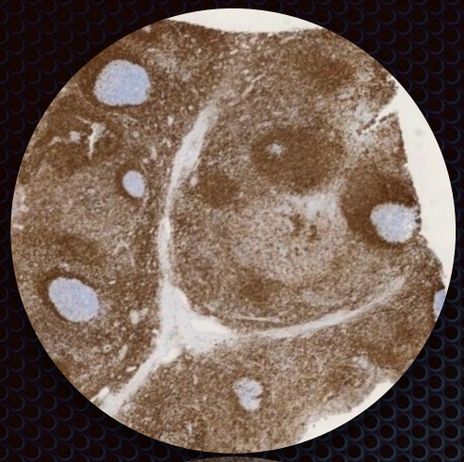


Immunhistochemical principles

The technical test approach
Pre-analytical phase (I-II)

NQC Workshop 2018

Ole Nielsen,
Dept. of Pathology
Odense University Hospital

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

Preanalytic

Prefixation
Fixative
Fixation
Postfixation
Processing
Dehydration and clearing
Paraffin impregnation
Paraffin sectioning
Storage

Analytic

Epitope retrieval
Blocking
Primary Antibody
Dilutent
Detection system
Chromogen
Counter stain
Mounting

Interpretive

Design of controls
Positive controls
Negative controls
Interpretation
Critical Stain Indicator

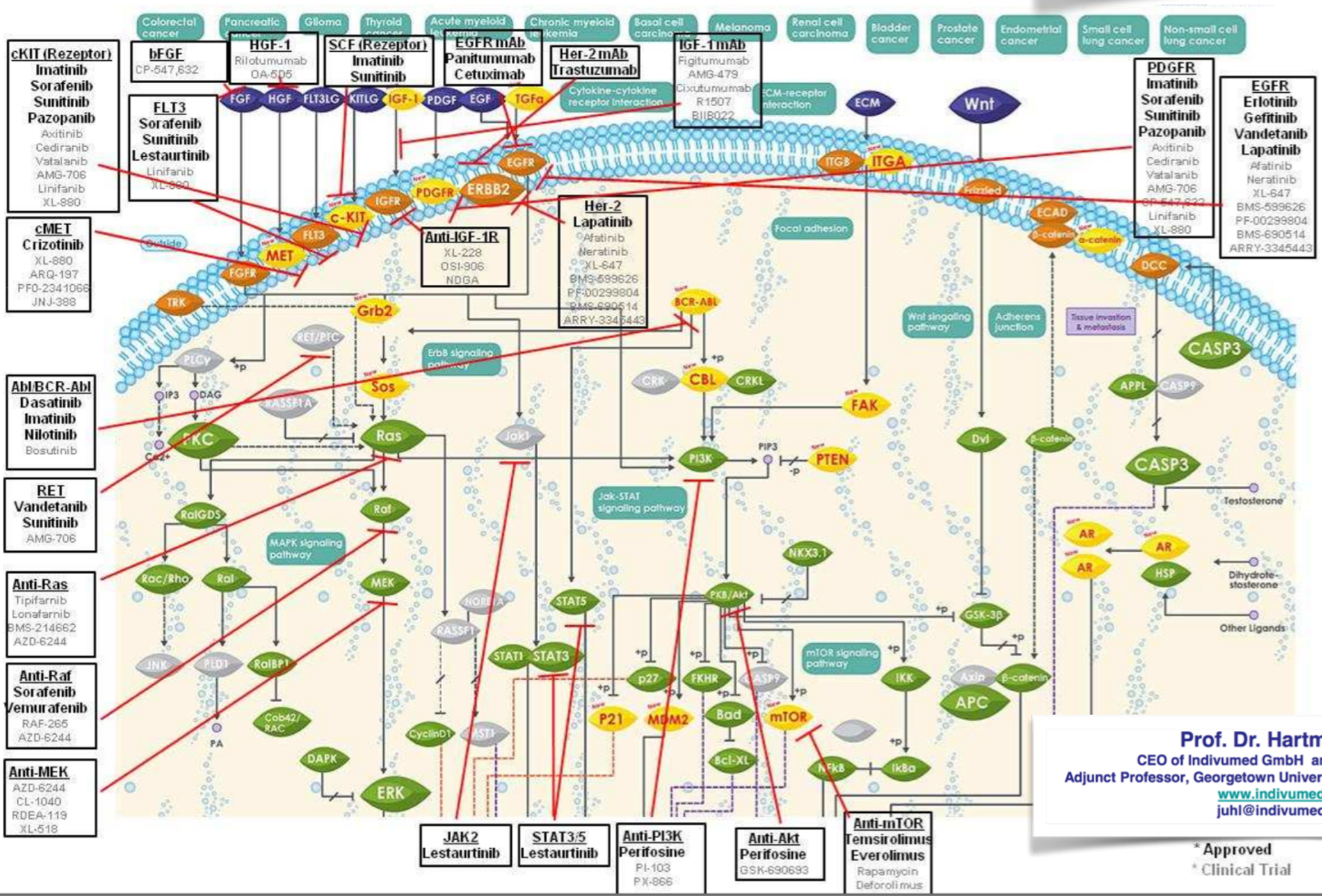
indivumed

NordiQC

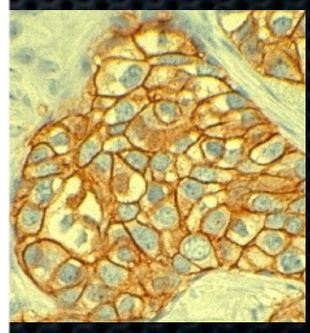
Ca. 60 „Targeted Therapies“ are Approved and >800 Compounds are in Clinical Trial

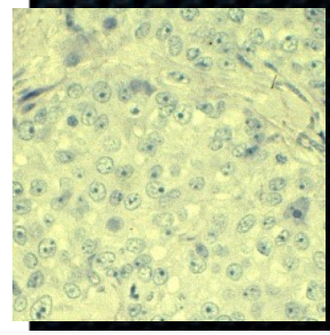
(Status 2012/01)

Some require IHC-based Companion Diagnostics!



HER2

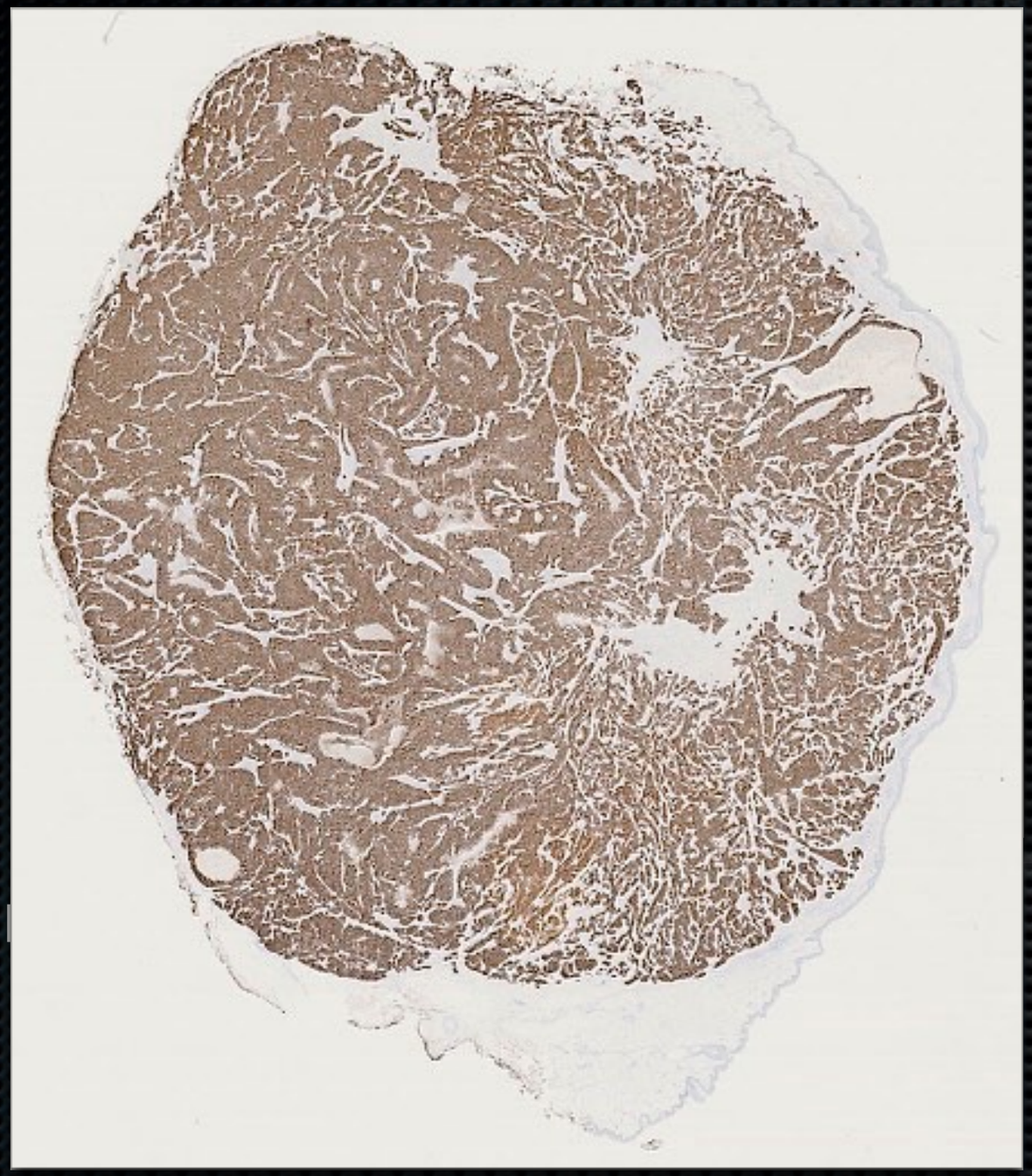




Prof. Dr. Hartmut Juhl
CEO of Indivumed GmbH and Inostics GmbH
Adjunct Professor, Georgetown University and Hamburg University
www.indivumed.com
juhl@indivumed.com

* Approved
* Clinical Trial

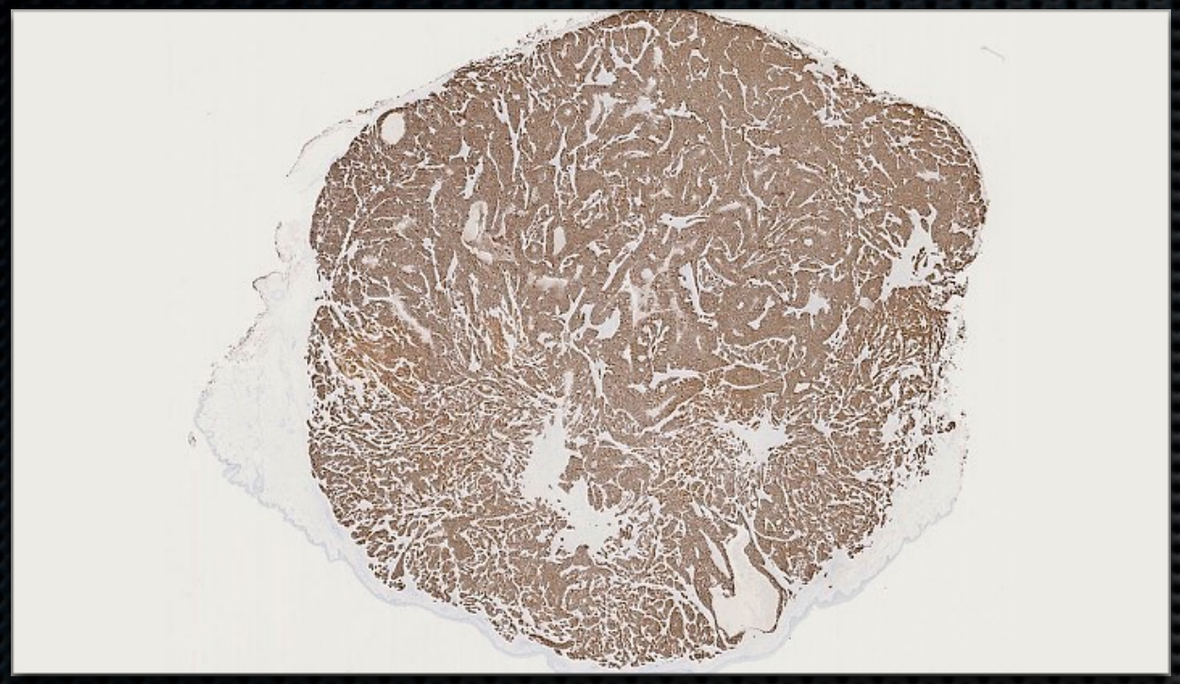
Thyroid carcinoma: Biology or artefact?



TTF-1, SPT24

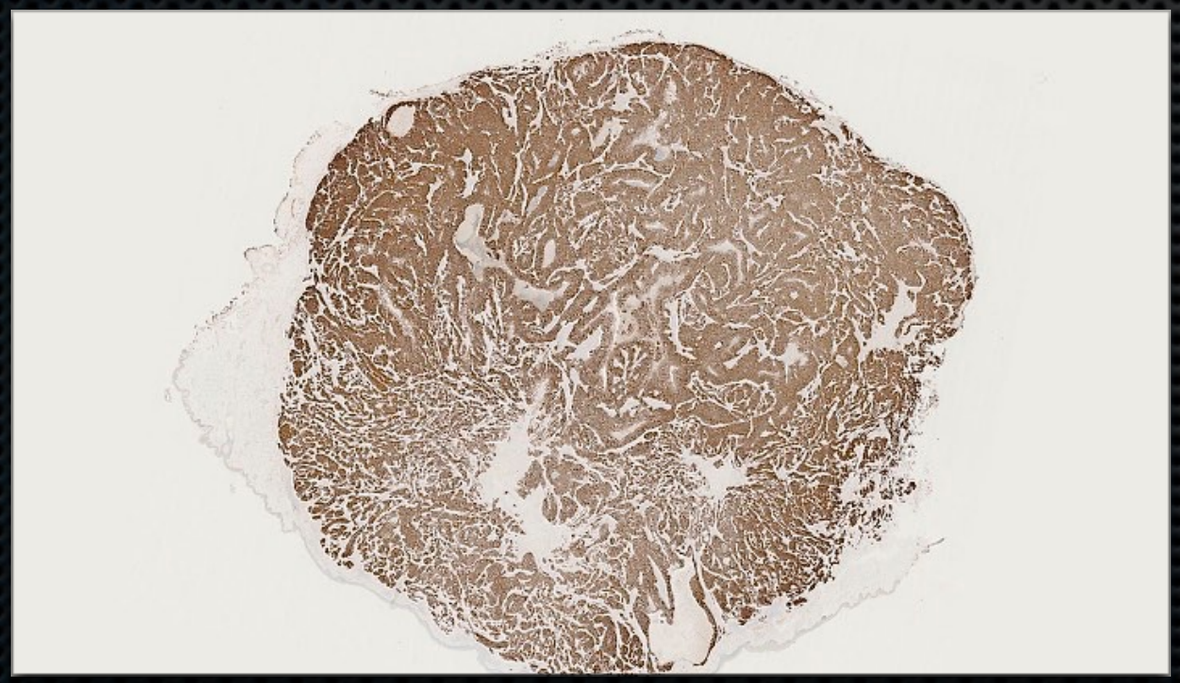
CD138, B-A38

Thyroid carcinoma: Biology or artefact?



TTF-1, SPT24

CD138, B-A38



PAX-8, ZR-1

PAX-8, pAb (CM363A)

A tissue quality index: an intrinsic control for measurement of effects of preanalytical variables on FFPE tissue

Veronique M Neumeister[1], Fabio Parisi[1], Allison M England[1], Summar Siddiqui[1], Valsamo Anagnostou[1], Elizabeth Zarrella[1], Maria Vassilakopolou[1], Yalai Bai[1], Sasha Saylor[1], Anna Sapino[2], Yuval Kluger[1,2], David G Hicks[3], Gianni Bussolati[2], Stephanie Kwei[4] and David L Rimm[1]

Laboratory Investigation (2014) 94, 467–474

If we cannot control pre-analytical variables can we quantify the damage or tissue degradation caused by them?

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

Decalcification:
Type, Time, Temperature

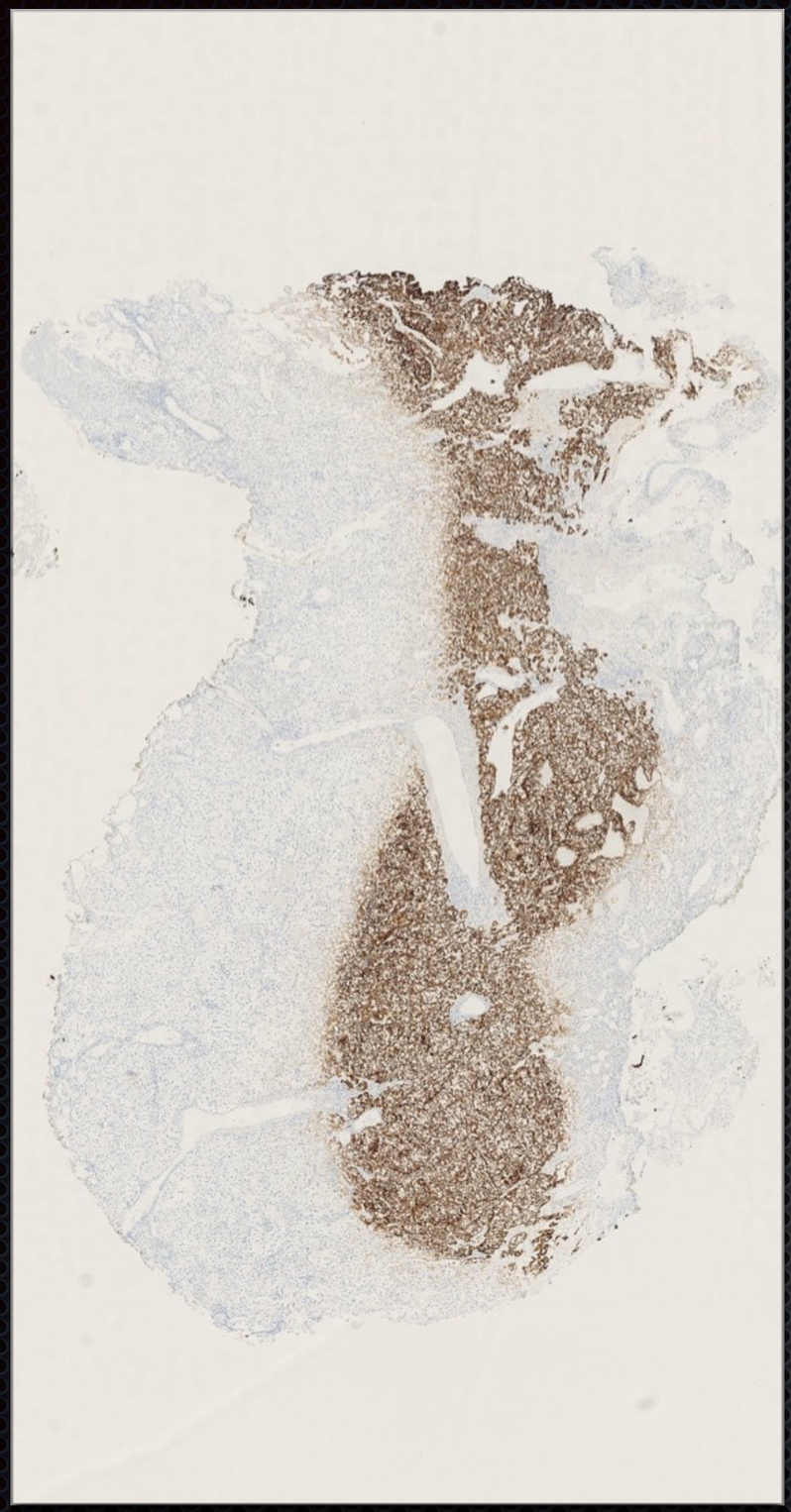
Prefixation

- Surgical procedures
- Fixation delay / ischemia (time and temperature)
- Specimen size
- Specimen manipulation (pathology ink)

Surgical procedures - Impact on IHC

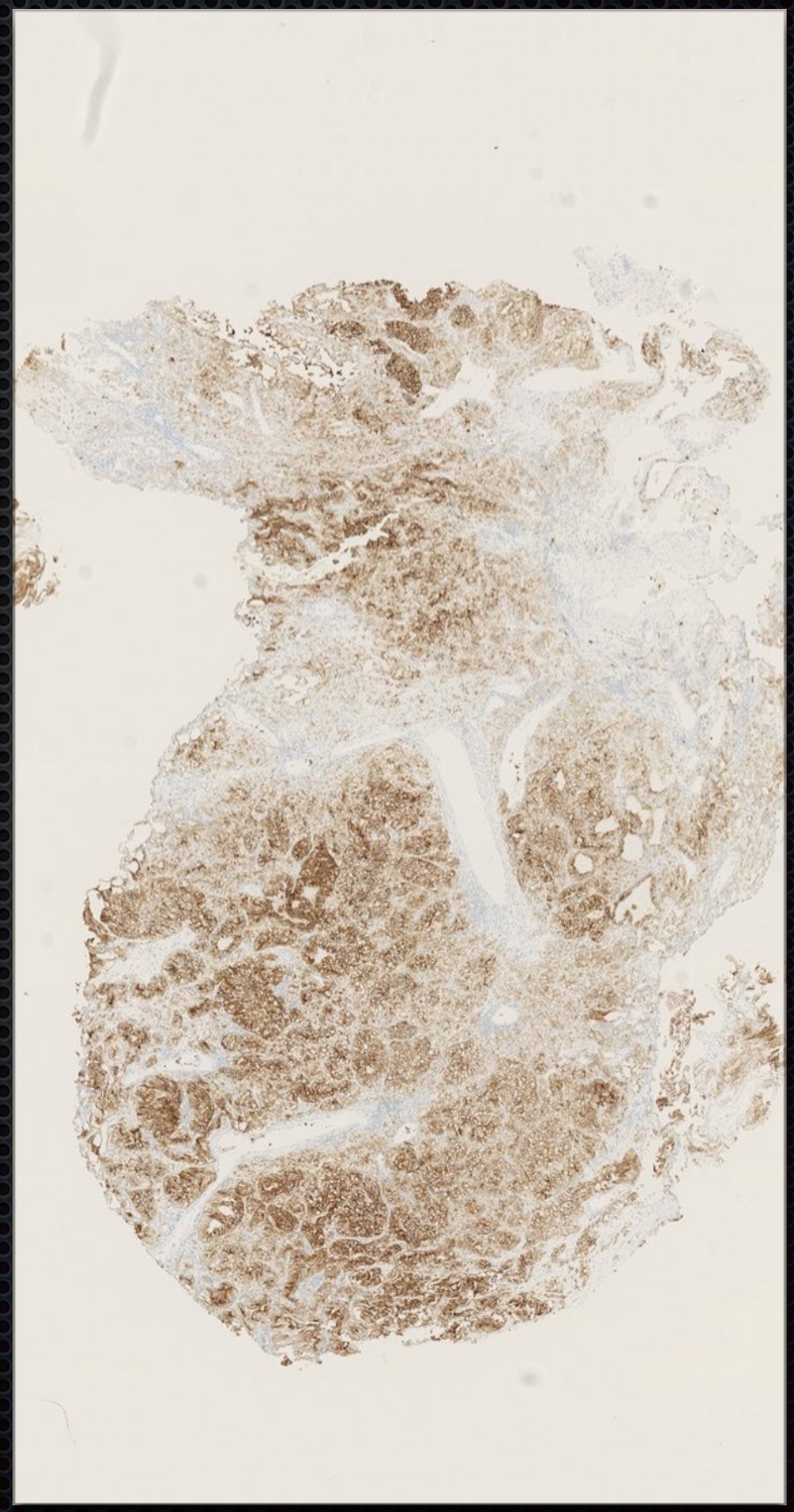
RCC

NordiQC



CK7/8, CAM5.2

CK8, EP17



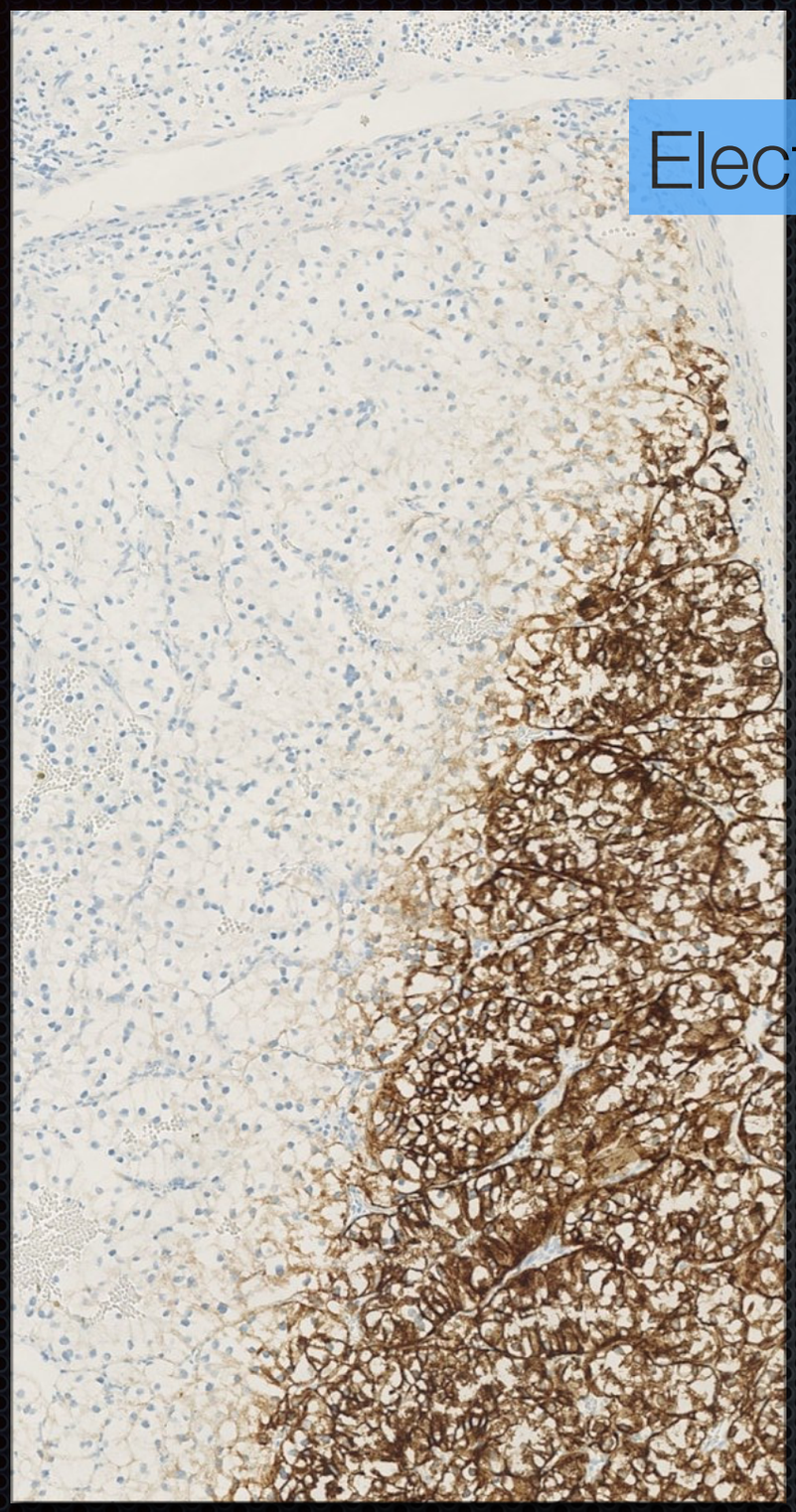
CD10, 56C6

Surgical procedures - Impact on IHC

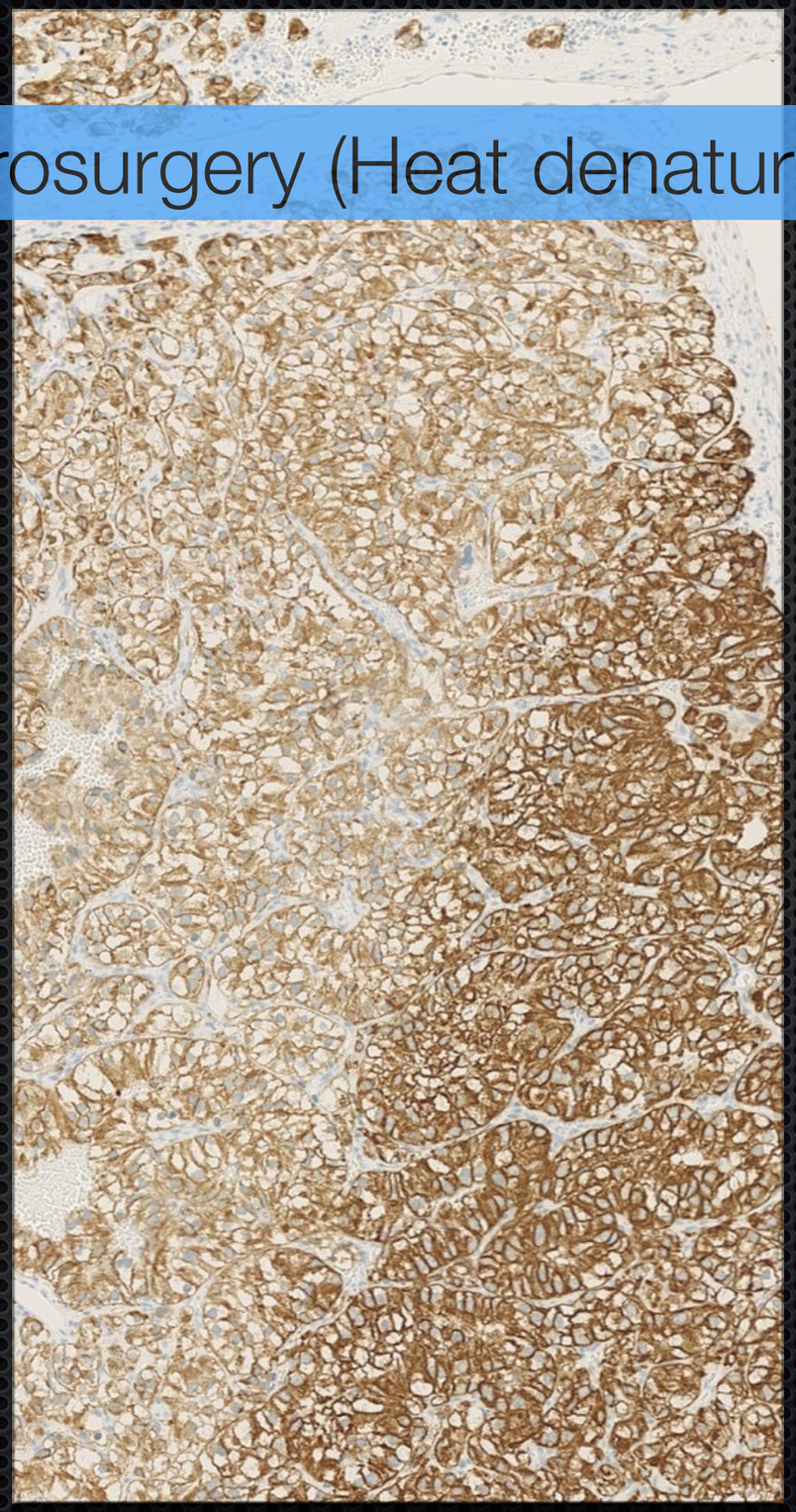
RCC

NordiQC

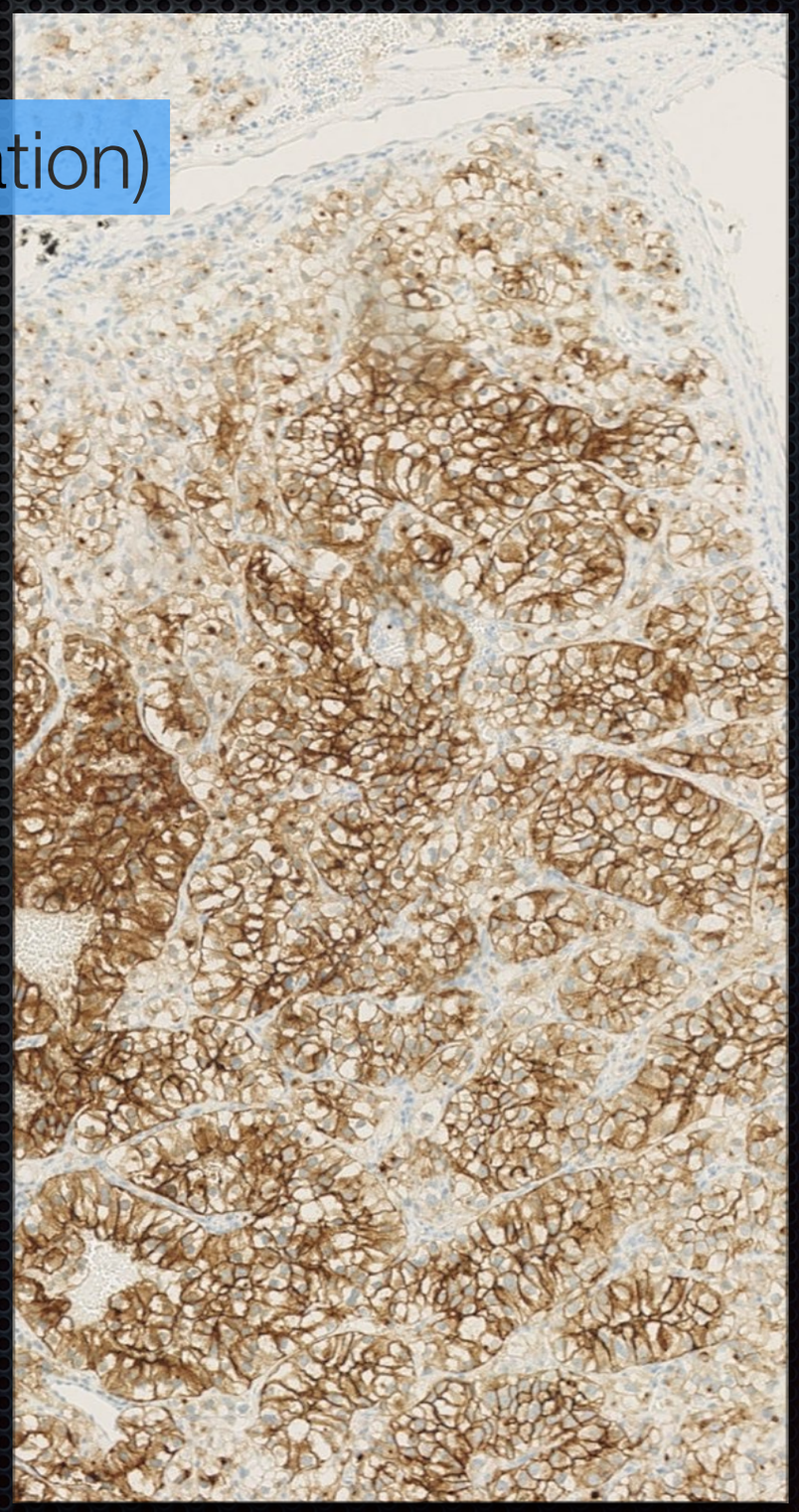
Electrosurgery (Heat denaturation)



CK7/8, CAM5.2

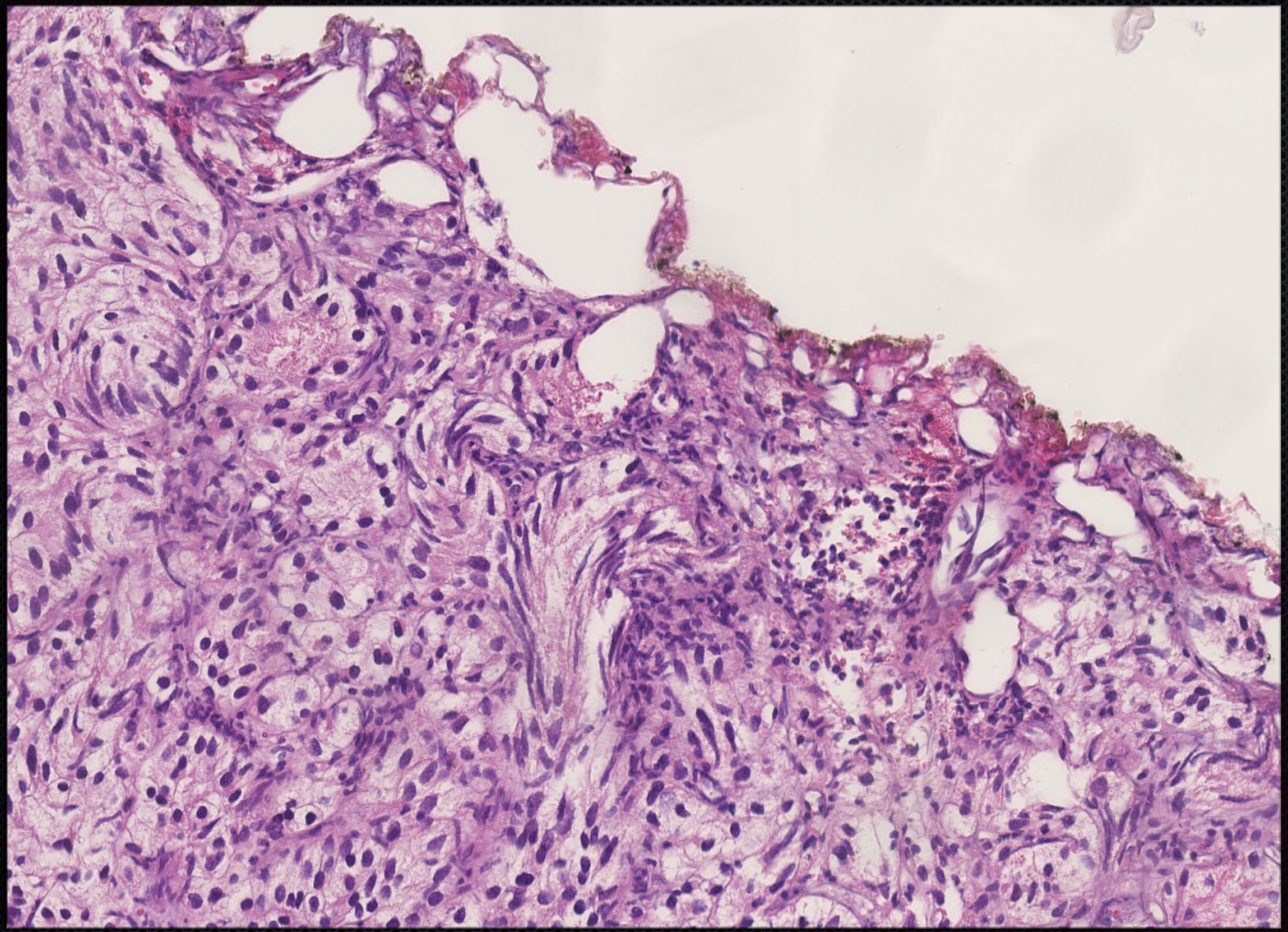


CK8, EP17

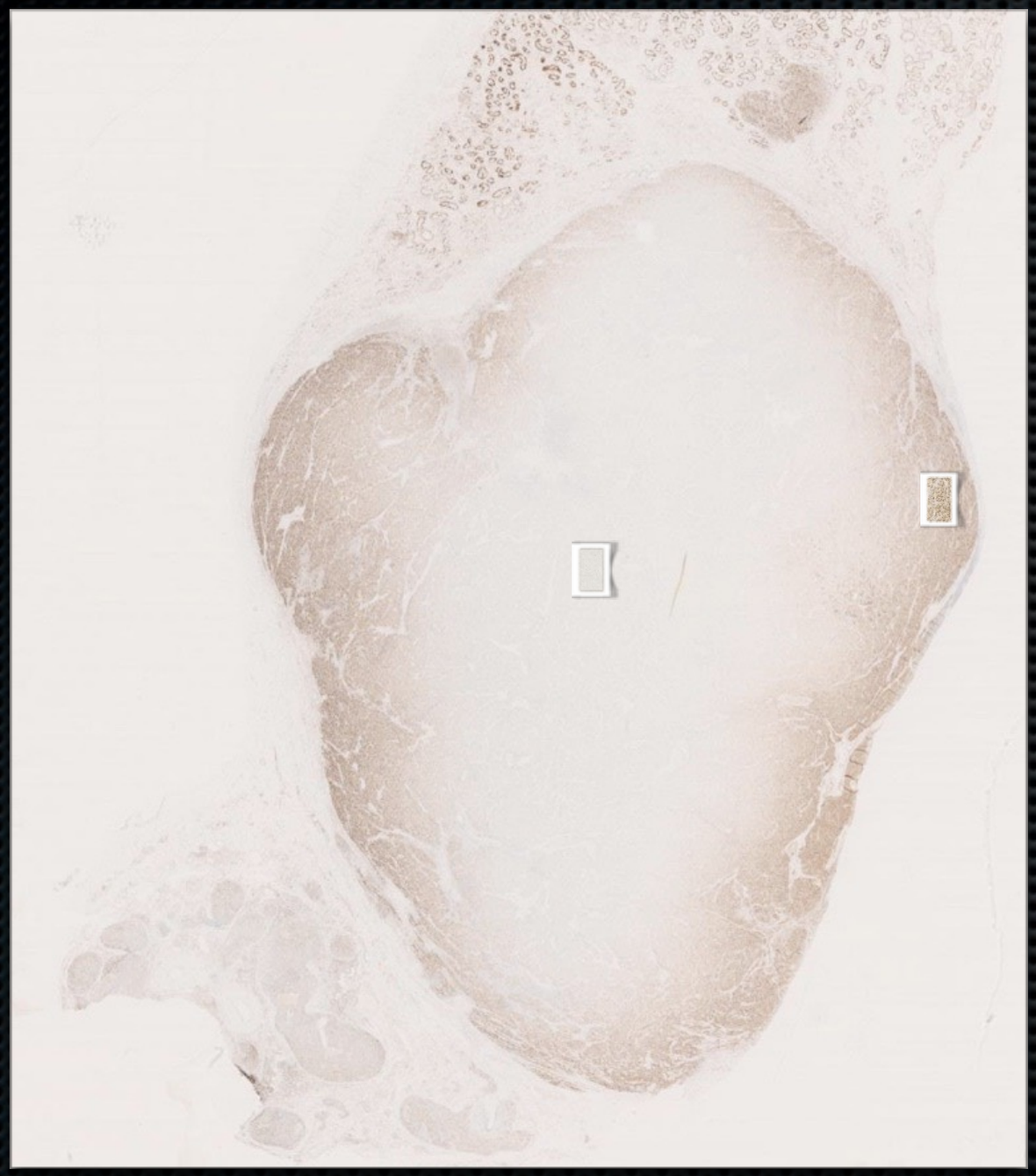


CD10, 56C6

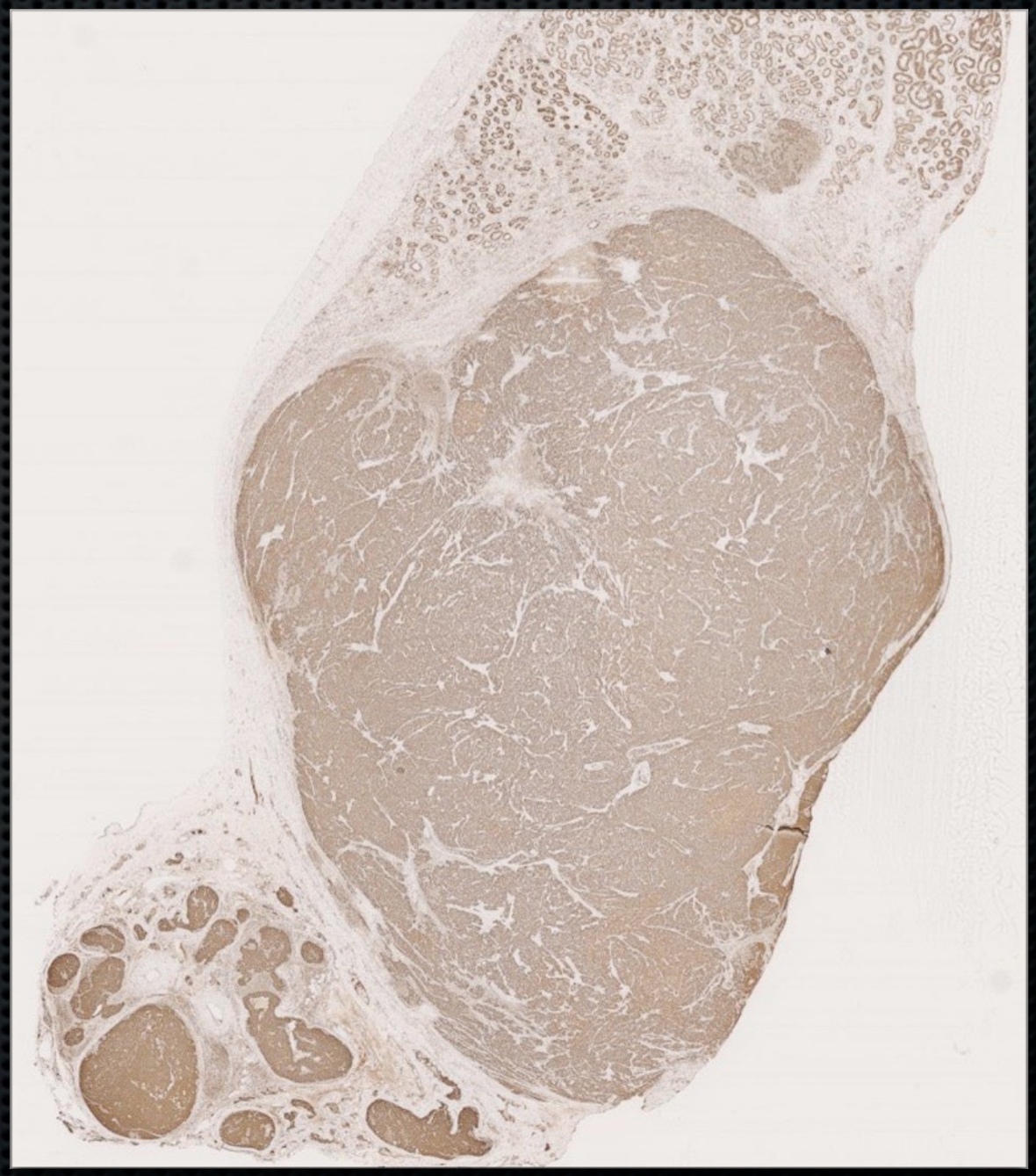
“Electrosurgery” (Heat) RCC



Seminoma: Biology or Artefact?



PMS2, EPR3947



MSH6, EP49

Seminoma: Biology or Artefact?

Delay of fixation?

Fixation procedure?

PMS2, EPR3947

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

A Study of Three Different Clones

Am J Clin Pathol 2010;134:813-819

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, MD[1]

Figure 1 Mean Q score decline for estro
clones 1D5 (DAKO), 6F11 (Leica), and SP
relation to time of fixation.

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

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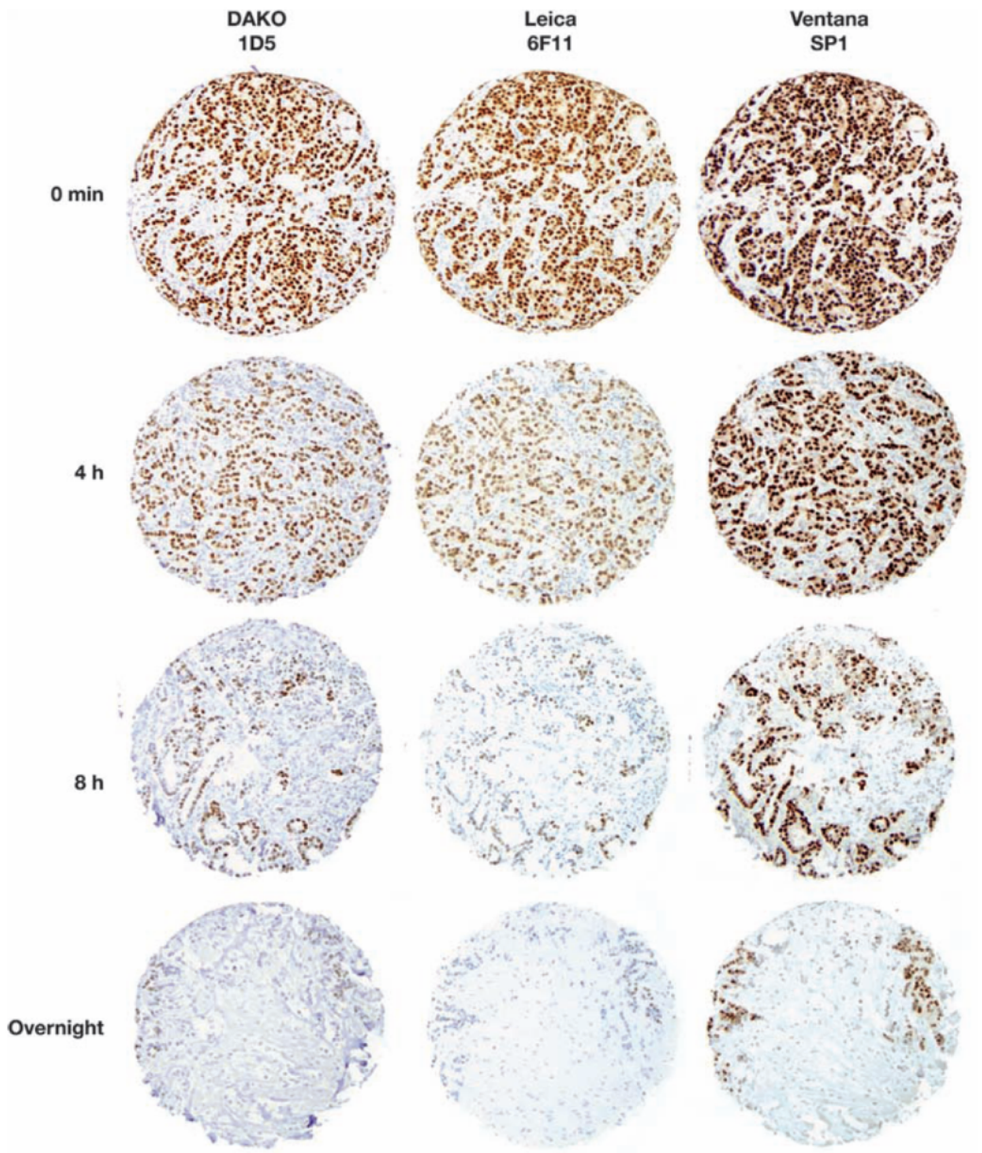


Image 1 (Case 9) Estrogen receptor expression by clones 1D5 (DAKO), 6F11 (Leica), and SP1 (Ventana) at different delayed formalin fixation times (0 minutes, 4 and 8 hours, and overnight). Note the decreased number/percentage of positive cells and the intensity of the stain with increased time of delayed fixation.

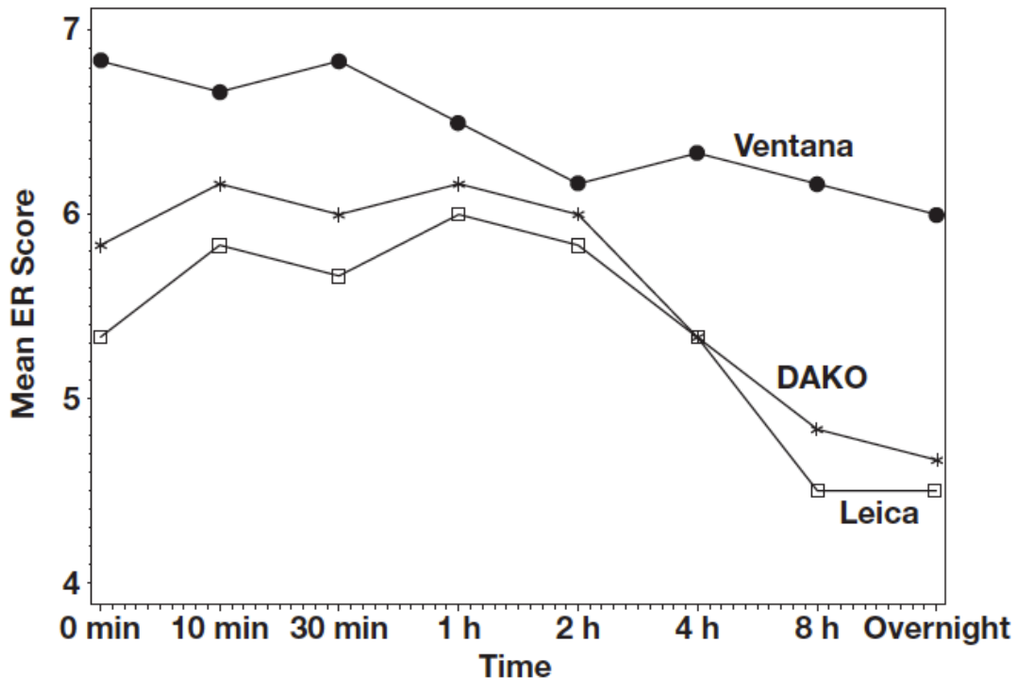


Figure 1 Mean Q score decline for estrogen receptor by clones 1D5 (DAKO), 6F11 (Leica), and SP1 (Ventana) in relation to time of fixation.

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

A Study of Three Different Clones

Am J Clin Pathol 2010;134:813-819

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, MD[1]

Based on our findings, it appears that regardless of the antibody clones evaluated, delayed formalin fixation has a negative effect on hormone receptors.

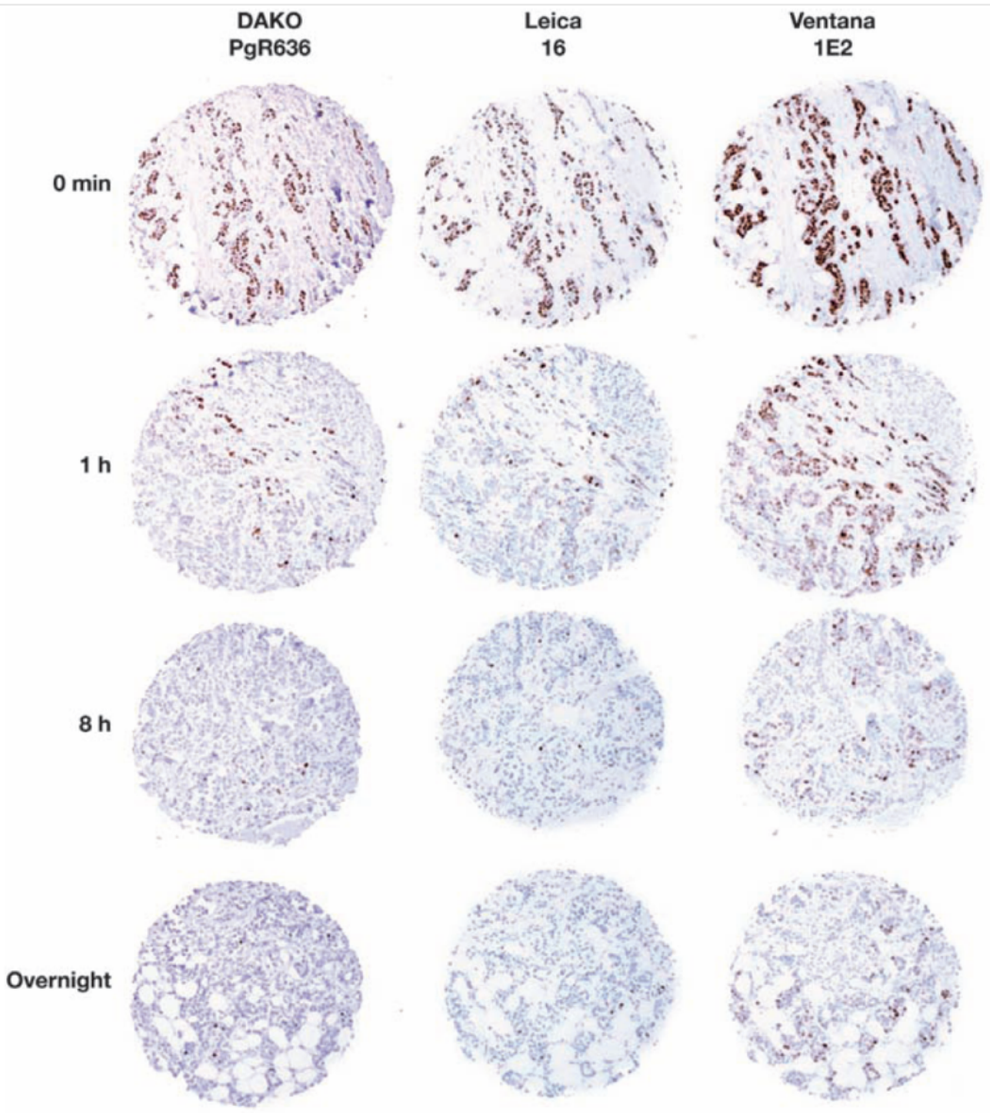


Image 3 (Case 8) Progesterone receptor expression by clones PgR636 (DAKO), 16 (Leica), and 1E (Ventana) at different delayed formalin fixation times (0 minutes, 1 and 8 hours, and overnight).

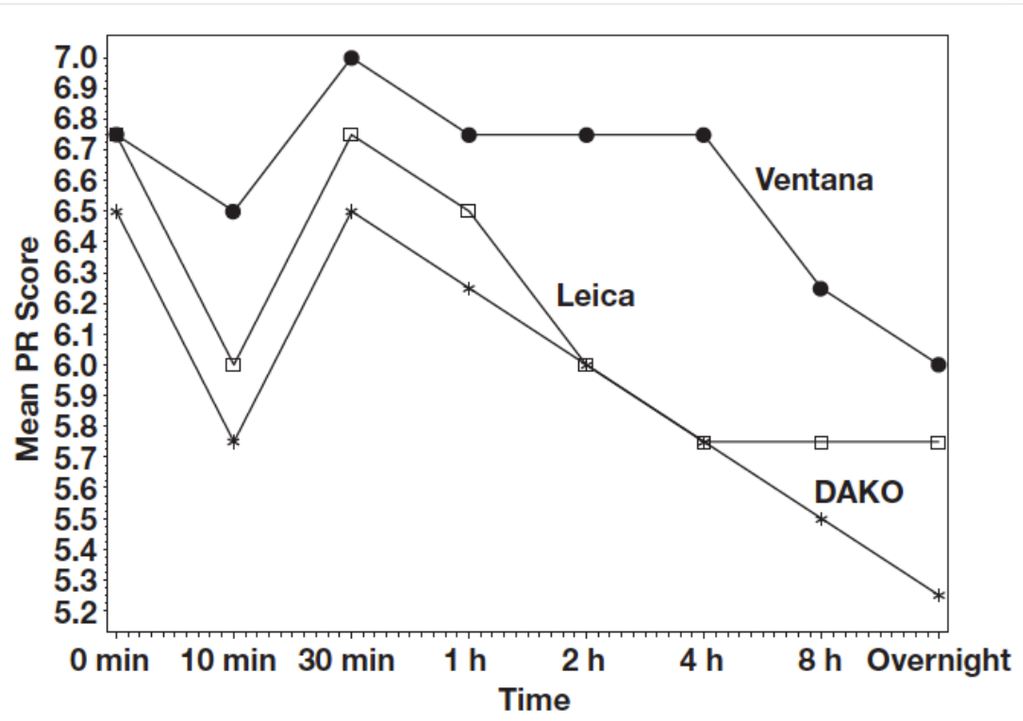
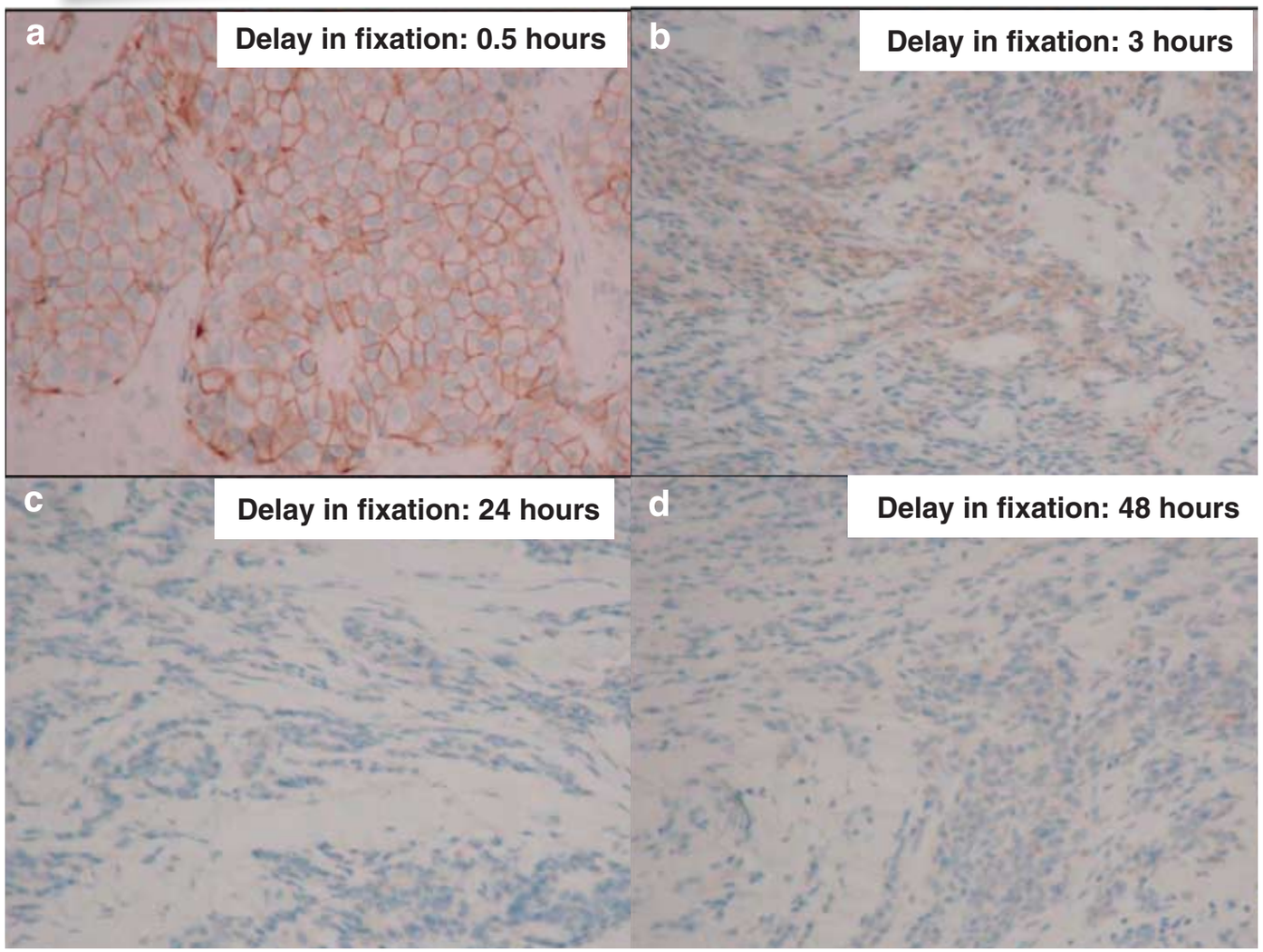


Figure 2 Mean Q score decline for progesterone receptor (clones PgR636, 16 and 1E2) in relation to time of fixation. Values were not statistically significant by the Page L test.

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

MODERN PATHOLOGY (2012) 25, 1098–1105



"Non-refrigerated samples are affected more by prolonged cold ischemic time than refrigerated samples. Cold ischemic time period of as short as one-half hour may occasionally impact the immunohistochemical (IHC) staining for progesterone receptor. Significant reduction in IHC staining for hormone receptors, and HER2, however, generally does not result until 4 h for refrigerated samples and 2 h for non-refrigerated samples. The ASCO/CAP guideline of cold ischemic time period of 1 h is a prudent guideline to follow".

Quantitative assessment shows loss of antigenic epitopes as a function of pre-analytic variables

Yalai Bai[1], Juliana Tolles[2], Huan Cheng[1], Summar Siddiqui[1], Arun Gopinath[1], Eirini Pectasides[1], Robert L Camp[1], David L Rimm[1] and Annette M Molinaro[2]

Laboratory Investigation (2011) 91, 1253–1261

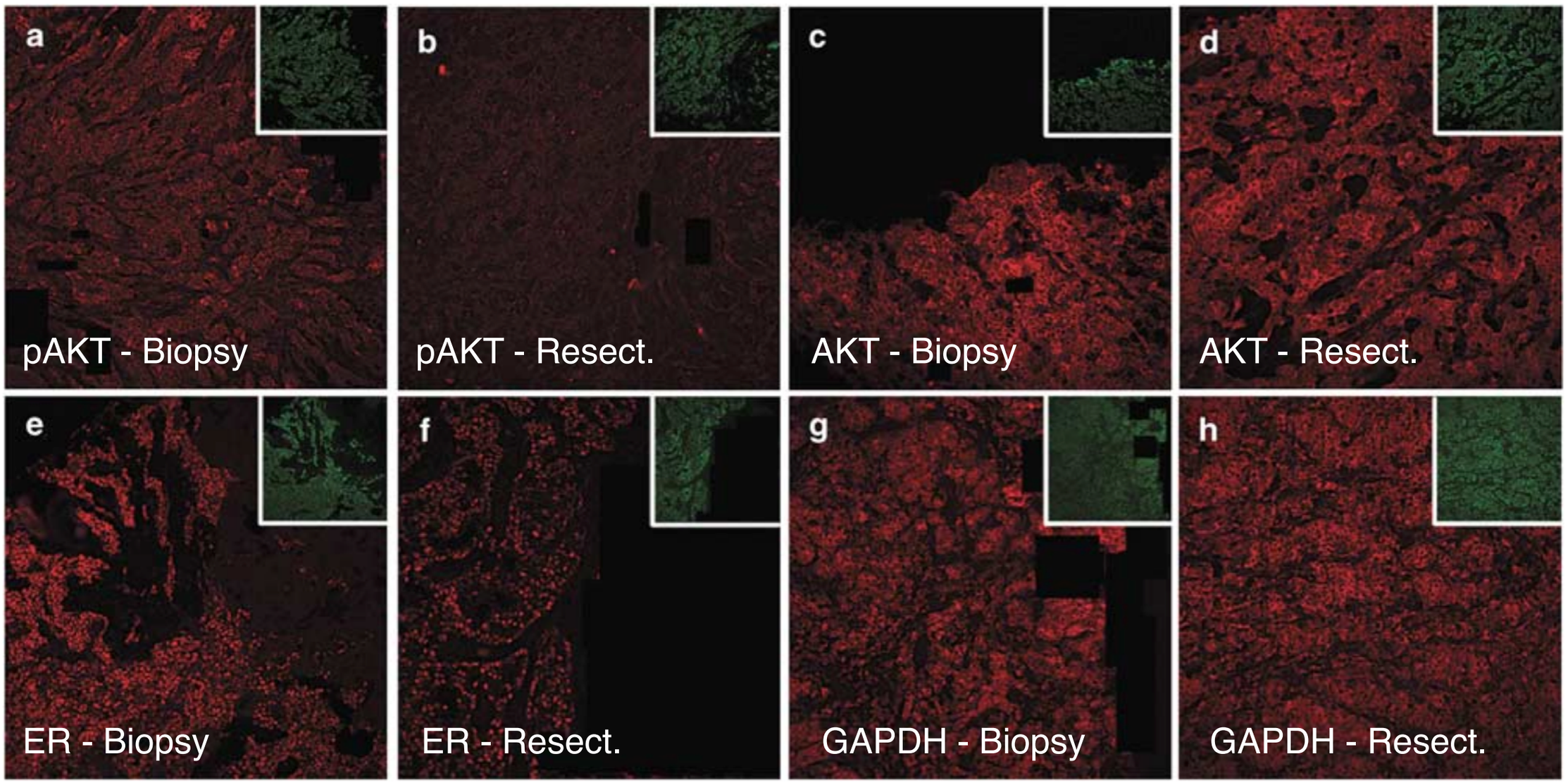


Figure 2 Comparison of biomarker staining images between biopsies *vs* tumor resections on whole tissue section slides. Representative immunofluorescence staining of pAKT (red) in CNB (**a**) and tumor resection (**b**), AKT (red) in CNB (**c**) and tumor resection (**d**), ER (red) in CNB (**e**) and tumor resection (**f**), and GAPDH (red) in biopsy (**g**) and tumor resection (**h**) was illustrated. Each corresponding cytokeratin staining is shown as inset (green). Photographs are shown at magnification of $\times 20$.

Quantitative assessment shows loss of antigenic epitopes as a function of pre-analytic variables

Yalai Bai[1], Juliana Tolles[2], Huan Cheng[1], Summar Siddiqui[1], Arun Gopinath[1], Eirini Pectasides[1], Robert L Camp[1], David L Rimm[1] and Annette M Molinaro[2]

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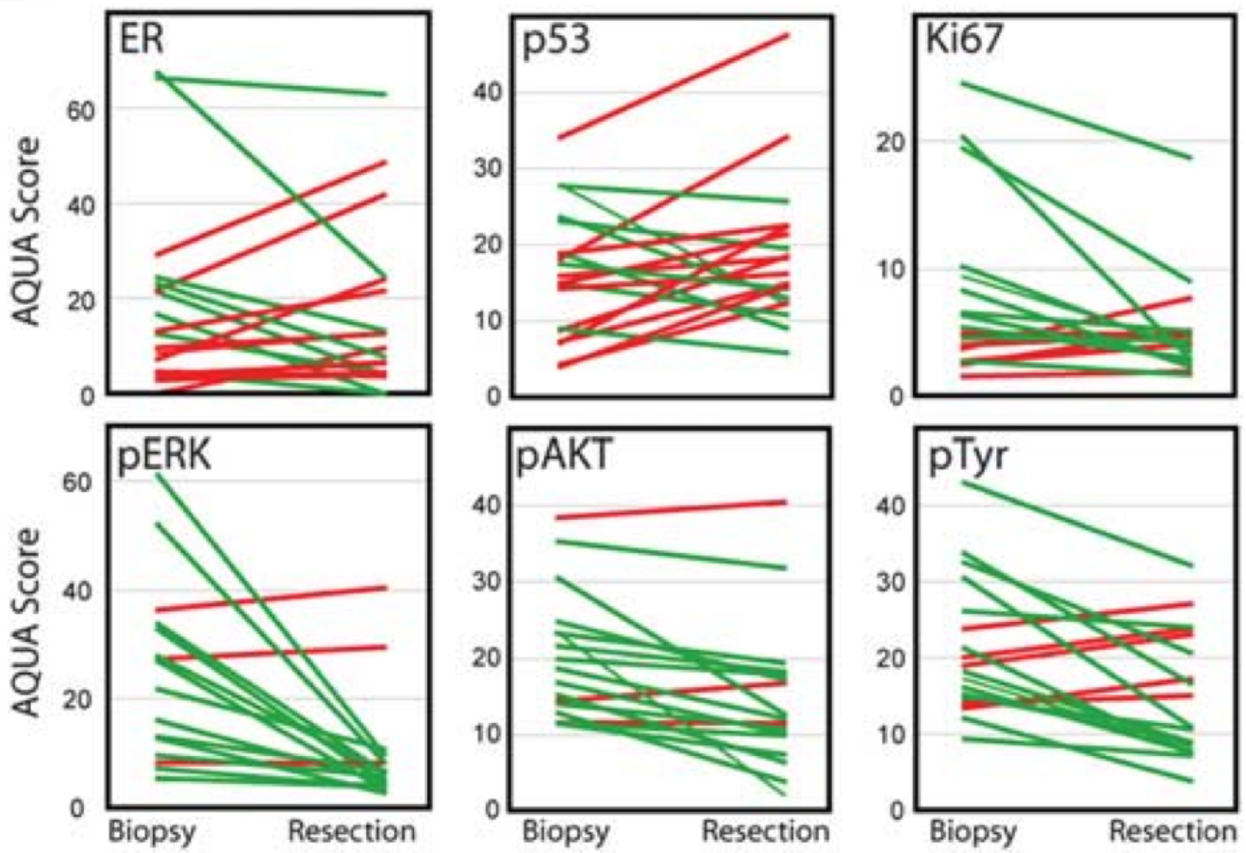


Figure 1 Differences in biomarker expression in core needle biopsies *vs* tumor resections. Twenty core needle biopsies and matched tumor resections were arrayed in TMA with two-fold redundancy. In all, 1.5 mm core from each tumor block was arrayed in a recipient block. The TMA was immunohistochemically stained with ER, p53, Ki67, pERK, pAKT and pTyr and the results were quantified using AQUA. Scores represent the average of two cores. Specimens that showed decreased staining in the resection relative to biopsy are shown in green; those with higher resection levels are shown in red.

Quantitative assessment shows loss of antigenic epitopes as a function of pre-analytic variables

Yalai Bai[1], Juliana Tolles[2], Huan Cheng[1], Summar Siddiqui[1], Arun Gopinath[1], Eirini Pectasides[1], Robert L Camp[1], David L Rimm[1] and Annette M Molinaro[2]

Laboratory Investigation (2011) 91, 1253–1261

- Detection levels for all phospho-epitopes were significantly decreased in tumor resections compared with biopsies while no significant change was seen in the corresponding total proteins.
- ER and cytokeratin showed significant loss of antigenicity.
- This data suggest that measurement of phospho-protein antigenicity in formalin-fixed tissue by immunological methods is dramatically affected by pre-analytic variables.
- This study suggests that core needle biopsies are more accurate for assessment of tissue biomarkers.

Vacuum Sealing and Cooling as Methods to Preserve Surgical Specimens

Thomas Kristensen, PhD, Birte Engvad* Torsten Pless, MD,†*
*Steen Walter, MD, DMSc ak, MD**

Appl Immunohistochem Mol Morphol. 2011 Oct;19(

Liver, kid nd breast

TABLE 1. Antibody Clones and Dilutions and Epitope Retrieval Procedures

Antibody	Clone	Source	Dilution	Retrieval
CD4	SP35	Ventana	RTU	CC1mild
CD8	C8/144B	Ventana	RTU	CC1std
CD10	56C6	Leica	1:10	CC1std
CD13	38C12	Leica	1:25	CC1mild
CD14	7	Leica	1:25	CC1std
CD34	QBEnd/10	Ventana	RTU	CC1mild
CD68	PG-M1	Dako	1:50	CC1std
CD138	B-A38	Ventana	RTU	CC1mild
CD138	BC/B-B4	Biocare	1:500	CC1mild
CDX2	EPR2764Y	Ventana	RTU	CC1std
CK7+8	CAM 5.2	BD	1:10	Protease 1: 8 min
CK7	SP52	Ventana	RTU	CC1std
CK7	OV-TL12/30	Ventana	RTU	CC1std
CK18	DC10	Dako	1:25	CC1std
CK20	SP33	Ventana	RTU	CC1std
E-Cad	ECH-6	Ventana	RTU	CC1std
E-Cad	HECD-1	Abcam	1:50	CC1std
ER	SP1	Ventana	RTU	CC1std
HEPA	OCH1E5	Ventana	RTU	CC1mild
HLA-DR	CR3/43	Dako	1:200	CC1mild
Ki67	30-9	Ventana	RTU	CC1std
Lamins A/C	EPR4100	Epitomics	1:4000	CC1std
PgR	1E2	Ventana	RTU	CC1mild
Villin	CWWB1	Ventana	RTU	CC1std

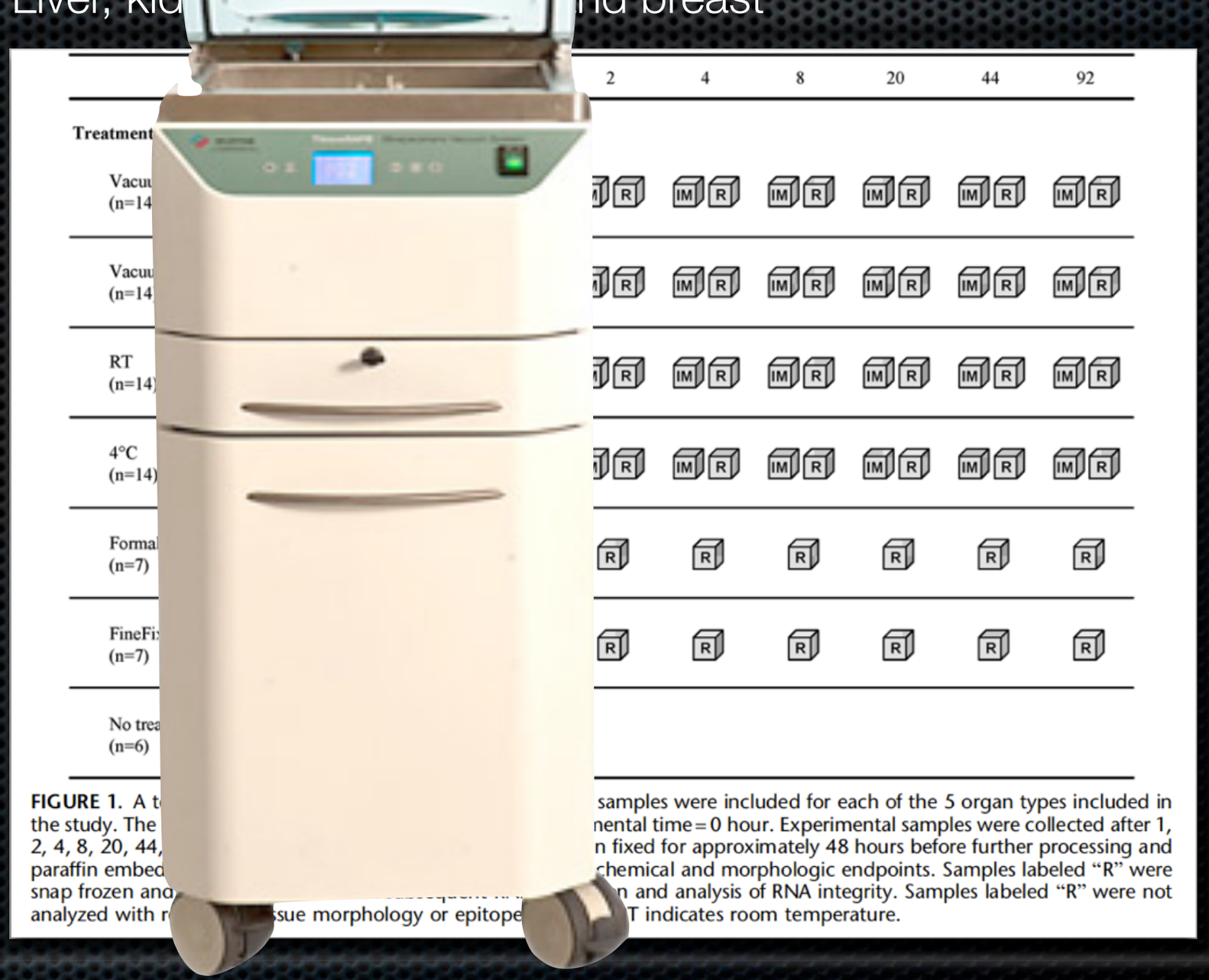


FIGURE 1. A t samples were included for each of the 5 organ types included in the study. The nental time = 0 hour. Experimental samples were collected after 1, 2, 4, 8, 20, 44, n fixed for approximately 48 hours before further processing and paraffin embed chemical and morphologic endpoints. Samples labeled "R" were snap frozen and n and analysis of RNA integrity. Samples labeled "R" were not analyzed with r ssue morphology or epitope T indicates room temperature.

Vacuum Sealing and Cooling as Methods to Preserve Surgical Specimens

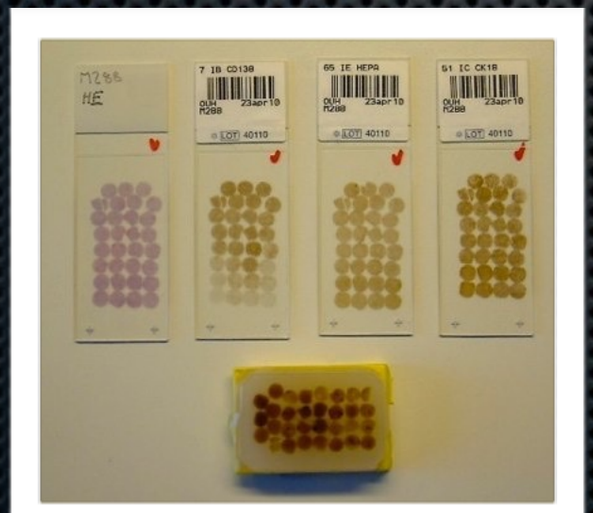
Thomas Kristensen, PhD, Birte Engvad, MD,* Ole Nielsen, MT,* Torsten Pless, MD,† Steen Walter, MD, DMSc, FEBU,‡ and Martin Bak, MD**

Appl Immunohistochem Mol Morphol. 2011 Oct;19(5):460-9.

Liver, kidney, spleen, colon and breast

TABLE 1. Antibody Clones and Dilutions and Epitope Retrieval Procedures

Antibody	Clone	Source	Dilution	Retrieval
CD4	SP35	Ventana	RTU	CC1mild
CD8	C8/144B	Ventana	RTU	CC1std
CD10	56C6	Leica	1:10	CC1std
CD13	38C12	Leica	1:25	CC1mild
CD14	7	Leica	1:25	CC1std
CD34	QBEnd/10	Ventana	RTU	CC1mild
CD68	PG-M1	Dako	1:50	CC1std
CD138	B-A38	Ventana	RTU	CC1mild
CD138	BC/B-B4	Biocare	1:500	CC1mild
CDX2	EPR2764Y	Ventana	RTU	CC1std
CK7+8	CAM 5.2	BD	1:10	Protease 1: 8 min
CK7	SP52	Ventana	RTU	CC1std
CK7	OV-TL12/30	Ventana	RTU	CC1std
CK18	DC10	Dako	1:25	CC1std
CK20	SP33	Ventana	RTU	CC1std
E-Cad	ECH-6	Ventana	RTU	CC1std
E-Cad	HECD-1	Abcam	1:50	CC1std
ER	SP1	Ventana	RTU	CC1std
HEPA	OCH1E5	Ventana	RTU	CC1mild
HLA-DR	CR3/43	Dako	1:200	CC1mild
Ki67	30-9	Ventana	RTU	CC1std
Lamins A/C	EPR4100	Epitomics	1:4000	CC1std
PgR	1E2	Ventana	RTU	CC1mild
Villin	CWWB1	Ventana	RTU	CC1std



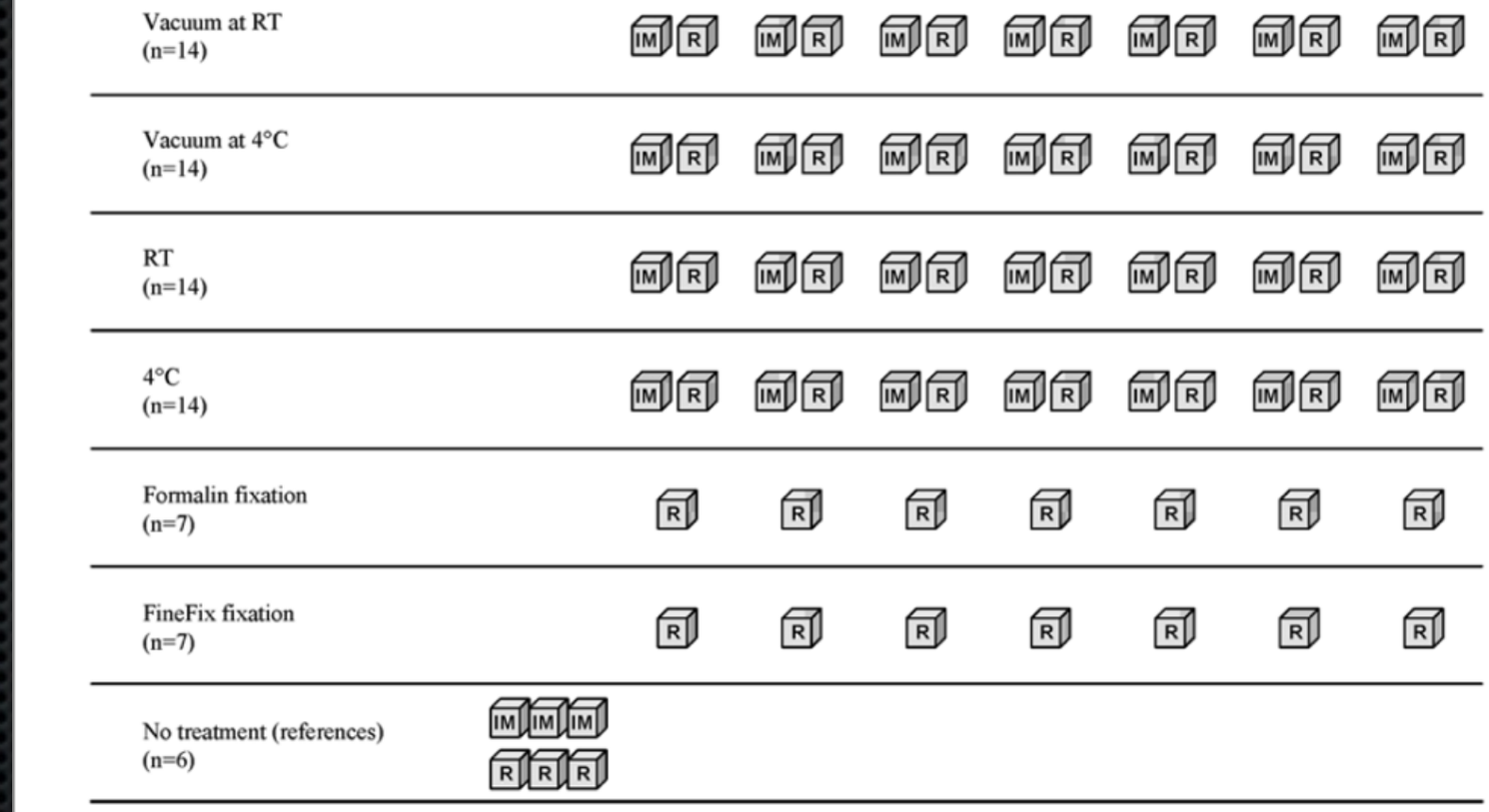


FIGURE 1. A total of 70 experimental samples and 6 reference samples were included for each of the 5 organ types included in the study. The 6 reference samples were collected at the experimental time = 0 hour. Experimental samples were collected after 1, 2, 4, 8, 20, 44, or 92 hours. Samples labeled "IM" were formalin fixed for approximately 48 hours before further processing and paraffin embedding and analyzed with respect to immunohistochemical and morphologic endpoints. Samples labeled "R" were snap frozen and stored at −80°C until subsequent RNA extraction and analysis of RNA integrity. Samples labeled "R" were not analyzed with respect to tissue morphology or epitope integrity. RT indicates room temperature.

Results - Morphology and IHC

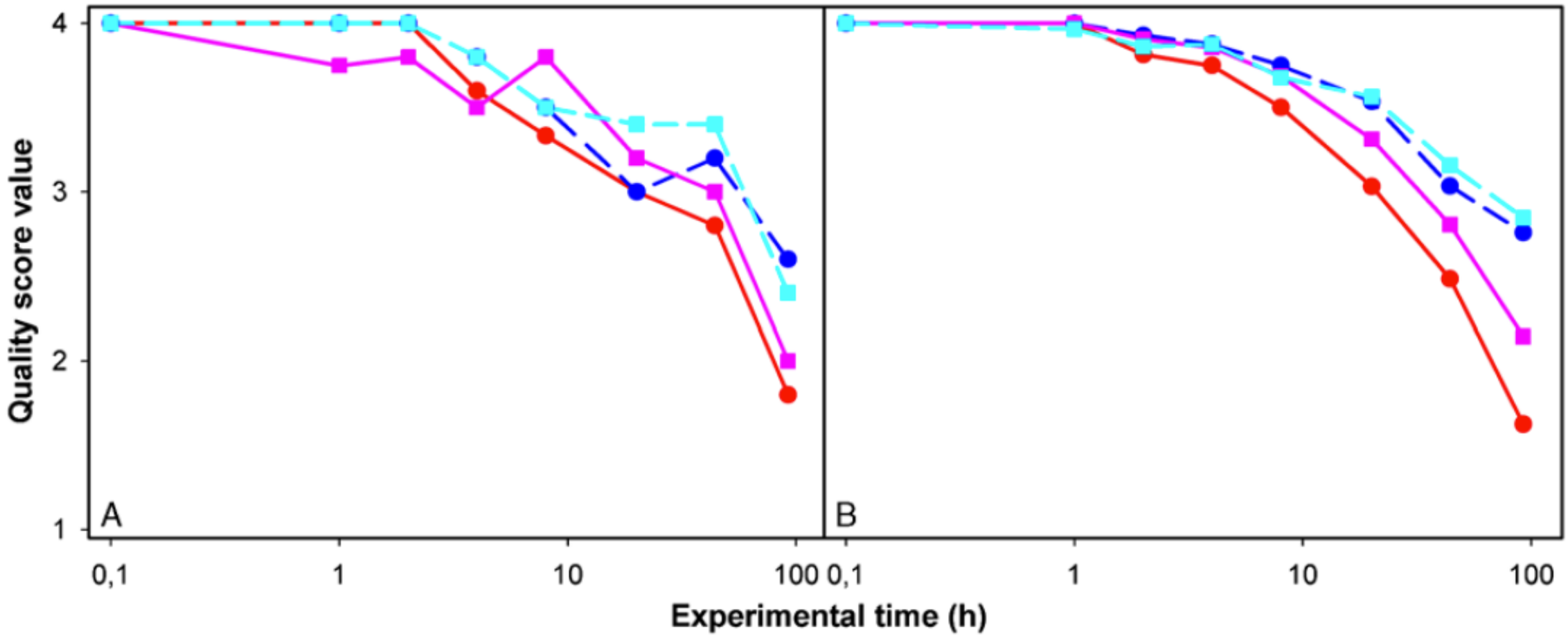
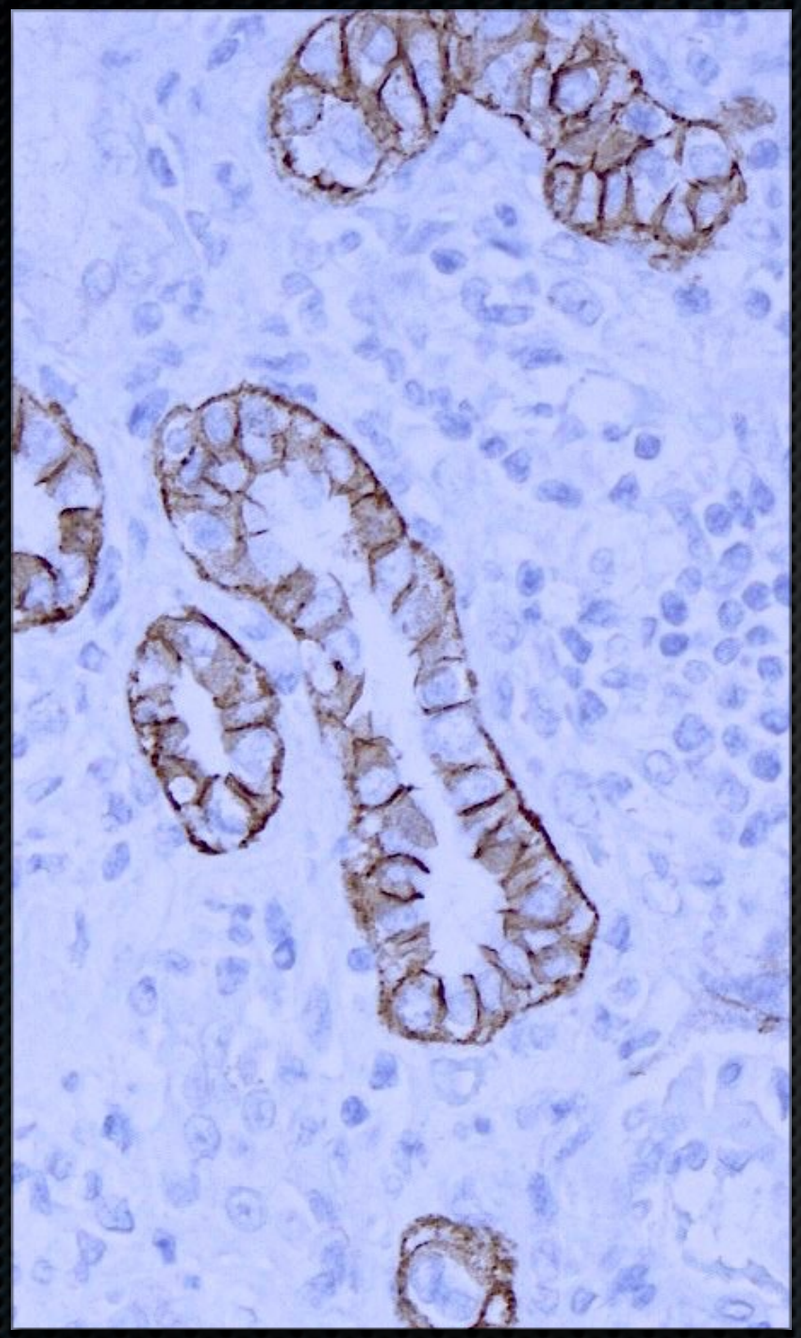
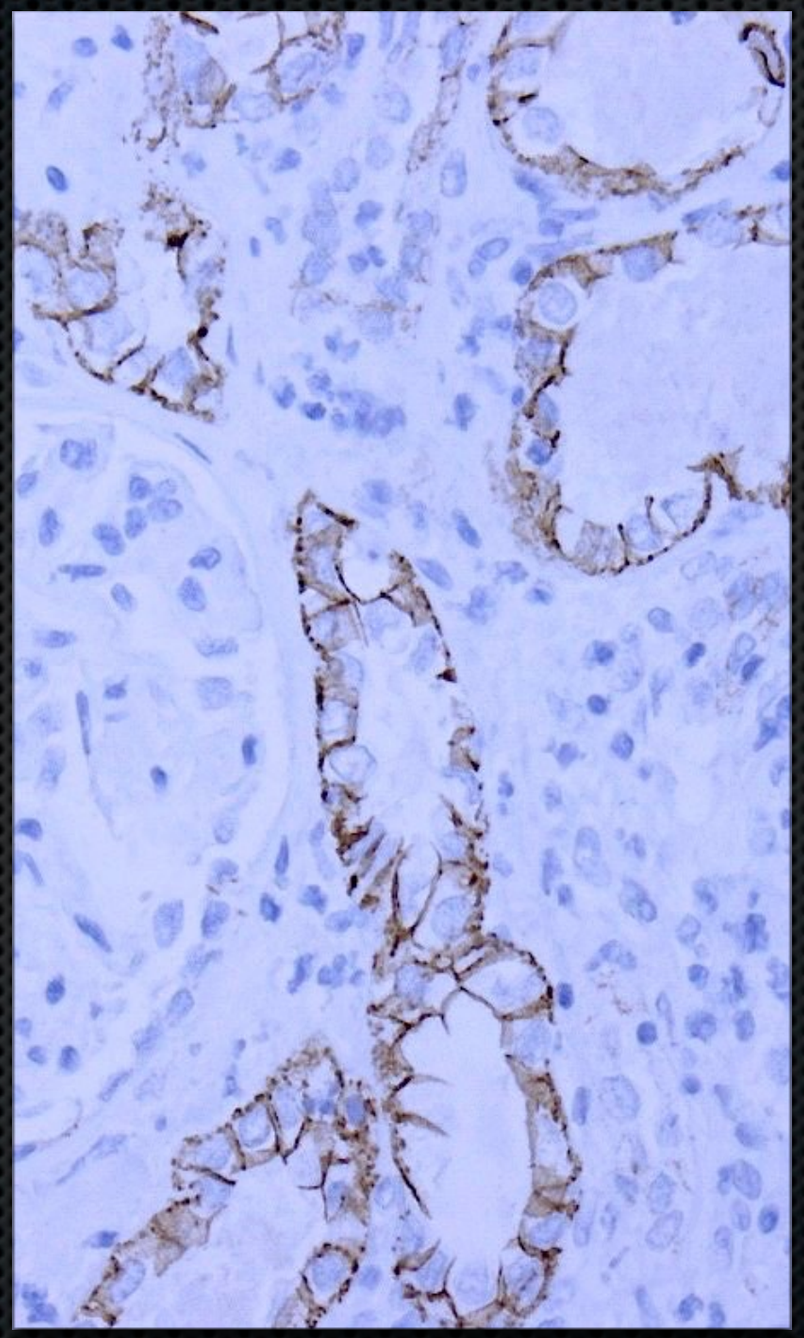


FIGURE 4. A, Morphologic integrity scores as a function of time in the 4 treatment groups: room temperature with vacuum ((—●—)), room temperature without vacuum ((—■—)), 4°C with vacuum ((- - ● - -)), and 4°C without vacuum ((- - ■ - -)). Each data point represents the mean of the score values in the 5 tissues. B, IHC staining quality scores as a function of time in the 4 treatment groups. Each data point represents the mean of all score values from all antibodies in all 5 tissues. Quality score value 4 corresponds to optimal, 3 corresponds to good, 2 corresponds to borderline, and 1 corresponds to poor morphologic integrity or IHC staining quality. Experimental time = 0 hour is depicted as 0.1 hour in both panels. IHC indicates immunohistochemical.

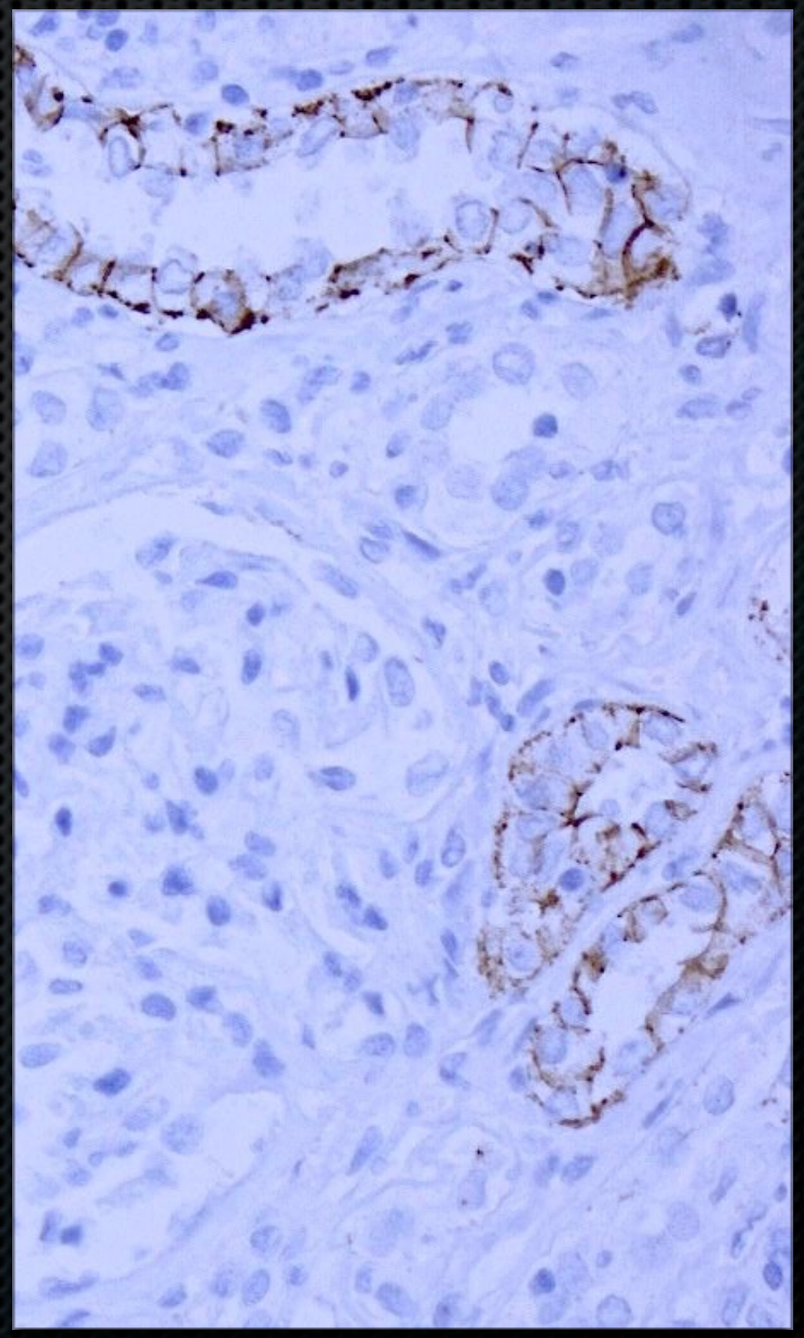
E-Cadherin, HECD1 - Kidney



Ref. No delay

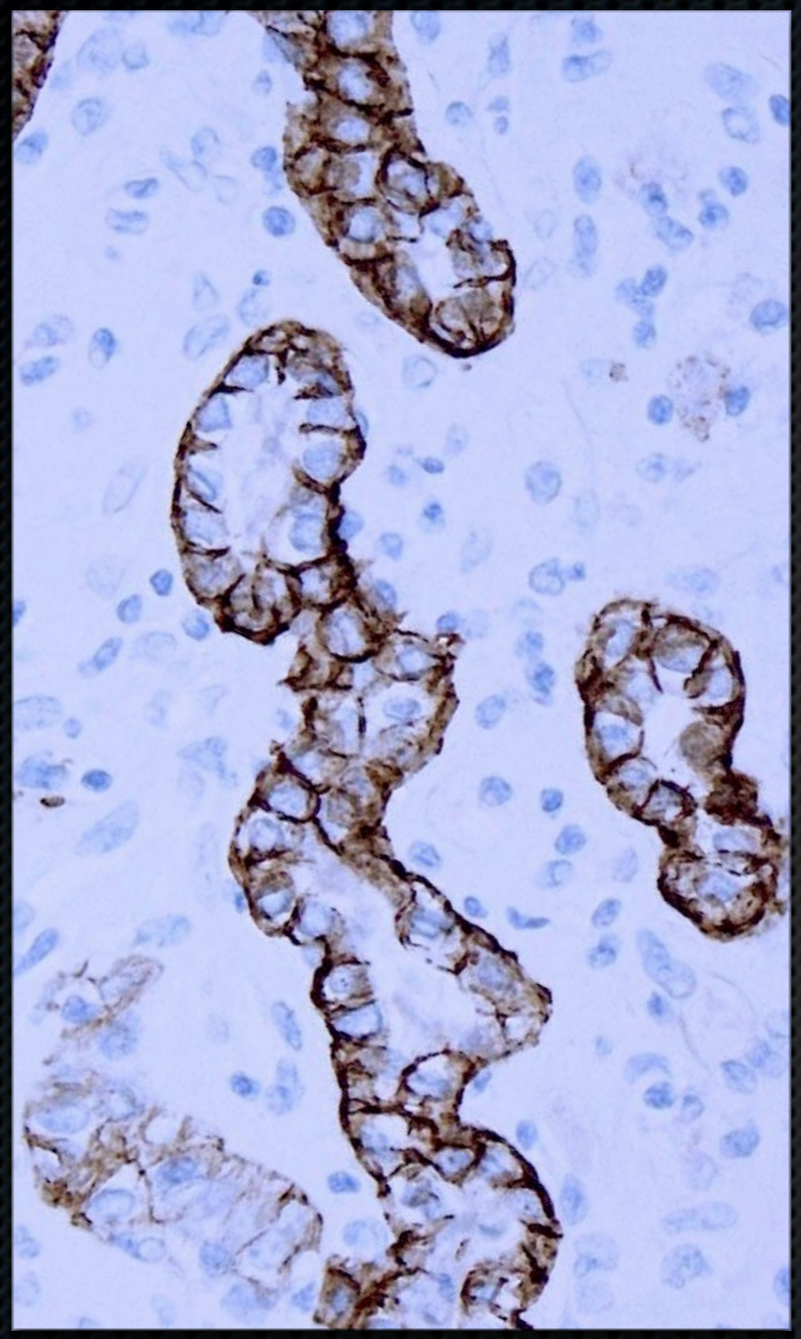


92hrs at 4°C/no vac

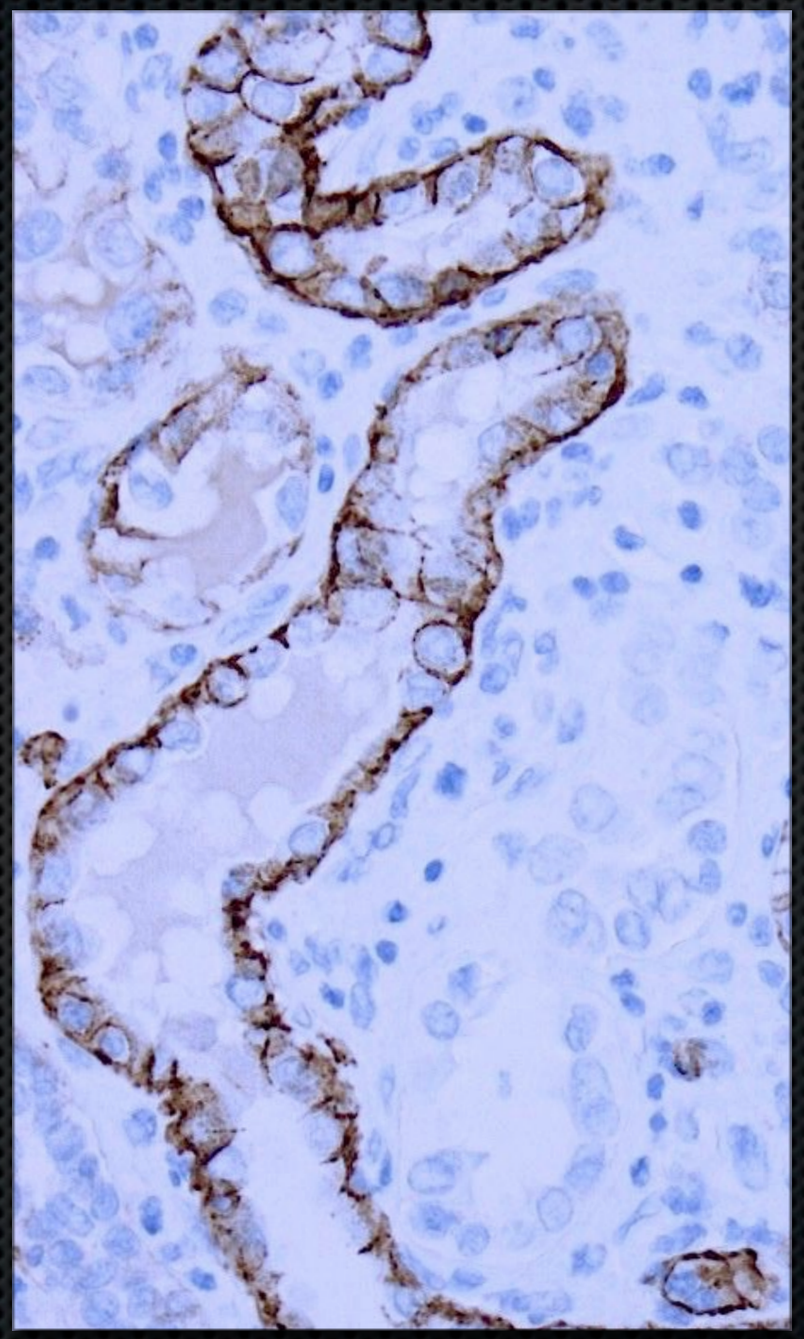


92hrs at 4°C/vac

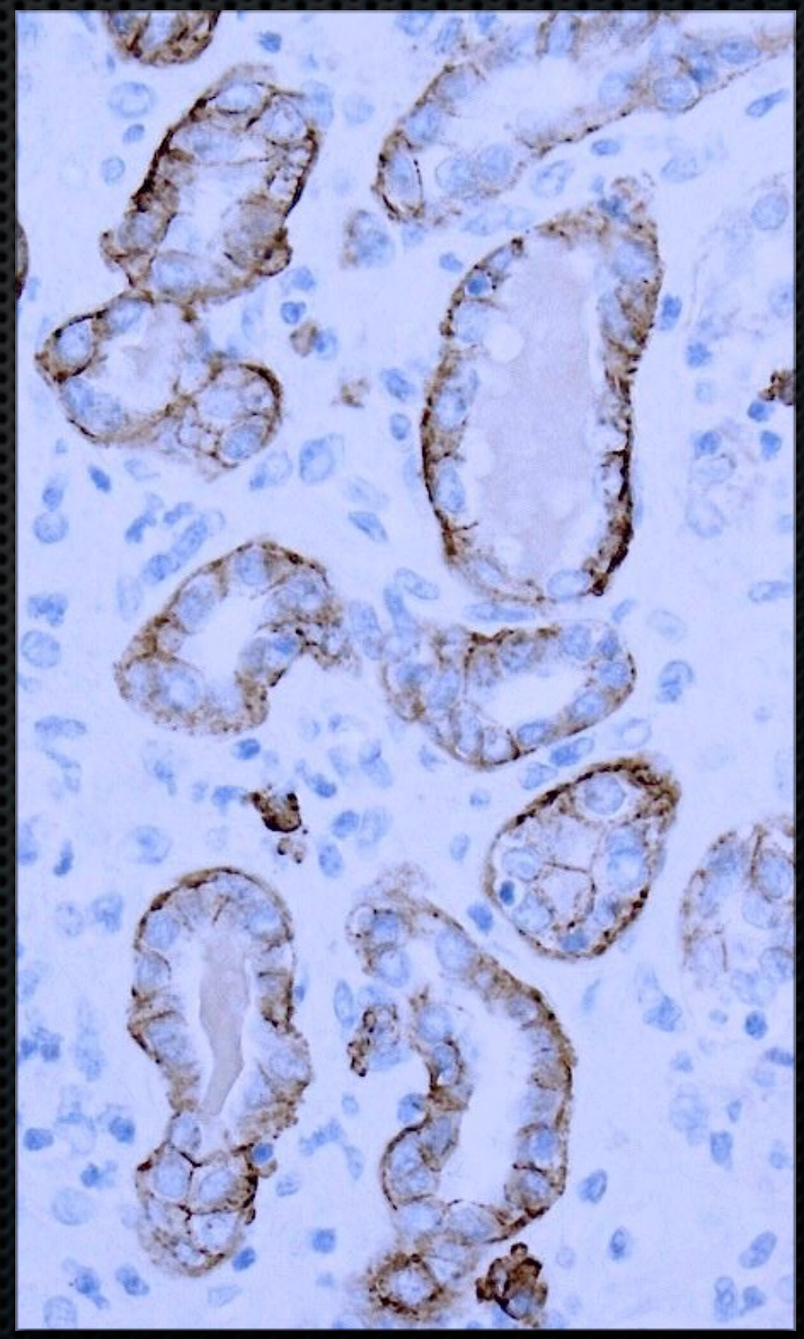
CD138, B-A38 - Kidney



Ref. No delay

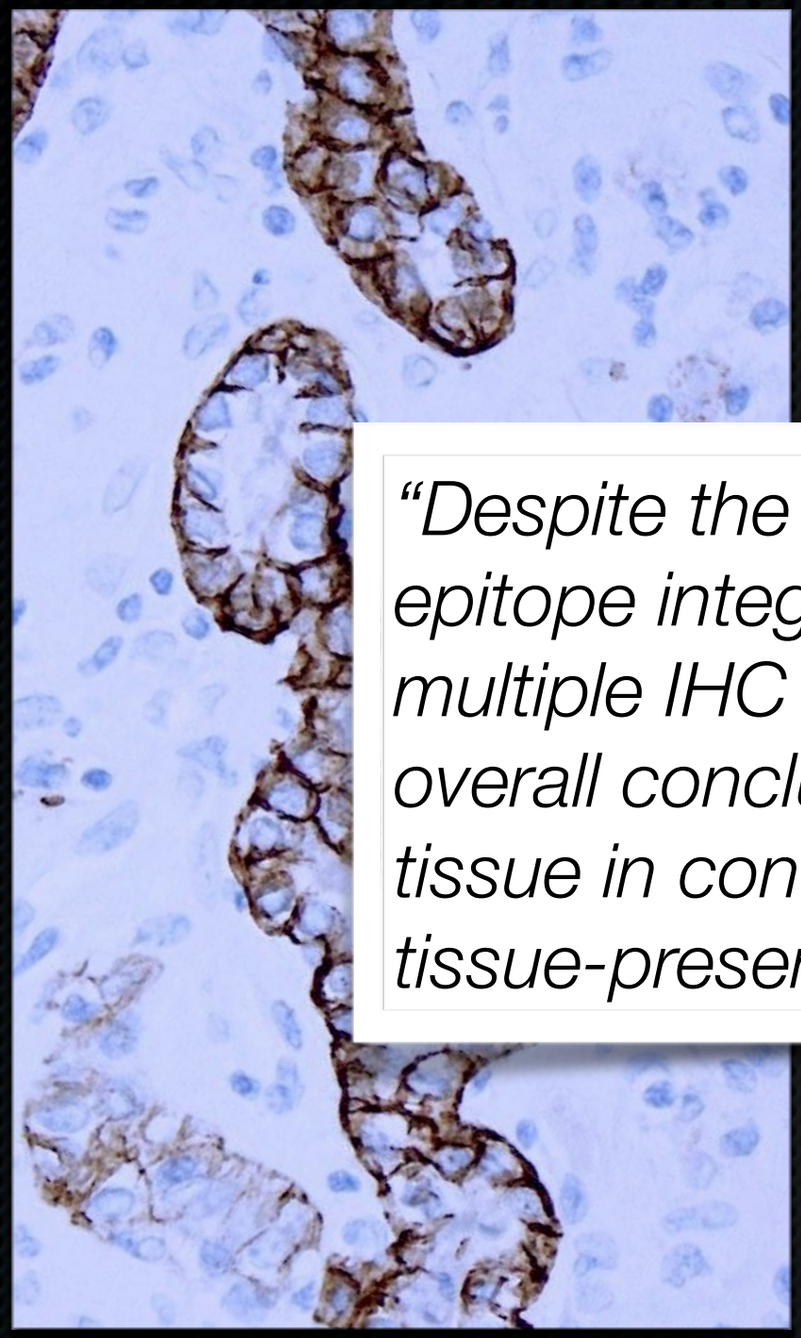


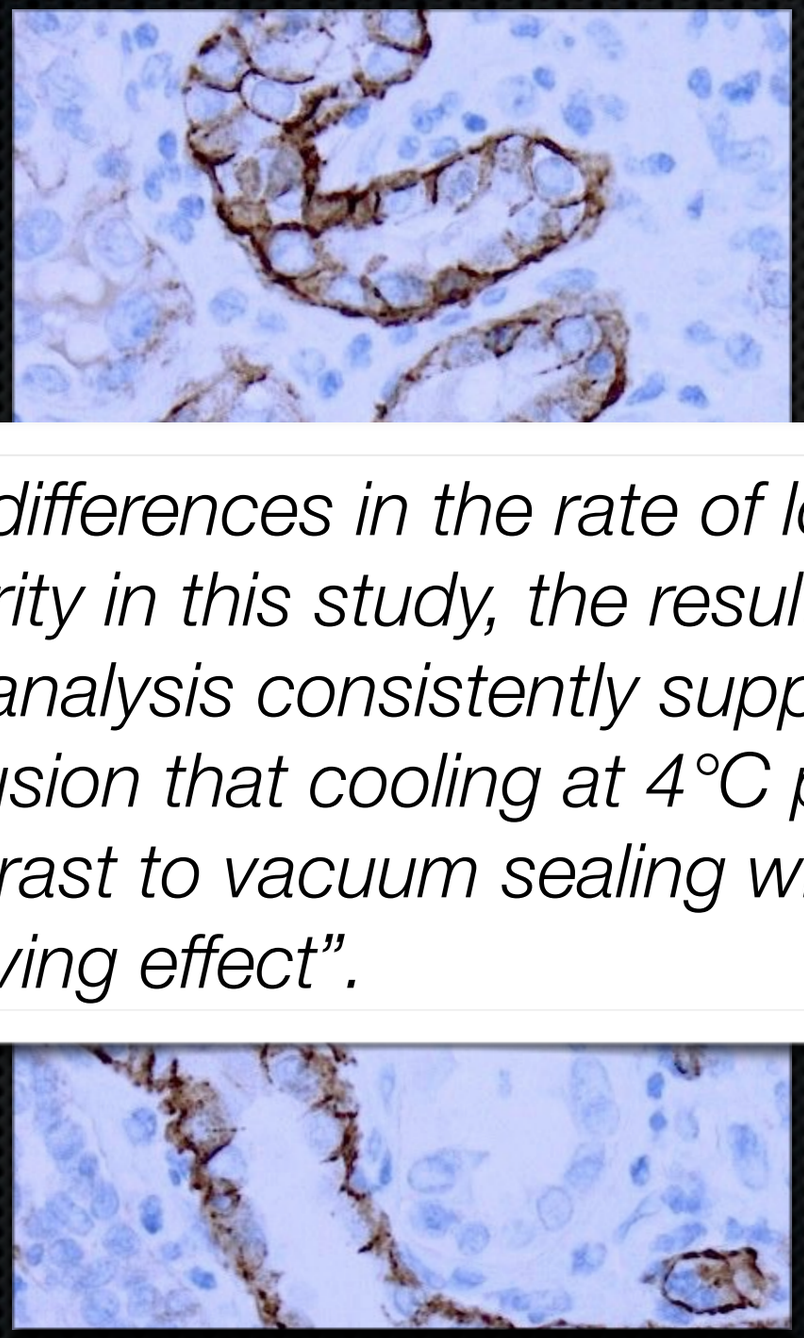
8hrs at 4°C/no vac

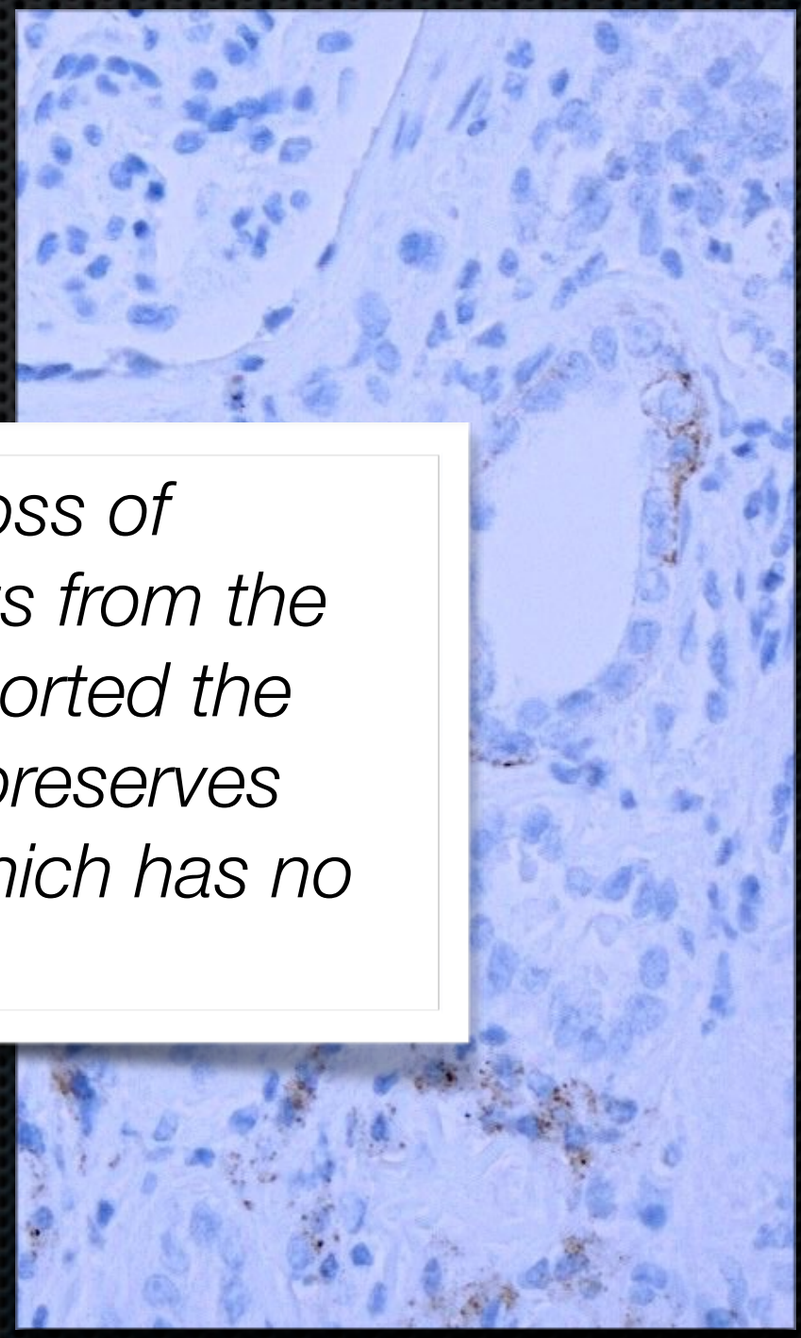


8hrs at 4°C/vac

CD138, B-A38 - Kidney







"Despite the differences in the rate of loss of epitope integrity in this study, the results from the multiple IHC analysis consistently supported the overall conclusion that cooling at 4°C preserves tissue in contrast to vacuum sealing which has no tissue-preserving effect".

Ref. No delay

8hrs at 4°C/no vac

92hrs at 4°C/vac

Under-Vacuum Sealed specimens and temperature

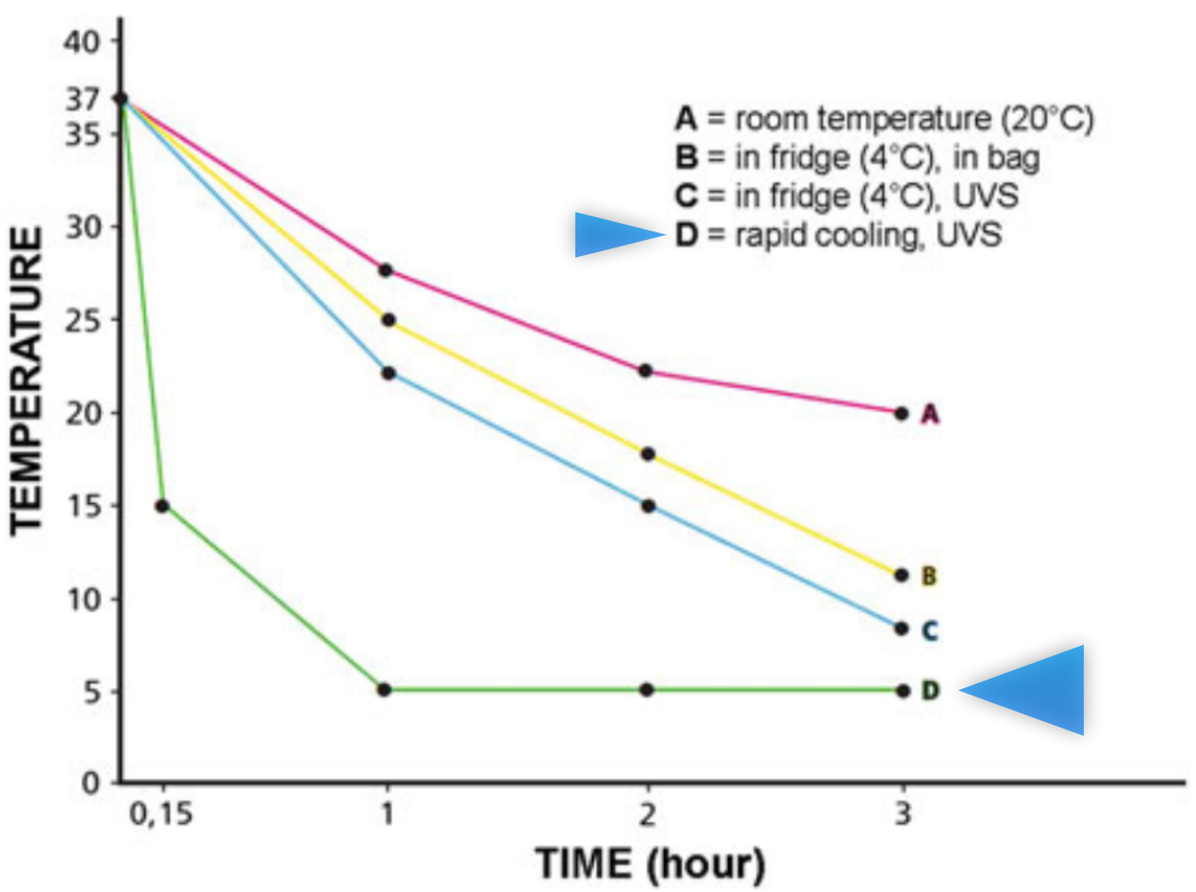


Fig. 3 Variations of the inner temperature in a surgical specimen (approximately 7 cm in diameter) in different conditions. From the body temperature, on surgical removal, if left at room temperature (*A*), it takes approximately 3 h for the specimen to stabilize at the external temperature. When the specimen is left, free, in a fridge (*B*), the inner temperature will gradually go down. The cooling process in Under-Vacuum Sealed (UVS) specimens (*C*) is facilitated by the lack of insulating air. By using cooling (-20°C) elements (*D*), the cooling of the UVS specimens is very rapid

Fixation delay

Preanalytic variable	Published Guidelines and Recommendations ASCO/CAP | CLSI	Literature-Based Recommendations
Fixation delay	**Less than 1 hr**	Less than 12 hrs **4°C is better than RT**

Engel KB, Moore HM. Arch Pathol Lab Med. 2011;135:537-543

ASCO/CAP: American Society of Clinical Oncology/College of American Pathologists

I/LA28-A2
Vol. 31 No. 4
Replaces MM04-A
Vol. 19 No. 26

Quality Assurance for Design Control and Implementation of Immunohistochemistry Assays; Approved Guideline—Second Edition

Stephen M. Hewitt, MD, PhD
Max Robinowitz, MD
Steven A. Bogen, MD, PhD
Allen M. Gown, MD
Krishan L. Kalra, PhD
Christopher N. Otis, MD
Betsy Spaulding
Clive R. Taylor, MD, DPhil

+ A long list of experts and advisors

This document provides guidelines for the development of validated diagnostic, prognostic, and predictive immunohistochemical assays.

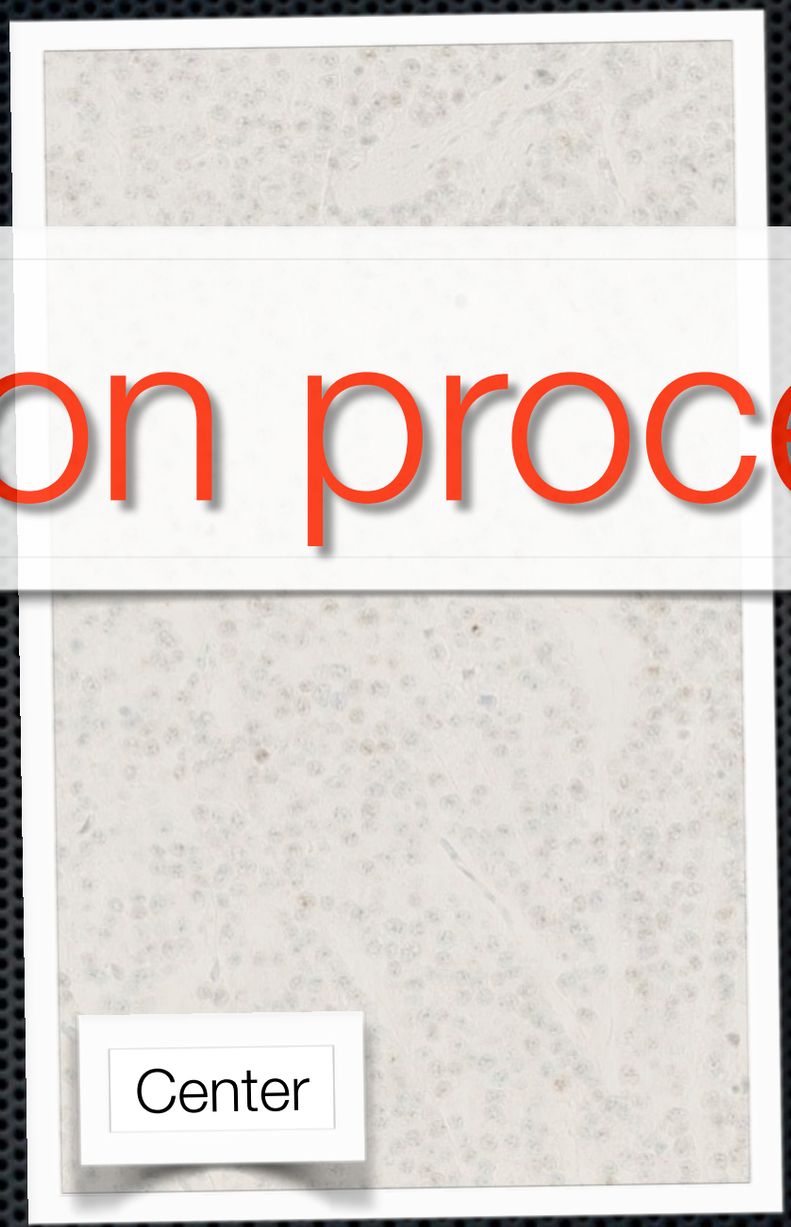
A guideline for global application developed through the Clinical and Laboratory Standards Institute consensus process.

CLINICAL AND LABORATORY STANDARDS INSTITUTE®

(CLSI)

Seminoma: Biology or Artefact?

Fixation procedure?

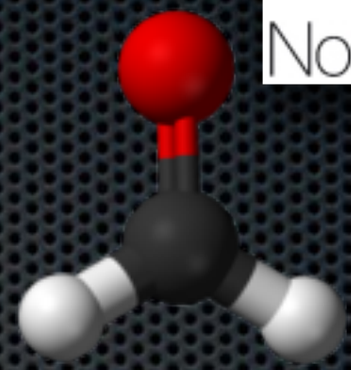


PMS2, EPR3947

Fixation procedure

- Fixative
  - Formula
  - Concentation
  - pH
- Fixation
  - Tissue to fixative ratio
  - Method (Immersion, MWO, sonication, movement etc)
  - Time
  - Temperatur
- Postfixation
  - Washing conditions and duration
  - Storage reagent and duration

Formaldehyde fixation



Phase 1	Penetration	Very fast
Phase 2	Binding	Very slow
Phase 3	Cross-linking	Slow

Formaldehyde obey the diffusion laws, that is, the depth penetrated is proportional to the square root of time.

Penetration rate can be determind using the equation:

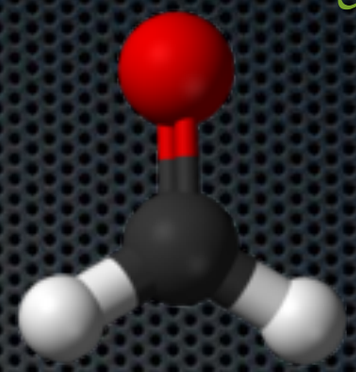
$$d = K\sqrt{t}$$

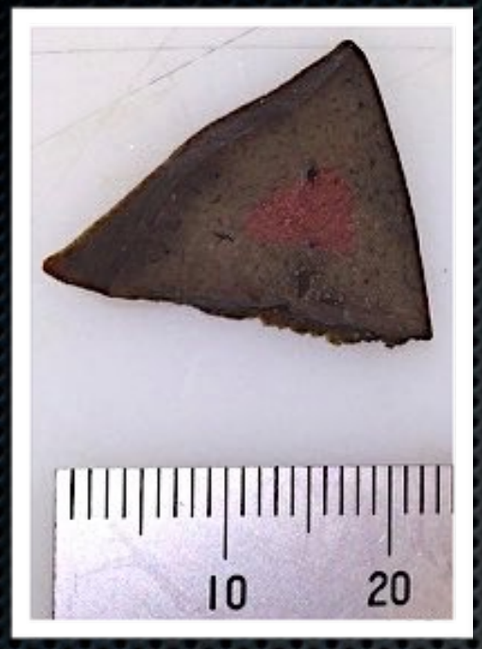
d = Distance penetrated in mm
K = Medawar's coefficient of diffusibility
t = Time in hours

Medawar's K = 5,5
Alternative:
Baker's K = 3,6
Hewletts K = 2,0

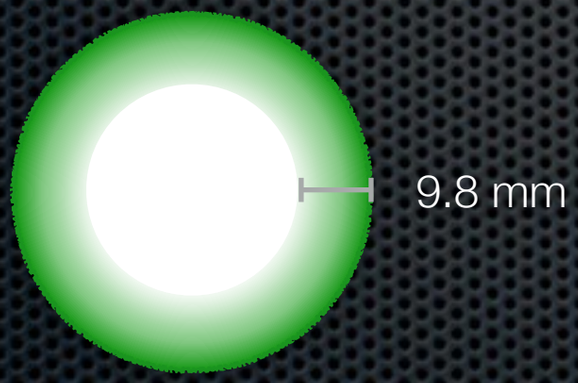
Formaldehyde fixation

Region Syddanmark
OUH
Afd. f.
Klinisk
Patologi
O. Nielsen





Fixation:
NBF 24 hrs



Penetration rate can be determind using the equation: $d = K\sqrt{t}$

Medawar's K = **5,5**
Baker's K = **3,6**

Hewletts K = **2,0**:

1 second d = 0.033 mm (124 mm/hr)
1 minute d = 0.26 mm (15.5 mm/hr)
4 minutes d = 0.52 mm (7.8 mm/hr)
16 minutes d = 1.04 mm (3.9 mm/hr)
1 hour d = 2.0 mm (2.0 mm/hr)
4 hours d = 4.0 mm (averages to 1.0mm/hr),
8 hours d = 5.66 mm (averages to 0.7mm/hr),
16 hours d = 8.0 mm (averages to 0.5mm/hr),
24 hours d = 9.8 mm (averages to 0.41mm/hr),
96 hours d = 19.6 mm (averages to 0.2mm/hr).

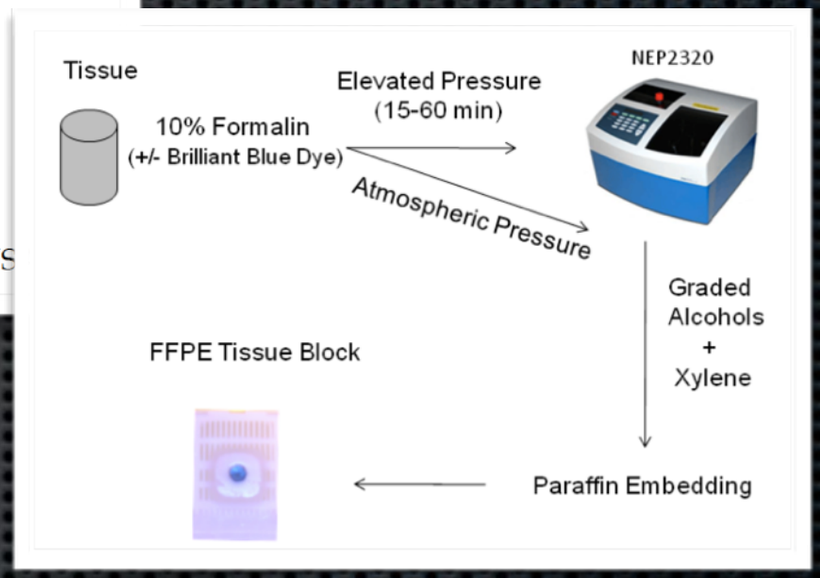
Journal of Cancer
2010; 1:178-183

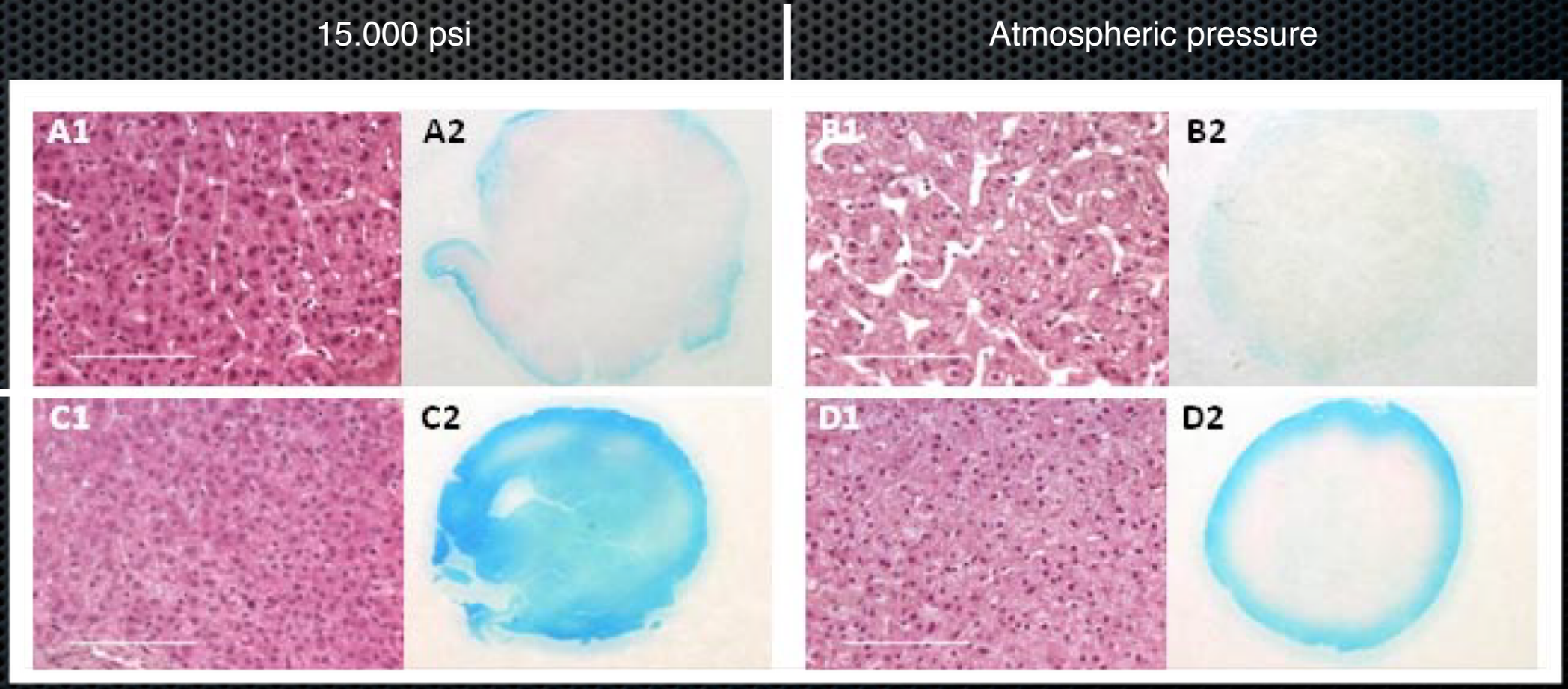
Short Research Communication

Elevated Pressure Improves the Rate of Formalin Penetration while Preserving Tissue Morphology

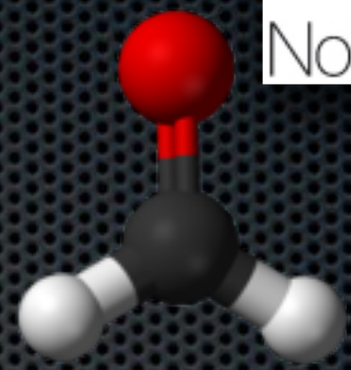
Ingrid E. Chesnick[1], Jeffrey T. Mason[1], Timothy J. O'Leary[2], Carol B. Fowler[1,2] ✉

1. Department of Biophysics, Armed Forces Institute of Pathology, Rockville, Maryland, USA;
2. Biomedical Laboratory Research and Development Service, Veterans Health Administration, Washington, DC, US



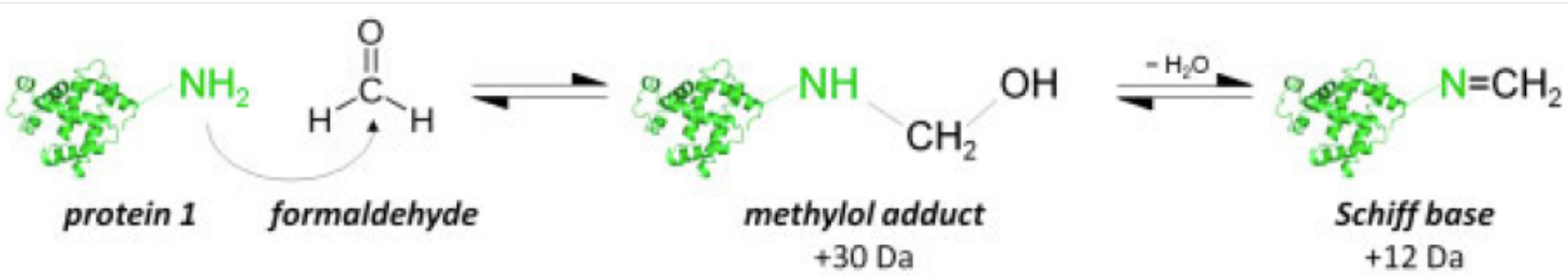


Formaldehyde fixation



Phase 1	Penetration	Very fast
Phase 2	Binding	Very slow
Phase 3	Cross-linking	Slow

Phase 2

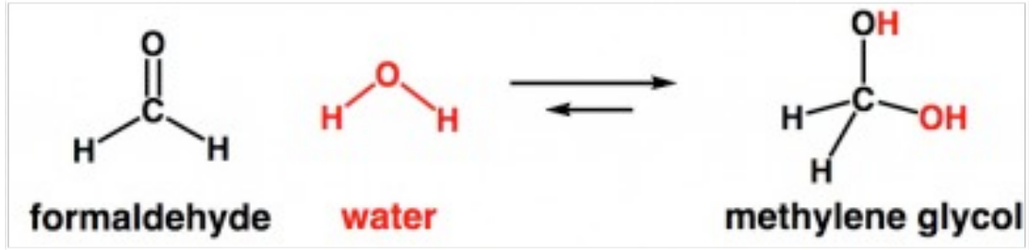


Phase 3

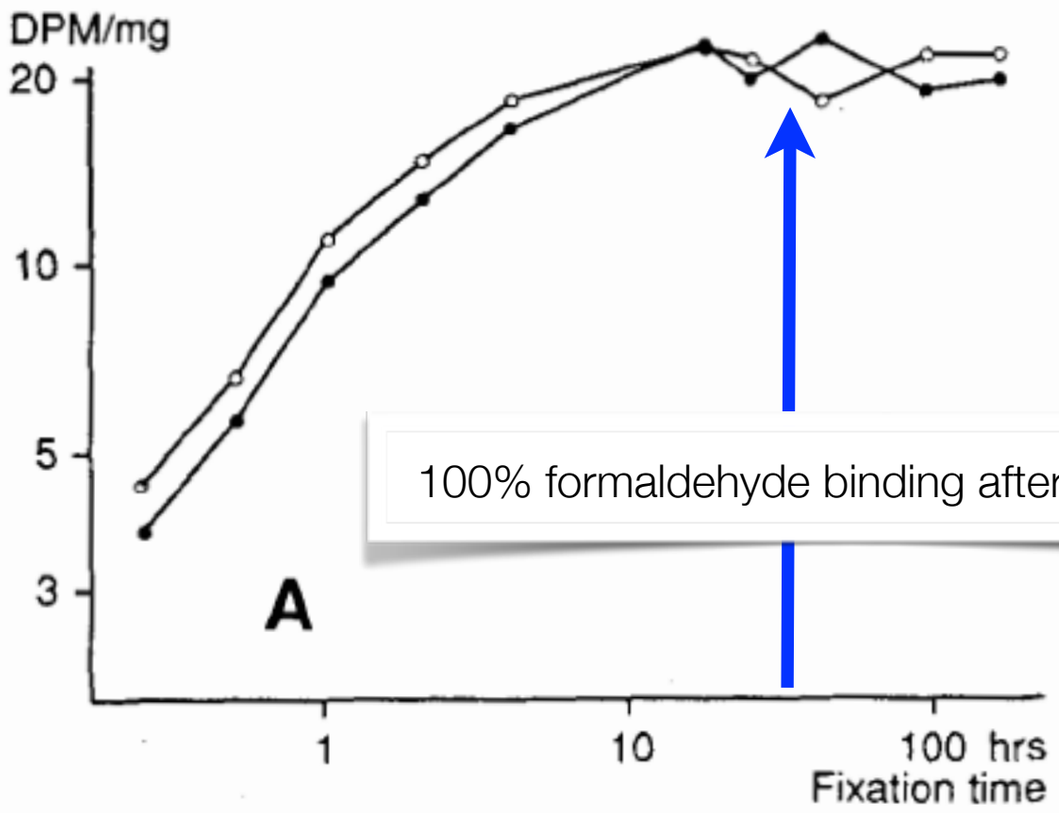
Kinetic Studies of Formaldehyde Binding in Tissue

Biotechnique and Histochemistry. 1994; **69**, 177-179

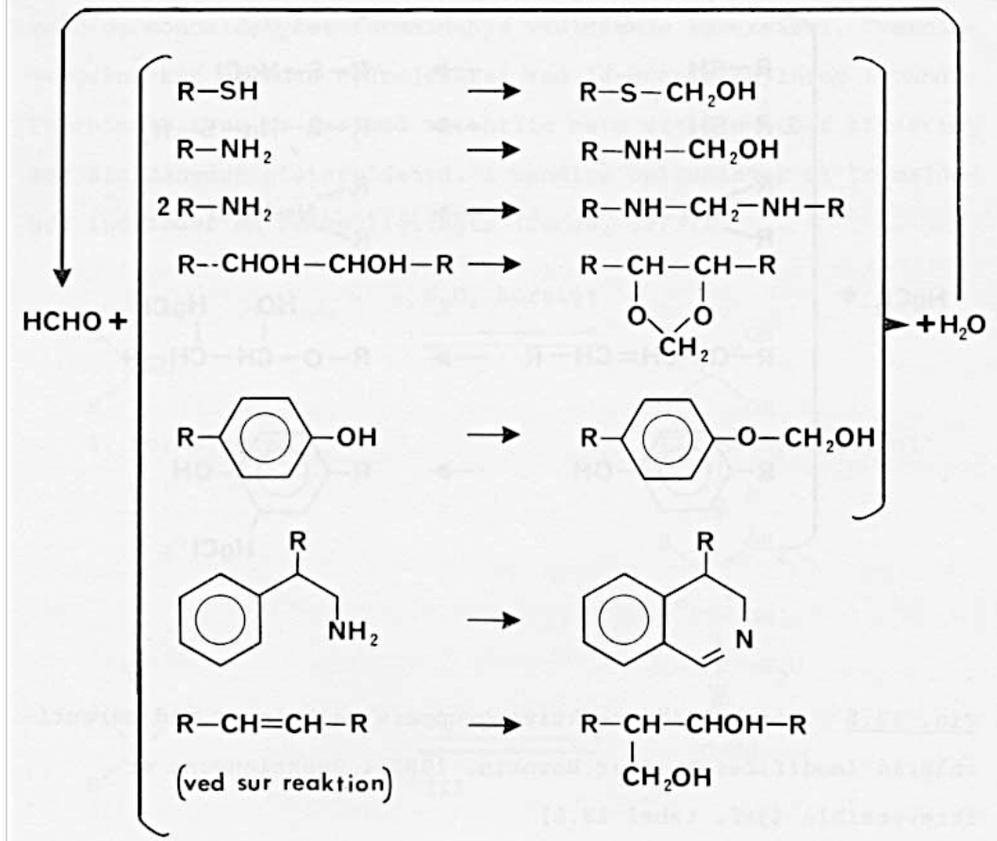
Kerstin G. Helander
Laboratory of Membrane Biology, Center for Ulcer Research and Education, University of California, Los Angeles, California 90073



4x4x4 mm liver tissue



100% formaldehyde binding after app. 25 hrs



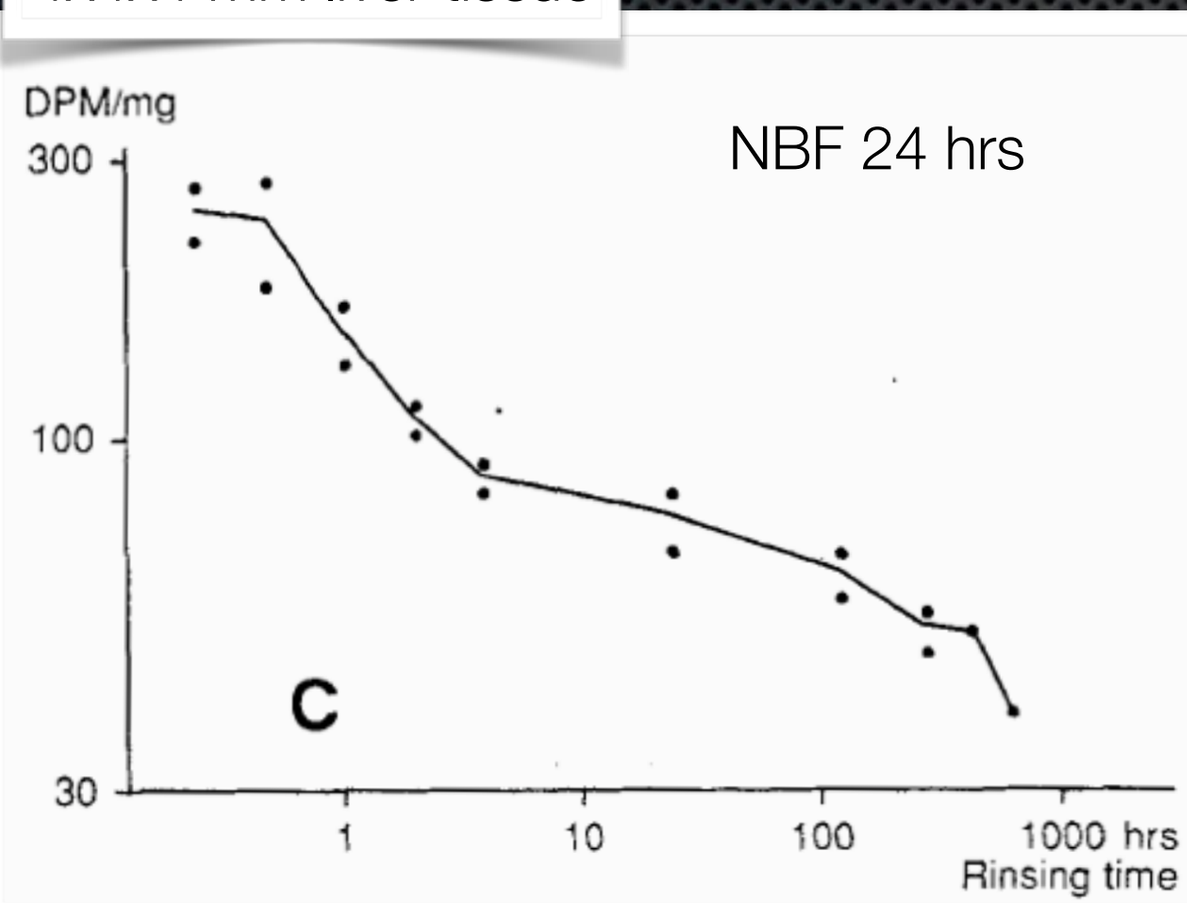
Kinetic Studies of Formaldehyde Binding in Tissue

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

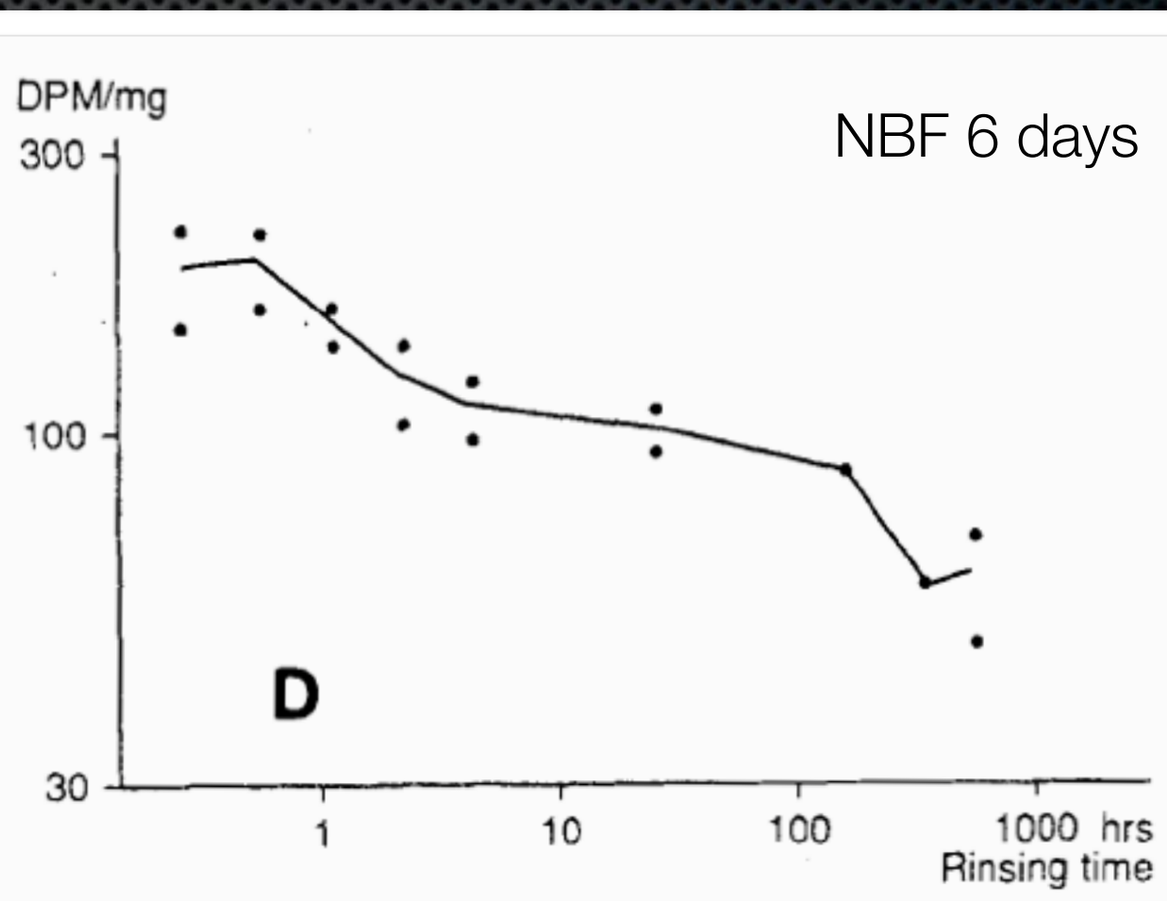
Biotechnique and Histochemistry. 1994; **69**, 177-179

Formaldehyde binding is reversible:

4x4x4 mm liver tissue



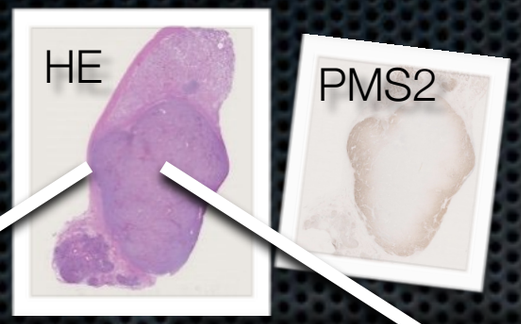
NBF 24 hrs

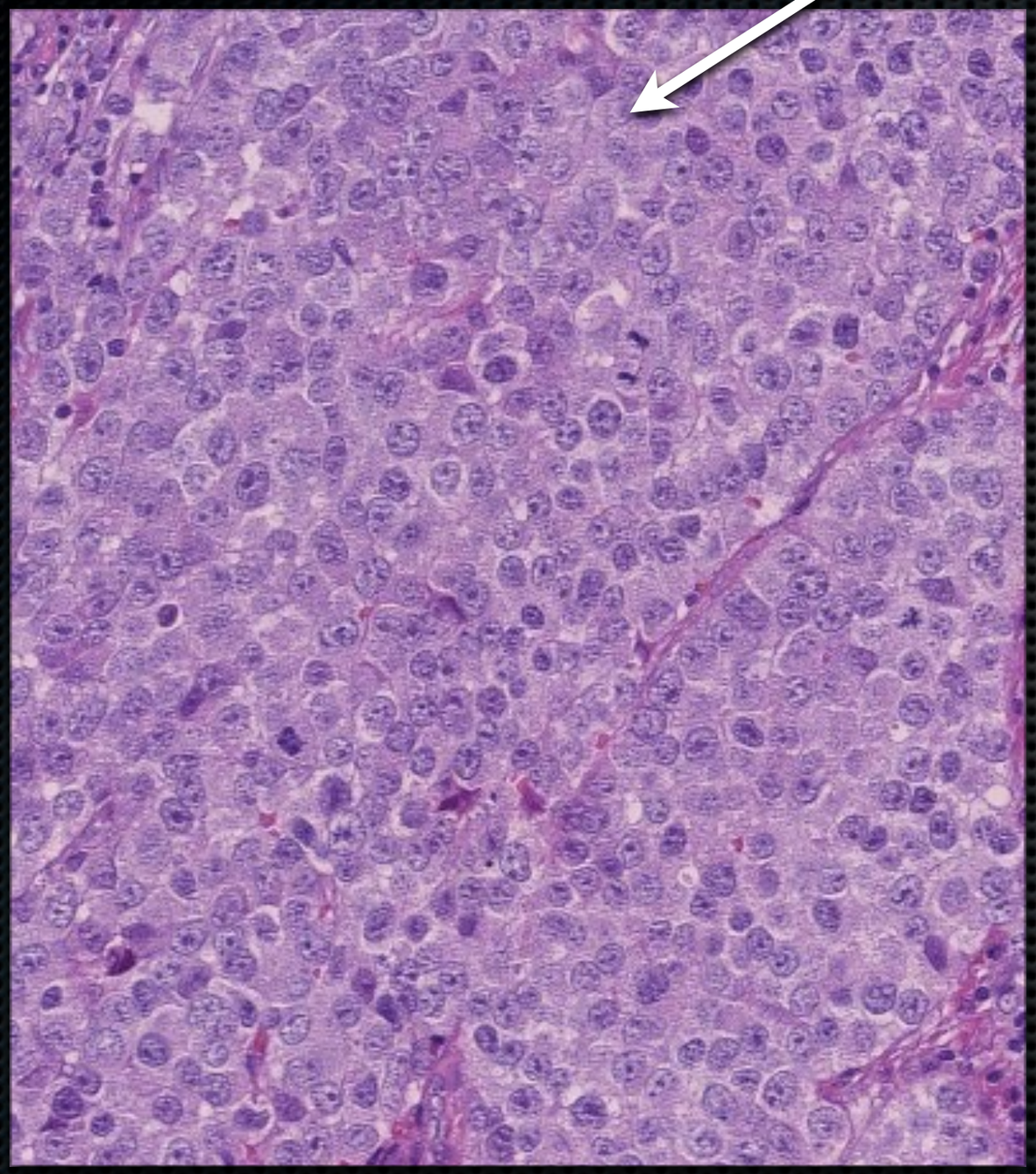


NBF 6 days

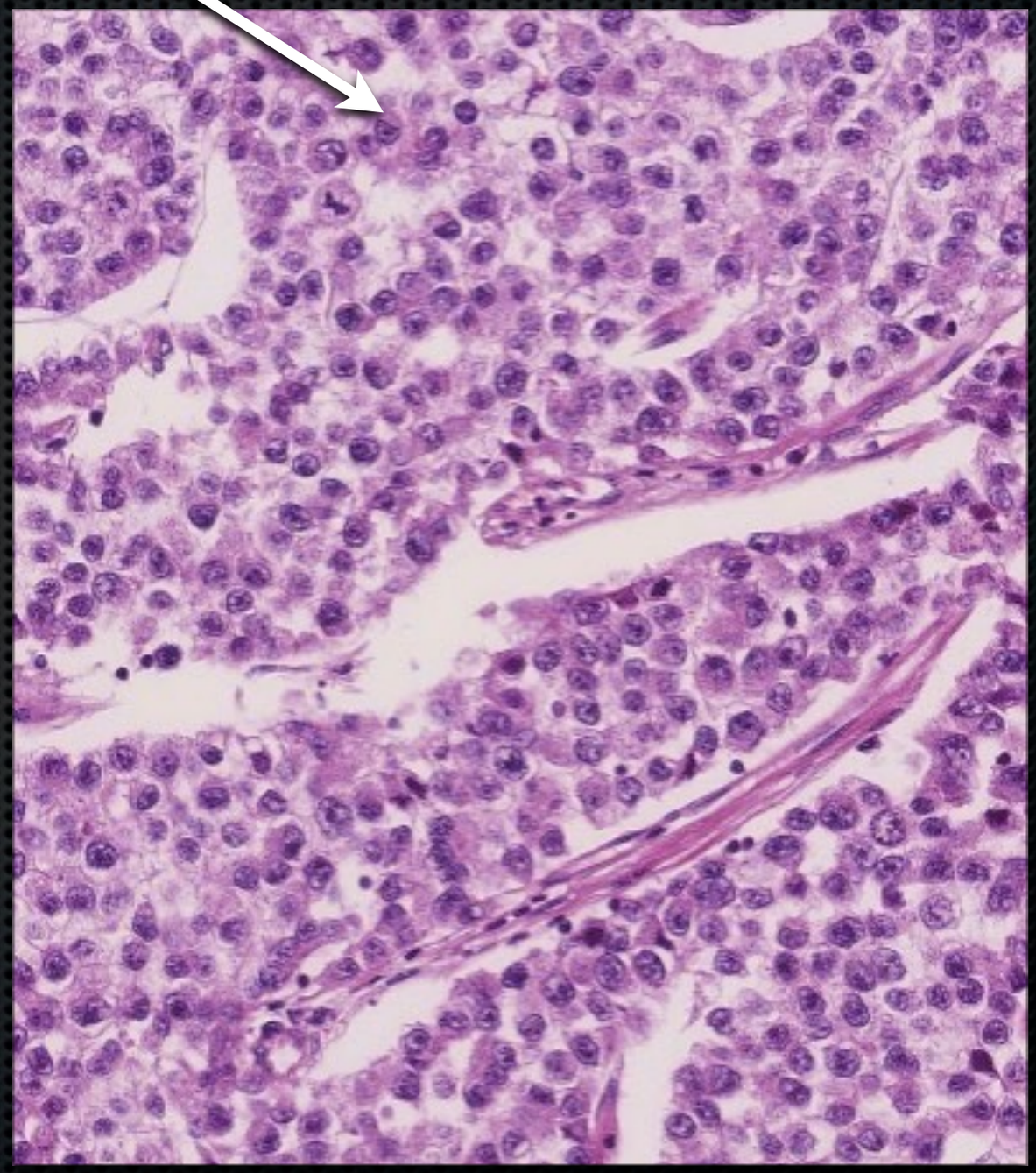
Rinsing with dH_2O

Seminoma





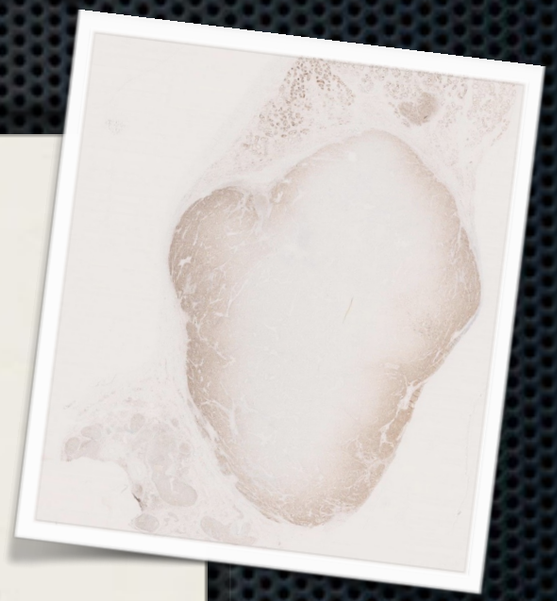
Edge



Center

PMS2, EPR3947 and fixatives

Clone EPR3947 can not be used on alcohol-fixed tissue



Bouin 24 hrs

Zamboni 24 hrs

Clarke 24 hrs

Carnoy 24 hrs

Methacarn 24 hrs

Form/Zn 24 hrs

Ethanol 24 hrs

NBF168 hrs

NBF 48 hrs

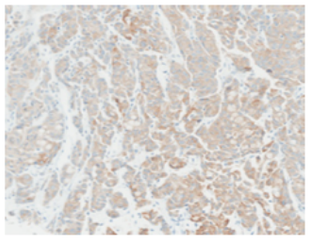
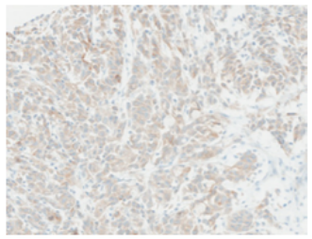
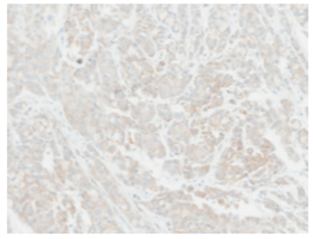
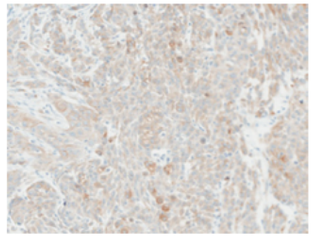
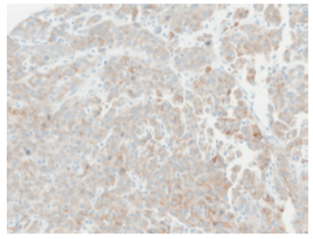
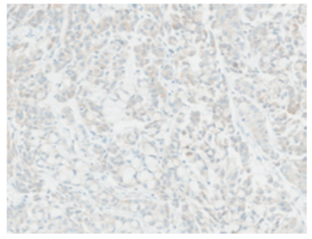
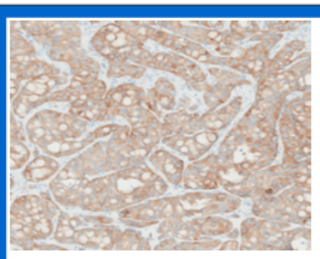
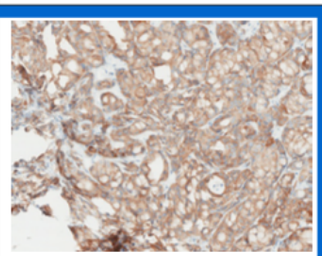
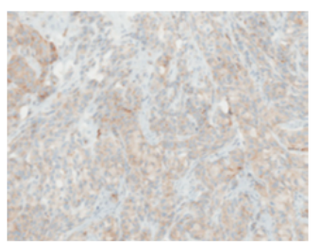
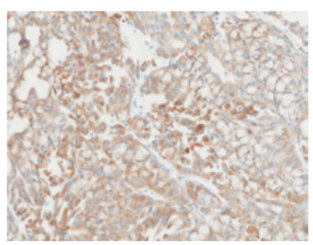
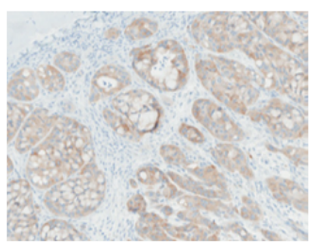
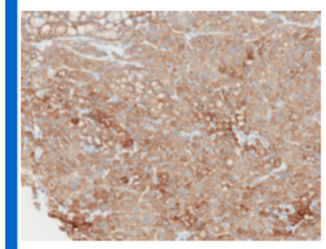
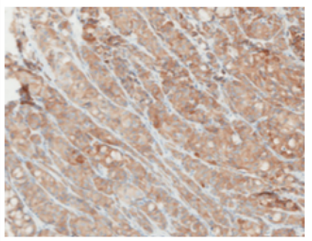
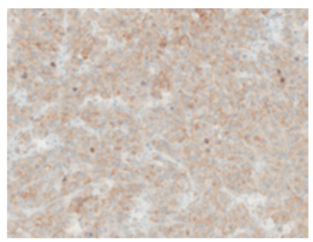
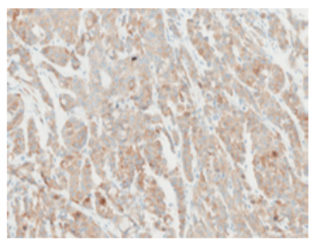
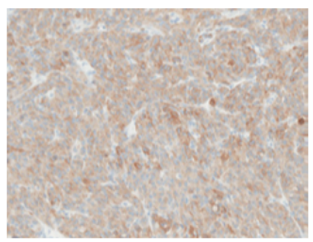
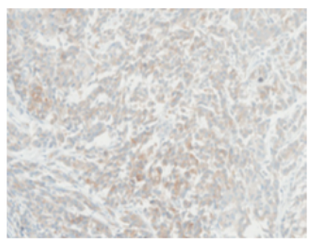
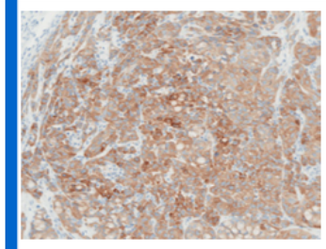
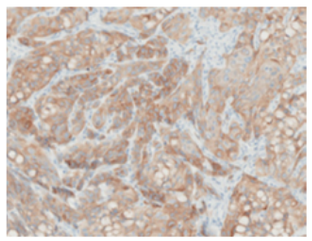
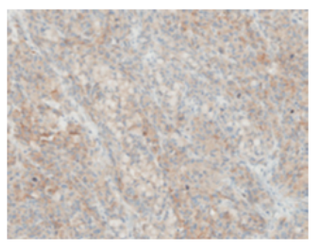
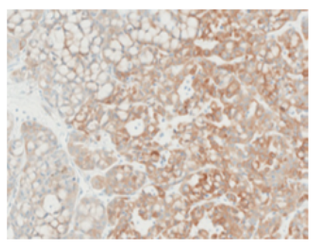
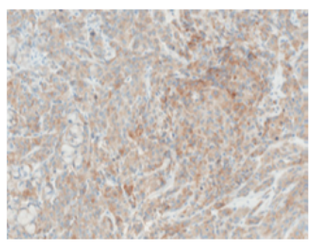
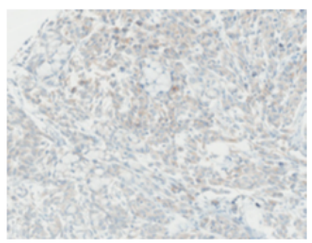
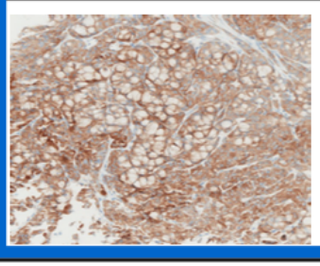
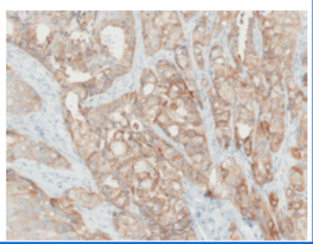
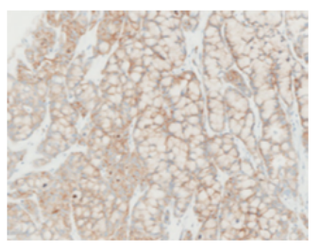
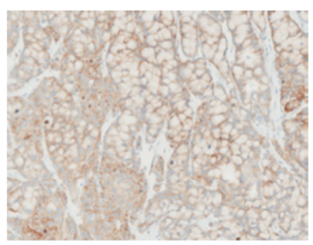
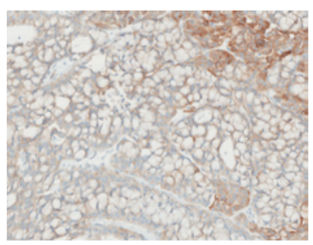
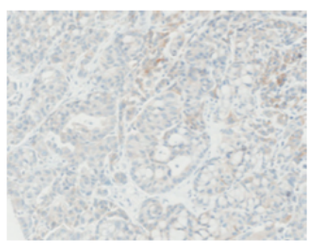
NBF 24 hrs

NBF 6 hrs

Impact of Pre-Analytical Conditions on VENTANA anti-ALK (D5F3) IHC Assay Staining

Fixative Recommendations to Achieve Optimal Staining Results with VENTANA anti-ALK (D5F3) IHC Assay

VENTANA anti-ALK (D5F3) IHC Assay Staining of Tissue Across Fixatives and Fixation Times

Fixation Time (Hrs)	Fixative					
	10% NBF	Zinc Formalin	PREFER	B5	AFA	95% Ethanol
1*						
8						Tumor cut through. No image available.
12						
24						
72						

Minimum Formalin Fixation Time for Consistent Estrogen Receptor Immunohistochemical Staining of Invasive Breast Carcinoma

Neal S. Goldstein, MD, Monica Ferkowicz, MT(ASCP), PathA(AAPA), Eva Odish, HTL(IHQ), Anju Mani, MD, and Farnaz Hastah, MD

Am J Clin Pathol 2003;120:86-92

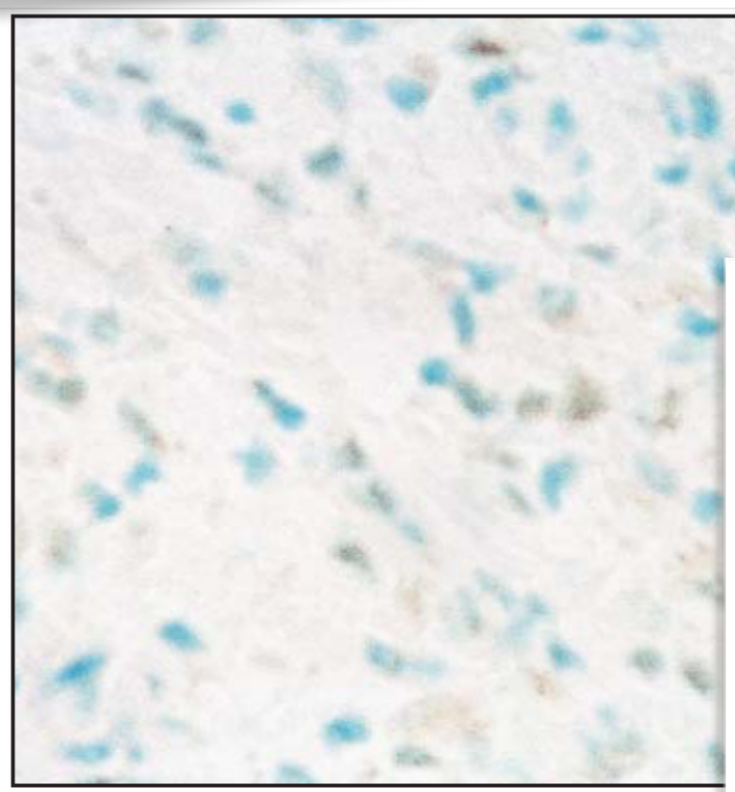


Image 1 Fixation, 3 h; antigen retrieval, 40 min.

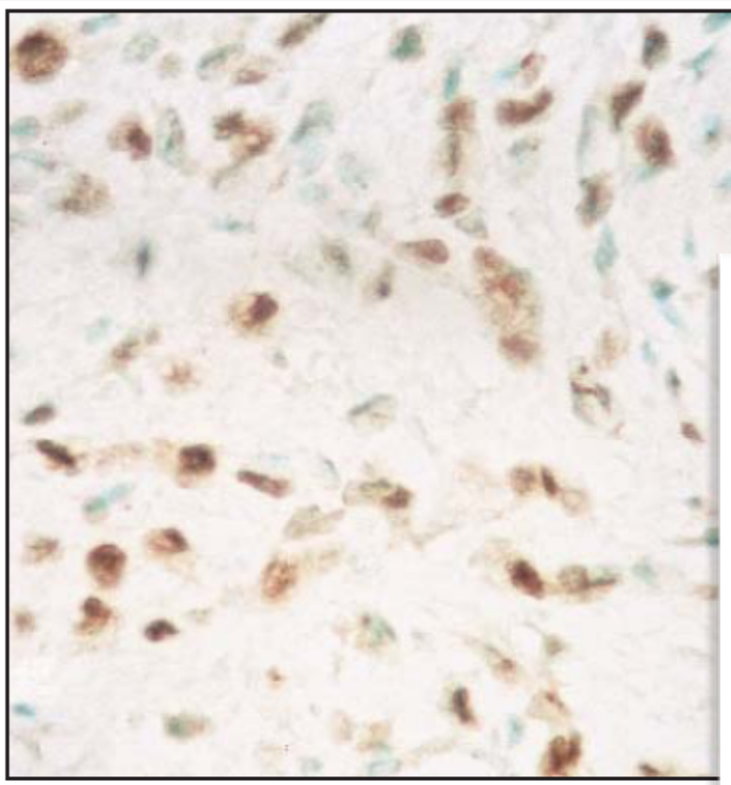


Image 2 Fixation, 6 h; antigen retrieval, 40 min.

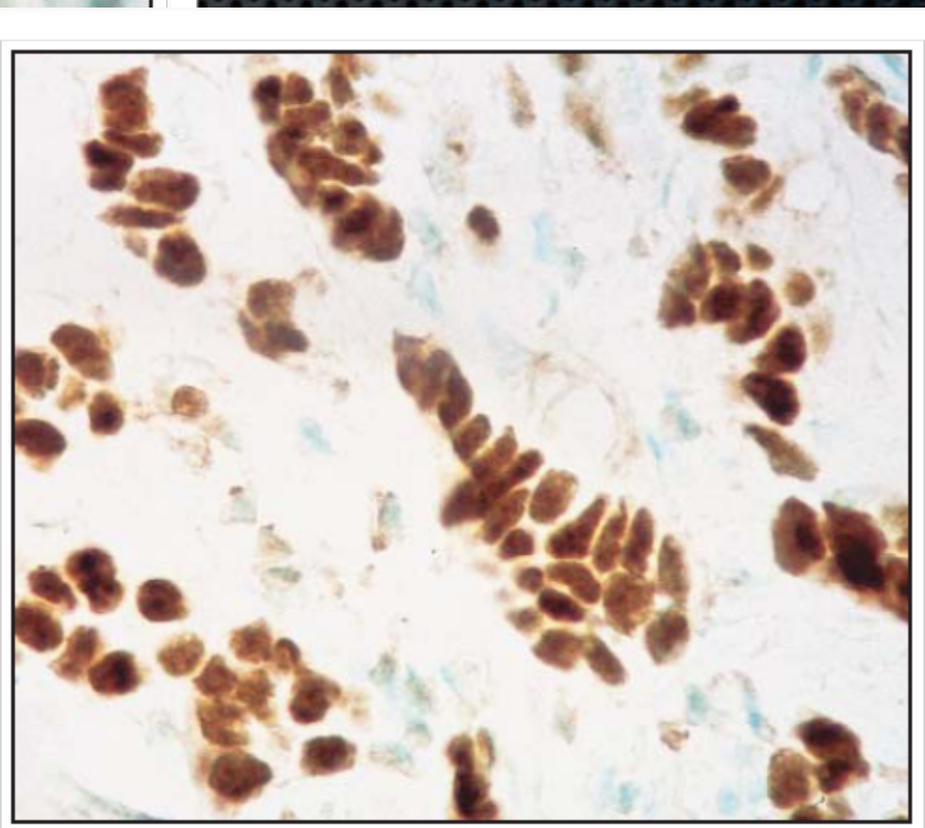


Image 3 Fixation, 8 h; antigen retrieval, 40 min.

"The minimum formalin fixation time for reliable immunohistochemical ER results is 6 to 8 hours in our laboratory, regardless of the type or size of specimen".

(*Am J Surg Pathol* 2011;35:545–552)

The Effect of Prolonged Fixation on the Immunohistochemical Evaluation of Estrogen Receptor, Progesterone Receptor, and HER2 Expression in Invasive Breast Cancer: A Prospective Study

Leung Chu Tong, BA, MD, Nahid Nelson, BSc, PhD,† Jim Tsourigiannis, BSc, M1*
and Anna Marie Mulligan, MB, MSc, FRCPath†*

TABLE 1. Antibodies and Conditions of Use

	Clone	Source	Antigen Retrieval Time
ER	SP1 (Monoclonal)	Ventana	30 min
PR	1E2 (Monoclonal)	Ventana	60 min
HER2	A0485 (Polyclonal)	DAKO	30 min

Fixation i 4% NBF for 13 hours versus 79 hours

Concordance between short fixation and long fixation:

99 % Concordance for ER

95 % Concordance for PR

98 % Concordance for HER2

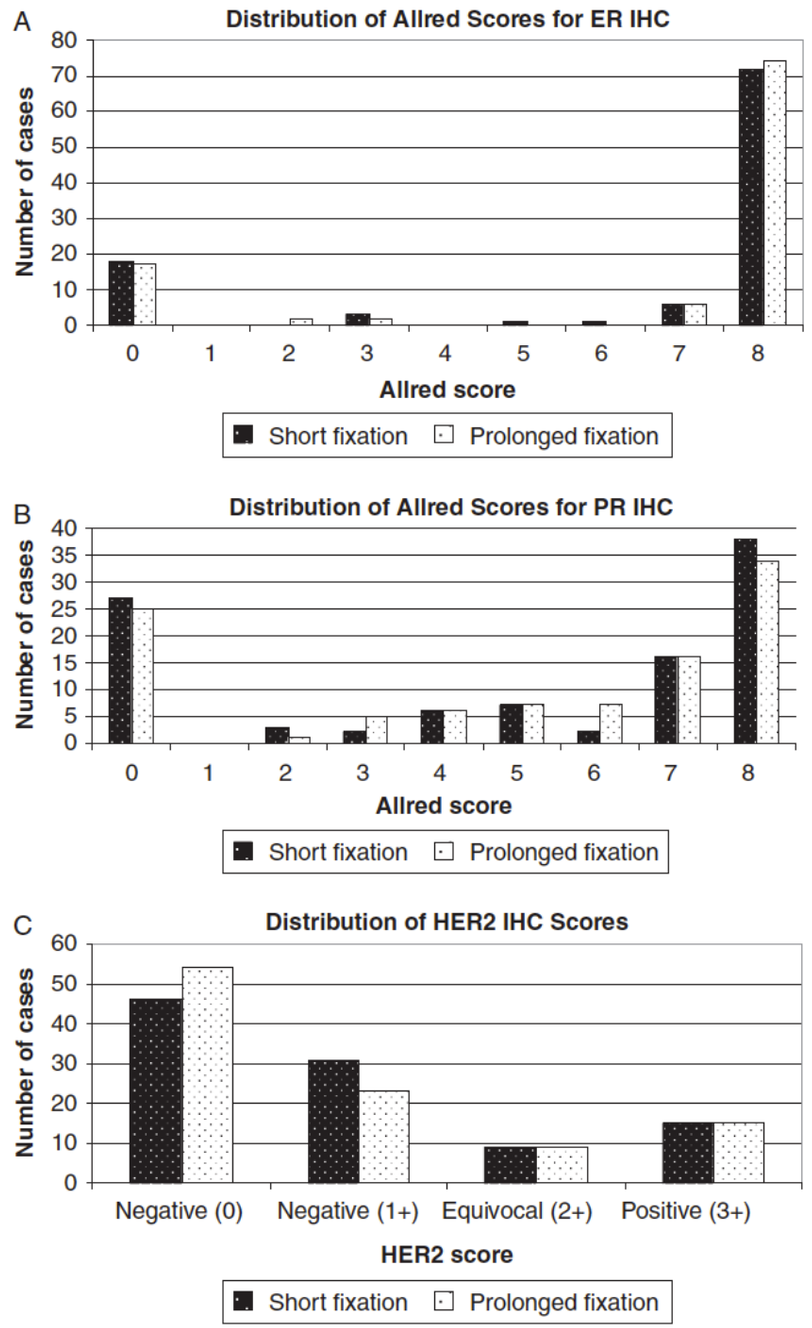
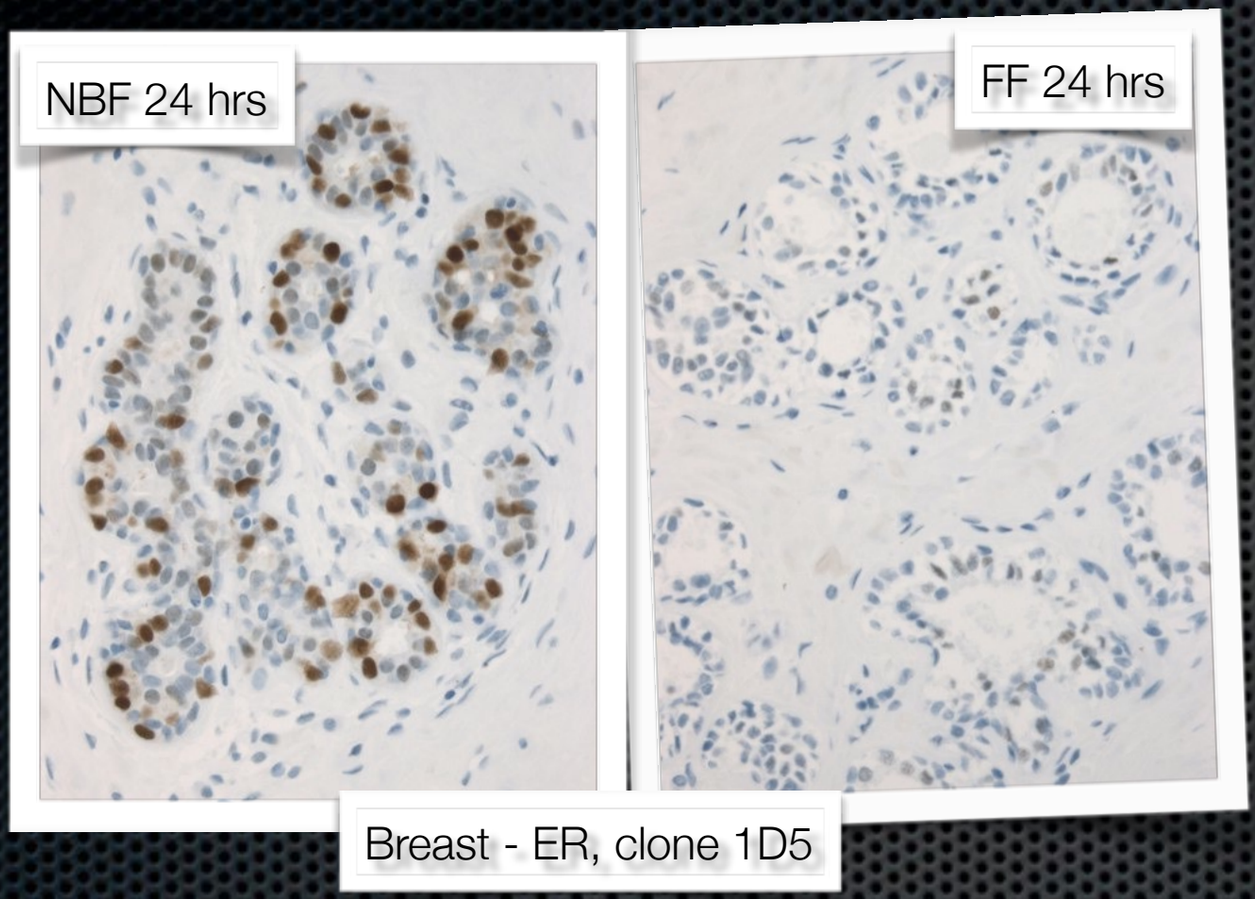
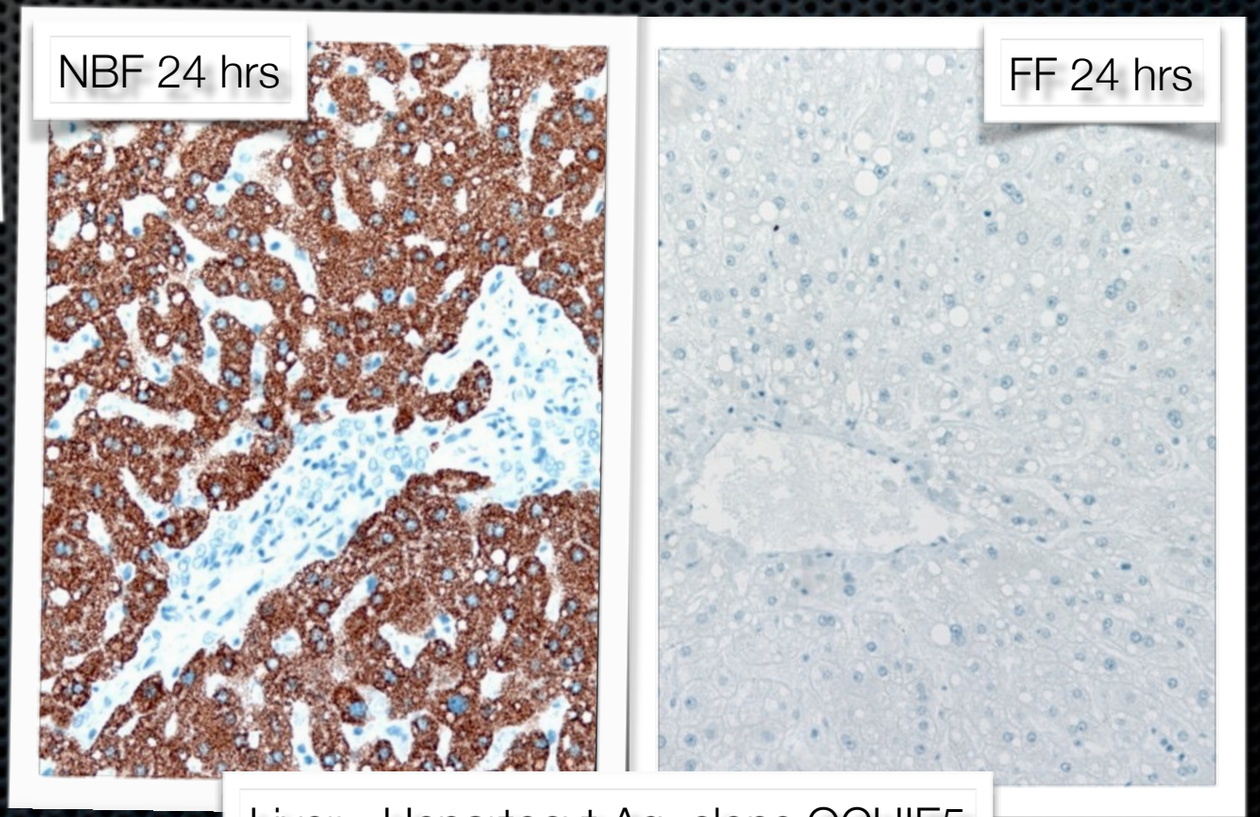


FIGURE 1. Distribution of (A) Allred scores for ER staining ($P = 1.0$), (B) Allred scores for PR staining ($P = 0.8625$), and (C) HER2 scores ($P = 1.0$) in the SF and PF groups.

4% NBF versus FineFix



Breast - ER, clone 1D5



Liver - Hepartocyt Ag, clone OCHIE5

"With existing IHC-protocols 35% (9 of 26) of the antibodies gave poor or borderline reactions on tissues fixed in FineFix"

(Unpublished data)

Alternatives to 4% NBF...

Name	Contains...	Company
F-solv	Denat. EtOH / Aldehyde derivate / Stabiliser	Yvsolab
UPM	Ethanol / Methanol / 2-Propanol / Formaldehyde	Copan
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
Pen-Fix	Formaldehyde / Ethanol / Buffer	Richard-Allen
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

PAXgene Tissue
New Tissue Fixation/Stabilization Technology

- Development began in 2007:
  - >1,500 compounds and combinations screened
  - >8,000 tissue samples tested to date
- Technology requirements
  - Histomorphology must be equivalent to FFPE tissue
  - RNA, DNA, miRNA must be preserved and of high quality
- Two-reagent system finalized in 2009
  - Fixation and stabilization reagents, both formalin-free
- First collection device
  - Container with two chamber one closure
- Under evaluation within SPIDIA

- Consortium: 7 public research organizations, 8 companies, 1 standards organization (CEN)
- Coordinator: QIAGEN GmbH

PreAnalytiX
A QIAGEN / BD Company

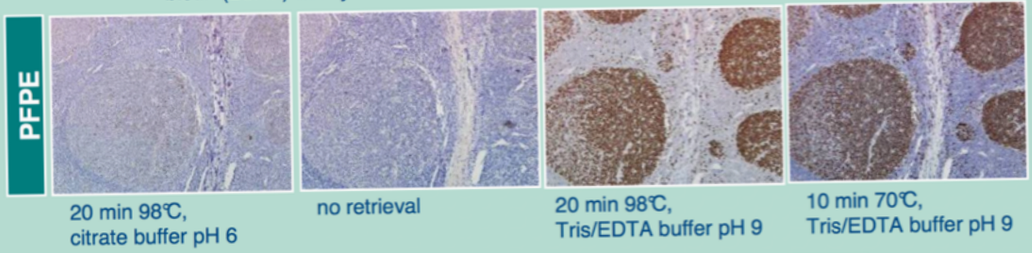
Summary
PAXgene Tissue …

- … is a standardized system for tissue fixation, stabilization and biomolecule purification.
- … preserves histomorphology and biomolecules.
- … works without crosslinking and chemical modification.
- … treated tissue can be stored within the stabilization reagent, or after processing.
- … results in comparable morphology but superior molecular results
- … requires protocol adaptations for immunhistochemistry staining

PAXgene Tissue enables multimodal analysis of biomolecules from the same sample, which is used for morphological analysis

Immunhistochemistry
Ki67 - Optimization of Epitope Retrieval

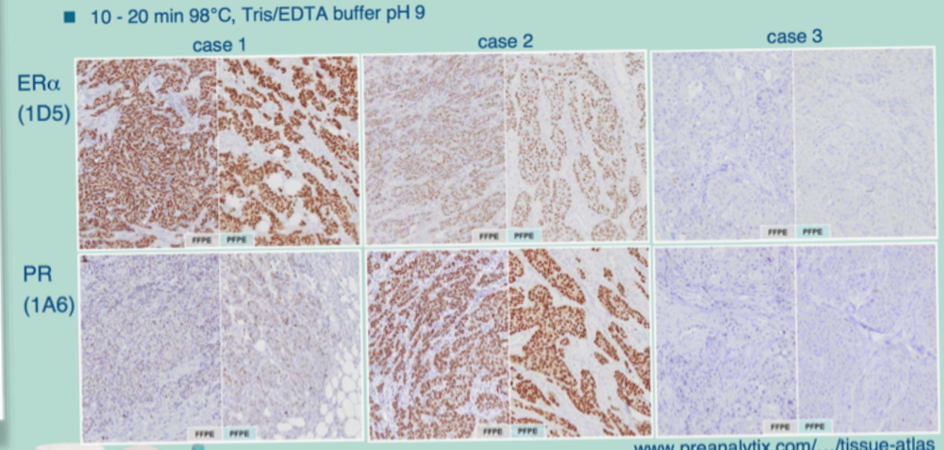
- Human tonsil
- Ki-67, clone MIB-1, Labelled streptavidin-biotin (LSAB) assay



PreAnalytiX
A QIAGEN / BD Company

Immunhistochemistry
Estrogen and Progesteron Receptor

- Human breast cancer cases (Cureline)
- Labelled streptavidin-biotin (LSAB) assays
- 10 - 20 min 98°C, Tris/EDTA buffer pH 9



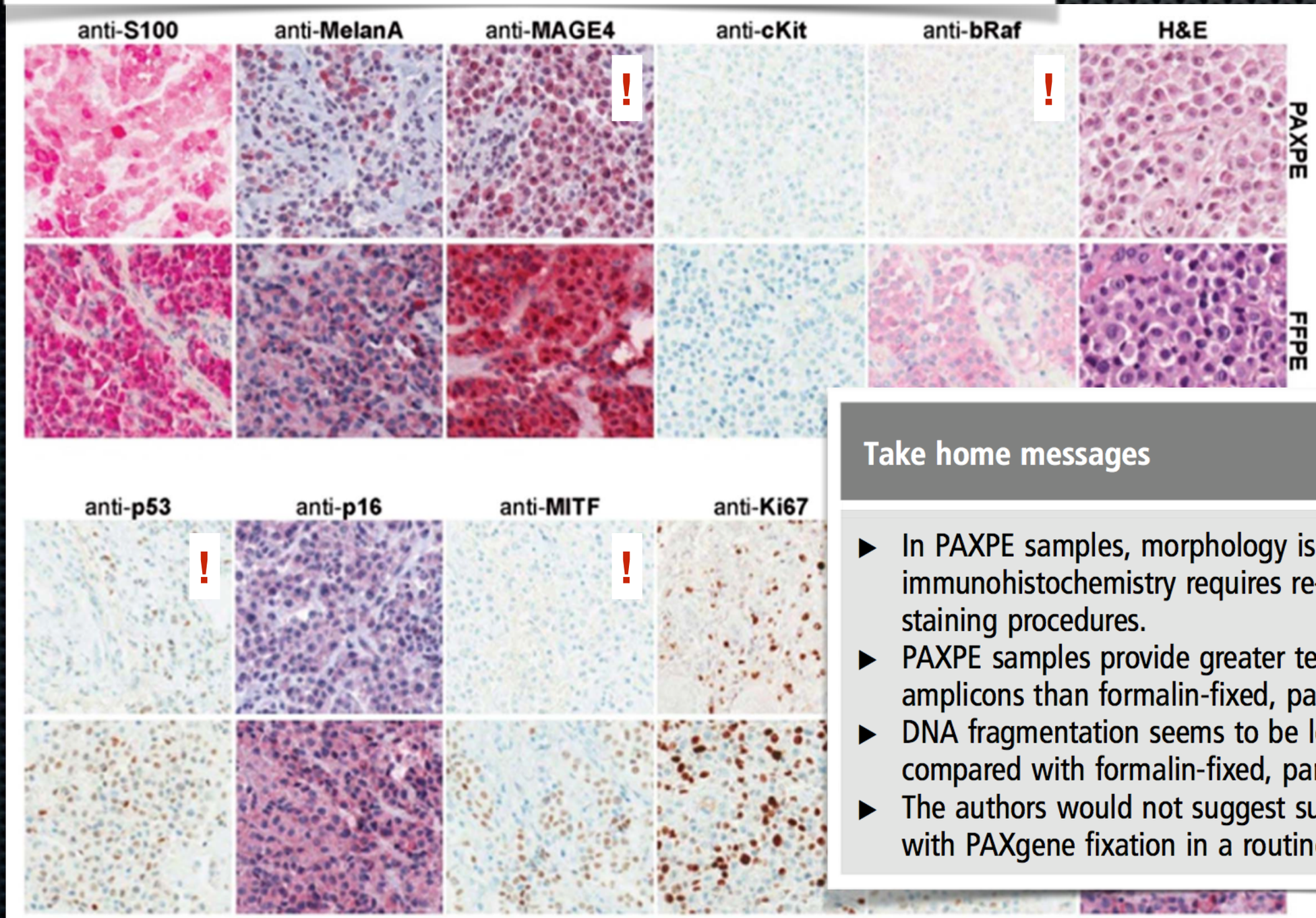
www.preanalytix.com/…/tissue-atlas

PreAnalytiX
A QIAGEN / BD Company

Will PAXgene substitute formalin? A morphological and molecular comparative study using a new fixative system

Benedetta Belloni,[1] Chiara Lambertini,[2] Paolo Nuciforo,[2] Jay Phillips,[3] Eric Bruening,[3] Stephane Wong,[3] Reinhard Dummer[1]

J Clin Pathol 2013;**66**:124–135.



Morphology was well preserved in PAXPE samples. However, 5 out of 11 IHC markers showed significantly lower overall staining and staining intensity with PAXPE tissues in comparison with formalin-fixed, paraffin-embedded (FFPE).

Take home messages

- In PAXPE samples, morphology is well preserved but immunohistochemistry requires re-evaluation of markers and staining procedures.
- PAXPE samples provide greater template integrity of mRNA amplicons than formalin-fixed, paraffin-embedded samples.
- DNA fragmentation seems to be lower in PAXPE samples compared with formalin-fixed, paraffin-embedded samples.
- The authors would not suggest substituting formalin fixation with PAXgene fixation in a routine pathology laboratory.

Choice of Fixative Is Crucial to Successful Immunohistochemical Detection of Phosphoproteins in Paraffin-embedded Tumor Tissues

(J Histochem Cytochem 57:257–264, 2009)

Janine A. Burns, Yuan Li, Carol A. Cheney, Yangsi Ou, Laura L. Franlin-Pfeifer, Nelly Kuklin, and Zhi-Qiang Zhang

Department of Biologics Research, Merck Research Laboratories, West Point, Pennsylvania

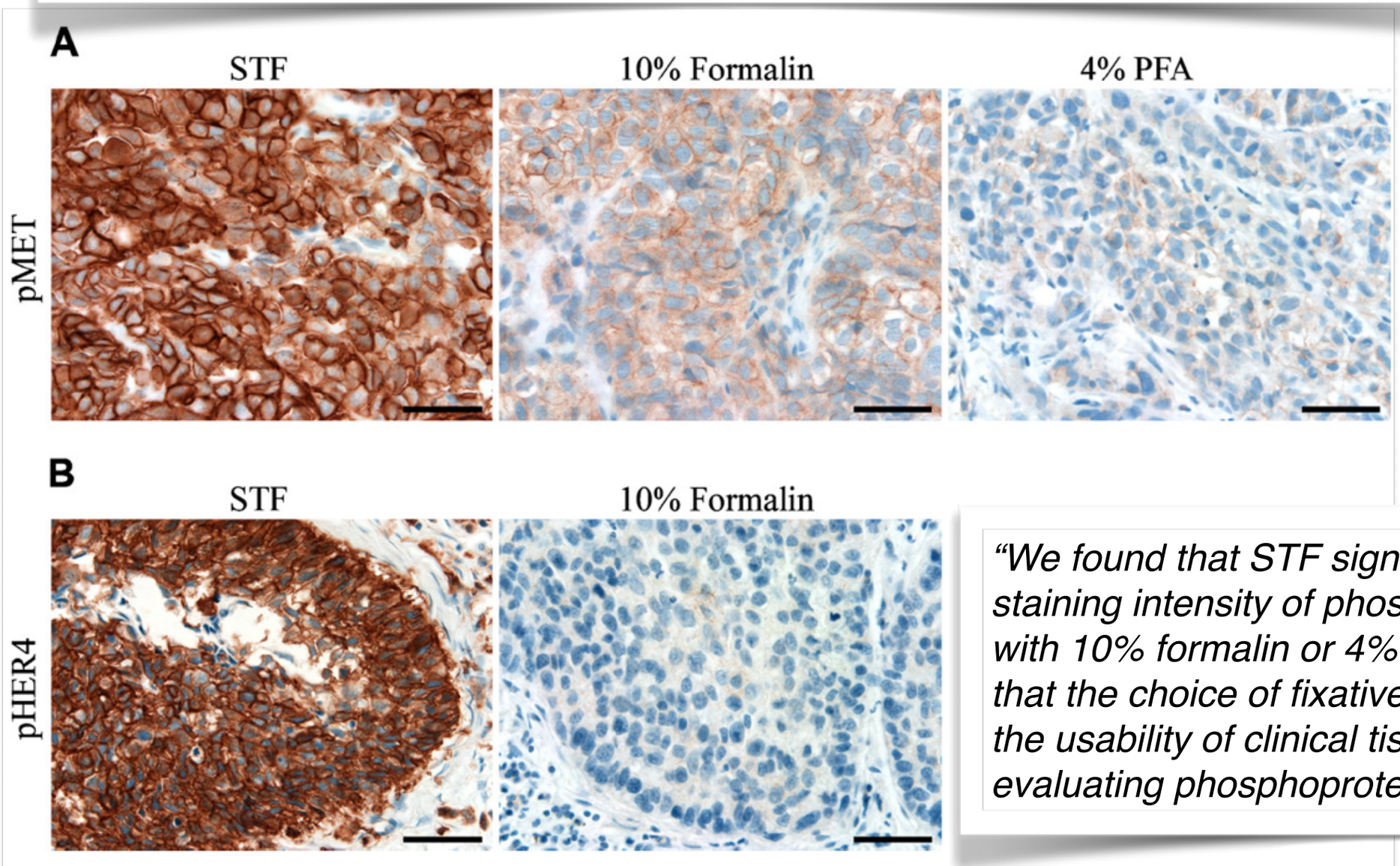


Figure 2 IHC staining of phosphoproteins in xenograft and human clinical tumor tissues. (**A**) SKOV-3 xenograft tumor tissues fixed in Streck's tissue fixative (STF), 10% formalin, and 4% paraformaldehyde (PFA) were stained with anti-pMet antibody. (**B**) Human lung tumor tissue fixed in 10% formalin and STF were stained with anti-pHER4 antibody. Bar = 50 μm.

Streck's tissue fixative (STF)

"We found that STF significantly enhanced the staining intensity of phosphoproteins compared with 10% formalin or 4% PFA. Our results indicate that the choice of fixative could significantly affect the usability of clinical tissue samples for evaluating phosphoprotein by IHC".

OPEN ACCESS Freely available online

PLOS ONE

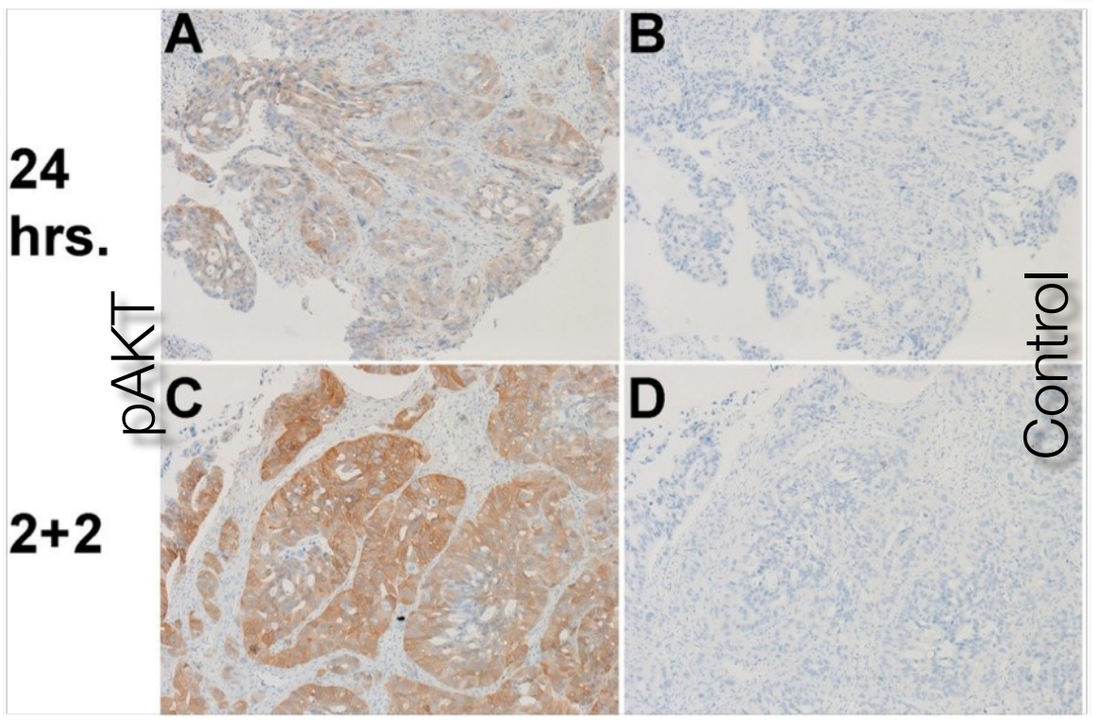
Rapid Two-Temperature Formalin Fixation

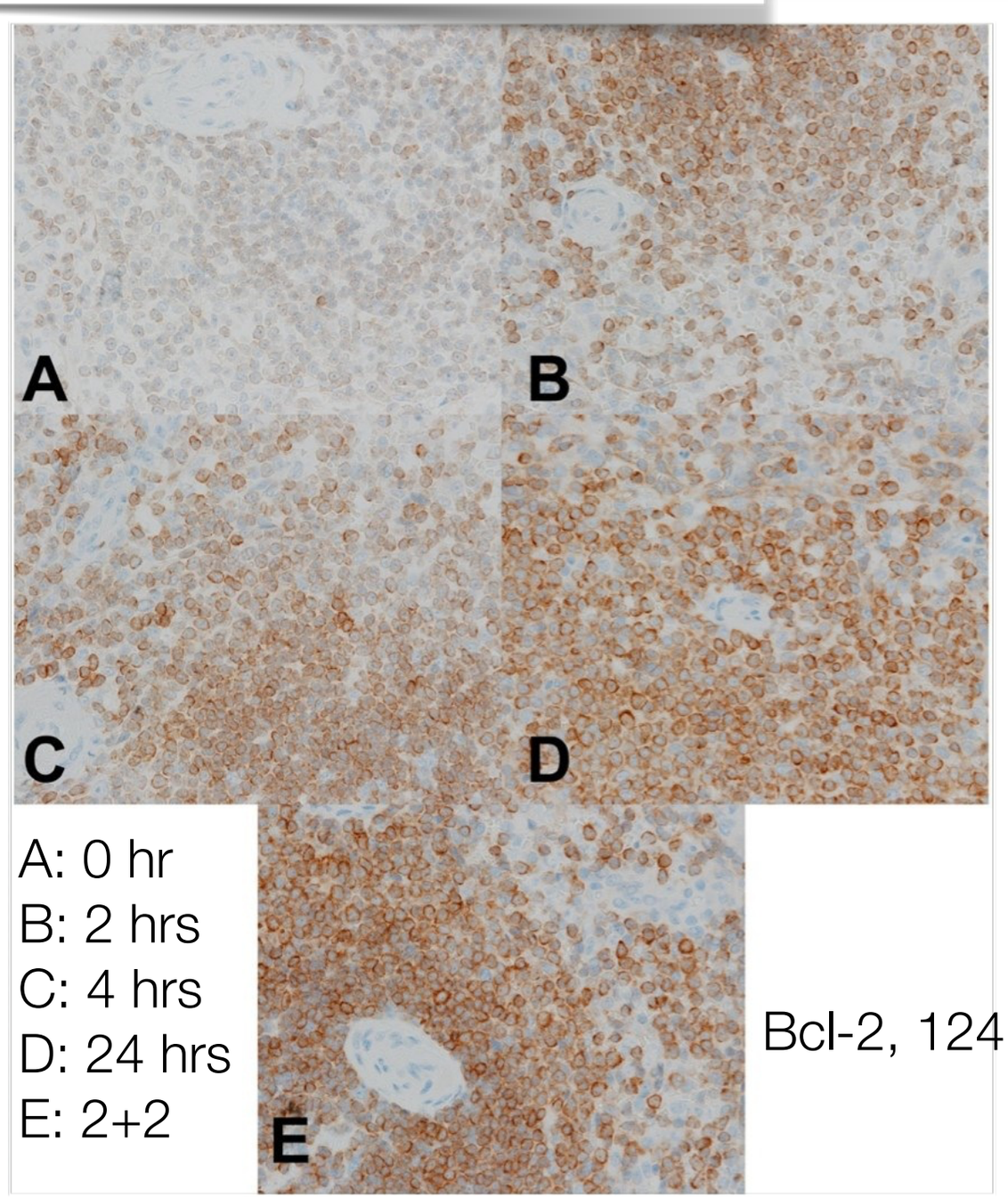
David Chafin[1☯], Abbey Theiss[1☯], Esteban Roberts[1], Grace Borlee[2¤], Michael Otter[1], Geoffrey S. Baird[2,3*]

1 Ventana Medical Systems, Inc., Tucson, Arizona, United States of America, **2** Department of Laboratory Medicine, University of Washington, Seattle, Washington, United States of America, **3** Department of Pathology, University of Washington, Seattle, Washington, United States of America

bcl-2 IHC after different fixation conditions

Control	No Pre-Soak				2 Hr Pre-Soak (4°C)			
RT 10% NBF	35° 10% NBF	40° 10% NBF	45° 10% NBF	50° 10% NBF	35° 10% NBF	40° 10% NBF	45° 10% NBF	50° 10% NBF
0 hr	0.5 hr	0.5 hr	0.5 hr	0.5 hr	0.5 hr	0.5 hr	0.5 hr	0.5 hr
2 hr	1 hr	1 hr	1 hr	1 hr	1 hr	1 hr	1 hr	1 hr
4 hr	2 hr	2 hr	2 hr	2 hr	2 hr	2 hr	2 hr	2 hr
8 hr	4 hr	4 hr	4 hr	4 hr	4 hr	4 hr	4 hr	4 hr
24 hr	6 hr	6 hr	6 hr	6 hr	6 hr	6 hr	6 hr	6 hr





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PLOS ONE

Immunohistochemistry of Colorectal Cancer Biomarker Phosphorylation Requires Controlled Tissue Fixation

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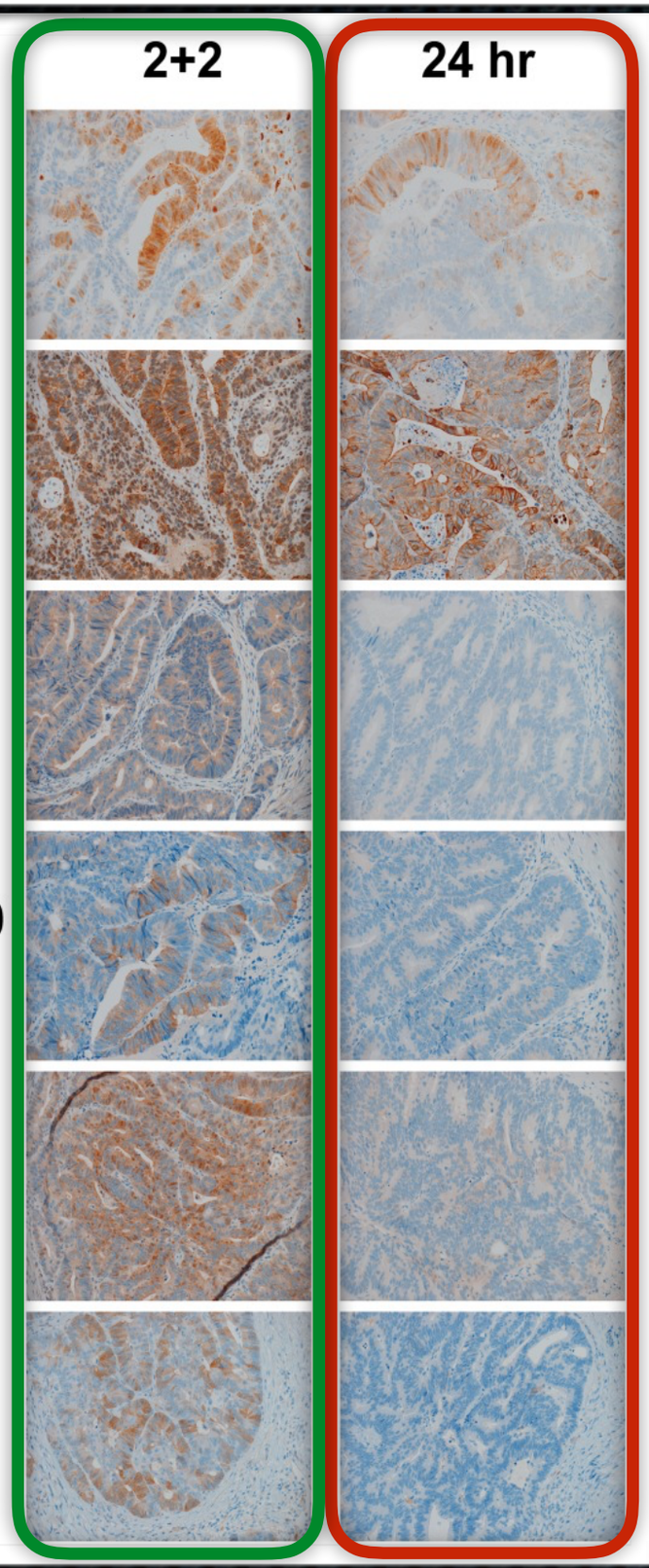
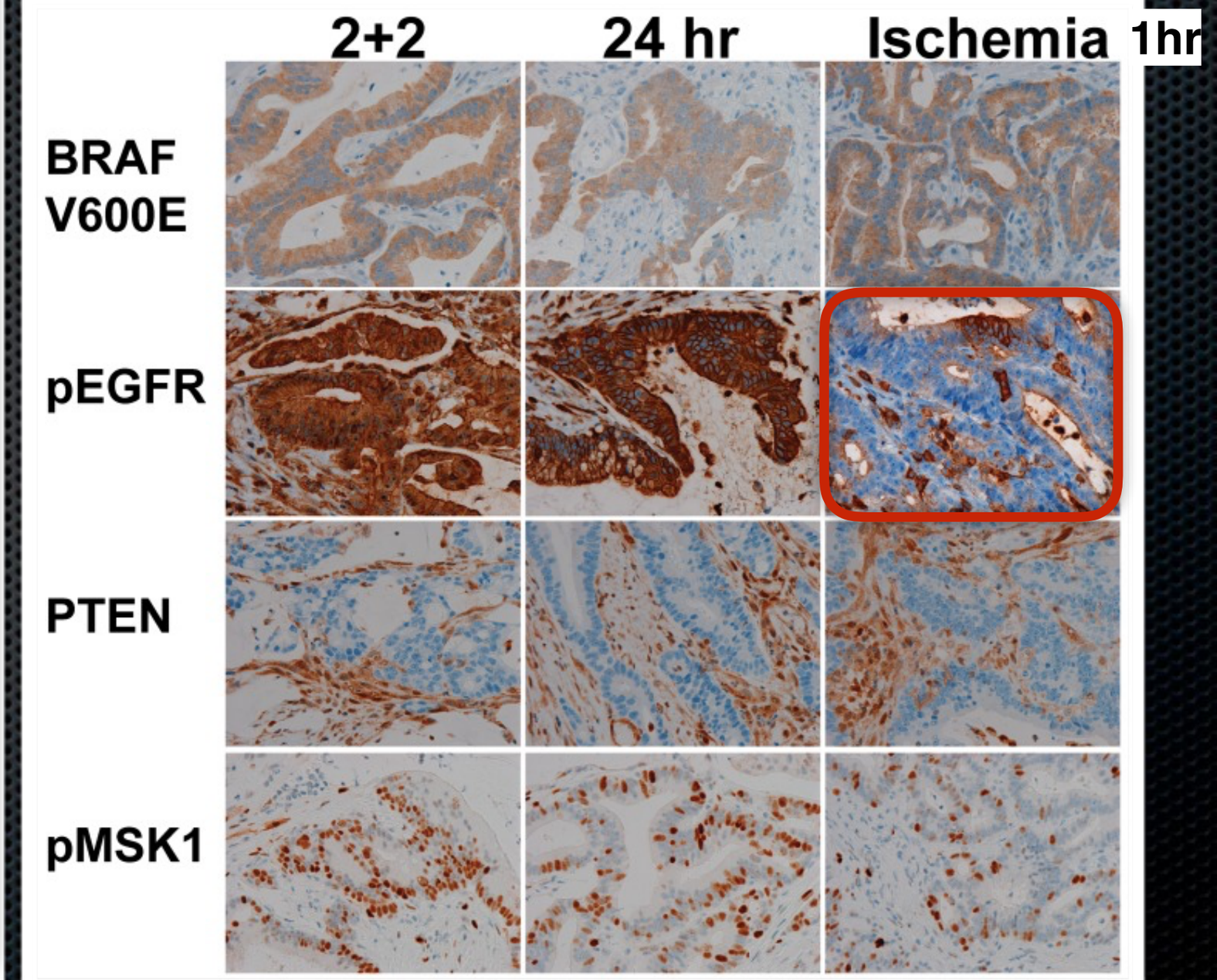




NBF fixation at 4°C ? - A practical solution ? 🤔

Evening Standard

LONDON, TUESDAY, 27 APRIL 2004 www.thisislondon.co.uk Incorporating THE EVENING NEWS 40p

Self-chilling beer

The can that turns into a mobile fridge

IT IS every beer lover's summer nightmare — stuck in the middle of a park with the sun warming your drink. Thankfully, scientists have come up with a solution: the self-cooling beer can.

Slightly longer than a normal drink can, it simply needs a twist to cool its content down. It can, its inventors claim, cool a beer to the perfect temperature of 3C within three minutes.

The I C (Instant Cool) Can works by using water evaporation. The top half is surrounded by a layer of watery gel. The base contains a water-absorbing material in a vacuum, and a special heat-absorbing chamber.

When the bottom is twisted, a seal between the two halves is broken. The vacuum draws the gel, and the heat, into the base. The gel is absorbed by the material, the heat is absorbed by the chamber — and the drink gets cold.

Trials showed that on average, the temperature of the liquid in the can fell 16C when the system was activated. Drinks stayed cool for up to an hour. The cans are already being tested by two large British brewers, who hope to have them on sale before the end of the summer.

According to Barney Guarino, chief executive of Tempra Technology, the company that developed the can,

By Mark Prigg
Science Correspondent

Britain is likely to get the te… first. "We are talking to seve… brewers, and we think we will … cans on sale before the end of … "We expect to do one final test … summers, then launch very soon …

The more expensive beers … tails are likely to be the first … from cooling cans, as — acco… Mr Guarino — consumers are … used to paying higher prices fo…

"All the market research we h… shows consumers don't mind … premium prices for this," he … "There simply aren't the econo… scale to make it cheap. But t… happen over time, and we ha… cans will become commonplace …

The system may also be devel… the hugely popular alcopop ma…

However one brewer was … technology could prove too e… for the average consumer.

A spokesman for Scottish … which brews beers including … Kronenbourg and John Smith's … would be unlikely to use the s… the present time. "The price p… is a problem, but this is an area … looking at for the future," he …

HOW IT WORKS

1. Twist can base to break seal
2. This causes sudden drop in pressure
3. That leads to evaporation of gel
4. The energy required results in rapid cooling of beer
5. Gel and heat absorbed in base

THE SELF-COOLING BEER CAN

After the widget, the next smart package for beer could be this cooling can from Tempra Technology. It is currently being tested by two UK brewers

The beer is surrounded by an outer compartment containing a watery gel

The bottom section, sealed from the top, contains a dessicant in a vacuum

The user twists the can to break the seal and the drop in pressure causes the water around the upper part to evaporate quickly, cooling the beer. The water is absorbed by the dessicant, and a sink absorbs the heat stopping the can feeling warm to the touch

The beer can be cooled by 16.7 °C in three minutes, the company claims

The beer can be cooled by 16.7 °C in three minutes, the company claims

Fixation

Preanalytic variable	Published Guidelines and Recommendations ASCO/CAP | CLSI	Literature-Based Recommendations
Fixative formula	**4% NBF** #	4% NBF
Time in fixative	**24 hrs***	24 hrs
Tissue to fixative ratio	**1:10**	1:1 to 1:20 **(1:2)**

Engel KB, Moore HM. Arch Pathol Lab Med. 2011;135:537–543

Arch Pathol Lab Med—Vol 131, January 2007

American Society of Clinical Oncology/College of American Pathologists Guideline Recommendations for Human Epidermal Growth Factor Receptor 2 Testing in Breast Cancer

Appl Immunohistochem Mol Morphol • Volume 16, Number 6, December 2008

Consensus Recommendations on Estrogen Receptor Testing in Breast Cancer By Immunohistochemistry

*6-48 hrs

*8-72 hrs 4% phosphate buffered formaldehyde, pH 7,0 - 7,4

4% NBF = 4% neutral buffet formaldehyde = 10% neutral buffet formalin

Decalcification

- Type
  - Strong acid (e.g. HCl)
  - Weak organic acid (e.g. formic acid)
  - Chelating agents (e.g. EDTA)
- Time, Temperature
- Time in fixative before decalcification

Call for a European programme in external quality assurance for bone marrow immunohistochemistry; report of a European Bone Marrow Working Group pilot study

J Clin Pathol 2009;**62**:547–551. doi:10.1136/jcp.2008.063446

E E Torlakovic,[1] K Naresh,[2] M Kremer,[3] J van der Walt,[4] E Hyjek,[5] A Porwit[6]

Take-home messages

- Immunohistochemistry tests are commonly if not regularly used in bone marrow trephine biopsies (BMTB) obtained for both primary and secondary bone marrow diseases, with or without morphological evidence of disease.
- Proficiency testing for BMTB immunohistochemistry (IHC) by extralaboratory quality assurance (EQA) programmes is not possible if the number of methods for tissue processing is not markedly reduced.
- The survey determined that almost all participants believed that their results were either "good" or "optimal" (90%) and that their daily QC/QA programmes were either "good" or "optimal" (93%); however, only 21% of laboratories were found to produce no poor results. This discrepancy is particularly important because it was shown with most commonly used IHC tests.
- The European Bone Marrow Working Group IHC Group is calling for a reduction in the number of methods used for BMTB processing and establishment of a unified EQA programme for BMTB IHC for all European countries.

Table 1 Tissue processing

Fixative	**n (28)**
10% NBF	15
5% NBF	1
B5	5
AZF	4
Schäffer's fixative	2
Burckhard fixative	1
F–G mixture	1
SUSA	1
Fixation time	**2 to >24 hours**
Exact and uniform	5
Variable	23
Decalcification	**n (28)**
None	2
EDTA	16
RDO (Rapid Decalcifier)	1
SUSA (acid)	1
10% nitric acid	1
Stieve solution*	1
Gooding and Stewart's†	4
Zenker/glacial acetic acid solution	1
Osteosoft	1
Decalcification time	**45 minutes to 3 days‡**
Exact and uniform timing	15
Variable	13

*Mercuric chloride/formaldehyde/acetic acid; †10% formic acid and 5% formaldehyde; ‡mostly depending of decalcifying reagent.
AZF, acetic acid–zinc–formalin; F–G mixture, formaldehyde–glutaraldehyde mixture; NBF, neutral buffered formalin; SUSA, sublimate mercury II chloride.

IHC and decalcification

- ✓Decalc™ (HCl-based)
- ✓Buffet formic acid (4M formic acid + 0.5M Na-formiat)
- ✓10% EDTA, pH 7

IHC and decalcification (2007)

24 hrs 4% NBF fixation prior to decalcification. 124 different antibodies on TMA's

Method \ Intensity	0/+	++	+++	++++
EDTA, 10% pH7	0	0	119	5
Formic acid (BFA)	2	13	103	6
Decalc™ (HCl)	101	21	2	0

Reference/No decalcification: ++++

Buffet formic acid (BFA): (4M formic acid + 0.5M Na-formiat)

IHC and decalcification (2007)

Antibody	Clone	Ref	Decalc	Formic	EDTA
Elastase, neutrophil , NP57	NP57	+++	0	0	+++
CD105, SN6h	SN6h	+++	0	+	+++
Bcl-2, 124 -Oncoprotein	124	+++	0	++	+++
Bcl-6, PG-B6p	PG-B6p	+++	0	++	+++
CD40, 11E9	1,1E+10	+++	0	++	+++
Factor XIII-a, poly		+++	+	++	+++
Oct-1, 12F11	12F11	+++	0	++	+++
Oct-2 (C20), poly		+++	0	++	+++
MUM1, MUM1p -Multiple Myeloma	MUM1p	+++	+	++	+++
Bob 1, TG14	TG14	+++	0	++	+++
CD4, 4B12	4B12	+++	0	++	+++
CD43, MT1	MT1	+++	0	++	+++
TCAR, BF1 -T-Cell Antigen	ßF1	+++	0	++	+++
CD16, 2H7- Fc Gamma Receptor	2H7	+++	0	++	+++
CD52, HI186	HI186	+++	0	++	+++

IHC and decalcification (2014)

24 hrs 4% NBF fixation prior to decalcification. 193 different antibodies on TMA's

Method \ Intensity	0/+	++	++(+)	+++	++++
EDTA, 10% pH7	0	0	5	185	3
Formic acid (BFA)	1	15	8	163	6
DecalcTM (HCl)	159	23	1	8	2

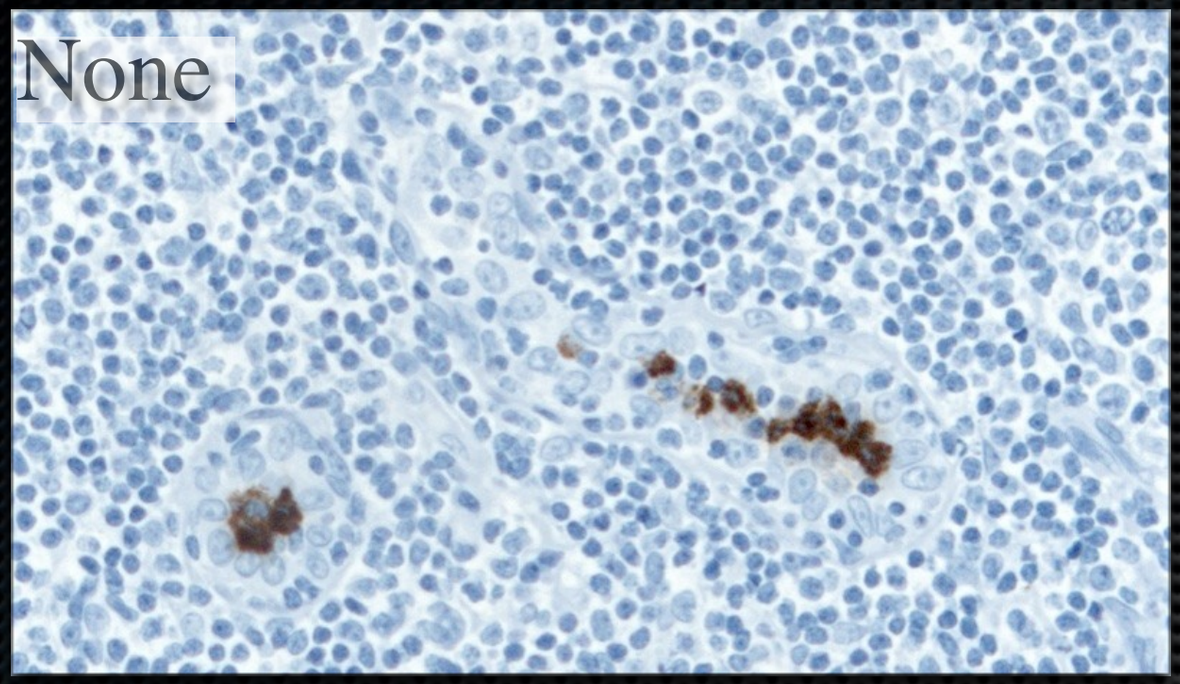
Reference/No decalcification: ++++

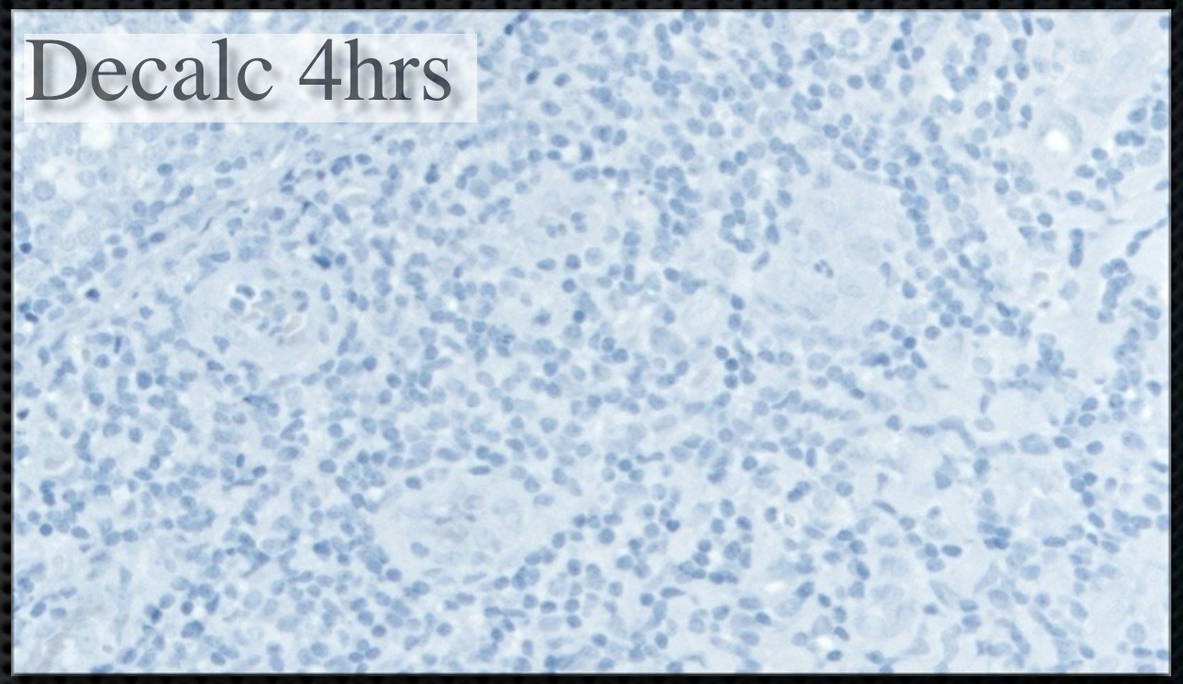
Buffet formic acid (BFA): (4M formic acid + 0.5M Na-formiat)

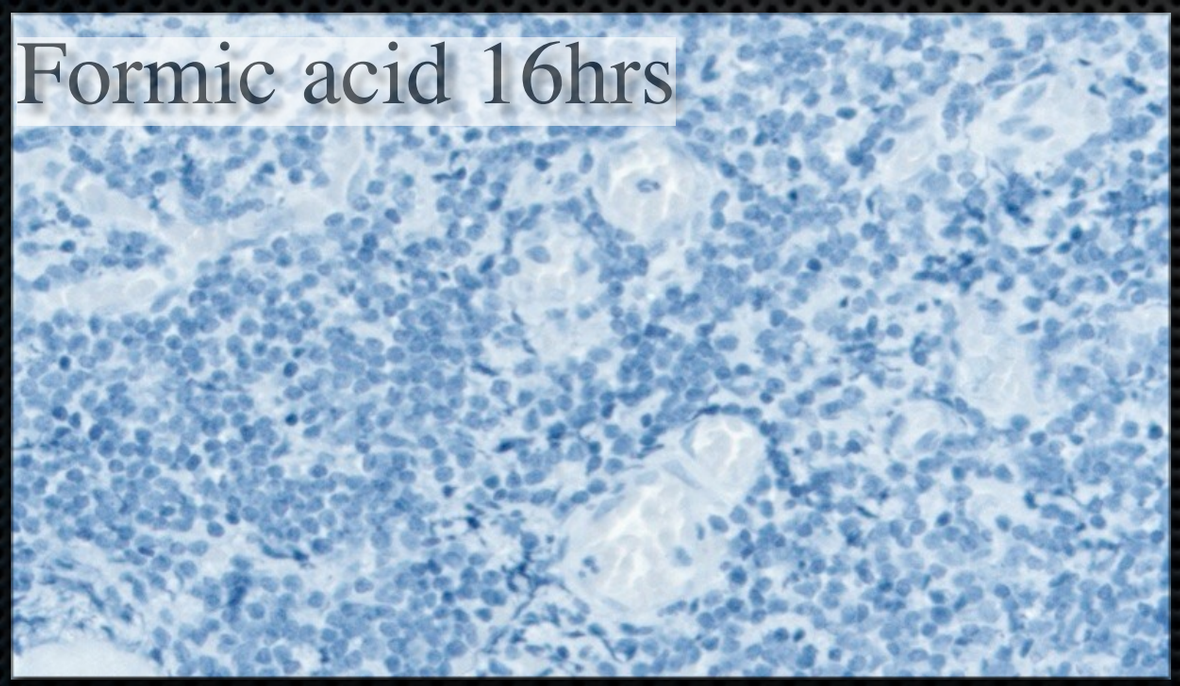
IHC and decalcification (2014)

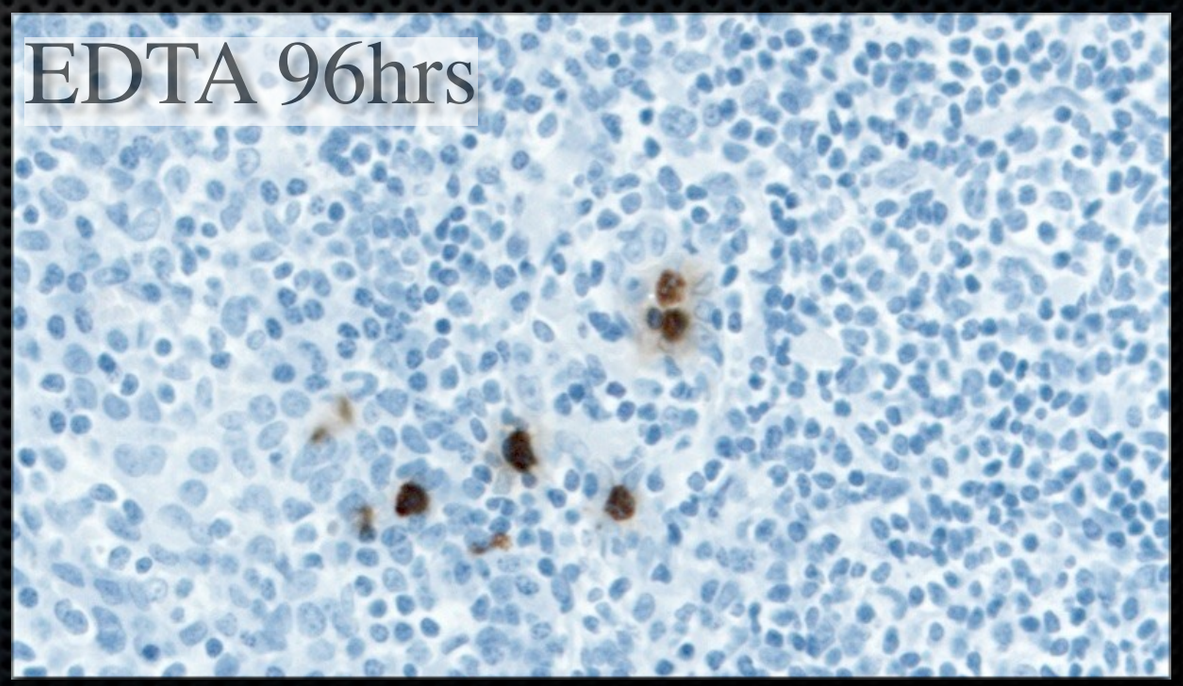
Antibody	Reference	DECAL	Formic	EDTA
CD303, 124B3.13	+++	+	+	+++
Makrofag, MAC 387	+++	0	++	++(+)
Bcl-2, 124 *	+++	0	++	+++
TCAR, BF1 *	+++	0	++	+++
Galectin-3, 9C4	+++	0	++	+++
Caveolin-1, 4D6	+++	0	++	+++
CD279, NAT105	+++	0	++	+++
Inhibin Alpha, R1	+++	0	++	+++
Bcl-2, E17	+++	0	++	+++
FOXP1, EPR4113	+++	0	++	+++
pHH3, E173	+++	0	++	+++
CD1a, EP3622	+++	0	++	+++
CD19, SP110	+++	0	++	+++
CD103, EPR4166(2)	+++	0	++	+++
CD123, 6H6	+++	0	++	++++
Neuroblastoma, NB84	+++	0	++/+	+++
MUM1, MUM1p *	+++	+	++(+)	++(+)
Podoplanin. D2-40 **	+++	+	++(+)	++(+)
Hairy Cell, DBA.44 **	+++	0	++(+)	+++
Oct-2 (C20), poly *	+++	0	++(+)	+++
CD27, 137B4 **	+++	0	++(+)	+++
CEA, Col-1	+++	0	++(+)	+++
NSE, H14	+++	+(+)	++(+)	+++
CD117, YR145	+++	++(+)	++(+)	+++

Decalcification and Elastase, neutrophilic, NP57

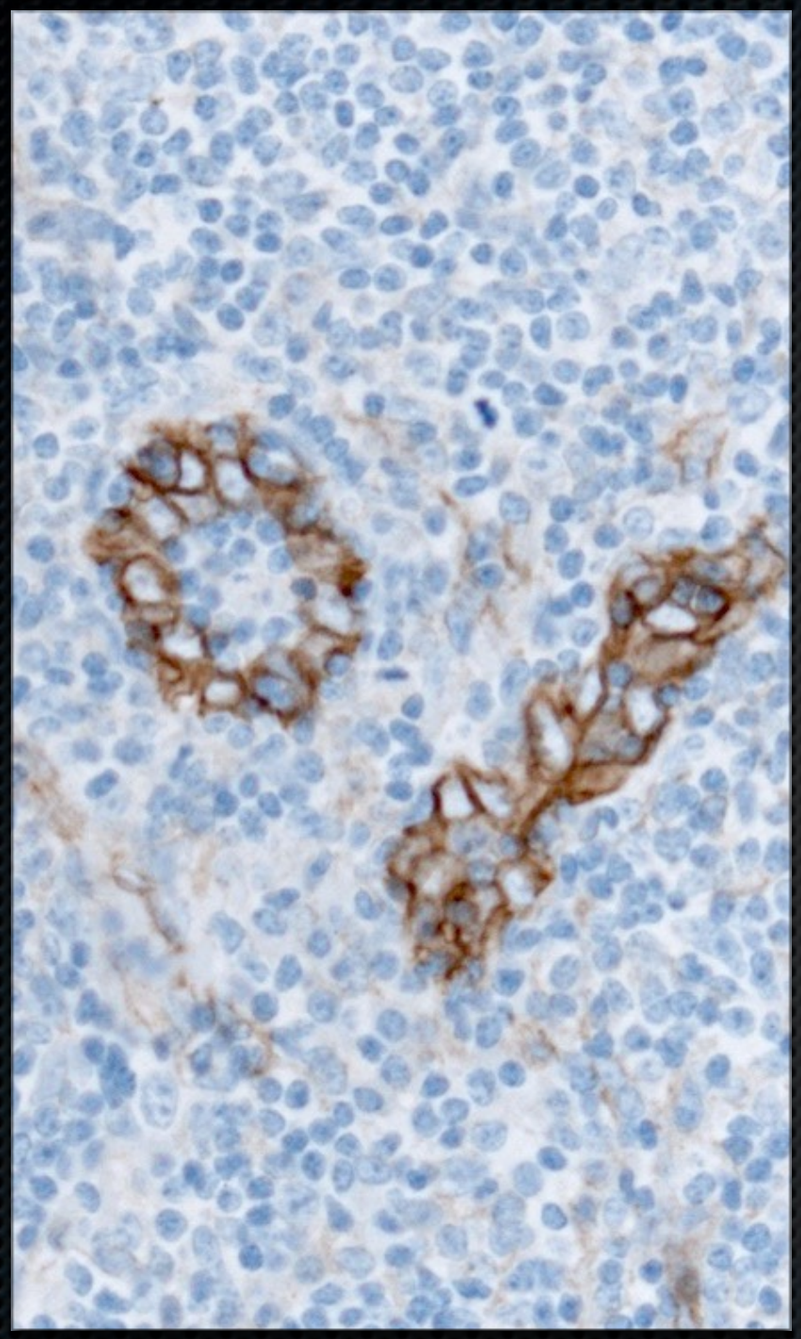




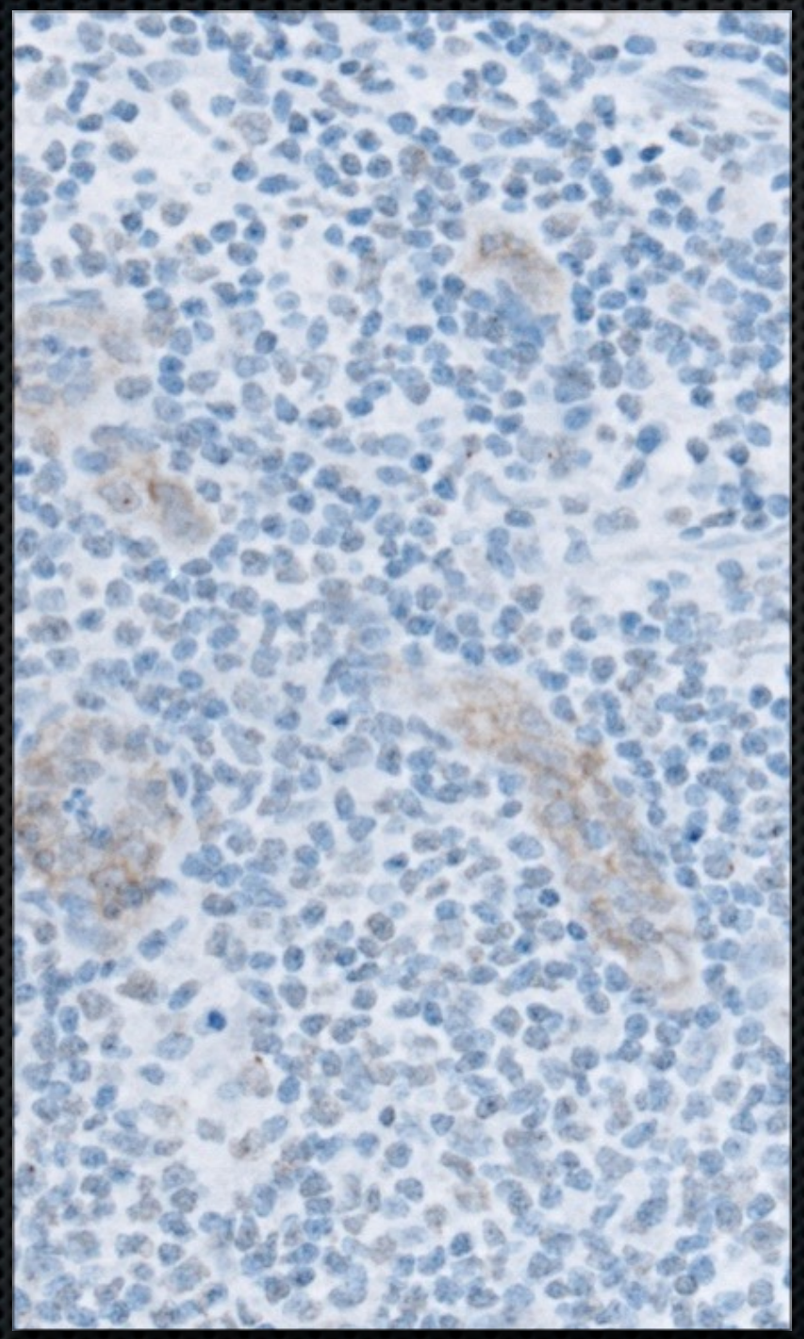




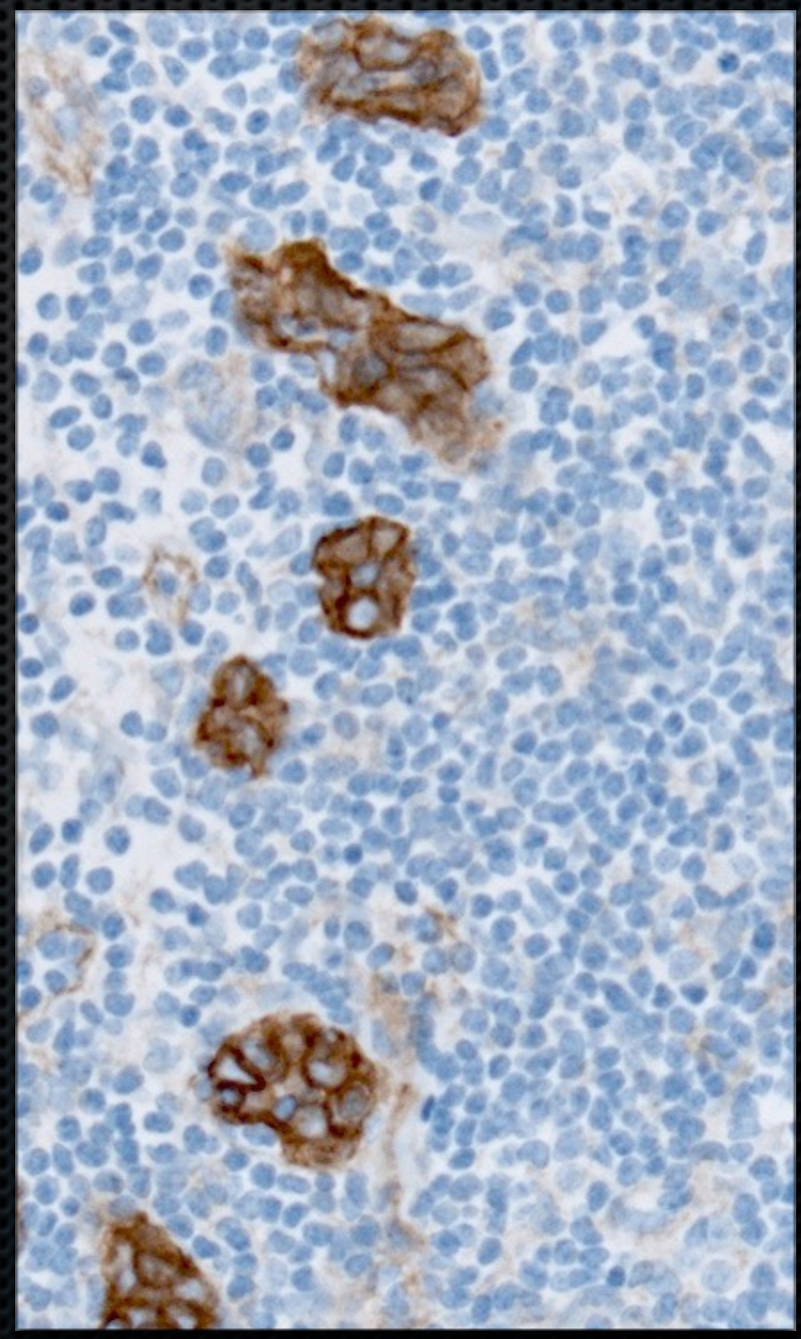
Decalcification and CD105, SN6h



No decalcification

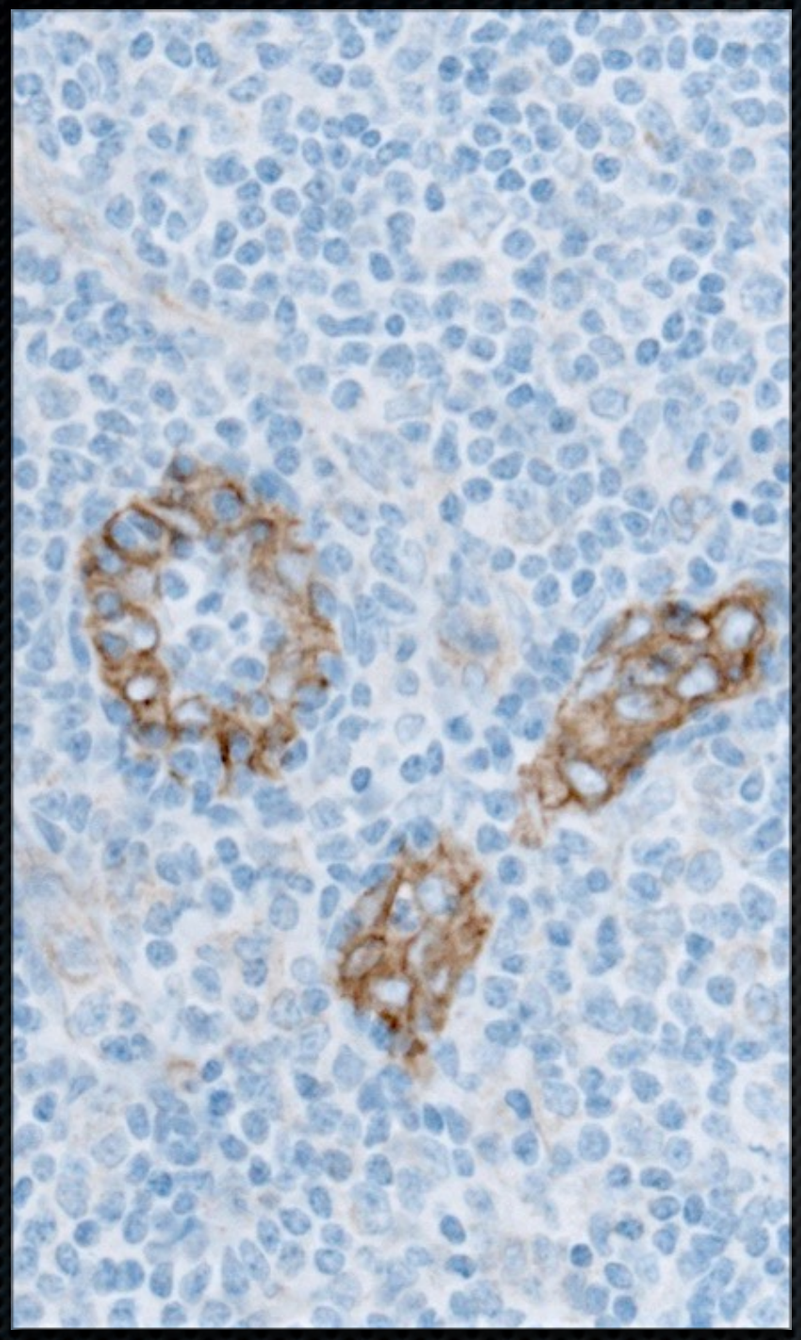


Formic acid 16hrs

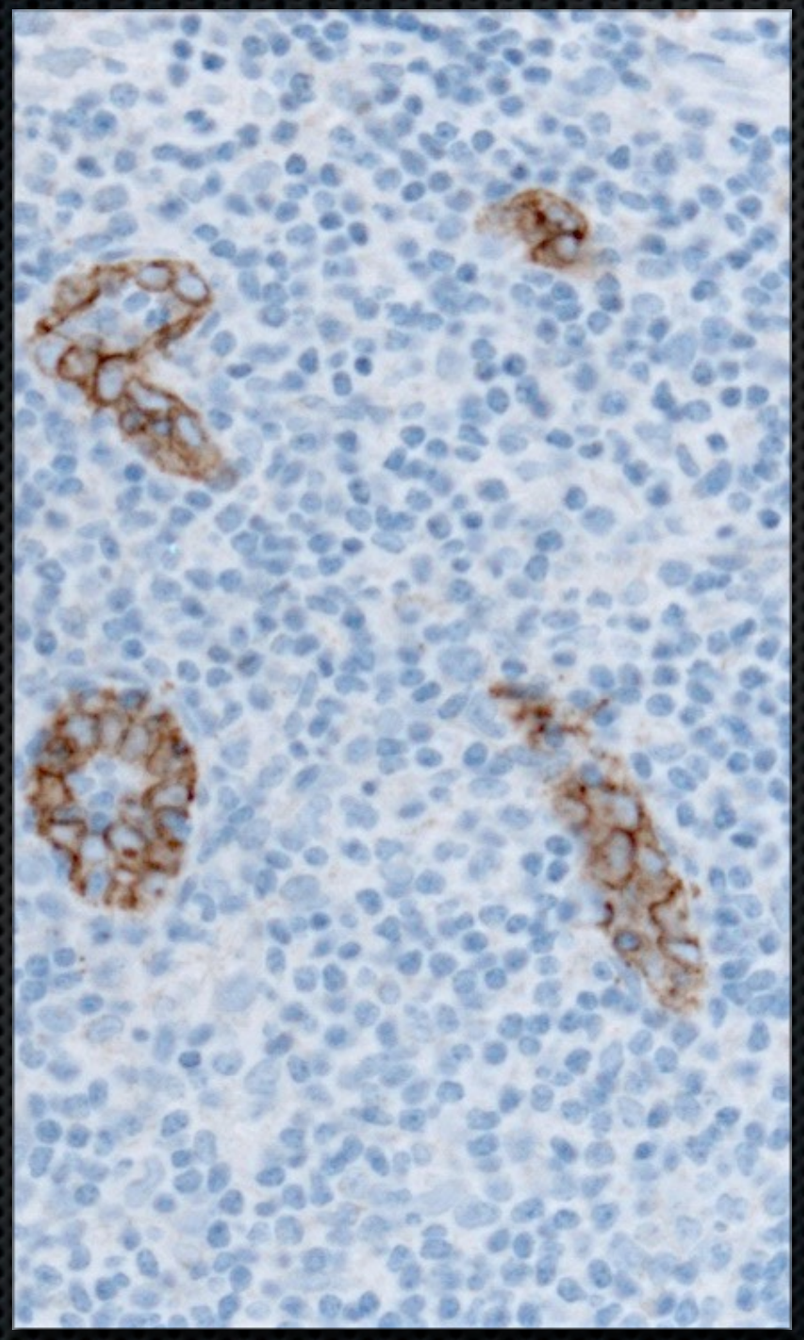


EDTA 96hrs

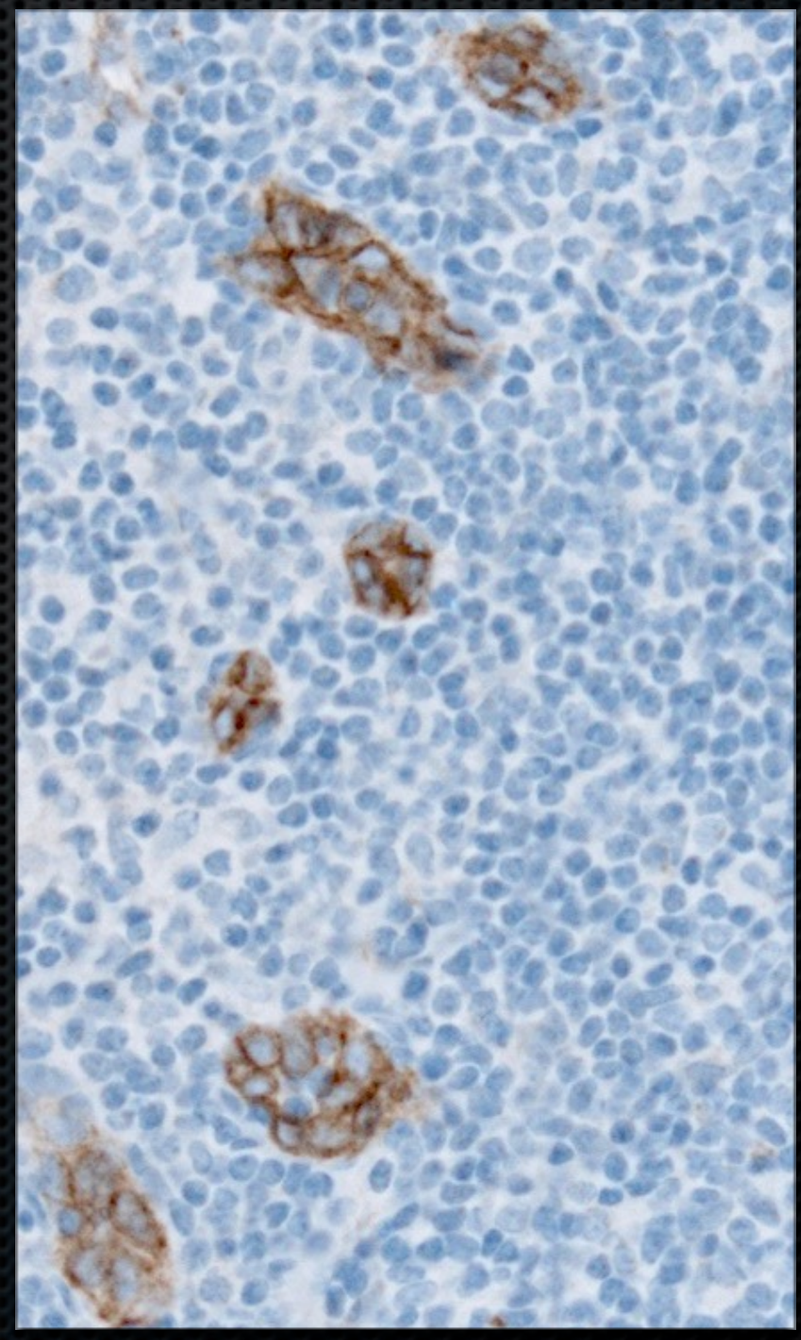
Decalcification and CD105, 4G11



No decalcification

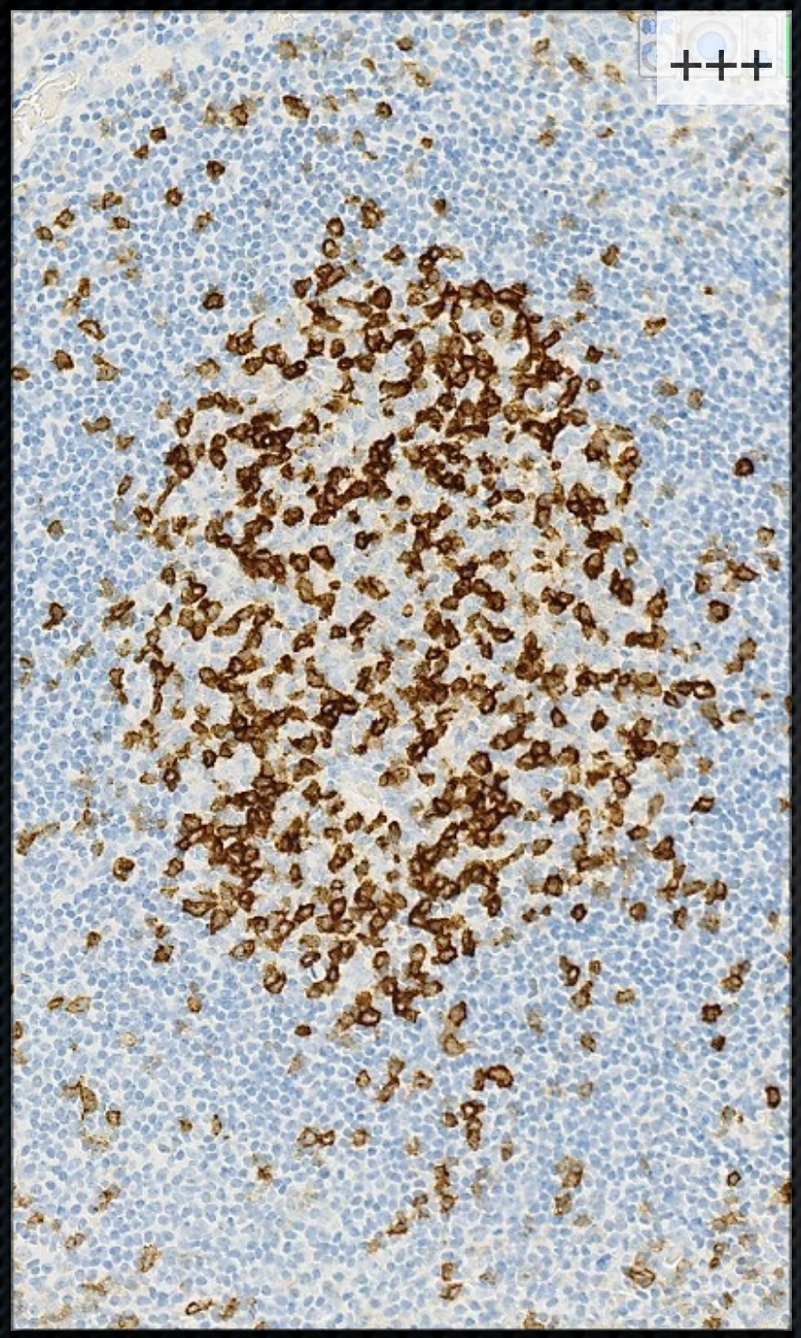


Formic acid 16hrs

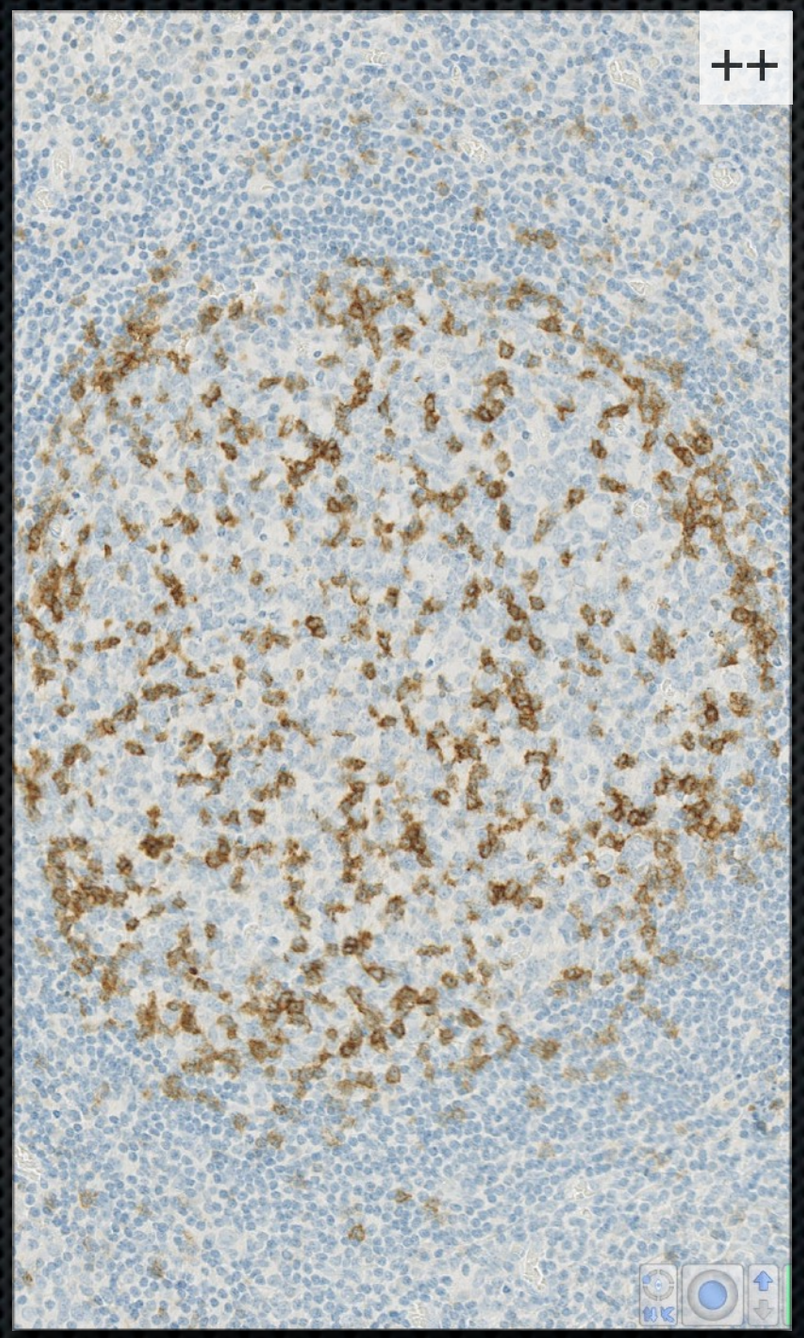


EDTA 96hrs

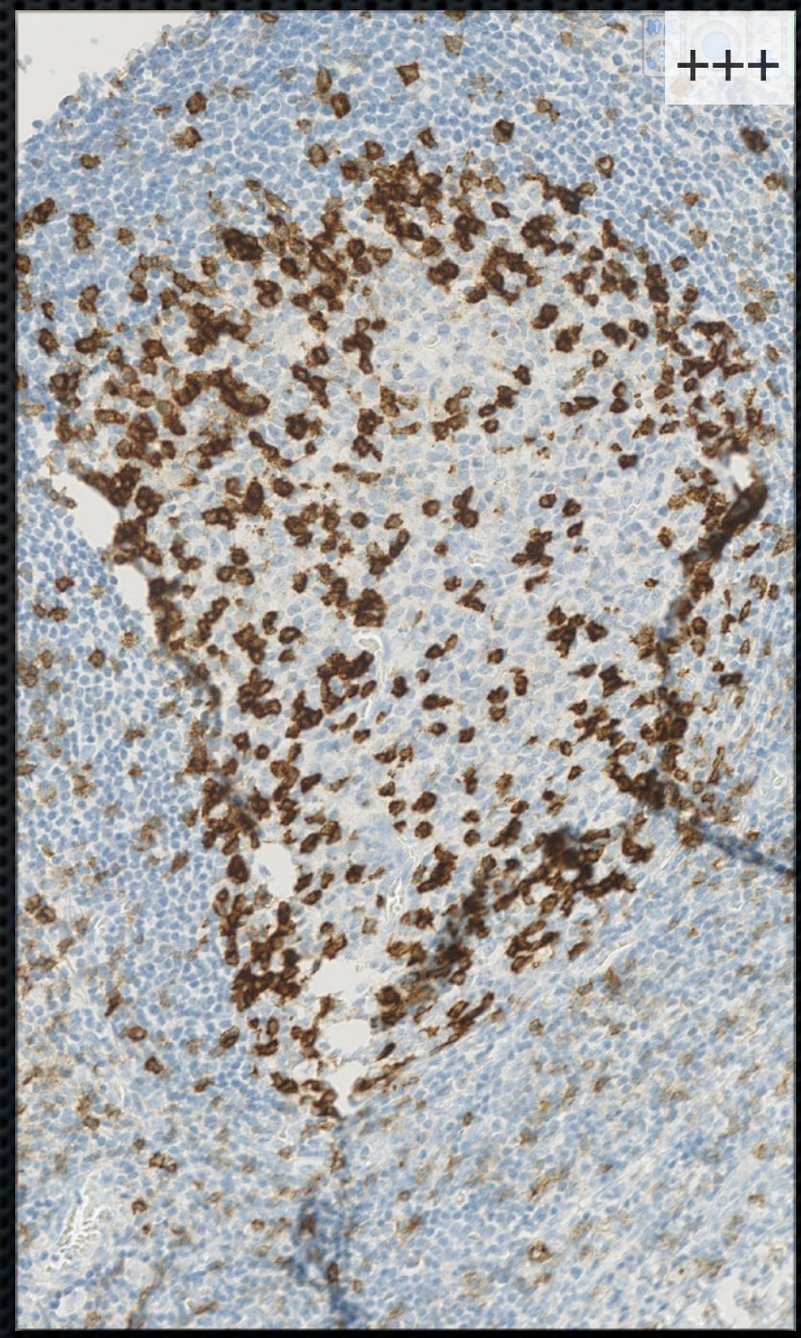
Decalcification and CD279 (PD-1), NAT105



No decalcification



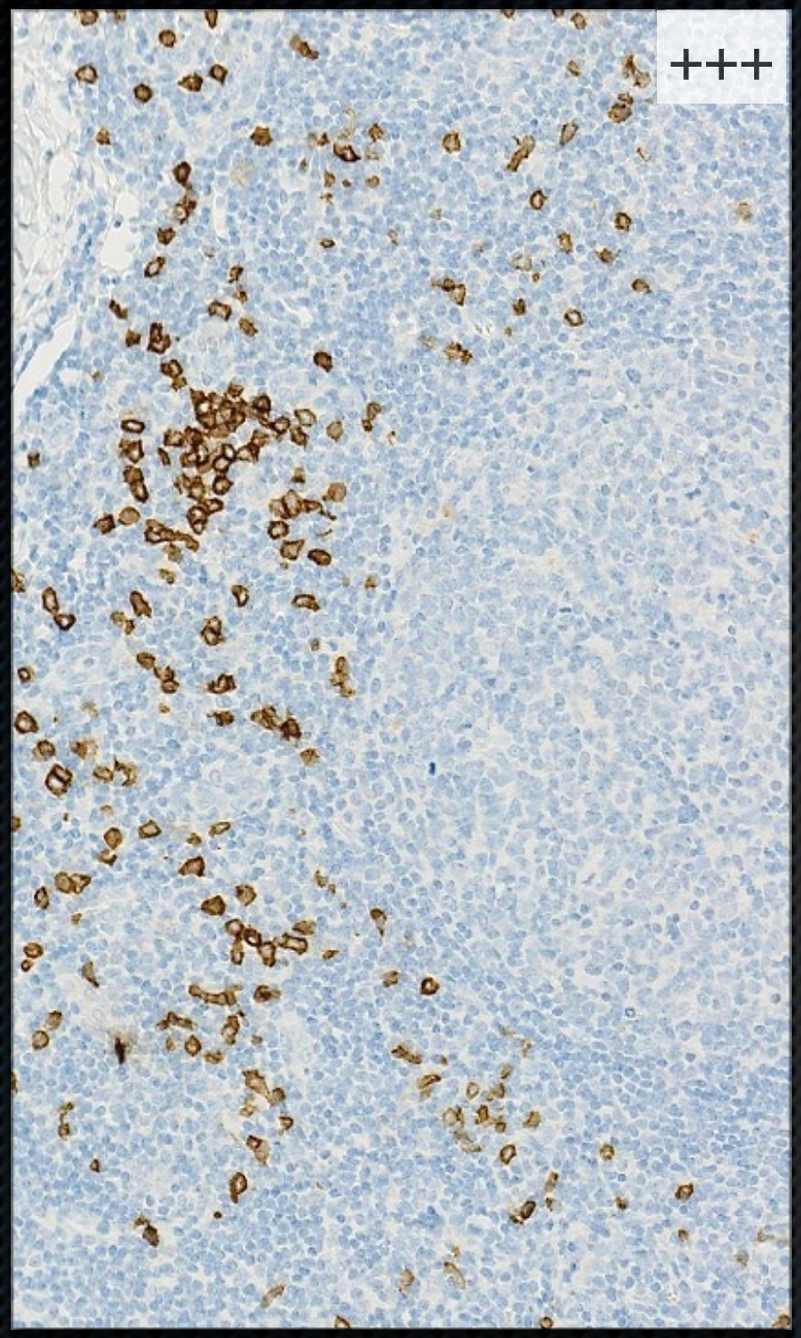
Formic acid 16hrs

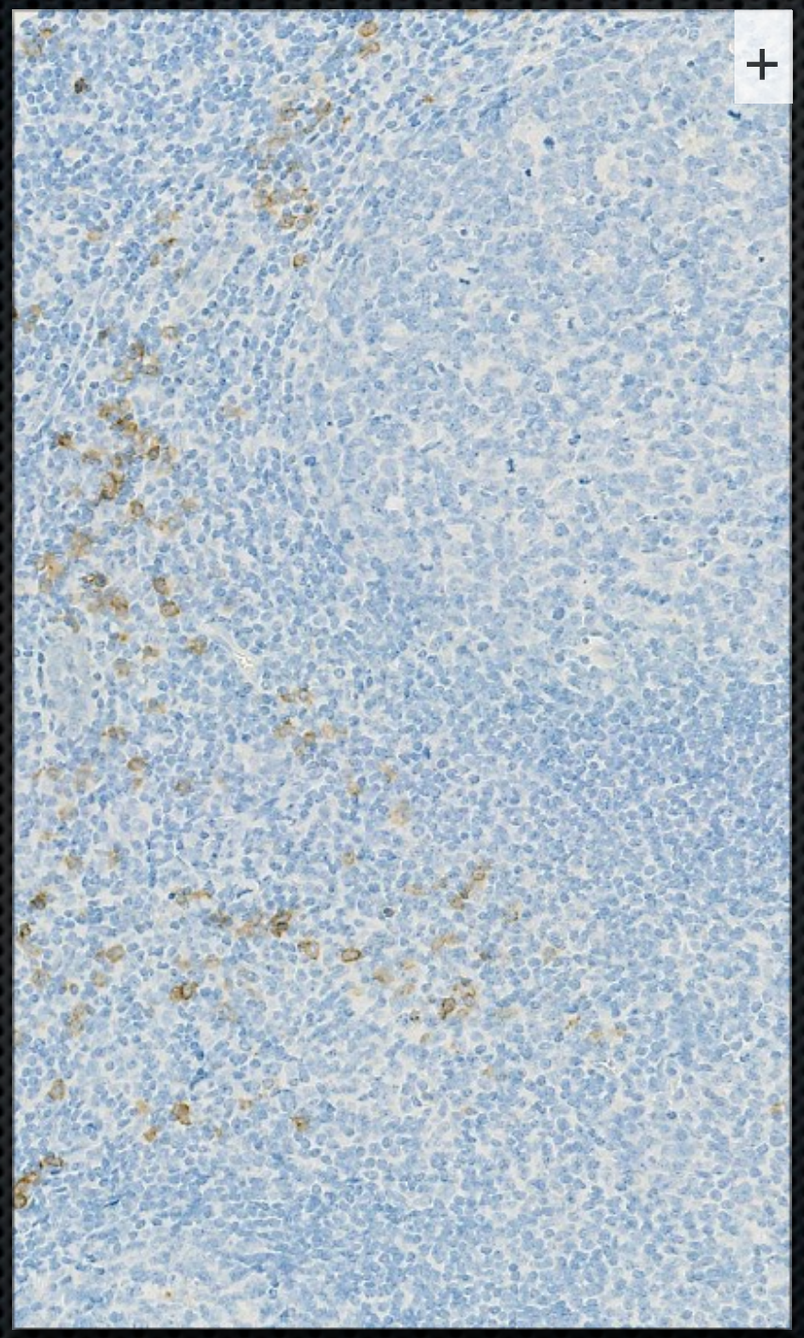


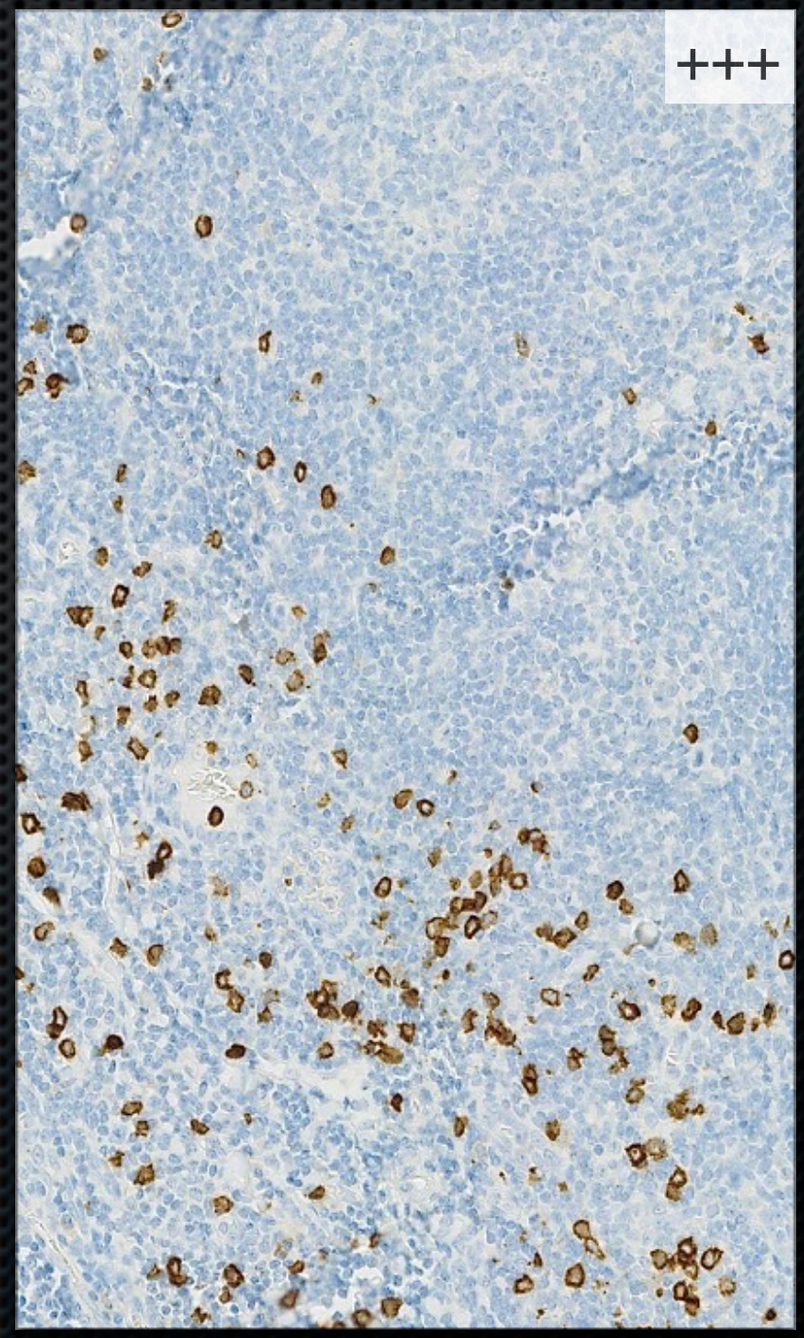
EDTA 96hrs

Decalcification and CD303, 124B3.13

CD303: Marker for Plasmacytoid dendritic cells







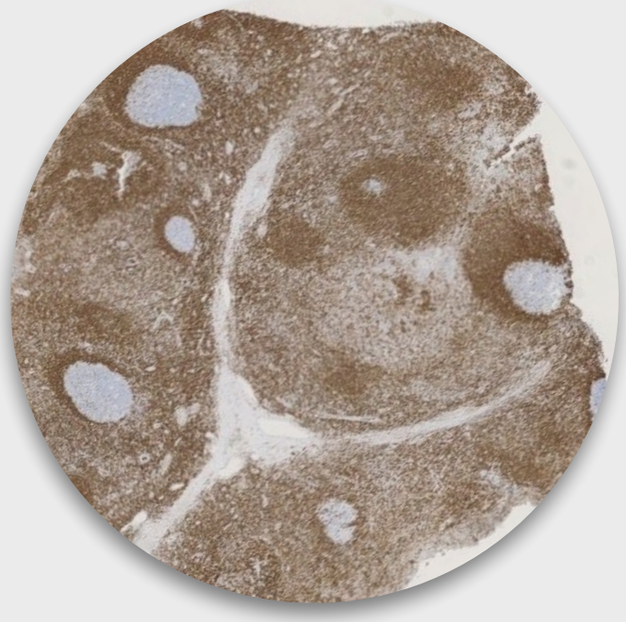
No decalcification Formic acid 16hrs EDTA 96hrs

Fixation time and decalcification in buffet formic acid (BFA)

Bcl2, clone124

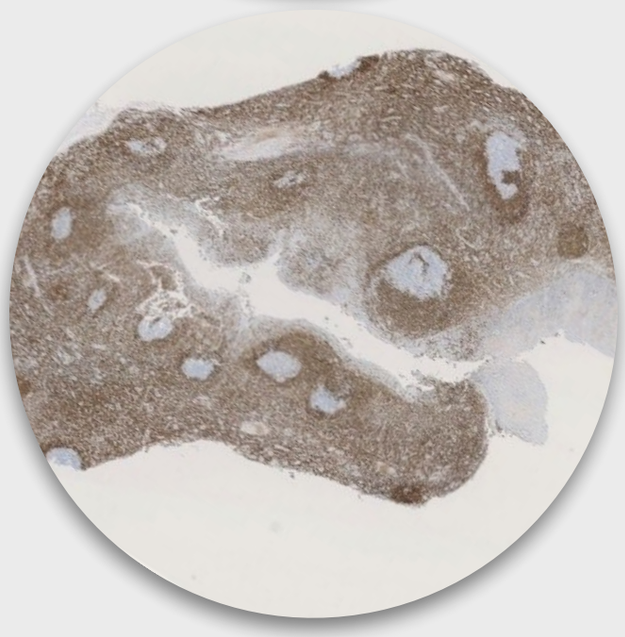
NBF 6 hrs | NBF 24 hrs | NBF 48 hrs

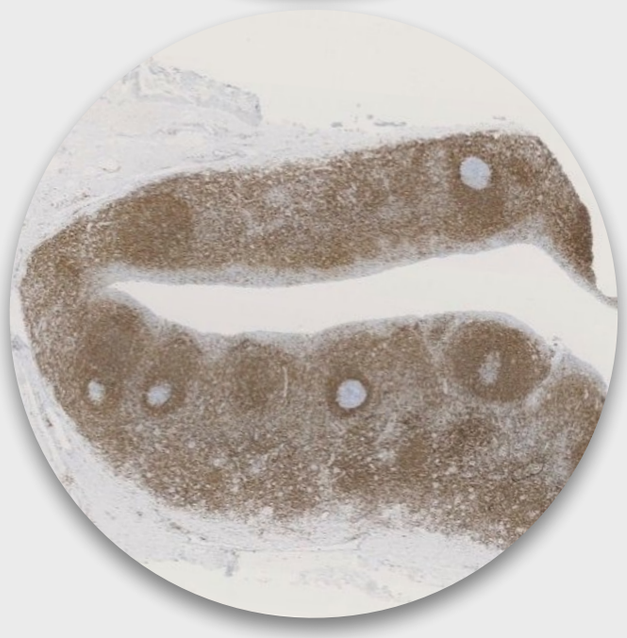
No decal



+

BFA 16 hrs





Decalcification

- Most antigens don't survive decalcification in strong acid (e.g. Decal™)
- All tested antigens survive decalcification in EDTA and show no, or minimal reduction in staining intensity
- Only very few antigens don't survive decalcification in formic acid, but app. 10% show a slight reduction in staining intensity - learn!

Effects of Decalcification on Immunohistochemistry Comparing: Immunocal®, Formical2000®, and EDTA Stat®

Philip E. Ferguson, M.D.[1,2,3] & Yolanda Sanchez, MS-CRM[4]

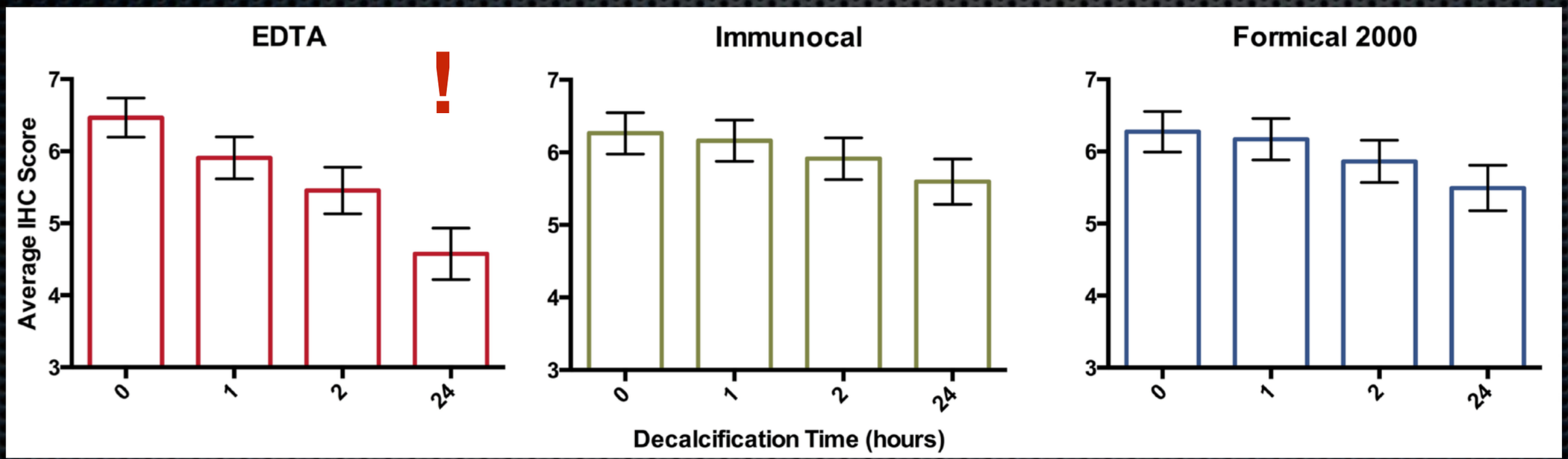
[1]PathMD, LLC, [2]Doctors' Anatomic Pathology Services, [3]Saint Bernards Medical Center, and [4]Leica Biosystems

Leica BIOSYSTEMS

NordiQC

Antibodies:

CD2, CD3, CD4, CD5, CD7, CD8, CD10, CD19, CD20, CD31, CD34, CD45, CD79a, CD138, Bcl-2, Bcl-6, Ki-67, AE1/AE3, BerEP4, CDX-2, CAM5.2, CK7, CK20, Desmin, E-Cadherin, MOC-31, S-100, Smooth Muscle Myosin-HC (SMM-HC), and CEA.



Conclusions: As expected, decalcification has negative effects on IHC staining. Weak acid decalcification reagents (Formical2000 and Immunocal) showed better performance characteristics compared to EDTA Stat*, (in contrast to Odense findings!) and nuclear transcription markers appear to be more sensitive to the effects of decalcification.

** The exact formulation of EDTA Stat solution is unknown*

Decalcification

Preanalytic variable	Published Guidelines and Recommendations CLSI	Literature-Based Recommendations
Decalcification	Interpret with caution - antigens could be lost!	**Tissue should be fixed 24 hrs in NBF prior to decalcification.** **EDTA < Formic acid < Strong acid**

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REVIEW INTERNATIONAL JOURNAL OF LABORATORY HEMATOLOGY

Int. Jnl. Lab. Hem. 2015, **37**, 431–449

ICSH guidelines for the standardization of bone marrow immunohistochemistry

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Table 1. Recommended protocols for bone marrow (BM) fixation and decalcification

Turnaround time (TAT)*	Fixative	Fixation time	Decalcification	Decal time	Comments
Very short TAT	Acetic acid–zinc–formalin (AZF)	2–72 h†	Shandon™ TBD-1™ Decalcifier	30–40 min†	Whenever possible, longer fixation (within the range) is preferred
Intermediate TAT	AZF	Overnight	Gooding and Stewart's decalcification fluid (10% formic acid and 5% formaldehyde)‡	6 h	So-called 'Hammersmith Protocol'
Standard TAT	10% buffered formalin = 3.7% formaldehyde	8–72 h (overnight fixation is preferred)†	14% EDTA	16–24 h†	Preferred protocol for BM biopsy fixation and decalcification

*Consideration of agitation and warming to 37 °C of the decalcifying solutions are recommended for each protocol. Ultrasonic decalcification may also be employed. These methods were shown to significantly shorten TAT.
†The timing may vary based on ancillary use of stirrers, ultrasound energization, microwave or other heating methods, or their combination.
‡Although decalcifying fixative is not recommended to be used alone, decalcifying fixative can produce superior results when used after the BM biopsy was already properly fixed in AZF or formalin.

Tissue-processing

Region Syddanmark
OUH
Afd. f.
Klinisk
Patologi
O. Nielsen

Implementation of a Microwave-assisted Tissue-processing System and an Automated Embedding System for Breast Needle Core Biopsy Samples: Morphology, Immunohistochemistry, and FISH Evaluation

Enrico Pegolo, MD, Maura Pandolfi, BSc, and Carla Di Loreto, MD

(*Appl Immunohistochem Mol Morphol* 2012;00:000–000)

Material: *233 consecutive needle core breast biopsies.*
The fixation time was strictly standardized, ranging from 18 to 24 hours.
After fixation, half of the core specimens from each case were randomly assigned to the conventional processing system (Leica ASP 300S 16-hrs program) and the other half to the MW-assisted tissue-processing system Sakura Tissue-Tek Xpress 120 (1-hr program).

TABLE 2. Immunohistochemical Analysis

Antibodies	Clone, Species	Manufacturer	Pretreatment	Dilution/Time
CK 5/6	D5/16B4, mouse	Dako	Tris/EDTA buffer (pH 9) at 97°C for 15 min	1:50/30 min
CK 19	RCK108, mouse	Dako	Citrate buffer (pH 6.1) at 97°C for 20 min	1:50/20 min
E-cadherin	NCH-38, mouse	Dako	Citrate buffer (pH 6.1) at 97°C for 20 min	1:100/20 min
p63	4A4, mouse	Dako	Citrate buffer (pH 6.1) at 97°C for 20 min	1:100/20 min
SMA	1A4, mouse	Dako	Tris/EDTA buffer (pH 9) at 97°C for 15 min	1:200/20 min
ER	SP1, rabbit	Aczonpharma (Bologna, Italy)	Citrate buffer (pH 6.1) at 97°C for 20 min	1:200/40 min
PR	PgR 636, mouse	Dako	Citrate buffer (pH 6.1) at 97°C for 20 min	1:100/40 min
Ki-67	Mib-1, mouse	Dako	Citrate buffer (pH 6.1) at 97°C for 20 min	1:50/20 min
HercepTest	Polyclonal, rabbit	Dako	Dako Epitope Retrieval Solution	Predil/30 min

CK indicates cytokeratin; ER, estrogen receptor; PR, progesterone receptor; Predil, prediluted; SMA, smooth muscle actin.

Implementation of a Microwave-assisted Tissue-processing System and an Automated Embedding System for Breast Needle Core Biopsy Samples: Morphology, Immunohistochemistry, and FISH Evaluation

Enrico Pegolo, MD, Maura Pandolfi, BSc, and Carla Di Loreto, MD

(*Appl Immunohistochem Mol Morphol* 2012;00:000–000)

TABLE 3. Estrogen Receptor Status in the Conventionally Processed and in the Matched MW-assisted Processed NCBs of Breast Carcinomas

	ER Status (Conventional)		
ER Status (MW)	**Positive**	**Negative**	**Total**
Positive	62	0	62
Negative	0	16	16
Total	62	16	78

Cohen κ test = 1.

ER indicates estrogen receptor; MW, microwave-assisted processing system; NCB, needle core biopsy.

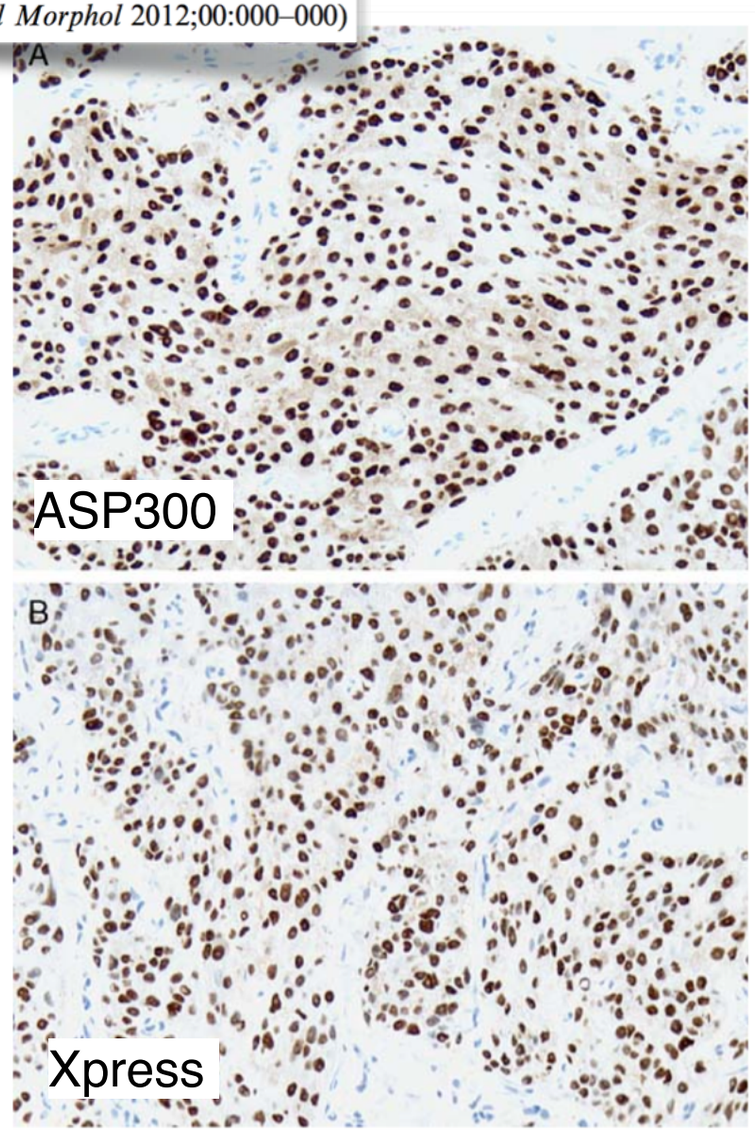


FIGURE 2. Needle core biopsy: invasive ductal carcinoma. Immunohistochemical reaction for estrogen receptor in the nuclei of tumor cells. The reaction is the same in the specimens prepared using the conventional processing method (A) and the microwave-assisted processing method (B) (A and B, immunoperoxydase for estrogen receptor, hematoxylin counterstain, original magnification ×200). full color online

Implementation of a Microwave-assisted Tissue-processing System and an Automated Embedding System for Breast Needle Core Biopsy Samples: Morphology, Immunohistochemistry, and FISH Evaluation

Enrico Pegolo, MD, Maura Pandolfi, BSc, and Carla Di Loreto, MD

(*Appl Immunohistochem Mol Morphol* 2012;00:000–000)

TABLE 6. HER2 Immunohistochemical Results in the Conventionally Processed and in the Matched MW-assisted Processed NCBs of Breast Carcinomas

	HER2 IHC (Conventional)			
HER2 IHC (MW)	**Negative**	**Equivocal**	**Positive**	**Total**
Negative	50	0	0	50
Equivocal	2	11	0	13
Positive	0	0	8	8
Total	52	11	8	71

Cohen κ test = 0.93. $P = 0.88$, χ^2 test.

IHC indicate immunohistochemistry; MW, microwave-assisted processing system; NCB, needle core biopsy.

The quality of H&E and immunohistochemical tissue sections provided by the new system is comparable to that obtained after the conventional processing method; this system also reduces the turnaround time for surgical pathology reports. Moreover, this is the first study that validates the assessment of the main prognostic and predictive biomarkers in breast NCBs processed by a MW-assisted system and automatically embedded.

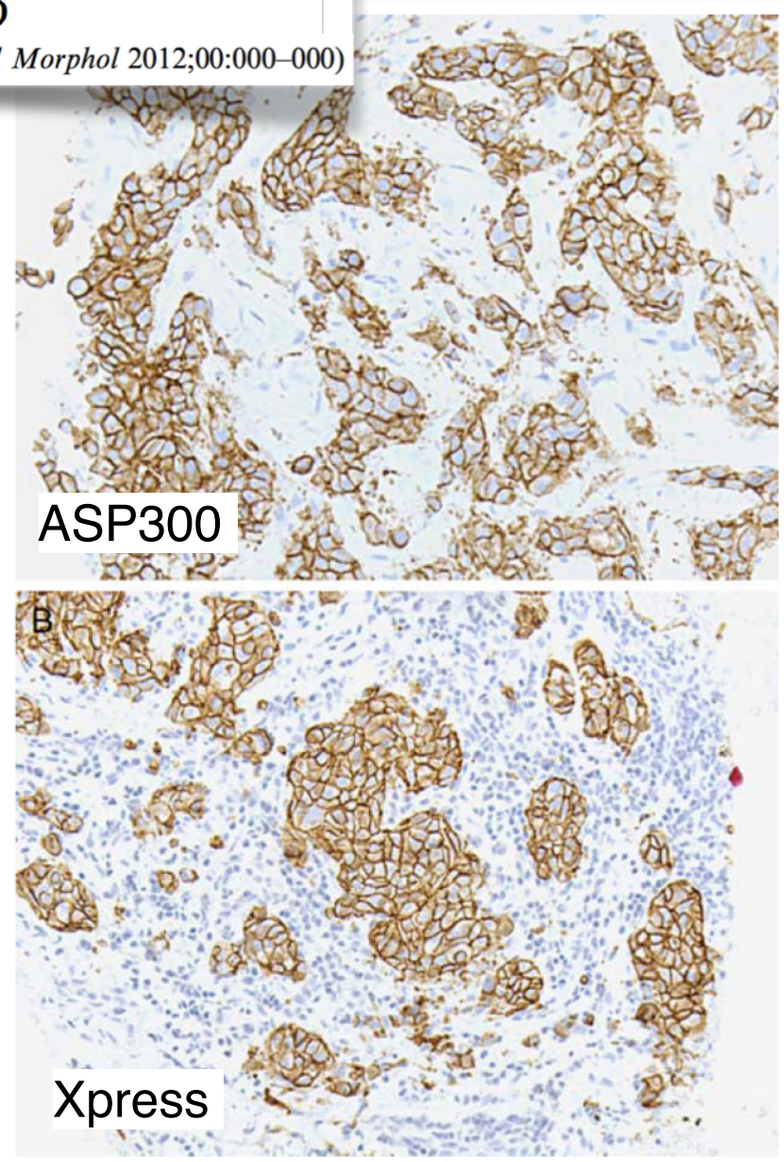


FIGURE 5. Needle core biopsy: invasive ductal carcinoma. Immunohistochemical reaction for HER2 (HercepTest) in the cell membranes of tumor cells. The same strong complete membrane staining (score 3+) is observed in the specimens prepared using the conventional processing method (A) and the microwave-assisted processing method (B) (A and B, HercepTest, original magnification ×200). full color online

Processing

Preanalytic variable	Published Guidelines and Recommendations ASCO/CAP | CLSI	Literature-Based Recommendations
Dehydration	1.25 - 15 hrs	10 hrs
Type of paraffin	Paraffin (55°C-58°C)	Paraffin (45°C)
Time in paraffin	0.5 - 4.5 hrs	1 - 2 hrs or 8 hrs

Engel KB, Moore HM. Arch Pathol Lab Med. 2011;135:537–543

Paraffin sectioning

- Type of blade and frequency of replacement
- Frequency of servicing and wax replacement
- Temperature of block during sectioning
- Slide pretreatment
- Water bath conditions, if used
- Chemical adhesives, if used
- Temperature and duration of slide drying

Immunocytochemistry 2008; Volume 6 Issue 3

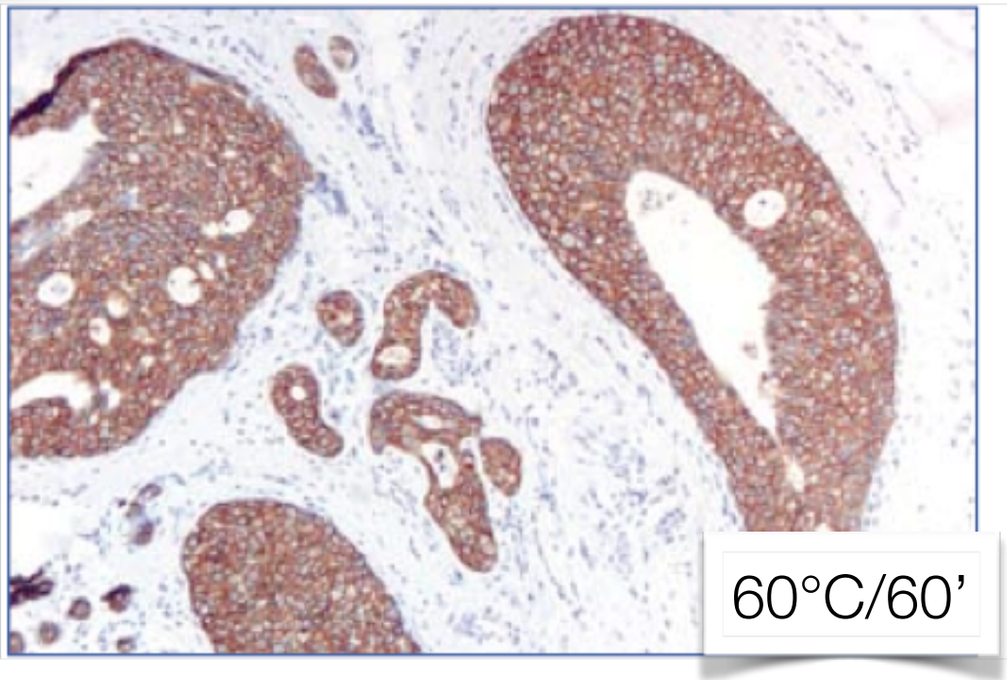
TECHNICAL ARTICLE

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

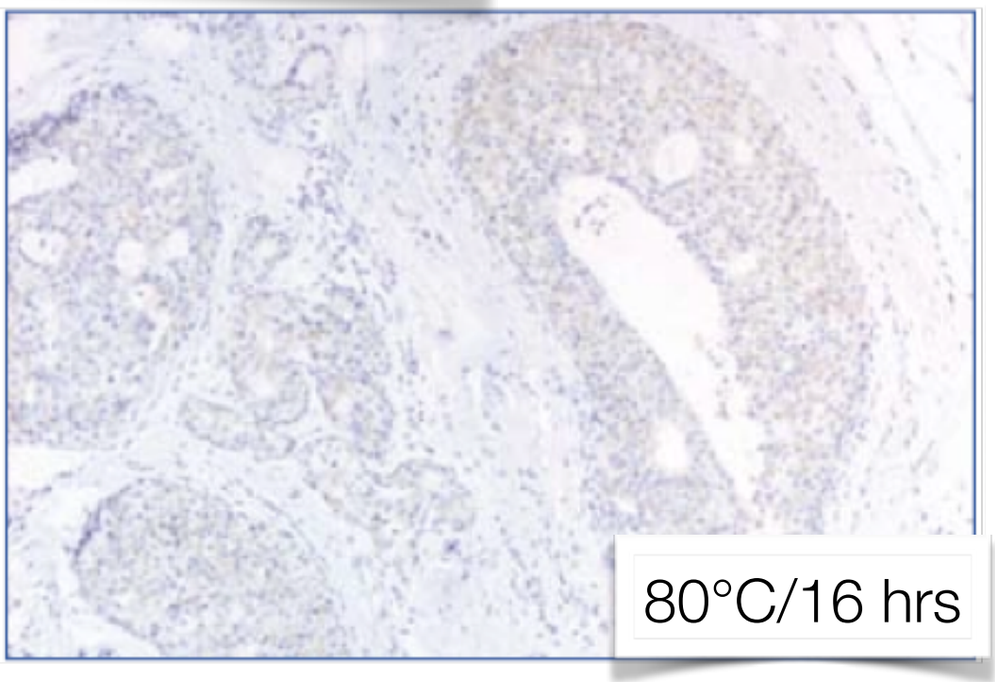
Bent Lundgaard Hansen, Henrik Winther and Kristian Moller

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

Antibodies:
a. HercepTest
b. Clone 4B5
c. Clone CB11

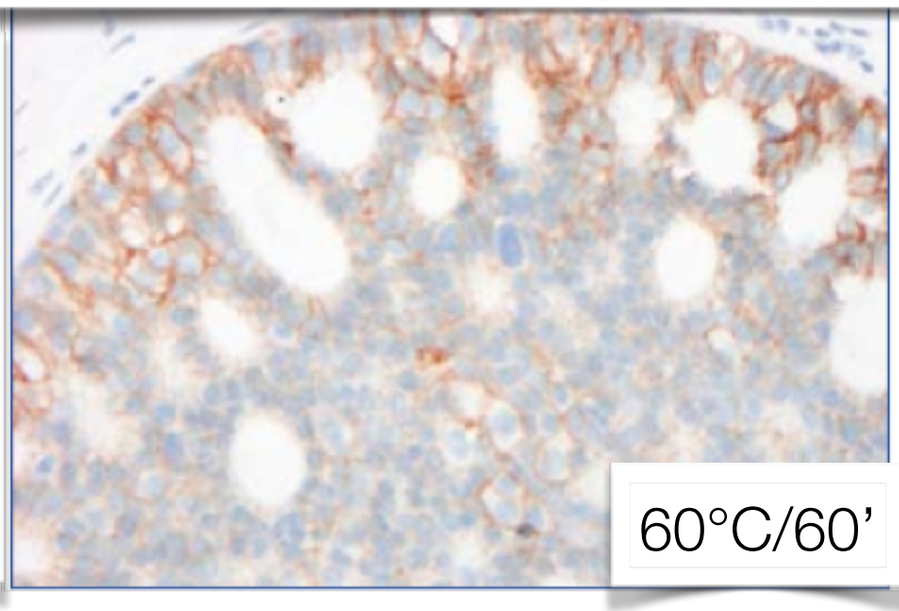


60°C/60’

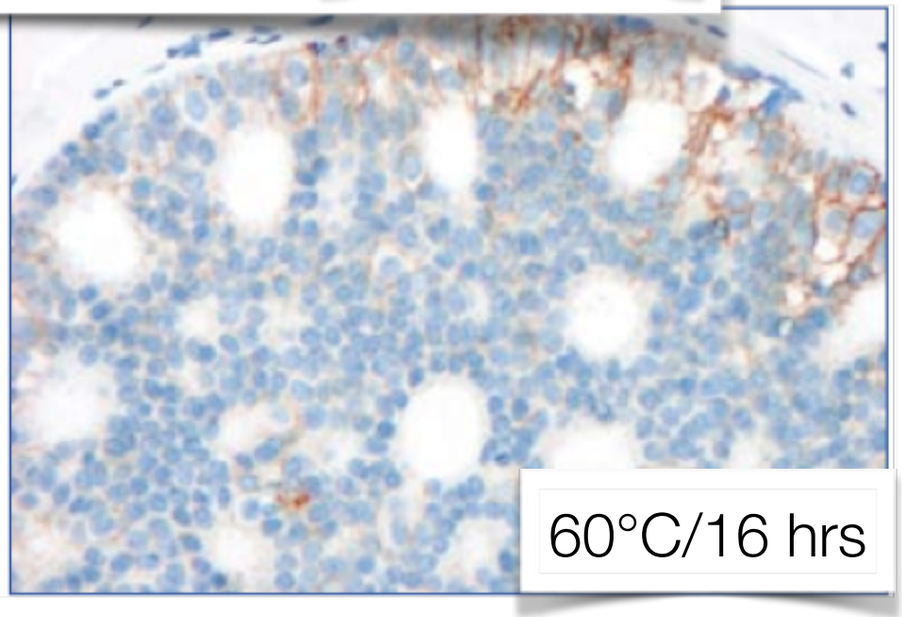


80°C/16 hrs

“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”.

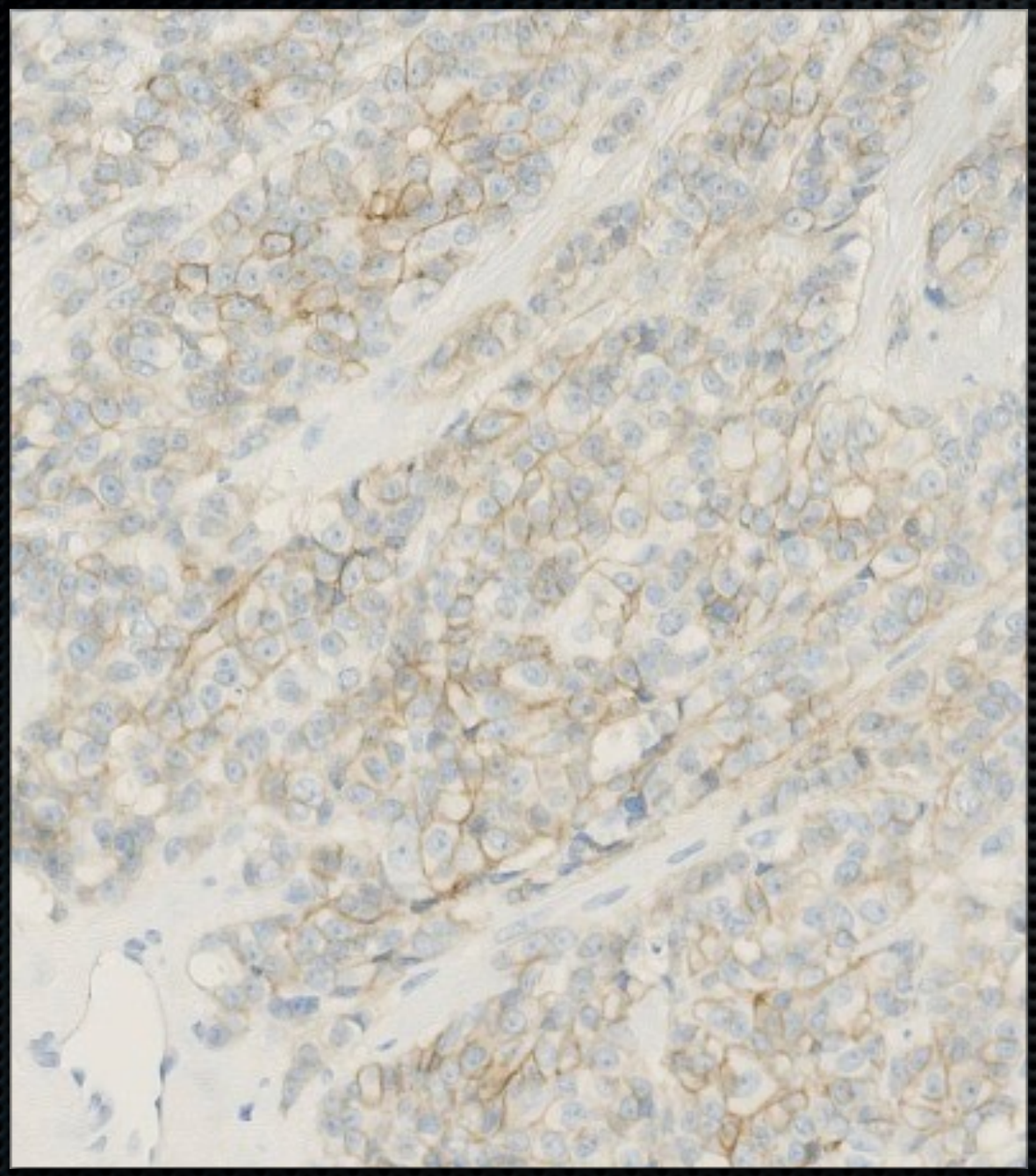


60°C/60’

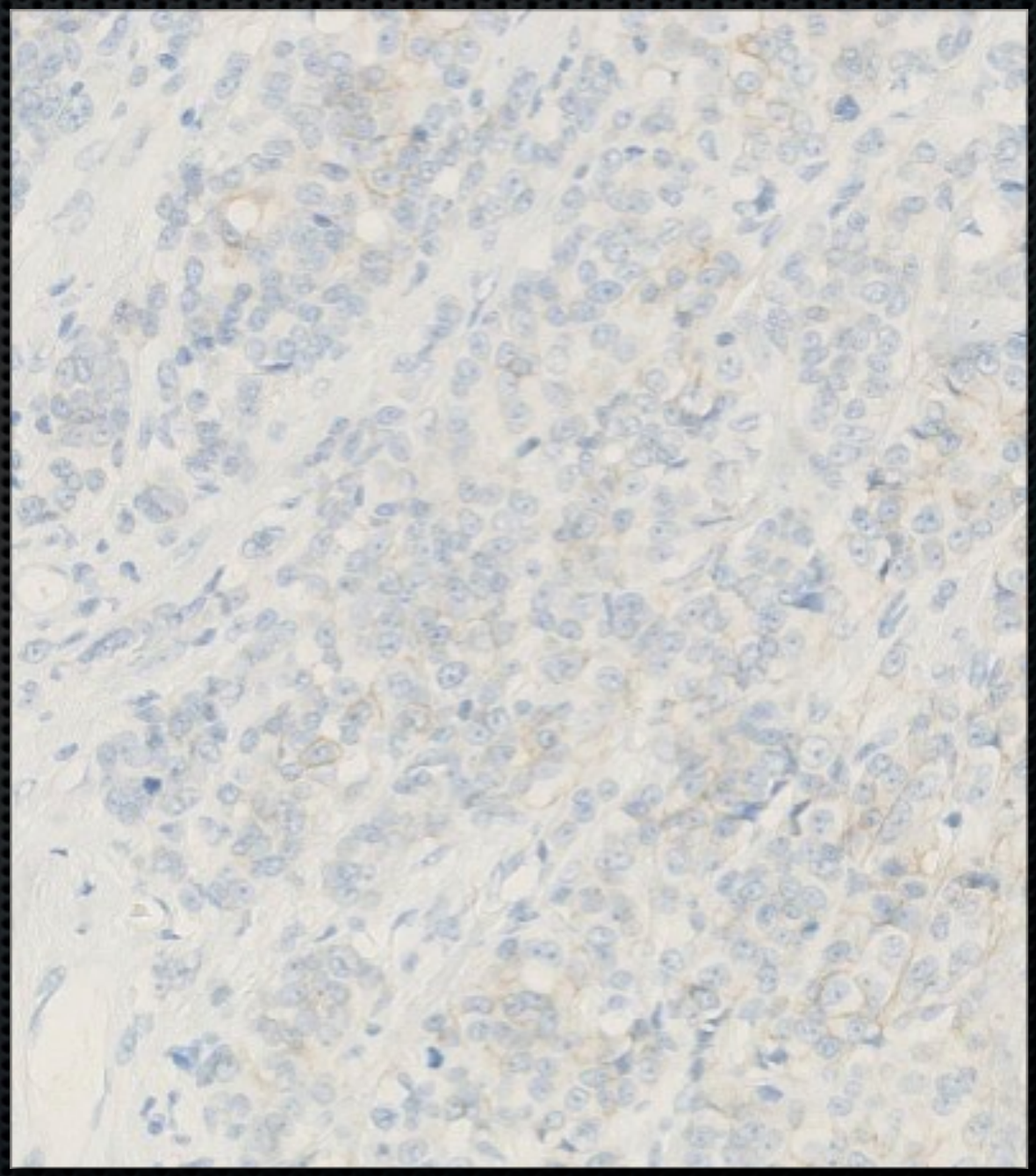


60°C/16 hrs

Drying of sections - HER2, 4B5

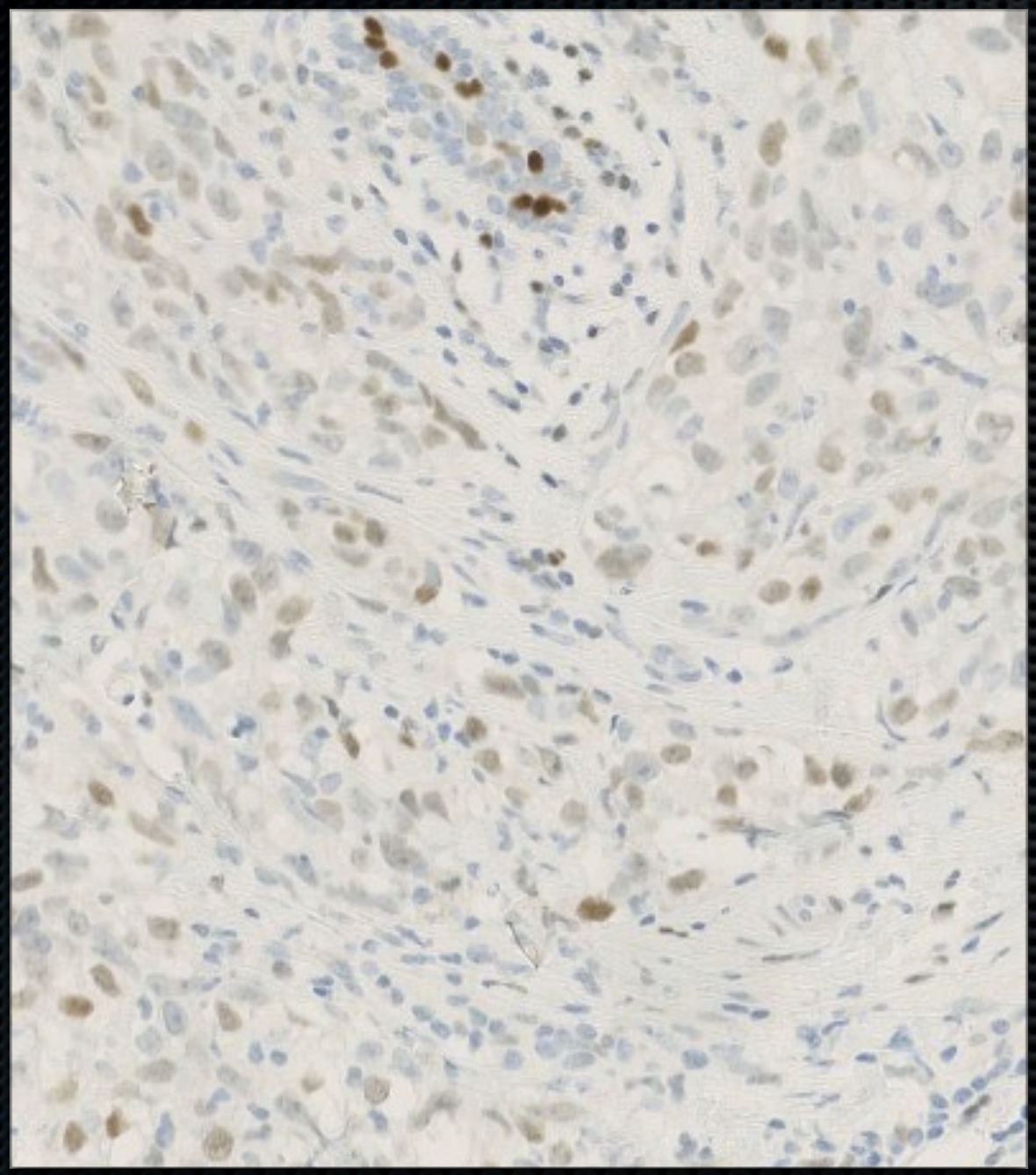


60 min at 60°C

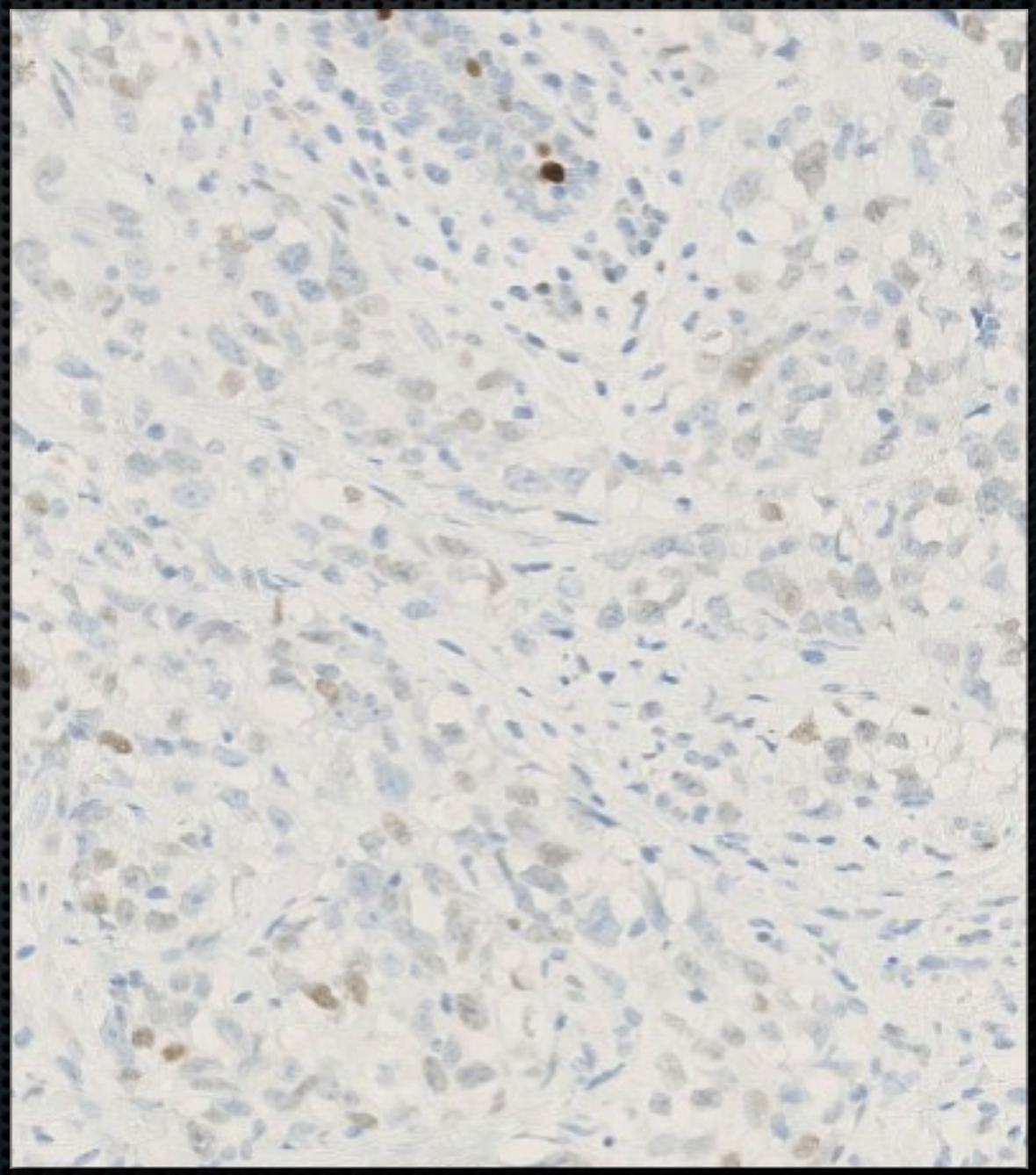


16 hrs at 80°C

Drying of sections - ER, SP1



60 min at 60°C



16 hrs at 80°C

Drying of sections (Baking)

Preanalytic variable	Published Guidelines and Recommendations ASCO/CAP | CLSI	Literature-Based Recommendations
Drying of sections	**<u>24 hrs at RT or 1 hr at 50°C - 60°C</u>**	24 hrs at RT or overnight at 37°C

Engel KB, Moore HM. Arch Pathol Lab Med. 2011;135:537–543

Storage

- Temperature and duration of paraffin block storage

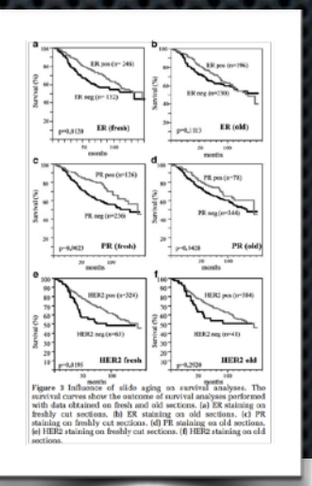
- Temperature, duration, and manipulation of slide-mounted tissue sections

Modern Pathology (2004) 17, 1414–1420

www.modernpathology.org

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)

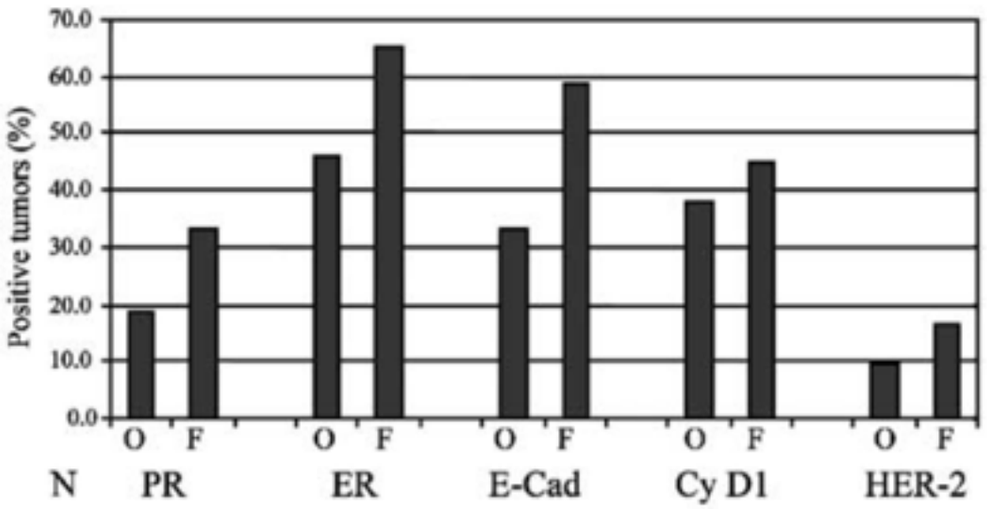
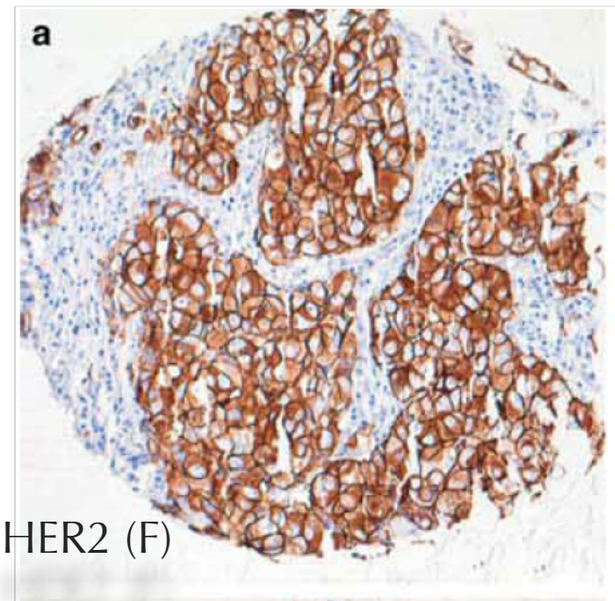
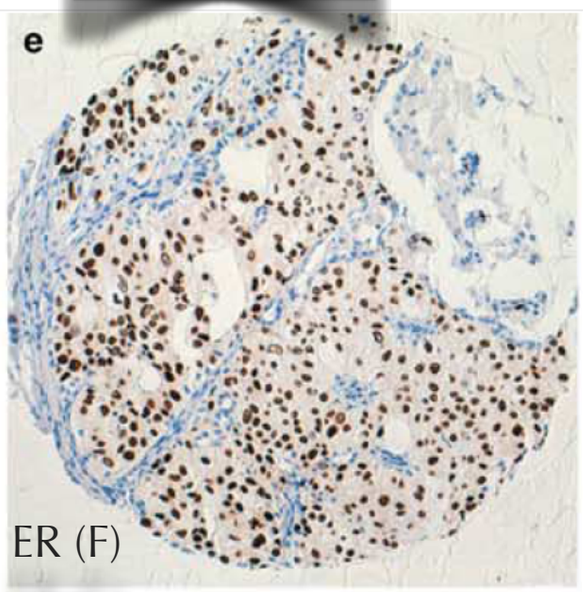


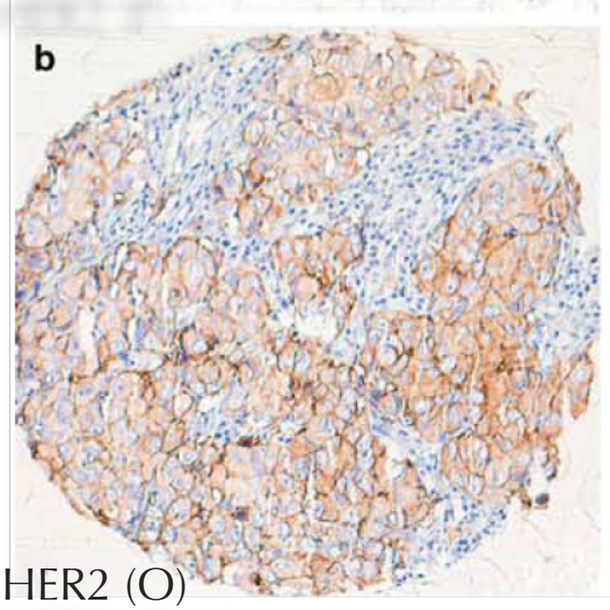
Figure 2 Influence of slide aging on the fraction of positive cases. For each antibody, the frequency of positive cases is shown as separate bars for old (O) and fresh (F) sections.



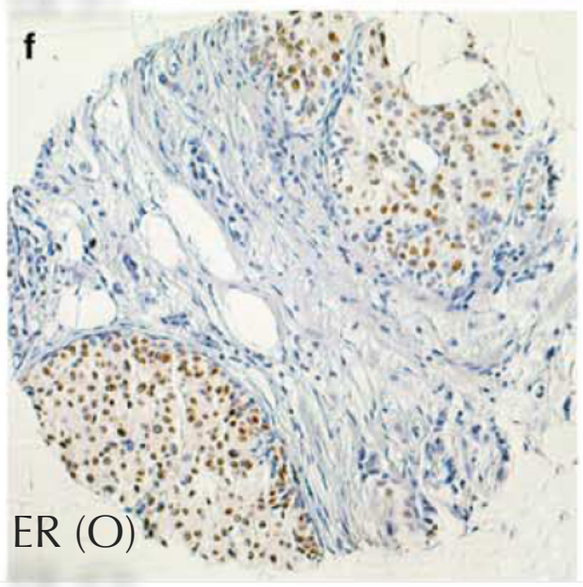
HER2 (F)



ER (F)



HER2 (O)



ER (O)

Influence of Storage Temperature and High-Temperature Antigen Retrieval Buffers on Results of Immunohistochemical Staining in Sections Stored for Long Periods

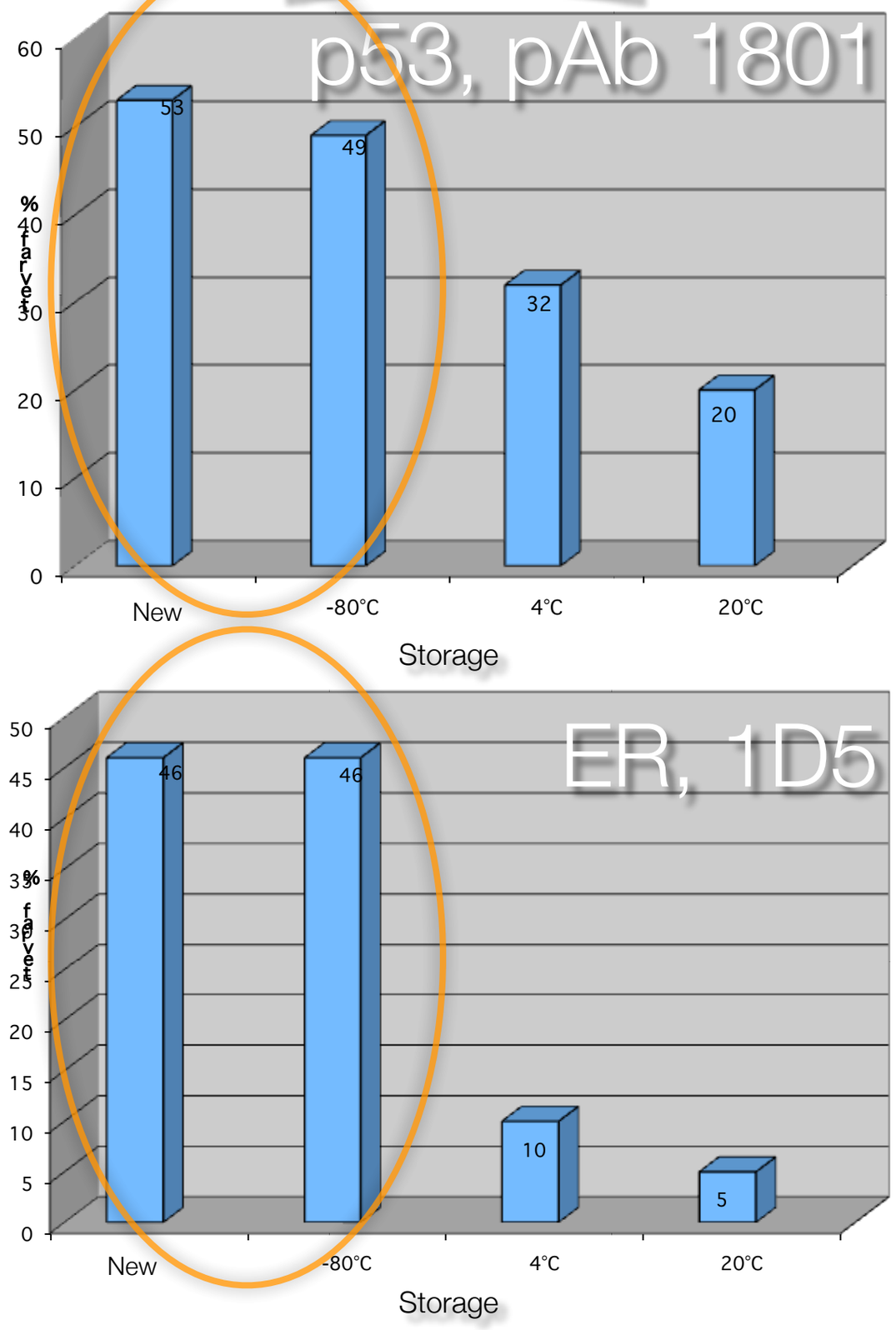
Applied Immunohistochemistry *6(4): 209–213, 1998*

Dorthe A. Grabau, M.D., Ph.D., Ole Nielsen, H.T., Steinbjørn Hansen, M.D., Mette M. Nielsen, M.D., Anne-Vibeke Lænkholm, M.D., Ann Knoop, M.D., and Per Pfeiffer, M.D., Ph.D.

Estrogen Receptor, 1D5

Storage at -80°C gave superior results

NordiQC



Factors Influencing the Degradation of Archival Formalin-Fixed Paraffin-Embedded Tissue Sections

Ran Xie, Joon-Yong Chung, Kris Ylaya, Reginald L. Williams, Natalie Guerrero, Nathan Nakatsuka, Cortessia Badie, and Stephen M. Hewitt

Tissue Array Research Program, Laboratory of Pathology, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, Maryland.

(J Histochem Cytochem 59:356–365, 2011)

Water?

Aquaporin 1 (AQP1)

Factors Influencing the Degradation of Archival Formalin-Fixed Paraffin-Embedded Tissue Sections

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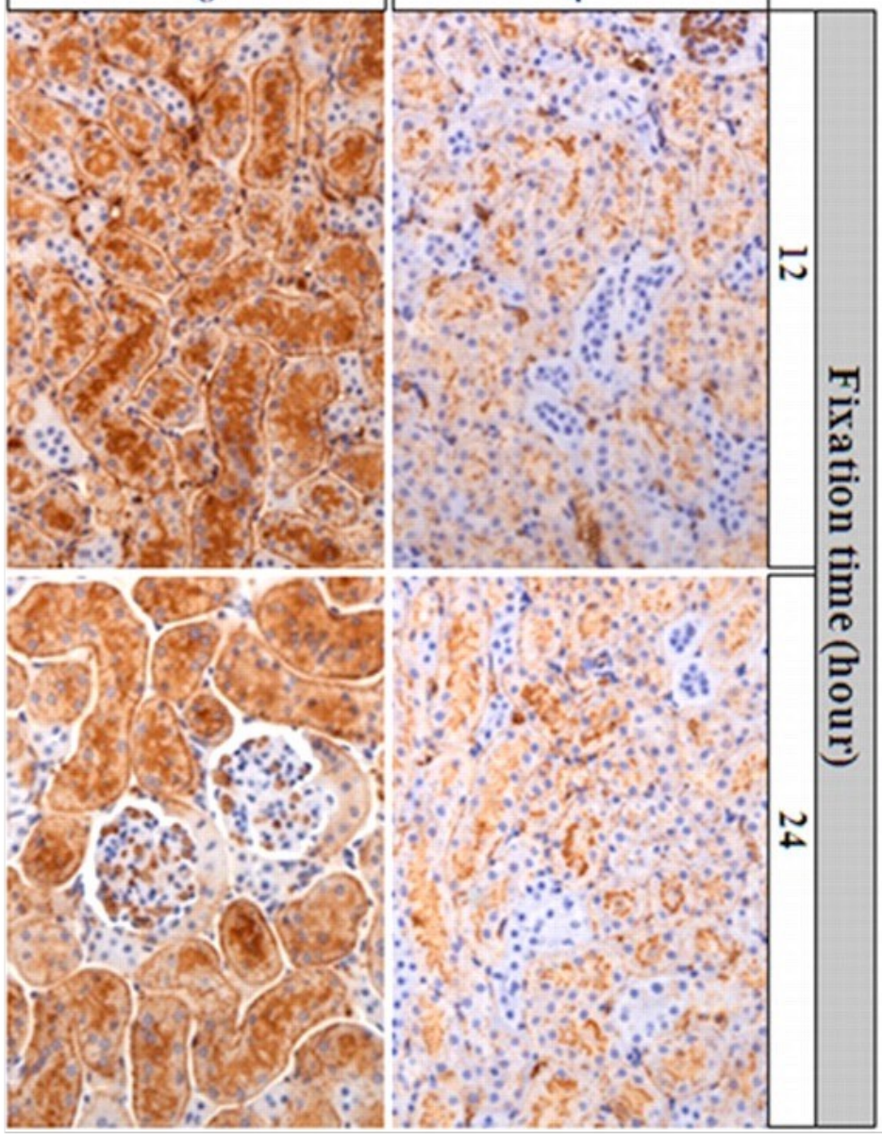
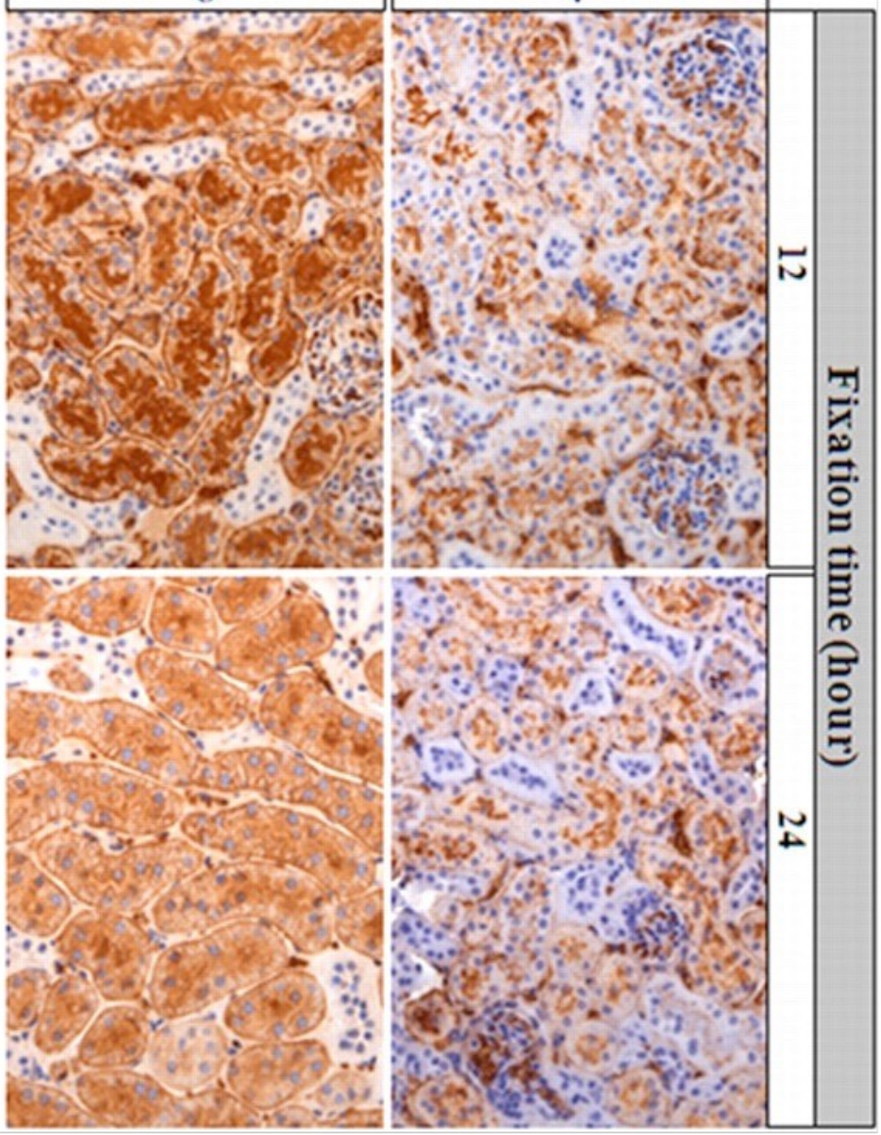
Tissue Array Research Program, Laboratory of Pathology, Center for Cancer Research. National Cancer Institute. National Institutes of Health. Bethesda, Maryland.

(J Histochem Cytochem 59:356–365, 2011)

Slide storage 2-weeks



Slide storage 0



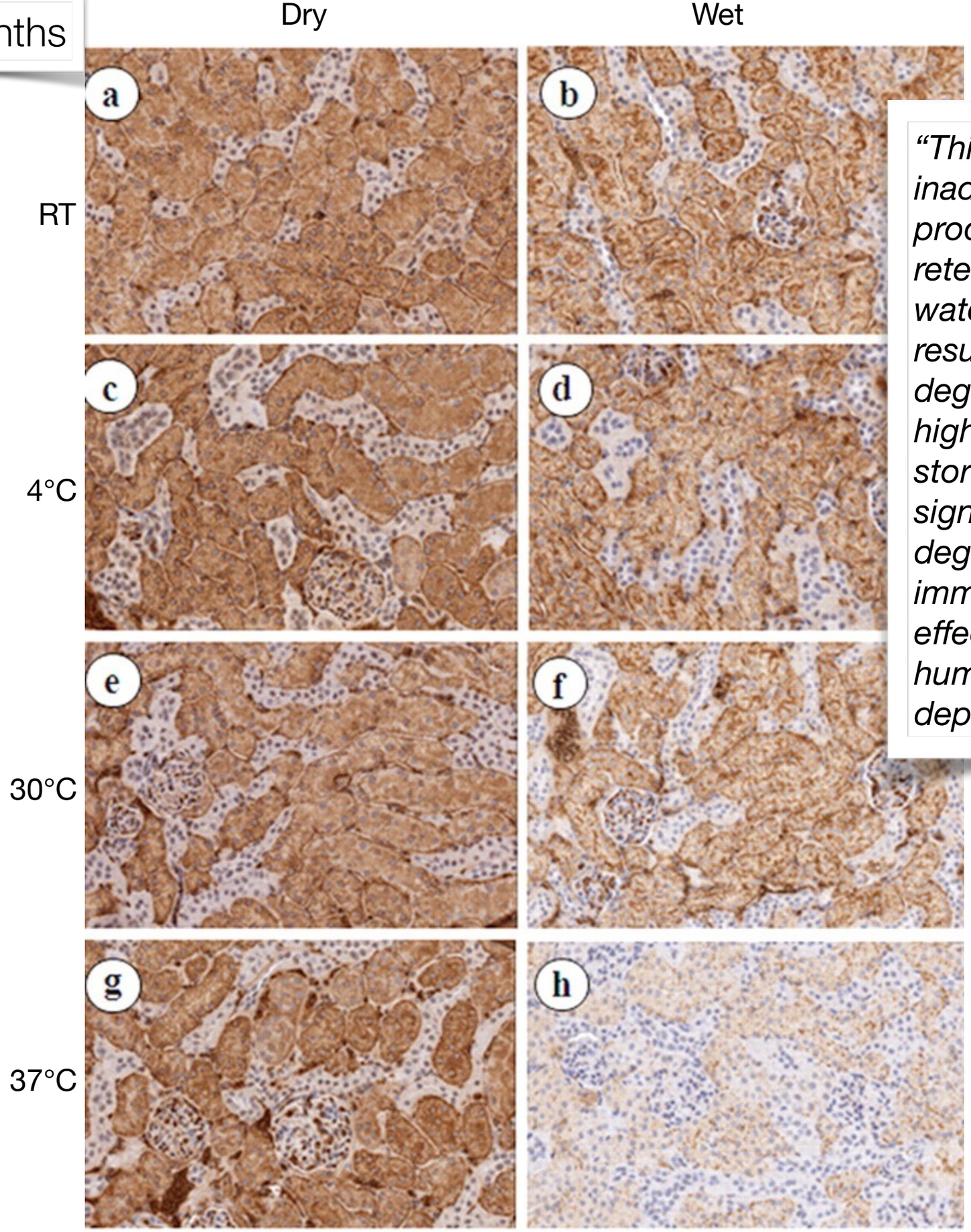
NordiQC

Storage 3 months

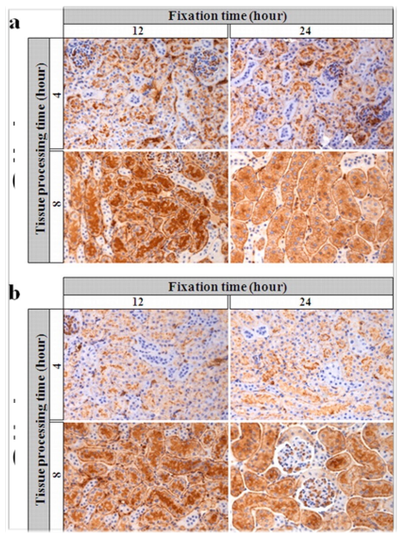
Factors Influencing the Degradation of Archival Formalin-Fixed Paraffin-Embedded Tissue Sections

Ran Xie, Joon-Yong Chung, Kris Ylaya, Reginald L. Williams, Natalie Guerrero, Nathan Nakatsuka, Cortessia Badie, and Stephen M. Hewitt
Tissue Array Research Program, Laboratory of Pathology, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, Maryland.

(J Histochem Cytochem 59:356–365, 2011)



"This study revealed that inadequate tissue processing, resulting in retention of endogenous water in tissue sections, results in antigen degradation. Exposure to high humidity during storage results in significant protein degradation and reduced immunoreactivity, and the effects of storage humidity are temperature dependent".



Loss of antigenicity with tissue age in breast cancer

Susan E Combs[1], Gang Han[1], Nikita Mani[1], Susan Beruti[2], Michael Nerenberg[3] and David L Rimm[1]

Laboratory Investigation (2016) 96, 264–269

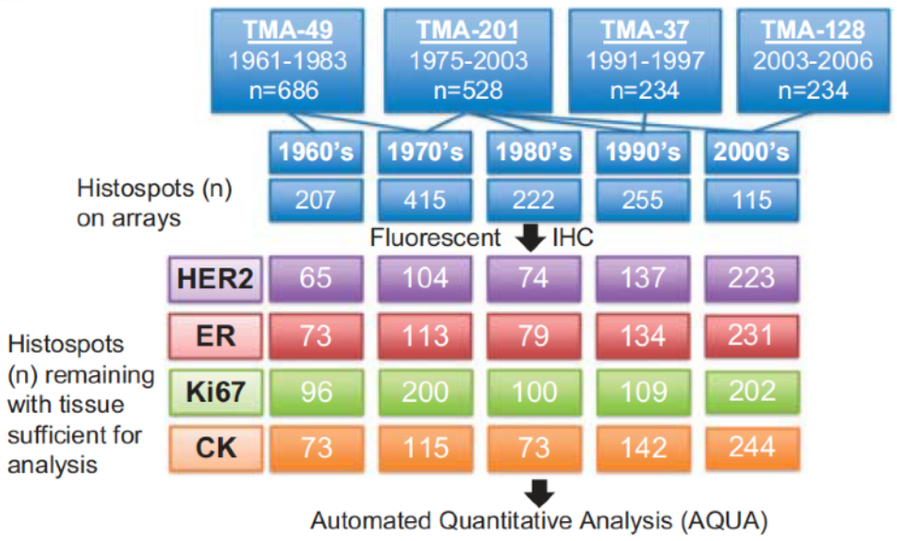


Figure 1 A consort diagram showing the cohorts from which the tissues were derived and the date ranges for each followed by the number of cases analyzed for each biomarker. IHC, immunohistochemistry; TMA, tissue microarray.

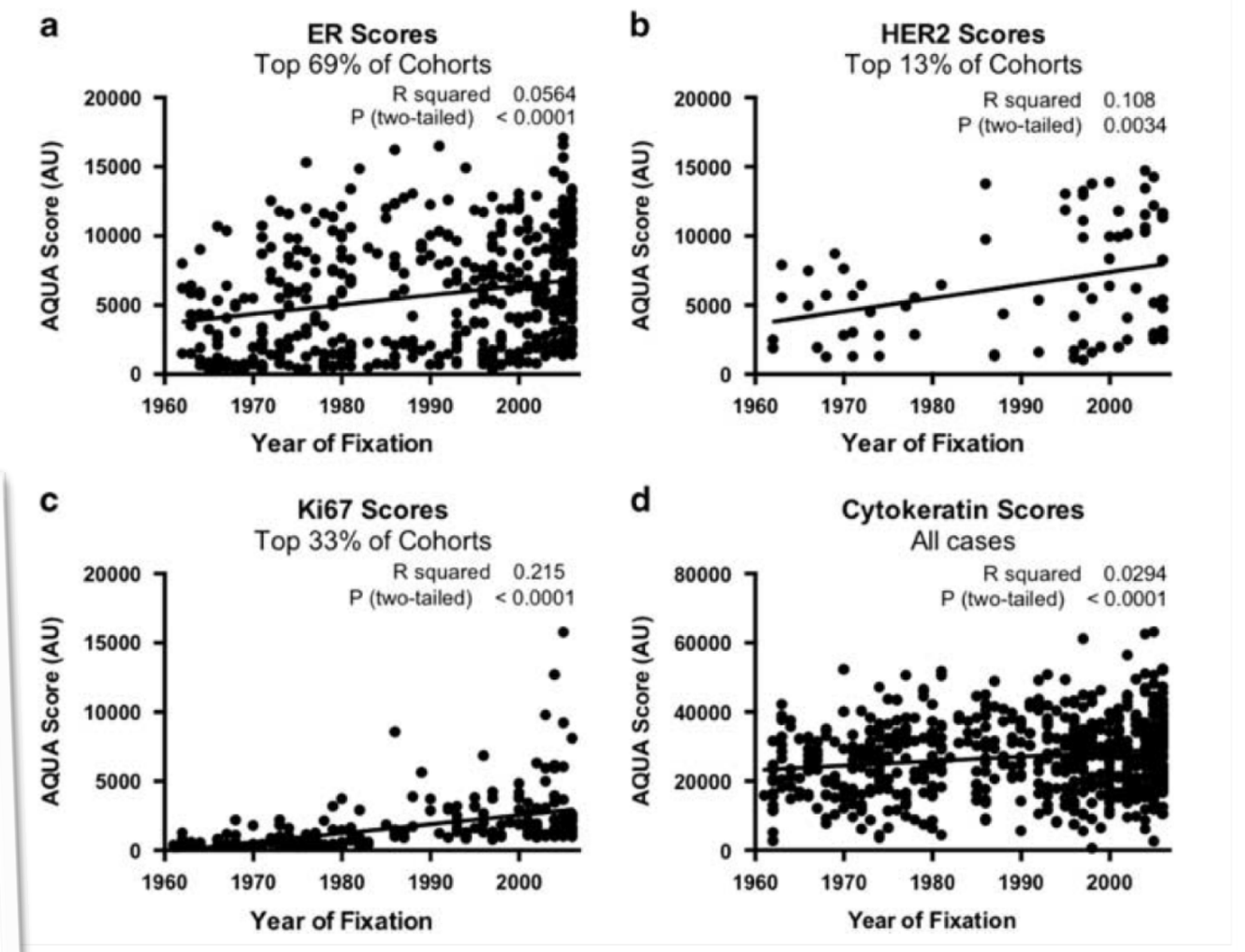


Figure 2 The distribution of scores for each biomarker as a function of tissue age after omitting the fraction of expected negative cases. (**a**) ER, (**b**) HER2, (**c**) Ki67 and (**d**) cytokeratin. The fraction of positive cases is shown by percentage beneath the biomarker in the title. The regression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

Storage of specimen

Preanalytic variable	Published Guidelines and Recommendations ASCO/CAP | CLSI	Literature-Based Recommendations
Storage of paraffin blocks	Indefinitely *	< 25 years *
Storage of sections (slide)	7 days or < 6 weeks	< 6 days

Engel KB, Moore HM. Arch Pathol Lab Med. 2011;135:537–543

* new data indicates up to 10% loss in 5 years

Days	20°C
Weeks	4°C
Months	-20°C
Years	-80°C

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

Decalcification:
Type, Time, Temperature

A tissue quality index: an intrinsic control for measurement of effects of preanalytical variables on FFPE tissue

Veronique M Neumeister[1], Fabio Parisi[1], Allison M England[1], Summar Siddiqui[1], Valsamo Anagnostou[1], Elizabeth Zarrella[1], Maria Vassilakopolou[1], Yalai Bai[1], Sasha Saylor[1], Anna Sapino[2], Yuval Kluger[1,2], David G Hicks[3], Gianni Bussolati[2], Stephanie Kwei[4] and David L Rimm[1]

Laboratory Investigation (2014) 94, 467–474

With focus on delay of fixation

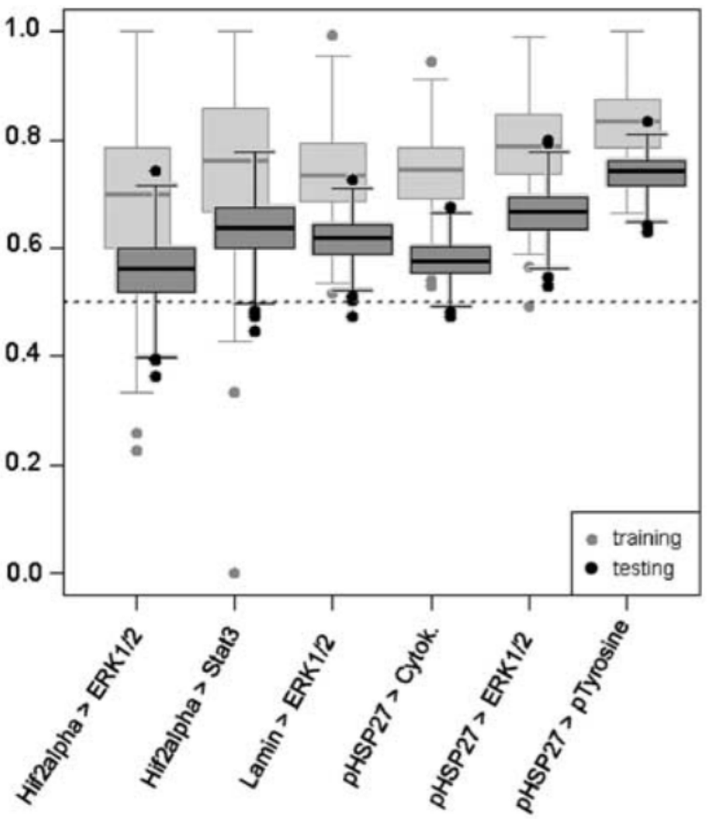
- Aim: Developing a quantitative intrinsic control that can measure the degree of degradation of any FFPE sample.
- If we cannot control pre-analytical variables can we quantify the damage or tissue degradation caused by them?
- Can we disqualify specimens for Companion dx testing?

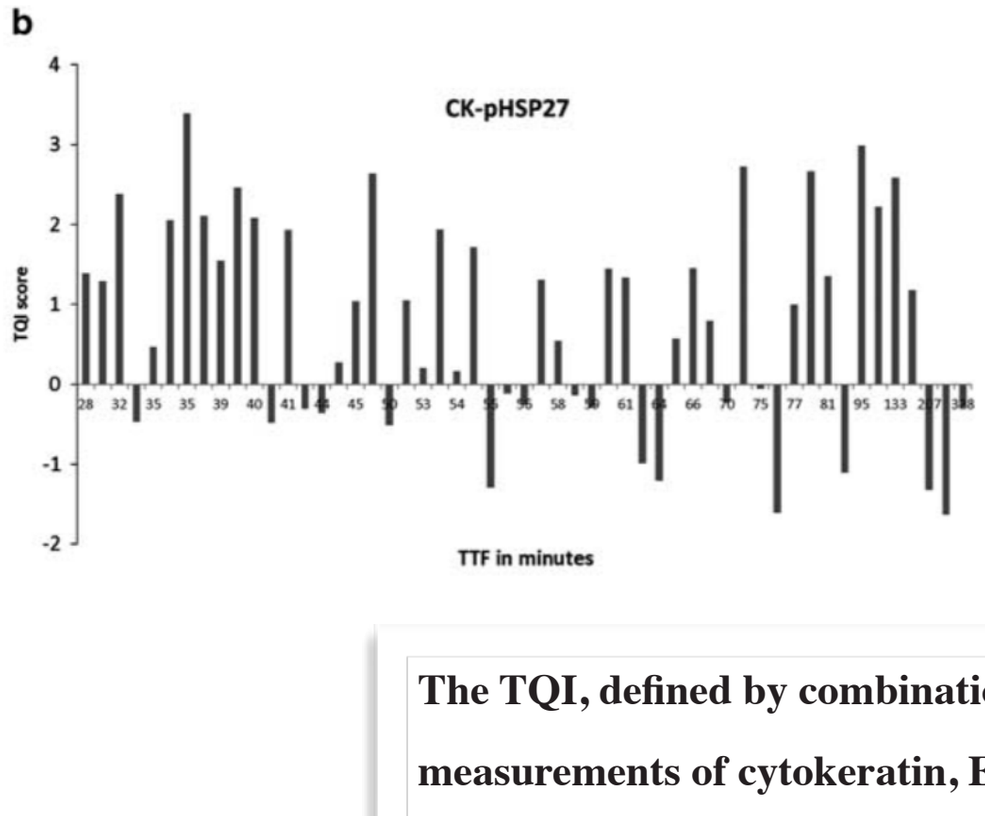
TQI: Tissue Quality Index

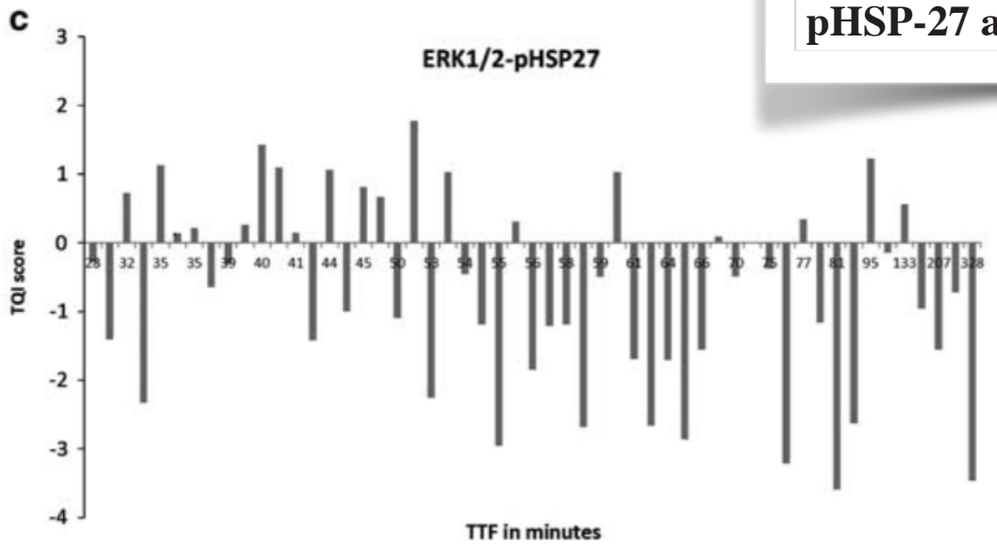
Table 1 Antibodies tested for the TQI

Symbol	Description	Antibody Clone/Isotype	Supplier
Markers of Cold Ischaemia			
ACTB	Beta-Actin	13E5/IgG	Cell Signaling Technology
TUBB	Beta-Tubulin	pF3/IgG	Cell Signaling Technology
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	14C10/IgG	Cell Signaling Technology
HIST4	Histone 4	L64C1	Cell Signaling Technology
HIST3	Histone 3	96C10/IgG1, kappa	Cell Signaling Technology
LMNA/C	Lamin A/C	Polyclonal	Cell Signaling Technology
LDHA	Lactat Dehydrogenase	IgG, C4B5	Cell Signaling Technology
ERalpha	Estrogen Receptor alpha	SP1/IgG	Thermo Scientific
CK	Cytokeratin	AE1/AE3/IgG1	DAKO
CK	Cytokeratin	Polyclonal	DAKO
ERK1/2	P44/42MAPK (Erk1/2)	137F5, IgG	Cell Signaling Technology
p53	Anti-Human p53 protein	IgG2b. DO-7	DAKO
Markers of Hypoxia			
CCND1	Cyclin D1	IgG/SP4	Thermo Fisher Fremont
Caspase	Cleaved Caspase 3 (Asp175)	Polyclonal	Cell Signaling Technology
HIF1	Hypoxia Inducible Factor 1	Polyclonal	Novus Biological
AKAP13	A-kinase anchoring protein 13	IgG2a/ZX-18	
CDC42		IgG3/B-8	
CCNB1	Cyclin B1	GNS-11/IgG2	
HIF-2alpha	Hypoxia inducible factor-2α	ep190b/IgG1	
CA9	Carbonic Anhydrase IX	Polyclonal(aa581-592	
Markers of phosphorylated proteins			
pAKT 473	phospho-Akt (ser473)	D9E/IgG	Cell Signaling Technology
ERK1/2	Phospho-p44/43MAPK (Erk1/2) (Thr292/Tyr204)	IgG	Cell Signaling Technology
pER	Phospho-Estrogen Receptor alpha (Ser118)	16J4/IgG2b	Cell Signaling Technology
Anti-Phosphotyrosine	4G10 Anti-Phosphotyrosine	IgG2b	Millipore
Anti-Phosphotyrosine		p-Tyr-100	Cell Signaling Technology
pHSP27 (pS78)	Phosphorylated Heat Shock Protein 27	Y175	Epitomics
pHer2 (Tyr1248)	Phospho-Her2/ErbB2 (Tyr1248)	PN2A	Thermo Scientific
Phospho-Stat3 (Tyr705)	Phospho-Stat3 (Tyr705)	D3A7/IgG	Cell Signaling Technology
p-S6 Ribosomal Protein (Ser235/236)	Phospho-S6 Ribosomal Protein (Ser235/236)	D52.2.2E/IgG	Cell Signaling Technology
Phospho-Jak2 (Tyr1007/1008)	Phospho-Jak2 (Tyr1007/1008)	Polyclonal	Cell Signaling Technology
Phospho-Met (Tyr1234/1235)	Phospho-Met (Tyr1234/1235)	IgG	Cell Signaling Technology
Phospho-Sapk/Jnk	Phospho-Sapk/Jnk	IgG	Cell Signaling Technology
Phospho mTor (Ser2448)	Phospho mTor (Ser2448)	49F9/IgG	Cell Signaling Technology
Markers of posttranslational modification			
Sumo1	small ubiquitin related modifier 1	Y299/IgG	Abcam
Acetylated-Lysine	Proteins posttranslat. Modified by acetylation	Polyclonal, purified	Cell Signaling Technology
NEDD8	Neural precursor cell-expr. devel. Downreg. protein9	IgG, 19E3	Cell Signaling Technology

TQI: Tissue Quality Index







The TQI, defined by combinations of measurements of cytokeratin, ERK1/2 and pHSP-27 and their relationship to cold ischemic

Figure 1 (**a**) The performance of six marker combinations on the testing and validation subgroup of the time to fixation breast cancer series as measured by receiver-operator characteristic (ROC) curves and area under the curve (AUC) values. The tissue quality index (TQI) was then calculated on the complete time to fixation breast cancer series. (**b**) TQI values of cytokeratin:pHSP27 and (**c**) ERK1/2:pHSP27 in relationship with increasing cold ischemic time.

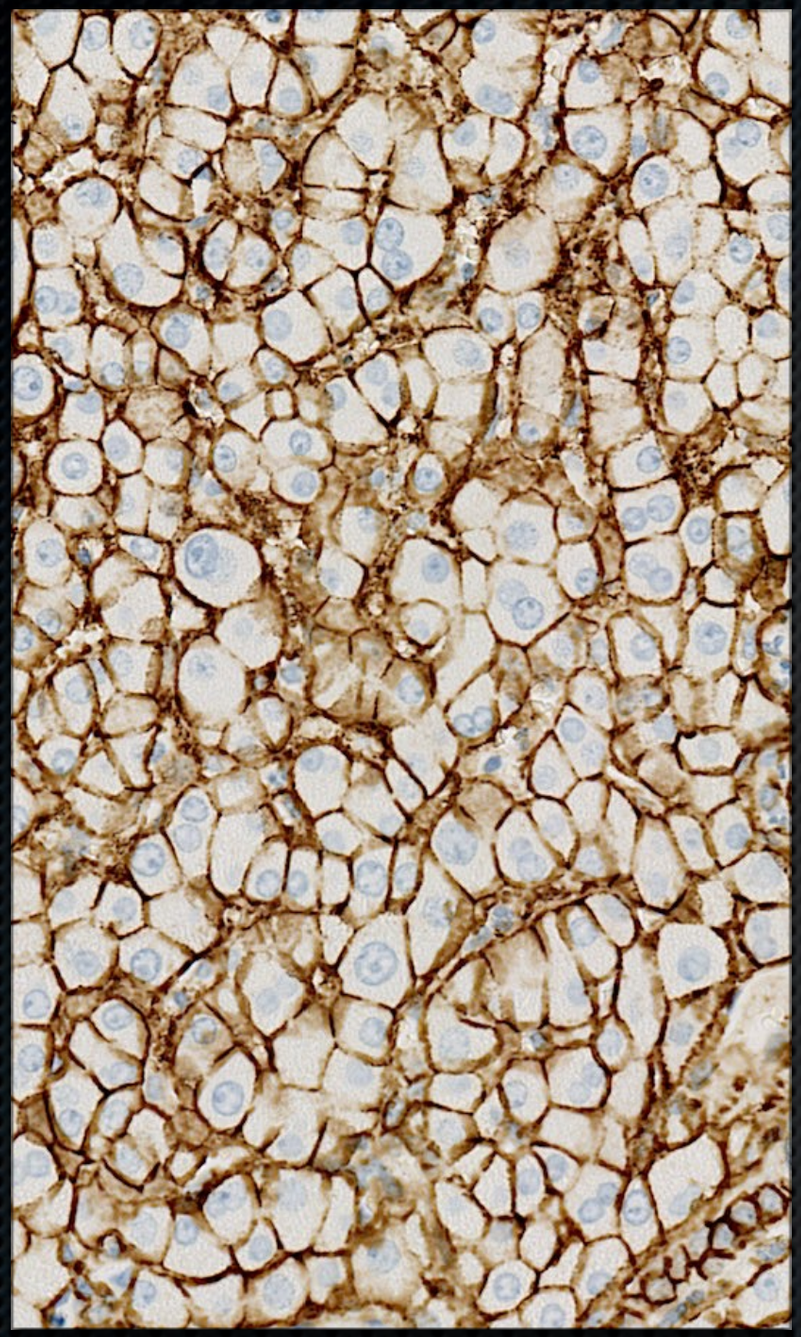
“The poor man’s TQI”

“Damage Controls”

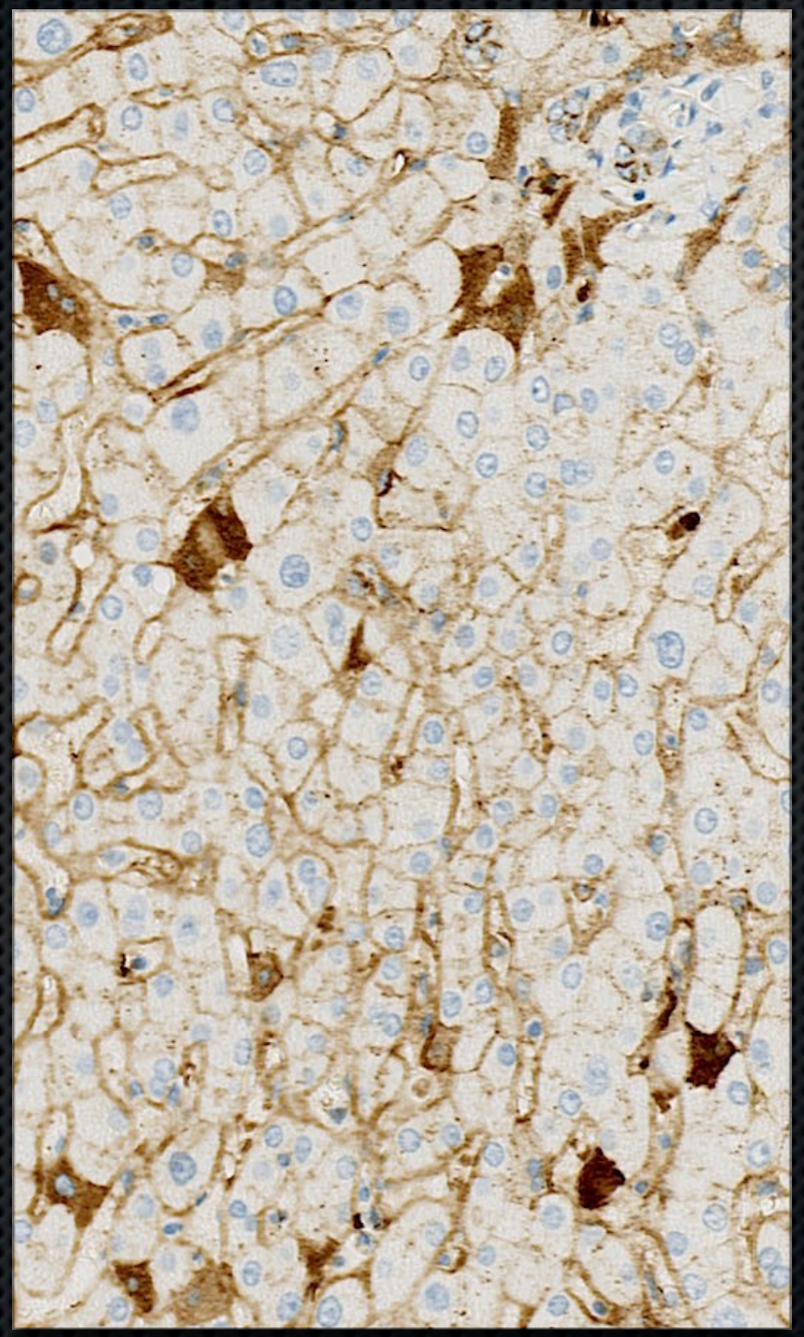
- **Fixation delay/Cold ischemia**
  - CD138, B-A38
- **Poor/short fixation in NBF**
  - MLH1, ES05
  - PMS2, EPR3947
  - BCL6, LN22
  - BCL2, 124
- **Electrosurgery**
  - CK, CAM5.2

CD138: Simple marker of fixation delay

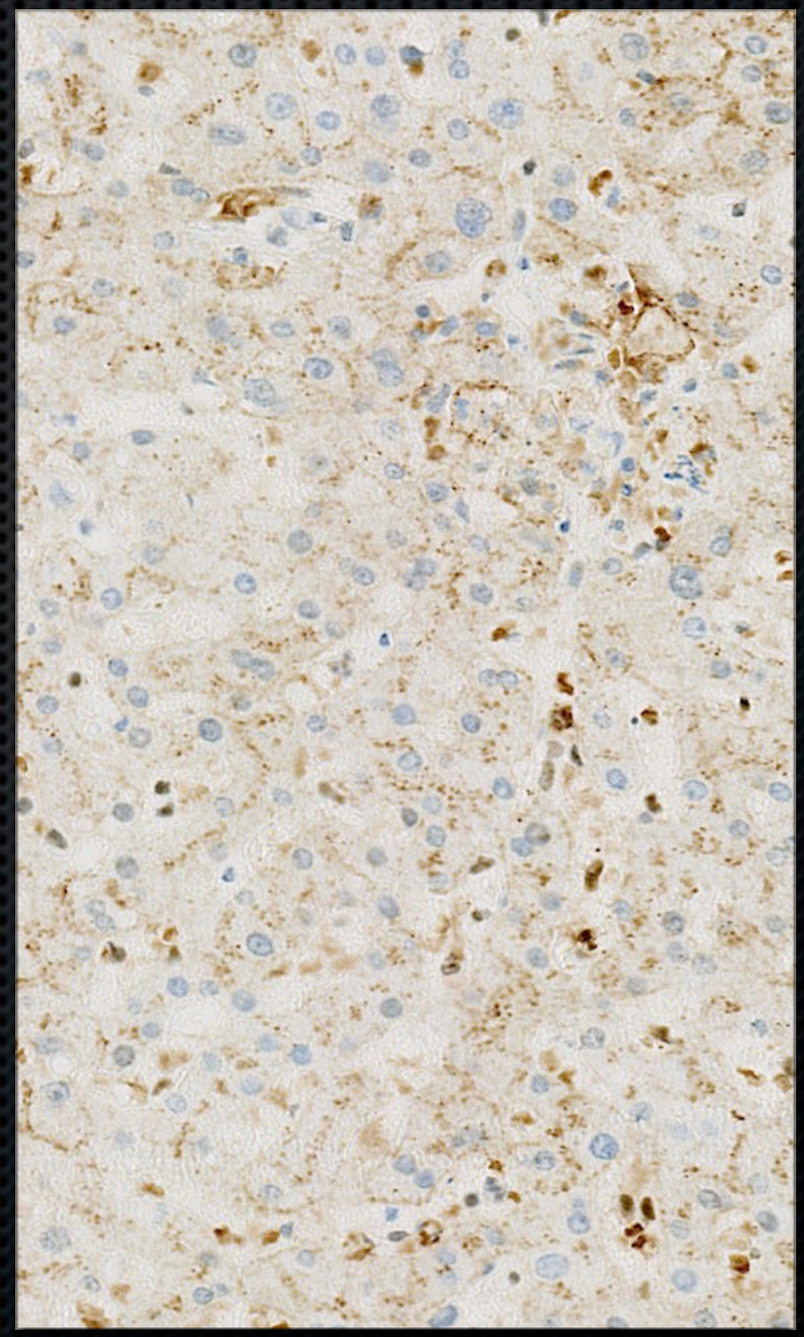
Odense data



Liver: No Fix delay



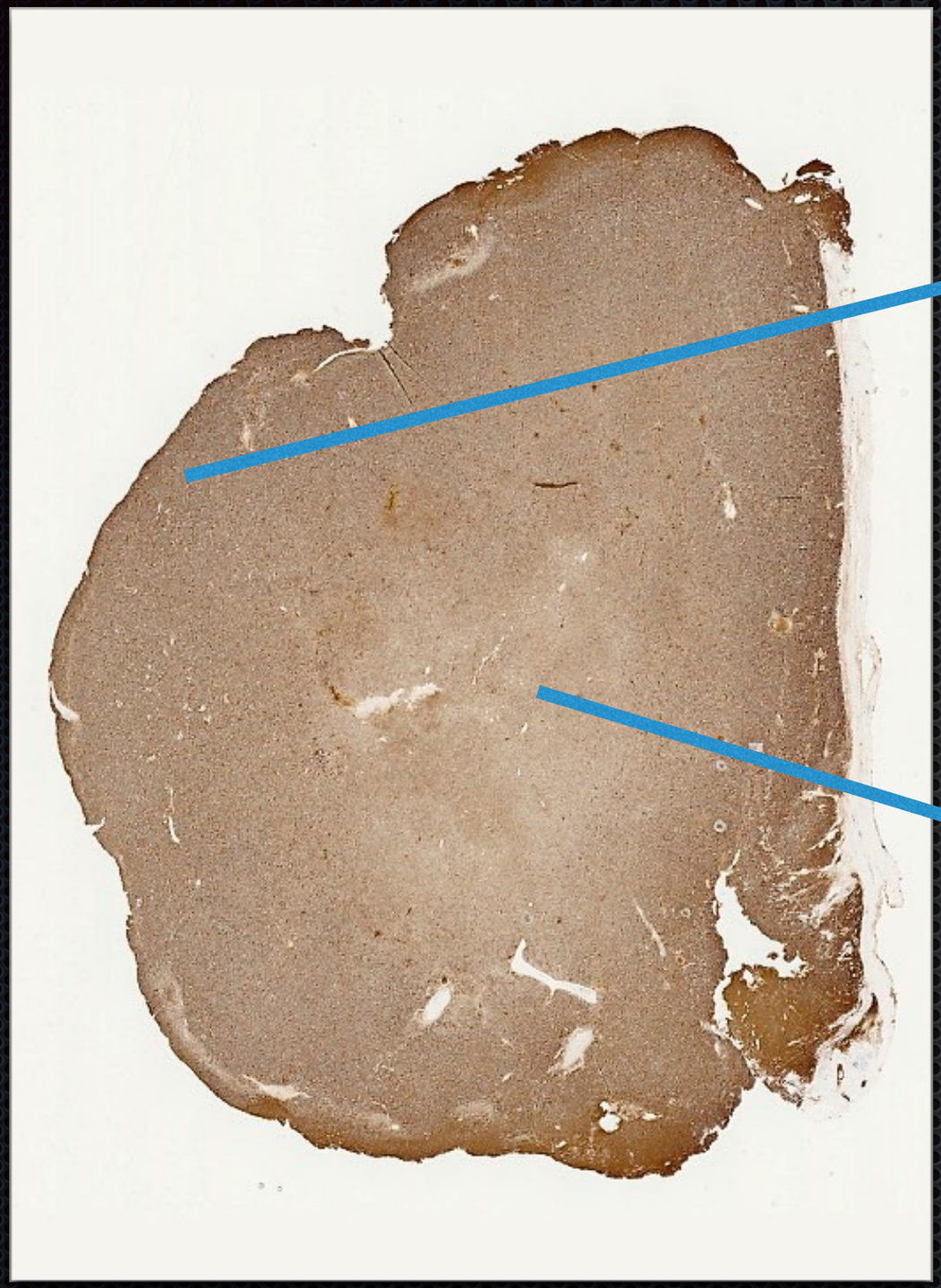
Liver 16 hrs delay

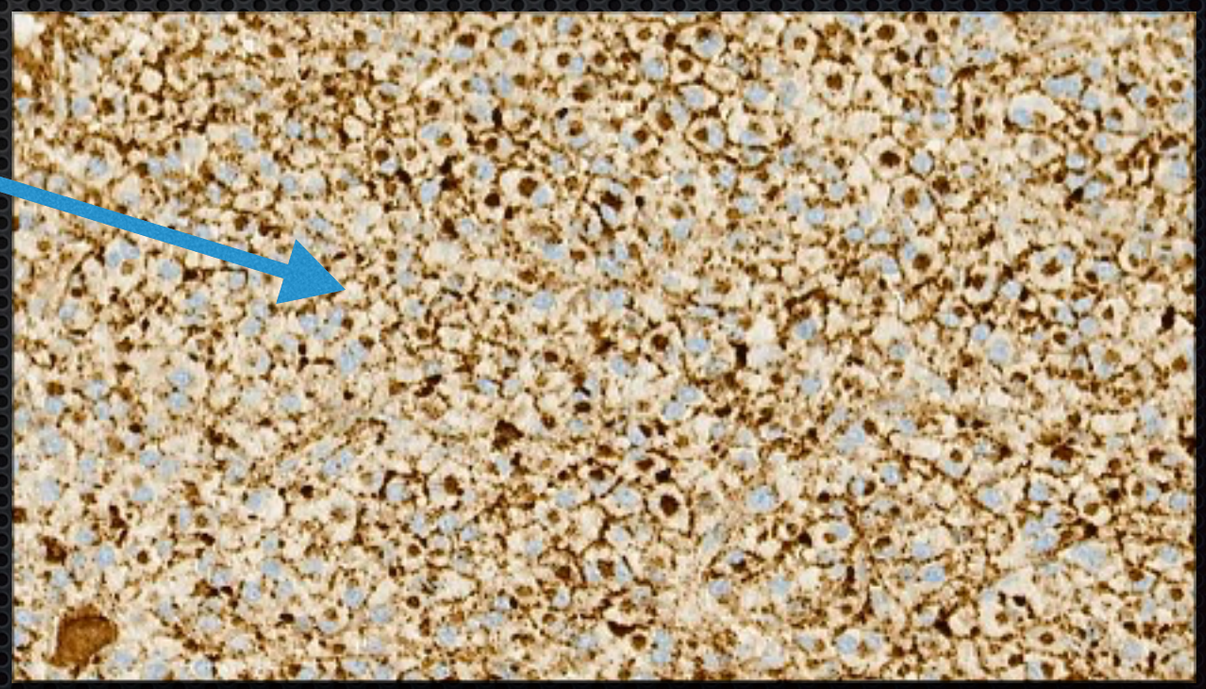


Liver 48 hrs delay

CD138 (B-A38): Simple marker of fixation delay

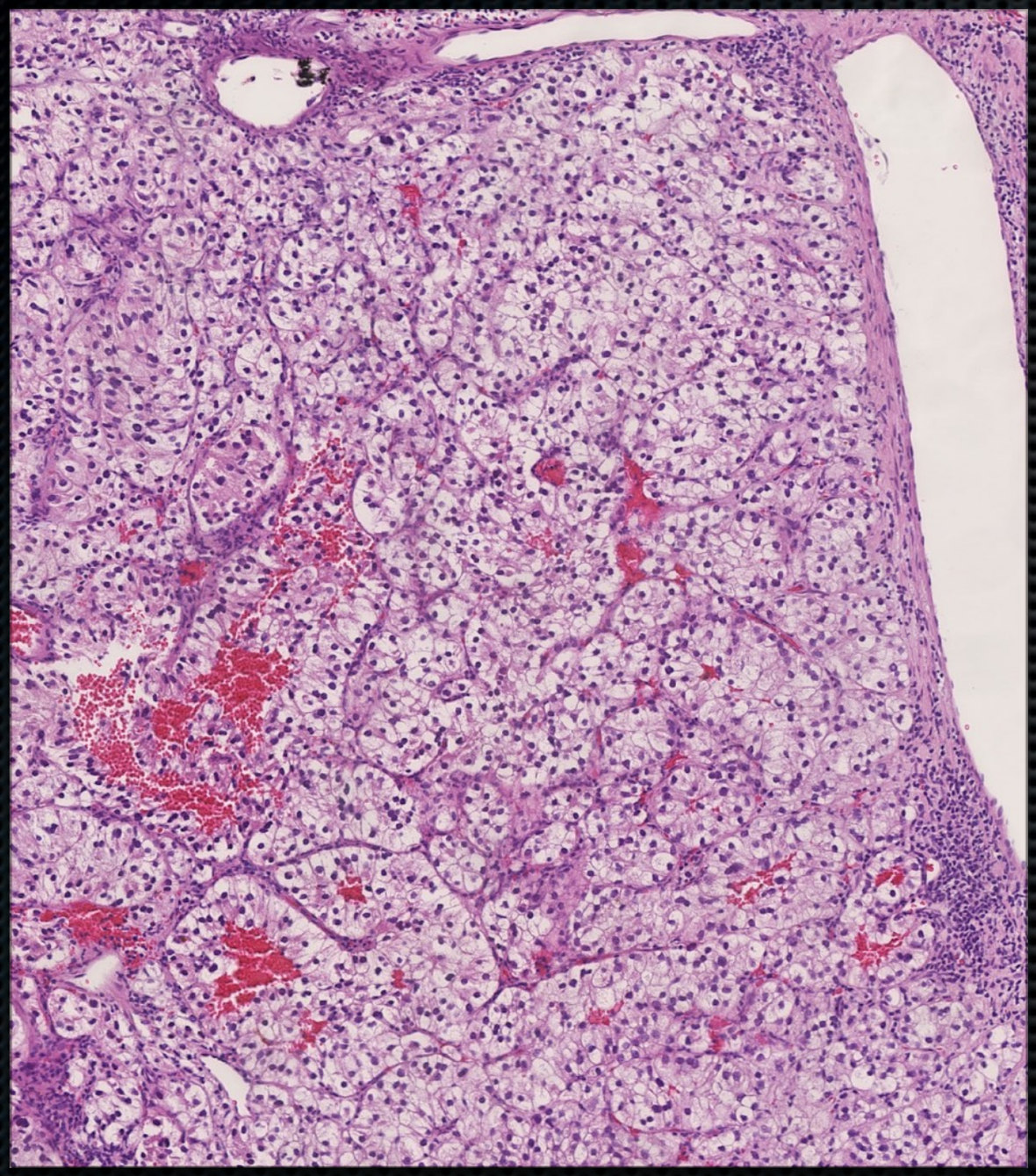
Odense data





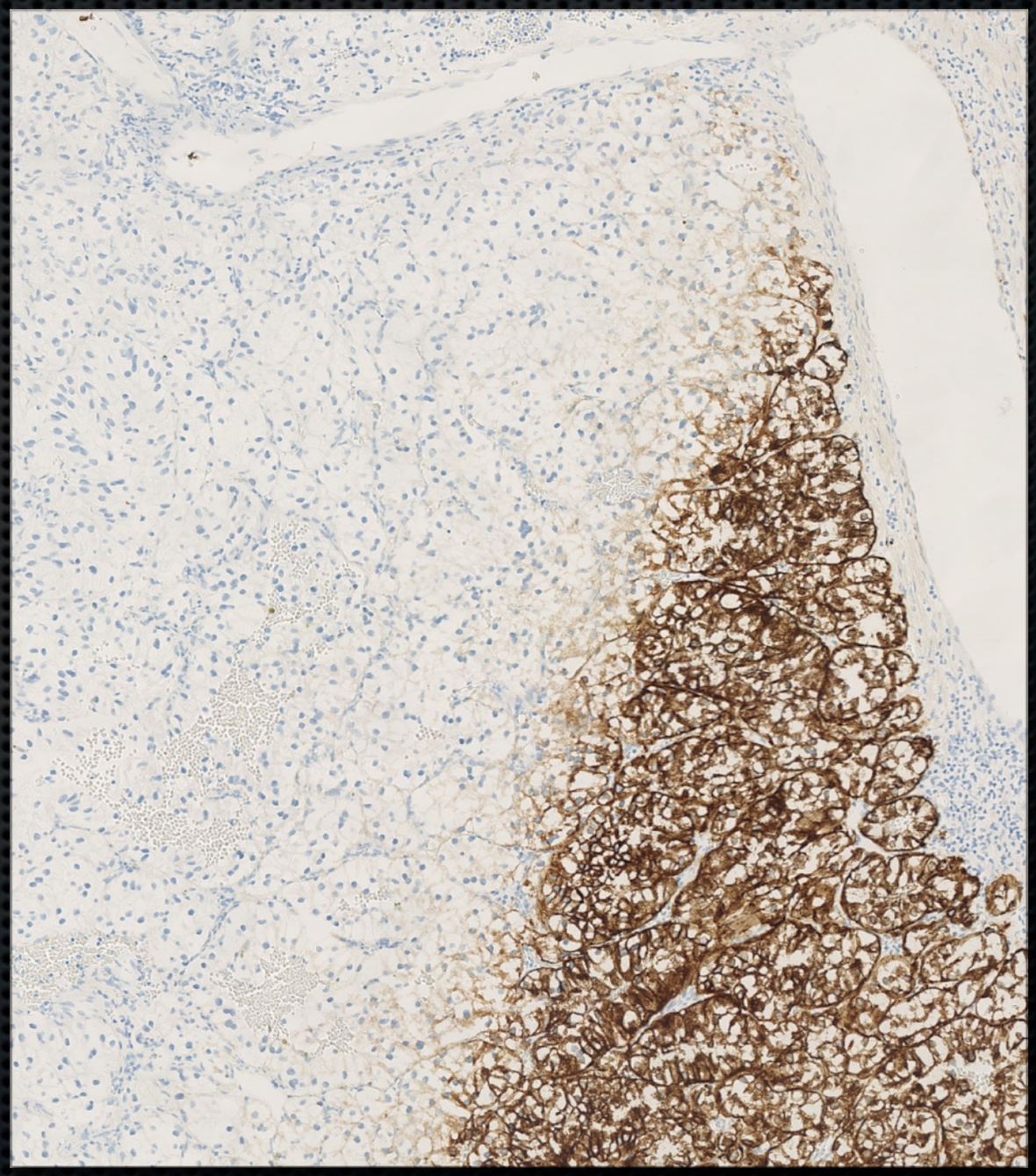
Plasmacytoma

CK, CAM5.2 simple marker of electrosurgery



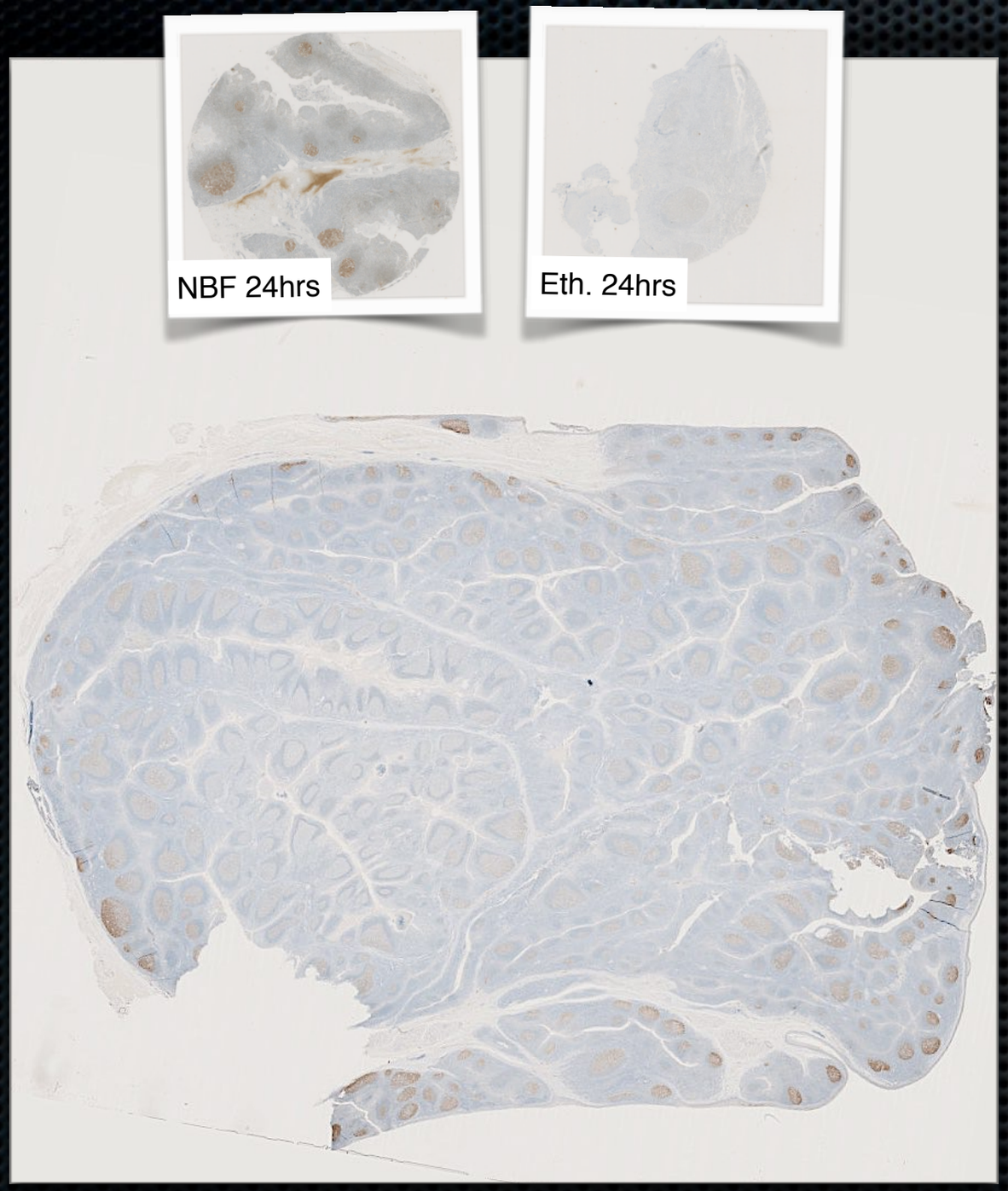
HE

RCC



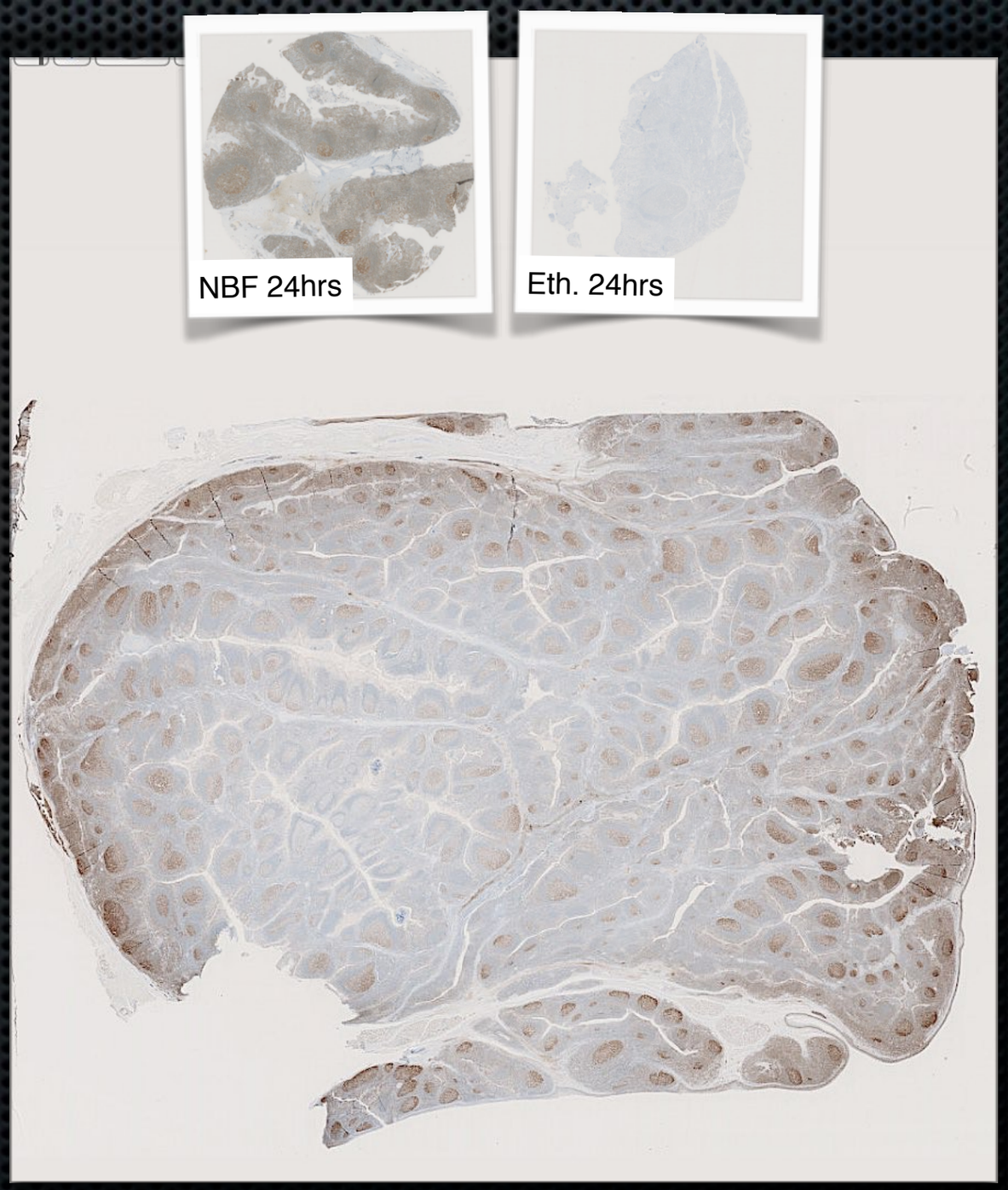
CK, CAM5.2

Markers of poor/short NBF fixation



BCL6, LN22

Tonsil



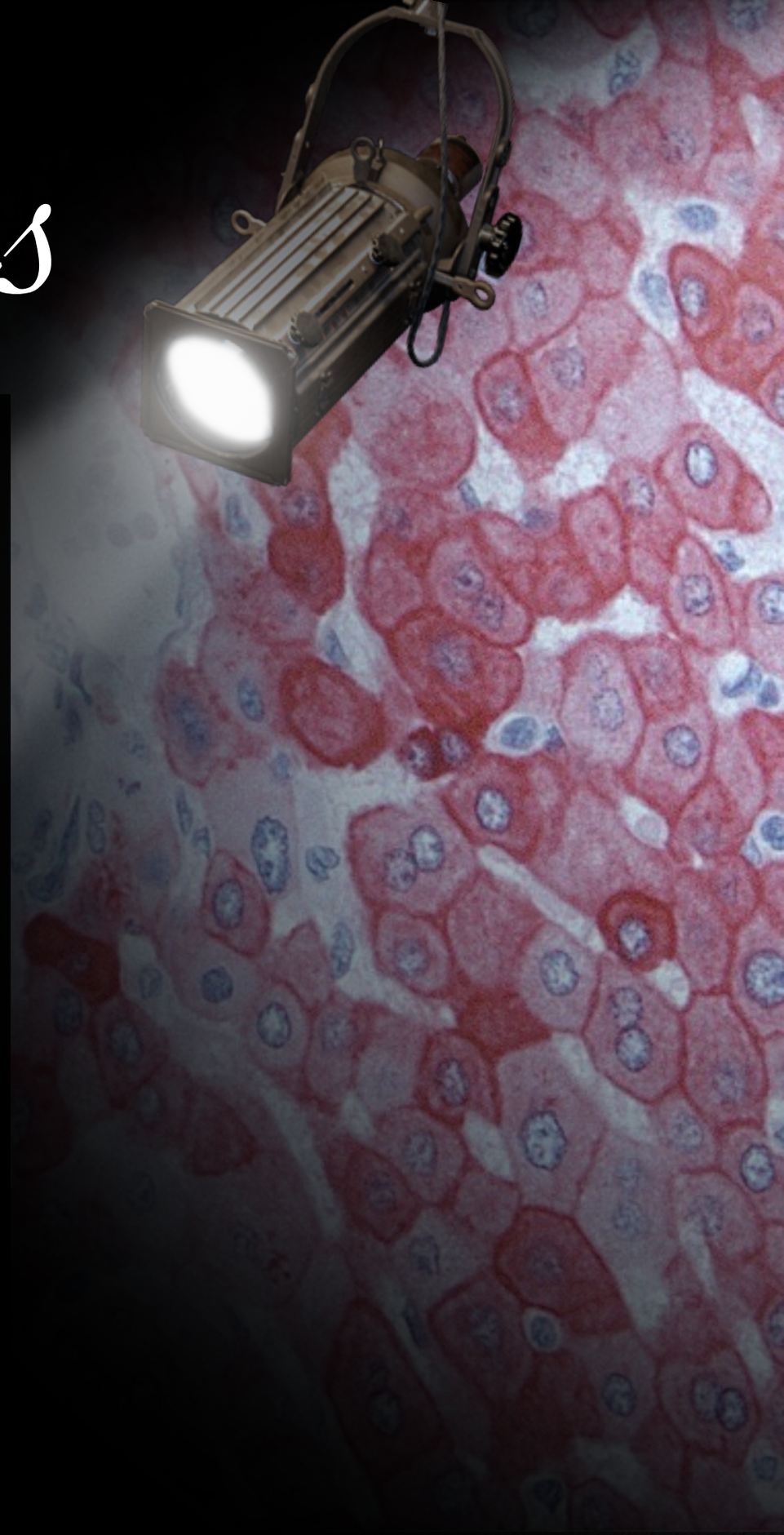
PMS2, EPR3947

Conclusions

- Less than half of the identified preanalytical variables in IHC have been examined in published research

- The majority of tested preanalytical variables impact the final IHC results

- There is a continued need for rigorous research and comprehensive guidelines on specimen fixation, processing, and storage



NordiQC

Thank you for your attention!