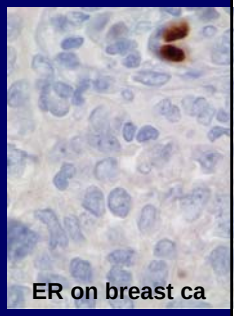
Don't forget about internal controls!!

Internal positive controls:

Look for reactivity in cells that should be positive

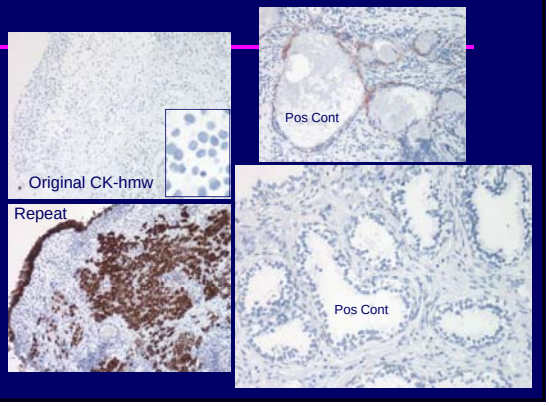
Internal negative controls:

Make sure expected negative cells are indeed negative



ER on breast ca

Control issues – CK-HMW stains



Elements of Successful Quality Control in IHC

- **Must be continuous & ongoing**
- **Importance of good controls**
- **Evaluate all Abs before & during use**
- **Determine optimal titers of all Abs**

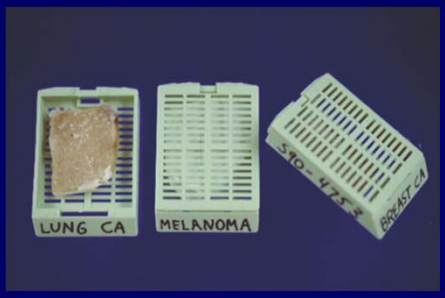
MANUFACTURER'S TITERS ARE GUIDELINES ONLY!

THERE IS NO SUCH THING AS A "PREDILUTE READY-TO-USE Ab!

IHC Quality Control

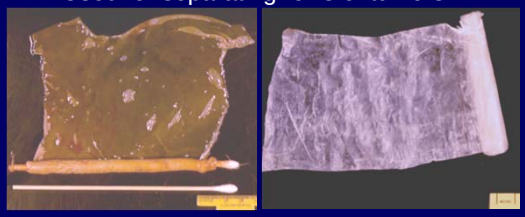
MULTITUMOR SANDWICH BLOCKS

MTSB: Collecting Tissue



MTSB: Collecting Amnion

Used for separating rows of tumors



Multitumor blocks

Strips in mold on hot plate

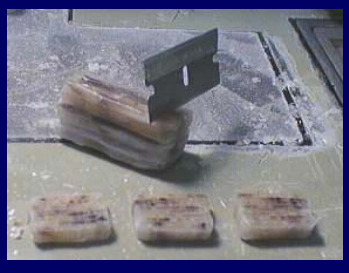
Transfer to Cold Plate

Add paraffin and cool

Multitumor Blocks

Multitumor Blocks

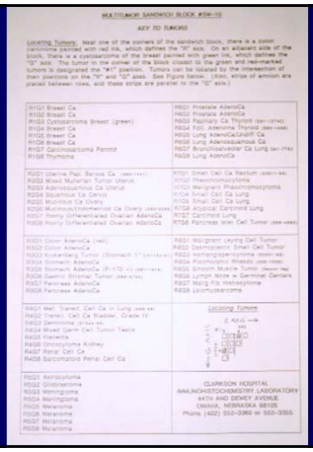
Multitumor Blocks



1 “loaf”: 4-6 blocks – 3000-4000 sections

Multitumor Blocks

MTSB: Key of tumors



Multitumor Block Sections

- Easy to make using your own tissue – 1 block can serve as pos control for MANY different Abs
- 1 “loaf” good for 3000-4000 slides
- Continuous monitor of quality control – contain multiple external PTC and NTC to demonstrate sensitivity and specificity.
- Best if mounted on same slide

Workup of a New Antibody

- What is the best antigen retrieval method?
- What is the optimal titer?

Day1: Select a titer, run a slide with no retrieval, several with proteases of choice, several in AR solutions of choice.

Day 2: Using the optimal antigen retrieval method, run a set of 4 to 6 serial two-fold dilutions of the primary antibody.

Workup of New Antibody

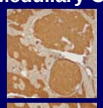
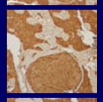
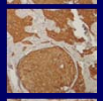
Use of Multitumor Sandwich Blocks

- **Many different tumor of all types**
- **Wide range of expected pos AND expected neg tumors (often with different antigen densities)**
- **Perfect for determining optimal titers of primary antibodies to maximize true-positive staining and minimize false-positive staining**

IHC: Titering of Primary Ab

- Must evaluate BOTH known positive cases and known negative cases
- Serial 2-fold dilutions convenient
  e.g., 1:100, 1:200, 1:400, etc.)
- Readily allows detection of contaminated antibodies from vendors (e.g., CD7 Ab contaminated with CK-LMW)

Importance of Proper Titers: Calcitonin

Ab Titer	Medullary Ca	Prostate Ca's
1:2000		
1:4000		
1:8000		
1:16,000		

Lame excuses for using Predilutes

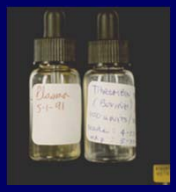
- "We don't have time to do that: we're really busy."
- "My sales rep says it's unnecessary."
- "They're so convenient."
- "Doing titers is confusing."
- "We've been doing fine in the past without titering."
- "We use an automated stainer so it's not necessary."

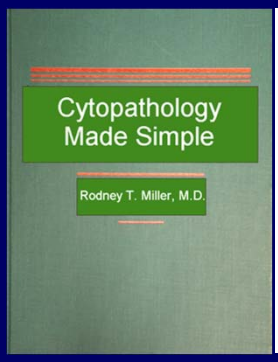
IHC on Cytologic Specimens

Rule #1

GET A CELL BLOCK!!!

- Use of Thrombin +/- Plasma to assist in obtaining cell block material





Get a good cell block and do immunostains on it if you can't figure it out on the H&E.

- The End

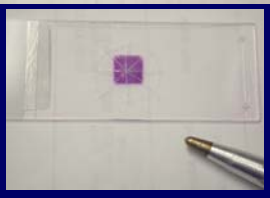
IHC on Cytologic Specimens

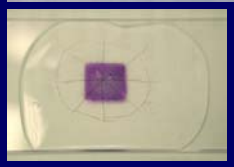
Rule #2

Use NON-ADHESIVE slides to make your smears

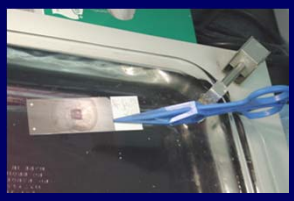
Allows use of tissue transfer techniques

Tissue Transfer IHC: Preparation





Tissue Transfer IHC: Soak & Peel




Tissue Transfer IHC: Peeling



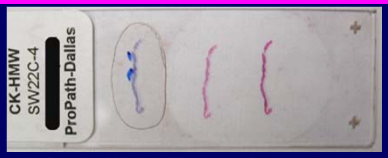
Tissue Transfer IHC

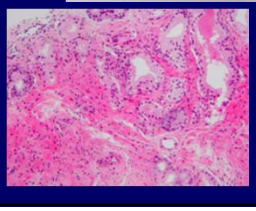
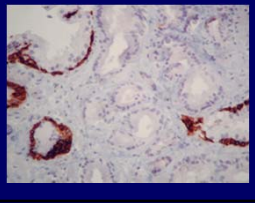
What if it's an adhesive slide? Try Tissue Protection IHC


Tissue Protection IHC: Prostate bx


Appearance of slide after Antigen Retrieval

Tissue Protection IHC: Prostate bx

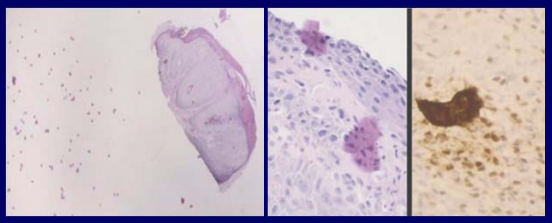


IHC: Artifacts

- Endogenous biotin
- "Desquamartifact"
- Bubble Artifact
- Drying Artifact
- Trapping Artifact
- Edge Artifact
- "Bleeding" Artifact
- Nuclear Artifact
- Bad Titer Artifact
- Poor Fixation
- Microbial Contam.
- B-5 Artifact
- Pencil Artifact
- Waterbath Artifact
- Checkerboard Artifact
- "CK Juice" Artifact

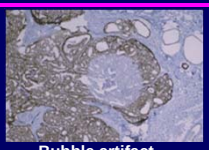
IHC: Desquamartifact



Low power | High power | CK AE1/AE3

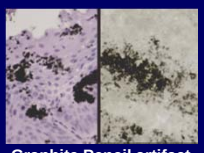
Desquamartifact Remedy

IHC: Artifacts

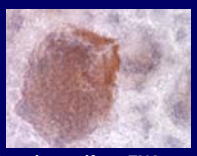


Bubble artifact

Drying artifact



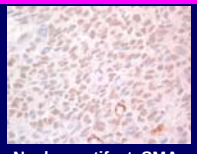
Graphite Pencil artifact



Trapping artifact, FNA smear

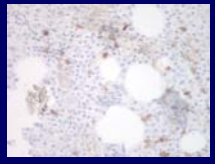
IHC: Artifacts

Bleeding artifact



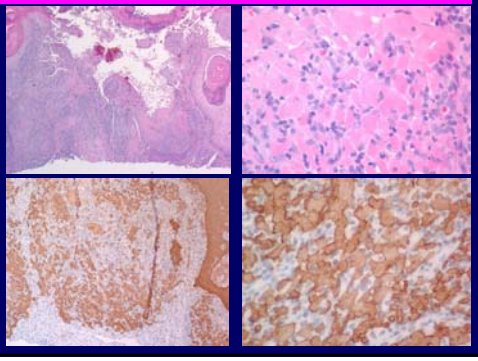
Nuclear artifact, SMA

Checkerboard artifact 100x



Checkerboard artifact 400x

IHC: Artifacts – "CK Juice"



IHC: Junk Artifact

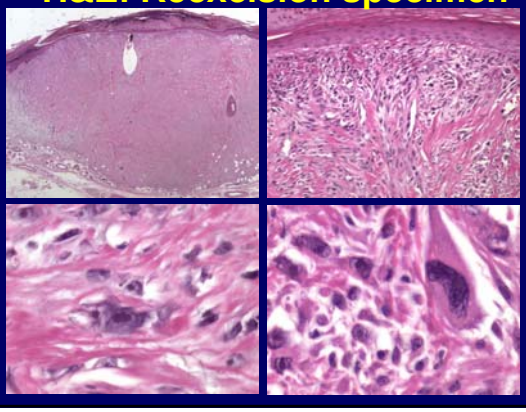
OOPS……..

84 year old female underwent shave biopsy of a 1 cm nodule on the arm. H&E showed malignant spindle cell tumor. Bx sent to UFMC, reported as spindle cell melanoma based on pos S100 and neg keratin.

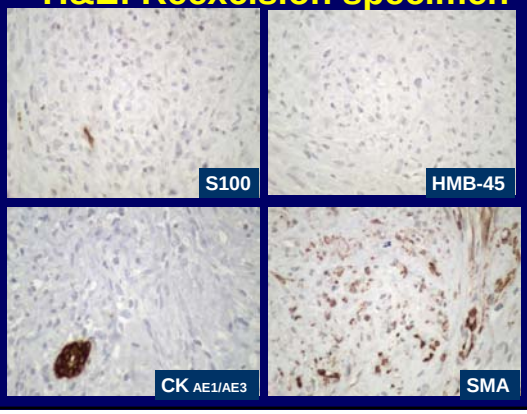
OOPS……..

Wide local excision subsequently performed, and immunostains repeated at ULCH. Now it is neg for both S100 and keratin. Sent to ProPath for workup – S100 and keratin (and some other stains) neg. DX: AFX

H&E: Reexcision specimen



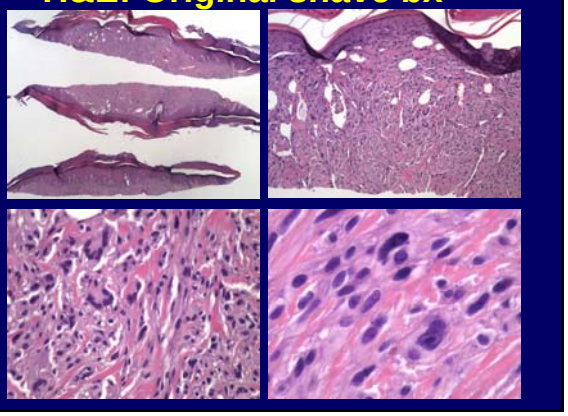
H&E: Reexcision specimen



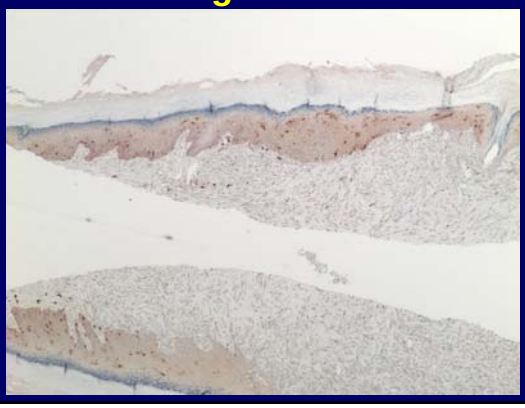
OOPS……..

Surgeon, who trained at UFMC, refuses to believe that UFMC may have made a mistake. Original slides from the UFMC obtained for review.

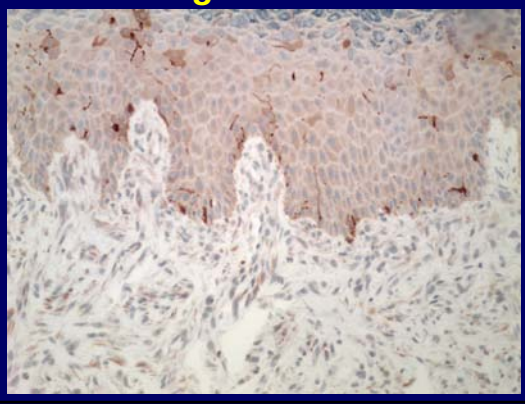
H&E: Original shave bx



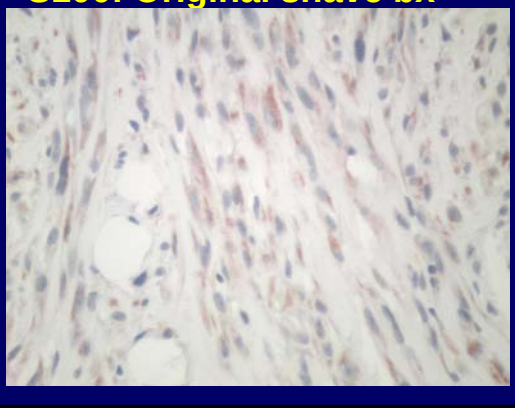
S100: Original shave bx



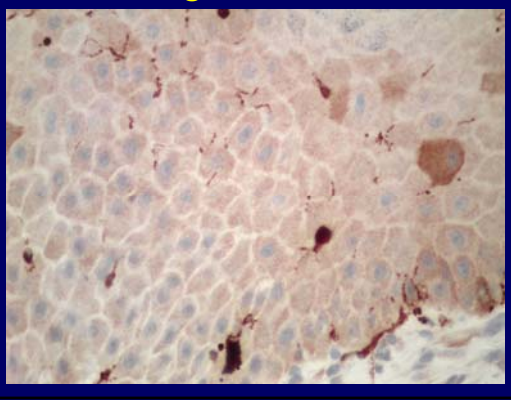
S100: Original shave bx



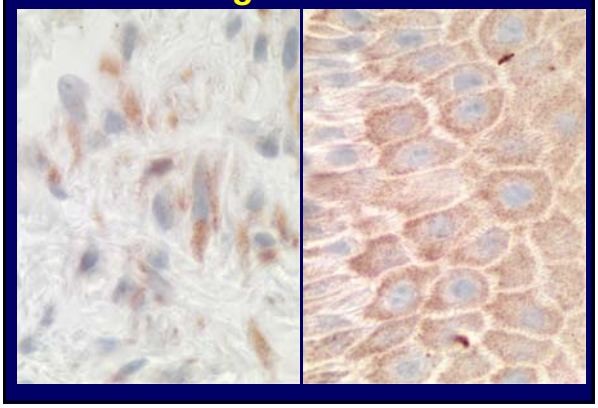
S100: Original shave bx



S100: Original shave bx



S100: Original shave bx



Mistakes that were made

1. Positive control tissue not on same slide (lab uses “batch” positive controls)
2. No negative control slide with case
3. Lack of attention to expected pattern of reactivity (S100: nuclear and cytoplasmic)
4. Lack of recognition of characteristics of true positive and false positive stains
5. Lack of attention to internal positive and negative controls

Miller’s Rules of IHC: 1

- Generate a logical DDx, and use PANELS
- Treat your tissue right (Garbage in……)
- IHC is not perfect, and tumors do not read textbooks. Correlate with H&E
- Make multitumor blocks and use them (and mount on same slides with patient tissue)
- Determine your own optimal titers

(PREDILUTE = BAD)

Miller’s Rules of IHC: 2

- Know spectrum and expected pattern of reactivity of markers used, know true positive & false positive stains, and don’t ignore controls (internal and external)
- Block endogenous biotin if using avidin-biotin-based methods
- For cytologic IHC, GET A CELL BLOCK, and put smears on NON-ADHESIVE slides
- DON’T RUN A STAGNANT LAB

Immunohistochemistry