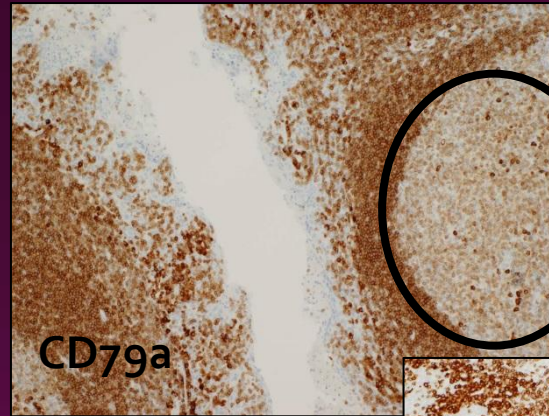


Maritime Pathology Conference: Update in Immunohistochemistry 2014

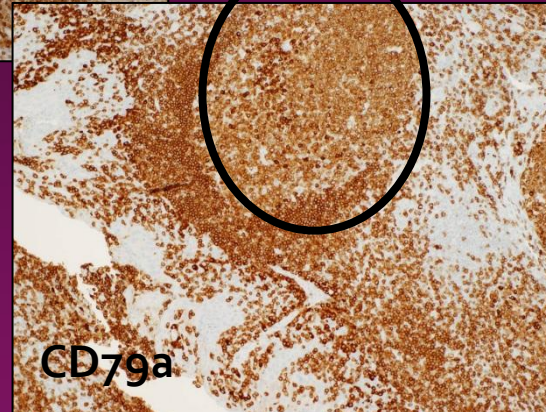
Kelly Dakin Haché, MD, PhD, FRCP(C)

Capital District Health Authority

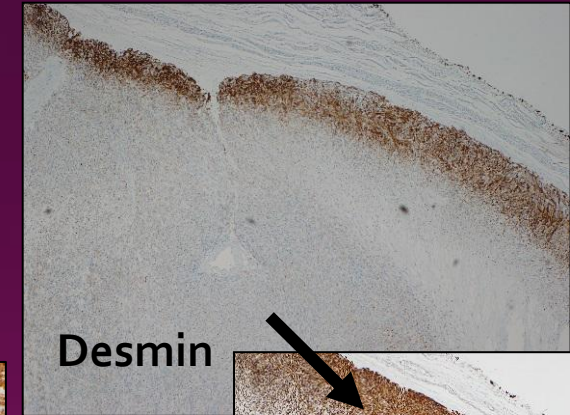
November 29, 2014



CD79a



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Desmin



Desmin

Disclosure Information

Kelly Dakin Haché

- I have no financial relationships to disclose
- I will not discuss off label use and/or investigational use in my presentation

Introduction

- Immunohistochemistry is an important tool to supplement histopathology
- Effectiveness of this tool depends on:
 - How well the tool is working
 - How well we are able to use the tool







Don't let inappropriate immunohistochemical results lead you down the garden path!



Outline

- Standard Terminology
- Technical Aspects of the Immunohistochemical Test
 - Pre-Analytical Phase
 - Analytical Phase
 - Post-Analytical Phase
- External Quality Assurance Programs



Learning Objectives

- 1) List variables that influence the results of immunohistochemical tests
- 2) Understand selection of appropriate normal tissue controls and the concept of quality indicators
- 3) Define and know specific recommendations for use of
 - 1) Positive Controls (internal and external)
 - 2) Negative Controls (internal and external)
 - 1) Specific negative control
 - 2) Non-specific negative control
 - 1) Negative reagent control for primary antibody (NRC-primAb)
 - 2) Negative reagent control for detection system (NRC-detSys)
- 4) Know recommendations for validation of
 - 1) Class I IHC tests
 - 2) Class II IHC tests



**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-Ordóñez, 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, FRCPC¹³*

Am J Clin Pathol 2010;133:354-365

Standard Terminology

Standard Terminology

- Immunohistochemical Test:
 - An immunochemical reaction that results in a color change (commonly using chromogen diaminobenzidine or DAB) that co-localizes with an epitope of interest in a tissue section
- Class I Immunohistochemical Test
 - An immunohistochemical test used by a pathologist within the context of morphology and clinical information to help establish a diagnosis
- Class II Immunohistochemical Test
 - An immunohistochemical test reported by a pathologist and used by a clinician treating a patient for the purpose of establishing prognosis, guiding management decisions (treatment selection, genetic testing, etc.), or predicting response to therapy

Current: HER2/neu, ER, PR, C-kit (CD117), CD20, Ki-67

Future: C4d, DOG1, MSI markers, NPM1, FOXP1, GCET1, IgG/IgG4, c-Myc

Phases of the Immunohistochemical Test

- **Pre-Analytical Phase** – all steps in tissue handling from the time the sample is removed from the patient to the production of the unstained slide
- **Analytical Phase** – all steps in tissue handling from the unstained slide to the time the cover-slip is placed on the stained slide
- **Post-Analytical Phase** – all steps in interpretation of the stained slide to the reporting of the results

Variables in Immunohistochemistry

Pre-Analytical (surgery → unstained section)

- Ischemic time
- Type of fixation
- Length of fixation
- Decalcification
- Processing
- Dehydration and clearing
- Paraffin impregnation
- Paraffin sectioning
- Storage

Analytical (unstained section → coverslip)

- Antibody supplier
- Clone
- Epitope retrieval
- Dilution
- Amplification
- Chromogen
- Counterstain
- Detection
- Blocker
- Mounting

Post-Analytical (interpretation)

- Design of controls
- Positive controls (internal and external)
- Negative controls
- Interpretation
- Critical indicators
- Reporting

With this many variables, there are millions of options for test conditions !!!!

Variables in Immunohistochemistry

Pre-Analytical (surgery → unstained section)

- Ischemic time
- Type of fixation
- Length of fixation
- Decalcification
- Processing
- Dehydration and clearing
- Paraffin impregnation
- Paraffin sectioning
- Storage

Standardize

Analytical (unstained section → coverslip)

- Antibody supplier
- Clone
- Epitope retrieval
- Dilution
- Amplification
- Chromogen
- Counterstain
- Detection
- Blocker
- Mounting

Optimize

Post-Analytical (interpretation)

- Design of controls
- Positive controls (internal and external)
- Negative controls
- Interpretation
- Critical indicators
- Reporting

Minimize

With this many variables, there are millions of options for test conditions !!!!

Effects of Preanalytical Variables on the Detection of Proteins by Immunohistochemistry in Formalin-Fixed, Paraffin-Embedded Tissue

Kelly B. Engel, PhD; Helen M. Moore, PhD

(Arch Pathol Lab Med. 2011;135:537–543)

Pre-Analytical Phase

Technical Aspects of the Immunohistochemical Test

Pre-Analytical Variables in Immunohistochemistry

Pre-Analytical (surgery → unstained section)

- Ischemic time
- Type of fixation
- Length of fixation
- Decalcification
- Processing
- Dehydration and clearing
- Paraffin impregnation
- Paraffin sectioning
- Storage

Most variables will influence IHC results to some degree:

- Only 44% of possible variables have been assessed in the literature
- Some variables influence IHC results while others seem less important
- Effects are often epitope dependent so suggestions in literature based on one type of IHC test may not be suitable for other IHC tests
- Older published literature may not reflect extent of problem if IHC tests were not compared using critical indicators (low expressing normal tissues for each epitope)
- IHC protocols can be optimized to control for each of these variables, but it is best to standardize pre-analytic variables as best as possible to avoid unexpected results in IHC

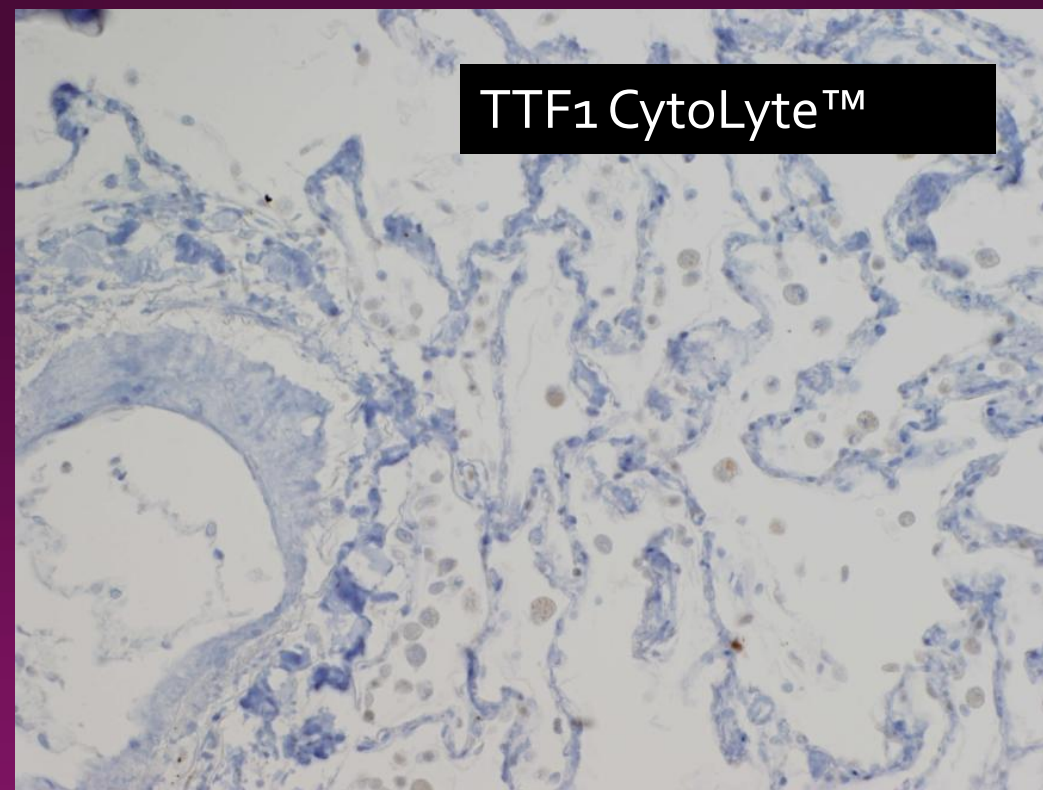
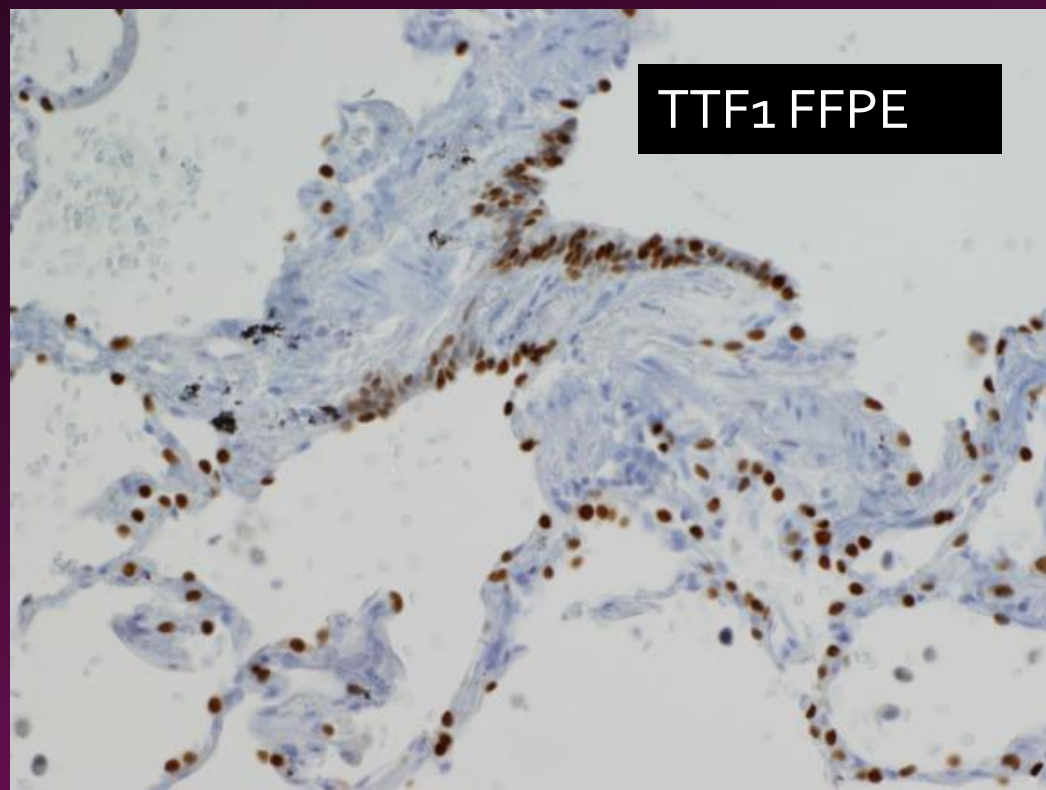
Pre-Analytical (surgery → unstained section)

- Ischemic time
 - Delayed fixation results in permanent loss of antigenicity
 - Document time out of patient, time in formalin, time cut and exposed to formalin
 - Some literature suggests up to 12 hours delay does not influence fixation, but that is antibody dependent
 - Aim for as short as possible (<30 minutes) to ensure all epitopes preserved
 - CAP-ASCO guidelines suggest <1 hour
- Type of fixation
 - Concentration: 10% neutral buffered formalin
 - pH: 5 to 7
 - Buffer: phosphate buffered saline (most commonly)
 - Ratio: at least 1:10 and preferably 1:20

TTF1 Expression in Normal Lung Tissue

CytoLyt® fixation and decalcification pretreatments alter antigenicity in normal tissues compared to standard formalin fixation. Jennette R. Gruchy, Penny J. Barnes, Kelly A. Dakin Hache.

Applied Immunohistochemistry and Molecular Morphology, 2014, in press.

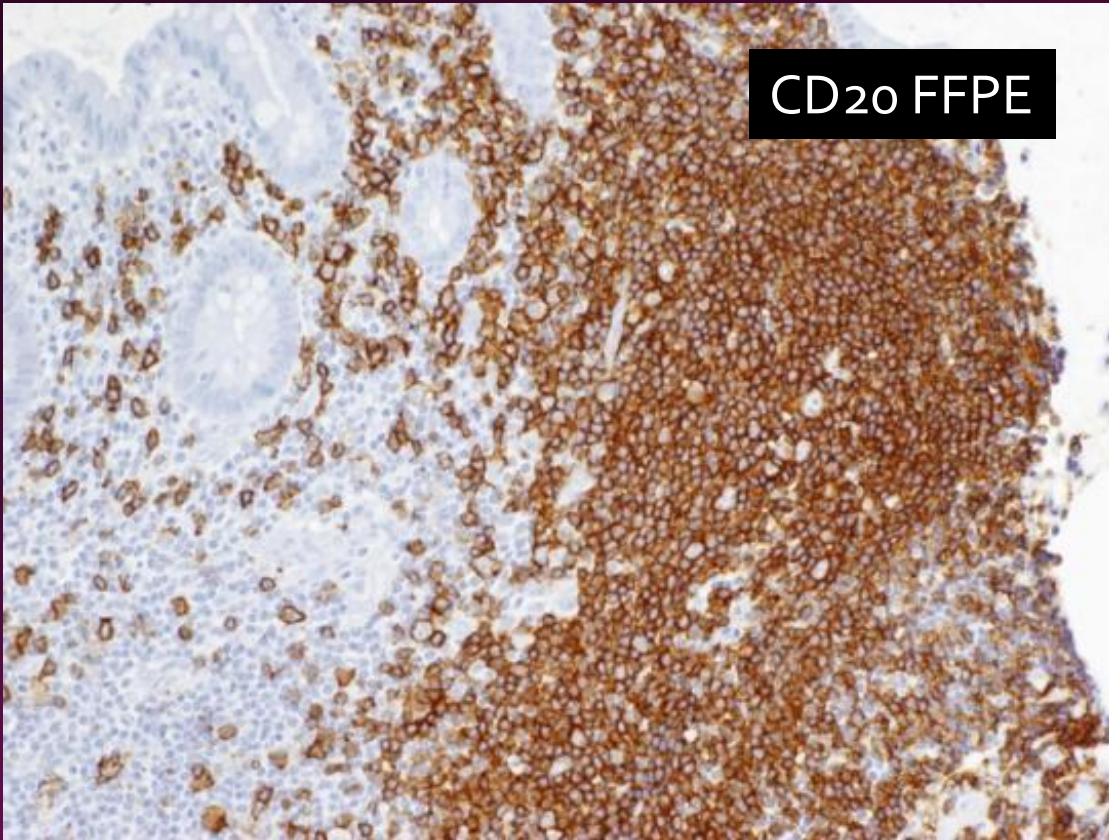


CD20 Expression in Normal Appendix

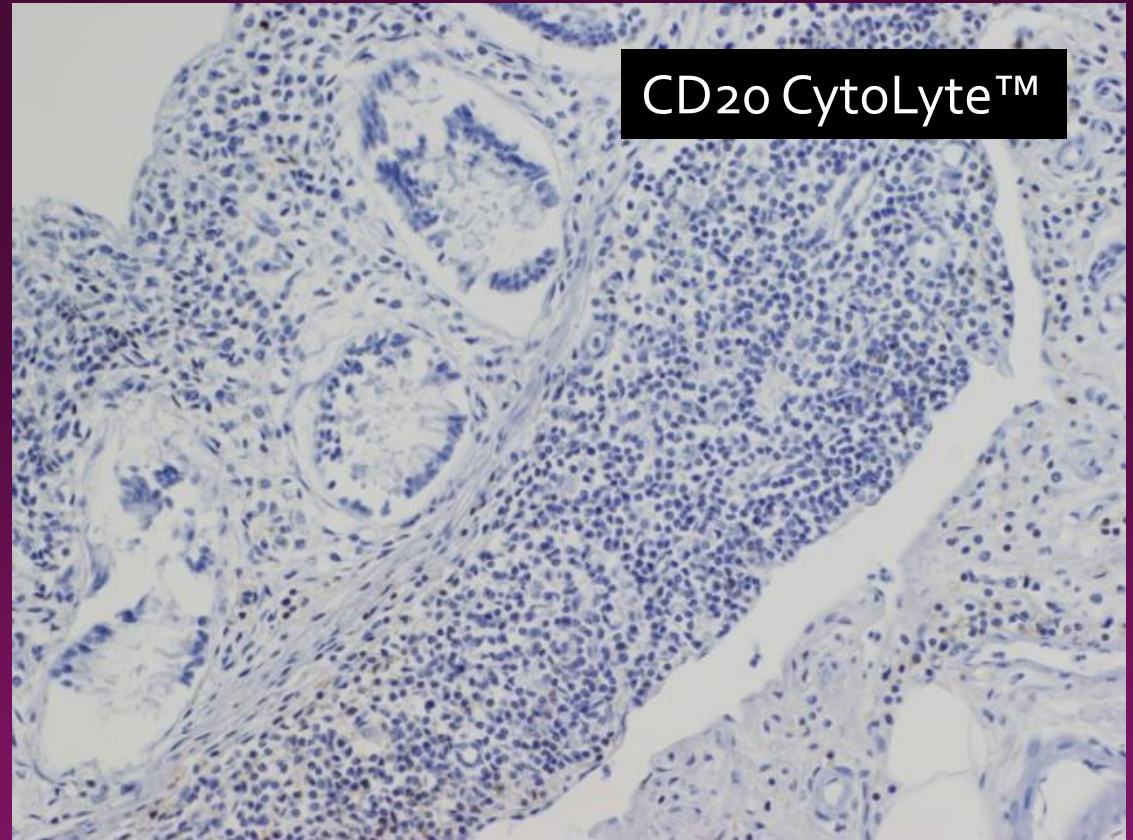
CytoLyt® fixation and decalcification pretreatments alter antigenicity in normal tissues compared to standard formalin fixation. Jennette R. Gruchy, Penny J. Barnes, Kelly A. Dakin Hache.

Applied Immunohistochemistry and Molecular Morphology, 2014, in press.

CD20 FFPE



CD20 CytoLyte™

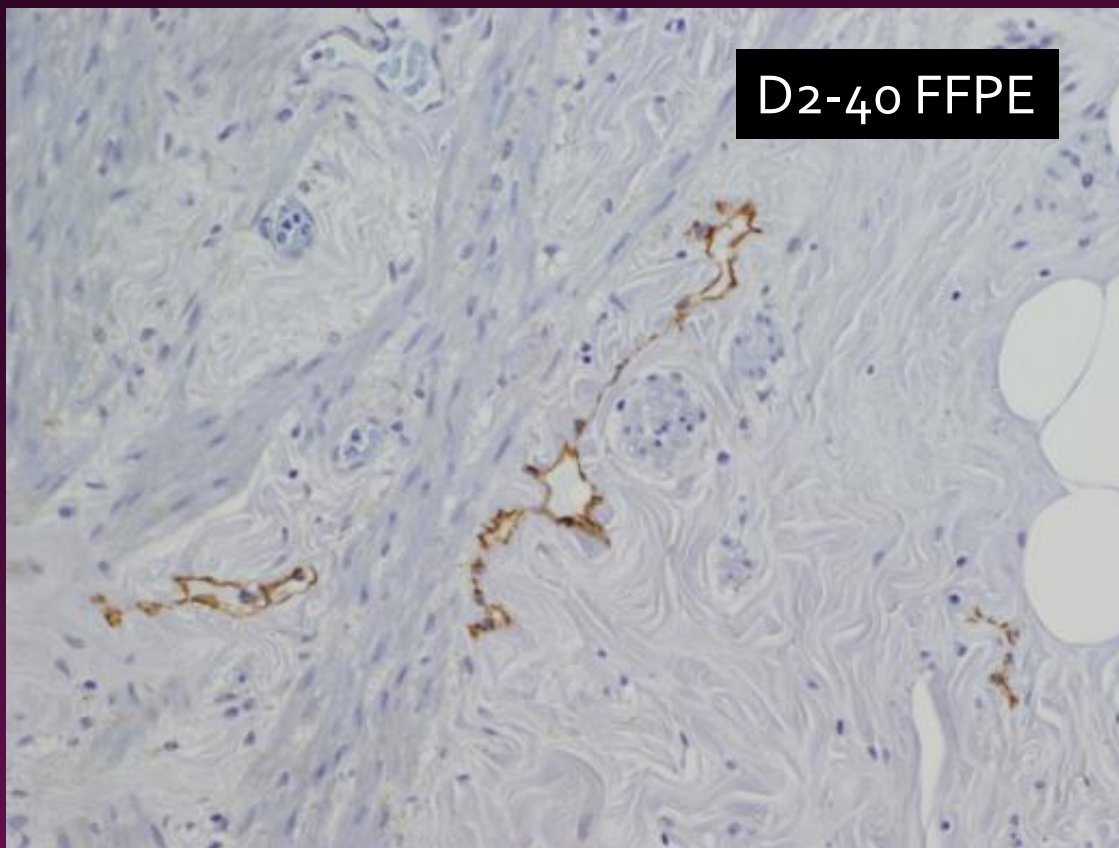


D2-40 Expression in Normal Appendix

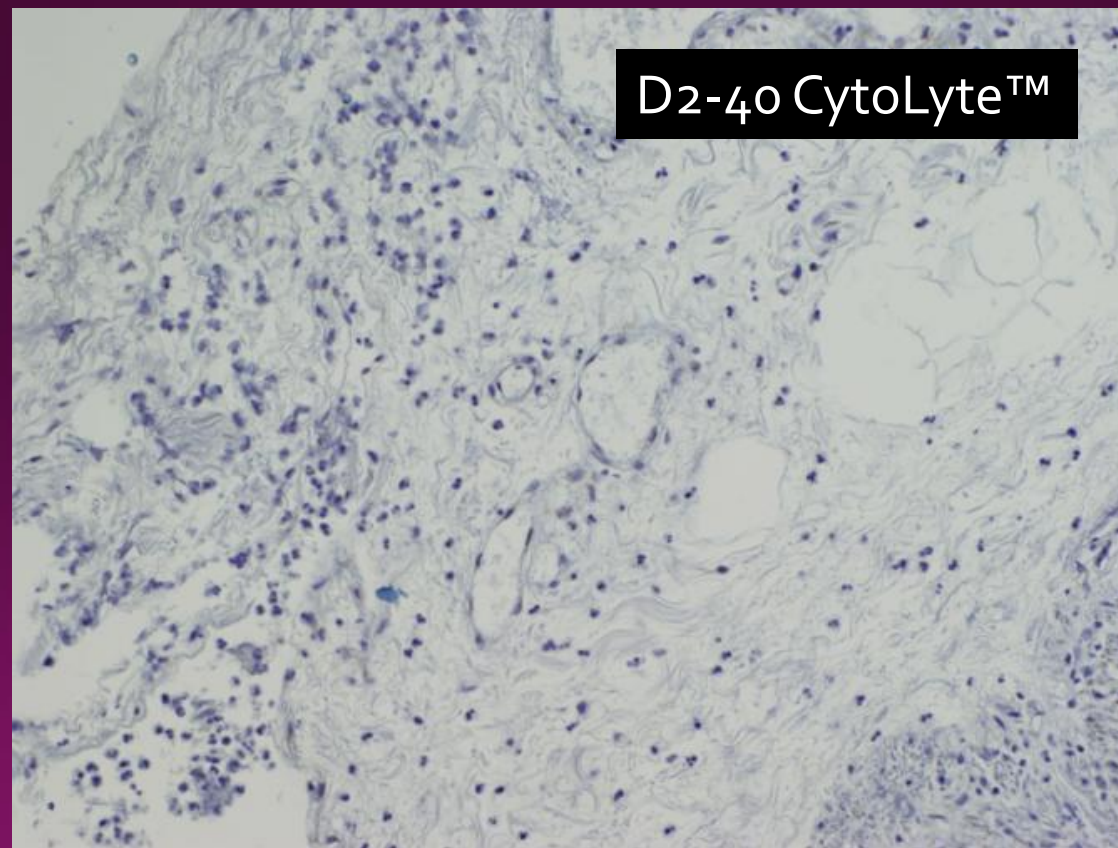
CytoLyt® fixation and decalcification pretreatments alter antigenicity in normal tissues compared to standard formalin fixation. Jennette R. Gruchy, Penny J. Barnes, Kelly A. Dakin Hache.

Applied Immunohistochemistry and Molecular Morphology, 2014, in press.

D2-40 FFPE



D2-40 CytoLyt™



Pre-Analytical (surgery → unstained section)

- Length of fixation
 - 24 hour formalin fixation is minimum fixation time to ensure optimal IHC for all antibodies
 - Penetration and binding occur relatively rapidly, but chemical cross-linking requires 24 hours no matter what the specimen size (biopsy or resection)
 - Under-fixation manifests as loss of antigenicity for certain antibodies
 - Decalcification procedures damage the unprotected epitopes in poorly fixed tissues
 - Over-fixation is not a problem and can be overcome using HIER

Pre-Analytical (surgery → unstained section)

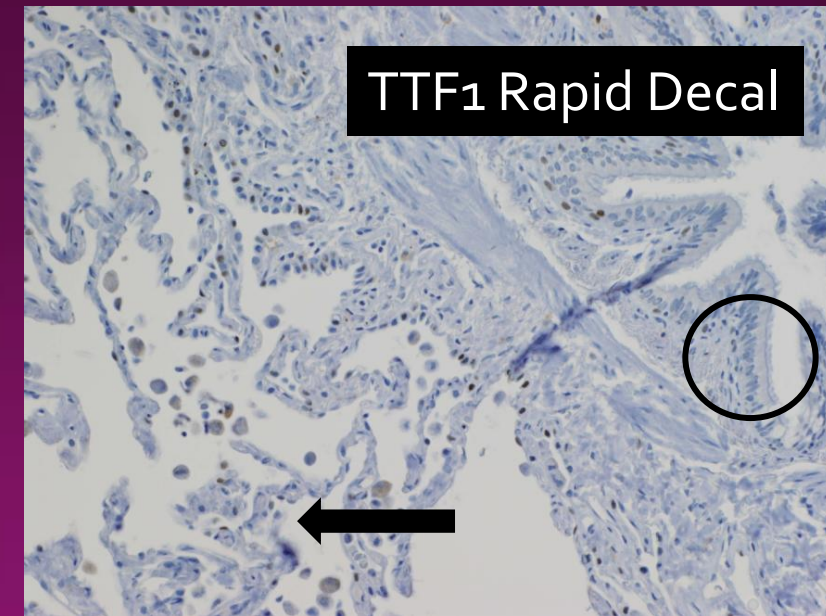
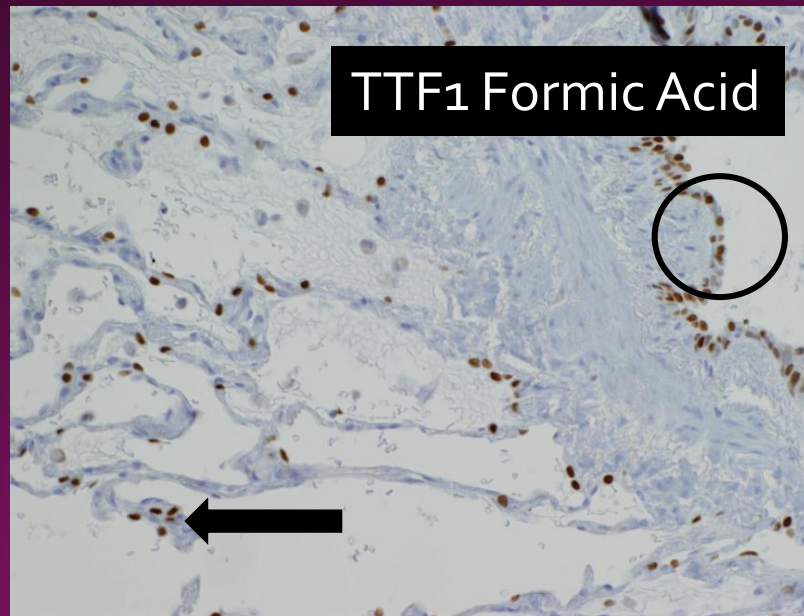
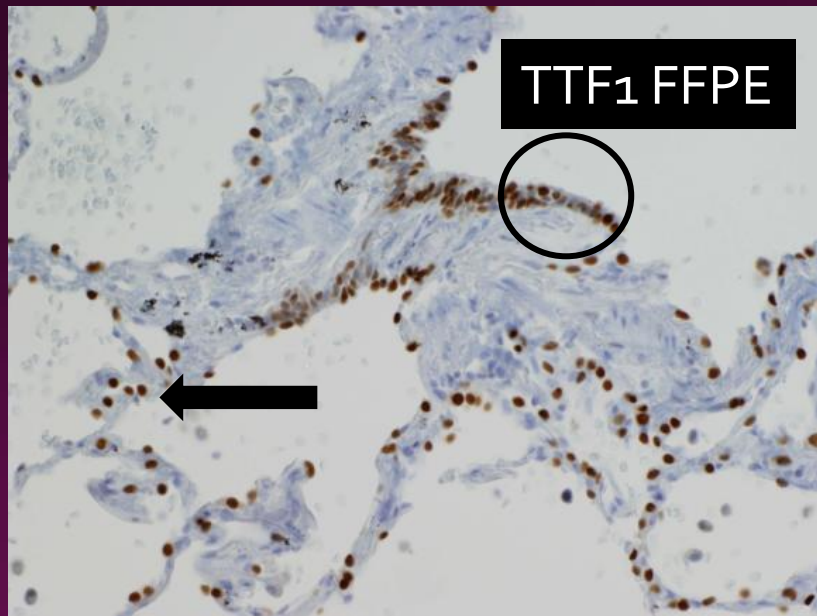
- Decalcification
 - Decalcification using strong acid (Rapid Decal - HCl) damages many epitopes
 - Decalcification using weak acid (formic acid) or EDTA is relatively harmless provided that the tissue has been properly formalin fixed in advance.
 - Bones and calcified specimens must be sectioned with a bone saw and completely fixed for at least 24 to 48 hours prior to placing in decalcification solutions
 - Failure to fix the tissue prior to decalcification results in loss of antigenicity – fixation is the key!

TTF1 Expression in Normal Lung Tissue

CytoLyt® fixation and decalcification pretreatments alter antigenicity in normal tissues compared to standard formalin fixation. Jennette R. Gruchy, Penny J. Barnes, Kelly A. Dakin Hache.

Applied Immunohistochemistry and Molecular Morphology, 2014, in press.

High expressor retained in Formic Acid
Low expressor reduced in Formic Acid
High and Low expressors lost in HCl

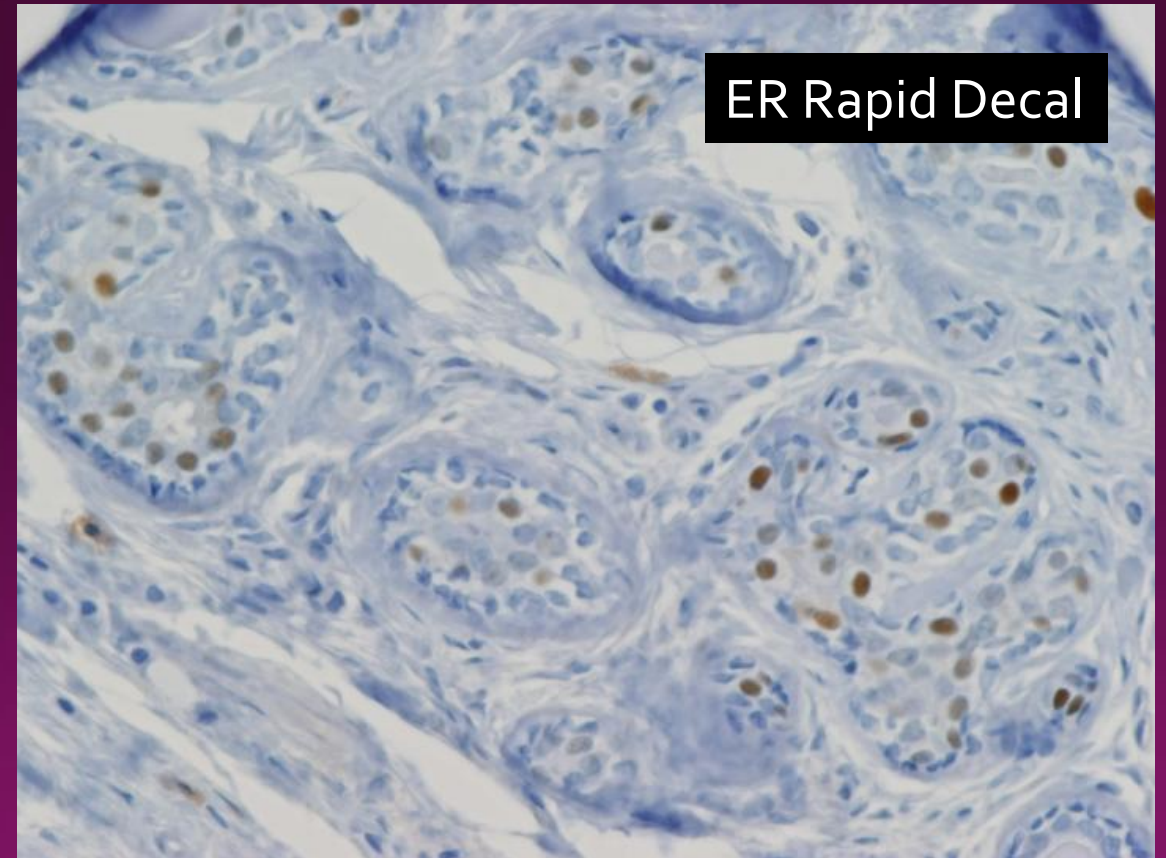
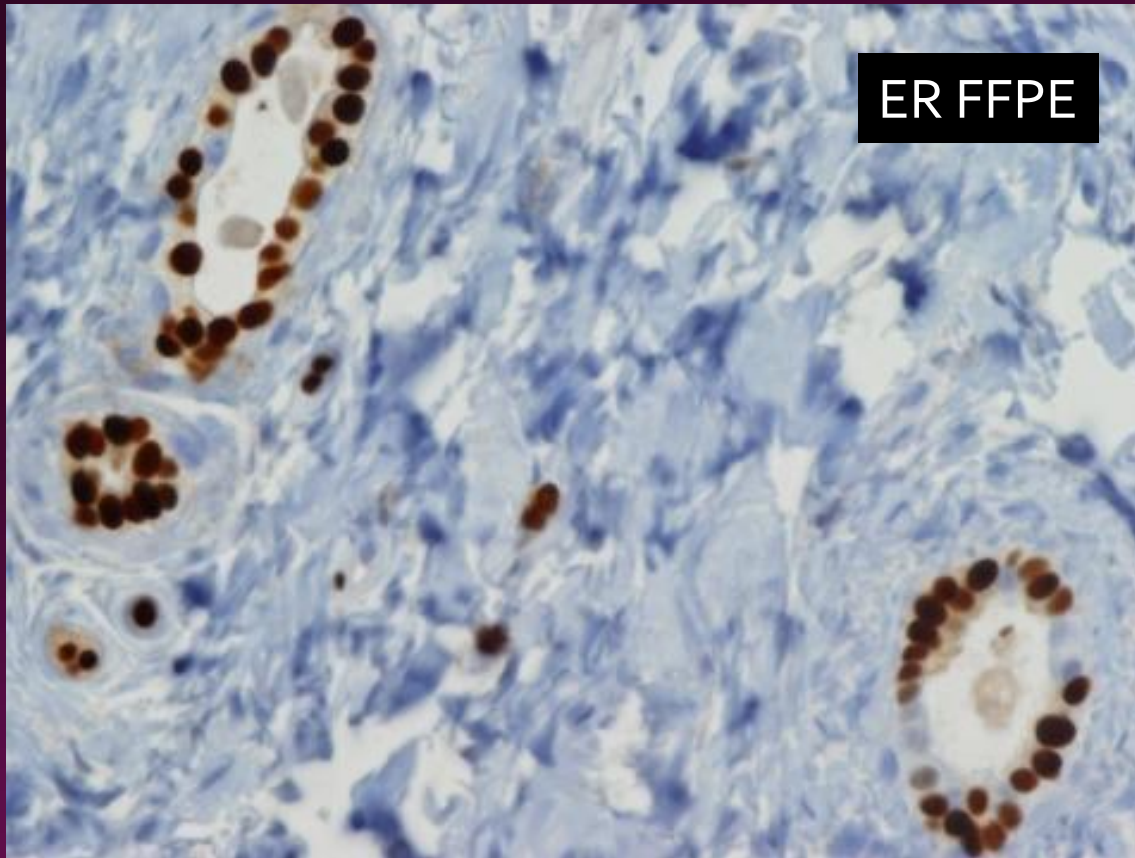


ER Expression in Normal Breast Tissue

Cervix is now accepted as an appropriate control for ER and may have been more informative in this study

CytoLyt® fixation and decalcification pretreatments alter antigenicity in normal tissues compared to standard formalin fixation. Jennette R. Gruchy, Penny J. Barnes, Kelly A. Dakin Hache.

Applied Immunohistochemistry and Molecular Morphology, 2014, in press.



Pre-Analytical (surgery → unstained section)

- Post-fixation specimen washing
 - Will not alter IHC if tissue is completely fixed (cross-linked)
 - Formalin that is “bound” but not “cross-linked” can be washed out
- Processing
 - Type of processor may not influence IHC as long as the tissue is appropriately fixed prior to processing
 - Number of cassettes on processor and position of cassette on processor does not appear to influence IHC
 - Control tissue and specimen tissue must be processed in similar fashion

Pre-Analytical (surgery → unstained section)

- Dehydration

- IHC results can be influenced by
 - Type of dehydration agent (ie. Isopropanol, ethanol, methanol, etc)
 - Duration of the dehydration step (literature suggests ~10 hours optimal)
 - QEII Small – 3 1/2 hours
 - QEII Large - 9 hours
 - Temperature of dehydration step (high = bad)
- Methods must be standardized for procedures in that lab

- Clearing

- IHC results can be influenced by
 - Type of clearing agent (ie. Xylene, isopropanol, etc.)
 - Temperature of clearing step (high = bad)
- Methods must be standardized for procedures in that lab

- Short dehydration step can leave water in the tissue and cause hydrolysis and loss of antigenicity over time
- Are IHC tests on small biopsies comparable to large resection specimens at our institution?

Pre-Analytical (surgery → unstained section)

- Paraffin impregnation
 - Low melting paraffin (55-58°C) for short duration (1-2 hours) is best
 - Higher temperatures for longer duration can reduce antigenicity
- Slide drying
 - Loss of antigenicity occurs with high heat and prolonged drying time
 - 4 hours at 60°C
 - Antigenicity is preserved under following conditions:
 - 24 hours at room temperature
 - Overnight at 37°C
 - 1 hour at 50-60°C

Pre-Analytical (surgery → unstained section)

- Storage of slide mounted sections (unstained slides)

- Few days at 20°C
- ~ week at 4°C
- ~ month at -20°C
- ~ year at -80°C

Consultation cases: Better to have block so that we can control important pre-analytic variables

- Bath temperature
- Temperature and duration of unstained section drying
- Temperature and duration of unstained section storage

- Paraffin block storage

- Paraffin blocks can be stored indefinitely without significant loss in antigenicity
- Cool dry location is preferred

Principles of Analytic Validation of Immunohistochemical Assays

Guideline From the College of American Pathologists Pathology and Laboratory
Quality Center

Patrick L. Fitzgibbons, MD; Linda A. Bradley, PhD; Lisa A. Fatheree, BS, SCT(ASCP); Randa Alsabeh, MD; Regan S. Fulton, MD, PhD; Jeffrey D. Goldsmith, MD; Thomas S. Haas, DO; Rouzan G. Karabakhtsian, MD, PhD; Patti A. Loykasek, HT(ASCP); Monna J. Marolt, MD; Steven S. Shen, MD, PhD; Anthony T. Smith, MLS; Paul E. Swanson, MD

Analytical Phase

(Arch Pathol Lab Med. doi: 10.5858/arpa.2013-0610-CP)

Technical Aspects of the Immunohistochemical Test

Standard Terminology

- Primary Antibody
 - The primary antibody is the reagent that targets the epitope of interest. The selection of the primary antibody (clone and commercial supplier) should be based on results from external QA/QC programs and mature literature that establishes the reliability of a particular primary antibody on a particular platform
- Platform
 - Platform refers to the type of automated instrument that performs the immunohistochemical test (Dako, Leica, Ventana – BenchMark Ultra, etc.)



Caution.....

- **Poor antibody clone** – does not work no matter what you do so recommend not using.
- **Clone good / vendor bad** – some vendors are more reliable for certain clones
- **Clone good / pre-dilute formulation bad** – some pre-dilute antibodies are difficult to optimize
- **Mouse Ascites Golgi (MAG) Reaction** – specific clones for CK5/6, synaptophysin, and cdx2 can give a false positive reaction in patients with blood group A due to non-specific antibody reaction
- **Platform dependent antibodies** – some antibodies will not perform on certain platforms no matter what you do so there are recommended clones for certain platforms
- **Misleading data-sheets** – some data-sheets supplied by vendors are just frankly wrong. They may state no retrieval necessary, recommend wrong retrieval, suggest inappropriate concentration, or state wrong control tissue.

Standard Terminology

- Pre-Treatment
 - Tissue proteins are protected from degradation by fixation. Fixation with formalin (aqueous formaldehyde) cross-links proteins via methylene bridges which can “mask” epitopes. Pre-treatment refers to the “unmasking” of epitopes that is achieved using heat or proteolysis. This step is also called epitope retrieval.
- Quenching
 - Most detection systems use a peroxidase-based reaction for precipitating the chromogen. Endogenous peroxidase must be quenched using hydrogen peroxide prior to completing the immunohistochemical reaction
- Biotin Blocking
 - Immunohistochemical tests that use a biotin-based detection system (ABC or LSAB) can give a false positive reaction due to the presence of endogenous biotin in some tissues. The endogenous biotin can be blocked by adding agents that bind endogenous biotin.

Antigen Retrieval (Demasking)

- Heat induced epitope retrieval (**HIER**)
 - Most important demasking method
 - Applicable for more than 80% of all antibodies
- Efficiency of HIER is a function of temperature and time (inverse relationship)
 - 120°C in a pressure cooker for 5-10 minutes
 - 100°C in a microwave oven for 20 minutes
 - 80°C in an incubator for 30 to 60 minutes ★
 - 60°C in an incubator for 24 hours

High temperature damages antigens especially with poor fixation

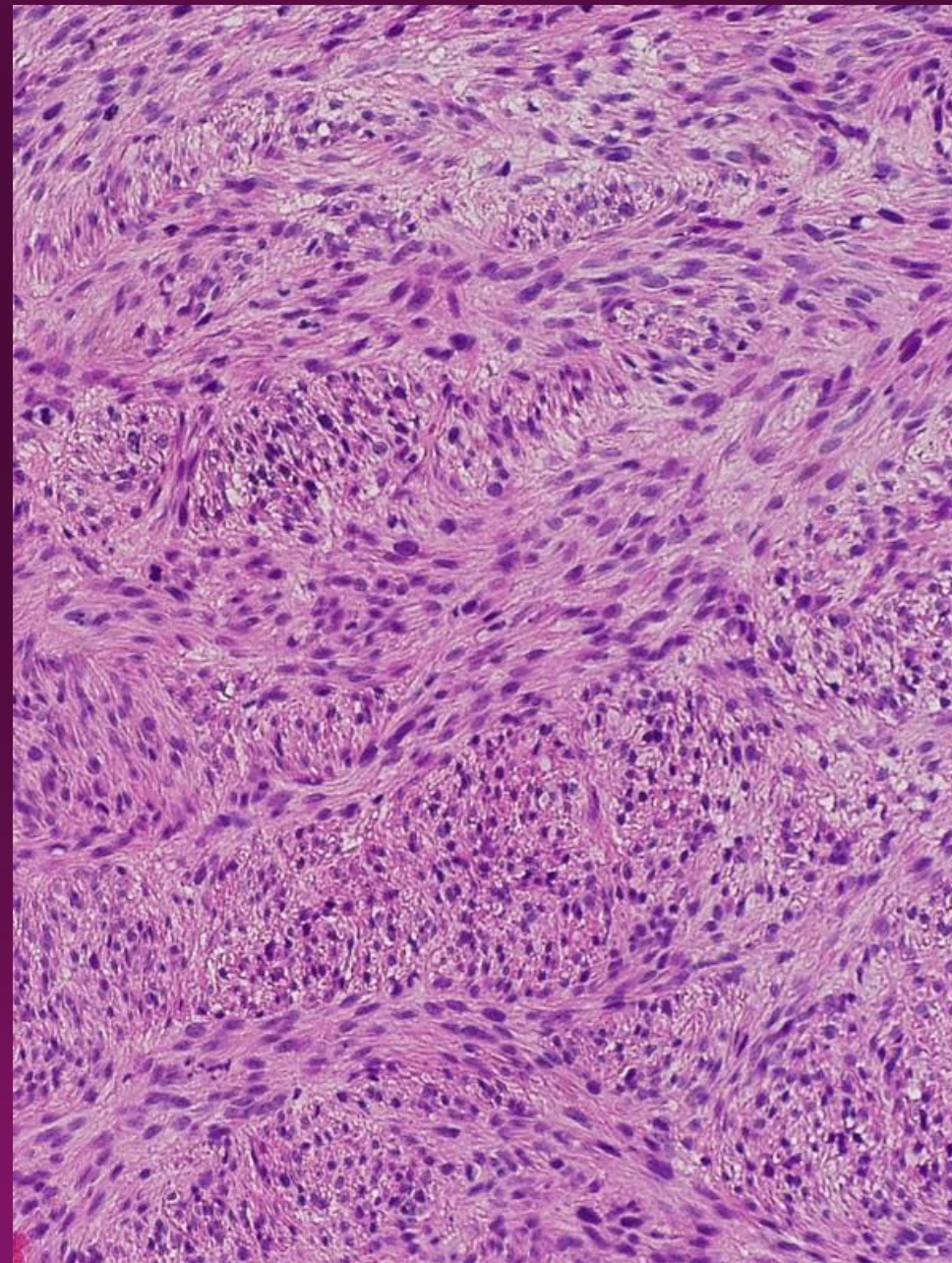
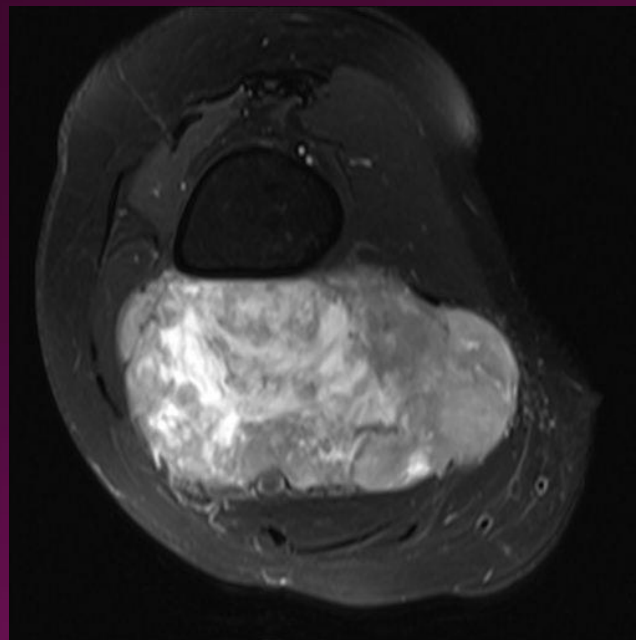
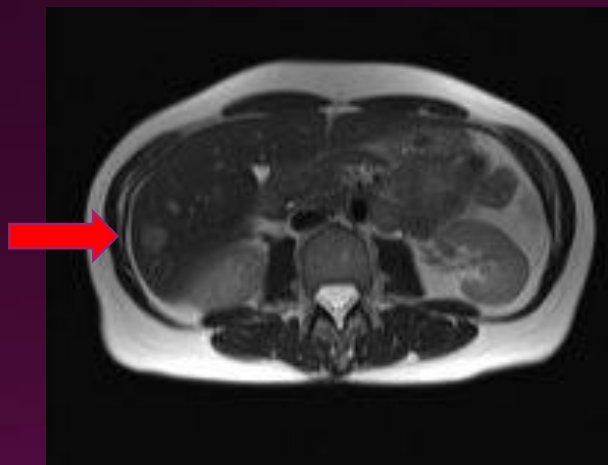
Too long to wait
- Efficiency of HIER is a function of pH and chemical composition of the buffer
 - High pH recommended for majority of antibodies (pH 8-9) ★
 - Low pH (citrate buffer) recommended for rare antibodies (pH 6) – EpCAM, PrP

Antigen Retrieval (Demasking)

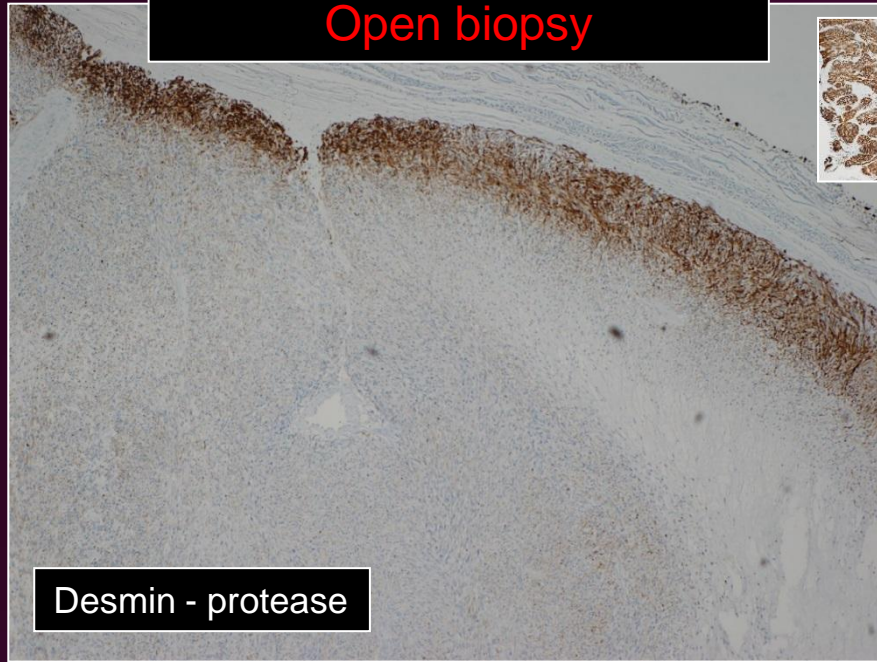
- Proteolysis
 - May require a higher Ab concentration to give an optimal result
 - May be difficult to control result as it is significantly affected by pre-analytical phase variables
 - Fixation
 - Decalcification
- HIER is recommended to replace proteolytic retrieval

Case

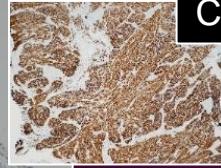
- 56 year old female with right posterior thigh mass and liver nodules



Open biopsy



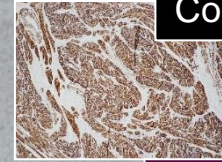
Control



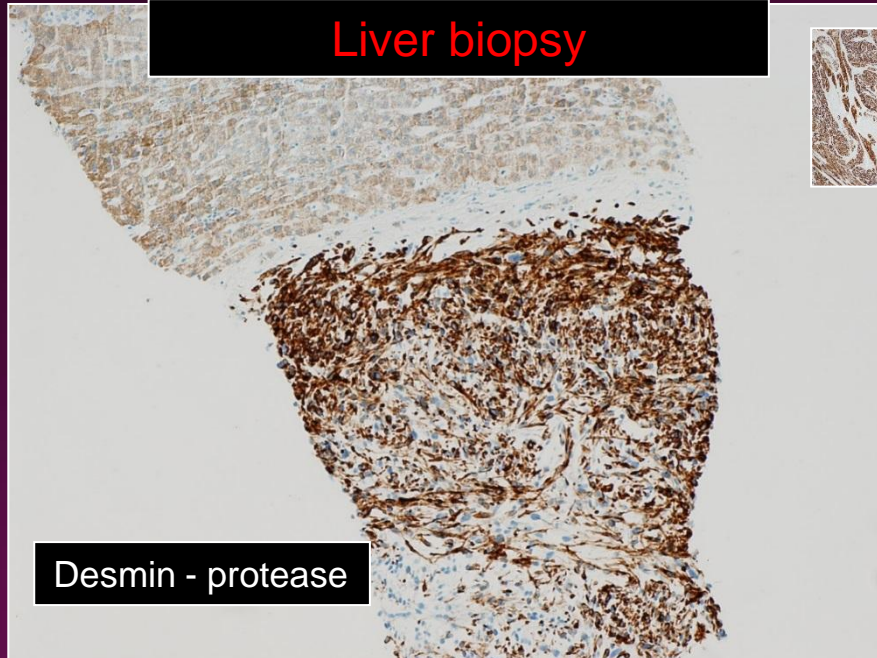
Resection specimen



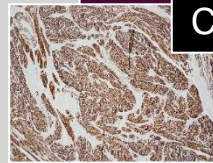
Control



Liver biopsy



Control



External Quality Assurance Programs may not identify this problem if the provided tissue is well fixed!