

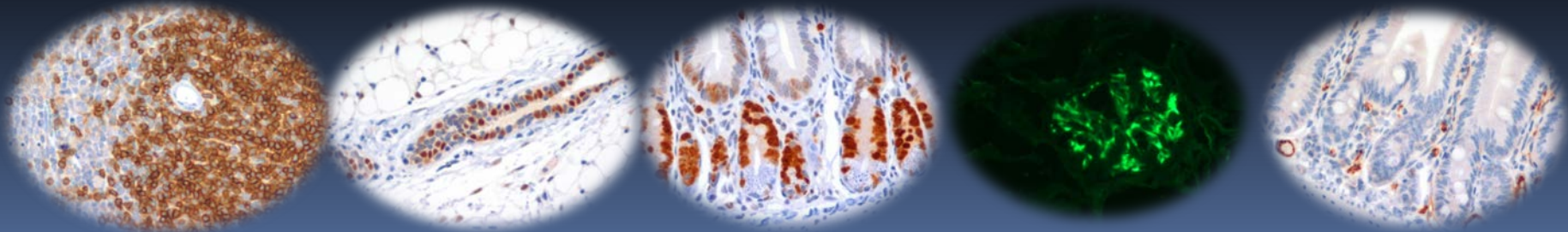
Protocols Seminar

Optimizing Immunohistochemistry (IHC) The Antigen Retrieval Technique

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(MHPL)



Outline

1. History and Mechanisms
2. Review of AR methods, options and reagents
3. What we do

Protocol of antibody work-up

Examples - MHPL

Section-1

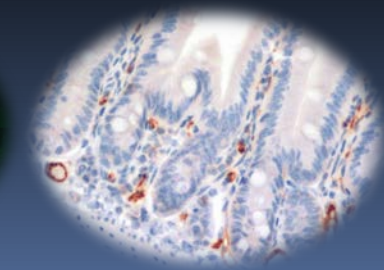
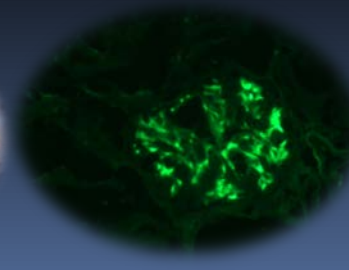
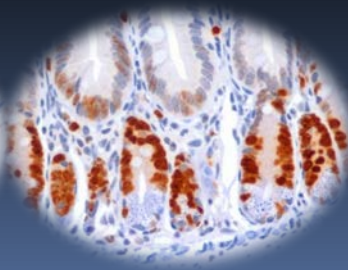
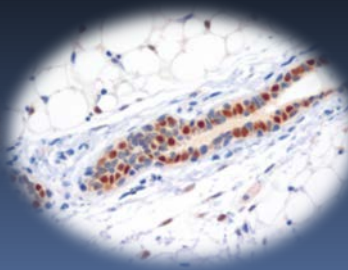
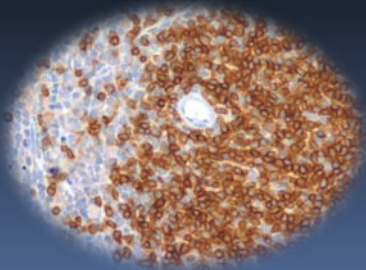
1. History and Mechanisms

2. Review of AR methods, options and reagents

3. What we do

Protocol of antibody work-up

Examples - MHPL



History

- In the 1940s, IHC was first introduced.
 - In the 1970s, with *digestive enzymes*, IHC staining was improved. **PIER**.
 - In 1991, Dr. Shi described the *high-temperature-heating antigen retrieval (AR)* technique. **HIER**.
- “**PIER**”: **P**roteolytic-**I**nduced **E**pitope **R**etrieval
 - “**HIER**”: **H**eat-**I**nduced **E**pitope **R**etrieval
 - “**FFPE**”: **F**ormalin-**F**ixed and **P**araffin-**E**mbedded

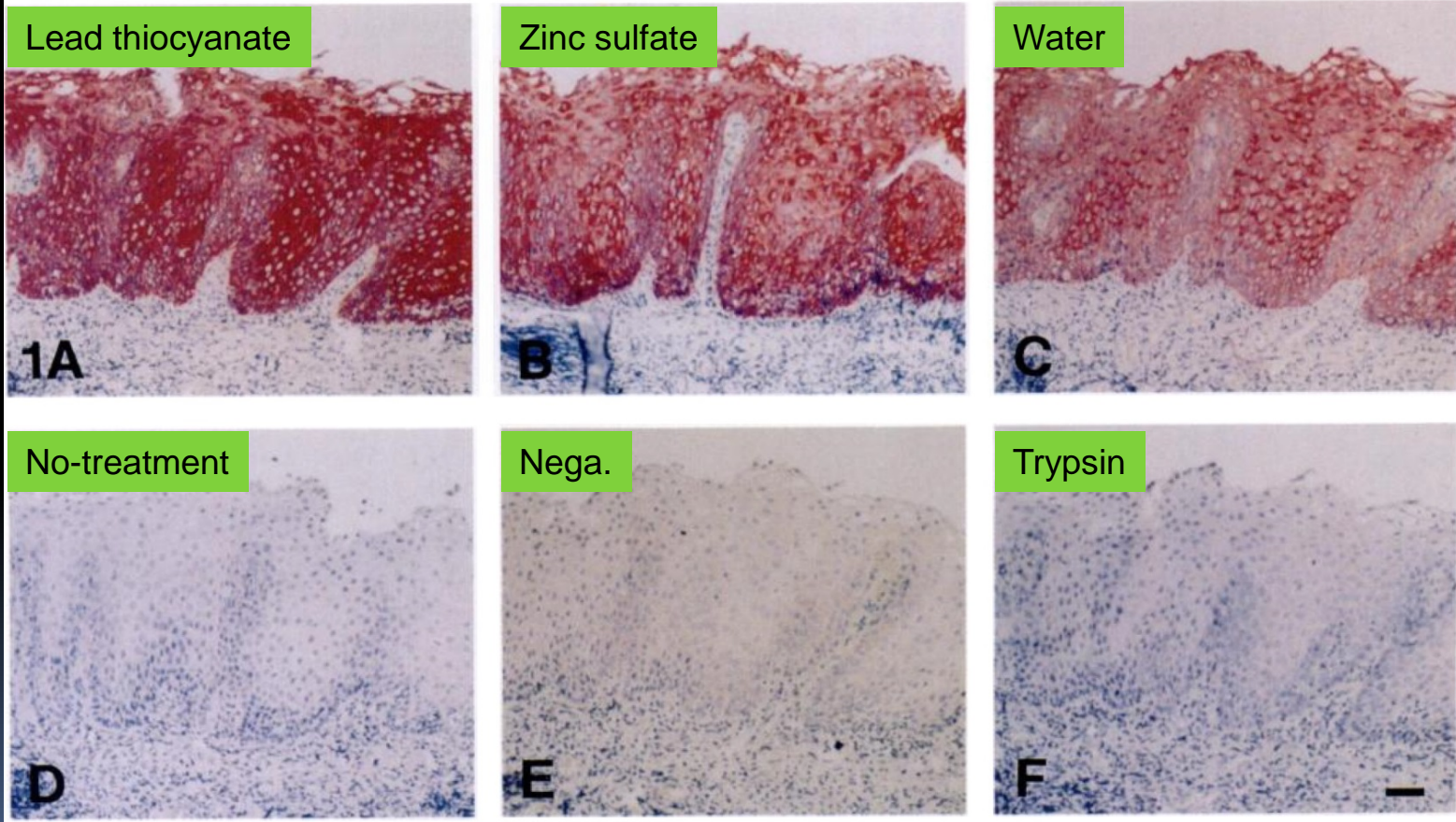


History-HIER

Rapid Communication

Antigen Retrieval in Formalin-fixed, Paraffin-embedded Tissues: An Enhancement Method for Immunohistochemical Staining Based on Microwave Oven Heating of Tissue Sections

SHAN-RONG SHI, MARC E. KEY,¹ and KRISHAN L. KALRA
BioGenex Laboratories, San Ramon, California 94583.



pan-cytokeratin , Human tonsil, Microwave

History-HIER

J Clin Pathol 1994;47:448-452
Microwave antigen retrieval in immunocytochemistry: a study of 80 antibodies
E C Cuevas, A C Bateman, B S Wilkins, P A Johnson, J H Williams, A H S Lee, D B Jones, D H Wright

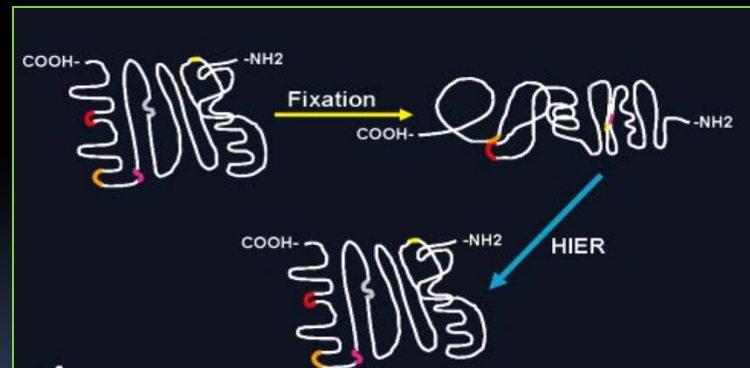
	Shi's: 1991	Cuevas's: 1994
No change	9	39
Increase	39	21
Reduce	4	
Previously only frozen		20
Total	52	80

Definition: predominantly defined as a high-temperature heating method to recover the antigenicity of tissue section that had been masked by formalin fixation.

Unknown 'Mechanism' for HIER

-Excerpted from Shi, et al's 2011 review in the JHC

1. Loosening or breaking of cross-linkages (Shi et al., 1991; Werner et al., 1996).
 - Another group observed that nine antibodies showed negative IHC staining results in unfixed tissues but were clearly positive after HIER treatment (Kakimoto et al. 2008)



2. Releases calcium from the cage-like calcium complex formation with proteins in FFPE.

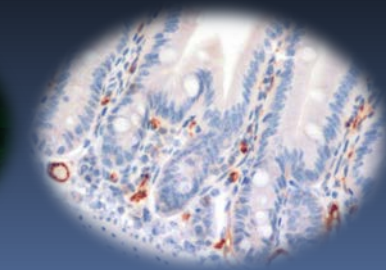
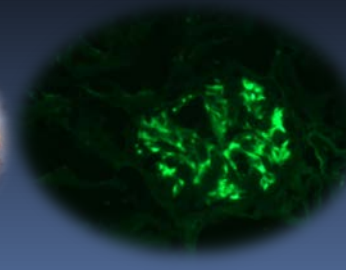
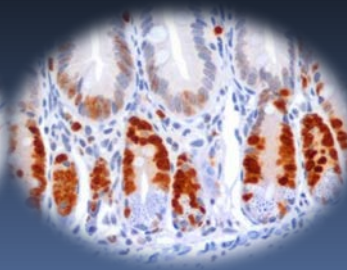
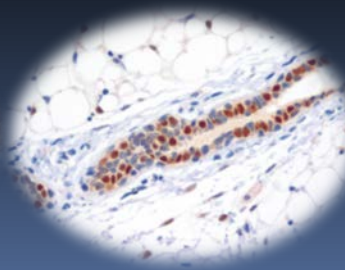
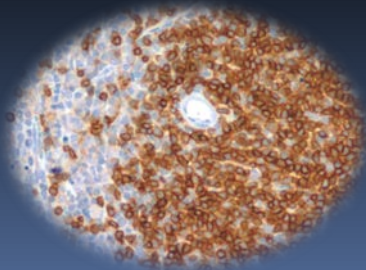
Section-2

1. History and Mechanisms
2. Review of AR methods, options and reagents

3. What we do

Protocol of antibody work-up

Examples - MHPL



Methods of AR

HIER	PIER
Citrate buffer pH 6.0 Tris-EDTA buffer pH 9.0 EDTA buffer pH 8.0	Proteinase K Trypsin Chymotrypsin Pepsin Pronase

“HIER”: Heat-Induced Epitope Retrieval.

“PIER”: Proteolytic-Induced Epitope Retrieval.

Whenever possible, use HIER instead of PIER

Factors Affecting Degree of HI ER

➤ Heating conditions: (T), (t)

- The higher the temperature, the shorter the heating time, and vice versa.

➤ pH value of the AR solution

➤ Fixation

- *There is no universal method of AR that is optimal for all antigens.*
- *There is no single HI ER solution that is best for all antigens.*

HIER Devices



HIER Devices

➤ Advantage

- Fast, convenient
- Standardization
- Less fall-off

➤ Disadvantage

- Heat artifacts
- Careful handling



MHPL's routine HIER device

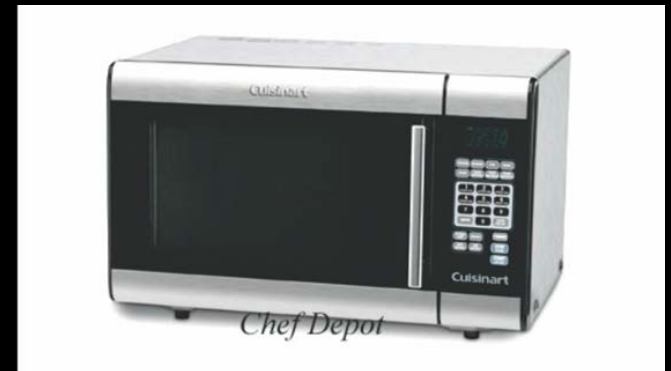
HIER Devices

➤ Advantage

- Fast, Convenient

★ ➤ Disadvantage

- Fall-off
- Difficulty in standardization
- Needs calibration



★ *MHPL doesn't use it for HIER*

HIER Devices

➤ Advantage

- Consistent
- Standardization
- Morphology

➤ Disadvantage

- Slow
- Not good for over fixed tissues



MHPL's routine HIER device

HIER Devices

➤ Advantage

- Consistent
- Standardization
- Morphology

➤ Disadvantage

- Slow
- Not good for over fixed tissues



Good for bone and cartilage tissues such as mouse ear, ankle, etc.

HIER Devices

Comparison of devices

Comparing	Pressure Cooker	Microwave	Vegetable Steamer	Oven
Temperature	25-110 C	100-105 C	85-95 C	60 C
Time (equivalent)	5-10min	10-20min	30-60min	O/N
Heat-source Regulation	Excellent	Good	Significant	Significant

Reagent and Specimen 'Handling'

➤ Reagents

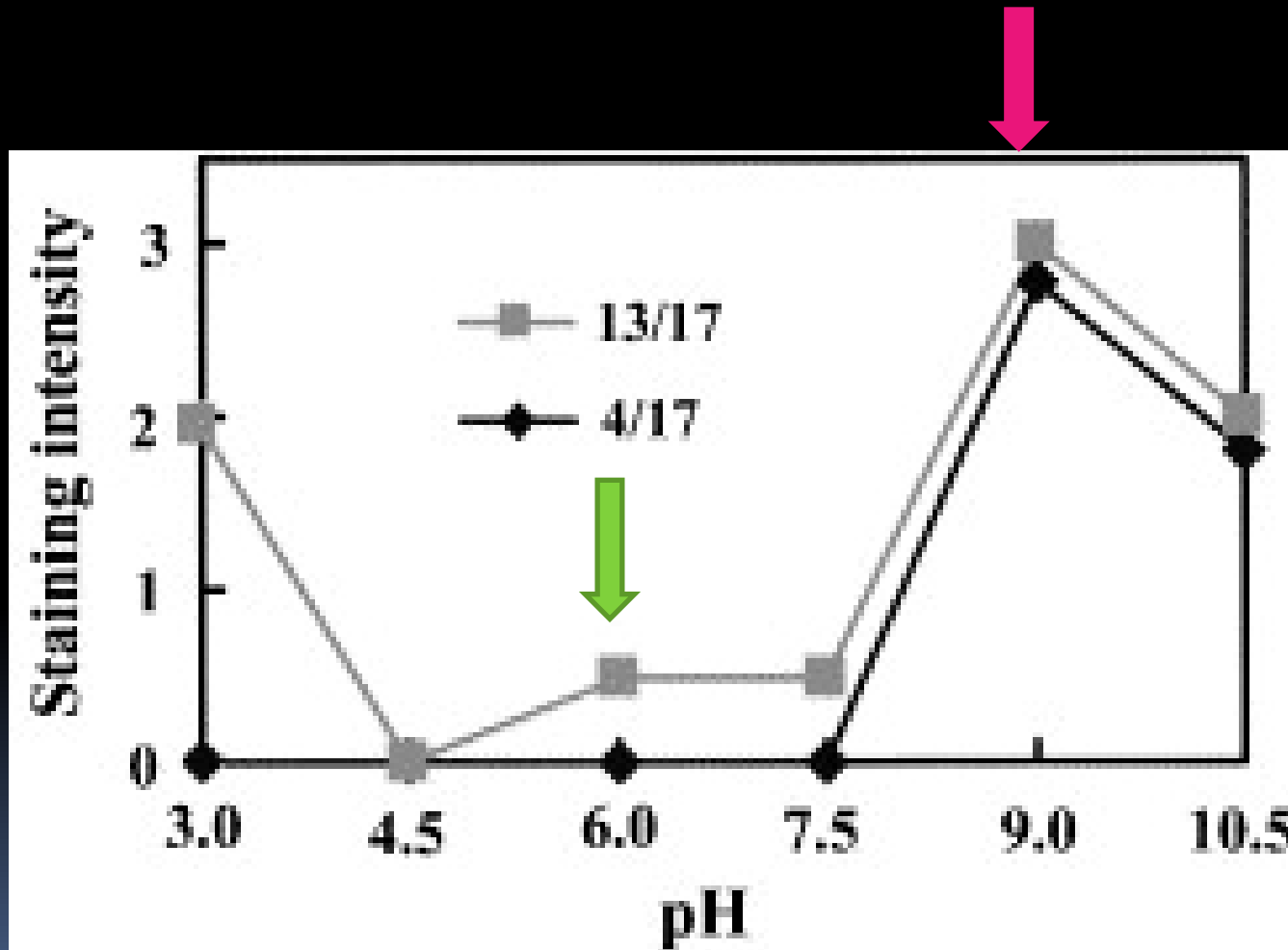
- AR buffers, stable months at 4 C
- One-time use

➤ Specimen

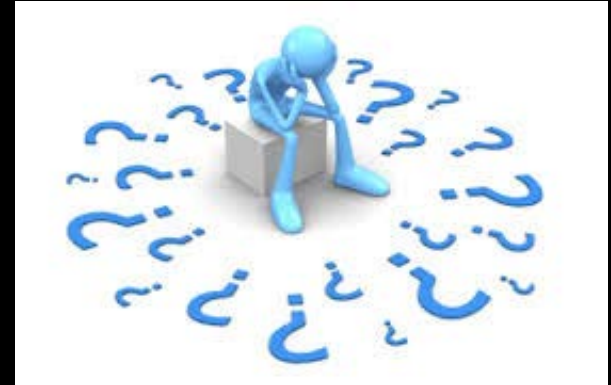
- Dewax
- 'Cooling' the specimen – actually extended retrieval
 - remove at 90 C from pressure cooker
 - let stand on the counter for 30 min
 - gradually replace a hot solution with RT DI water

*The key is consistency of technique.
Everyone must perform 'post-heating' procedures the same way!*

pH-Dependent Patterns of HIER



To Fix or Not to Fix



➤ To fix

- Better morphology
- Prevent antigen elution or degradation
- Preserve position of antigen
- Must standardize fixation protocol!

➤ Not to fix

- Antibody will not detect fixed antigen
- Antigen retrieval does not work

We recommend preparing initial samples in both fixative and OCT, If you are not sure.

How and What to Fix With

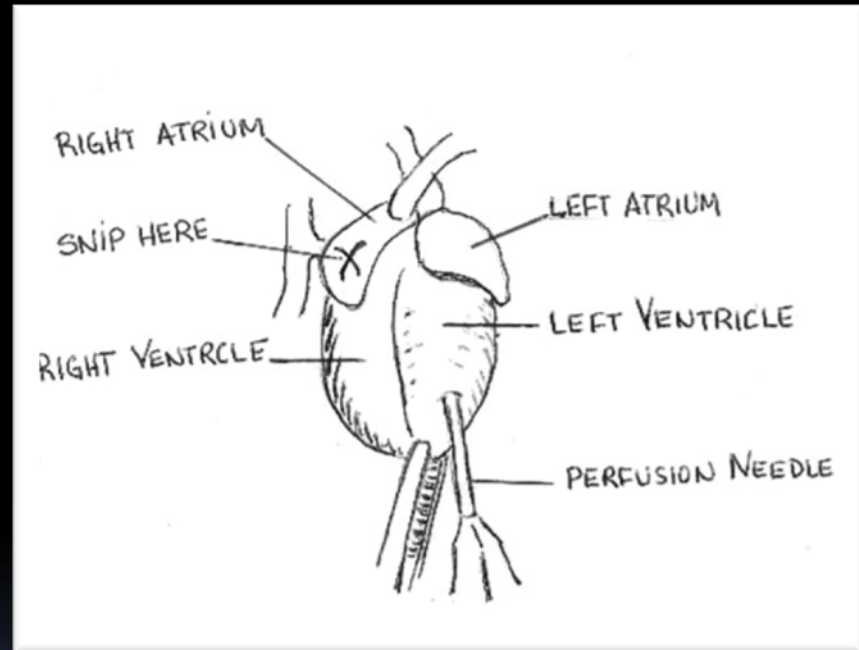
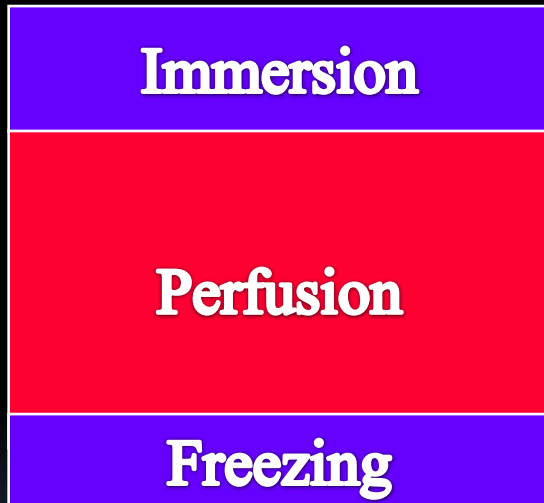
There is no universal fixative to fit all, but 10% **NBF** suitable for 95% of application.

- increasing fixation time causes increasing amounts of antigen damage
- referred to as "antigen masking"
- Tissue size: approximately **3 mm thickness, <24hrs.**

NBF: Neutral Buffered Formalin

Fixative preparation and protocol must remain consistent

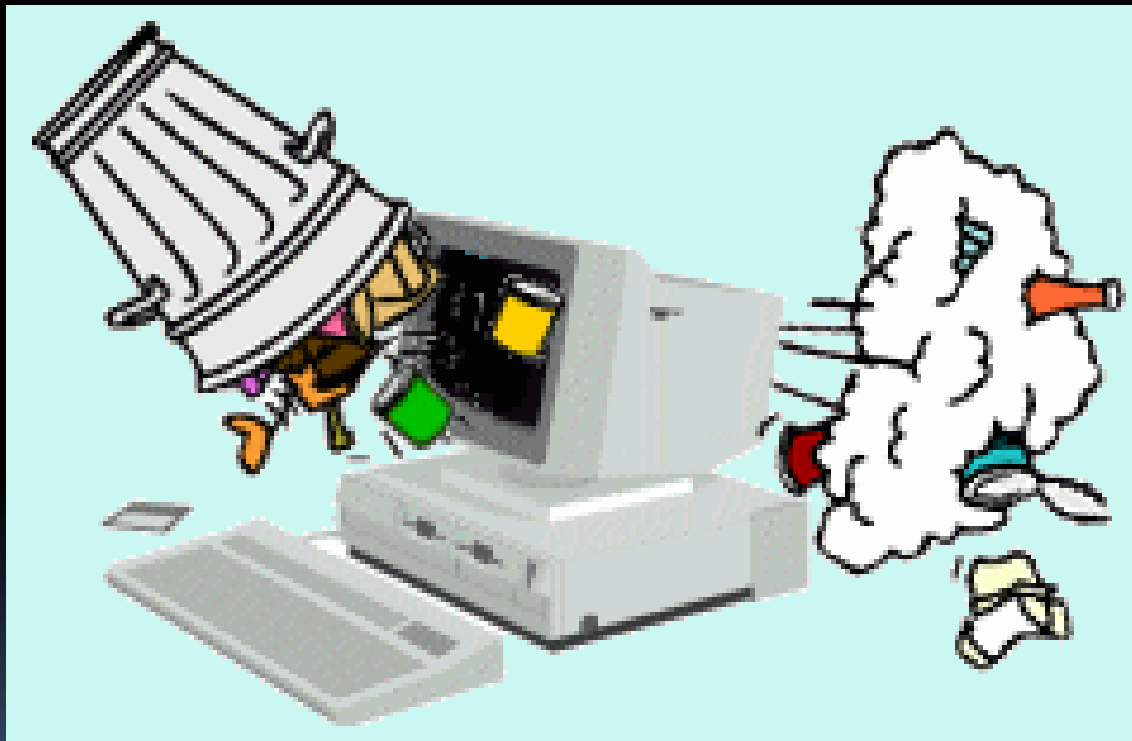
Methods of Fixation



Transcardiac perfusion

Allowing rapid fixation of deep tissue

Garbage In, Garbage Out!

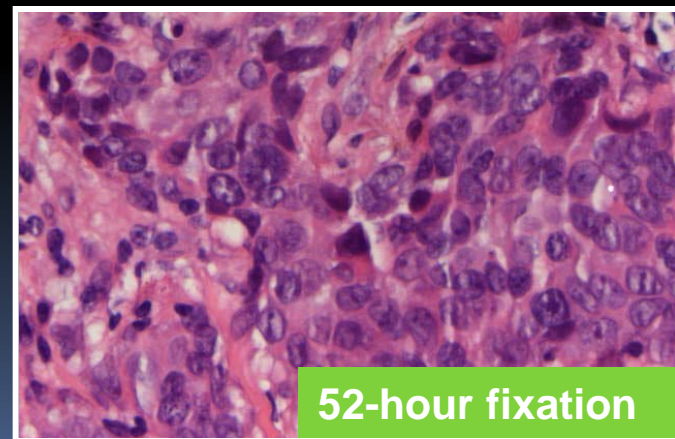
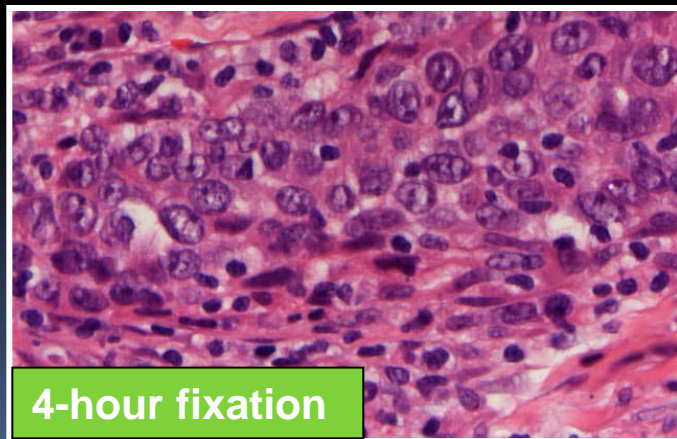
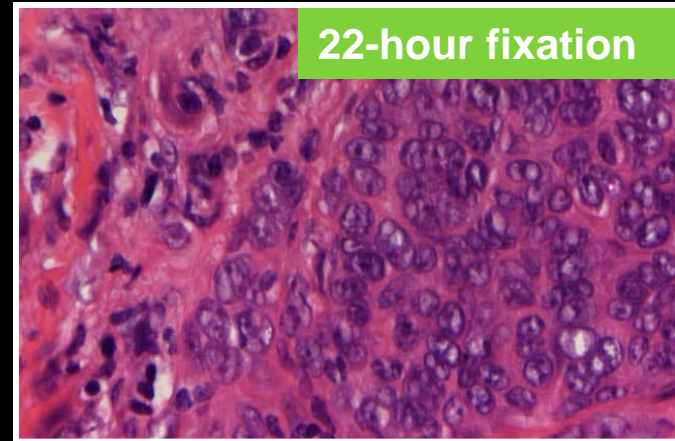
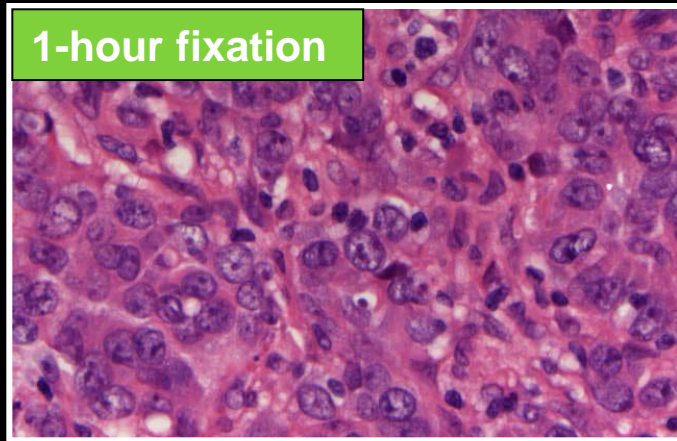


Under-fixed

Duration of fixation should be 6-48 hours



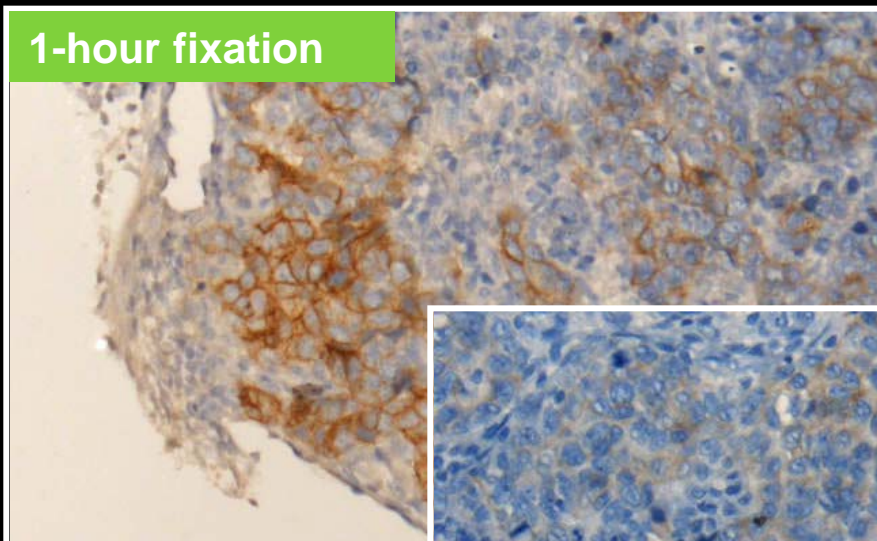
H&E staining does not reveal poor fixation



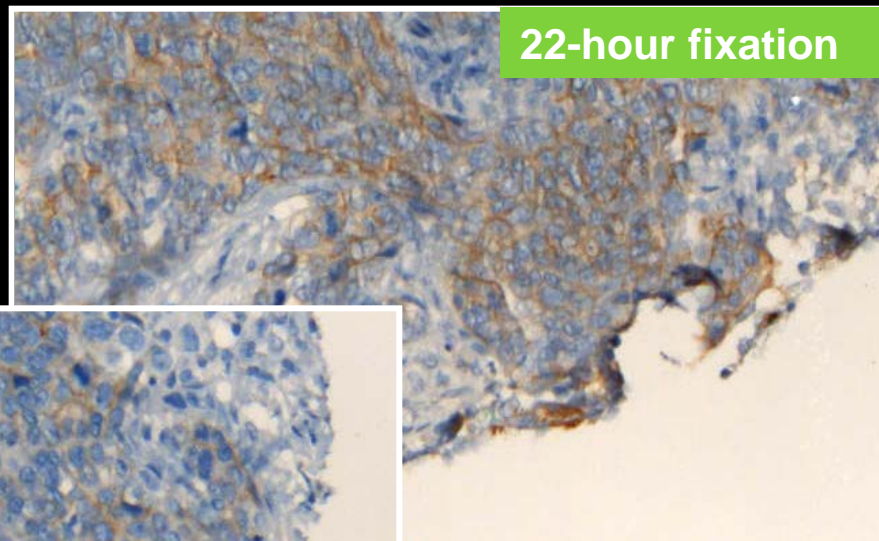
Duration of fixation impacts on HER2-staining pattern



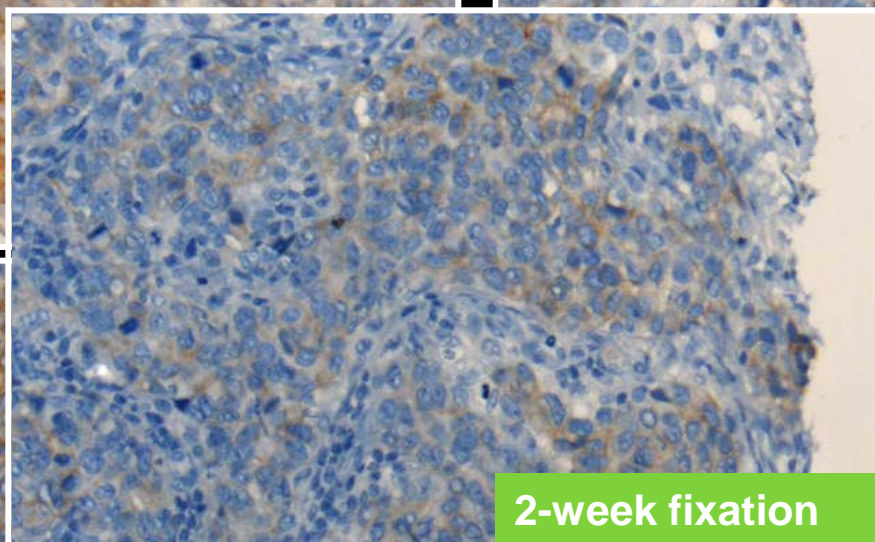
1-hour fixation



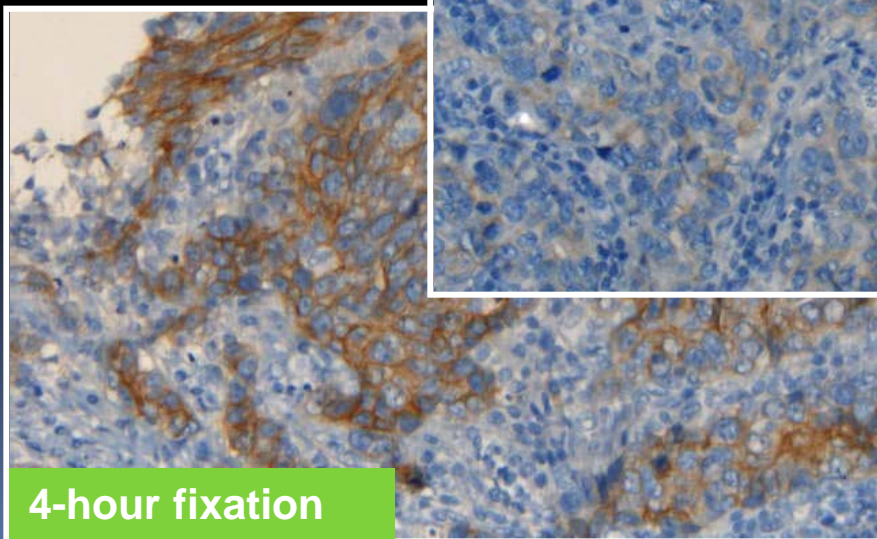
22-hour fixation



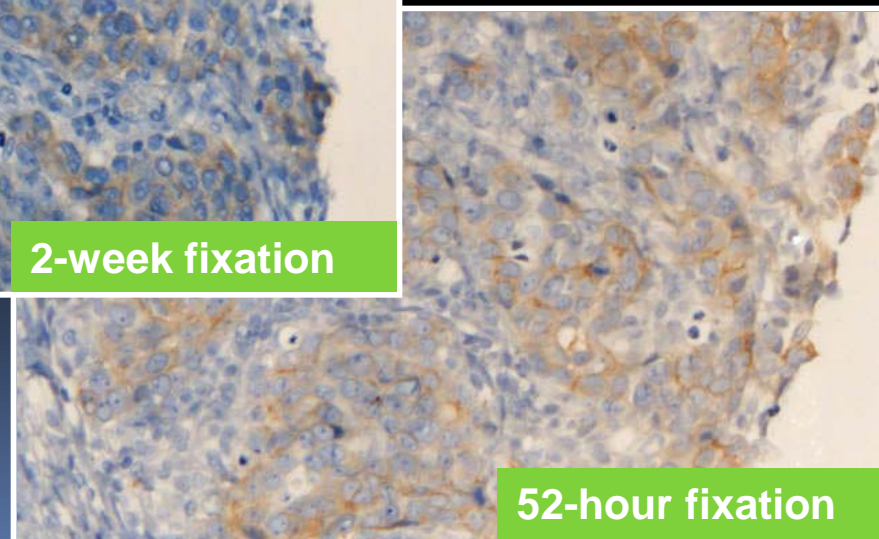
2-week fixation



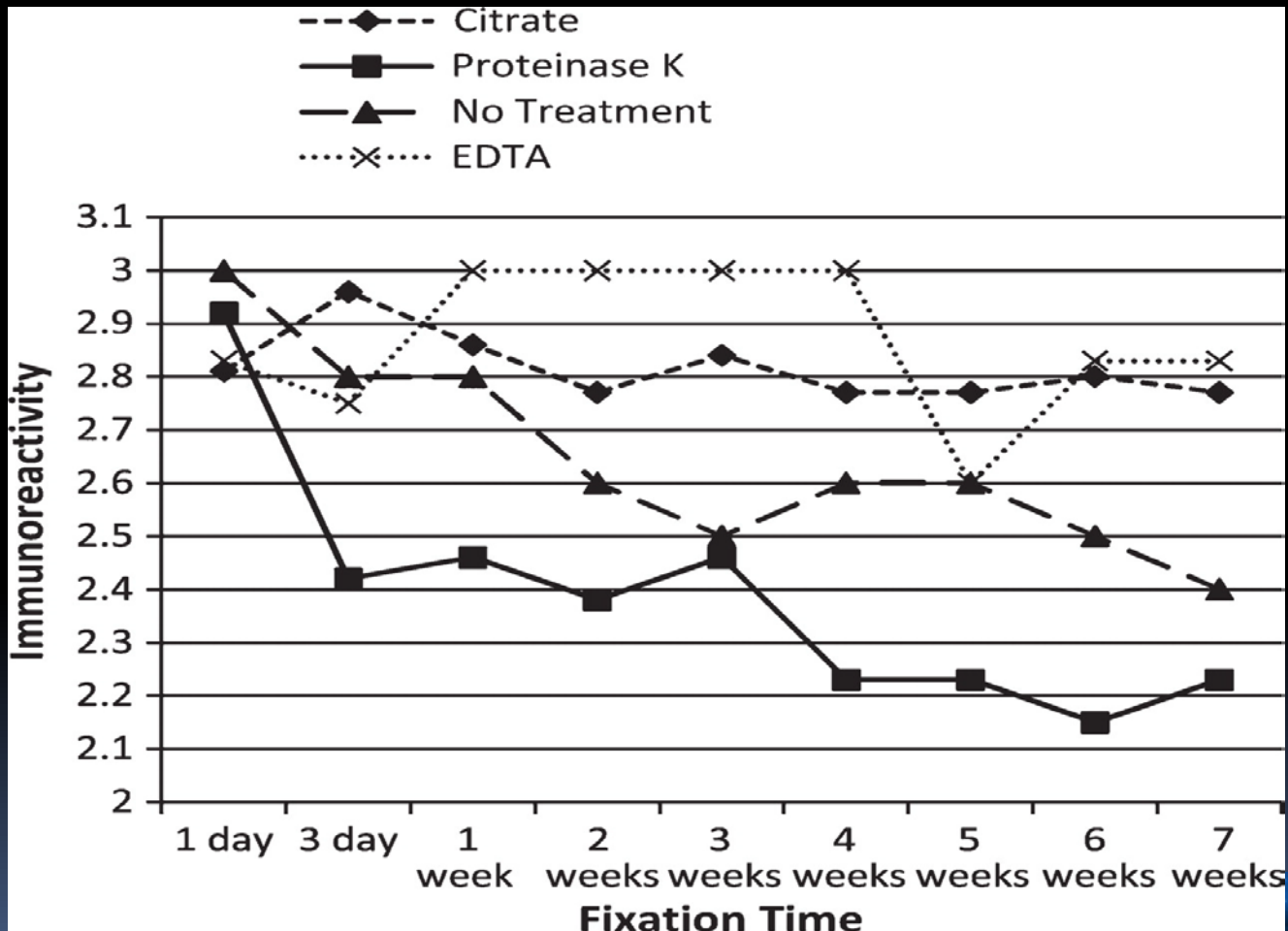
4-hour fixation



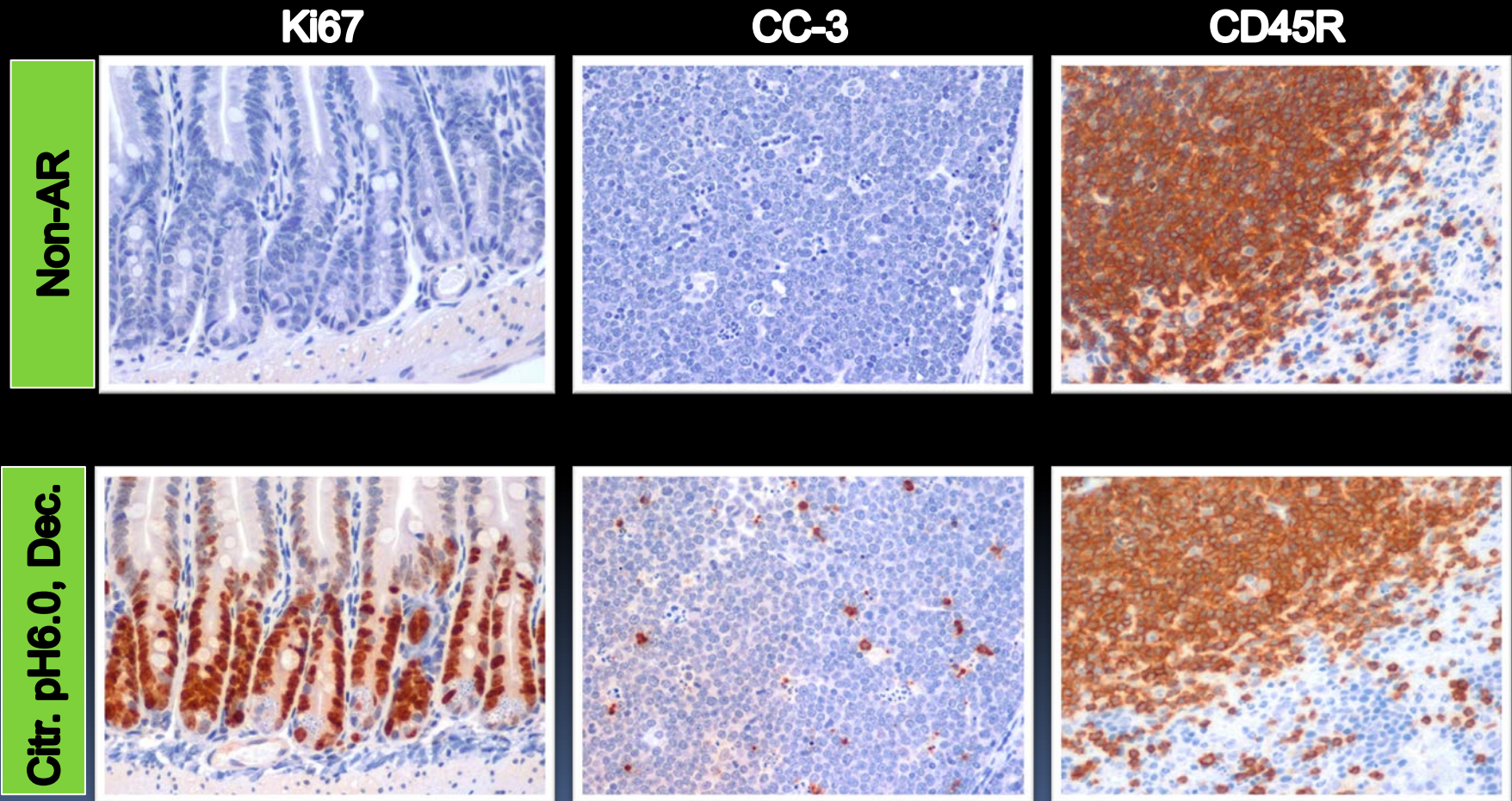
52-hour fixation



Influence of Reagents on AR

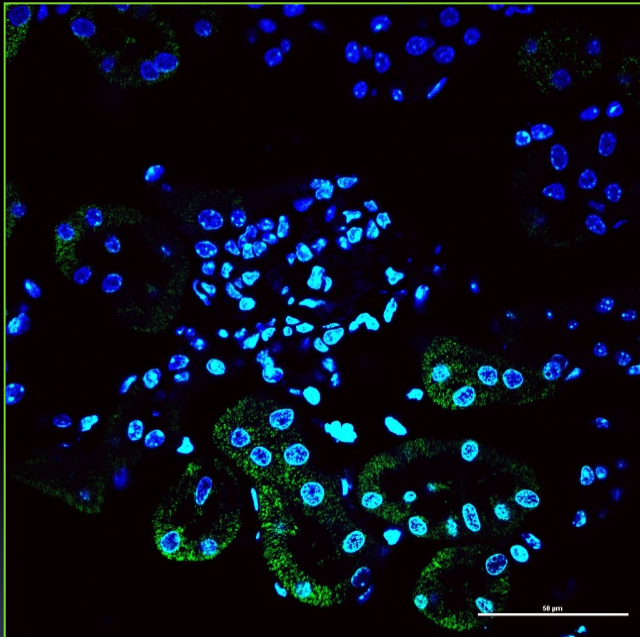


AR Has a Significant Effect on IHC Results

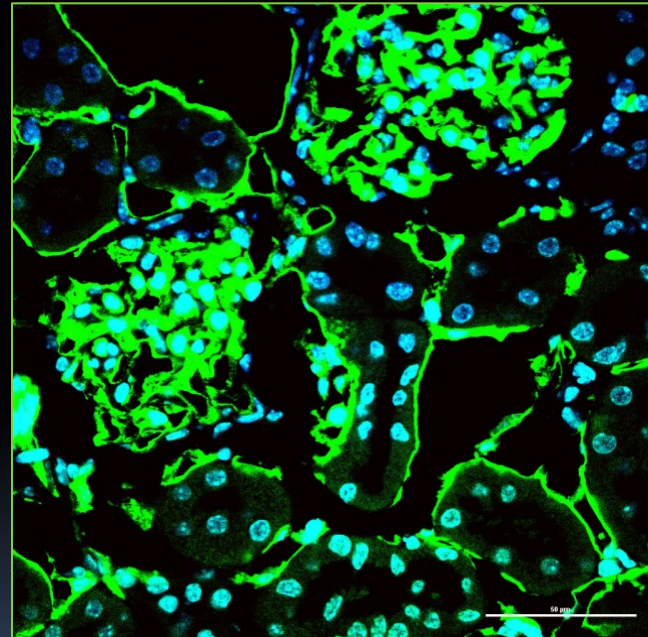


AR Has a Significant Effect on IHC Results

Non-AR

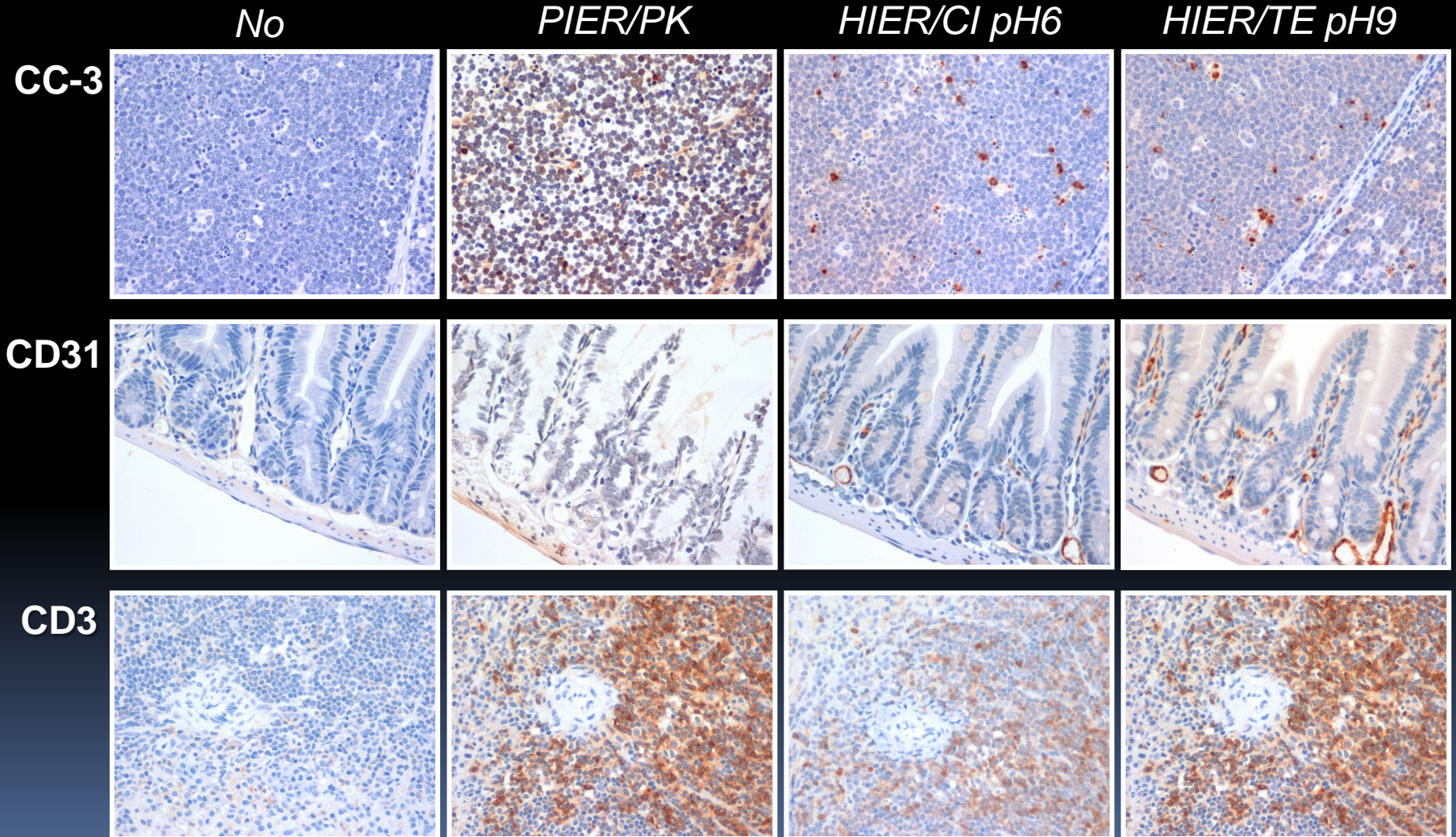


+AR



CD31 stained, mouse kidneys

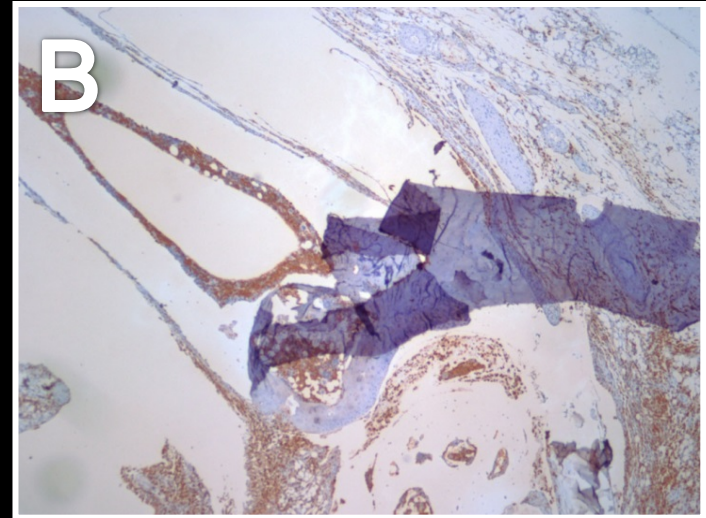
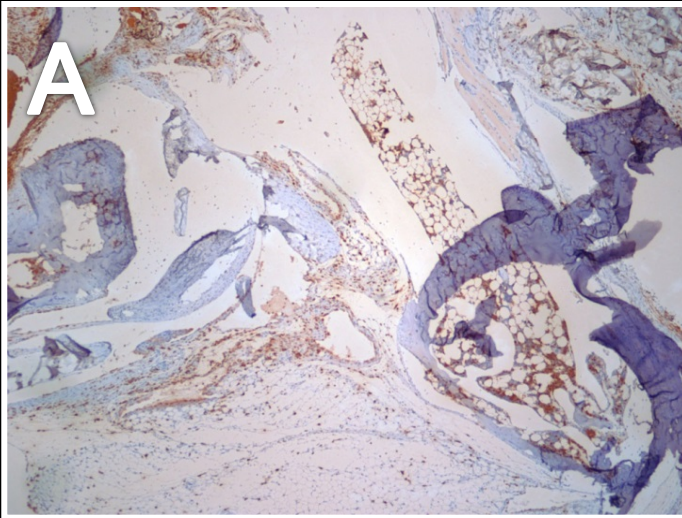
Inappropriate AR Procedure



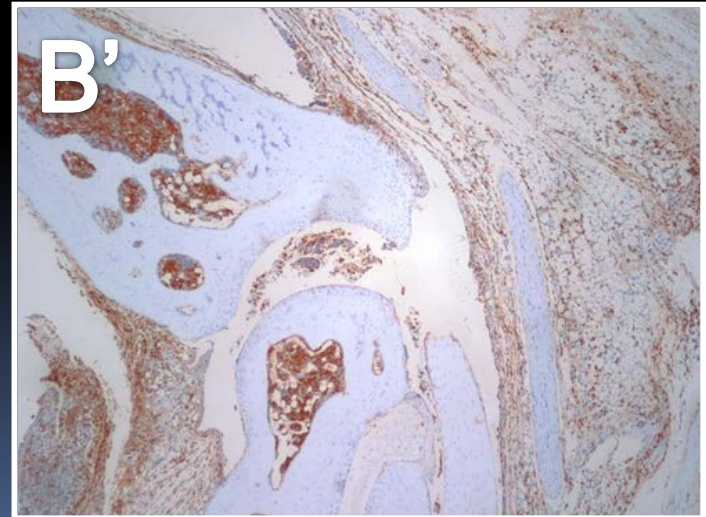
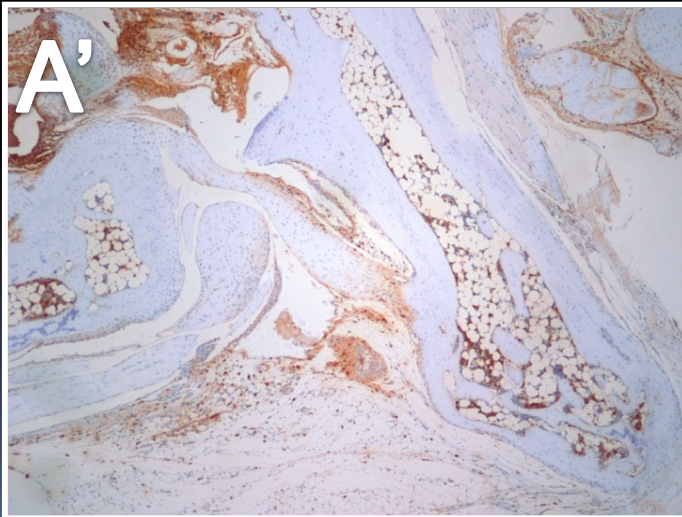
>95% of all commonly used antibodies require HIER

Inappropriate AR Procedure

Decloaker



Oven

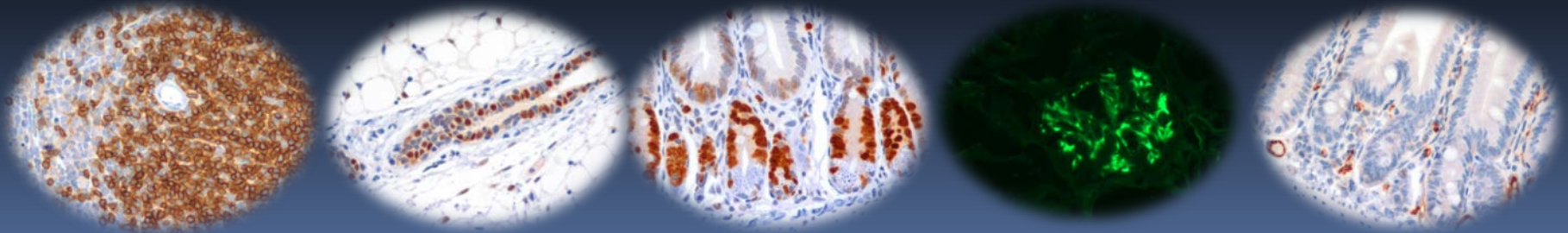


Decloaker vs. Oven - Ym-1, on mouse ankle

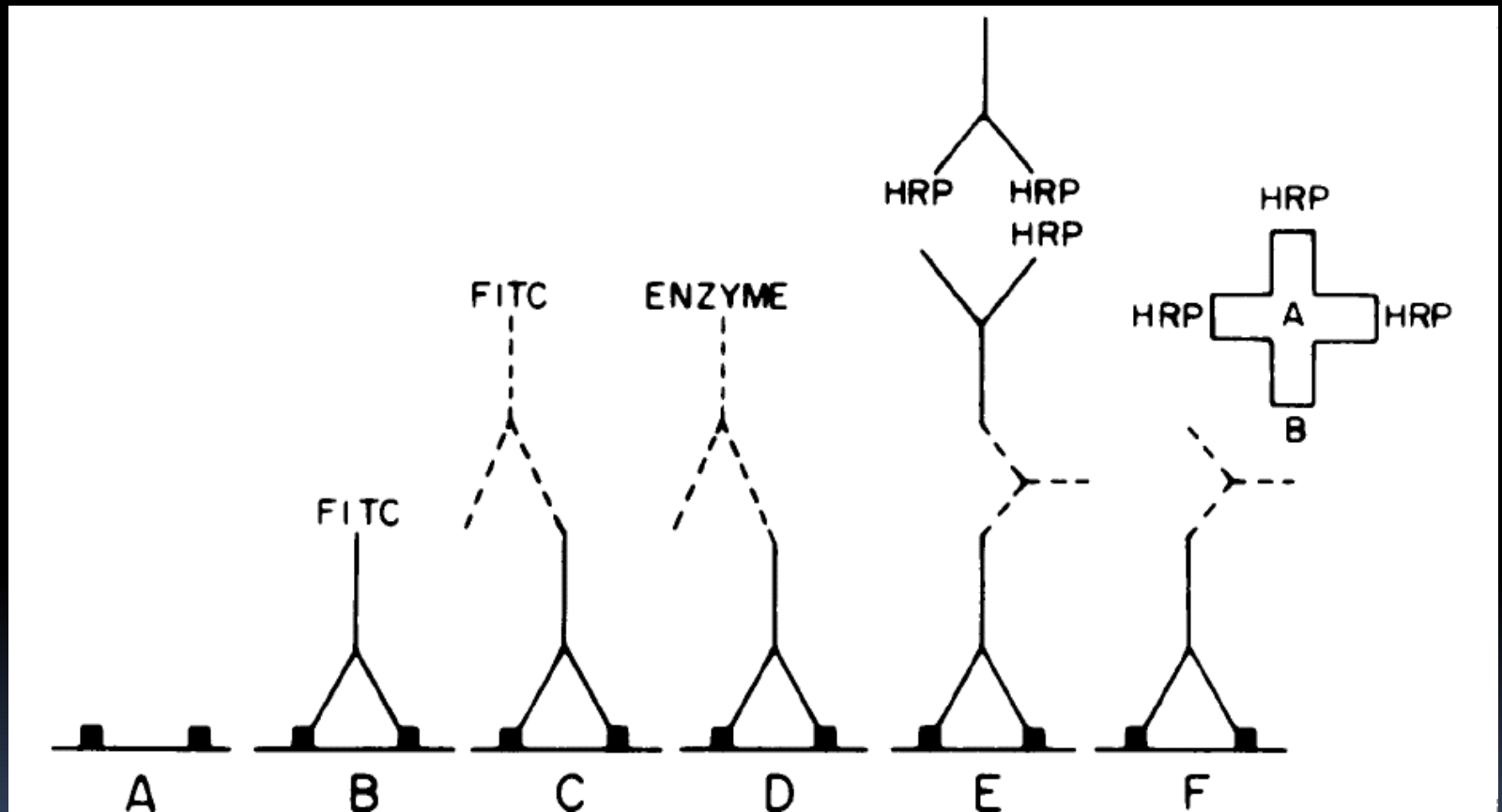
SECTION-3

1. History and Mechanisms
2. Review of AR methods, options and reagents
3. What we do

Protocol of antibody work-up
Examples - MHPL



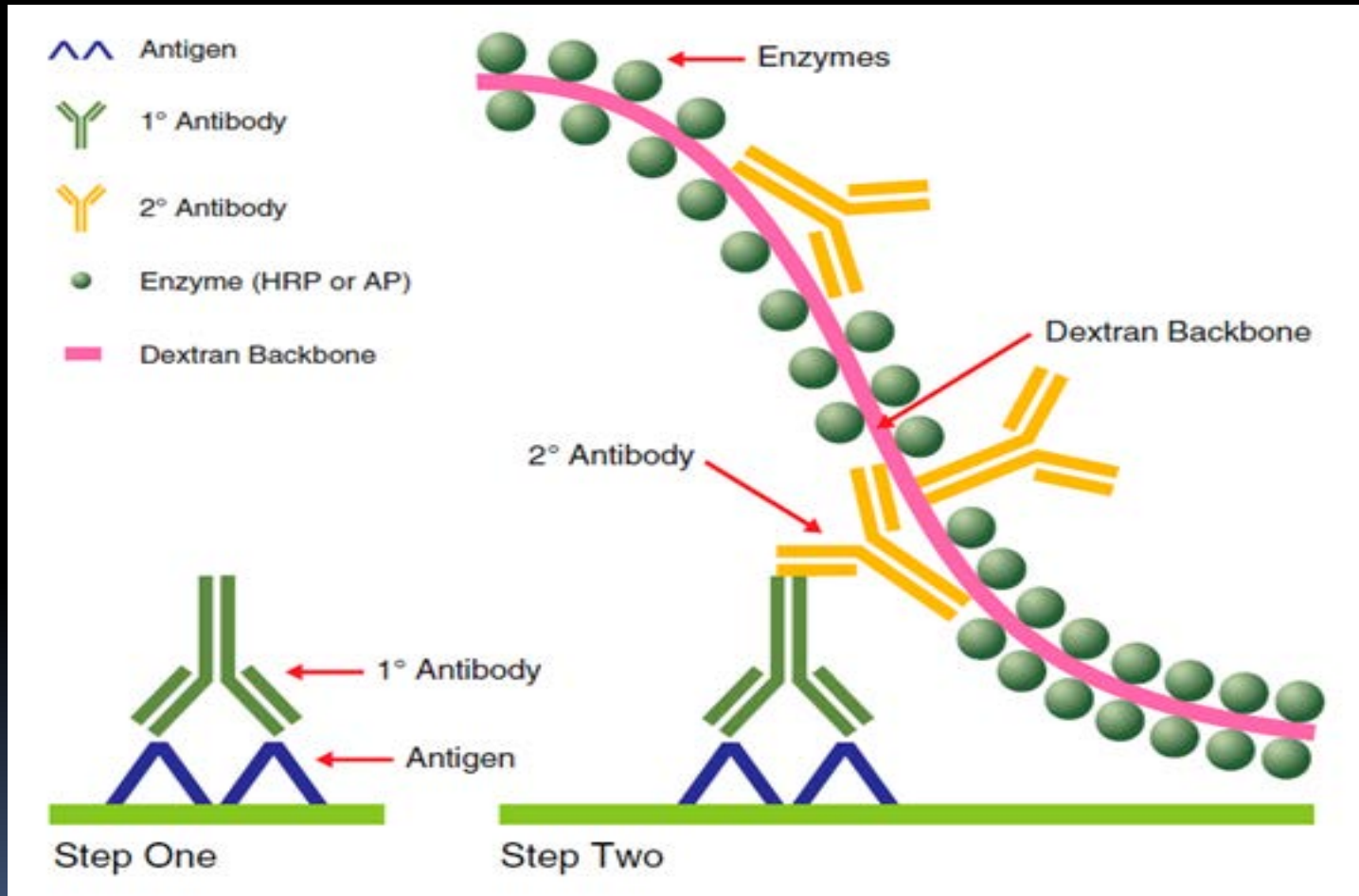
Detection Systems



Direct

indirect

Detection Systems



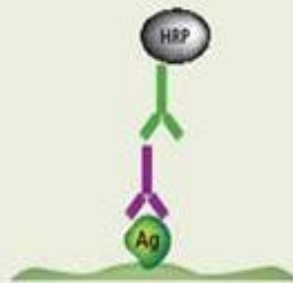
Polymer-based Technology

Detection Systems

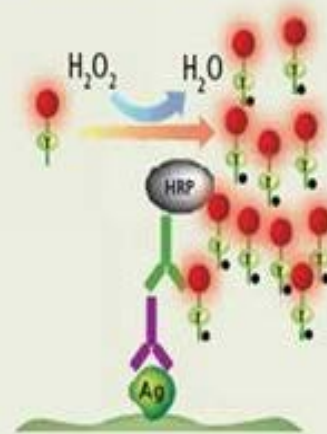
1. Incubate with primary antibody



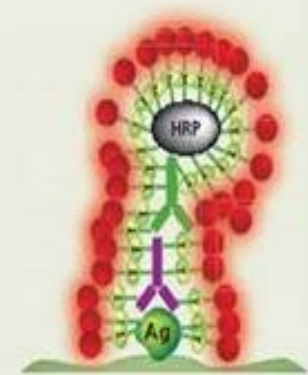
2. Introduce HRP



3. Incubate with TSA reagent (3-10 min). HRP catalyzes formation of TSA free radicals.



4. TSA free radicals form covalent bonds with tyrosine residues proximal to HRP. Unbound TSA radicals form dimers that are washed away.



Tyramide Signal Amplification (TSA) Systems

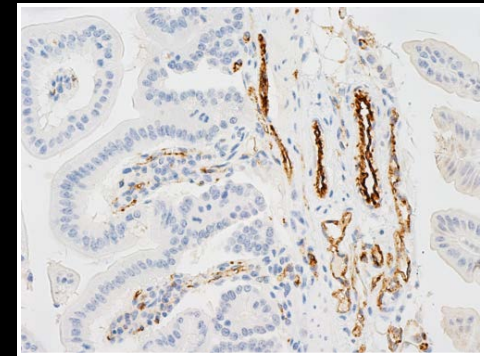
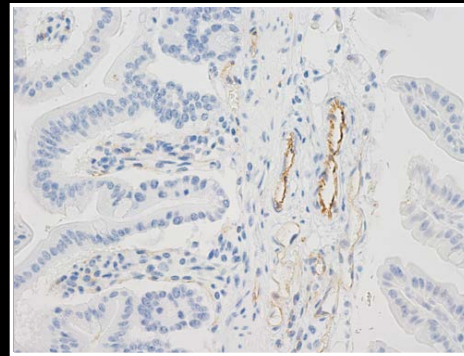
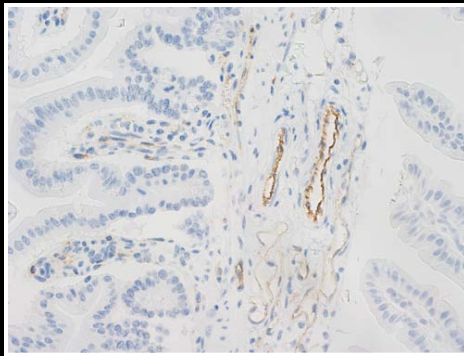
Comparison of Detection Systems

ABC

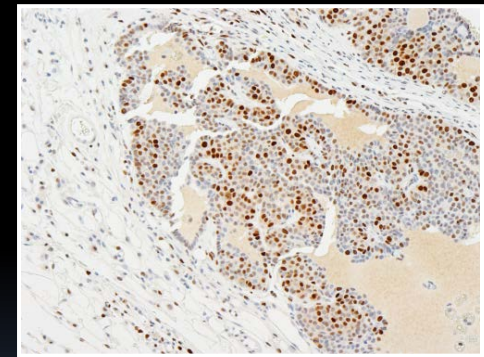
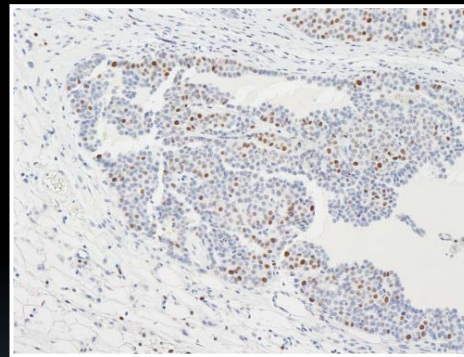
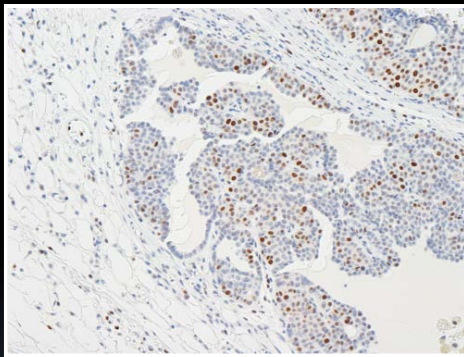
Polymer

ABC+TSA

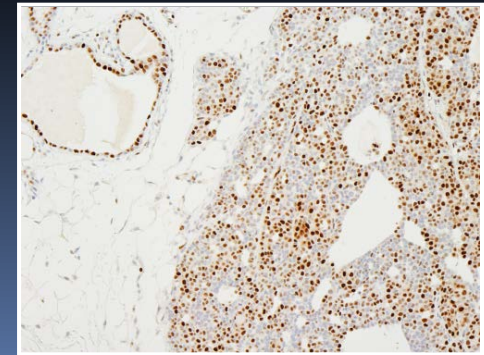
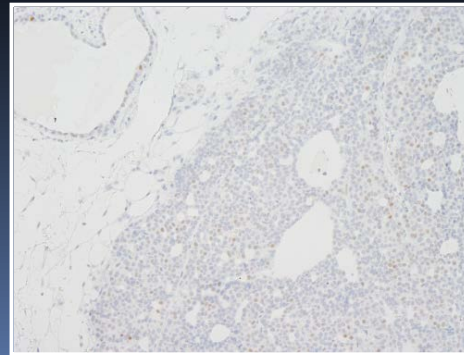
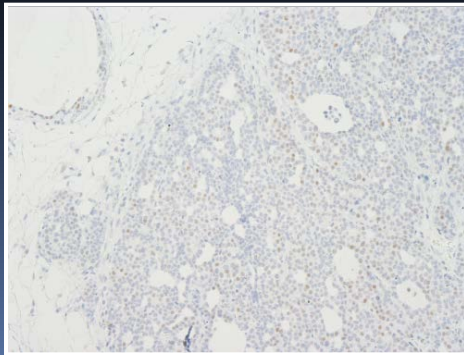
CD31



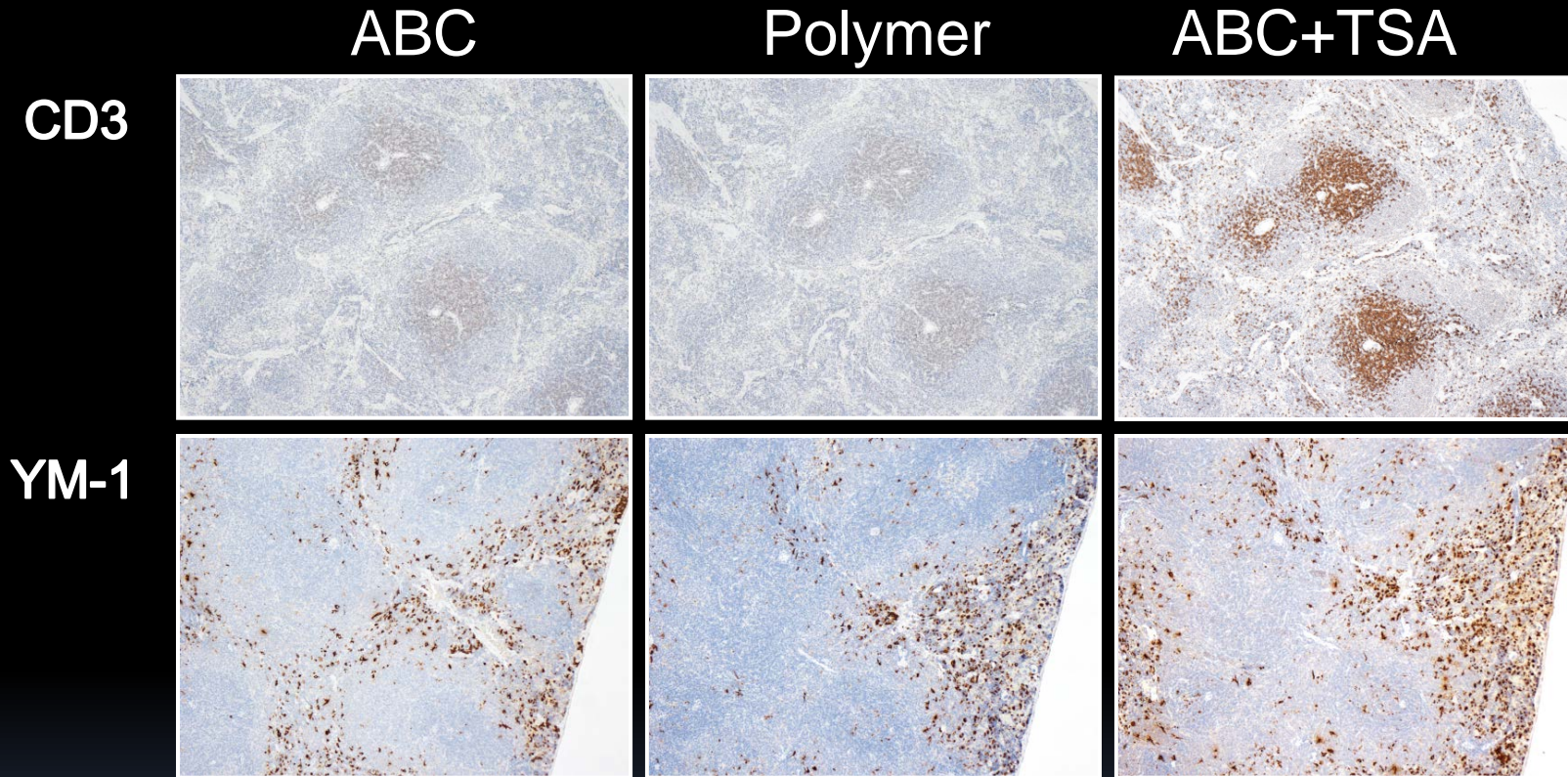
PCNA



Cyclin D-1



Comparison of Detection Systems



Protocol of Antibody Work-up

Step 1. Identify potential antibody

- Reactivity, host, positive control, review papers...

Step 2. Optimal AR methods

- No Ag Retrieval
- Citrate buffer, pH 6.0
 - pressure cooker
 - 60°C, oven, O/N
- Tris-EDTA, pH9.0
- Proteinase K

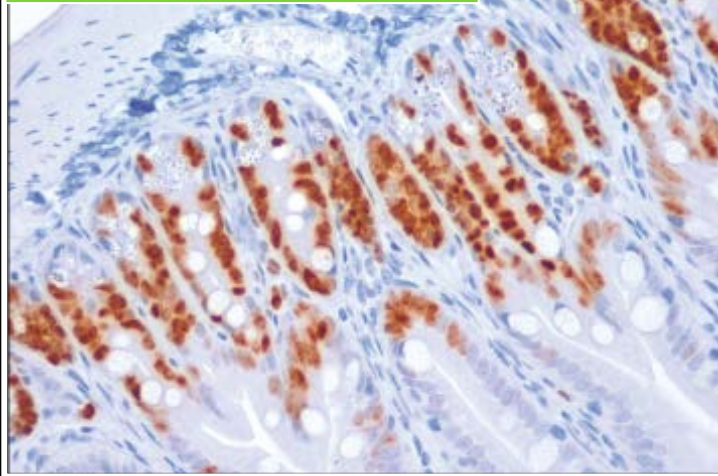
Step 3. Titer antibody

- 2- 3 different antibody concentrations

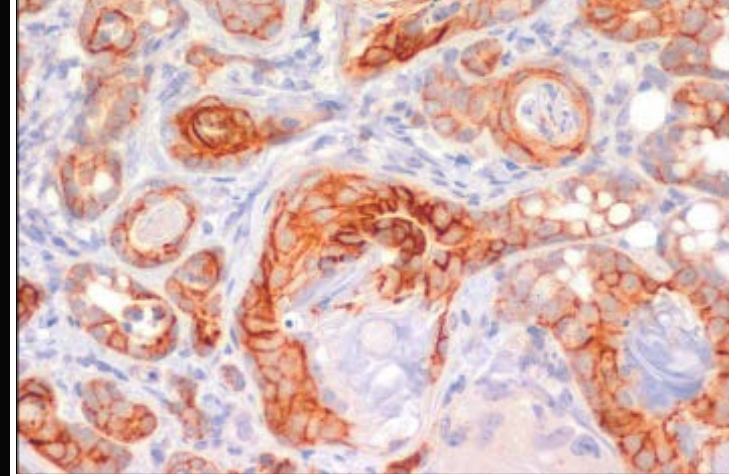
At least 10 slides total required

Cellular Location of Antigens

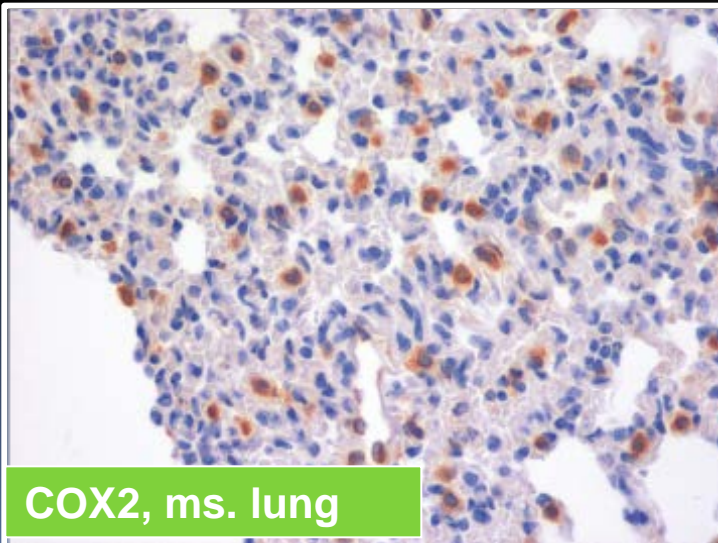
Ki67, ms. intestine



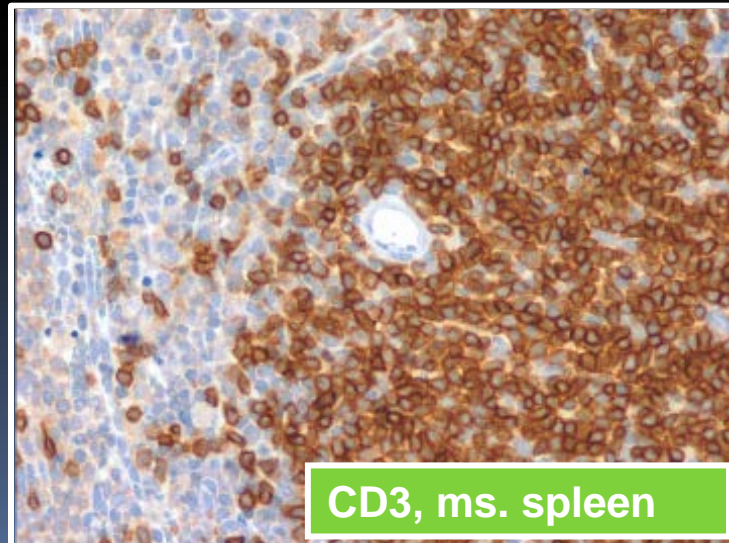
Her-2 ms. mammary cancer



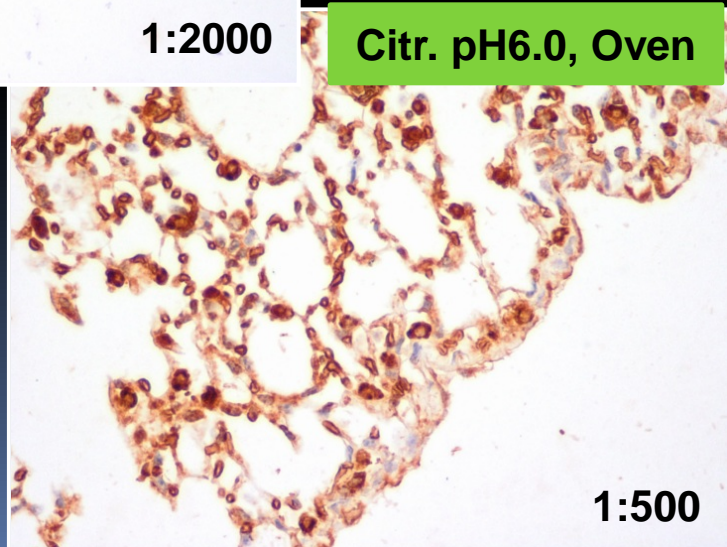
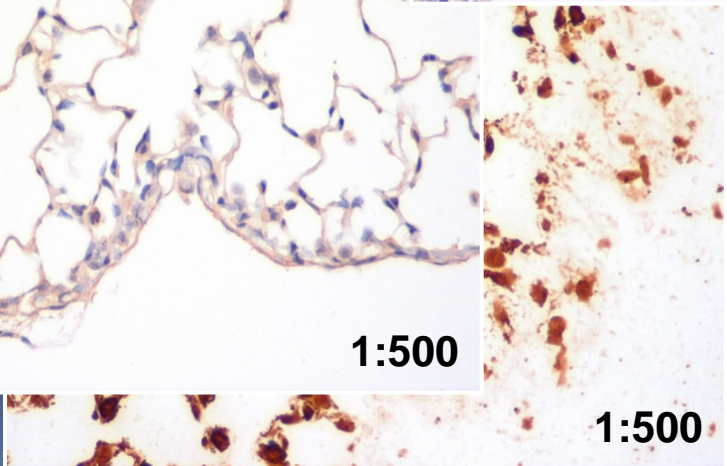
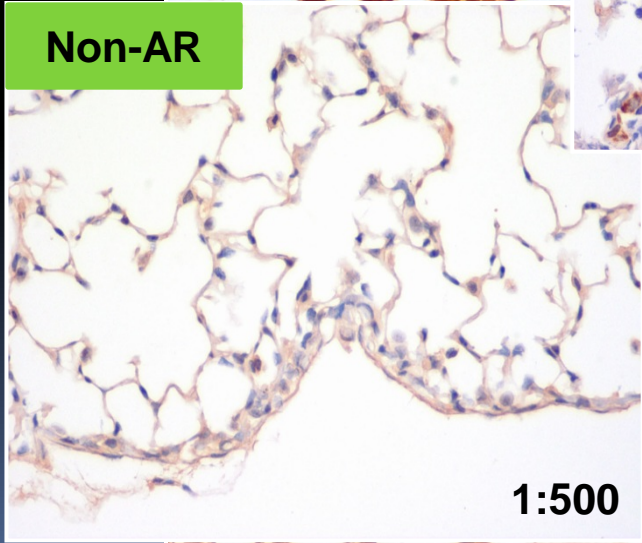
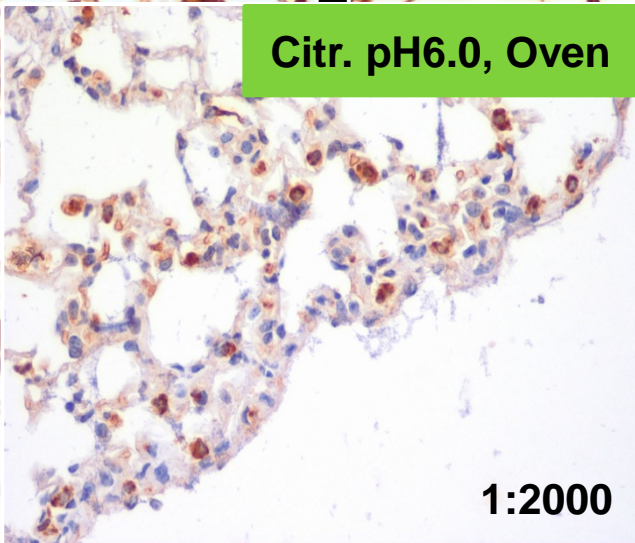
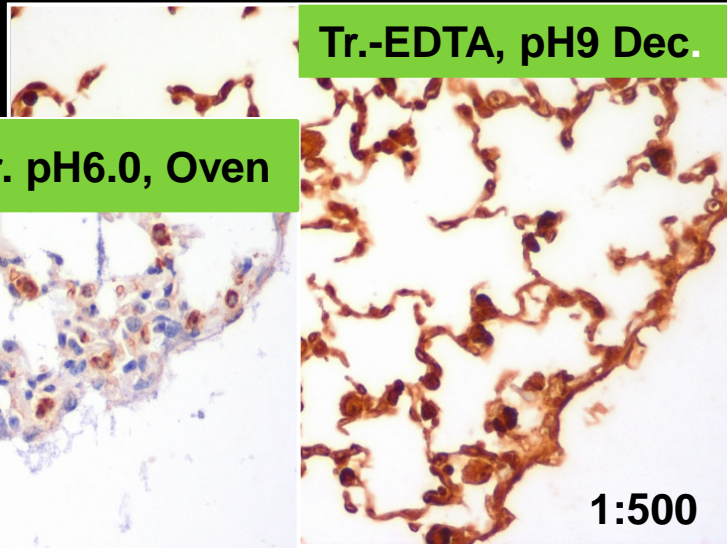
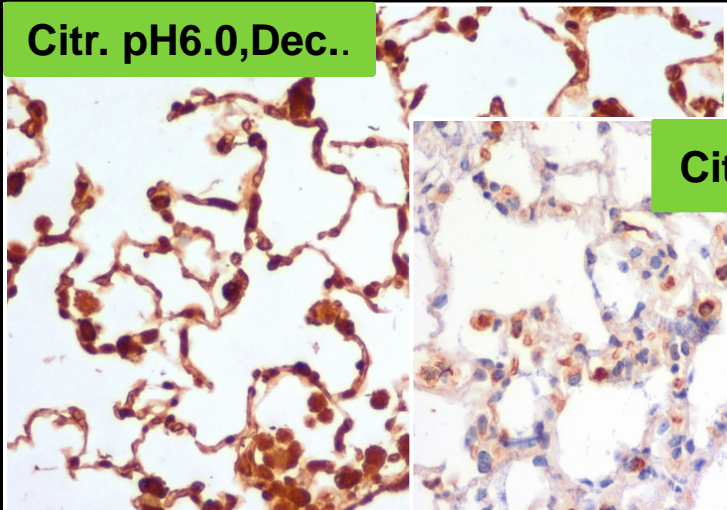
COX2, ms. lung



CD3, ms. spleen

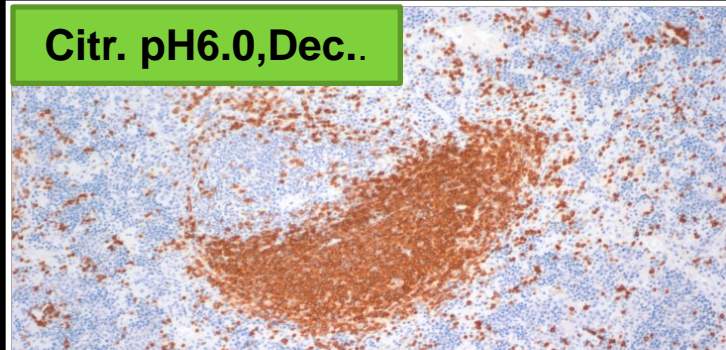


Examples-1 proSP-C

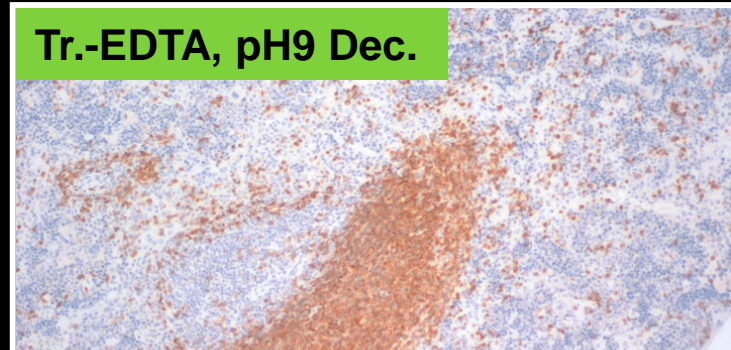


Examples-2 CD45R

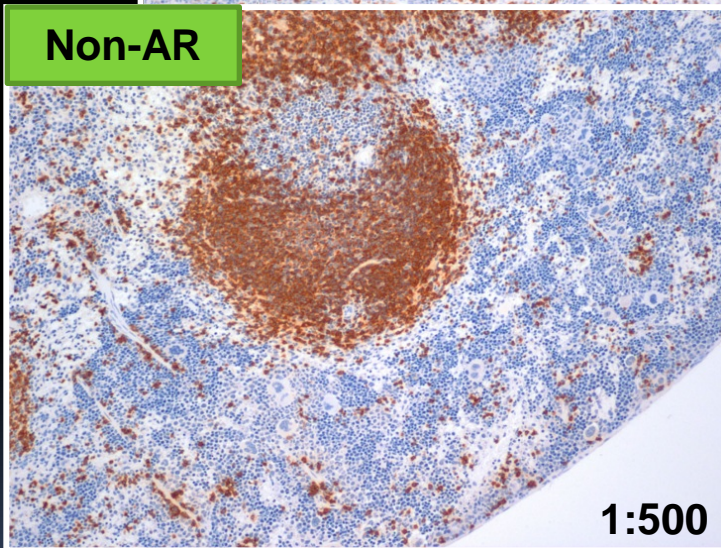
Citr. pH6.0,Dec..



Tr.-EDTA, pH9 Dec.

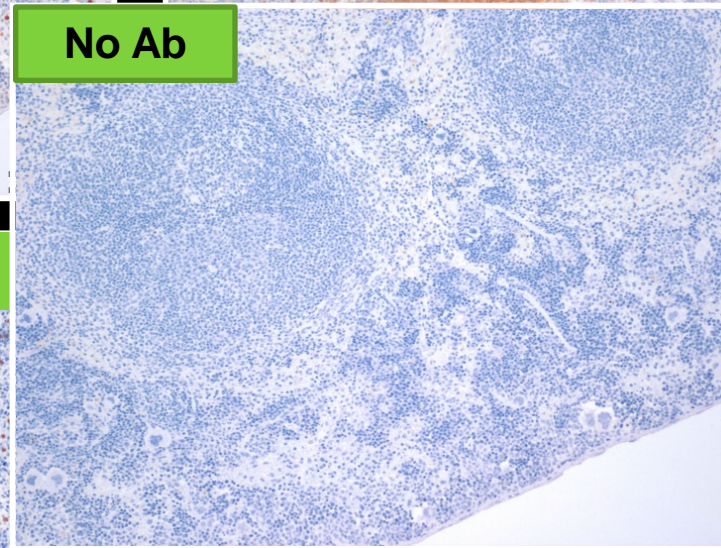


Non-AR



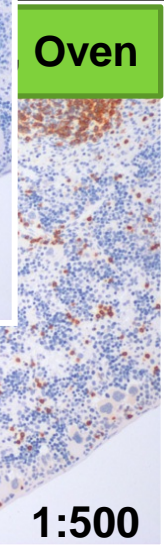
1:500

No Ab

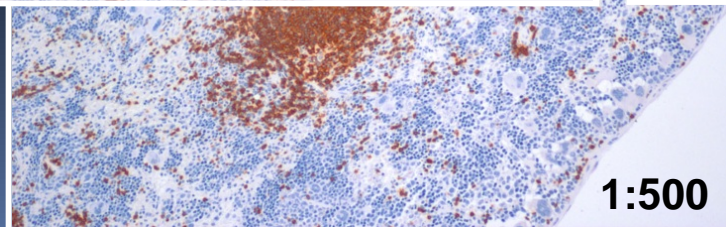


1:500

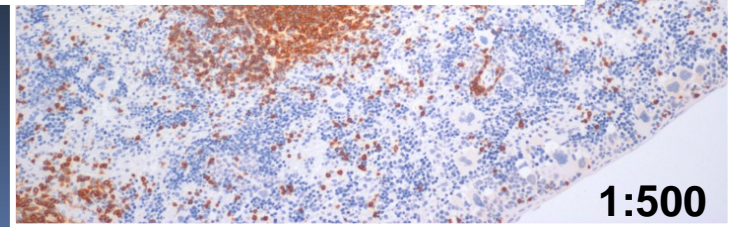
Oven



1:500



1:500



1:500

What We Do

- **More sensitive detection system:**
 - Biotin tyramide conjugates
 - DyLight 488 tyramide conjugates
 - DyLight 555 Tyramide conjugates
- **Cost efficient:**
 - More dilution primary antibody
 - Reduced cost of reagents
- **Immunoperoxidase method:**
 - We currently have 66 antibodies optimized
 - In addition we have 19 typical antibodies work-up for investigators.
- **Developing protocols for double and triple labeling IFC**

Final Note: “We are Here to Help”

MHPL website

<http://www.feinberg.northwestern.edu/research/cores/units/mouse-histology.html>

The screenshot shows the website for the Mouse Histology and Phenotyping Laboratory (MHPL) at Northwestern University Feinberg School of Medicine. The page features a purple header with the university's logo and name. Below the header is a navigation menu with options: Home, Clinical Trials, Graduate Research, Research Cores, Services & Support, News & Events, and Contact Us. A search bar is located in the top right corner. The main content area is titled "Mouse Histology and Phenotyping Laboratory" and includes a "Mission" section. The mission statement describes the lab's role in assisting investigators with mouse histopathology, including necropsy, phenotyping, dissection, and tissue processing. It also mentions the lab's involvement in special staining, immunohistochemistry (IHC), and pathologist consultations. A sidebar on the right contains a "MHPL Home Page" button and a section for "Hours of Operation".

**NORTHWESTERN UNIVERSITY
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Mouse Histology and Phenotyping Laboratory

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Mission

The mission of the Northwestern University Mouse Histology & Phenotyping Laboratory (MHPL) is to assist investigators with mouse histopathology. The laboratory provides comprehensive histology services for all rodent species (i.e., mouse, rat, etc.) which include necropsy, phenotyping of organs and tissues, dissection and tissue processing, and one-on-one consultation. The lab generates unstained paraffin and frozen sections for investigators to be used for special staining and immunohistochemistry (IHC). Pathologist consultation can also be provided to help develop strategies to elucidate phenotypes and gain mechanistic insight regarding the biologic actions of the targeted molecule or the toxicity of exogenously administered substances. In addition, the laboratory provides training opportunities for learning histology techniques and phenotyping analysis.

MHPL Home Page

Hours of Operation:

This site contains a list of services, documents, and protocols relevant to the procedures performed at the MHPL.

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Amelia C Cobbs, HT-ASCP, Histology Technologist

Faculty advisory committee

- David Engman, MD, PhD (Chair)
- Leonidas Plataniias, MD, PhD
- Andrew Mazar, PhD
- Alexander Stegh, PhD
- Chyung-Ru Wang, PhD
- Richard M. Pope, MD
- Raymond Bergan M.D

HAPPY HALLOWEEN

