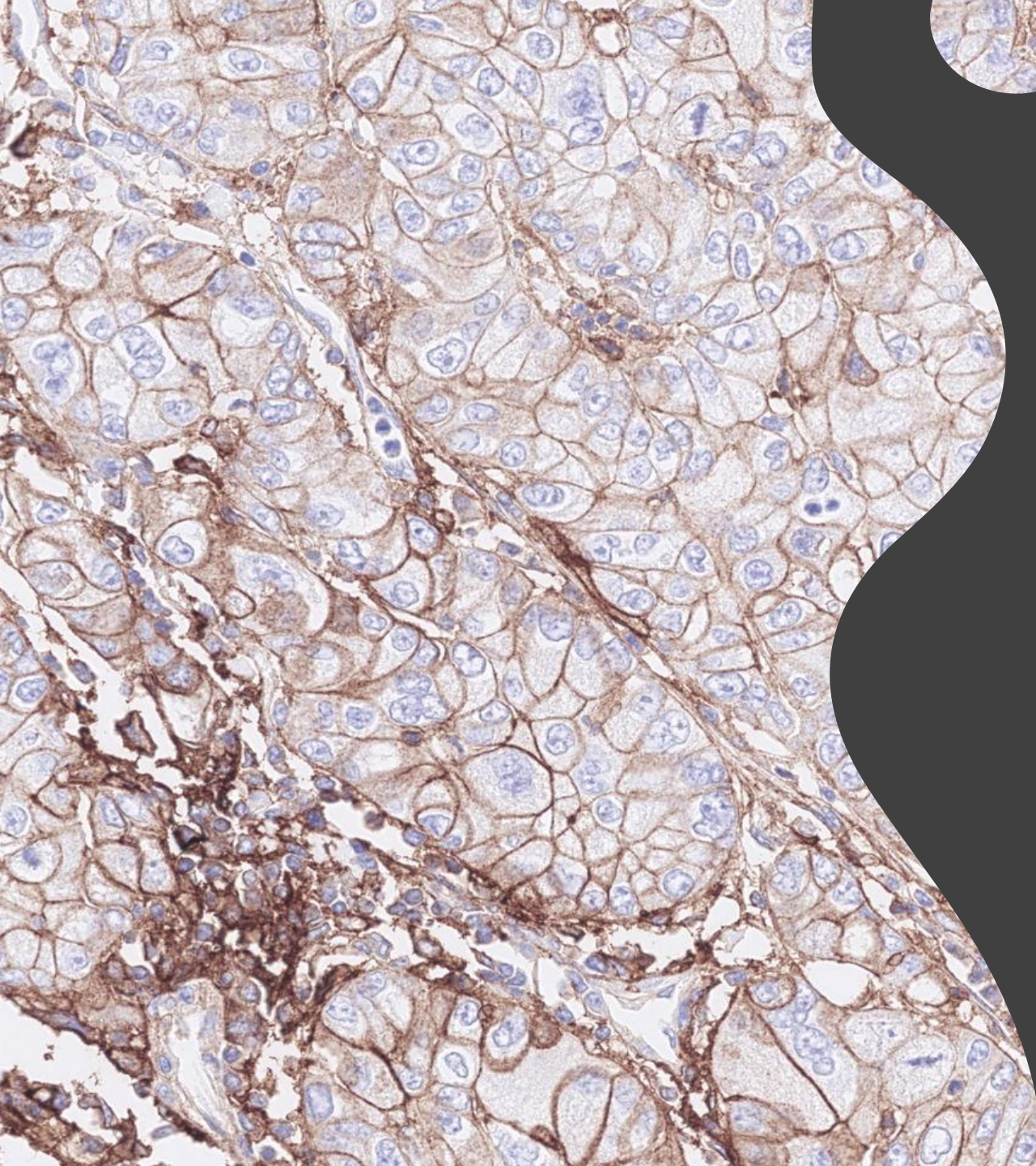




The technical approach Pre-analytical phase NordiQC seminar 2025

By Tanya Julio
Histotechnologist
Dept. of Pathology
Aarhus University Hospital, DK

The total test paradigm

“Immunohistochemistry is technically complex, and no aspect of this complexity can be ignored, from the moment of collecting the specimen to issuance of the final report”

Taylor CR. Arch Pathol Lab Med 2000; 124:945

Preanalytical

- Operating method
- Ischemia
- Fixative and fixation
- Tissue processing
- Paraffin embedding
- Paraffin sectioning
- Slide choice
- Storage



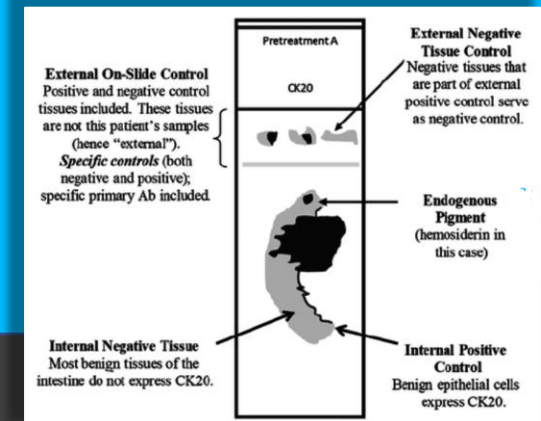
Analytical

- Choice of platform
- Epitope retrieval
- Blocking
- Primary Antibody
- Diluent
- Detection system
- Chromogen
- Counter stain
- Mounting



Interpretation

- Design of controls
- Positive controls
- Negative controls
- Interpretation
- Critical stain indicator



60-70% Errors in pathology estimated to be related to preanalytical...

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—Vol 135, May 2011

Table 1. Potential Sources of Preanalytic Variation During Specimen Fixation and Processing

Prefixation	Dehydration and clearing
Duration and delay of temperature	Reagent
Specimen size	Temperature
Specimen manipulation (pathology ink)	No. of changes
	Duration (total and change-specific)
Fixative	Paraffin impregnation
Formula	Type and melting point of wax
Concentration	No. of changes
pH	Duration (total and change-specific)
Age of reagent	Method (immersion and sonication or microwave acceleration)
Preparation source	
Fixation	Paraffin sectioning
Tissue to fixative volume ratio	Type of blade and frequency of replacement
Method (immersion, injection, and sonication or microwave acceleration)	Frequency of servicing and wax replacement
Conditions of primary and secondary fixation	Temperature of block during sectioning
Movement	Slide pretreatment
Light exposure	Water bath conditions, if used
Primary container	Chemical adhesives, if used
No. and position of cofixed specimens	Temperature and duration of slide drying
Postfixation	Storage
Washing conditions and duration	Temperature and duration of paraffin block storage
Storage reagent and duration	Temperature, duration, and manipulation of slide-mounted tissue sections
Processing	
Type of processor, frequency of servicing and reagent replacement	
Tissue to reagent volume ratio	
No. and position of coprocessed specimens	

Decalcification:
Type, Time, Temperature

By courtesy Ole Nielsen

Preanalytics and Precision Pathology

Pathology Practices to Ensure Molecular Integrity of Cancer Patient Biospecimens for Precision Medicine

Carolyn C. Compton, MD, PhD; James A. Robb, MD; Matthew W. Anderson, MD, PhD; Anna B. Berry, MD; George G. Birdsong, MD; Kenneth J. Bloom, MD; Philip A. Branton, MD; Jessica W. Crothers, MD; Allison M. Cushman-Vokoun, MD, PhD; David G. Hicks, MD; Joseph D. Khoury, MD; Jordan Laser, MD; Carrie B. Marshall, MD; Michael J. Misialek, MD; Kristen E. Natale, DO; Jan Anthony Nowak, MD, PhD; Damon Olson, MD; John D. Pfeifer, MD, PhD; Andrew Schade, MD; Gail H. Vance, MD; Eric E. Walk, MD; Sophia Louise Yohe, MD

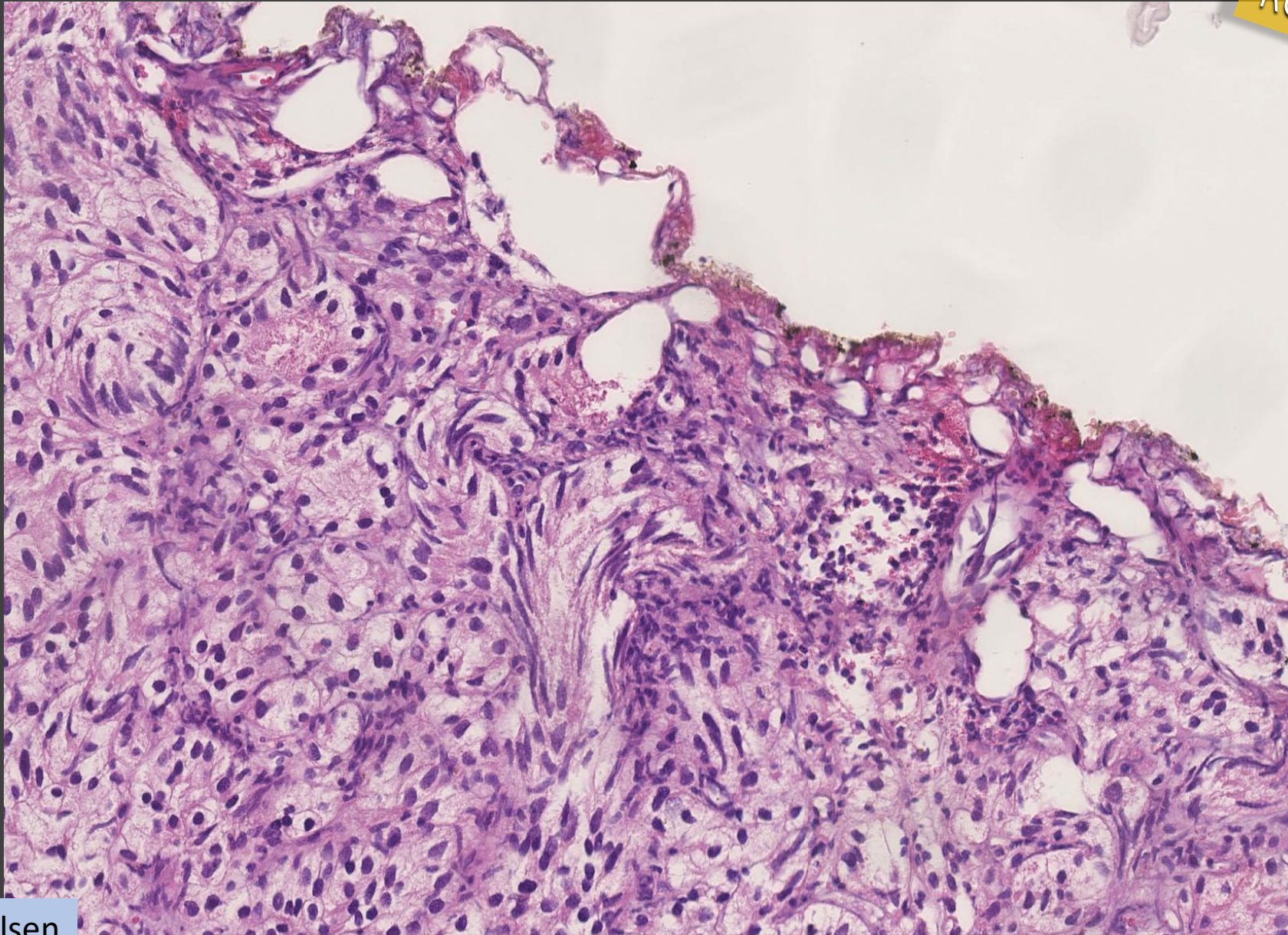


Prefixation

- Surgical procedure
- Fixation delay (cold ischemia)
- Specimen size
- Specimen manipulation (pathology ink)

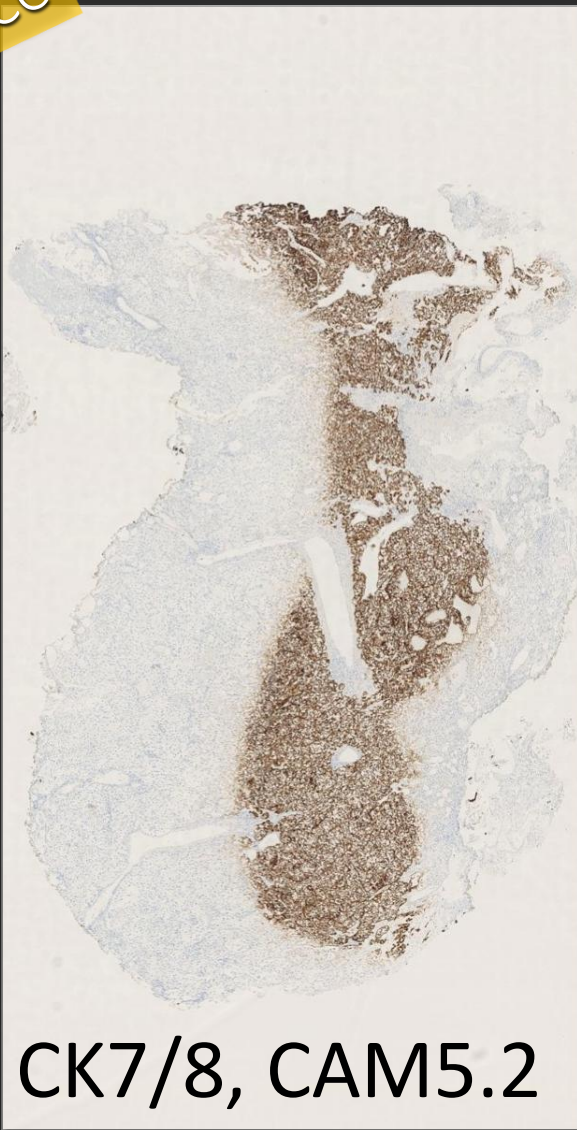
Electrosurgery – Heat impact

RCC



Surgical procedures - Impact on IHC

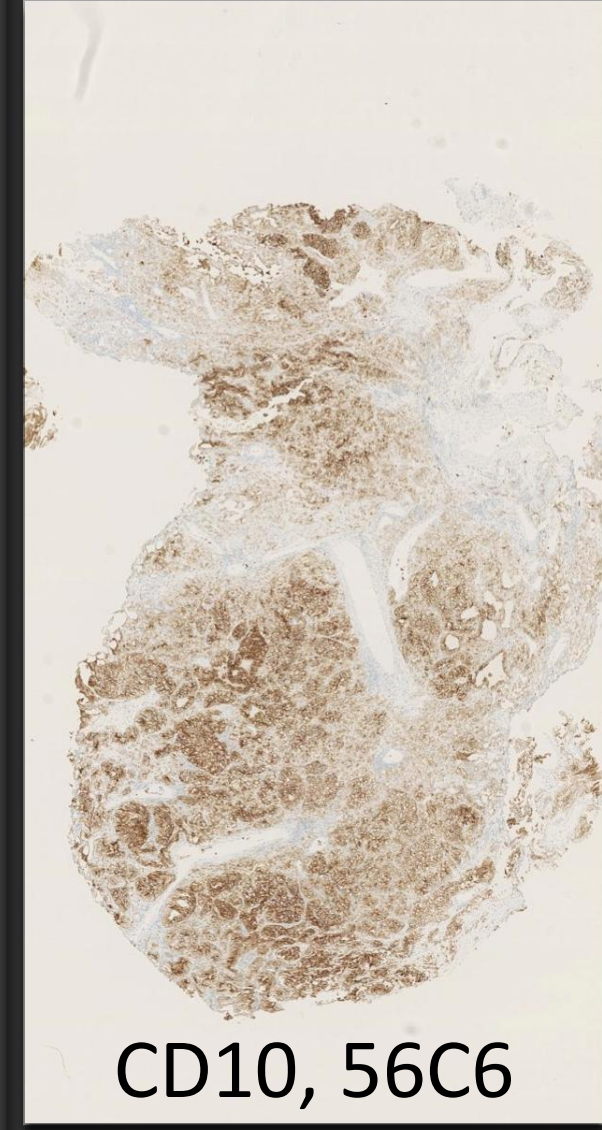
RCC



CK7/8, CAM5.2



CK8, EP17



CD10, 56C6

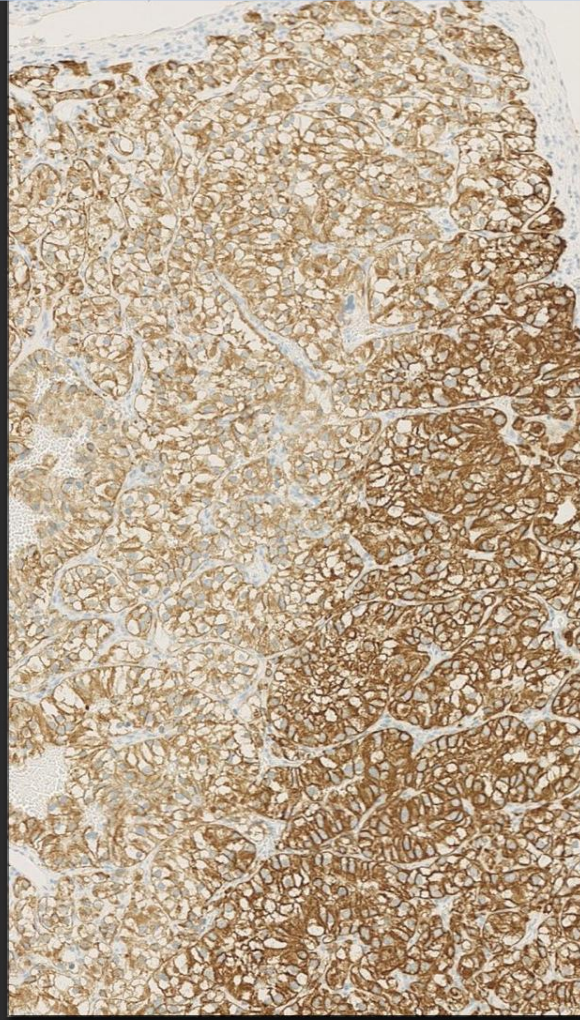
Surgical procedures - Impact on IHC

RCC

CK7/8, CAM5.2



CK8, EP17

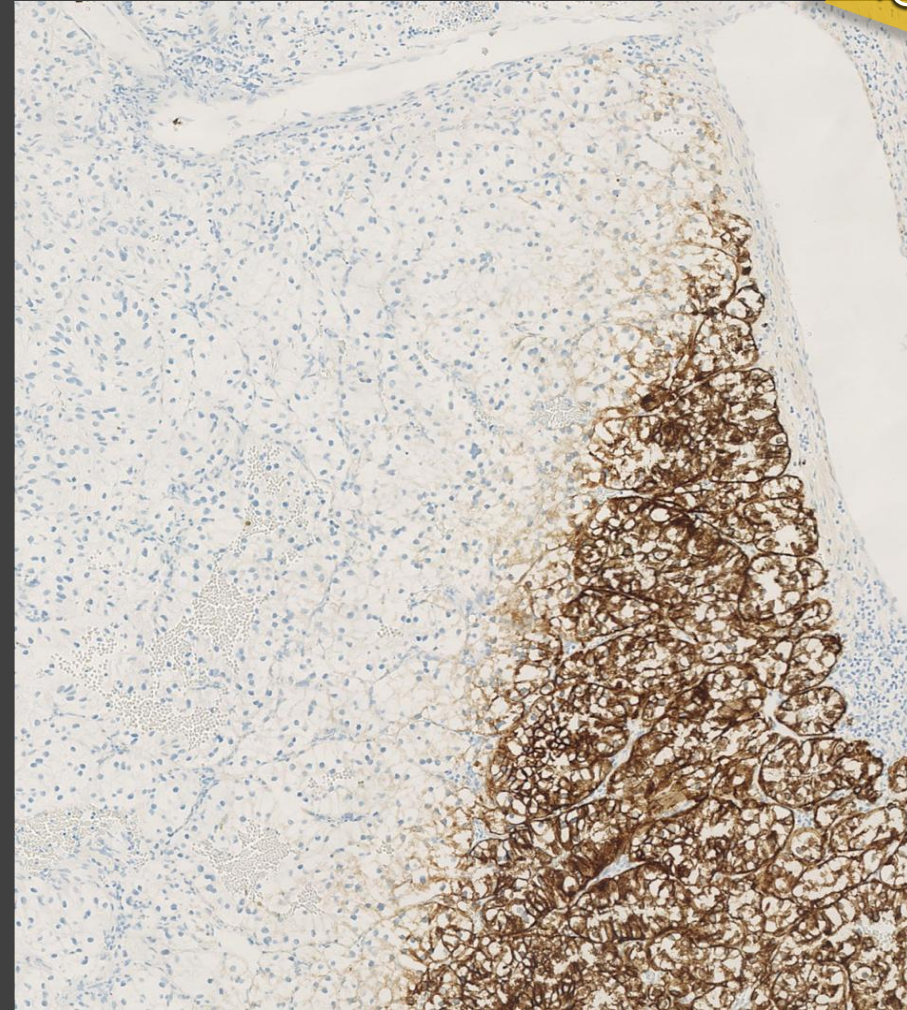
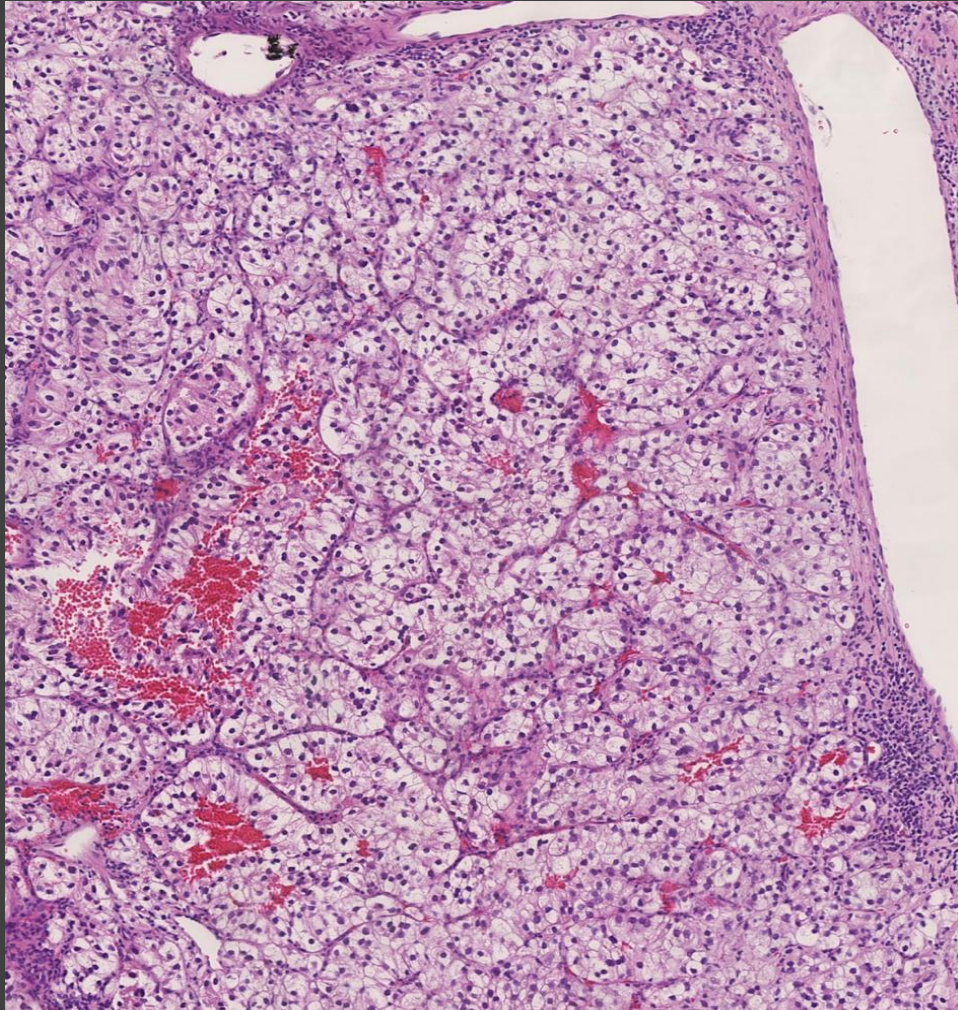


CD10, 56C6

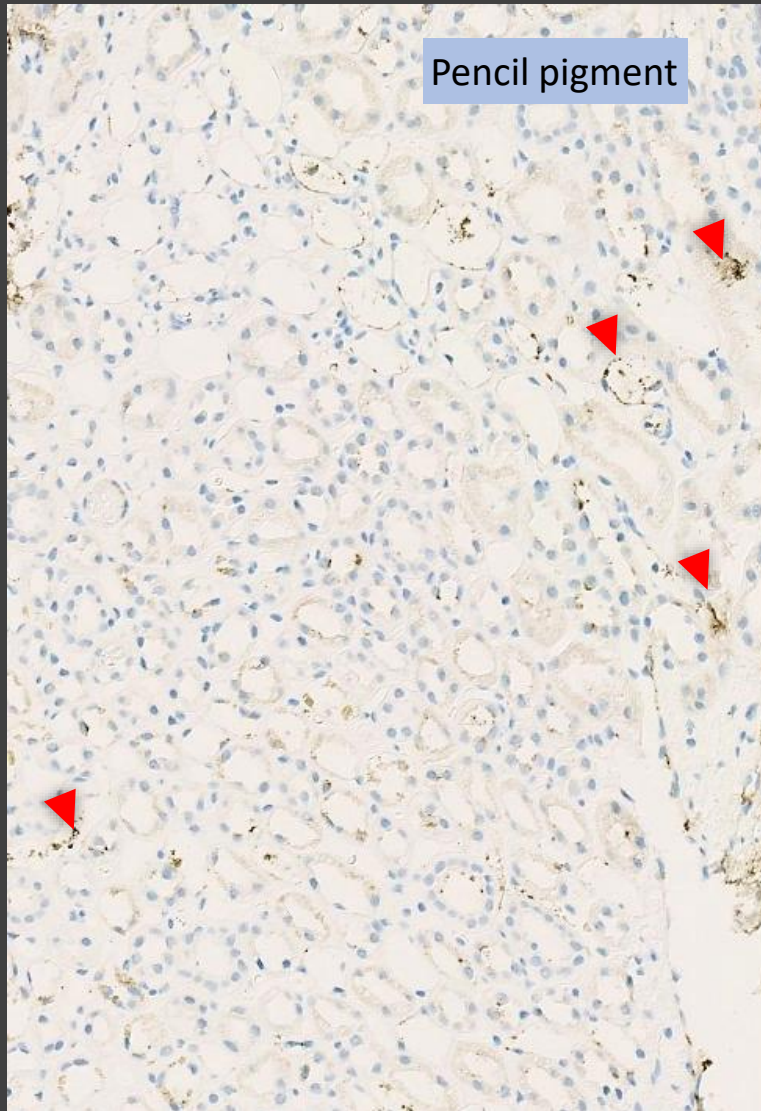


CK, CAM5.2 simple marker of electrosurgery

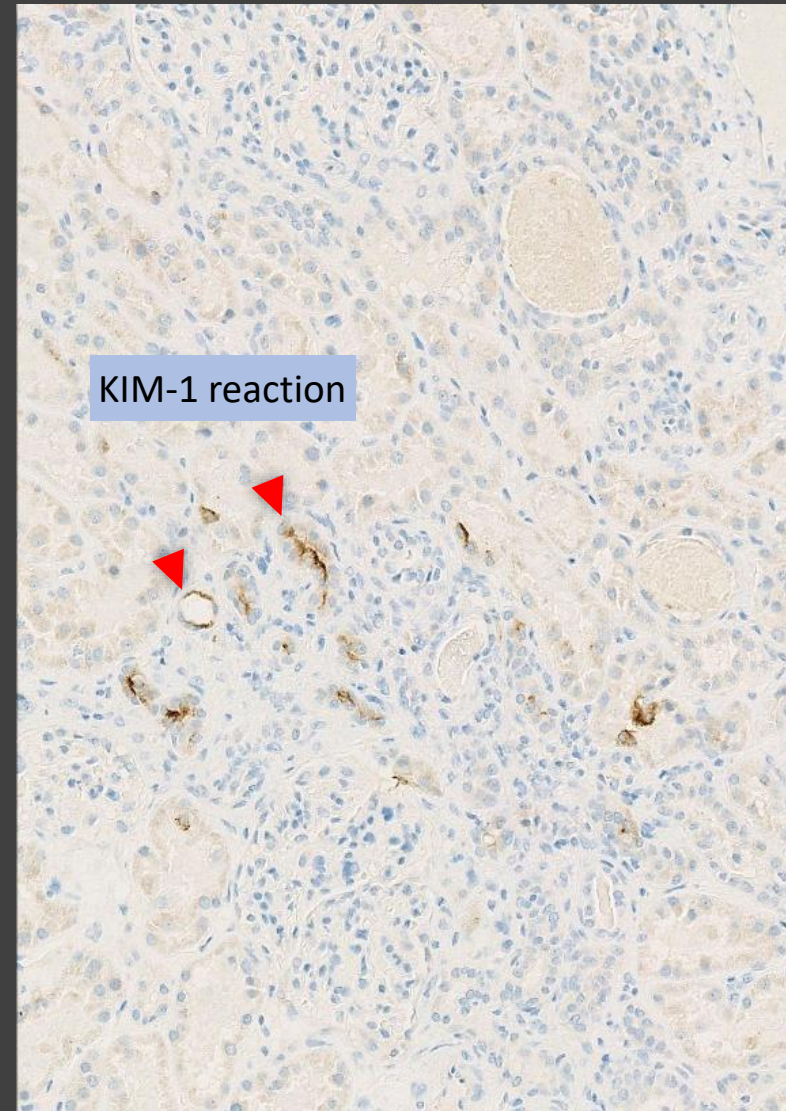
RCC



Pencil marking of small biopsies



KIM-1 (Kidney with marking)



KIM-1 (Kidney without marking)

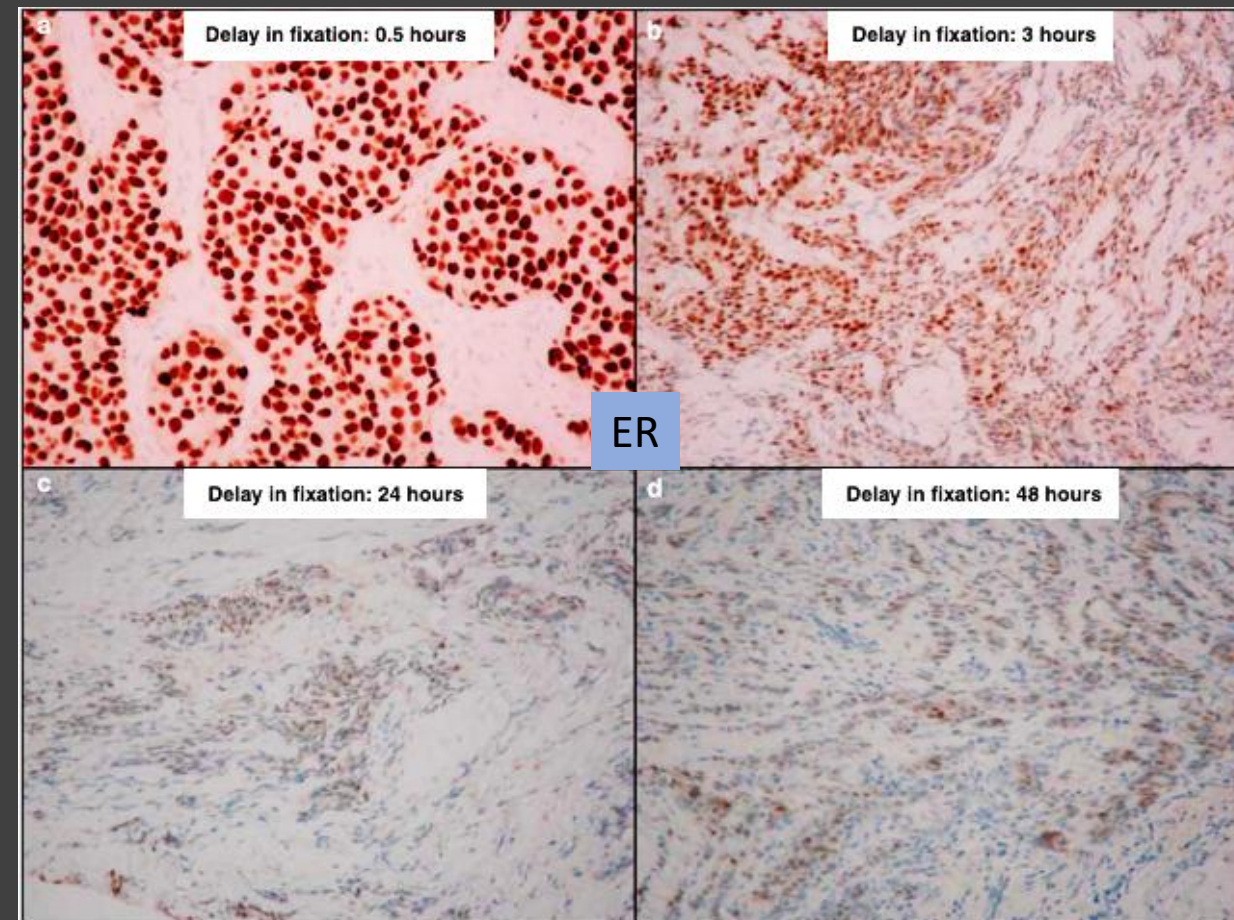
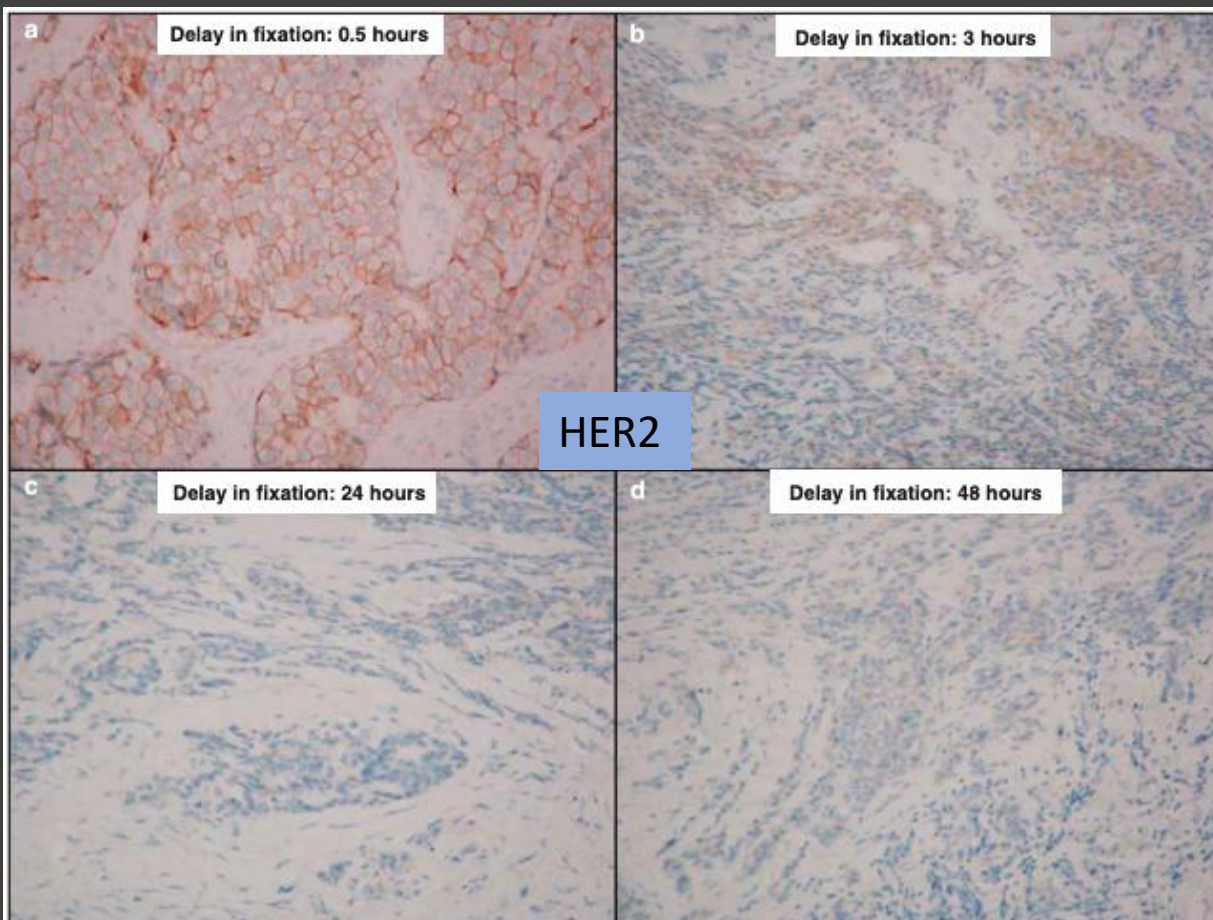
Cold ischemia – time from removal to fixation

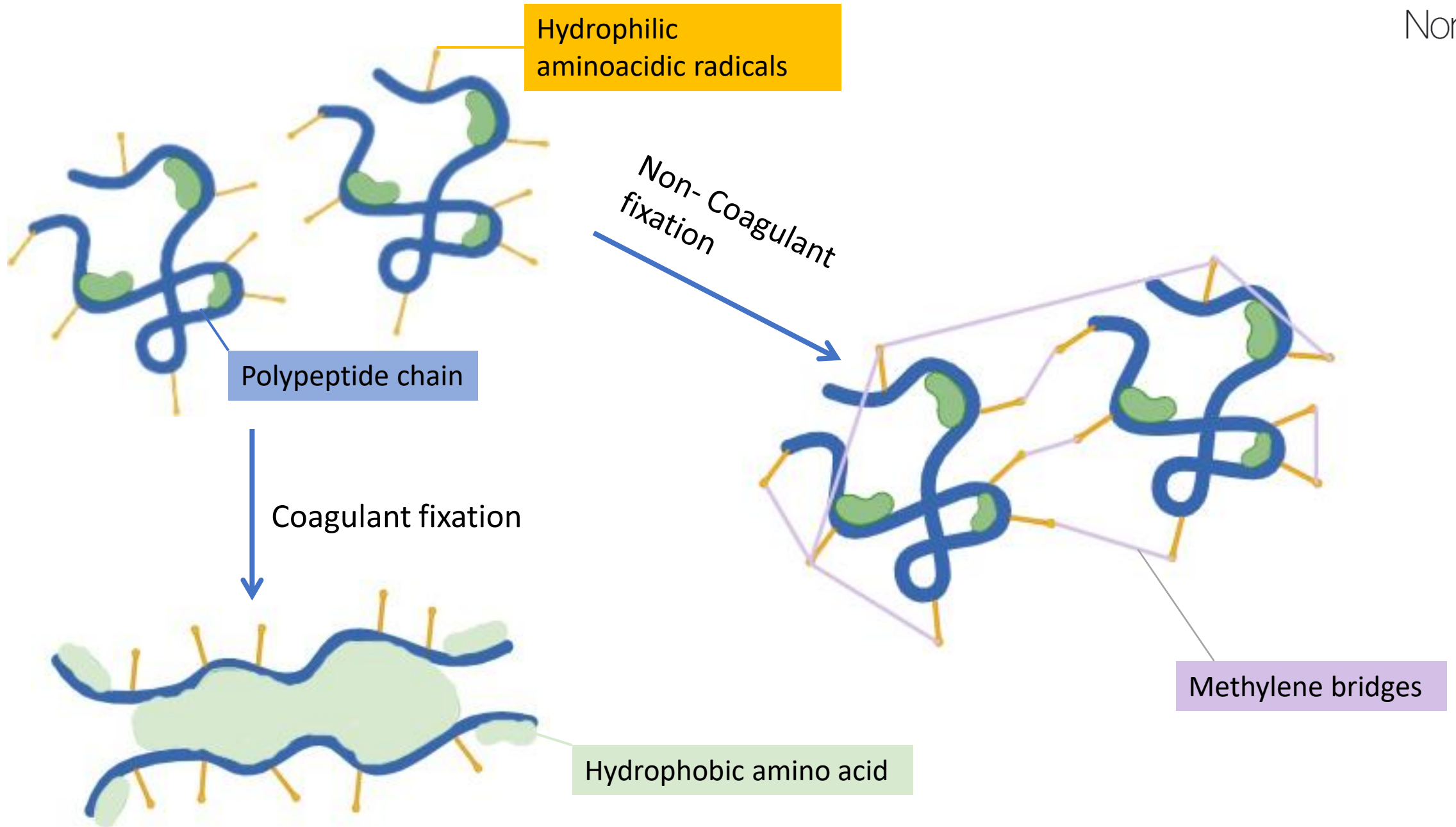
The effect of cold ischemic time on the immunohistochemical evaluation of estrogen receptor, progesterone receptor, and HER2 expression in invasive breast carcinoma

Isil Z Yildiz-Aktas, David J Dabbs and Rohit Bhargava

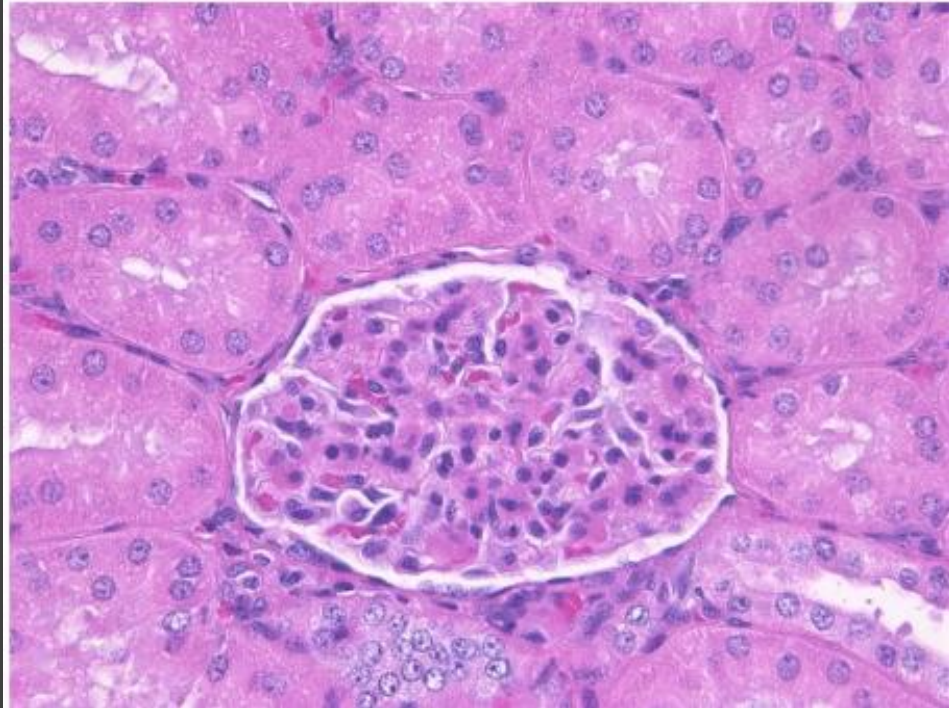
Department of Pathology, Magee-Womens Hospital, University of Pittsburgh Medical Center, Pittsburgh, PA, USA

ASCO/CAP: time before fixation < 1h room temperature

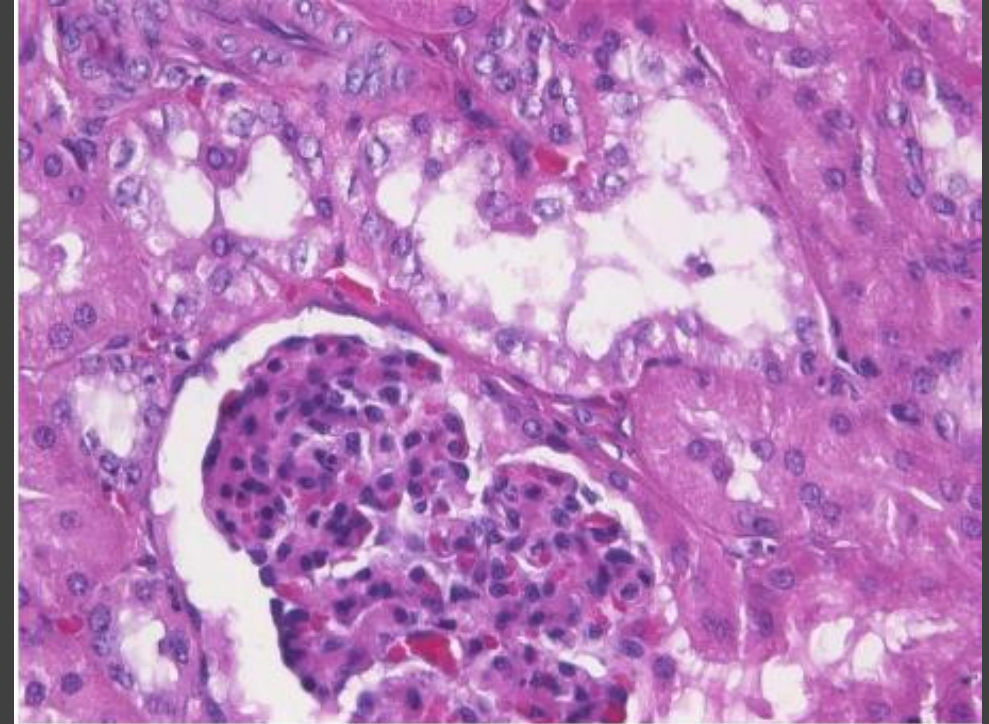




Noncoagulating vs coagulating fixative



Kidney fixated in neutral buffered formaldehyde. The structures are well preserved.



Kidney fixated in neutral buffered formaldehyde but too short time. The tissue display excessive shrinkage and poorly defined cell structures.

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

Fixation

Detection of Changes in Immunohistochemical Stains Caused by Postmortem Delay and Fixation Time

Lundström, Yasmin Med Stud*; Lundström, Patrik Med Stud*; Popova, Svetlana N. MD, PhD*; Lindh...

Impact of delayed and prolonged fixation on the evaluation of immunohistochemical staining on lung carcinoma resection specimen

Maartje van Seijen^{1,2} • Luka Brcic³ • Atilio Navarro Gonzales⁴ • Irene Sansano⁵ • Matyas Bendek^{6,7} • Iva Brcic
Birgit Lissenberg-Witte⁸ • H. Ibrahim Korkmaz¹ • Thomas Geiger⁹ • Rosita Kammler⁹ • Rolf Stahel^{9,10} •
Erik Thunnissen¹ • On behalf of ETOP⁹

The Influence of Tissue Ischemia on Biomarker Expression in Colorectal Cancer

Havelund, Birgitte M. MD*; Olsen, Dorte A. MSc*; Andersen, Rikke F. PhD*; Spindler, Karen-Lise G. MD,
PhD*; Brandslund, Ivan MD, DMSc*; Jakobsen, Anders MD, DMSc*; Soerensen, Flemming B. MD,
DMSc*⁵

Author Information

Applied Immunohistochemistry & Molecular Morphology: July 2013 - Volume 21 - Issue 4 - p 298-307
doi: 10.1097/PAI.0b013e31826f4475

Effect of Delayed Formalin Fixation on Estrogen and Progesterone Receptors in Breast Cancer

A Study of Three Different Clones

Jingxin Qiu, MD, PhD,¹ Swati Kulkarni, MD,² Rameela Chandrasekhar,³ Mark Rees, PhD,^{4,6}
Kathryn Hyde,⁵ Gregory Wilding, PhD,³ Dongfeng Tan, MD,⁶ and Thaer Khoury, MD¹

Key Words: Breast cancer; Biomarkers; Delay to formalin fixation

The effect of cold ischemic time on the immunohistochemical evaluation of estrogen receptor, progesterone receptor, and HER2 expression in invasive breast carcinoma

Isil Z Yildiz-Aktas, David J Dabbs and Rohit Bhargava

Delay to formalin fixation effect on breast biomarkers

Thaer Khoury¹, Sheila Sait², Helena Hwang¹, Rameela Chandrasekhar³, Gregory Wilding³,
Dongfeng Tan⁴ and Swati Kulkarni⁵

[Delay in formalin fixation and HER2 testing in gastric cancer]

[Article in Chinese]

Lixia Zeng¹, Junqi Huang, Yun Ma, Yixiao Liu, Yuying Wei, Qian Zheng, Hongtao Ye²

Delay to formalin fixation 'cold ischemia time': effect on ERBB2 detection by *in-situ* hybridization and immunohistochemistry

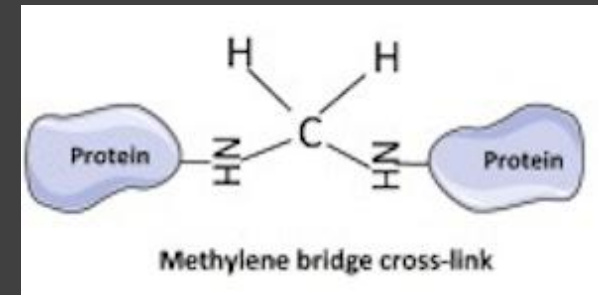
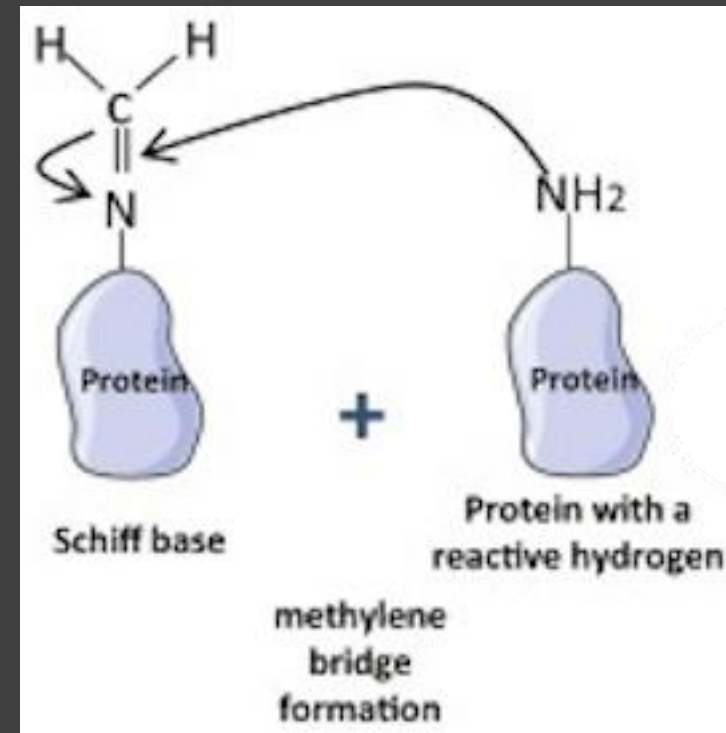
Bryce P Portier, Zhen Wang, Erinn Downs-Kelly, Jordi J Rowe, Deepa Patil, Chis Lanigan, G Thomas
Budd, David G Hicks, David L Rimm & Raymond R Tubbs

Modern Pathology 26, 1–9 (2013) | [Cite this article](#)

Fixation

- 10% Neutral buffered formaldehyde
- For more than 70 years
- You should insure enough liquid for your sample size (10-20:1)
- Formaldehyde is one of the fastest solutions regarding tissue penetration but one of the slowest regarding fixation

Phase I	Penetration	Fast
Phase II	Binding	Moderate
Phase III	Cross-linking	Slow



Fixation

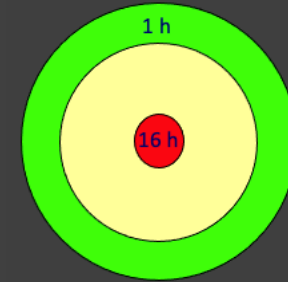
Penetration time at $K = 3.6$ (Baker's coefficient)

d = penetration in mm

$K = 3.6$ (Bakers coefficient)

t = time in hours

$$d = K \times \sqrt{t}$$



3 cm



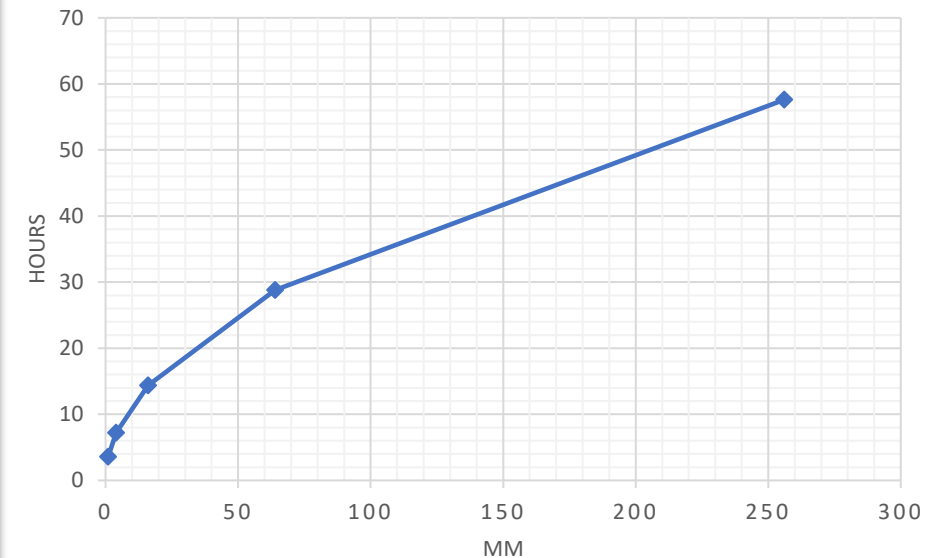
1 hour = 3.6 mm

4 hours = 7.2 mm (1.8 mm/hr)

16 hours = 14.4 mm (0.9 mm/hr)

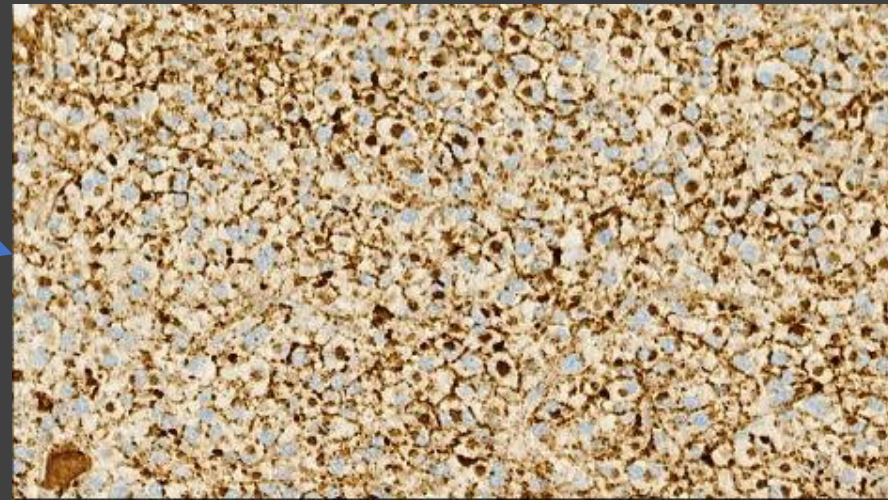
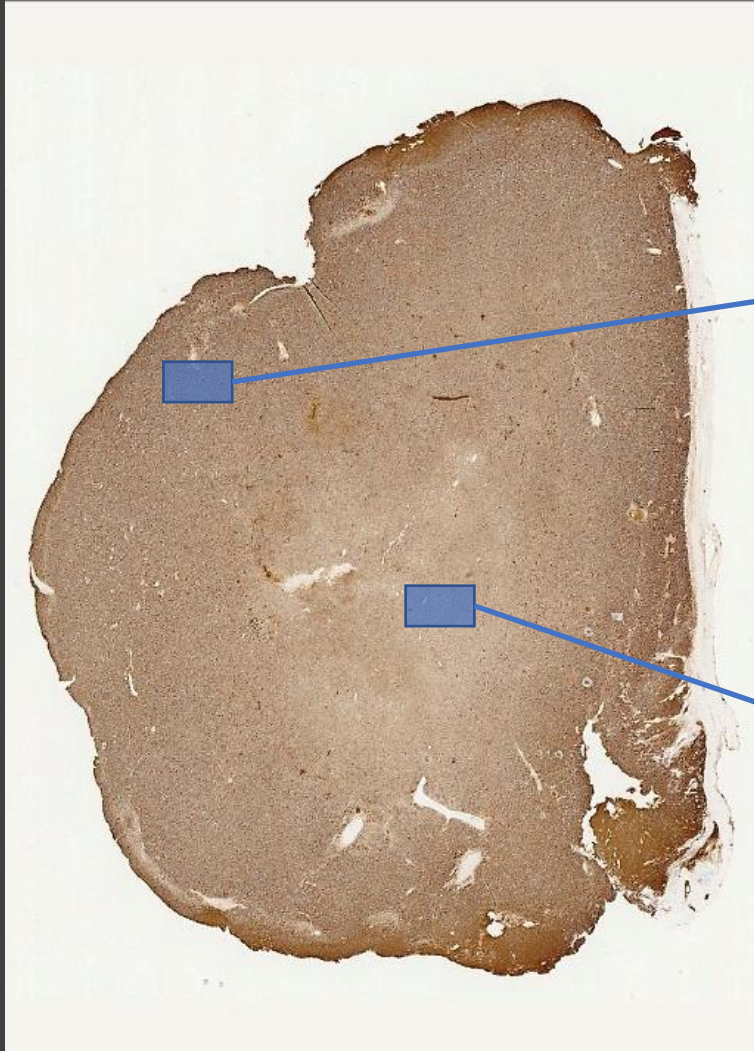
64 hours = 28.8 mm (0.45 mm/hr)

256 hours = 57.6 mm (0.225 mm/hr)



Plasmacytoma

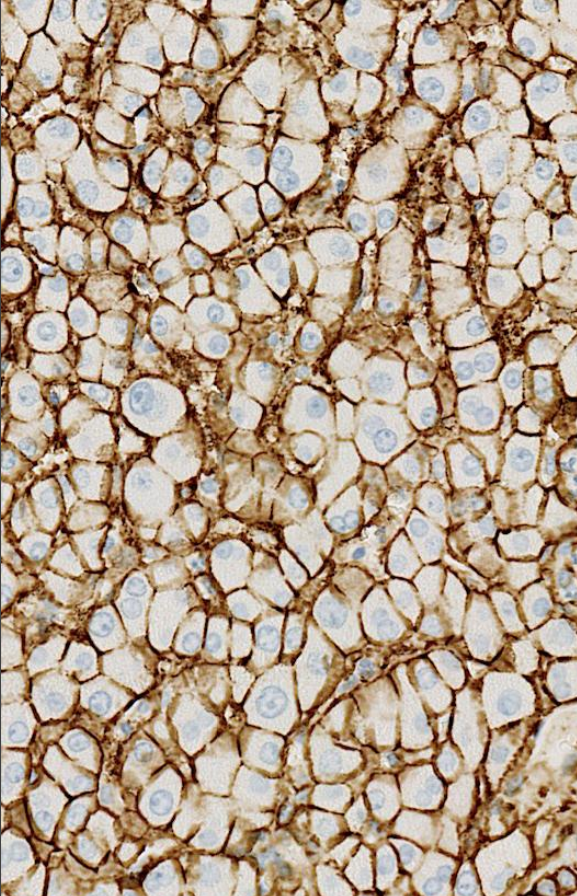
Edge



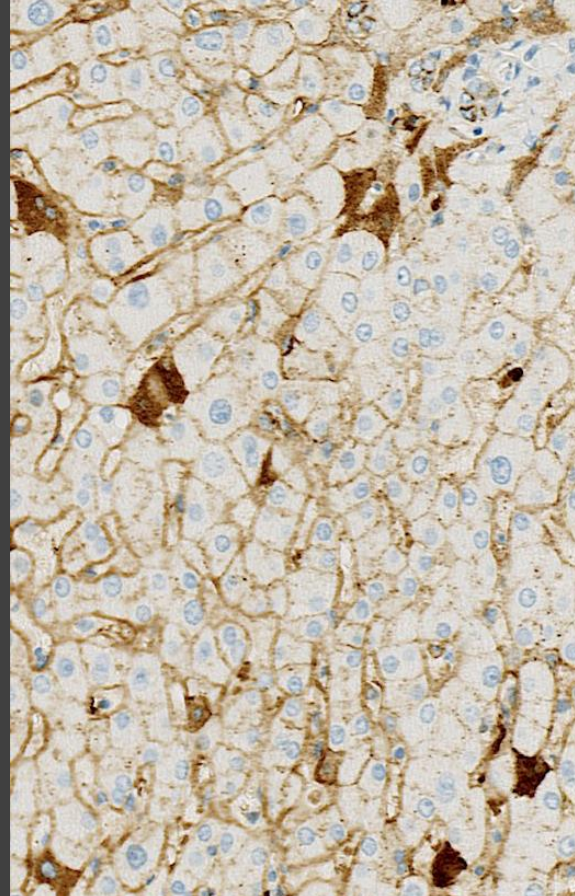
CD138

Center

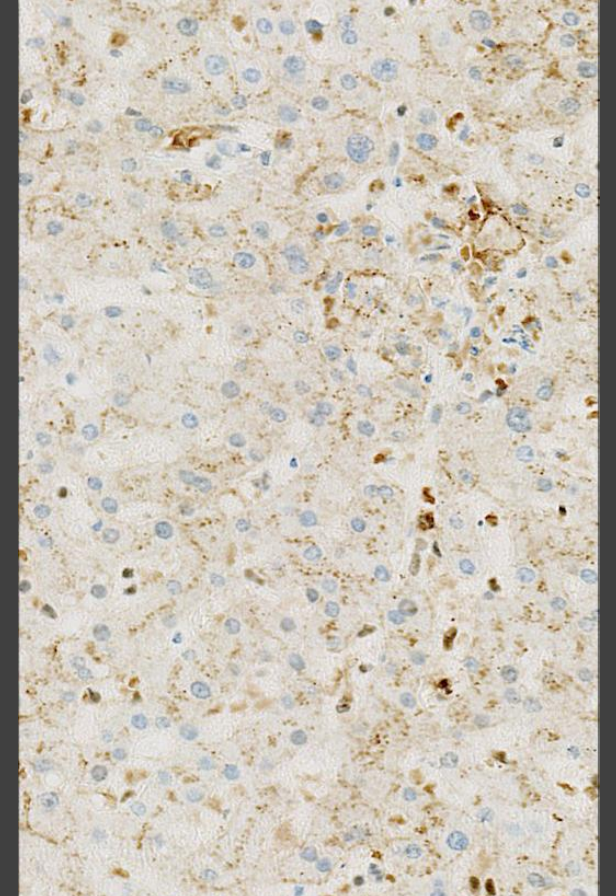
CD138: Simple marker of fixation delay



Liver: No Fix delay



Liver 16 hrs delay

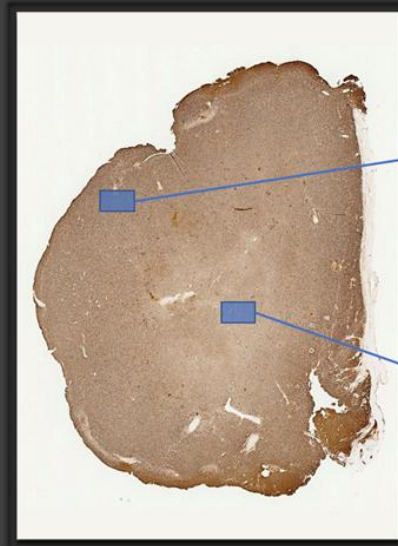


Liver 48 hrs delay

Targets sensitive to short formalin fixation / delayed time to formalin;

Target
ALK
Bcl6
BRAF
CD138
Cyclin D1
MLH1
p53
PD-L1
PMS2
PAX8
PRAME
TTF1

Plasmacytoma

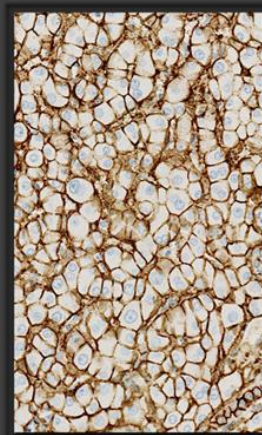


Edge

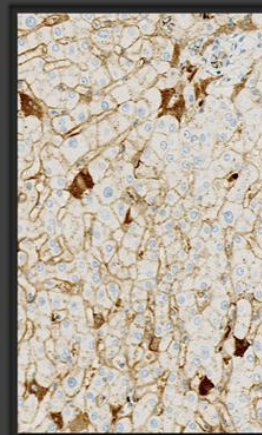


Clinical sample

CD138



Liver: No Fix delay



Liver 16 hrs delay



Liver 48 hrs delay

Control design

By courtesy Ole Nielsen

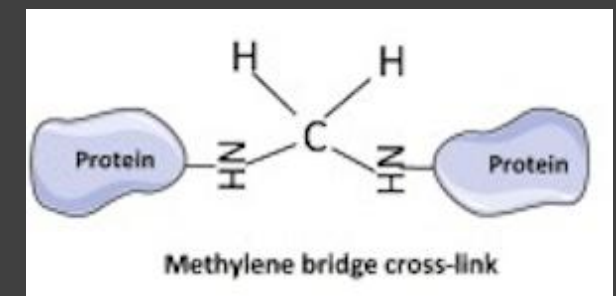
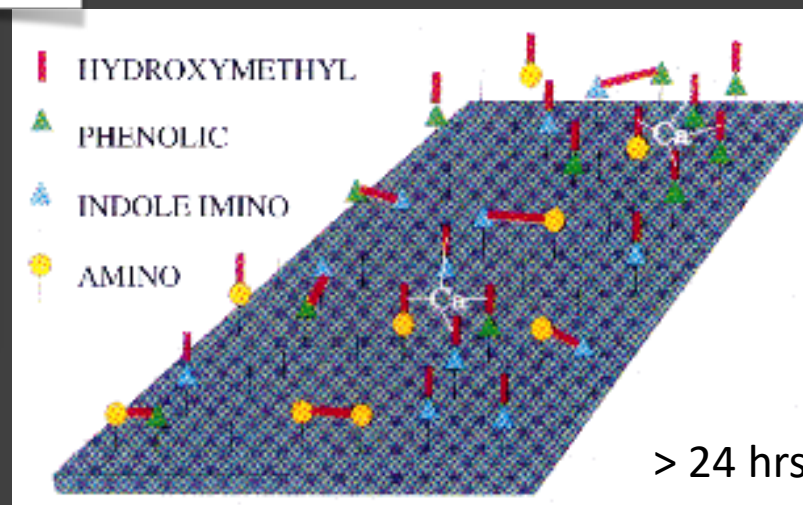
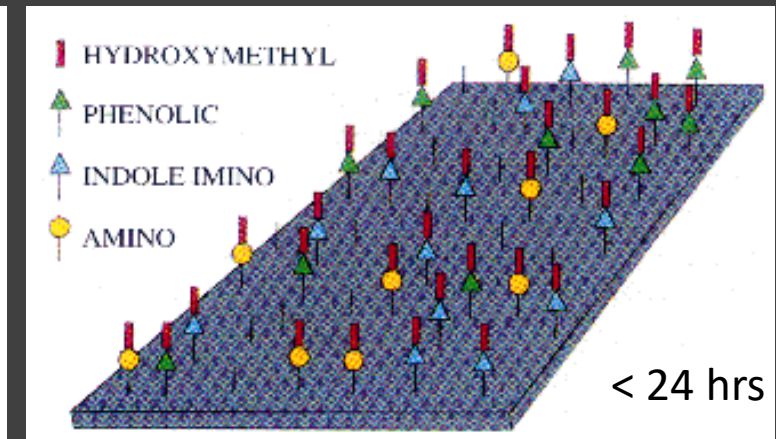
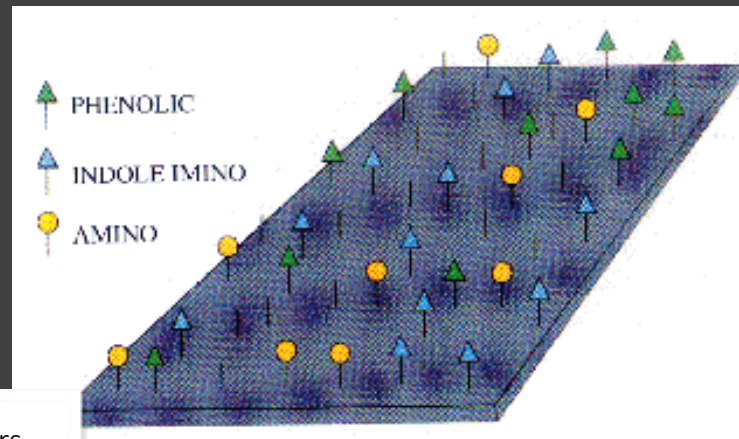
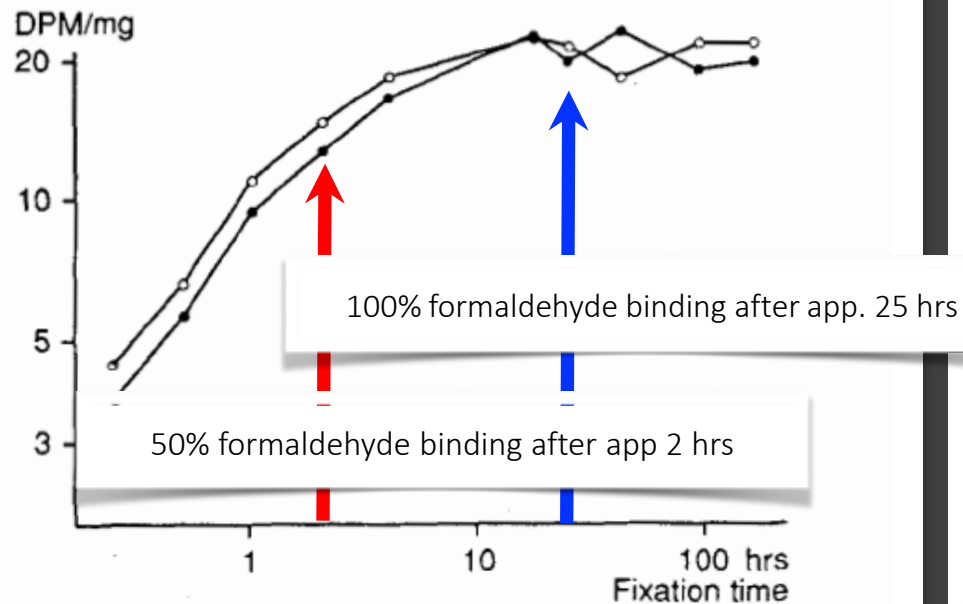
Kinetic Studies of Formaldehyde Binding in Tissue

Kerstin G. Helander

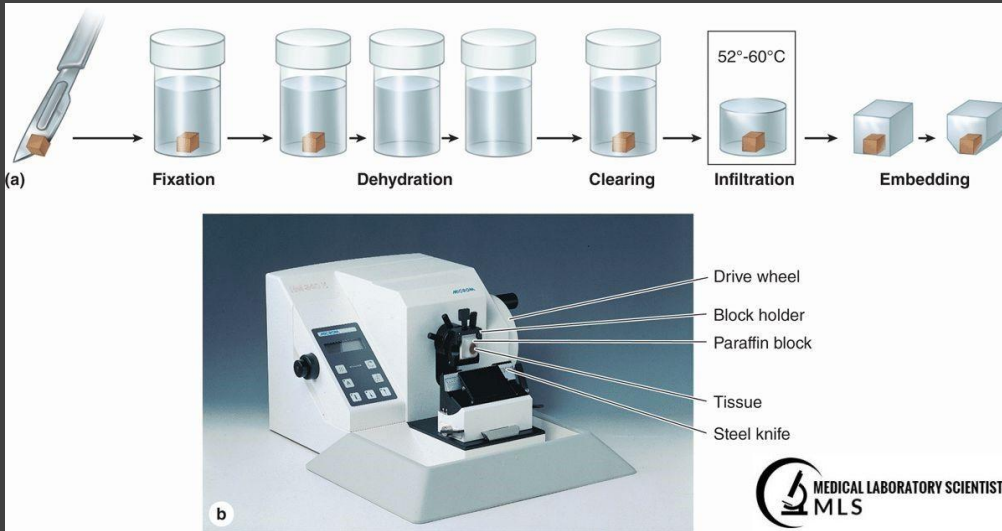
Laboratory of Membrane Biology, Center for Ulcer Research and Education, University of California, Los Angeles, California 90073

“There is a misconception that smaller biopsy samples will fix more quickly than larger resection specimens and therefore require less time in formalin.”

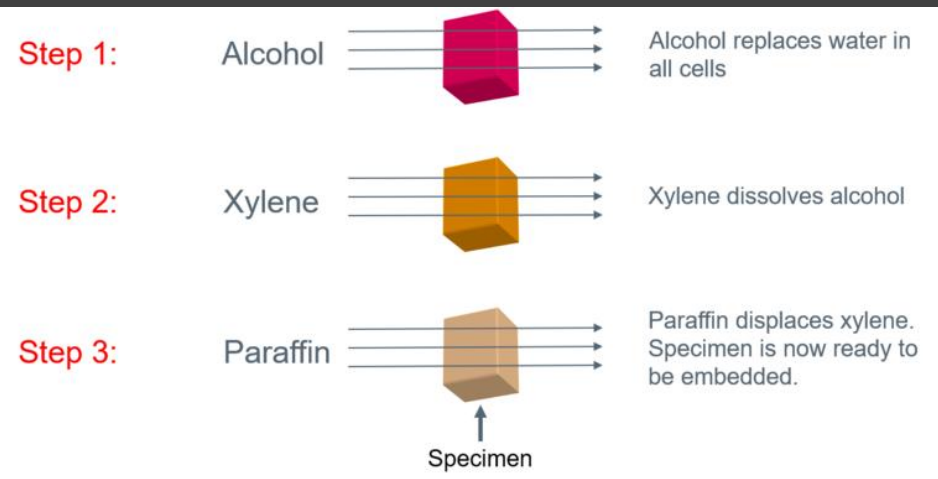
David G. Hicks



Tissue processing



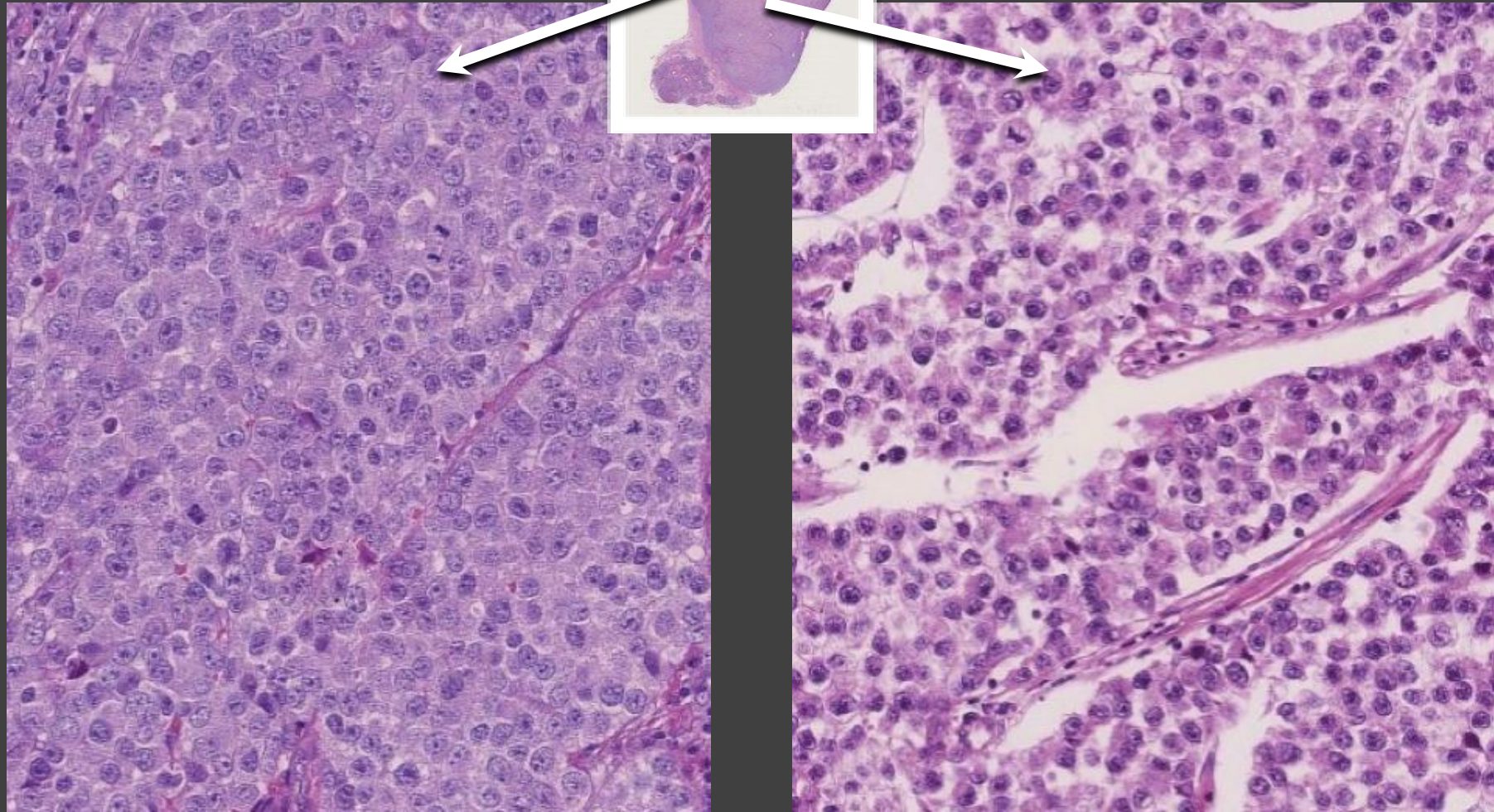
Sta	Solution	Time	Temp (°C)	Mix
1	Formalin	0:10	37	Slow
2	Alcohol (70%)	1:00	37	Slow
3	Alcohol (96%)	0:45	37	Slow
4	Alcohol (96%)	1:00	37	Slow
5	Alcohol (99%)	1:00	37	Slow
6	Alcohol (99%)	1:15	37	Slow
7	½ Alcohol (99%) ½ Histo-clear	1:00	37	Slow
8	1/3 Alcohol (99%) 2/3 Histo-clear	1:00	37	Slow
9	Histo-clear	1:30	37	Slow
10	Histo-clear	2:00	40	Slow
11	Paraffin	0:45	65	Slow
12	Paraffin	1:00	65	Slow
13	Paraffin	1:00	65	Slow
14	Paraffin	1:15	65	Slow



Too short time in formalin induces a hybrid fixation with alcohol affecting some antigens / targets



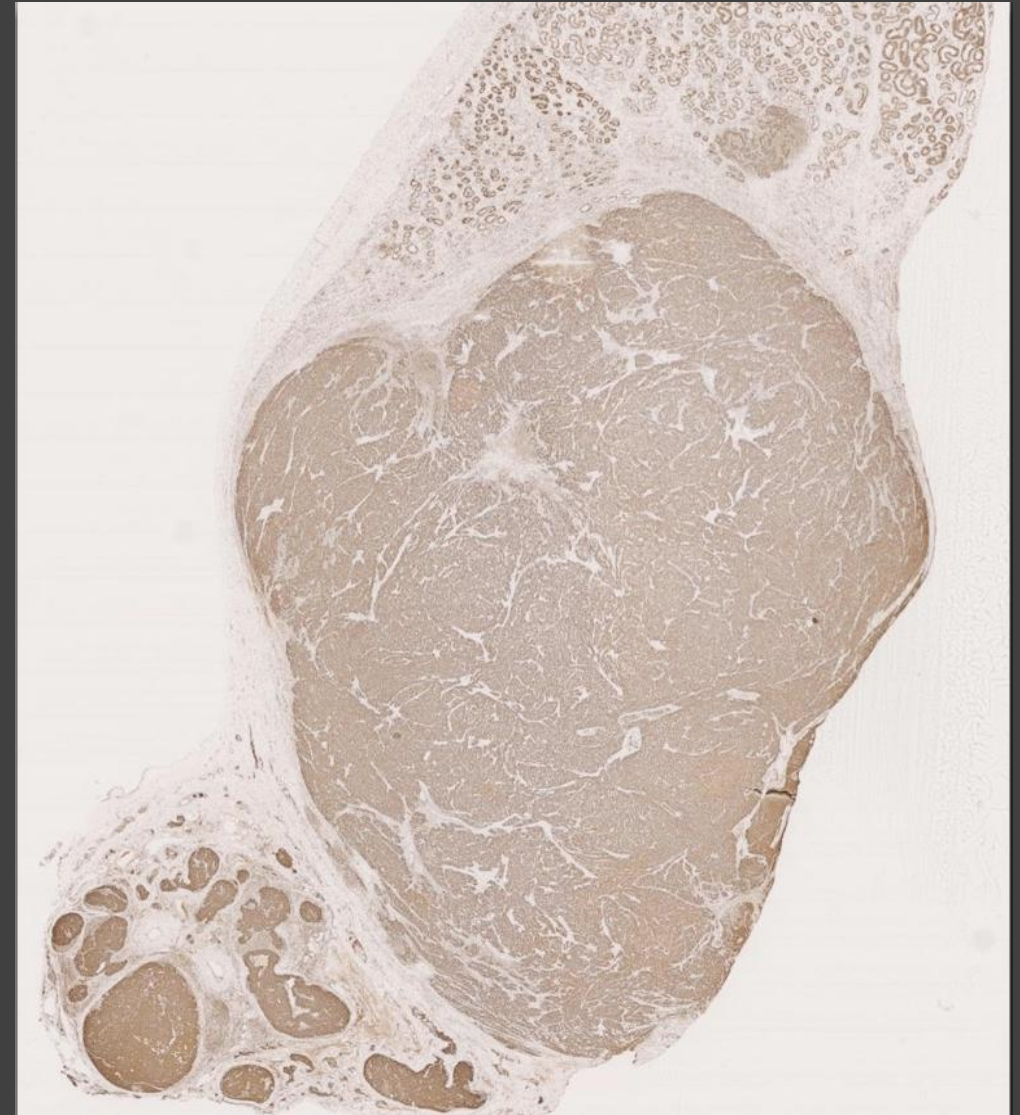
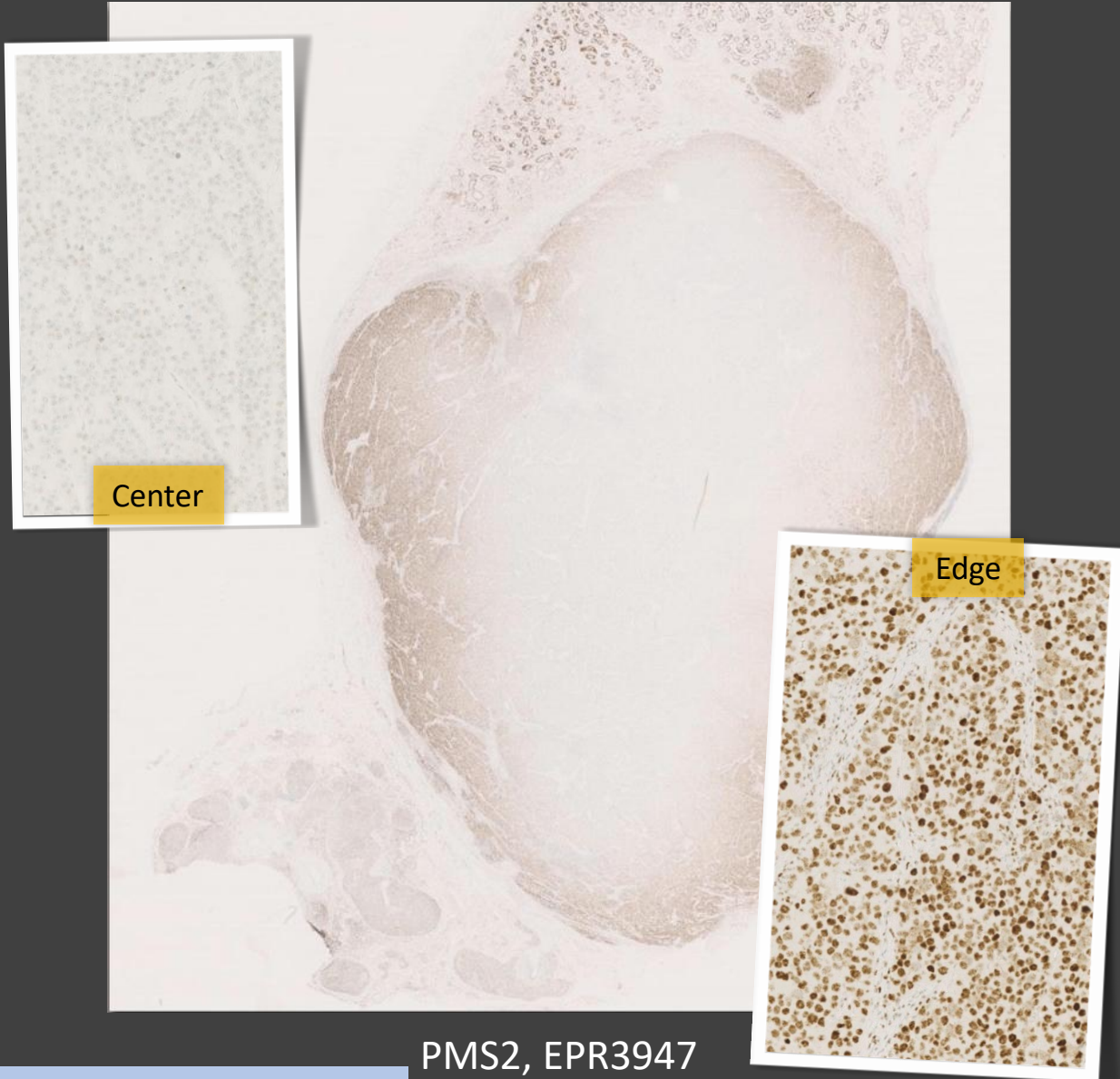
Seminoma



Edge

Center

Seminoma



Cytology cell blocks are suitable for immunohistochemical testing for PD-L1 in lung cancer

H. Wang^{1,2,3*}, J. Agulnik^{3,4}, G. Kasymjanova^{3,4}, A. Wang⁴, P. Jiménez⁵, V. Cohen^{3,4}, D. Small³, C. Pepe³, L. Sakr³, P. O. Fiset^{1,2}, M. Auger^{1,2}, S. Camilleri-Broet^{1,2}, M. Alam El Din^{1,2}, G. Chong^{1,2}, L. van Kempen^{1,2,3} & A. Spatz^{1,2,3,4}

Annals of Oncology 29: 1417–1422, 2018

doi:10.1093/annonc/mdy126

Published online 12 April 2018

Cytology/EBUS isn't clinical validated and NOT included in the labeling for PD-L1 IHC 22C3 pharmDx

EBUS

- In their 86 paired samples of NSCLC with both histological blocks and cytology material, they reported **an overall agreement of 94% with the 22C3 clone**. It is not clear why the cytology cell blocks were more often associated with high PD-L1 expression than surgical resections in our study.
- In this study, they assessed the effect on PD-L1 IHC of 10% buffered formalin only, methanol/alcohol only versus prefixation with Cytolyt/alcohol followed by 10% formalin. **They did not find difference of PD-L1 expression**, nor in morphology, with or without Cytolyt/alcohol pre-fixation.

Cell block procedure – pros and cons

Received: August 20, 2020; Revised: September 28, 2020; Accepted: September 30, 2020

Formalin Fixation for Optimal Concordance of Programmed Death-Ligand 1 Immunostaining Between Cytologic and Histologic Specimens From Patients With Non-small Cell Lung Cancer

Bregje M. Koomen, MD ¹; Jose van der Starre-Gaal, MD, PhD²; Judith M. Vonk, PhD³; Jan H. von der Thüsen, MD, PhD⁴; Jacqueline J. C. van der Meij, MD⁵; Kim Monkhorst, MD, PhD⁶; Stefan M. Willems, MD, PhD^{1,7}; Wim Timens, MD, PhD⁷; and Nils A. 't Hart, MD, PhD^{2,7}

Reduced PD-L1 TPS expression in Cellient processed and alcohol fixed cell blocks compared to Agar / Plasma trombin and formalin processed cell blocks

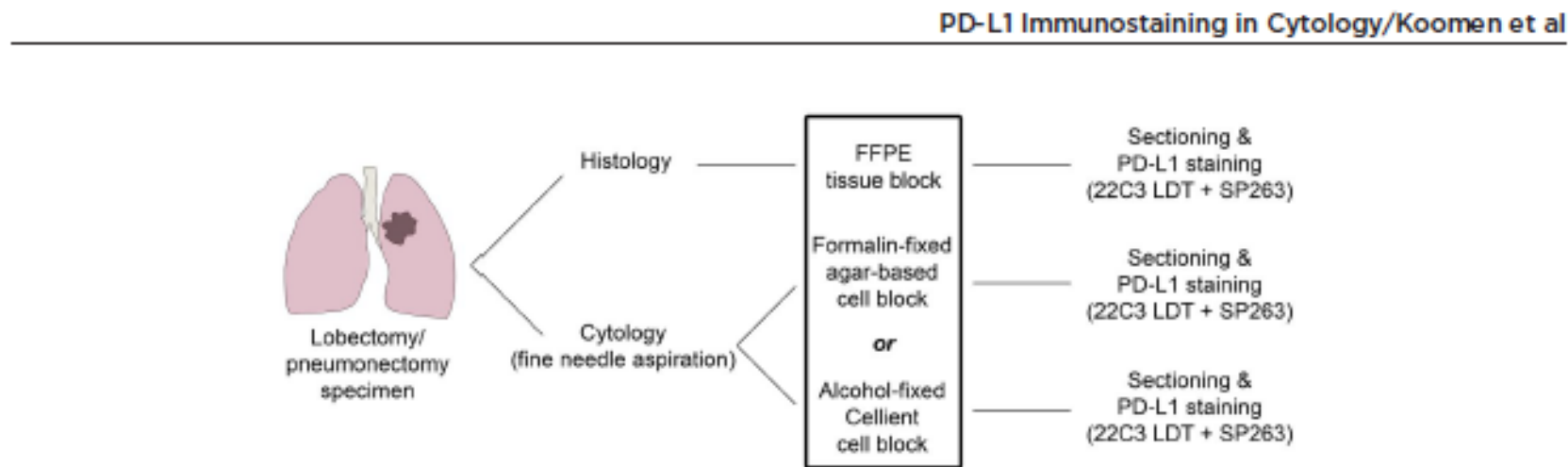


Figure 1. This is a schematic representation of the study design. FFPE Indicates formalin-fixed, paraffin-embedded; LDT, laboratory-developed test (using the 22C3 antibody); PD-L1, programmed death-ligand 1; SP263, antibody used in the standardized assay.

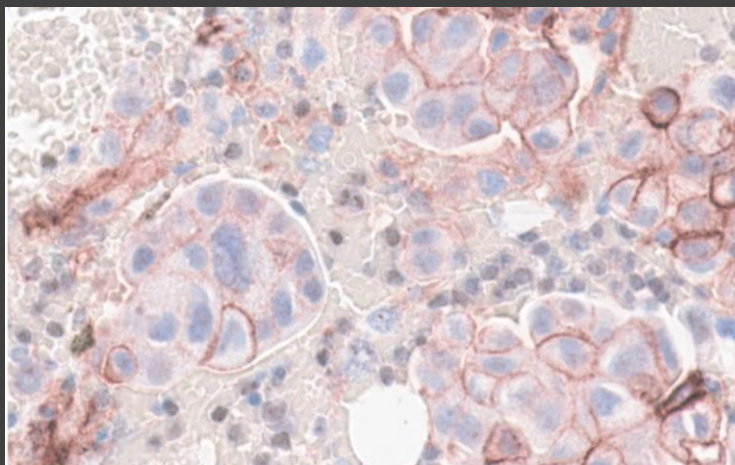
Cell block procedure – pros and cons

Received: August 20, 2020; Revised: September 28, 2020; Accepted: September 30, 2020

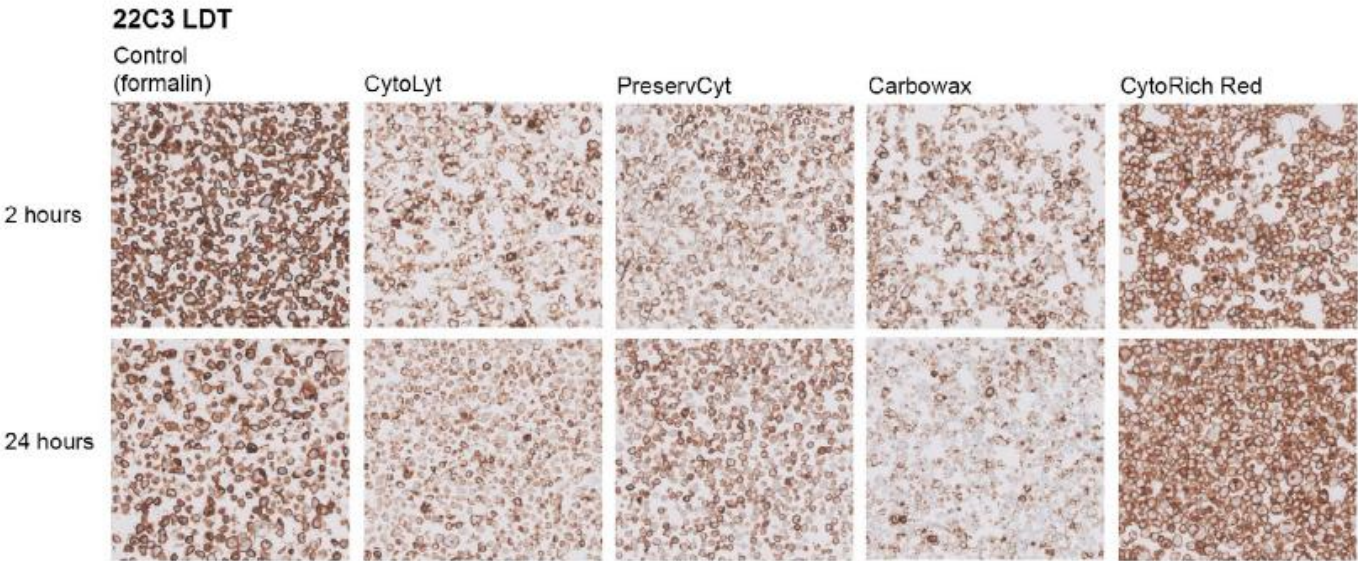


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Target
ALK
Bcl6
BRAF
CD138
Cyclin D1
MLH1
p53
PD-L1
PMS2
PAX8
PRAME
TTF1



CytoLyt® Fixation and Decalcification Pretreatments Alter Antigenicity in Normal Tissues Compared With Standard Formalin Fixation

Gruchy, Jennette R. MD, MSc; Barnes, Penny J. MD, FRCPC; Dakin Haché, Kelly A. MD, PhD, FRCPC
Applied Immunohistochemistry & Molecular Morphology 23(4):p 297-302, April 2015. | DOI: 10.1097/PAI.0000000000000082

Name	Contains...	Company
F-solv	Denat. EtOH / Aldehyde derivate / Stabiliser	Yvsolab
GreenFix	Ethandial / Ethanol	Diapath
CyMol	Ethanol / Methanol / 2-Propanol	Copan
RCL-2	Ethanol / Acetic acid / Complex carbohydrates	Alphelys
FineFix	Ethanol / Glycerol / PVA / Simple carbohydrates	Milestone
Formaldehyde-EtOH	Formaldehyde / Ethanol / Buffer	BBC Biochemical
Zn-Formalin	Formaldehyde / Methanol / Zn-sulfate	Richard-Allen
Prefer	Glyoxal / Ethanol	Anatech
Davidson's AFA	Formaldehyde / Ethanol / Acetic acid	Electron Micr. Sci.
Molecular Fixativ	Methanol / Polyethylenglycol	Sakura
All-Fix	Glyoxal / Ethanol	Cancer Diagnostic
Histochoice	Glyoxal / Zn-sulfate / Butandial	Ameresco-Inc.
O-Fix	Formaldehyd / Ethanol / Acetic acid	SurgiPath
GTF	Glyoxal / Ethanol	StatLab Medical
PAXgene Tissue-fix	Alcohols / Acid / A soluble organic compound	Qiagen- PreAnalytix

Alternatives to NBF??



Revelation in the Field of Tissue Preservation – A Preliminary Study on Natural Formalin Substitutes

[Shankargouda Patil](#), Senior lecturer, [BR Premalatha](#), Senior lecturer, [Roopa S Rao](#), Professor and Head, and [BS Ganavi](#), Postgraduate student

Probing natural substitute for formalin: Comparing honey, sugar, and jaggery syrup as fixatives

[Amritaksha Bhattacharyya](#), [Bhavana Gupta](#),¹ [Anil Singh](#),² [Kunal Sah](#),² and [Vivek Gupta](#)³

Table 5: Problems encountered with different fixatives and their remedies

PROBLEM	FIXATIVES	REMEDY
Breach in continuity of sections	- Honey - Sugar syrup - Jaggery syrup	- Re-impregnate the tissue for another hour - Use new blades - Handle the sections carefully
Intense staining with eosin	- Honey - Sugar syrup	Minimize the staining time with eosin
Folding of the tissue sections	Sugar syrup	- Difficult to avoid - Careful microtomy and floatation techniques

Table 4: The possible mechanism of fixation by honey, sugar & jaggery

Fructose present in honey, sugar & jaggery

↓ Low pH

Breakdown to form aldehydes

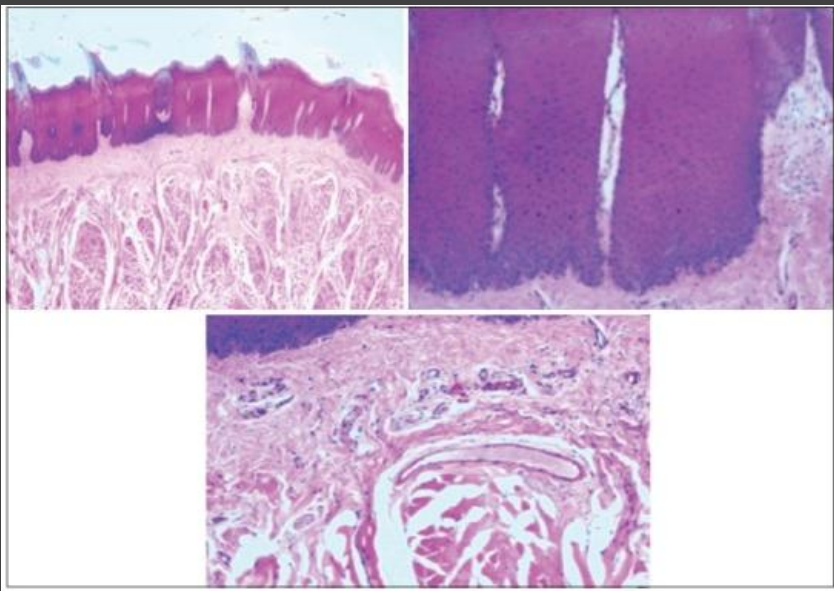
↓

Aldehydes cross-link with tissue amino acids
(Similar to the action of formaldehyde)

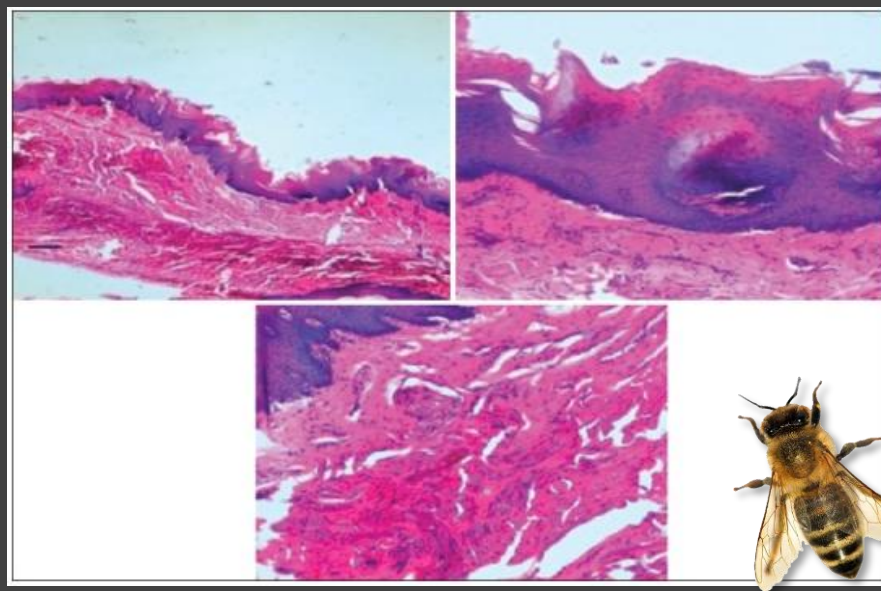
↓

Tissue fixation

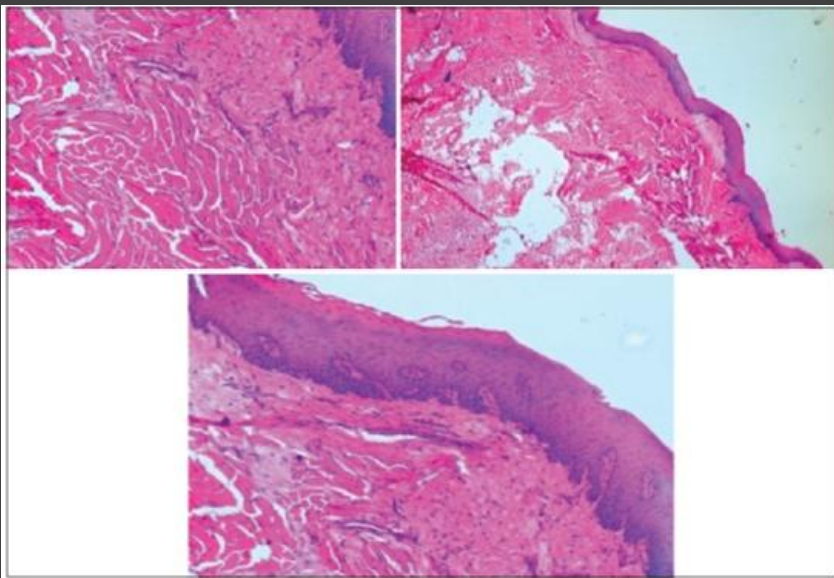
Table 4: The possible mechanism of fixation by honey, sugar & jaggery



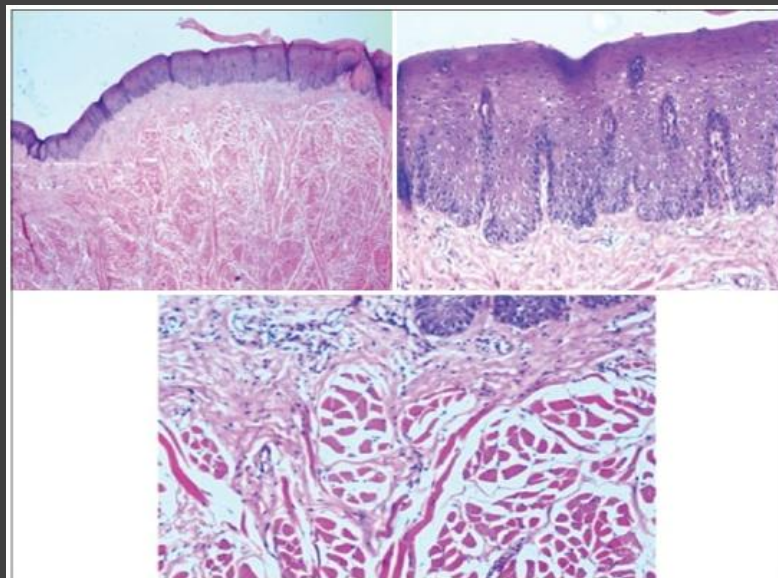
Formalin



Honey



Jaggery

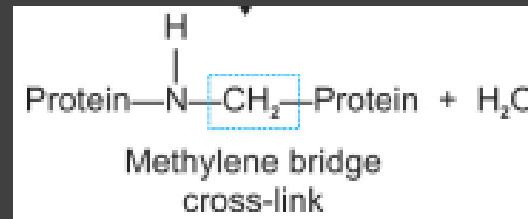


Water

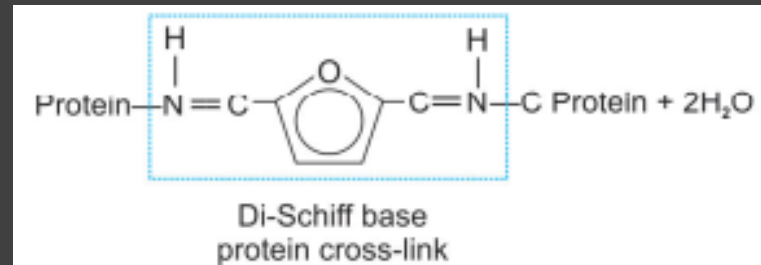


Tissue Preservation with Natural Fixatives: An Immunohistochemical Evaluation

¹Barnali Majumdar, ²Roopa S Rao, ³Shankargouda Patil



Formalin

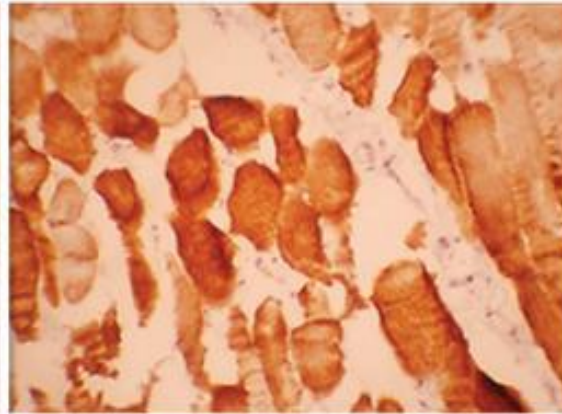
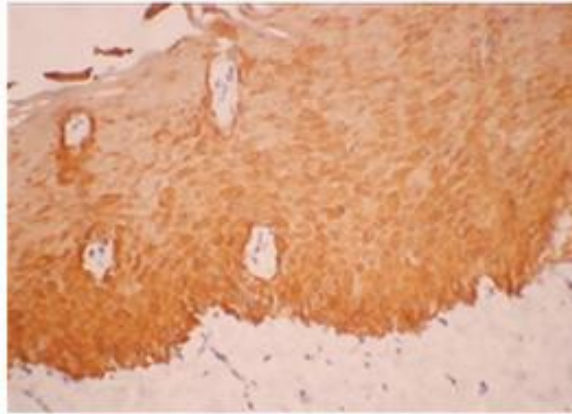


Natural
fixative

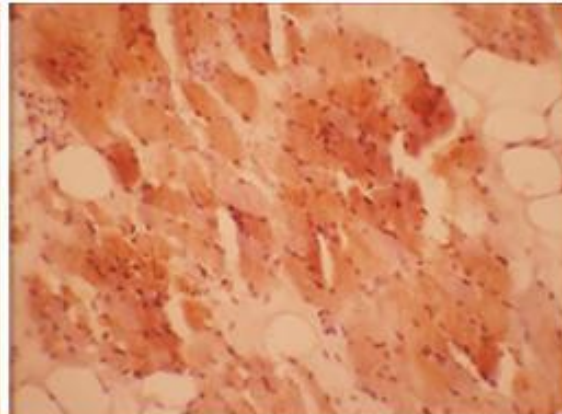
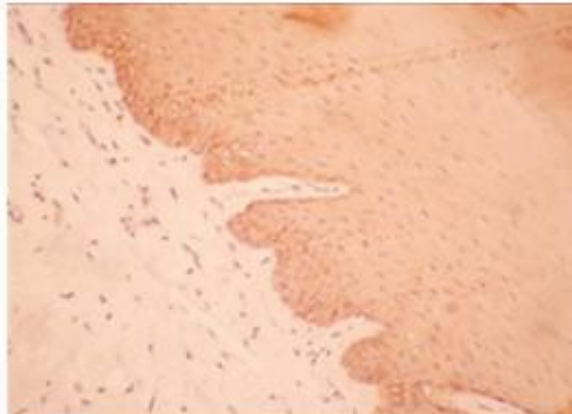
Pan-cytokeratin

Desmin

Jaggery



Honey



Formalin

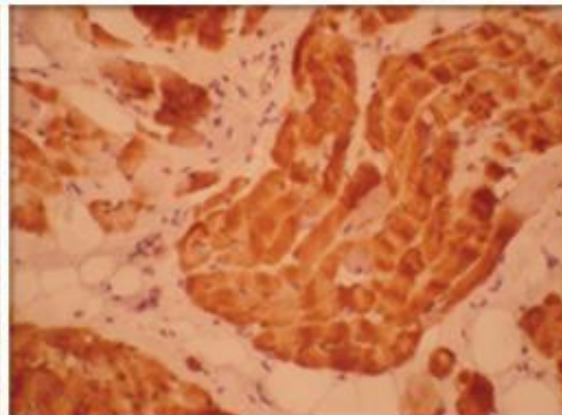
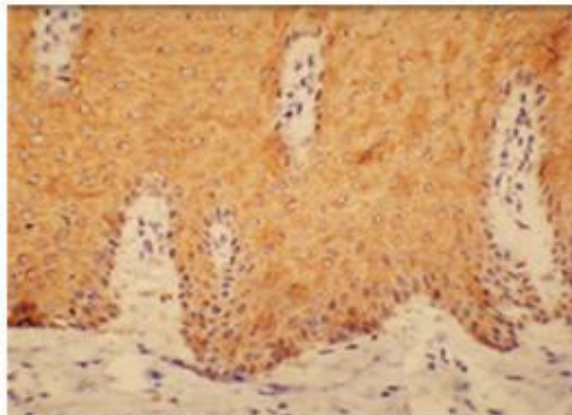


Fig. 1: Intensity of the immunohistochemical stains

Fixation – take home message



- Formaldehyde is at the moment the golden standard
- Fixation needs to happen <1h from collecting the specimen
- Fixation needs minimum 24-48 hours dependent on the sample size
- Overfilling your cassette will cause problems during processing
- If changing to other fixations types all immunohistochemistry needs reevaluation – be careful with alcohol fixatives

Virchows Arch. 2019; 475(2): 191–199.

Published online 2019 Jul 1. doi: [10.1007/s00428-019-02595-9](https://doi.org/10.1007/s00428-019-02595-9)

PMCID: PMC6647403

PMID: [31264038](https://pubmed.ncbi.nlm.nih.gov/31264038/)

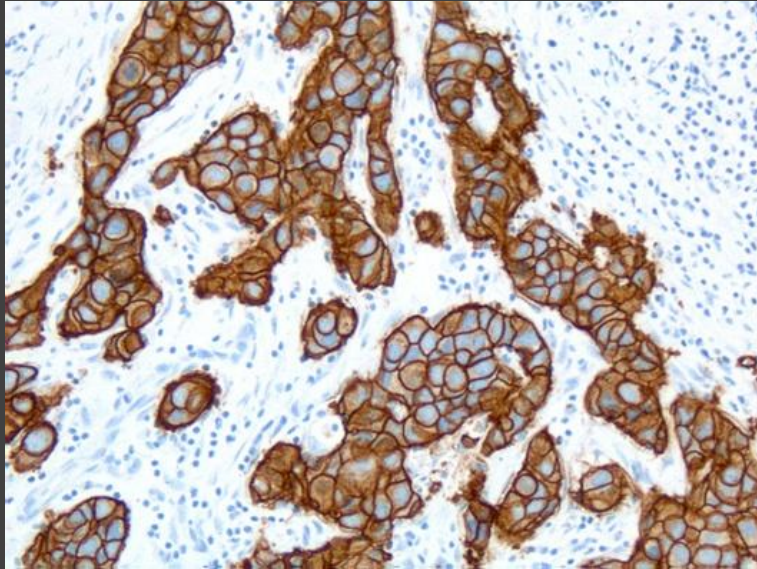
Impact of delayed and prolonged fixation on the evaluation of immunohistochemical staining on lung carcinoma resection specimen

Maartje van Seijen,^{1,2} Luka Brcic,³ Atilio Navarro Gonzales,⁴ Irene Sansano,⁵ Matyas Bendek,^{6,7} Iva Brcic,³ Birgit Lissenberg-Witte,⁸ H. Ibrahim Korkmaz,¹ Thomas Geiger,⁹ Rosita Kammler,⁹ Rolf Stahel,^{9,10} Erik Thunnissen,¹¹ and On behalf of ETOP⁹

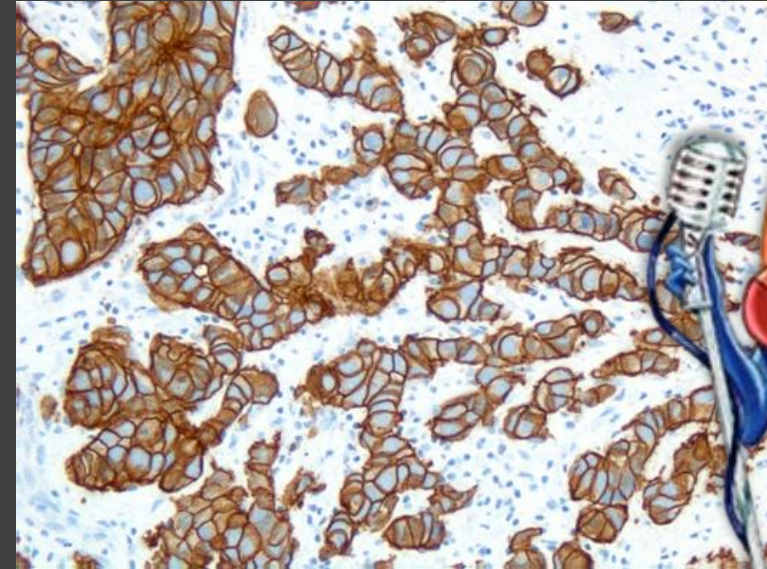
"Prolonged fixation had no influence on the performance of immunohistochemical stains. Delay of fixation negatively affects the expression of different immunohistochemical markers, influencing diagnostic (cytokeratins) and predictive (PD-L1) testing."

Prolong fixation - HER-2 PATHWAY, rmAb 4B5

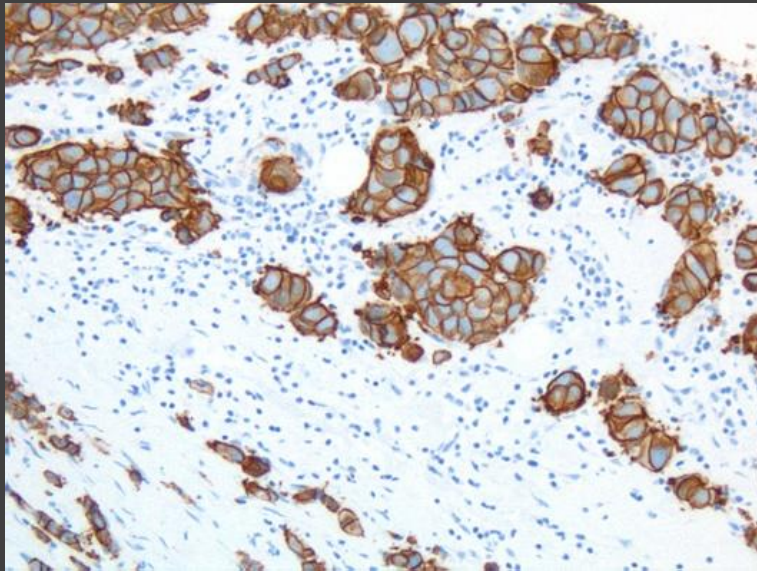
4 h



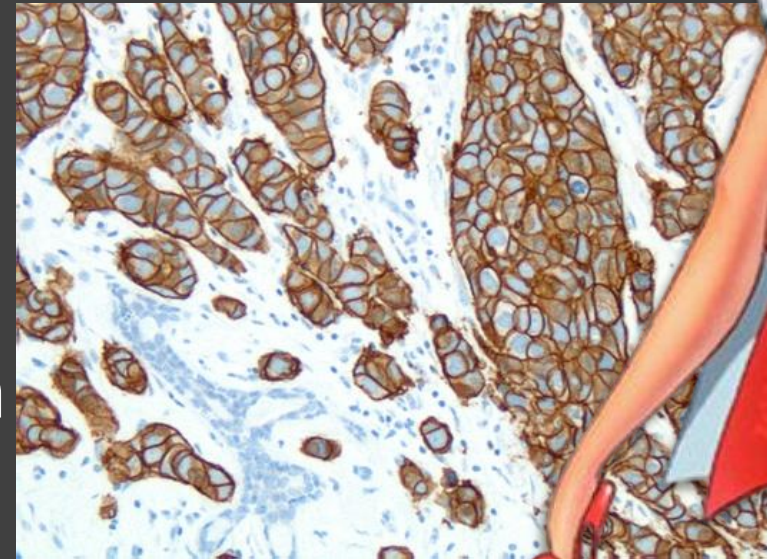
24 h



48 h



168 h



Breast carcinoma 3+, HER-2 PATHWAY, rmAb 4B5

Prolonged fixation

HER2
rmAb
4B5

Internal IHC validation	4 h. NBF	24 h. NBF	48 h. NBF	168 h. NBF
Tumour 1	1+	1+	1+	1+
Tumour 2 3+	3+	3+	3+	3+
Tumour 3	0	0	0	0
Tumour 4	1+	1+	1+	1+
Tumour 5	0	0	0	0
Tumour 6 3+	3+	3+	3+	3+
Tumour 7	0	0	0	0
Tumour 8	0	0	0	0
Tumour 9	0	0	0	0

ER
rmAb
SP1

Internal IHC validation	4 h. NBF	24 h. NBF	48 h. NBF	168 h. NBF
Tumour 1	+	+	+	+
Tumour 2	-	-	-	-
Tumour 3	+	+	+	+
Tumour 4	+	+	+	+
Tumour 5	+	+	+	+
Tumour 6	+	+	+	+
Tumour 7	-	-	-	-
Tumour 8	+	+	+	+
Tumour 9	+	+	+	+

PR
rmAb
1E2

Internal IHC validation	4 h. NBF	24 h. NBF	48 h. NBF	168 h. NBF
Tumour 1	+	+	+	+
Tumour 2	-	-	-	-
Tumour 3	+	+	+	+
Tumour 4	+	+	+	+
Tumour 5	+	+	+	+
Tumour 6+	+	+	+	+
Tumour 7	-	-	-	-
Tumour 8	+	+	+	+
Tumour 9	+	+	+	+

ECAD
mAb
NCH-36

Internal IHC validation	4 h. NBF	24 h. NBF	48 h. NBF	168 h. NBF
Tumour 1	+	+	+	+
Tumour 2	+	+	+	+
Tumour 3	+	+	+	+
Tumour 4	+	+	+	+
Tumour 5	+	+	+	+
Tumour 6	+	+	+	+
Tumour 7	+	+	+	+
Tumour 8	+	+	+	+
Tumour 9	+	+	+	+

Conclusion: IHC biomarkers not affected by NBF fixation time and patient material and control material can be fixed from 4 - 168h in 10% NBF **but**

Prolonged fixation

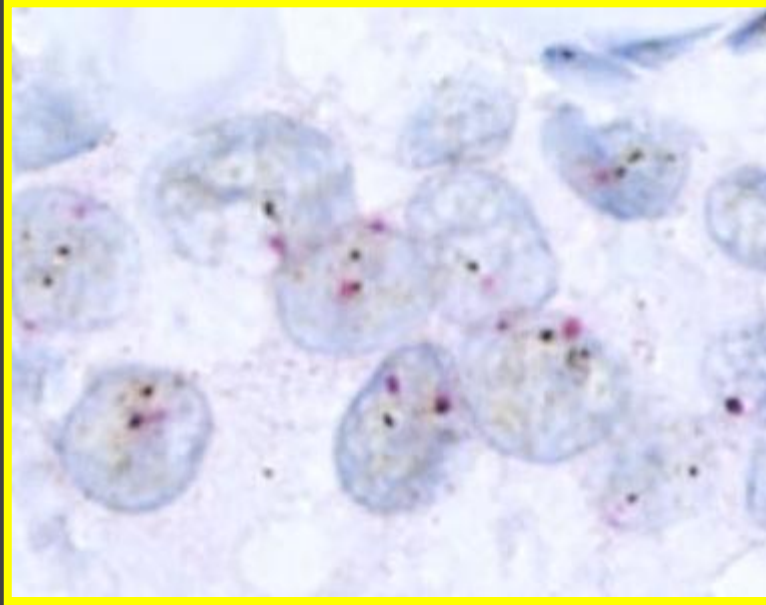
Internal SISH validation	4 h. NBF	24 h. NBF	48 h. NBF	168 h. NBF
Tumour 1	-	-	-	FN
Tumour 2 _{Amp}	+	+	+	+
Tumour 3	(?)	-	FN	FN
Tumour 4	-	-	FN	FN
Tumour 5	-	-	-	-
Tumour 6 _{Amp}	+	+	+	+
Tumour 7	-	-	-	FN
Tumour 8 _{poly.}	-	-	-	FN
Tumour 9 _{poly.}	-	-	-	FN

HER-2 ISH: 8/36 cores could not be assessed..!

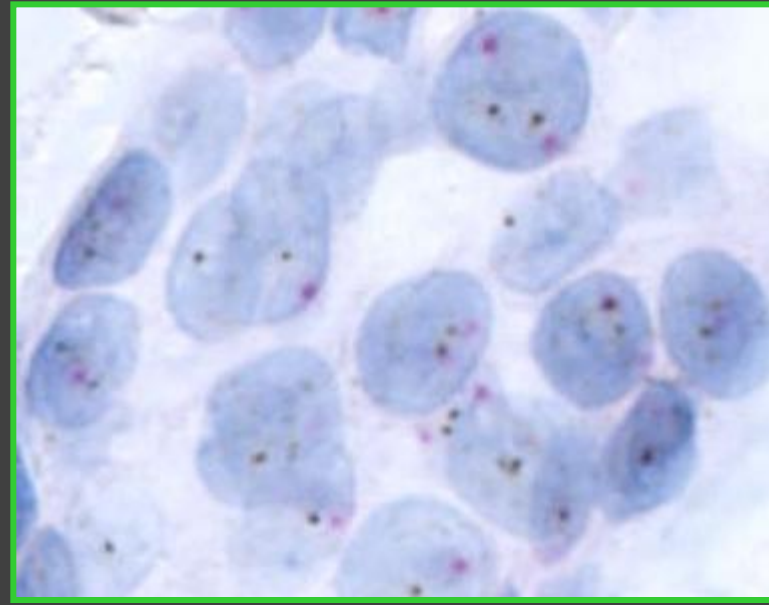
Breast carcinomas, Dual SISH

Prolonged fixation

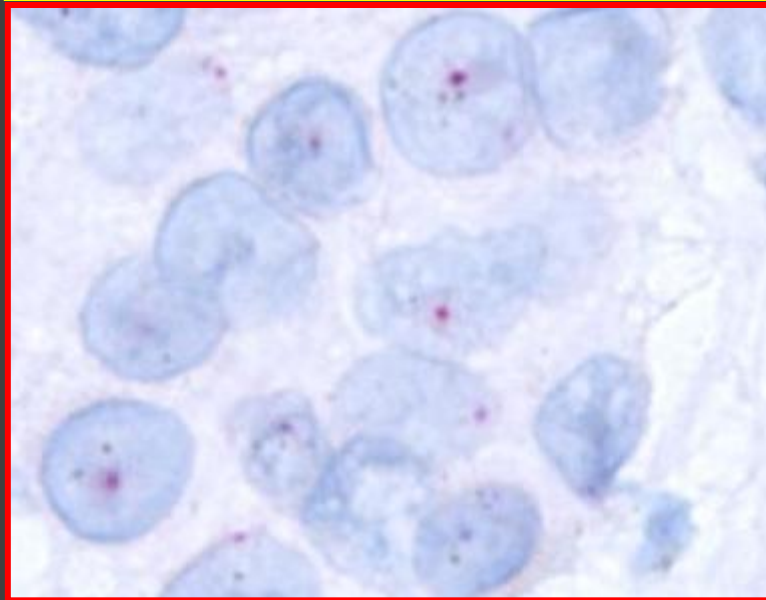
4 h



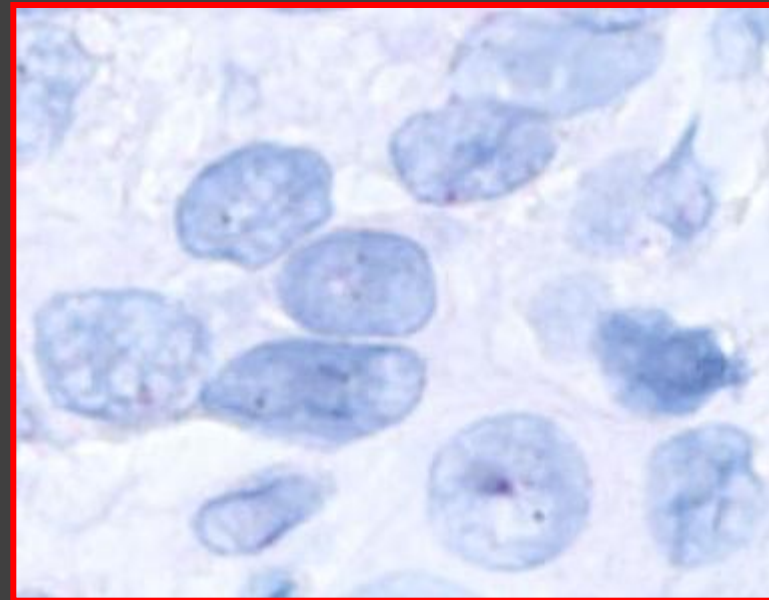
24 h



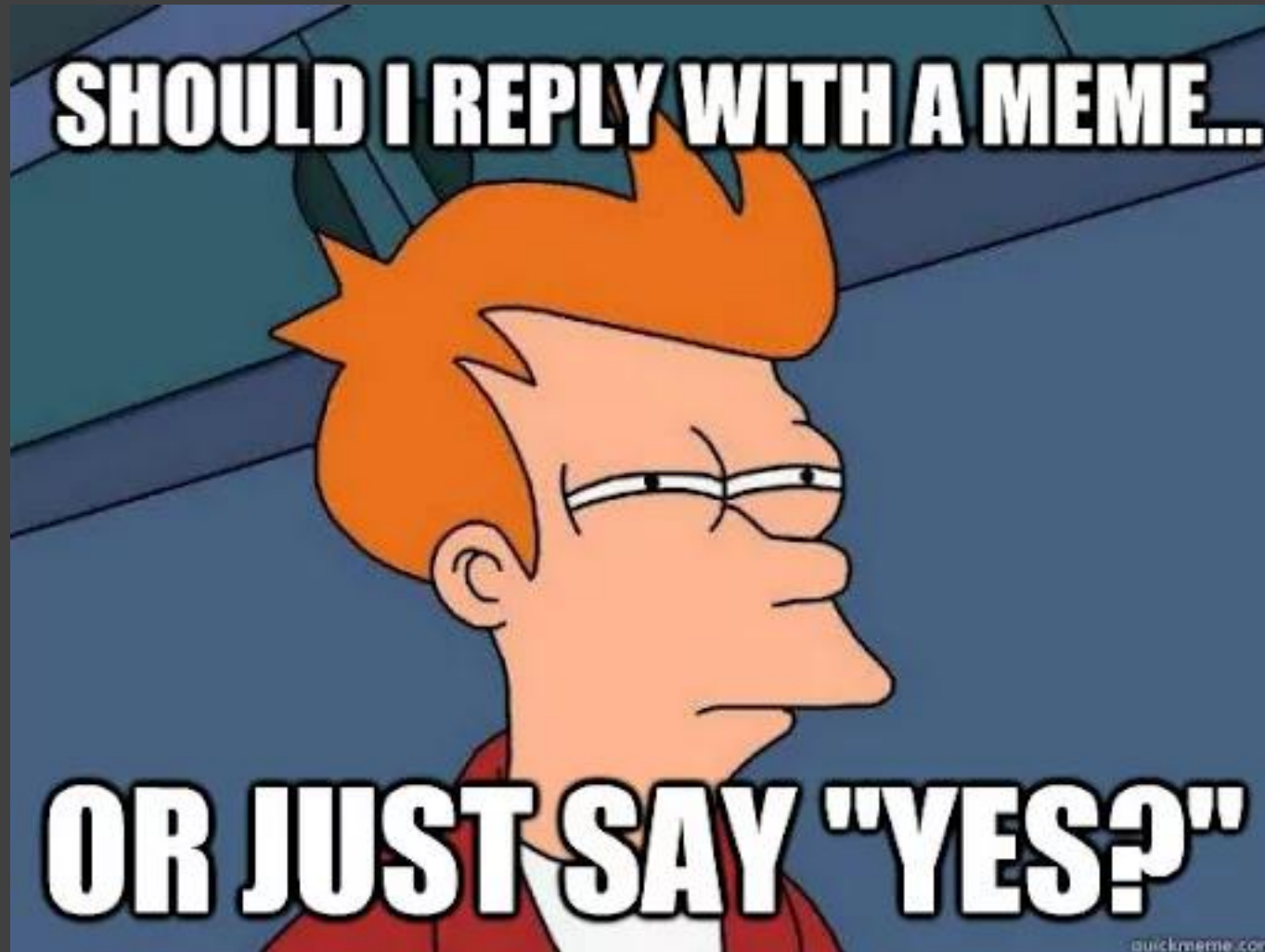
48 h



168 h



Did you realize we are more than halfway through and still talking about fixation.... Important?



Decalcification

✂ Type

✂ Strong acid (e.g. HCl)

✂ Weak organic acid (e.g. formic acid) 2-2,5 timer longer

✂ Chelating agents (e.g. EDTA) 8-16 times longer

✂ Time, Temperature

✂ Time in fixative before decalcification

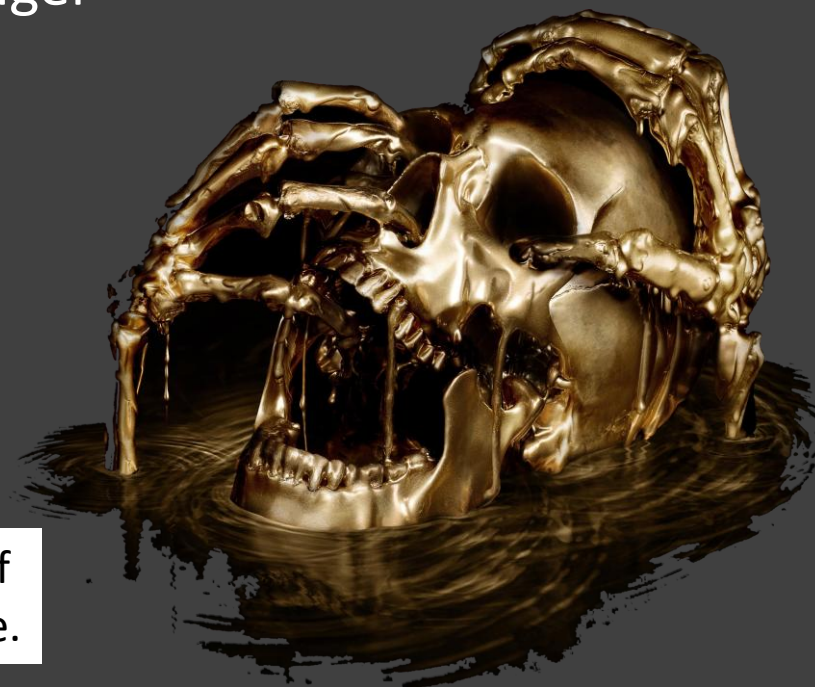
Modern Pathology (2020) 33:1505–1517
https://doi.org/10.1038/s41379-020-0503-6

USCAP

ARTICLE

Effect of decalcification protocols on immunohistochemistry and molecular analyses of bone samples

Elodie Miquelstorena-Standley^{1,2} · Marie-Lise Jourdan³ · Christine Collin³ · Corinne Bouvier⁴ · Frédérique Larousserie⁵ · Sébastien Aubert⁶ · Anne Gomez-Brouchet⁷ · Jean-Marc Guinebretière⁸ · Matthias Tallegas^{1,2} · Bénédicte Brulin⁹ · Louis-Romée Le Nail^{2,9,10} · Anne Tallet³ · François Le Loarer¹¹ · Jessica Massiere¹¹ · Christine Galant¹² · Gonzague de Pinieux^{1,2,9}



68% of the bones cancers are a result of metastasis from breast, lung or prostate.

ARTICLE

Effect of decalcification protocols on immunohistochemistry and molecular analyses of bone samples

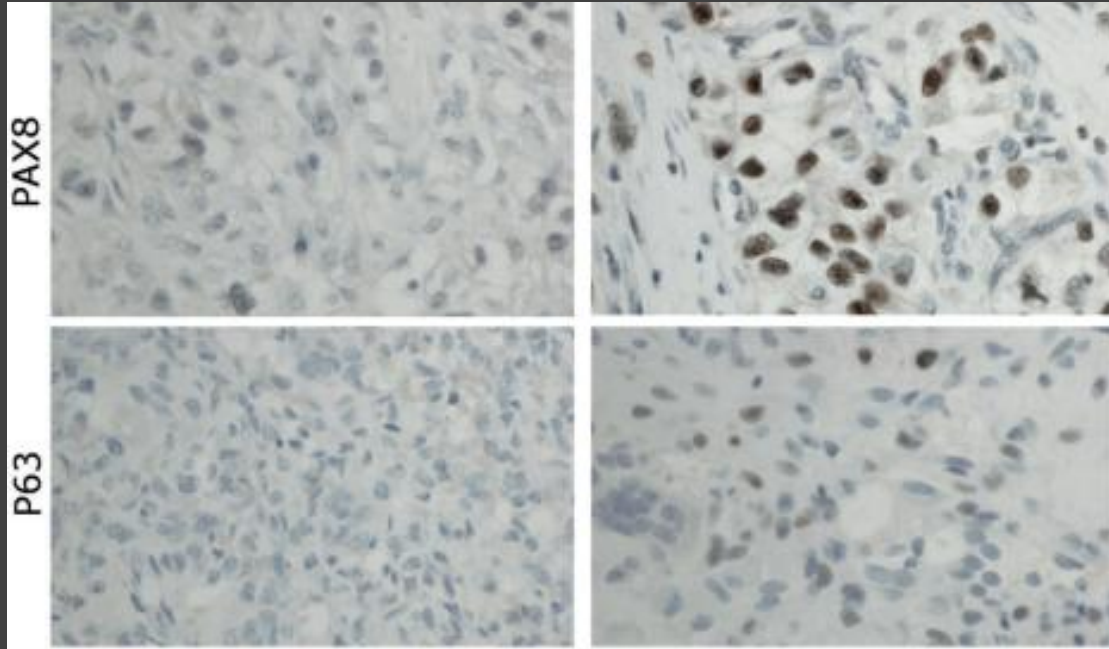
Elodie Miquelstorena-Standley^{1,2} · Marie-Lise Jourdan³ · Christine Collin³ · Corinne Bouvier⁴ · Frédérique Larousserie⁵ · Sébastien Aubert⁶ · Anne Gomez-Brouchet⁷ · Jean-Marc Guinebretière⁸ · Matthias Tallegas^{1,2} · Bénédicte Brulin⁹ · Louis-Romée Le Nail^{2,9,10} · Anne Tallet³ · François Le Loarer¹¹ · Jessica Massiere¹¹ · Christine Galant¹² · Gonzague de Pinieux^{1,2,9}

Table 1 Content and pH provided by manufacturers of commercial decalcifying agents.

	Decalc	DC2	DC3	DC1	TBD2	EDTA
Manufacturer	Histolab, Gothenburg, Sweden	VWR, Radnor, PA, USA	VWR, Radnor, PA, USA	VWR, Radnor, PA, USA	Thermo Fisher Scientific, Waltham, MA, USA	Promega, Madison, WI, USA
Content	Hydrochloric acid 10–20%	Hydrochloric acid 10–25%	Hydrochloric acid 5–10% Alcohols, C12–14, ethoxylated, propoxylated <1% EDTA disodium salt <0.1%	Formic acid 5–15% Formaldehyde 5–10%	Water 77–80% Formic acid 21–23% Fluorad >1% Sodium citrate >1% Polyvinyl pyrrolidone >1%	EDTA 0.5 M
pH	<1	<1	<1	1.3–2.7	2.3–2.4	8

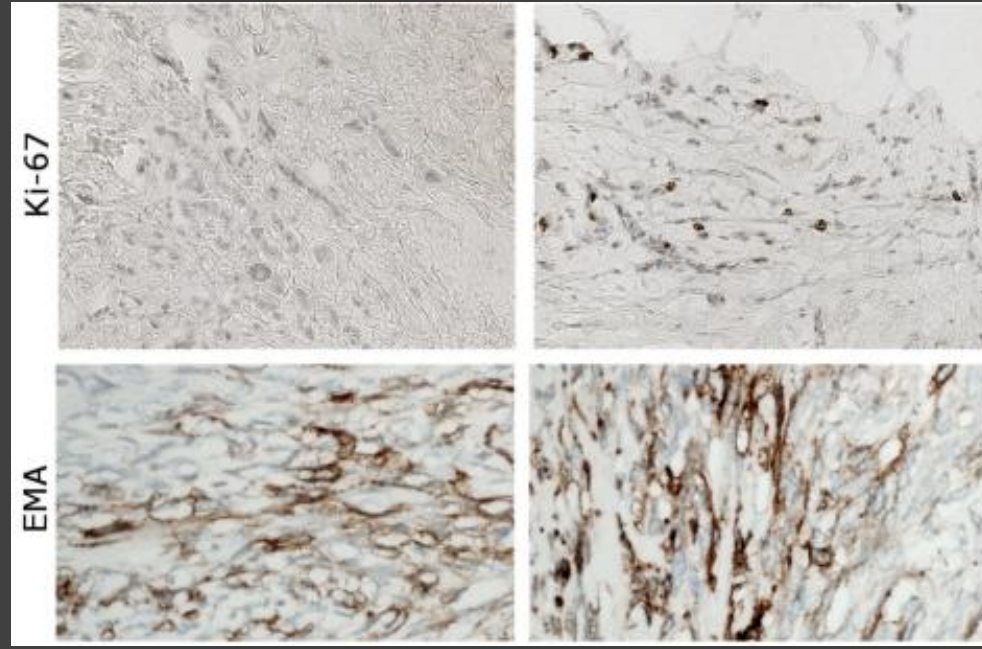
Hydrochloric acid

Formic acid

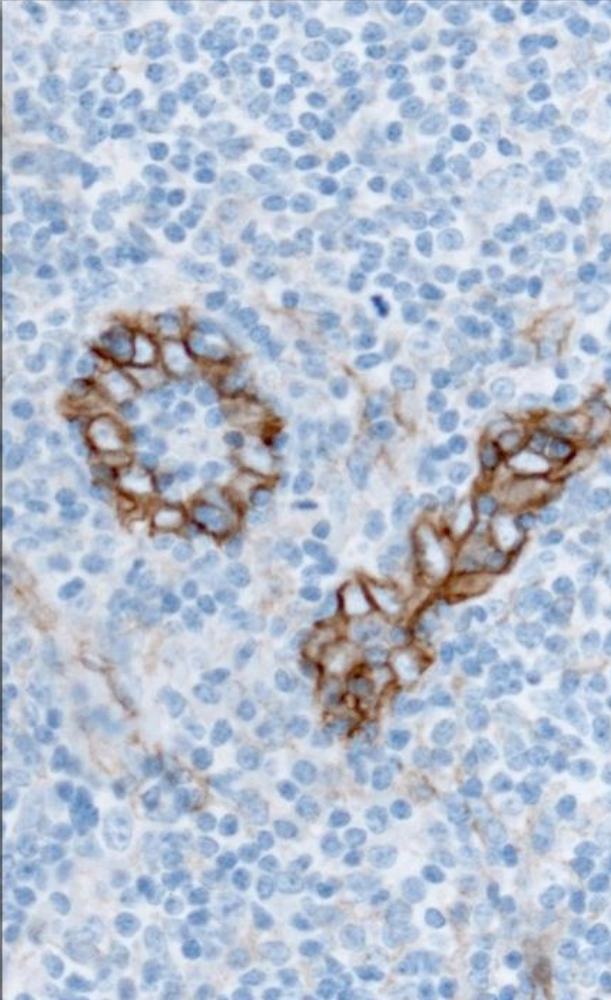


Hydrochloric acid

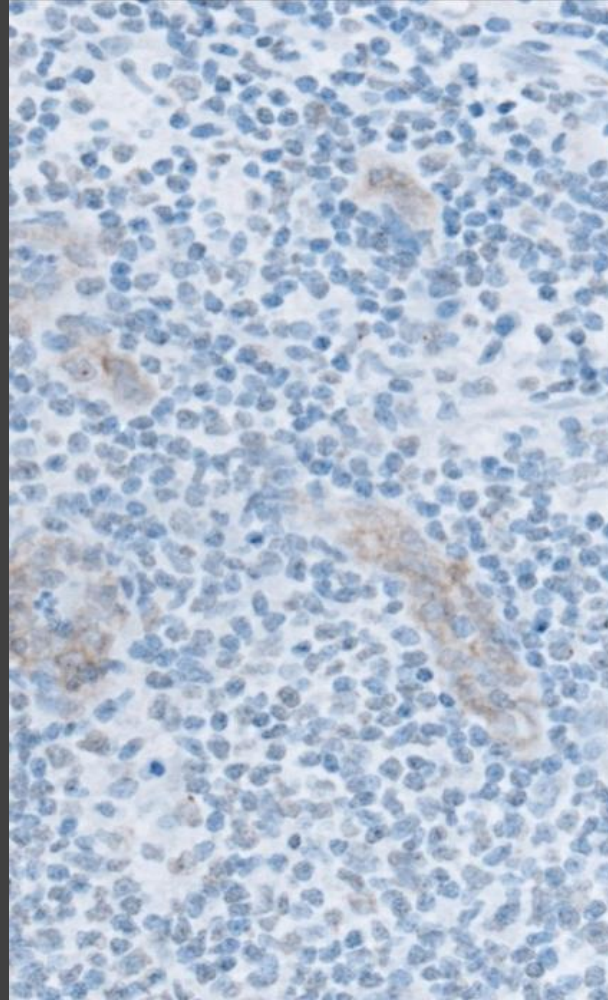
Formic acid



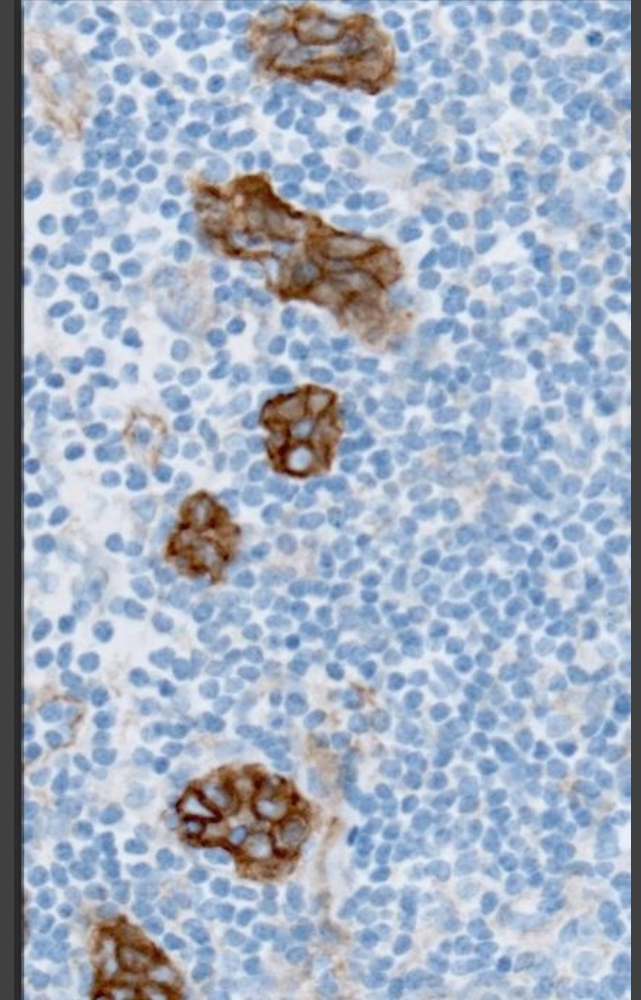
Decalcification and CD105, SN6h



No decalcification

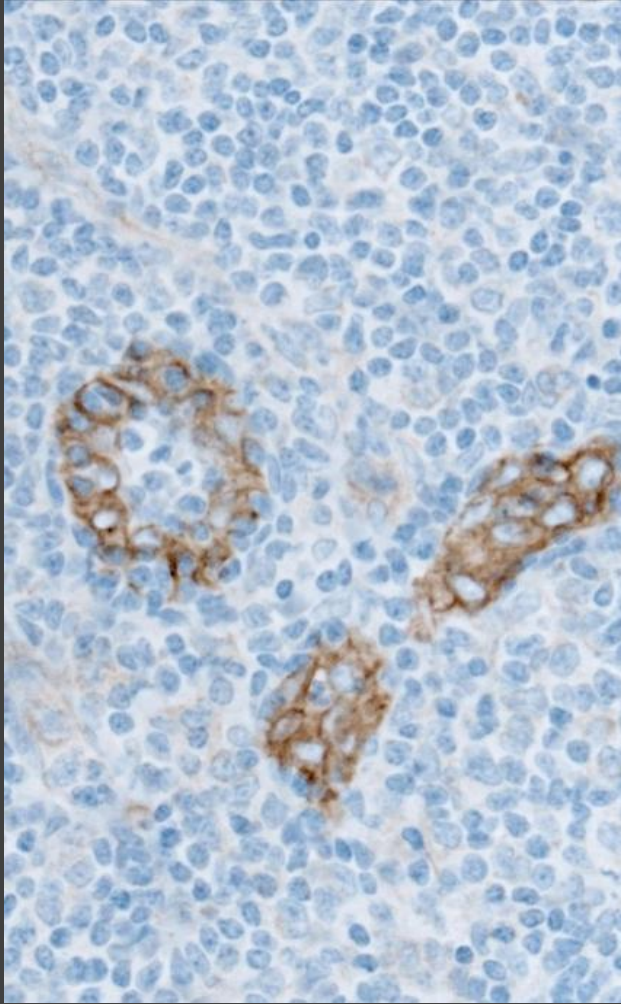


Formic acid 16hrs

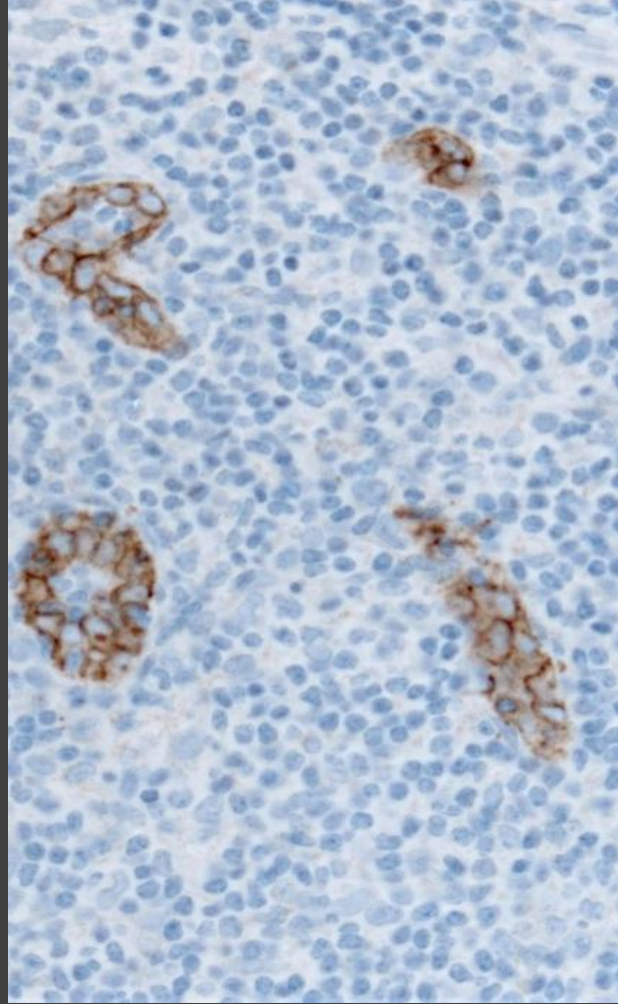


EDTA 96hrs

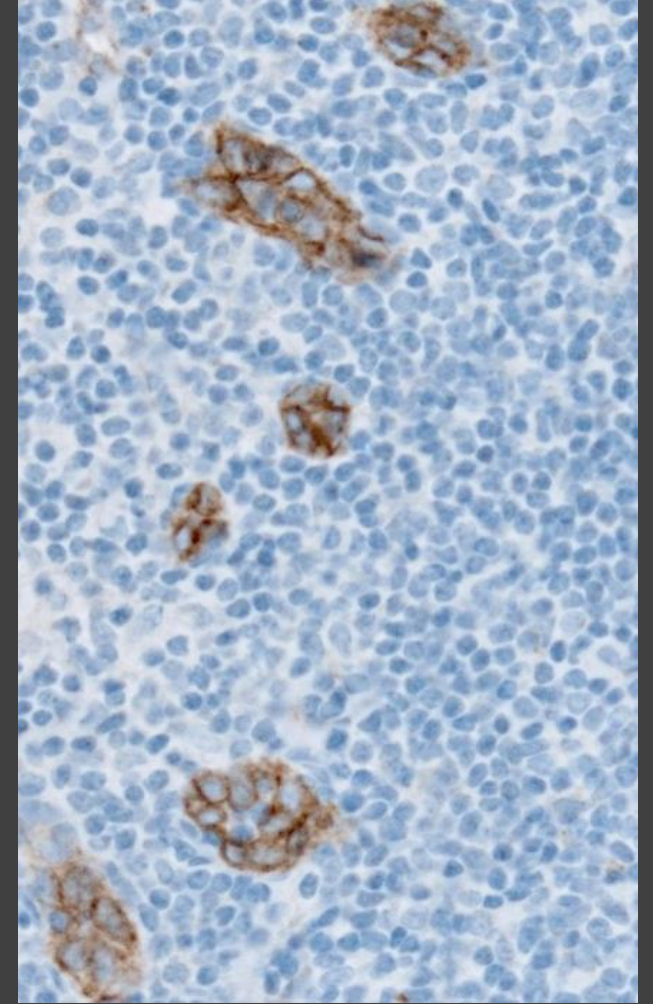
Decalcification and CD105, 4G11



No decalcification



Formic acid 16hrs



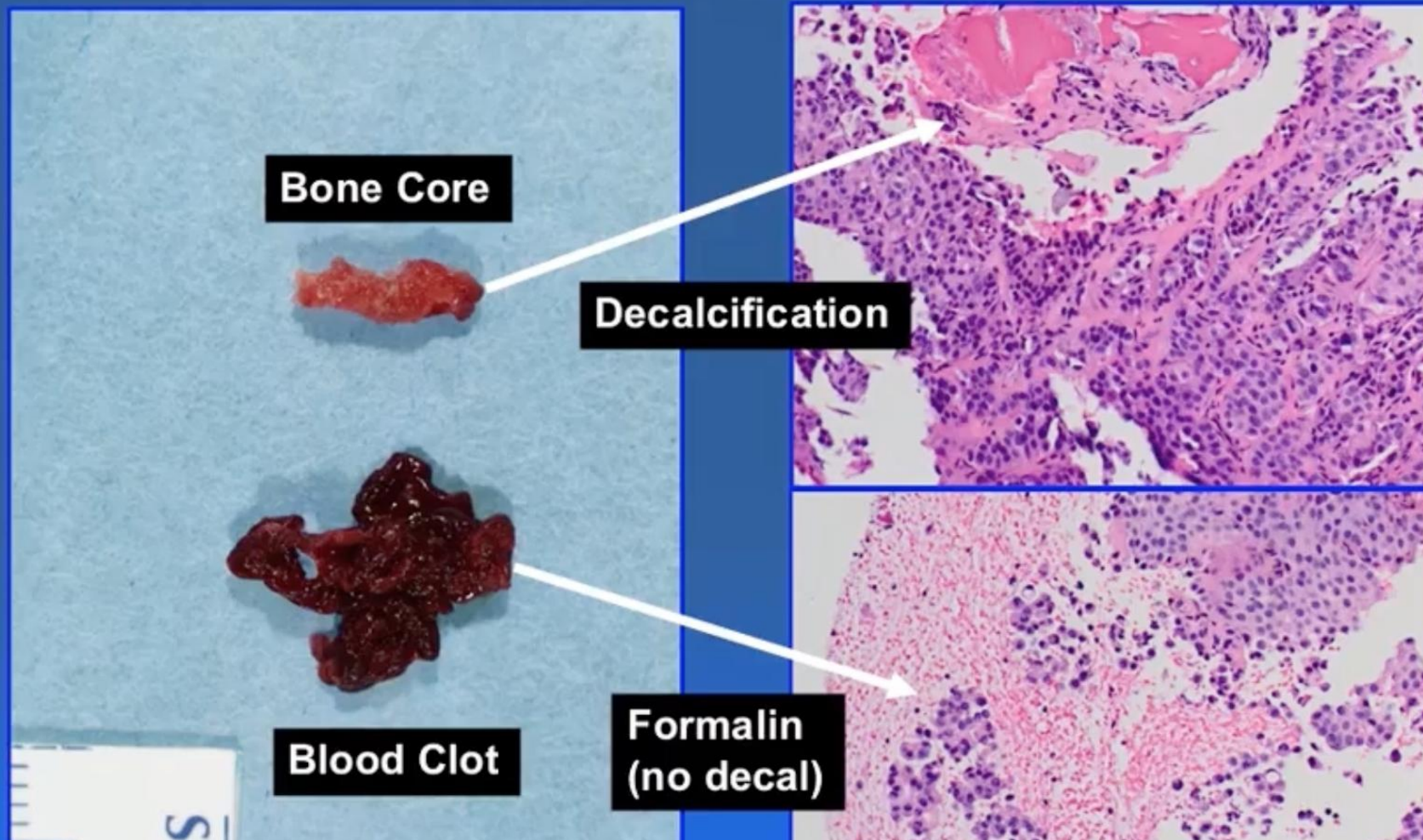
EDTA 96hrs



The Impact of Decalcification on Staining

Leica Biosystems

Core Biopsy of Bone for Suspected Breast Cancer



Courtesy of Lorelee McMahon

EDTA - slow and steady win the race



MODERN PATHOLOGY (2016) 29, 1460–1470
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Influence of decalcification procedures on immunohistochemistry and molecular pathology in breast cancer

Willemijne AME Schrijver¹, Petra van der Groep^{1,2,3}, Laurien DC Hoefnagel^{1,3}, Natalie D ter Hoeve¹, Ton Peeters¹, Cathy B Moelans¹ and Paul J van Diest¹

Human Pathology (2021) 117, 108–114



Human
PATHOLOGY

www.elsevier.com/locate/humpath

Original contribution

Effect of EDTA decalcification on estrogen receptor and progesterone receptor immunohistochemistry and HER2/neu fluorescence in situ hybridization in breast carcinoma[☆]

Erik Washburn MD*, Xiaoyu Tang MD, PhD¹, Carla Caruso MD², Michelle Walls, Bing Han MD, PhD



Modern Pathology (2020) 33:1505–1517
<https://doi.org/10.1038/s41379-020-0503-6>



ARTICLE

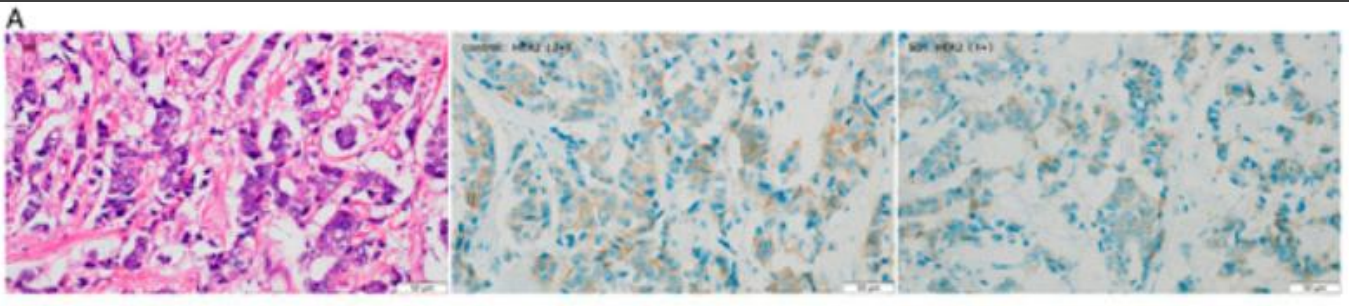
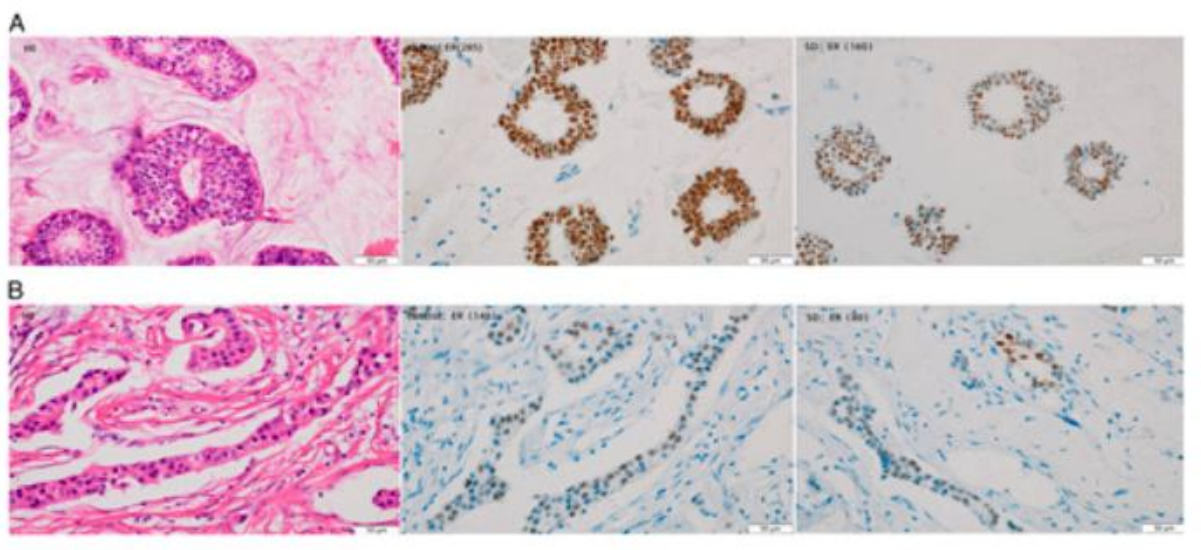
Effect of decalcification protocols on immunohistochemistry and molecular analyses of bone samples

Elodie Miquelstorena-Standley^{1,2} · Marie-Lise Jourdan³ · Christine Collin³ · Corinne Bouvier⁴ · Frédérique Larousserie⁵ · Sébastien Aubert⁶ · Anne Gomez-Brouchet⁷ · Jean-Marc Guinebretière⁸ · Matthias Tallegas^{1,2} · Bénédicte Brulin⁹ · Louis-Romée Le Nail^{2,9,10} · Anne Tallet³ · François Le Loarer¹¹ · Jessica Massiere¹¹ · Christine Galant¹² · Gonzague de Pinieux^{1,2,9}

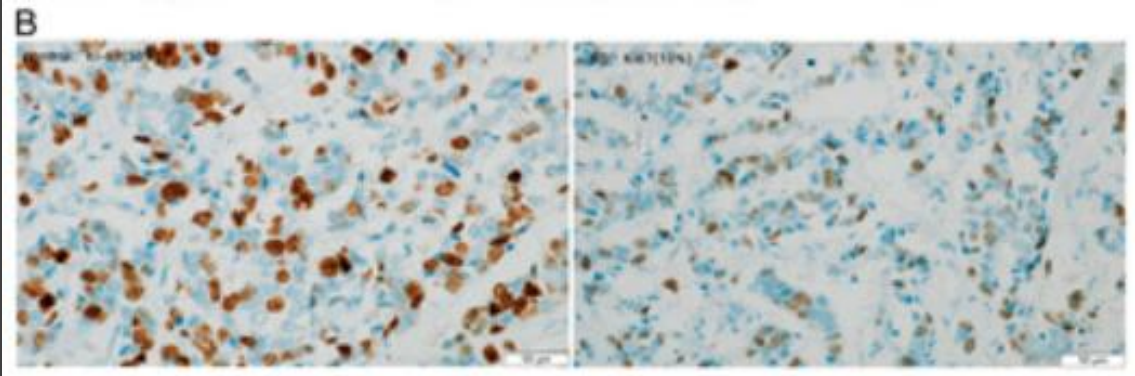
Longer incubation, but minimal negative effects on immunohistochemistry, molecular analysis (DNA/RNA) and CISH.

Effect of Surface Decalcification With Hydrochloric Acid on the Determination of Estrogen Receptor, Progesterone Receptor, Ki67, and Human Epidermal Growth Factor Receptor 2 Expressions in Invasive Breast Carcinoma Based on Immunohistochemistry and Fluorescence In Situ Hybridization

Wu Ping, MD,[†] Rao Xin, MD,^{‡§} Zhang Li, MD,[†] Chen Yupeng, MM,[†] Song Fangling, MM,[†] Ren Caihong, MD,[†] Hu Shun, MD,[†] and Zhang Sheng, MD^{✉†}



44 Breast metastasis to bone included



Ki67 decreased from 22% to 13% after HCL

ER – 9/31 cases decreased expression, but all still positive

Her2	0-1+	2+	3+
EDTA	23	12	8
HCL	27	8	8

4 cases no FISH

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—Vol 135, May 2011

Table 1. Potential Sources of Preanalytic Variation During Specimen Fixation and Processing

Prefixation	Dehydration and clearing
Duration and delay of temperature	Reagent
Specimen size	Temperature
Specimen manipulation (pathology ink)	No. of changes
	Duration (total and change-specific)
Fixative	Paraffin impregnation
Formula	Type and melting point of wax
Concentration	No. of changes
pH	Duration (total and change-specific)
Age of reagent	Method (immersion and sonication or microwave acceleration)
Preparation source	
Fixation	Paraffin sectioning
Tissue to fixative volume ratio	Type of blade and frequency of replacement
Method (immersion, injection, and sonication or microwave acceleration)	Frequency of servicing and wax replacement
Conditions of primary and secondary fixation	Temperature of block during sectioning
Movement	Slide pretreatment
Light exposure	Water bath conditions, if used
Primary container	Chemical adhesives, if used
No. and position of cofixed specimens	Temperature and duration of slide drying
Postfixation	Storage
Washing conditions and duration	Temperature and duration of paraffin block storage
Storage reagent and duration	Temperature, duration, and manipulation of slide-mounted tissue sections
Processing	
Type of processor, frequency of servicing and reagent replacement	
Tissue to reagent volume ratio	
No. and position of coprocessed specimens	

Decalcification:
Type, Time, Temperature

Paraffin sectioning

- Type of blade and frequency of replacement
- Frequency of servicing and wax replacement
- Temperature of block during sectioning
- Section thickness 3-5 μm
- Speed of cutting
- Tissue orientation
- Water bath conditions, if used
- Temperature and duration of slide drying



Water bath



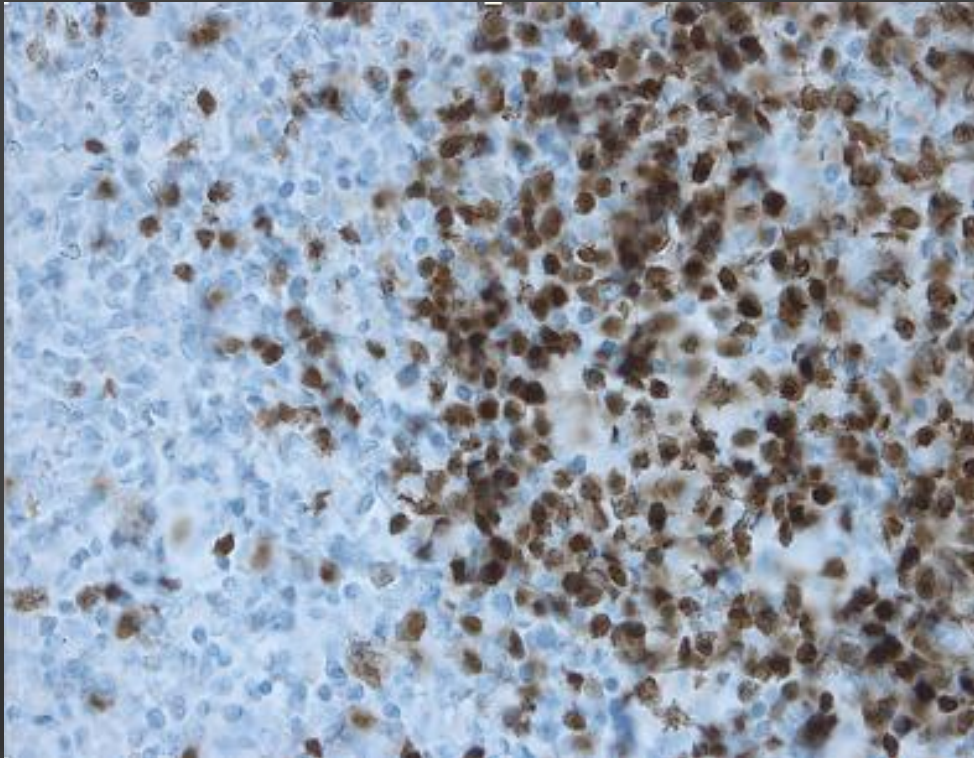
Flotation bath temperature is carefully checked. A temperature 4–9°C below the melting point of the wax is optimal. Sections should readily flatten but the wax should not melt.

Fatty tissues may need a lower temperature.

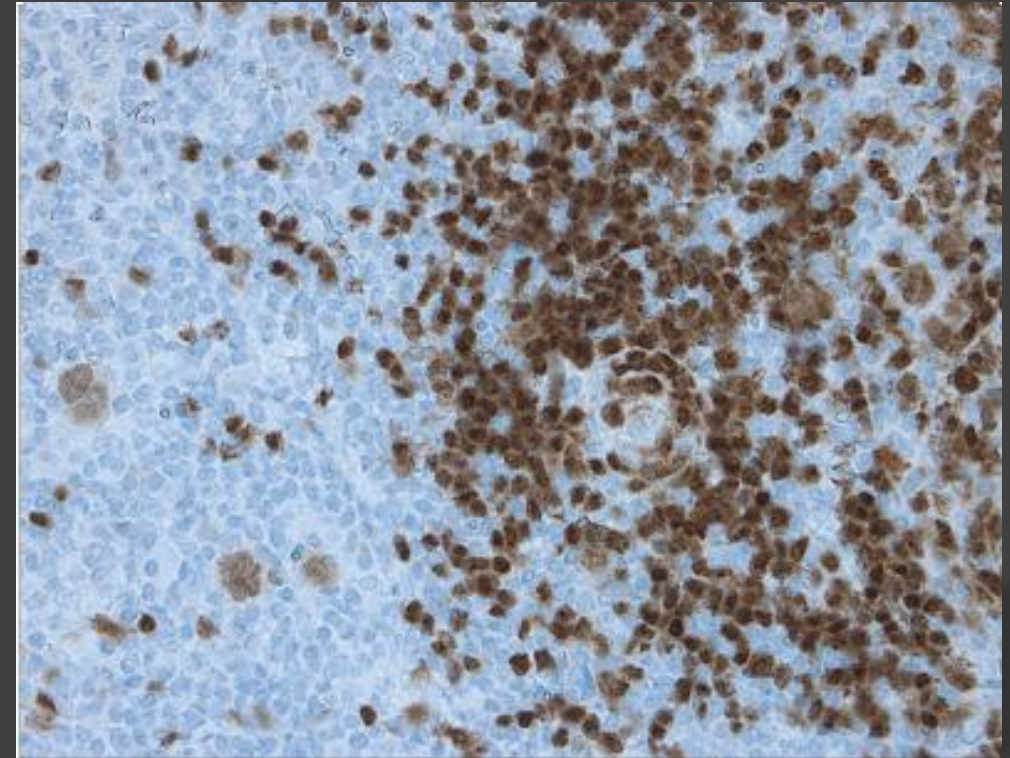


If sections are left on the flotation bath for more than 15 seconds, the wax melts. Although this may seem to make the process faster, it can rapidly cause over-expansion and tissue and cell damage.

Pax5 in HD



42 °C/5 sec



52 °C/10 sec

Sectioning – remember to stretch



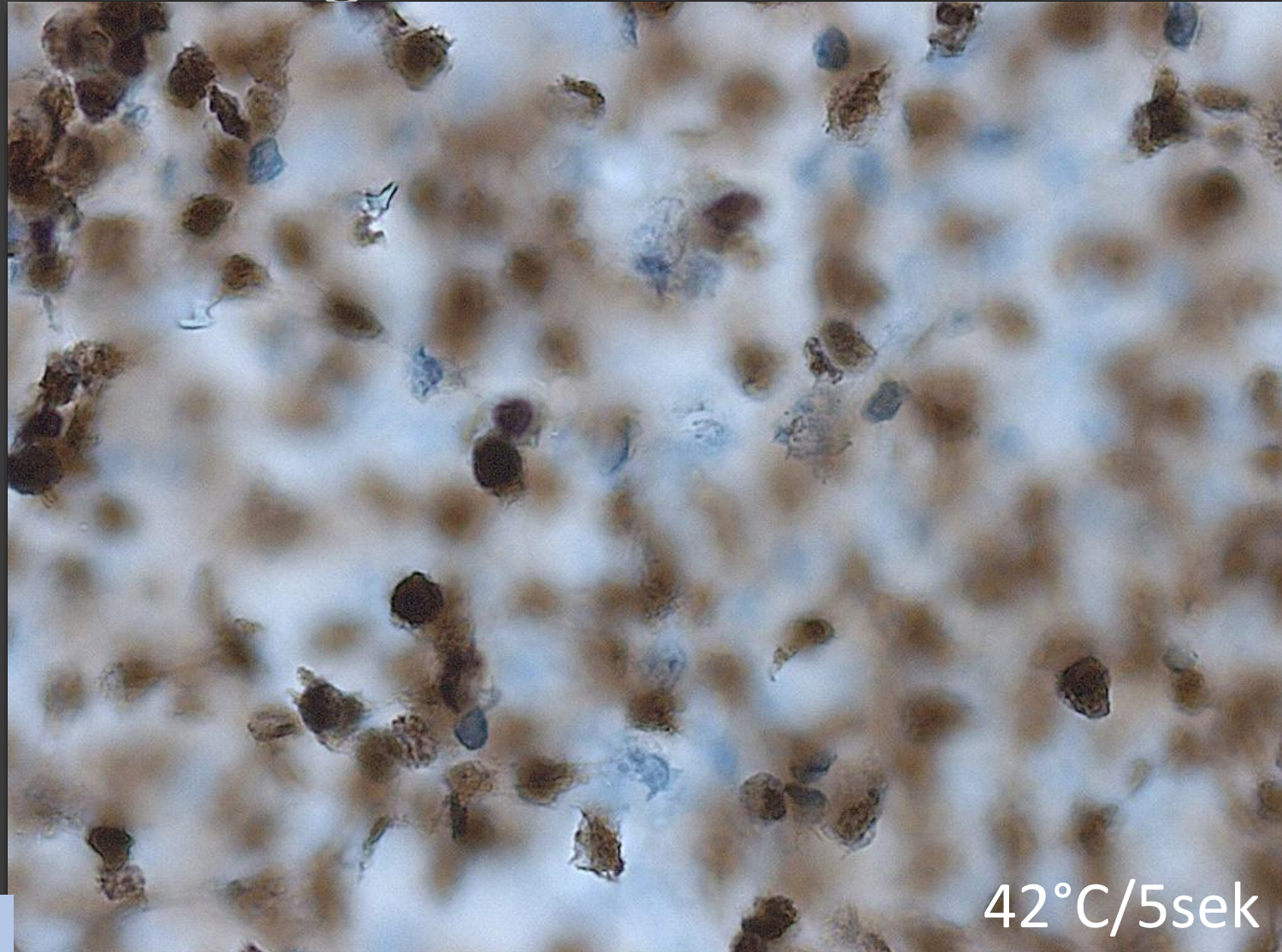
Sections



Slides

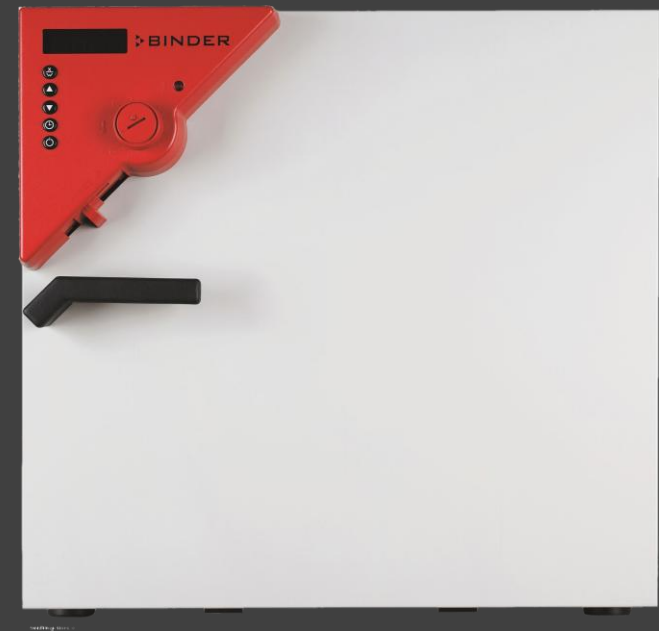
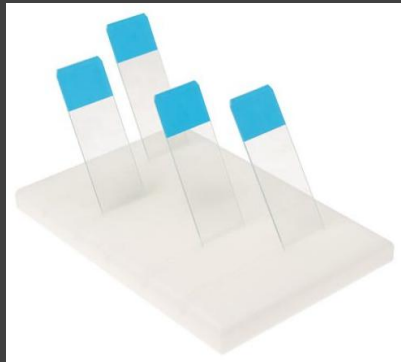


Pax5 in HD – is not ready for digital pathology
without stretching



Oven after cutting

- Because we have Omnis in our lab we never leave the slide on the rim of the water bath to stretch.
- We place it vertical immediately and insure as little as possible water is trapped under the tissue.
- Set to dry in oven with circulating heat at 40 °C/15 min
 - Different type of slides may need different conditions
- Baked in oven with circulating heat at 60 °C/45 min

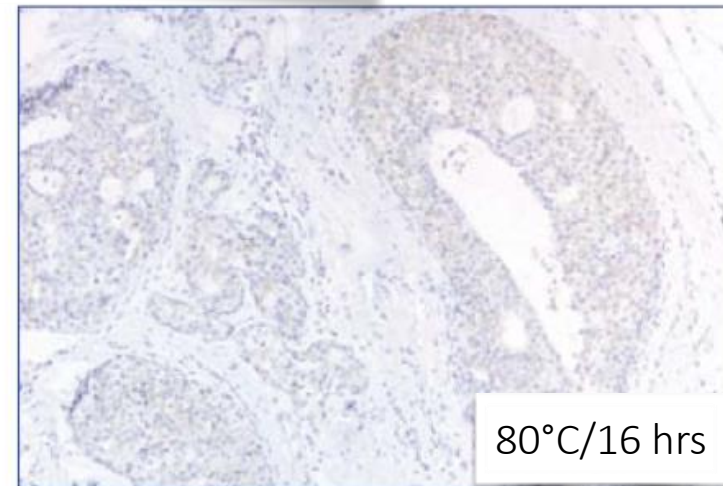
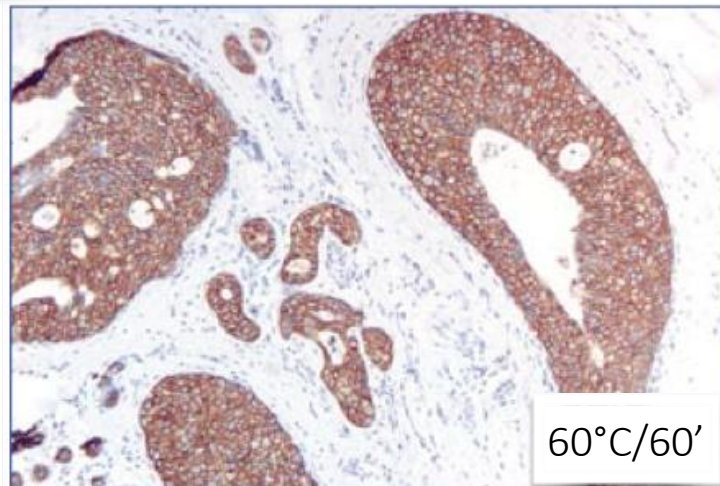


TECHNICAL ARTICLE

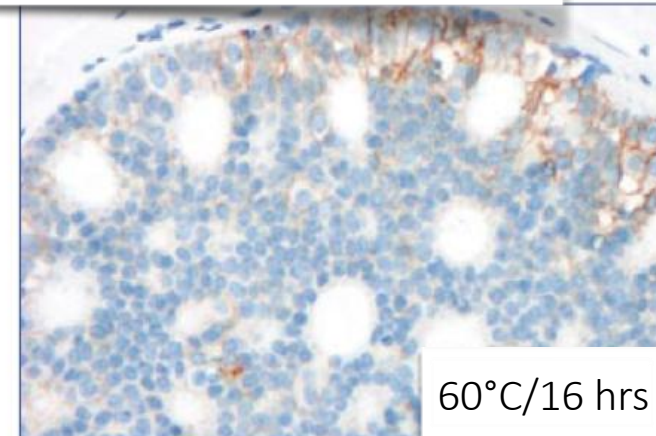
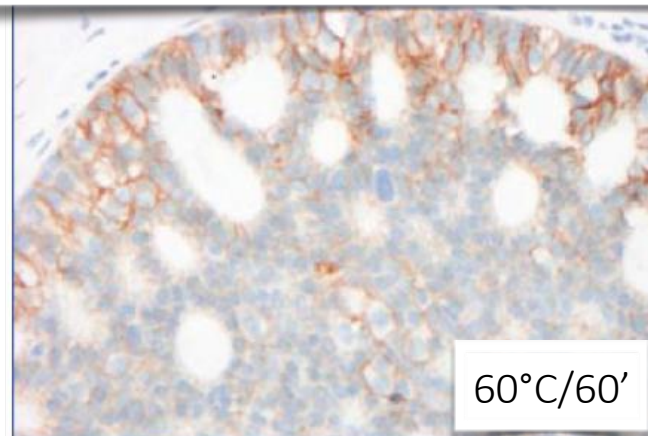
EXCESSIVE SECTION DRYING OF BREAST CANCER TISSUE PRIOR TO DEPARAFFINISATION AND ANTIGEN RETRIEVAL CAUSES A LOSS IN HER2-IMMUNO-REACTIVITY

Bent Lundgaard Hansen, Henrik Winther and Kristian Moller

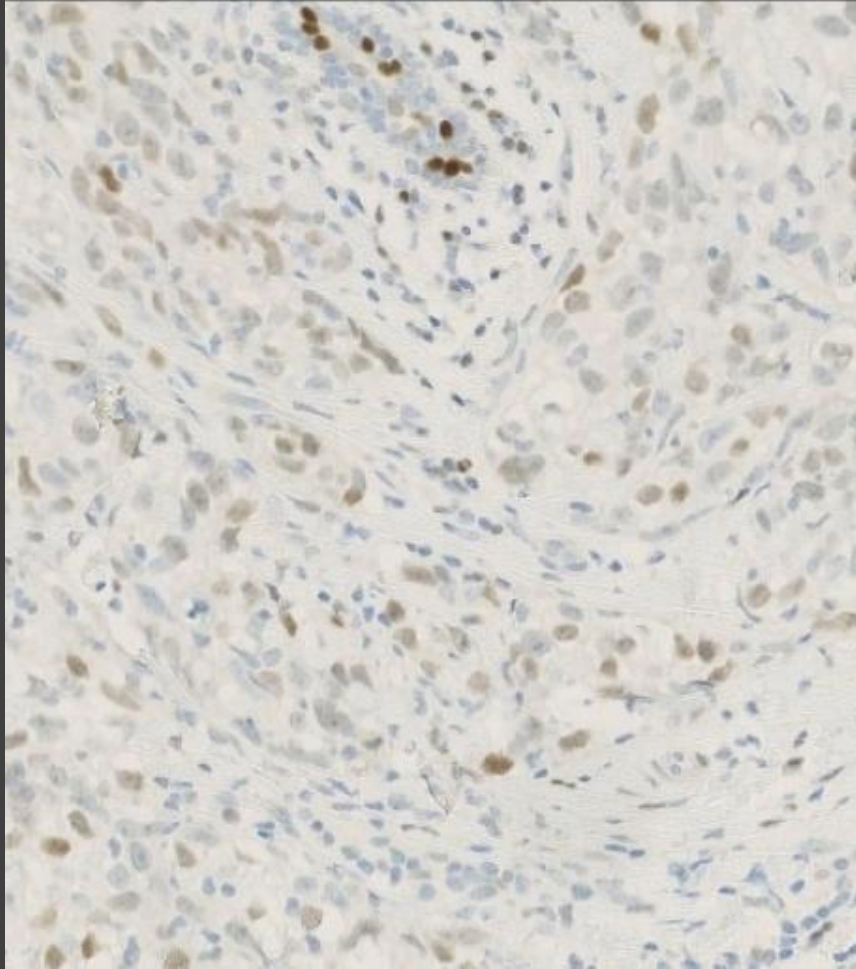
Dako A/S, DK-2600, Glostrup, Denmark



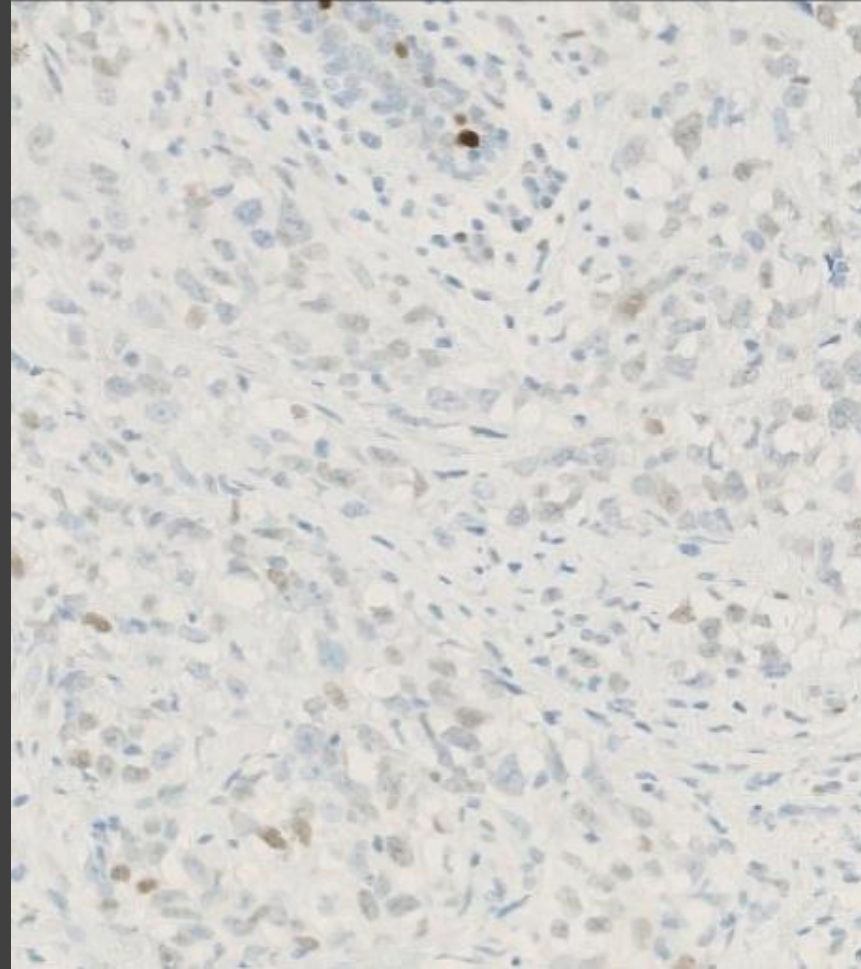
"Procedure for drying of tissue prior to deparaffinization: The drying temperature should be 60°C for a maximum of one hour, 37 °C for a maximum of 24 hours, or ambient temperature for 24 hours or longer".



Drying of sections - ER, SP1



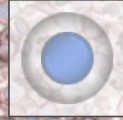
60 min at 60°C



16 hrs at 80°C

PD-L1 cell line from Histocyte, high

10x



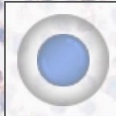
15 min at 40°C
45 min at 60°C

15 min at 40°C
18 hrs at 60°C

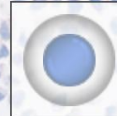
22c3.15.tør_45min.varme

PD-L1 cell line from Histocyte, low

10x



NordiQC



15 min at 40°C
45 min at 60°C

15 min at 40°C
18 hrs at 60°C

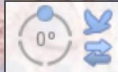
Placenta, normal tissue

15 min at 40°C
45 min at 60°C

15 min at 40°C
18 hrs at 60°C

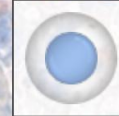
Patient sample, EBUS coagulum

20x



15 min at 40°C
45 min at 60°C

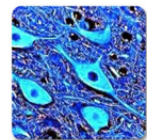
NordIQ



15 min at 40°C
18 hrs at 60°C

Drying of sections

Preanalytical variable	Published Guidelines and Recommendations	Literature-Based Recommendations	Aarhus University Hospital
Drying of sections	ASCO/CAP CLSI 24 hrs at RT or 1 hr at 50°C - 60°C	Engel KB, Moore HM. Arch Pathol Lab Med. 2011;135:537-543 24 hrs at RT or overnight at 37°C	15 min at 40 °C in circulating oven, then 45 min at 60°C



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Drying paraffin sections on hotplate inadvisable

Yang Guo, Yu Xiang and Zheng-Wei Yang*

“Taken together, we consider it inadvisable to dry paraffin sections (freshly floated onto slides from water bath) **on hotplate or at a horizontal position**. When the drying temperature is high, the section will be destructed and compressed; when the temperature is low, there will be no drying effect or that the section maybe deformed.”

Influence of slide aging on results of translational research studies using immunohistochemistry

Martina Mirlacher, Marlis Kasper, Martina Storz, Yvonne Knecht, Ursula Dürmüller, Ronald Simon, Michael J Mihatsch and Guido Sauter

Fresh sections (F) vs. sections stored at 4°C for 6 months (O)

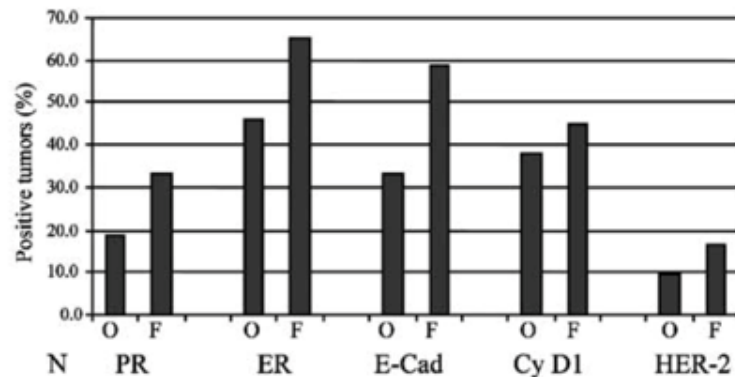
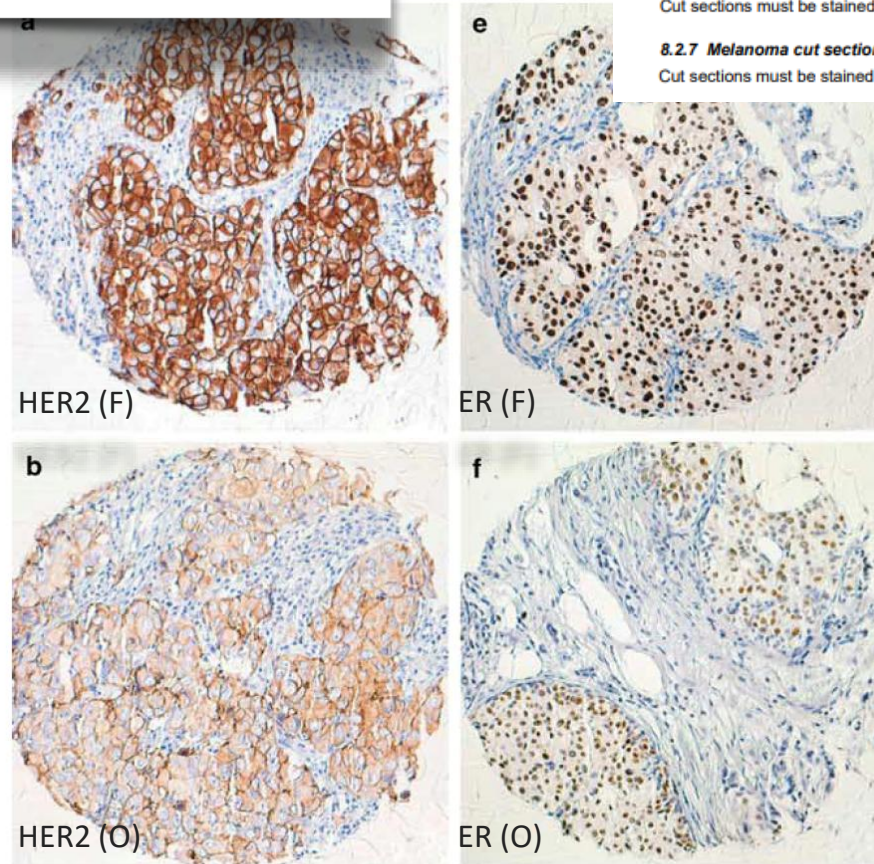


Figure 2 Influence of slide aging on the fraction of positive cases. For each antibody, the frequency of positive cases is shown as separate bars for old (O) and fresh (F) sections.



8.2 Cut section storage recommendation

To preserve antigenicity, tissue sections, once mounted on slides, should be held in the dark at 2-8 °C (preferred), or at room temperature up to 25 °C. Slide storage and handling conditions should not exceed 25 °C at any point post-mounting to ensure tissue integrity and antigenicity.

8.2.1 NSCLC cut section storage recommendation

Cut sections must be stained within 6 months when stored at 2-8 °C (preferred), or at 25 °C.

8.2.2 Urothelial carcinoma cut section storage recommendation

Cut sections must be stained within 6 months when stored at 2-8 °C (preferred), or at 25 °C.

8.2.3 Esophageal cancer cut section storage recommendation

Cut sections must be stained within 4.5 months when stored at 2-8 °C (preferred), or within 1 month when stored at 25 °C.

8.2.4 HNSCC cut section storage recommendation

Cut sections must be stained within 6 months when stored at 2-8 °C (preferred), or within 4 months when stored at 25 °C.

8.2.5 TNBC cut section storage recommendation

Cut sections must be stained within 7.5 months when stored at 2-8 °C (preferred), or within 4 months when stored at 25 °C.

8.2.6 Cervical cancer cut section storage recommendation

Cut sections must be stained within 2 months when stored at 2-8 °C (preferred), or within 1 month when stored at 25 °C.

8.2.7 Melanoma cut section storage recommendation

Cut sections must be stained within 4 months when stored at 2-8 °C (preferred), or within 2 months when stored at 25 °C.



Controls in storage

Negative factors influencing antigen preservation in cut sections

- Time
- Temperature
- Water amount in slide
- Moist / humidity in room
- Light

All with negative effects

Paraffin coating of single slides or
Paraplast sealing of boxes have not proven to be efficient



Storage time	Storage temp.
Days	Room temp.
Weeks	4°C
Months	-20°C
Years	-80°C
Cut sections, mount on charged slides and dry overnight or up to 48 hours and store in closed boxes without baking.	
Immediately before IHC bake 30-60 min at 60°C	

Is there an expiration date on the tissue blocks?

Loss of antigenicity with tissue age in breast cancer

Susan E Combs¹, Gang Han¹, Nikita Mani¹, Susan Beruti², Michael Nerenberg³ and David L Rimm¹

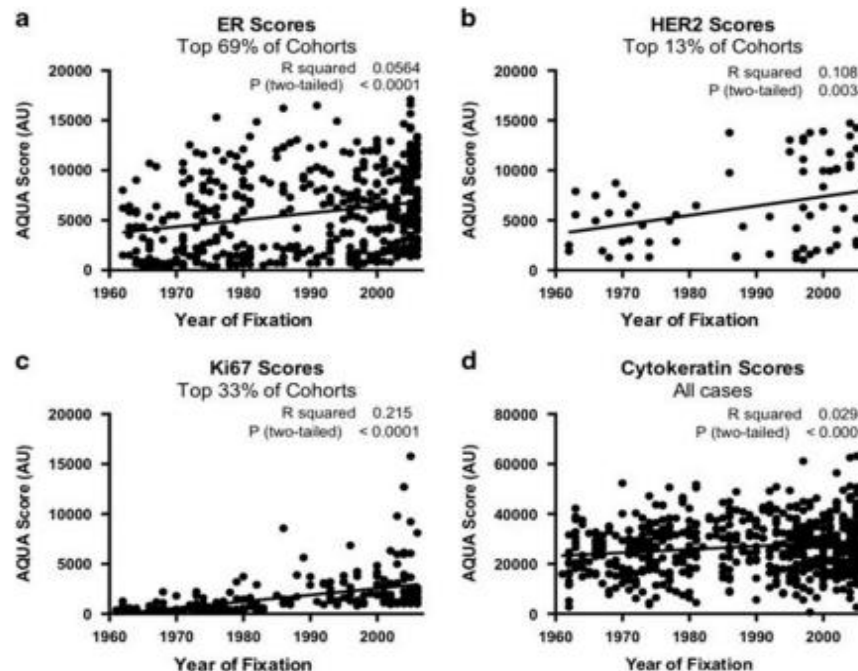


Figure 2 The distribution of scores for each biomarker as a function of tissue age after omitting the fraction of expected negative cases. (a) ER, (b) HER2, (c) Ki67 and (d) cytokeratin. The fraction of positive cases is shown by percentage beneath the biomarker in the title. The regression value and P-value are presented in the insets. Au, arbitrary unit.

The average signal decreased with preservation time for all biomarkers measured. For ER and **HER2**, there was an average of 10% signal loss after 9.9 years and **8.5 years**, respectively, compared with the most recent tissue. Detection of **Ki67** expression was lost more rapidly, with **10% signal loss in just 4.5 years**. Overall, these results demonstrate the need for adjustment of tissue age when studying FFPE biospecimens. **The rate of antigenicity loss is biomarker specific and should be considered as an important variable for studies using archived tissues.**

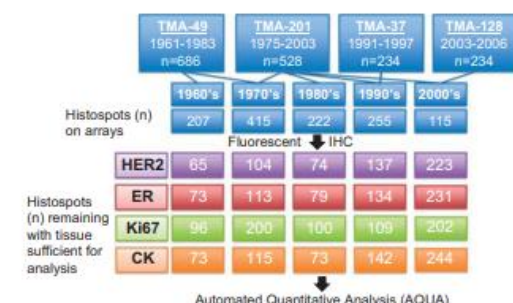


Figure 1 A consort diagram showing the cohorts from which the tissues were derived and the date ranges for each followed by the number of cases analyzed for each biomarker. IHC, immunohistochemistry; TMA, tissue microarray.

Correlation between PD-L1 expression and clinicopathological characteristics of non-small cell lung cancer: A real-world study of a large Chinese cohort

J Thorac Dis 2019;11(11):4591-4601

Yan Jin^{1,2}, Xuxia Shen^{1,2}, Yunjian Pan^{2,3}, Qiang Zheng^{1,2}, Haiquan Chen^{2,3}, Hong Hu^{2,3*}, Yuan Li^{1,2*}

The surgical resection group consisted of 827 recently resected and 329 archived (>5 years old) NSCLC samples

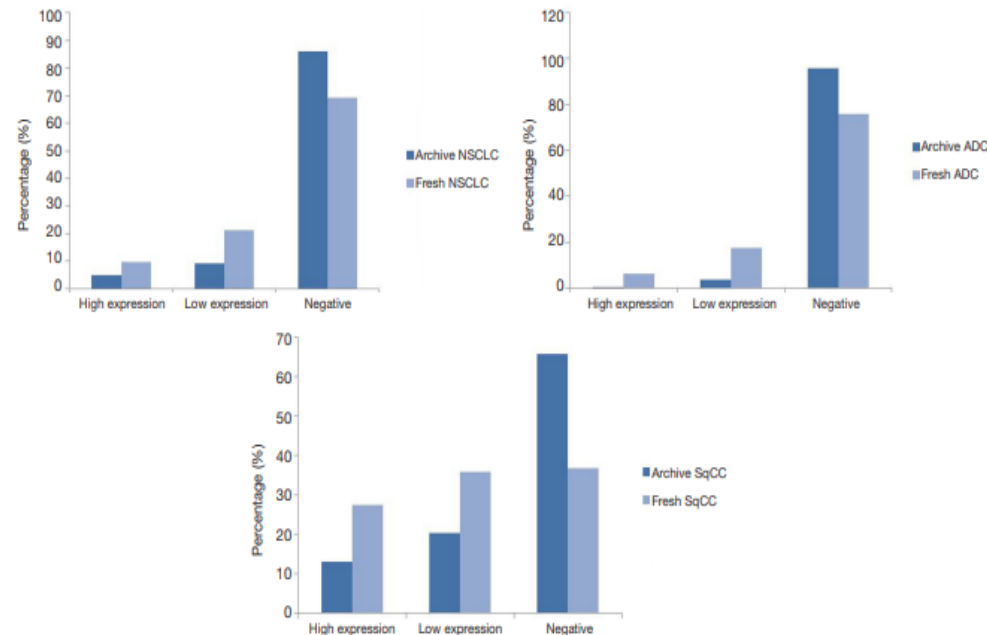


Figure 2 Comparison of PD-L1 expression in recently acquired samples and archived NSCLC samples. PD-L1, programmed death ligand-1; NSCLC, non-small cell lung cancer; ADC, adenocarcinoma; SqCC, squamous cell carcinoma.

PD-L1 high expression was observed in 9.7% of 827 NSCLC patients, including 6.5% with adenocarcinoma (ADC, n=690), and 27.4% with squamous cell carcinoma (SqCC, n=117). These results showed higher expression rates than those in archived samples (>5 years old, n=329).

Need to secure IHC testing quality – Guidelines pre-analytics

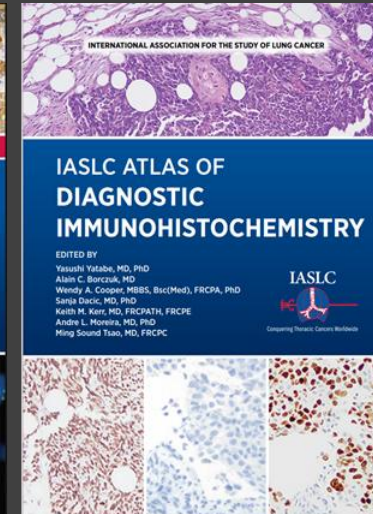
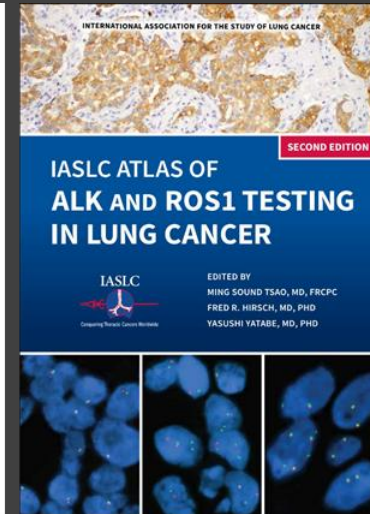
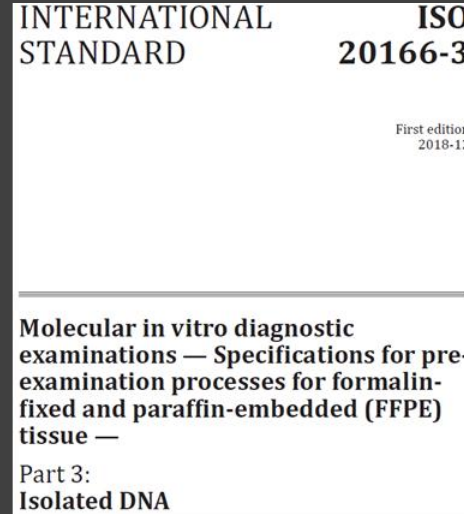
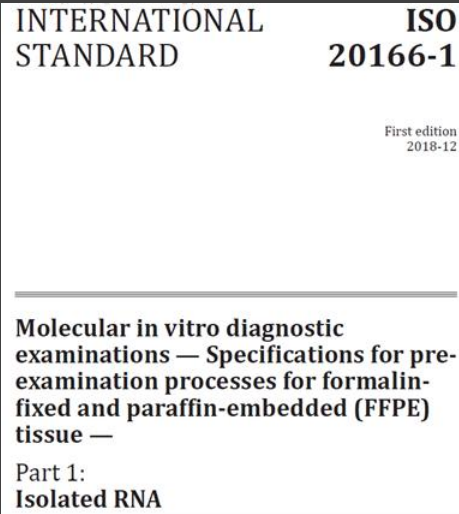


Table 2. Guidelines for core pre-analytical procedures for tissue from international and national authorities

Pre-analytical step	ASCO/CAP*	IASLC**	ISO/TC 212***
Biomolecule/method	ER-, PR-, HER2-IHC	PD-L1-IHC	Isolated DNA, RNA
Ischemic time	60 min. or less.	30 min. or less	Avoid or as short as possible
Type of fixative	10% NBF	10% NBF	10% NBF
Time in fixative	6-72 hours	6-48 hours	12-24 hours
Tissue thickness/fixative ratio	5 mm/-	-/10:1	5 mm/4-10:1
Storage time/temp. for slides	6 weeks at RT#	8 weeks at RT#	Avoid/short at 2-8°C
Storage time/temp. for blocks	-	3 years/2-8°C or RT#	/2-8°C or RT#

* American Society of Clinical Oncology (ASCO) and the College of American Pathologists (CAP), ** International Association for the Study of Lung Cancer (IASLC), *** European Committee for Standardization, ISO 20166, # Room temperature



That was
Preatalytical
– important?

Special thanks to the wizards of Immunohistochemistry

- Ole Nielsen, DK
- Michael Bzorek, DK
- Søren Nielsen, DK

what are other
words for
extremely important?



life or death, life-and-death,
life and death,
earth-shattering, earth-shaking,
vitally important

