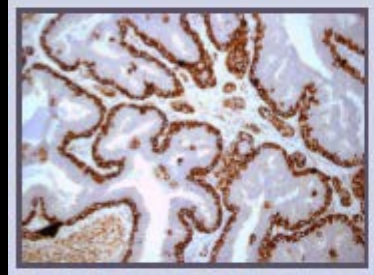
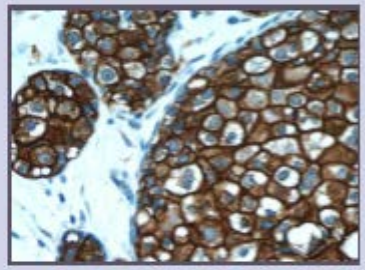




NordiQC

TATA MEDICAL CENTER

ISIMm
International Society for IHC and Molecular Morphology

International Symposium on Immunohistochemistry

January 4th - 7th, 2018

Immunohistochemical principles
The technical test approach

Pre-analytical parametres

Søren Nielsen
Global Pathology Manager
Agilent Technologies

(Former Scheme Manager, NordiQC)

IHC – The Technical Test Approach

CME - DAY 1 Thursday - 04.01.2018

09:00 – 13:00	***Morning session – the IHC and molecular laboratory. Moderator: CliveTaylor***
09:00 – 09:30	Søren Nielsen: Pre-analytical IHC parameters
09:35 – 10:10	Søren Nielsen: Analytical IHC parameters I (clone selection, protocol optimization, automation)
10:15 – 10:45	*Coffee break*
10:45 – 11:30	Mogens Vyberg: The impact of proficiency testing on lab immunoassays
11:35 – 12:10	Søren Nielsen: Analytical IHC parameters II (control selection)
12:15 – 12:55	T. S. Sridhar : Molecular studies on FFPE tissue
13:00 – 14.30	*Lunch break*
14:30 – 17:00	***Afternoon interactive Parallel IHC Session***
	IHC session, technicians | *IHC session, pathologists*
14:45 – 15:45	Søren Nielsen: Technical pitfalls, trouble shooting, internal quality control - **for technicians** Taylor, Bhargava, Vyberg: Diagnostic pitfalls, trouble shooting - **for pathologists**
15:45 – 16:15	*Coffee break*
16:15– 17:15	Søren Nielsen: Technical pitfalls, trouble shooting, internal quality control (cont'd) - **for technicians** Taylor, Bhargava, Vyberg:Diagnostic pitfalls, trouble shooting (cont'd) - **for pathologists**

IHC – The Technical Test Approach

IHC project coordinator at Institute of Pathology, Aalborg, Denmark & Scheme manager NordiQC – till 11.2017.

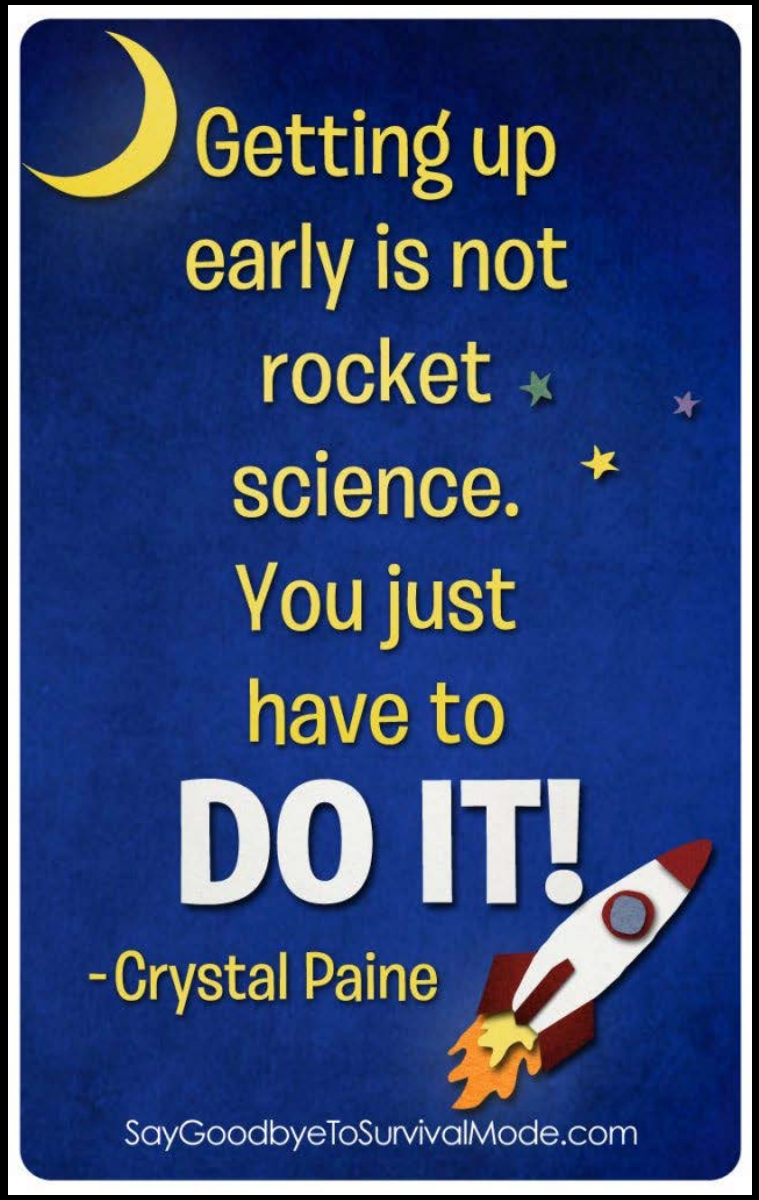
- > 70.000 IHC slides annually
  - BenchMark Ultra, Ventana
  - Autostainer Link 48, Dako
  - Omnis, Dako
  - Bond III, Leica
- IHC cooperation partners
  - Biocare
  - Cell Marque
  - Dako / Agilent
  - Leica
  - Thermo Fisher
  - Ventana / Roche
  - + Ad hoc projects/partners

IHC – The Technical Test Approach

Agenda:

1. Focus on the main IHC technical challenges
2. How to optimize IHC assays
3. How to set-up and use controls to calibrate, verify and validate IHC assays

IHC – The Technical Test Approach



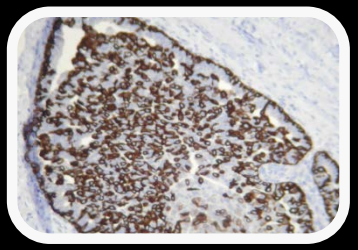
No formula I without basics – Tyres

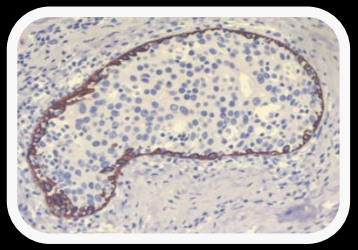
No IHC without high quality tissue

The Power of IHC – e.g. Breast Pathology

Hyperplasia or In-situ

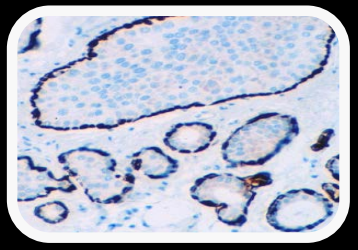
CK5, CK14, Heavy chain myosin, p63

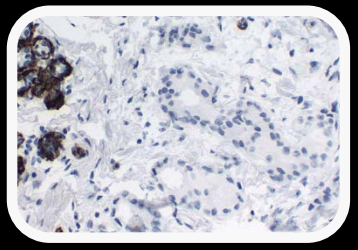




In-situ or invasive

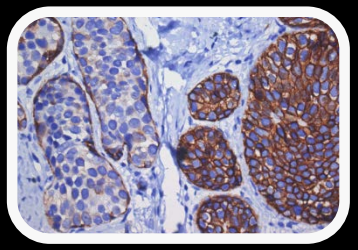
CK5, CK14, Heavy chain myosin, p63

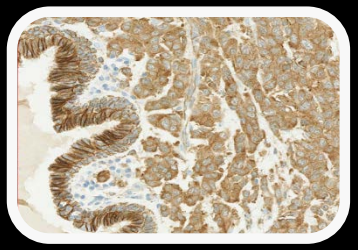




Lobular or ductal lesion

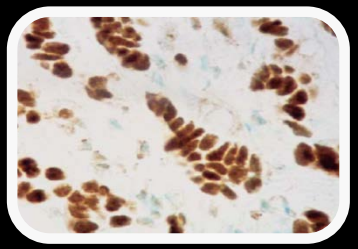
E-cadherin, p120

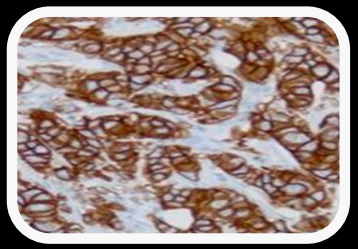




Predictive - Prognostic

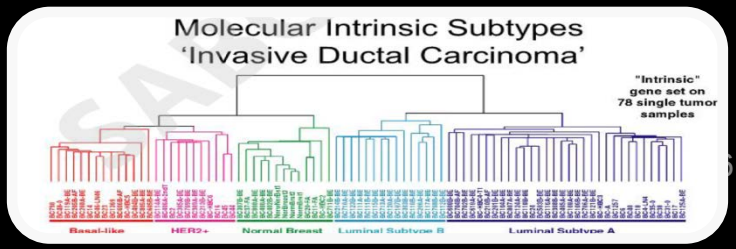
ER, PR, HER2, Ki67





Intrinsic subtype

PAM50 – *ER, PR, HER2, Ki67, CK5*

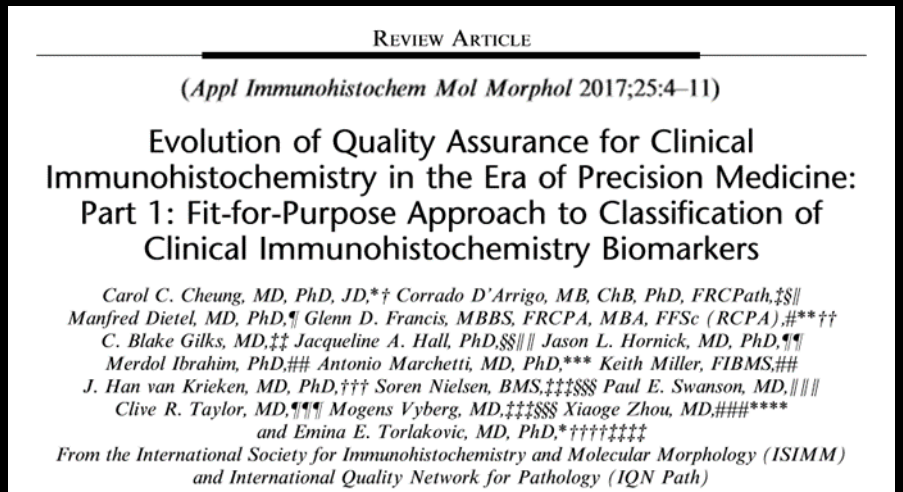


IHC – The Technical Test Approach

Original nomenclature and grouping of IHC tests:

- Class I / Type I IHC tests: Interpreted in the context of histo- or cytomorphologic and clinical data. Results interpreted and used by pathologists. E.g. CD45, TTF1, SOX10, CDX2, p40 etc

- Class III /Type II IHC tests: Stand-alone tests being interpreted (largely) to provide predictive and prognostic information. Results interpreted by pathologists and used by clinicians to give tailored treatment. E.g. ER, HER2, ALK, PD-L1 etc .



REVIEW ARTICLE

(*Appl Immunohistochem Mol Morphol* 2017;25:4–11)

Evolution of Quality Assurance for Clinical Immunohistochemistry in the Era of Precision Medicine: Part 1: Fit-for-Purpose Approach to Classification of Clinical Immunohistochemistry Biomarkers

Carol C. Cheung, MD, PhD, JD,† Corrado D'Arrigo, MB, ChB, PhD, FRCPath,‡§‖ Manfred Dietel, MD, PhD,¶ Glenn D. Francis, MBBS, FRCPA, MBA, FFSc (RCPA),#**†† C. Blake Gilks, MD,‡‡ Jacqueline A. Hall, PhD,§§‖‖ Jason L. Hornick, MD, PhD,¶¶ Merdol Ibrahim, PhD,## Antonio Marchetti, MD, PhD,*** Keith Miller, FIBMS,## J. Han van Krieken, MD, PhD,††† Soren Nielsen, BMS,‡‡‡§§§ Paul E. Swanson, MD,‖‖‖ Clive R. Taylor, MD,¶¶¶ Mogens Vyberg, MD,‡‡‡§§§ Xiaoge Zhou, MD,###**** and Emina E. Torlakovic, MD, PhD,*†††‡‡‡‡*

From the International Society for Immunohistochemistry and Molecular Morphology (ISIMM) and International Quality Network for Pathology (IQN Path)

AJCP / SPECIAL ARTICLE

Am J Clin Pathol 2010;133:354-365

CME/SAM

Canadian Association of Pathologists–Association canadienne des pathologistes National Standards Committee/Immunohistochemistry

Best Practice Recommendations for Standardization of Immunohistochemistry Tests*

Emina Emilia Torlakovic, MD, PhD,[1] Robert Riddell, MD, FRCPath, FRCPC,[2] Diponkar Banerjee, MBChB, FRCPC, PhD,[3] Hala El-Zimaity, MD, MS, FRCPC,[4] Dragana Pilavdzic, MD, FRCPC,[5] Peter Dawe, MS,[6] Anthony Magliocco, MD, FRCPC,[7] Penny Barnes, MD, FRCPC,[8] Richard Berendt, MD, FRCPC,[9] Donald Cook, MD, FRCPC,[10] Blake Gilks, MD, FRCPC,[11] Gaynor Williams, MD, PhD,[12] Bayardo Perez-Ordonez, MD, FRCPC,[13] Bret Wehrli, MD, FRCPC,[14] Paul E. Swanson, MD,[15] Christopher N. Otis, MD,[16] Søren Nielsen, HT, CT,[17] Mogens Vyberg, MD,[17] and Jagdish Butany, MBBS, MS, FRCPC[13]

IHC – The Technical Test Approach

... The biomarker protocol trap – Caution: not for faint-hearted lab personel !!!!!

Fixation
Time, Type, Volume

Decalcification
Preparation

Pre-analytic

Tissue
Type, Dimension,
Laser resection,
De-differentiation

Section
Thickness
Storage
Drying

Pre-treatment

Primary antibody
Clone, Dilution
Buffer, Time, Temp

Manual
Stainer

Analytic

Visualization
Sensitivity, Specificity

Development
Sensitivity,
Localization

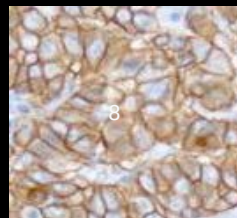
With 3 choices for 5 variables in each phase = > 4 million protocols….

Controlment

Quantification
Reporting

Post-analytic

Interpretation
Localization
Positive/Negative - cut-off level



IHC – The Technical Test Approach

The basal fundament for a technical optimal IHC performance:

- Appropriate tissue fixation and processing
- Appropriate and efficient epitope retrieval
- Appropriate choice & titre of antibody/clone
- Robust, specific & sensitive detection system
- Appropriate choice of control material

IHC – The Technical Test Approach

- Appropriate **tissue fixation** and processing
  - Problem 1: Delayed fixation – Cold ischemic time
  - Problem 2: Too short fixation in NBF
  - Problem 3: Other fixatives than NBF
  - *Too long fixation in NBF is not a problem !!!*
- Appropriate tissue fixation and **processing**
  - Problem 1: Agressive decalcification
  - Problem 2: Deviation from SOP – e.g. section baking

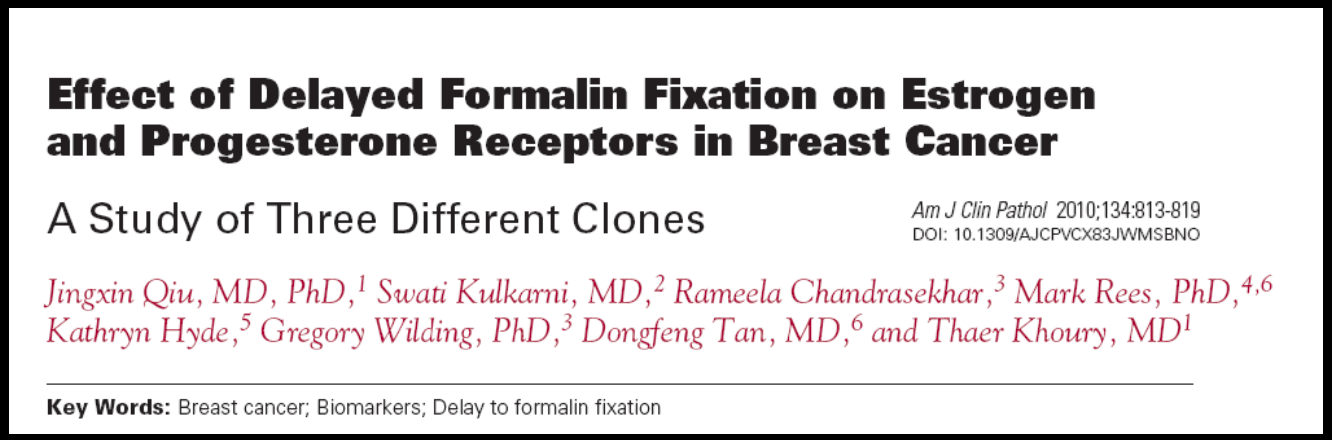
False negative or false positive

IHC – The Technical Test Approach

MODERN PATHOLOGY (2009) 22, 1457–1467

Delay to formalin fixation effect on breast biomarkers

Thaer Khoury[1], Sheila Sait[2], Helena Hwang[1], Rameela Chandrasekhar[3], Gregory Wilding[3], Dongfeng Tan[4] and Swati Kulkarni[5]



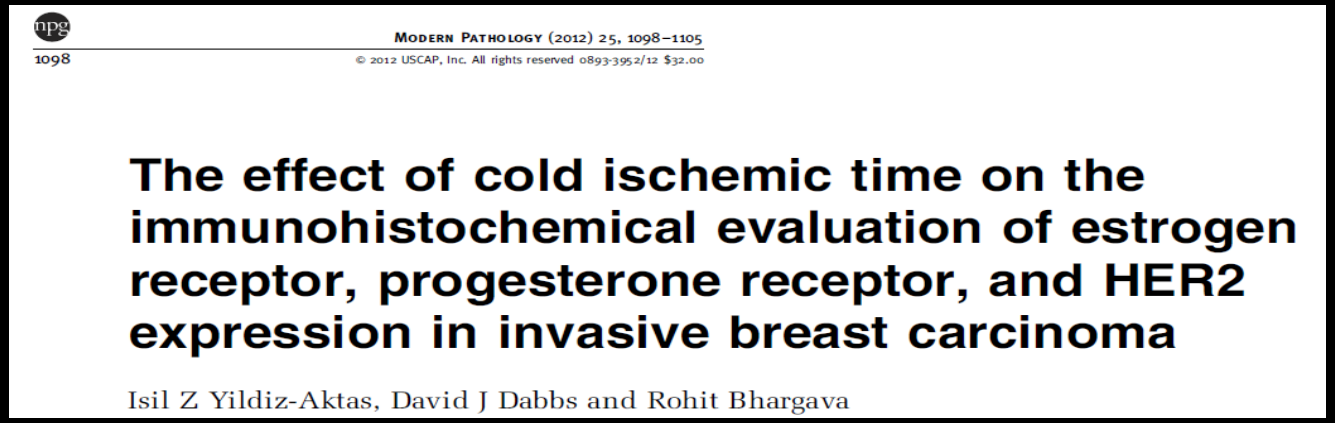
Effect of Delayed Formalin Fixation on Estrogen and Progesterone Receptors in Breast Cancer

A Study of Three Different Clones

Am J Clin Pathol 2010;134:813-819
DOI: 10.1309/AJCPVCX83JWMSBNO

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]

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



MODERN PATHOLOGY (2012) 25, 1098–1105

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

The vast majority of publications indicate inferior IHC/ISH performance in tissue subjected to delayed fixation.

But

To what degree ?
What is acceptable ?
What is best practice ?

IHC – The Technical Test Approach

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]

Time matters.....

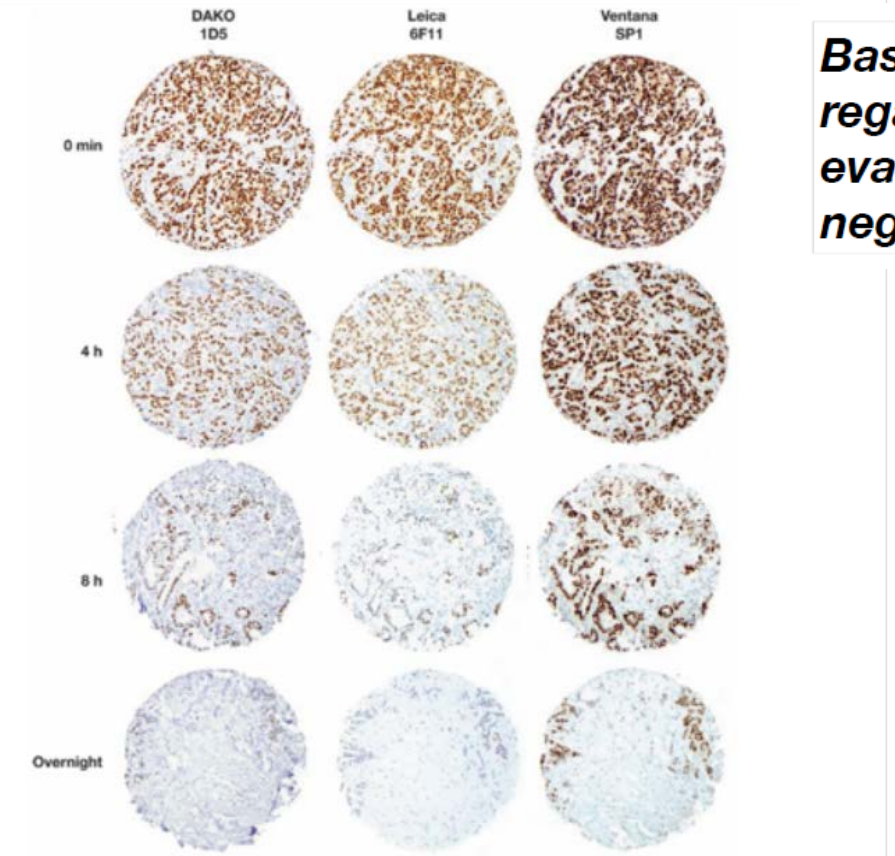


Image 1 (Case 9) Estrogen receptor expression by clones 1D5 (DAKO), 6F11 (Leica), and SP1 (Ventana) at different delayed formalin fixation times (0 minutes, 4 and 8 hours, and overnight). Note the decreased number/percentage of positive cells and the intensity of the stain with increased time of delayed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

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

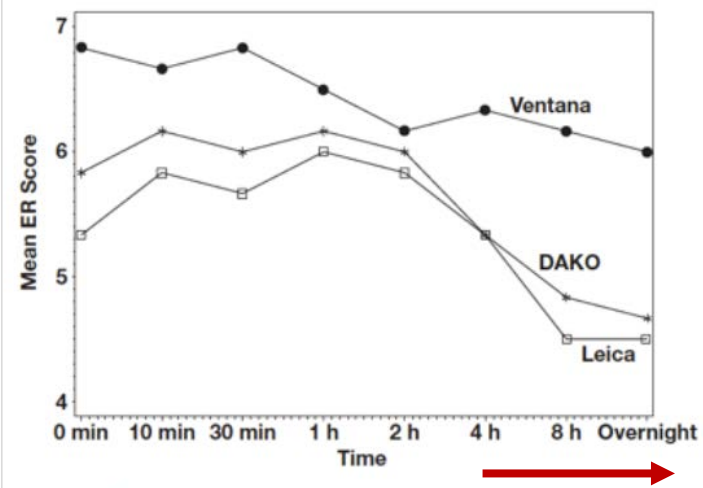


Figure 1 Mean Q score decline for estrogen receptor by clones 1D5 (DAKO), 6F11 (Leica), and SP1 (Ventana) in relation to time of fixation.

IHC – The Technical Test Approach

Modern Pathology (2012) 25, 1098–1105
1098
© 2012 USCAP, Inc. All rights reserved 0893-3952/12 $32.00

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

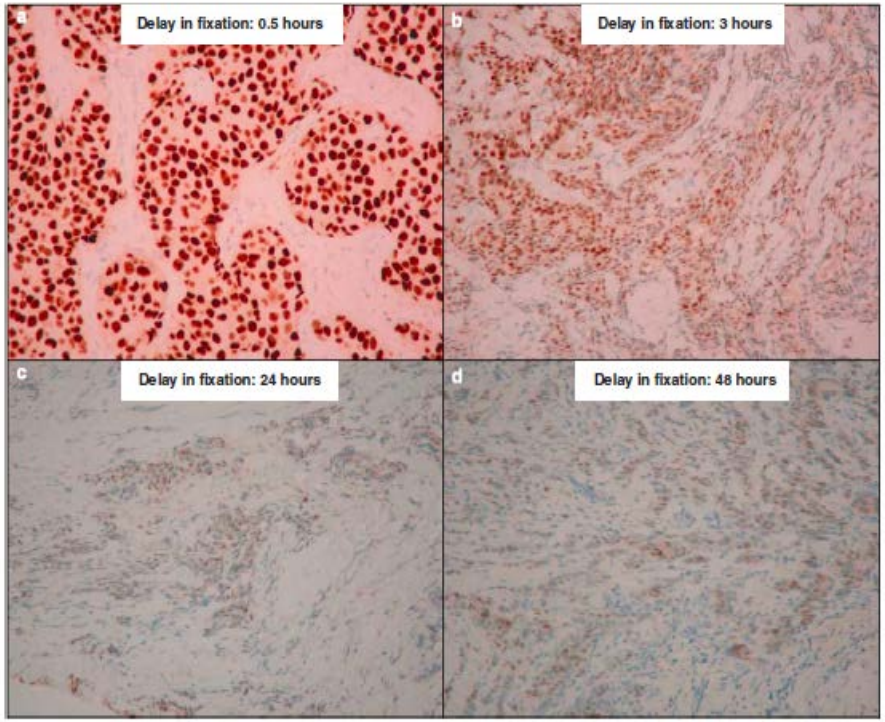


Figure 1 An example of a non-refrigerated case. The tumor was strongly positive for estrogen receptor (similarly to core biopsy) at 0.5 h of delayed fixation (**a**) but demonstrated significant reduction at 3 h (**b**), 24 h (**c**), and 48 h (**d**). All photomicrographs were taken at × 200.

H-score: intensity (0-3) x proportion (%)

Table 3 Average and median ER and PR H-scores for different cold ischemic time periods for refrigerated samples

4°C

Cold ischemic time period (h)	*ER H-score (mean and median)*	*PR H-score (mean and median)*	*ER H-score compared with core (P-value)*	*PR H-score compared with core (P-value)*
0.5	193; 230	129; 150	0.5608	0.9361
1	200; 230	128; 140	0.7301	0.9092
2	194; 220	132; 170	0.5762	0.9916
3	190; 220	120; 155	0.4967	0.7244
4	182; 215	104; 80	0.3365	0.3855
24	159; 210	100; 75	0.1146	0.3356
48	145; 160	77; 20	0.0637	0.1130

Table 4 Average and median ER and PR H-scores for different cold ischemic time periods for non-refrigerated (at room temperature) samples

20°C/RT

Cold ischemic time period (h)	*ER H-score (mean and median)*	*PR H-score (mean and median)*	*ER H-score compared with core (P-value)*	*PR H-score compared with core (P-value)*
0.5	200; 230	133; 160	0.7180	0.9827
1	195; 220	122; 120	0.6218	0.7875
2	178; 210	105; 60	0.2858	0.4217
3	146; 180	87; 70	0.0312	0.1448
4	146; 170	78; 50	0.0389	0.0877
24	115; 95	68; 20	0.0031	0.0467
48	118; 90	63; 20	0.0049	0.0366

IHC – The Technical Test Approach

Modern Pathology (2012) 25, 1098–1105

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

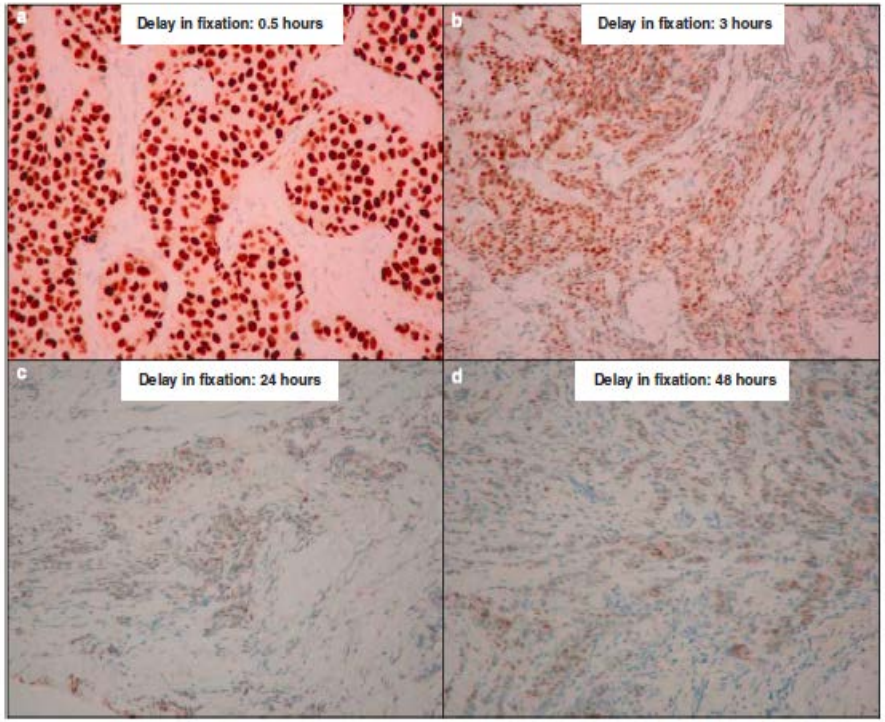


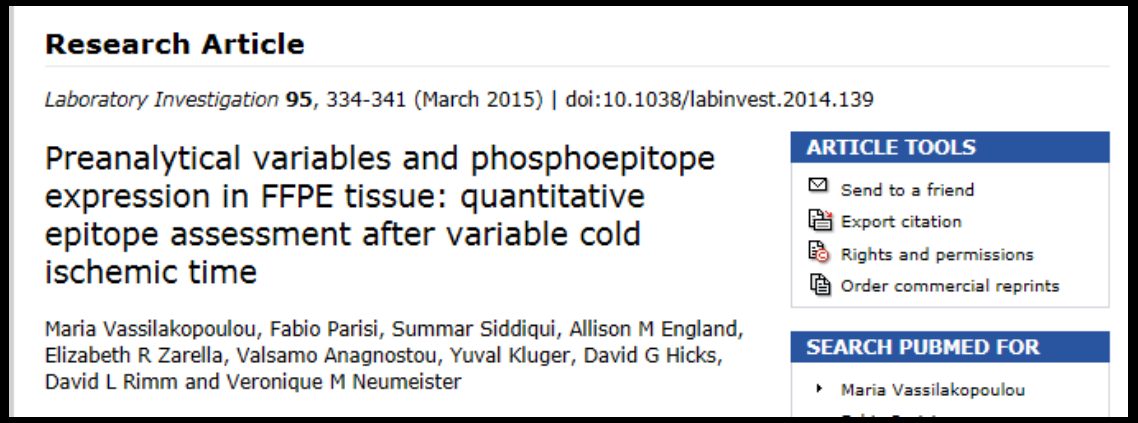
Figure 1 An example of a non-refrigerated case. The tumor was strongly positive for estrogen receptor (similarly to core biopsy) at 0.5 h of delayed fixation (**a**) but demonstrated significant reduction at 3 h (**b**), 24 h (**c**), and 48 h (**d**). All photomicrographs were taken at × 200.

H-score: intensity (0-3) x proportion (%)

Time and temp. matters.....

"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".

IHC – The Technical Test Approach



Research Article

Laboratory Investigation **95**, 334-341 (March 2015) | doi:10.1038/labinvest.2014.139

Preanalytical variables and phosphoepitope expression in FFPE tissue: quantitative epitope assessment after variable cold ischemic time

Maria Vassilakopoulou, Fabio Parisi, Summar Siddiqui, Allison M England, Elizabeth R Zarella, Valsamo Anagnostou, Yuval Kluger, David G Hicks, David L Rimm and Veronique M Neumeister

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SEARCH PUBMED FOR

Maria Vassilakopoulou

computed using bootstrapping. The majority of the epitopes tested revealed changes in expression levels with increasing time to formalin fixation. Some phosphorylated proteins, such as phospho-HSP27 and phospho-S6 RP, involved in post-translational modification and stress response pathways increased in expression or phosphorylation levels. Others (like phospho-AKT, phosphor-ERK1/2, phospho-Tyrosine, phospho-MET, and others) are quite labile and loss of antigenicity can be reported within 1–2h of cold ischemic time. Therefore specimen collection should be closely monitored and subjected to quality control measures to ensure accurate measurement of these epitopes. However, a few phosphoepitopes (like phospho-JAK2 and phospho-ER) are sufficiently robust for routine usage in companion diagnostic testing.

Cold ischemic time 1-2 hours:

Phospho-HSP27	Increased
Phospho-AKT	Reduced
Phospho-ER	Stable

Message; Consistency in tissue handling and transportation… if possible…☺

Central for precision testing for precision medicine

IHC – The Technical Test Approach

Concl.: Cooling preserved specimens, whereas vacuum sealing added no effect

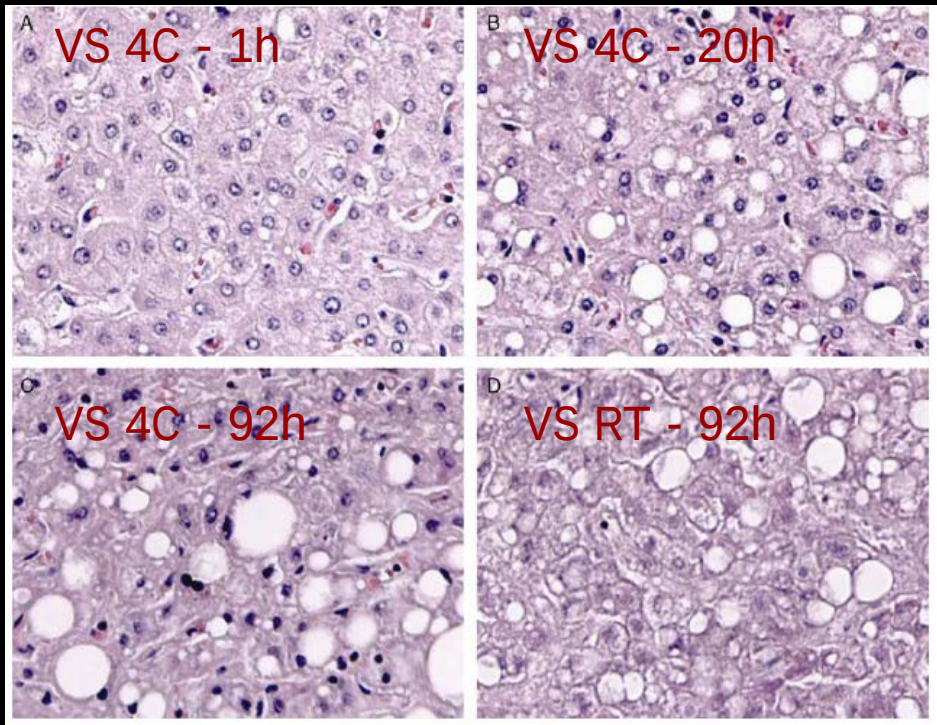
TECHNICAL ARTICLE

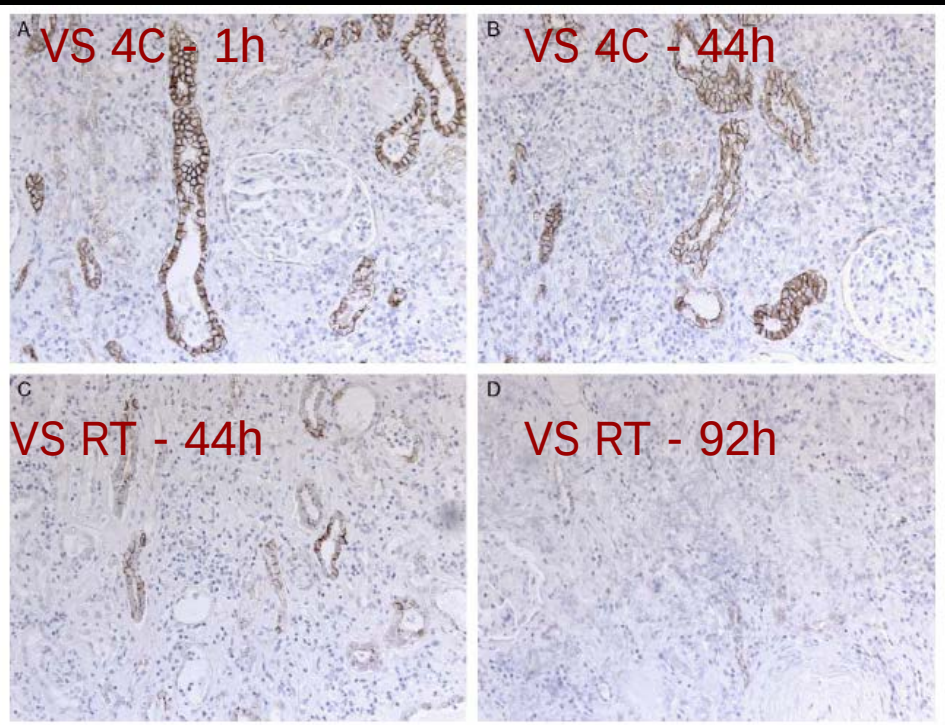
tochem Mol Morphol • Volume 19, Number 5, October 2011

Vacuum Sealing and Cooling as Methods to Preserve Surgical Specimens

(IHC and molecular assays)

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





IHC – The Technical Test Approach

Pre-analytical variable	Published guidelines ASCO/CAP - CLSI	Litterature based guidelines*
Fixation delay	1 hour	< 12 hours (*3-4 hours*)
Transportation temp.	No data	4°C better than RT
Vacuum sealing	No data	No data

* Engel and. Moore (*2011*) Effects of Preanalytical Variables on the Detection of Proteins by Immunohistochemistry in Formalin-Fixed, Paraffin-Embedded Tissue. Archives of Pathology & Laboratory Medicine: May 2011, Vol. 135, No. 5, pp. 537-543.

IHC – The Technical Test Approach

Published Ahead of Print on October 7, 2013 as 10.1200/JCO.2013.50.9984
The latest version is at http://jco.ascopubs.org/cgi/doi/10.1200/JCO.2013.50.9984

JOURNAL OF CLINICAL ONCOLOGY | ASCO SPECIAL ARTICLE

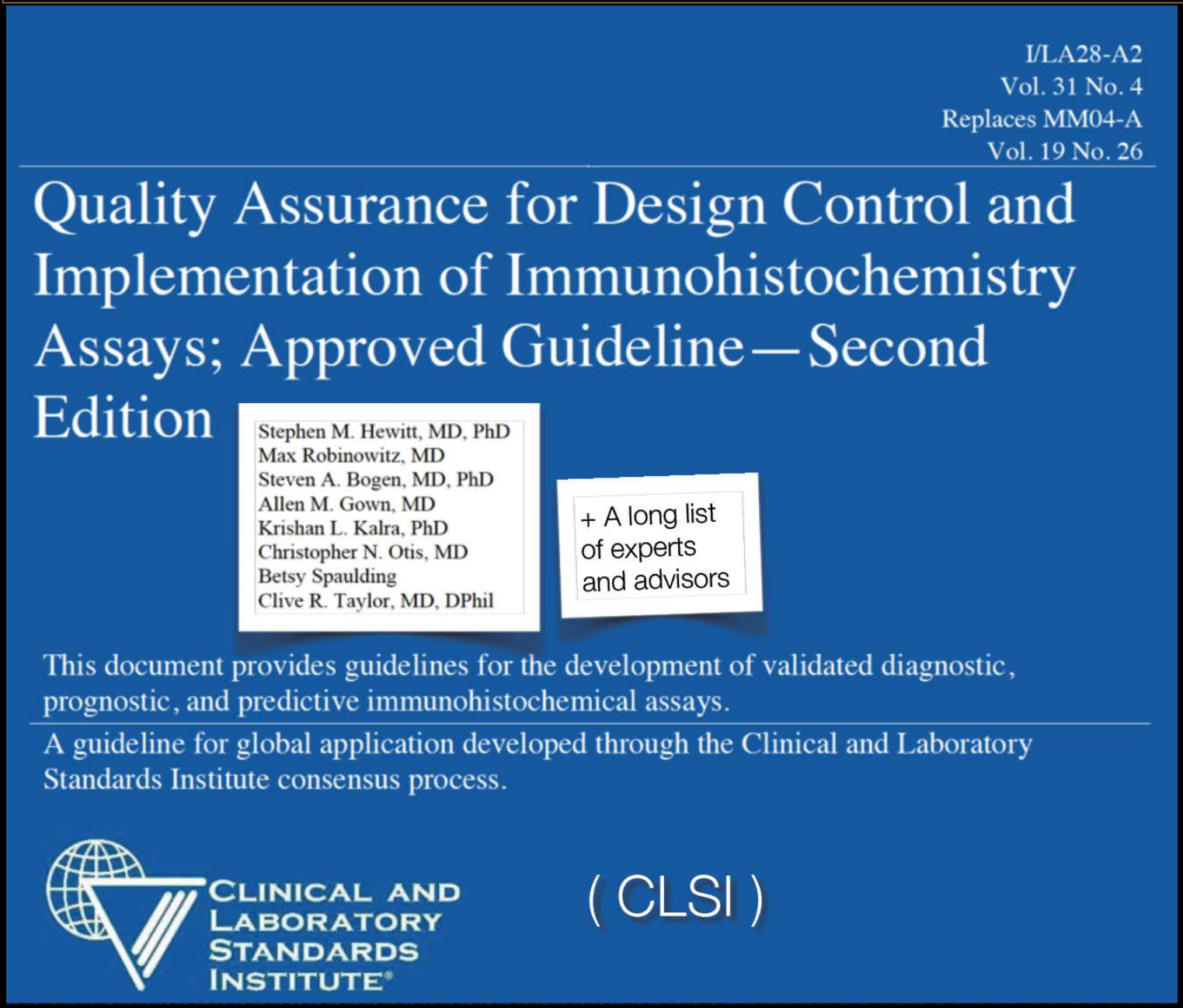
Recommendations for Human Epidermal Growth Factor Receptor 2 Testing in Breast Cancer: American Society of Clinical Oncology/College of American Pathologists Clinical Practice Guideline Update

Antonio C. Wolff, M. Elizabeth H. Hammond,* David G. Hicks,* Mitch Dowsett,* Lisa M. McShane,* Kimberly H. Allison, Donald C. Allred, John M.S. Bartlett, Michael Bilous, Patrick Fitzgibbons, Wedad Hanna, Robert B. Jenkins, Pamela B. Mangu, Soonmyung Paik, Edith A. Perez, Michael F. Press, Patricia A. Spears, Gail H. Vance, Giuseppe Viale, and Daniel F. Hayes**

Guidelines to;

Tissue handling
IHC / ISM methods
Interpretation
QA
.....

IHC – The Technical Test Approach



IHC – The Technical Test Approach

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

IHC – The Technical Test Approach

- For more than 70 years NBF has shown to have a bizarre effect

- Formaldehyde is one of the fastest solutions regrading tissue penetration but one of the slowest regarding fixation

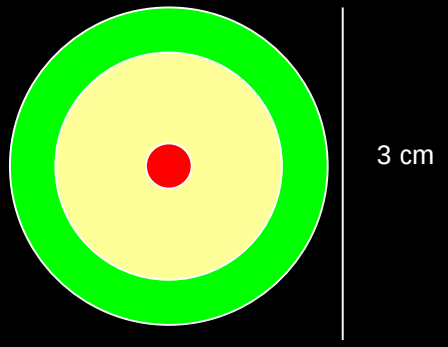
Phase I	**Penetration**	**Fast**
Phase II	**Binding**	**Moderate**
Phase III	**Cross-linking**	**Slow**

IHC – The Technical Test Approach

Formaldehyde fixation

How long will it take to fix?

How fast does Formalin penetrate in tissue....???



IHC – The Technical Test Approach

Formaldehyde fixation

How long will it take to fix?

Penetration time at K = 3.6 (Baker's coeeficient)

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

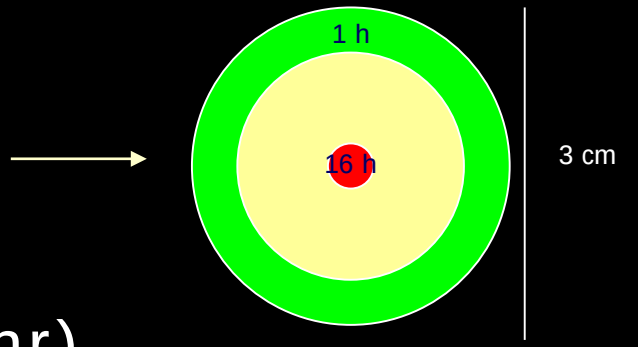
1 hour = 3.6 mm

4 hours = 7.2 mm (1.8 mm/hr)

16 hours = 14.4 mm (0.9 mm/hr)

64 hours = 28.8 mm (0.45 mm/hr)

256 hours = 57.6 mm (0.225 mm/hr)



(to double the depth takes 4x the time)

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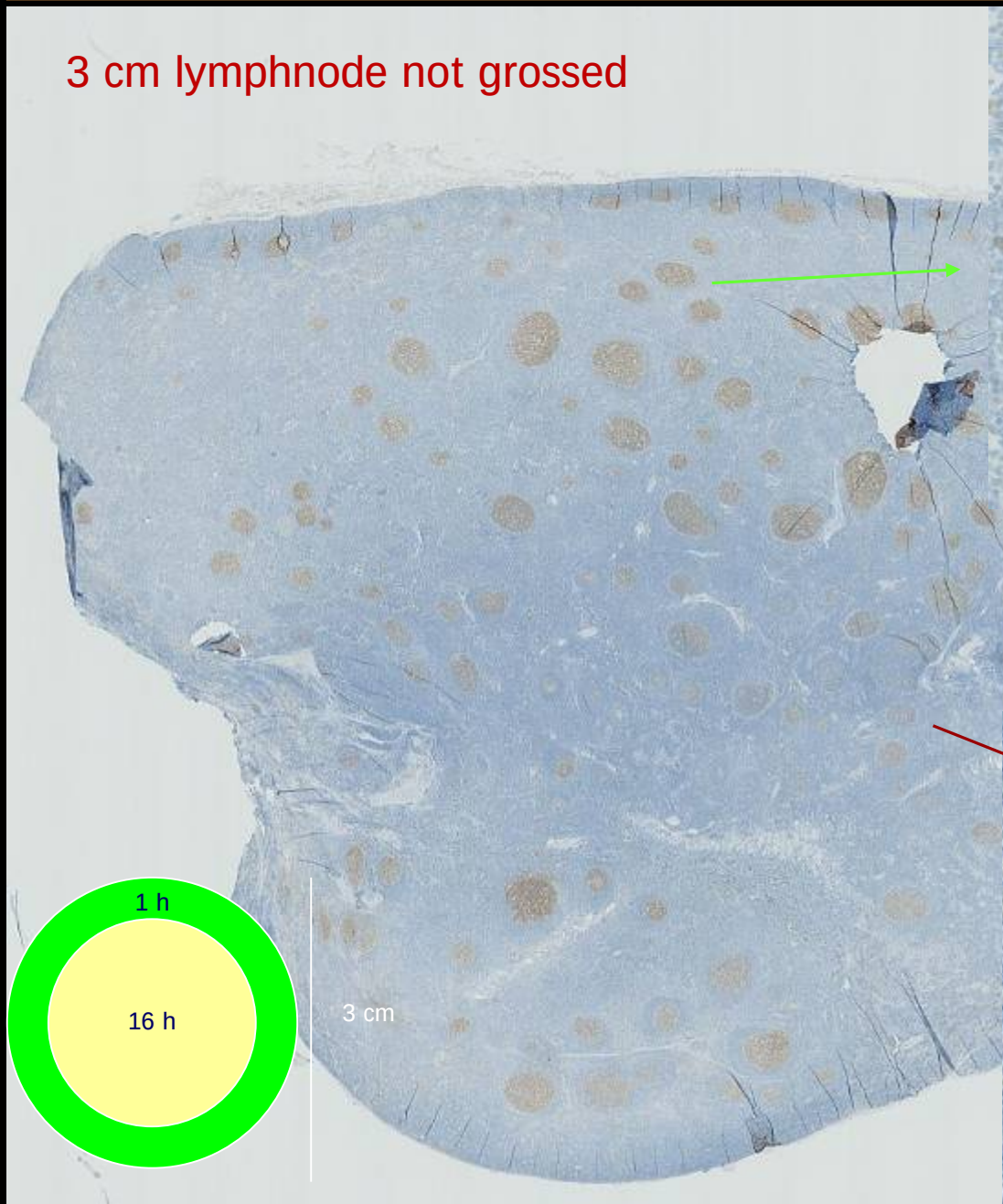
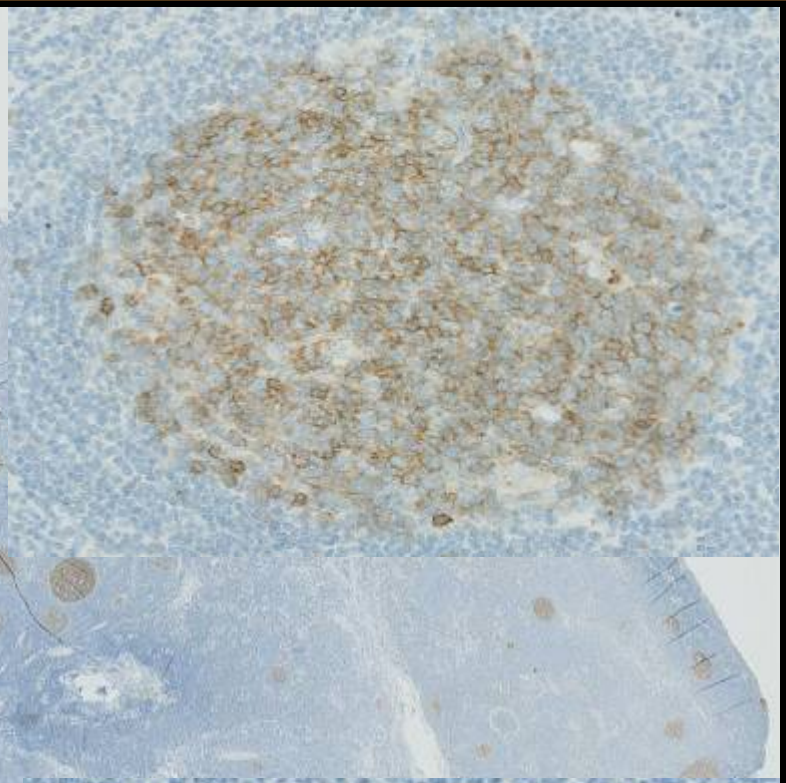
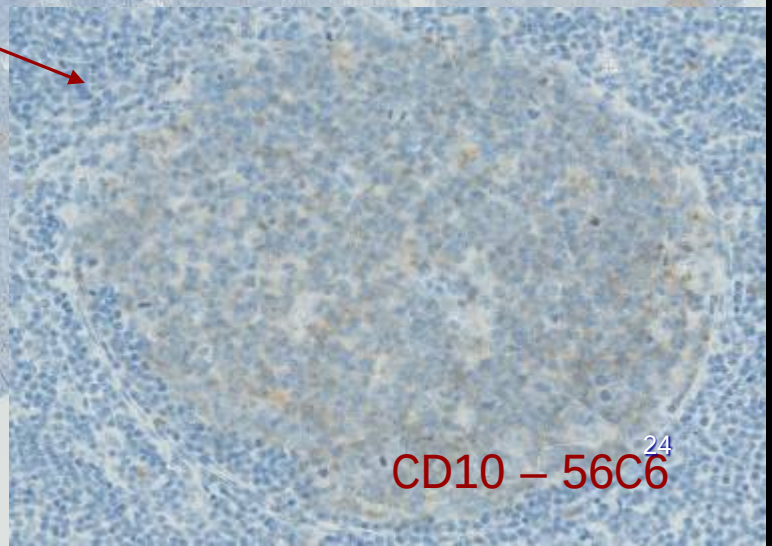
3 cm lymphnode not grossed







CD10 – 56C6

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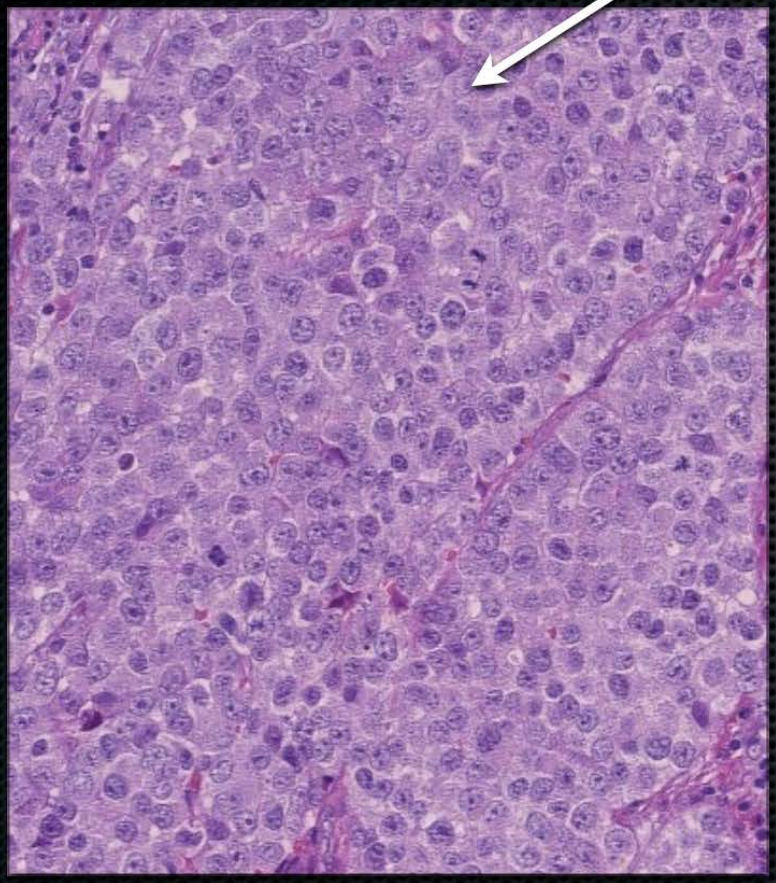
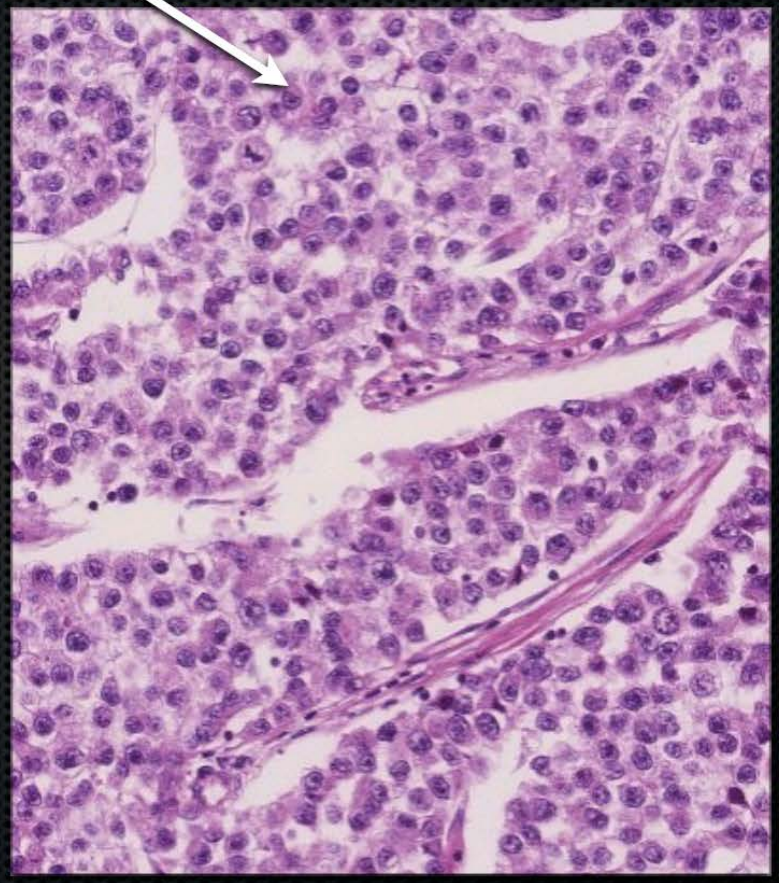
Seminoma

Poor tissue handling



Edge



Center

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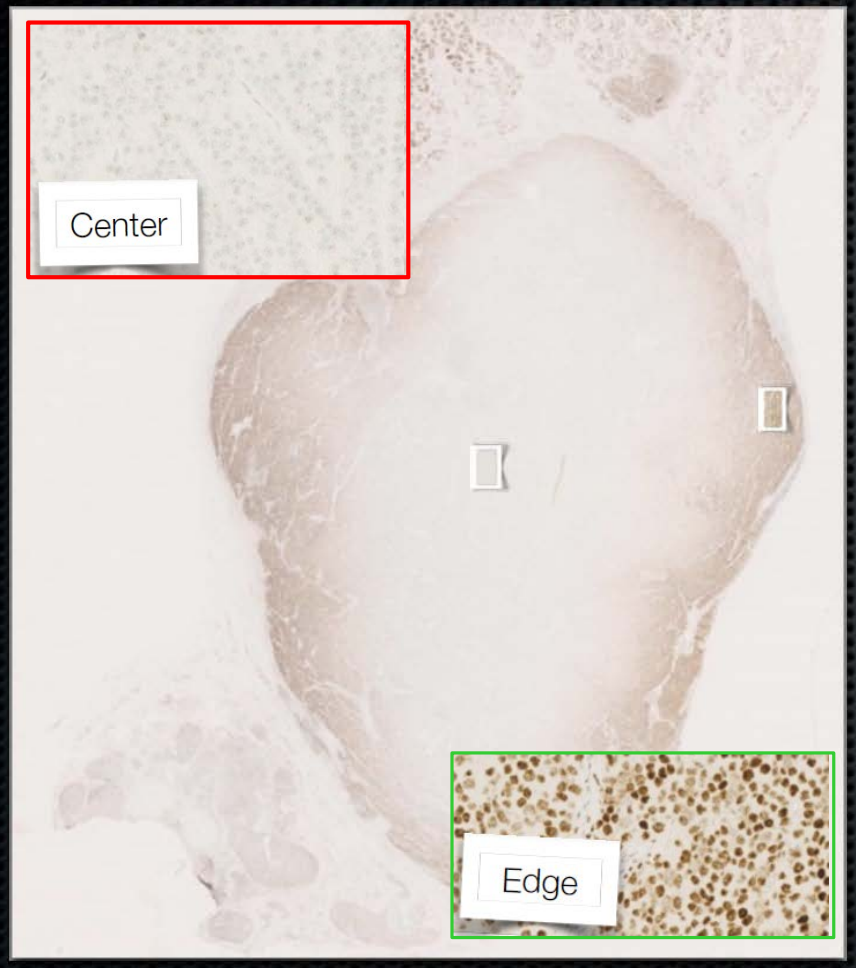
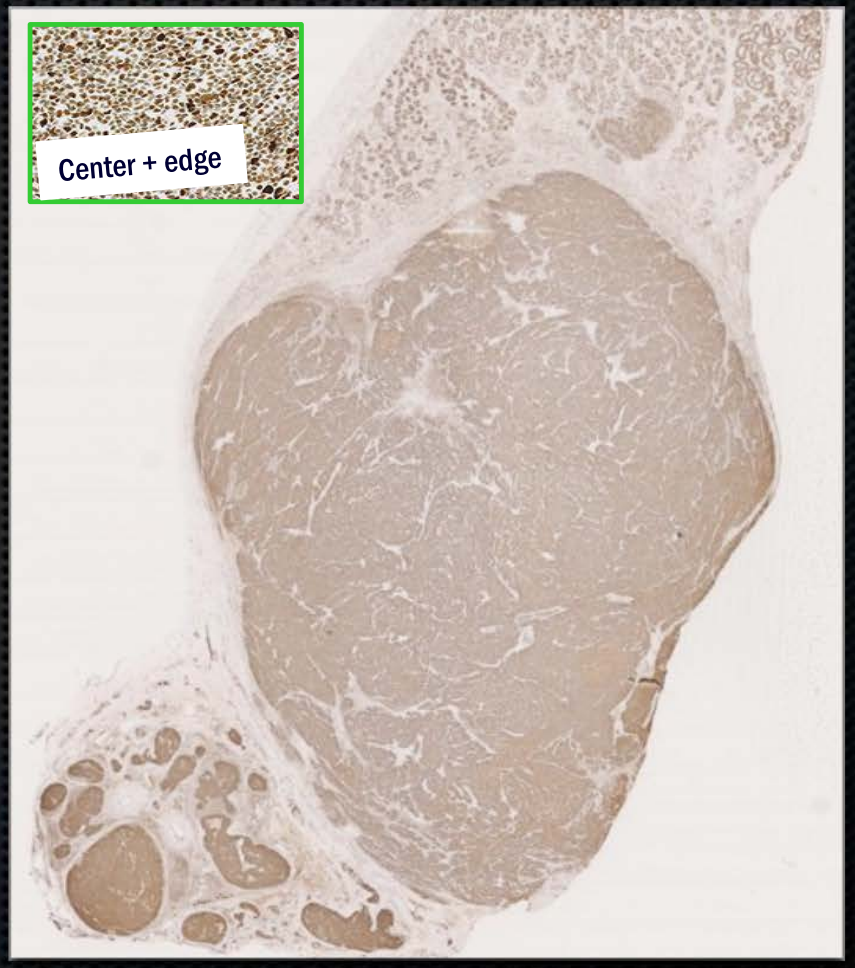


PMS2, EPR3947



MSH6, EP49

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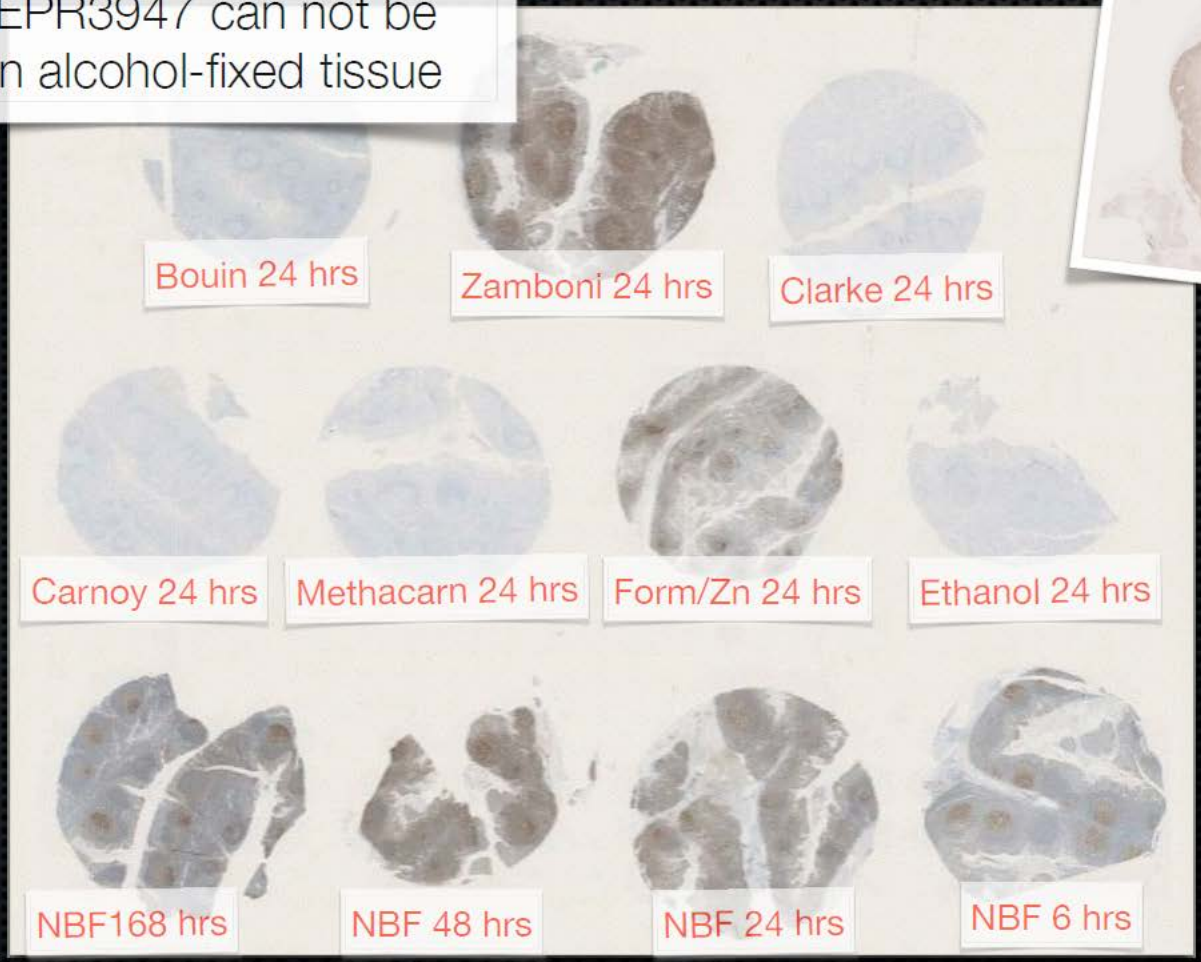
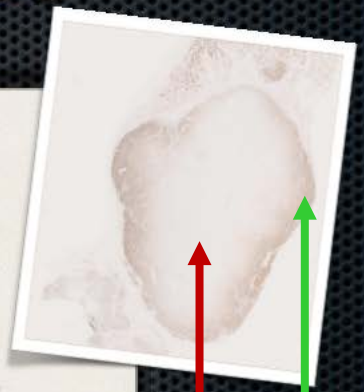
PMS2, EPR3947 and fixatives

Clone EPR3947 can not be used on alcohol-fixed tissue



Poor tissue handling



NBF fix

Alcohol fix

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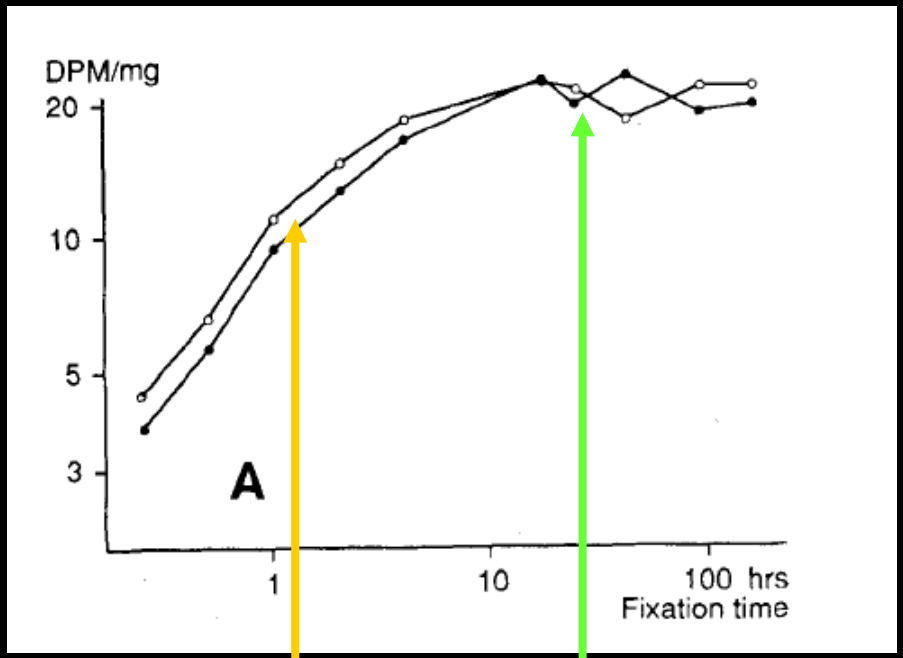
Kinetic Studies of Formaldehyde Binding in Tissue

1052-0295/94/6903-177/$3.00/0
BIOTECHNIC & HISTOCHEMISTRY®
Volume 69
Number 3

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



- room temp.
- 37°C

4 x 4 x 4 mm liver tissue

100 % binding of formaldehyde after 16-24 hours at 25°C

50 % binding of formaldehyde after 100 min. at 25°C

Helander, KG. Kinetic studies of formaldehyde binding in tissue. *Biotechnique and Histochemistry.* 1994; 69, 177 -179

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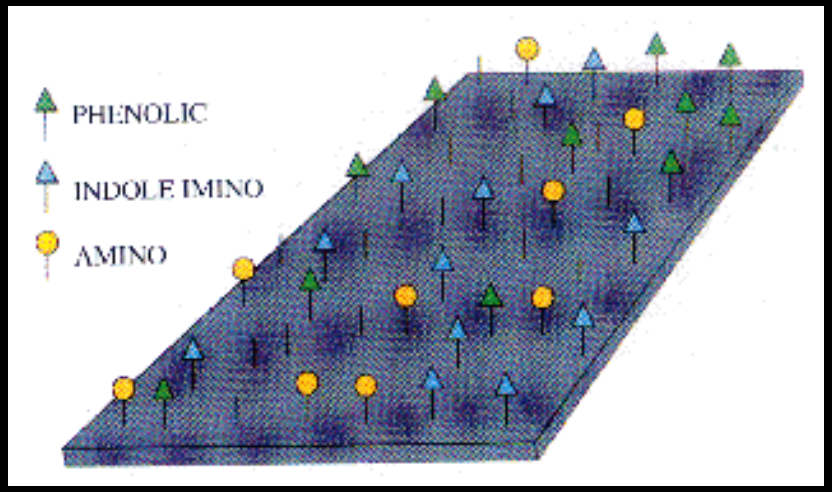
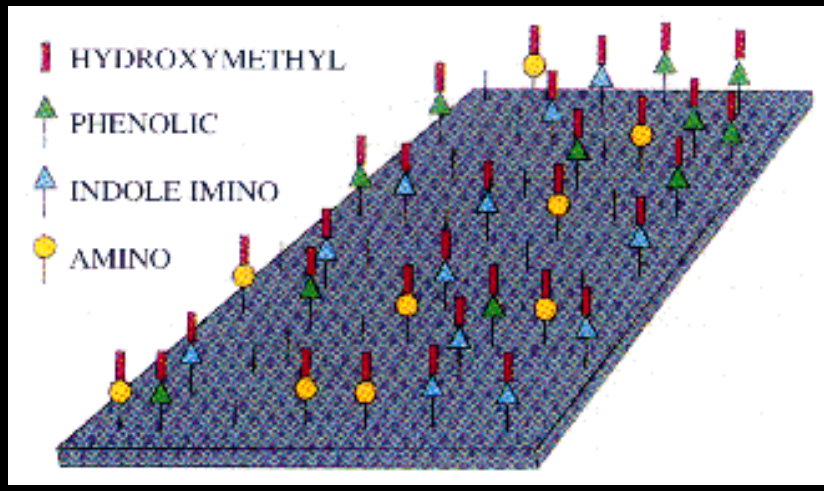
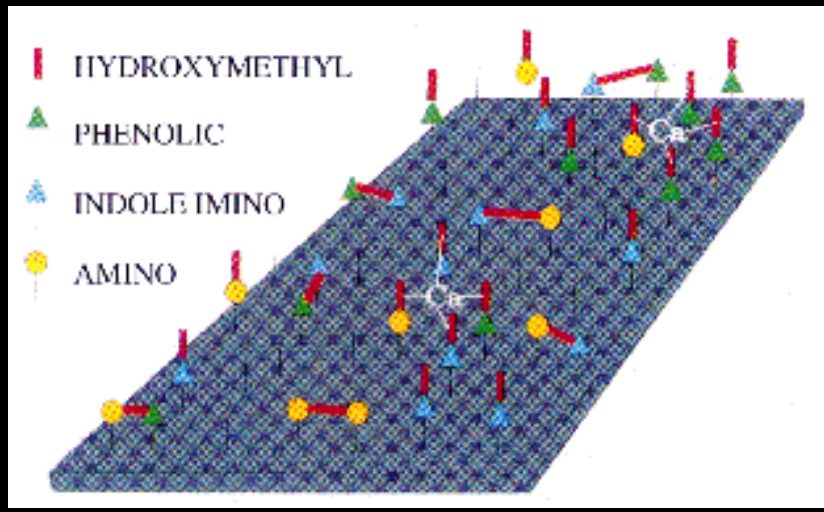
<16 - 24 h





Formaldehyde is a cross linking protein fixing agent, reacting ” clock-wise” - the longer the more effective !



>16 - 24 h

$$HC{=}CH.CHO + H_2N{-}P \longrightarrow H_2C{=}CH{-}CHOH{-}NH{-}P$$

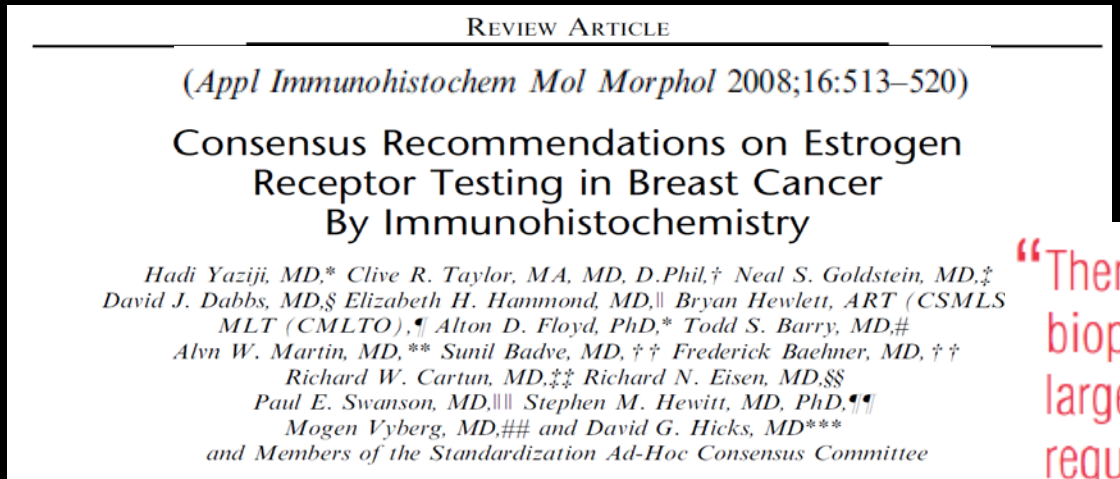
$$H_2C{=}CH{-}CHOH{-}NH{-}P + H_2N{-}P' \longrightarrow H_2C{=}CH{-}\underset{\displaystyle NH{-}P'}{\underset{|}{CH}}{-}NH{-}P + H_2O$$

Methylene glycol + free aldehyde = NBF

IHC – The Technical Test Approach

- To secure fixation and stabilization the fixation time is critical and not just the penetration time !!!
- 16 - 24 h minimum for a 1 mm biopsy
- 16 - 24 h minimum for a 4 mm specimen

Penetration-time + Binding-time => Reaction/fixation-time



REVIEW ARTICLE

(*Appl Immunohistochem Mol Morphol* 2008;16:513–520)

Consensus Recommendations on Estrogen Receptor Testing in Breast Cancer By Immunohistochemistry

Hadi Yaziji, MD, Clive R. Taylor, MA, MD, D.Phil,† Neal S. Goldstein, MD,‡ David J. Dabbs, MD,§ Elizabeth H. Hammond, MD,‖ Bryan Hewlett, ART (CSMLS MLT (CMLTO),¶ Alton D. Floyd, PhD,* Todd S. Barry, MD,# Alvn W. Martin, MD,** Sunil Badve, MD,†† Frederick Baehner, MD,†† Richard W. Cartun, MD,‡‡ Richard N. Eisen, MD,§§ Paul E. Swanson, MD,‖‖ Stephen M. Hewitt, MD, PhD,¶¶ Mogen Vyberg, MD,## and David G. Hicks, MD*** and Members of the Standardization Ad-Hoc Consensus Committee*

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

IHC – The Technical Test Approach

Published Ahead of Print on October 7, 2013 as 10.1200/JCO.2013.50.9984
The latest version is at http://jco.ascopubs.org/cgi/doi/10.1200/JCO.2013.50.9984

JOURNAL OF CLINICAL ONCOLOGY | ASCO SPECIAL ARTICLE

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

Antonio C. Wolff, M. Elizabeth H. Hammond,* David G. Hicks,* Mitch Dowsett,* Lisa M. McShane,* Kimberly H. Allison, Donald C. Allred, John M.S. Bartlett, Michael Bilous, Patrick Fitzgibbons, Wedad Hanna, Robert B. Jenkins, Pamela B. Mangu, Soonmyung Paik, Edith A. Perez, Michael F. Press, Patricia A. Spears, Gail H. Vance, Giuseppe Viale, and Daniel F. Hayes**

6 - 72h

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

Consensus Recommendations on Estrogen Receptor Testing in Breast Cancer By Immunohistochemistry

Hadi Yaziji, MD, Clive R. Taylor, MA, MD, D.Phil,† Neal S. Goldstein, MD,‡ David J. Dabbs, MD,§ Elizabeth H. Hammond, MD,‖ Bryan Hewlett, ART (CSMLS), MLT (CMLTO),¶ Alton D. Floyd, PhD,* Todd S. Barry, MD,# Alvn W. Martin, MD,** Sunil Badve, MD,†† Frederick Baehner, MD,†† Richard W. Cartun, MD,‡‡ Richard N. Eisen, MD,§§ Paul E. Swanson, MD,‖‖ Stephen M. Hewitt, MD, PhD,¶¶ Mogen Vyberg, MD,## and David G. Hicks, MD*** and Members of the Standardization Ad-Hoc Consensus Committee*

8 - 72h

IHC – The Technical Test Approach

Minimum formalin fixation time for consistent estrogen receptor immunohistochemical staining of invasive breast carcinoma.

Goldstein NS, Ferkowicz M, Odish E, Mani A, Hastah F

Am J Clin Pathol. 2003 Jul;120(1):86-92

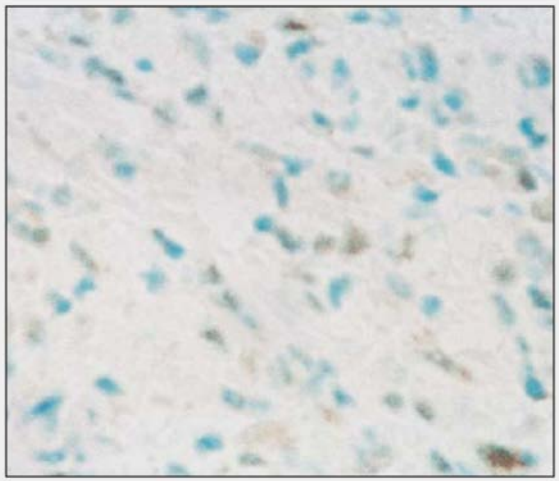


Image 1 Fixation, 3 h; antigen retrieval, 40 min.

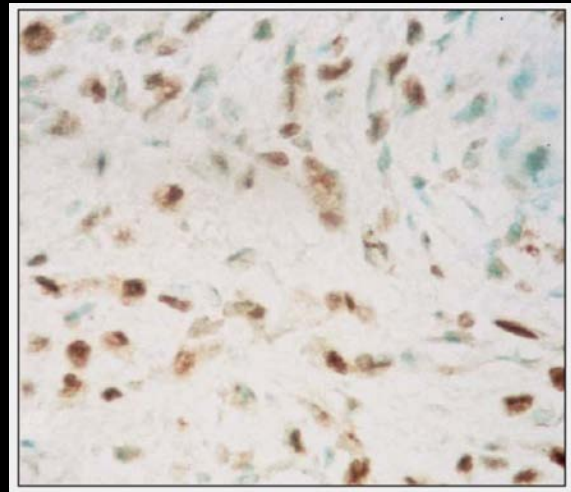


Image 2 Fixation, 6 h; antigen retrieval, 40 min.

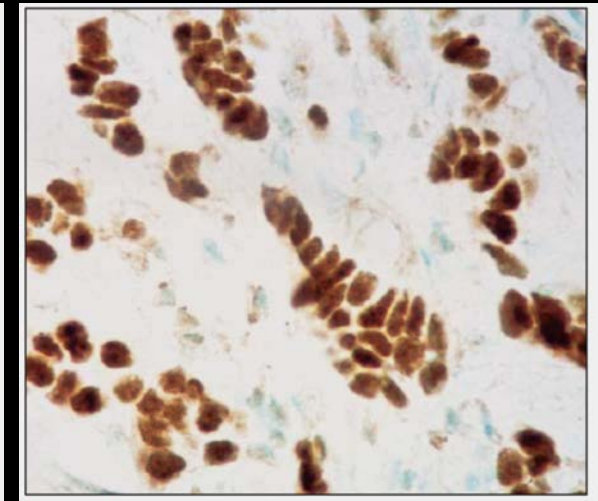


Image 3 Fixation, 8 h; antigen retrieval, 40 min.

Table 1

Formalin Fixation Times and Estrogen Receptor Staining With Standard, 40 Minutes of Antigen Retrieval Pretreatment

Formalin Fixation Time	Mean Q Score (Range)	Mean Difference in Q Score (Range)*	P†
3 h	2.46 (0-6)	4.36 (1-7)	<.001
6 h	5.75 (2-7)	1.14 (0-4)	<.001
8 h	6.70 (5-7)	0.04 (0-1)	.791
10 h	6.70 (5-7)	0.08 (0-1)	.791
12 h	6.70 (5-7)	0.04 (0-1)	1.000
1 d	6.70 (5-7)	0.04 (0-1)	1.000
2 d	6.70 (5-7)	0.04 (0-1)	.625
7 d	6.60 (5-7)	0.12 (0-1)	—

* Case maximum minus block.
† Compared with adjacent block fixed for a longer period.

Tissue sections of 24 ER-positive, invasive breast carcinomas were fixed for 3, 6, 8, and 12 hours and 1, 2, and 7 days. ER values were quantified using the Q score (0-7).

" 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 (core biopsy or resection)". (mAb clone 1D5)

IHC – The Technical Test Approach

Fixation Time Does Not Affect the Expression of Estrogen Receptor

Julio A. Ibarra, MD,[1] Lowell W. Rogers, MD,[2] Ainura Kyshtoobayeva, MD,[3] and Kenneth Bloom, MD[3]

Key Words: Fixation time; Estrogen receptor; Immunohistochemistry

Am J Clin Pathol 2010;133:747-755
DOI: 10.1309/AJCPPIUHS4GVAR0I

Abstract

The purpose of this pilot study was to determine the impact of the length of fixation in 10% buffered formalin on the expression of estrogen receptor by immunohistochemical analysis. We studied tissue samples from 10 invasive breast cancer cases after fixation for 1, 3, 6, and 9 to 10 hours. The tissue was processed immediately after fixation, resembling routine practice. Then the 40 blocks were incubated with antiestrogen receptors SP1, 6F11, and 1D5. The stained slides were reviewed and scored.

We found no significant difference in the intensity of the stain or the percentage of cells stained regardless of the time in fixation or the antibody used. Fixation times between 1 and 9 hours in 10% formalin do not seem to have an impact on the expression of estrogen receptor by immunohistochemical analysis, at least in these high-expressing tumors.

Table 1
Estrogen Receptor Clones Used*

Antibody Clone	Vendor	Staining Platform	Antigen Retrieval Method	Antibody Dilution/Diluent	Antibody Incubation Time (min)	Detection System
SP1	Ventana	Ventana Autostarter	CC1 at 95°C, 30 min	Prediluted	32	Ventana Detection System
6F11	Novocastra	DAKO Autostainer	Citrate, decloaker (5 min at 125°C; 10 s at 90°C)	1:500/DAKO background reducing	30	PowerVision+ (Vision BioSystems), 30 min; DAB for 2 min
1D5	Zymed	DAKO Autostainer	Citrate, decloaker (5 min at 125°C; 10 s at 90°C)	1:3,000/DAKO background reducing	30	PowerVision+, 30 min; DAB for 2 min

DAB, diaminobenzidine.
* DAKO, Carpinteria, CA; Novocastra, Newcastle upon Tyne, England; Ventana, Tucson, AZ; Vision BioSystems, Norwell, MA; Zymed, Carlsbad, CA.

IHC – The Technical Test Approach

Table 2
Combined Results for Four Pathologists by Case Number, Antibody, and Fixation Time*

	Fixation Time (h)							
	1		3		6		9-10	
Case No./Antibody	Positive Cells (%)	Intensity	Positive Cells (%)	Intensity	Positive Cells (%)	Intensity	Positive Cells (%)	Intensity
1								
SP1	100	3	100	3	100	3	100	3
6F11	95-100	2-3	100	3	100	2-3	100	2-3
1D5	95-100	2-3	100	2-3	100	2-3	100	2-3
2								
SP1	95-100	2-3	90-100	2-3	95-100	1-3	ND	ND
6F11	90-95	2-3	90-95	2-3	95-100	2-3	ND	ND
1D5	70-80	1-2	30-40	1-2	90	1-2	ND	ND
3								
SP1	100	3	100	3	100	3	100	3
6F11	100	2-3	100	2-3	100	2-3	100	2-3
1D5	90-100	2-3	100	2-3	100	2-3	100	2-3
4								
SP1	100	3	100	3	100	3	100	3
6F11	95-100	2-3	100	2-3	100	2-3	100	2-3
1D5	95-100	2-3	100	2-3	100	2-3	100	2-3
5								
SP1	100	2-3	90-100	2-3	90-100	1-3	95-100	2-3
6F11	90-95	2-3	80-95	1-3	90-95	1-3	95-100	2-3
1D5	90-95	1-3	70-75	1-3	70-75	1-3	70-75	1-3
6								
SP1	100	3	100	3	100	3	100	3
6F11	95-100	2-3	95-100	2-3	95-100	2-3	95-100	2-3
1D5	95-100	1-3	95-100	2-3	95-100	2-3	95-100	2-3
7								
SP1	100	3	100	3	100	3	100	3
6F11	100	3	100	3	100	3	100	3
1D5	100	2-3	95-100	2-3	100	2-3	100	2-3
8								
SP1	100	3	100	3	100	3	100	3
6F11	100	3	100	3	100	3	100	3
1D5	100	3	100	3	100	3	100	3
9								
SP1	100	3	100	3	100	3	100	3
6F11	100	3	100	3	100	3	100	3
1D5	100	3	100	3	100	3	100	3
10								
SP1	100	3	100	3	100	3	100	3
6F11	100	2-3	100	2-3	100	2-3	100	2-3
1D5	95-100	2-3	95-100	2-3	95-100	2-3	100	2-3

ND, not done.
* Staining intensity was graded as follows: 1, weak; 2, intermediate; and 3, strong.

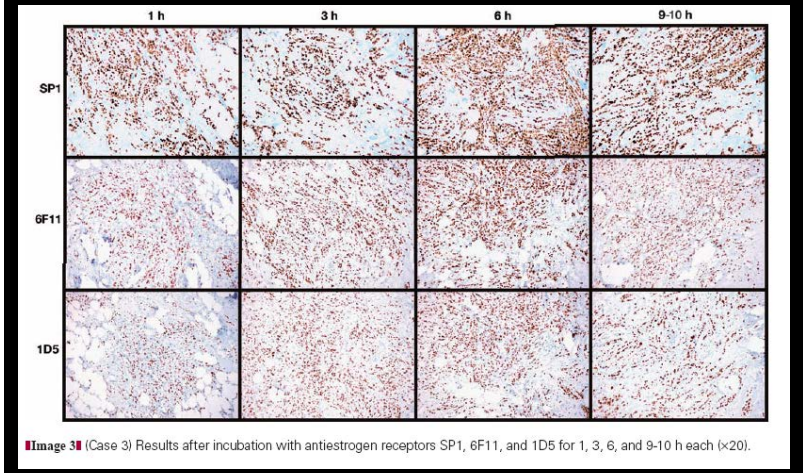


Image 3 (Case 3) Results after incubation with antiestrogen receptors SP1, 6F11, and 1D5 for 1, 3, 6, and 9-10 h each (×20).

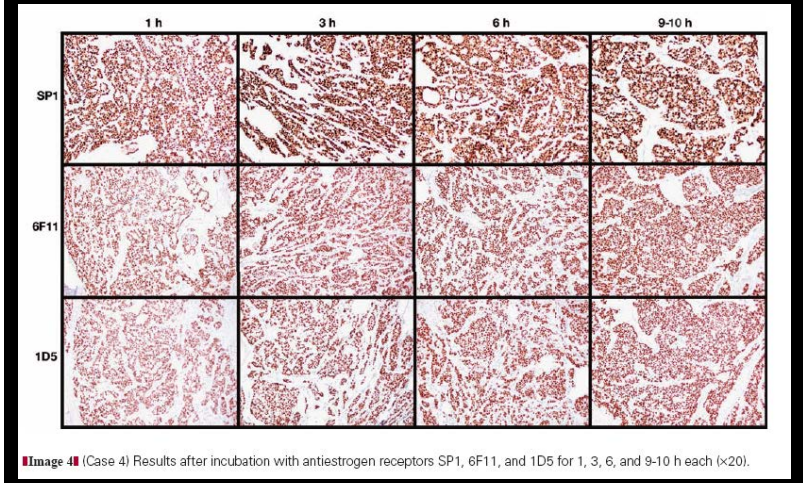


Image 4 (Case 4) Results after incubation with antiestrogen receptors SP1, 6F11, and 1D5 for 1, 3, 6, and 9-10 h each (×20).

Only tumours with a high ER expression…… to be validated in a larger study > 100 pts.

IHC – The Technical Test Approach

ORIGINAL ARTICLE

(Am J Surg Pathol 2014;38:1071–1078)

Brief Fixation Does Not Affect Assessment of Hormone Receptor Expression in Invasive Breast Carcinoma Biopsies

Paving the Road for Same-day Tissue Diagnostics

Shona Kalkman, MD, Maarten W. Barentsz, MD,† Arjen J. Witkamp, MD, PhD,‡ Elsken van der Wall, MD, PhD,§ Helena M. Verkooijen, MD, PhD,† and Paul J. van Diest, MD, PhD**

CNB: 45 min. in NBF
Res.: 8-72 h. in NBF

TABLE 1. Agreement of ERα Expression Between Ultrashort Fixed CNB and Conventionally Fixed Resection Specimens of Invasive Breast Carcinoma Patients (98.6%, κ = 0.85; 95% CI = 0.56-1.00)

	Resection Specimen		
	ERα⁻	ERα⁺	Total
CNB			
ERα⁻	3	0	3
ERα⁺	1	70	71
Total	4	70	74

TABLE 3. Agreement of PR Expression Between Ultrashort Fixed CNB and Conventionally Fixed Resection Specimens of Invasive Breast Carcinoma Patients (92.0%, κ = 0.81; 95% CI = 0.66-0.96)

	Resection Specimen		
	PR⁻	PR⁺	Total
CNB			
PR⁻	19	6	25
PR⁺	0	50	50
Total	19	56	75

CNB: mean average 91% pos. cells
Res.: mean average 88% pos. cells.

CNB: mean average 44% pos. cells
Res.: mean average 58% pos. cells.

Caution: **Primarily only ER/PR high expressing tumour**
Each biomarker/antibody must be evaluated!!!

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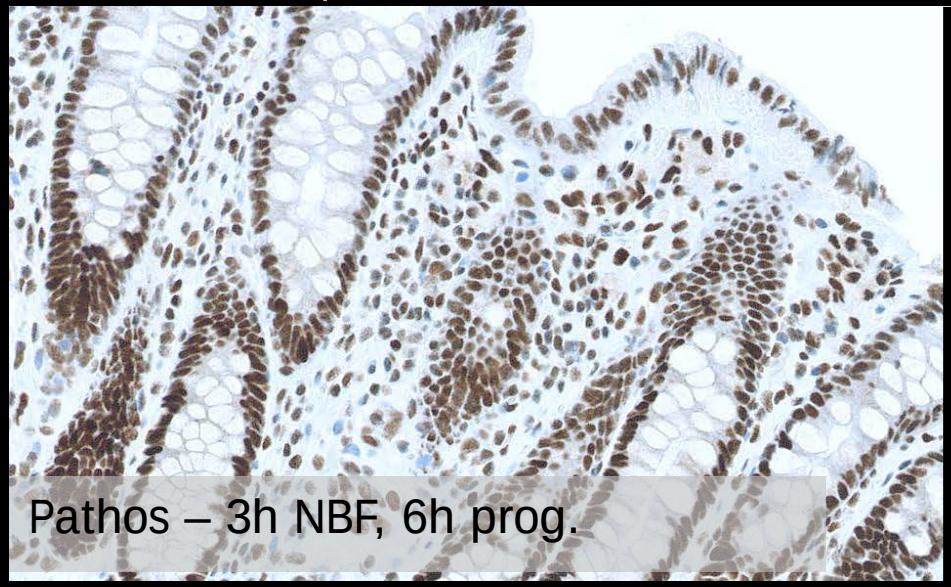
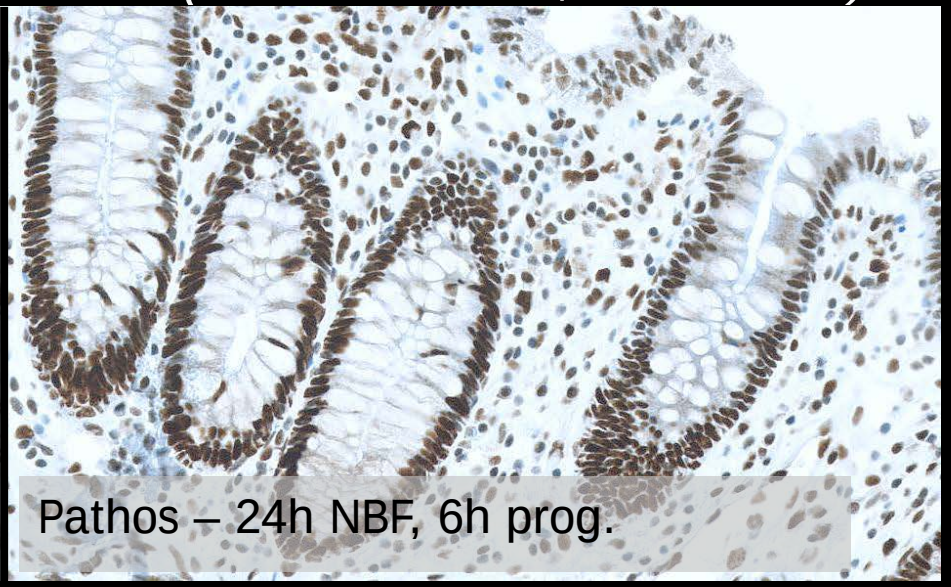
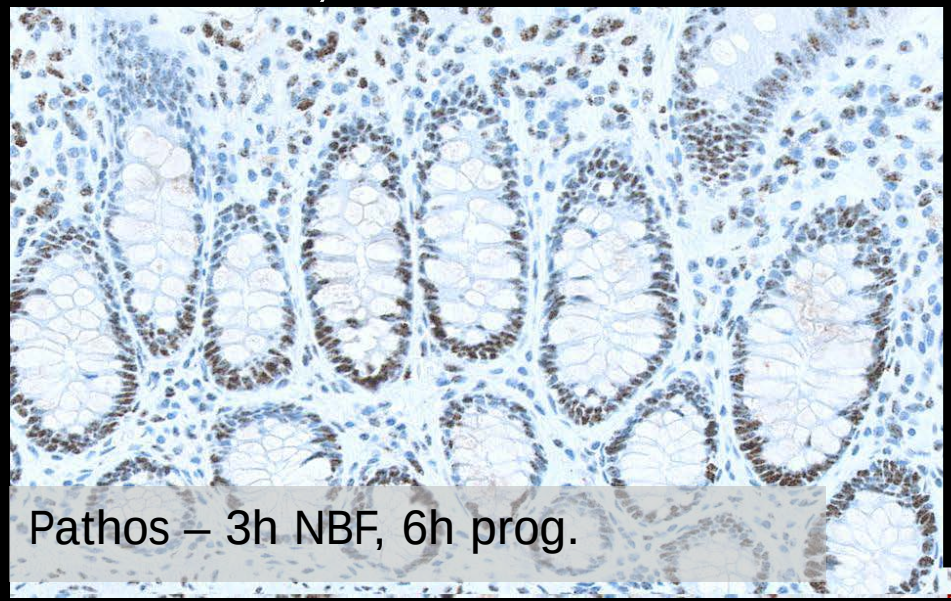
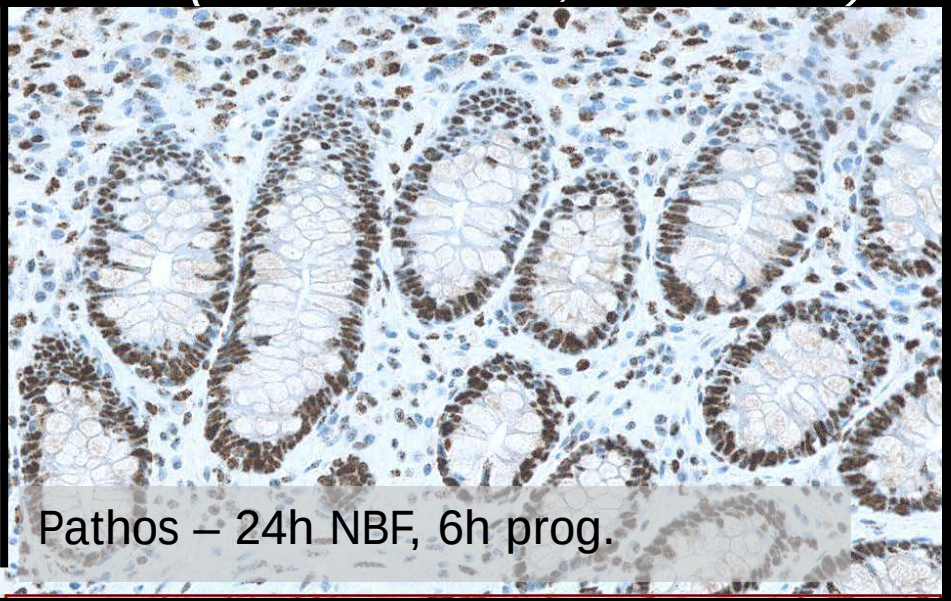
Colon: MSH2, mAb clone G219-1129 *(same for MSH6, clone EP49)*





Colon: MLH1, mAb clone ES05 *(same for PMS2, clone EP51)*





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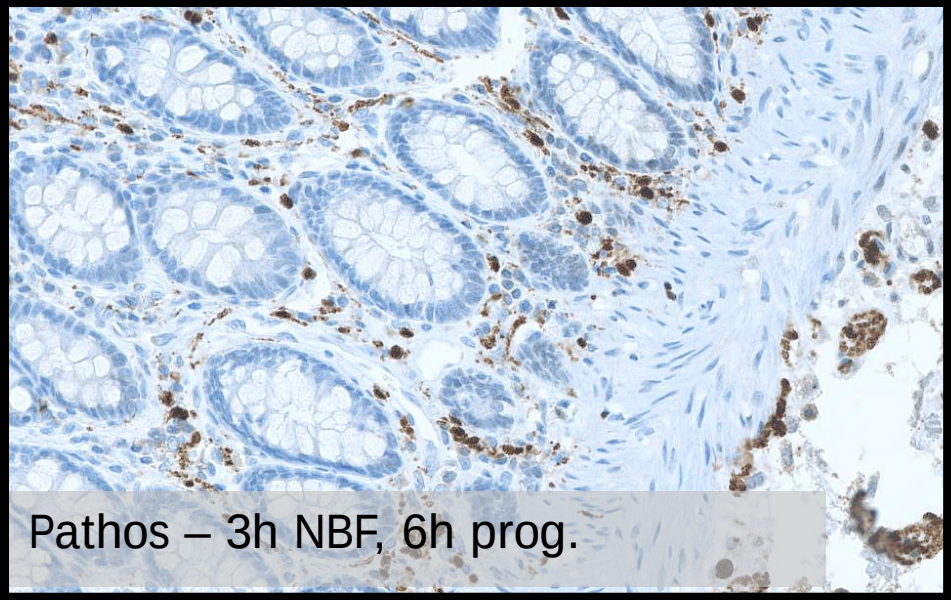
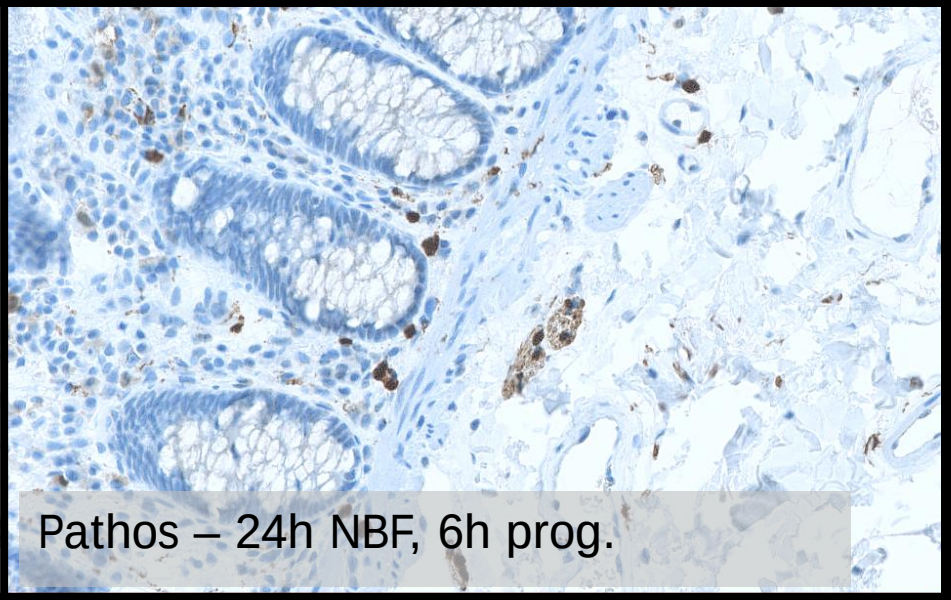
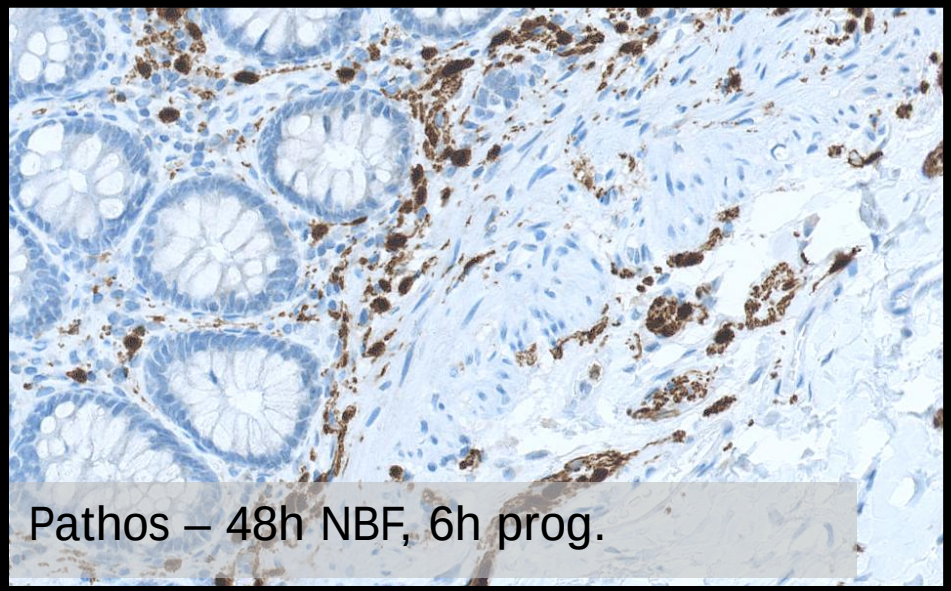
Colon: S100, polyclonal



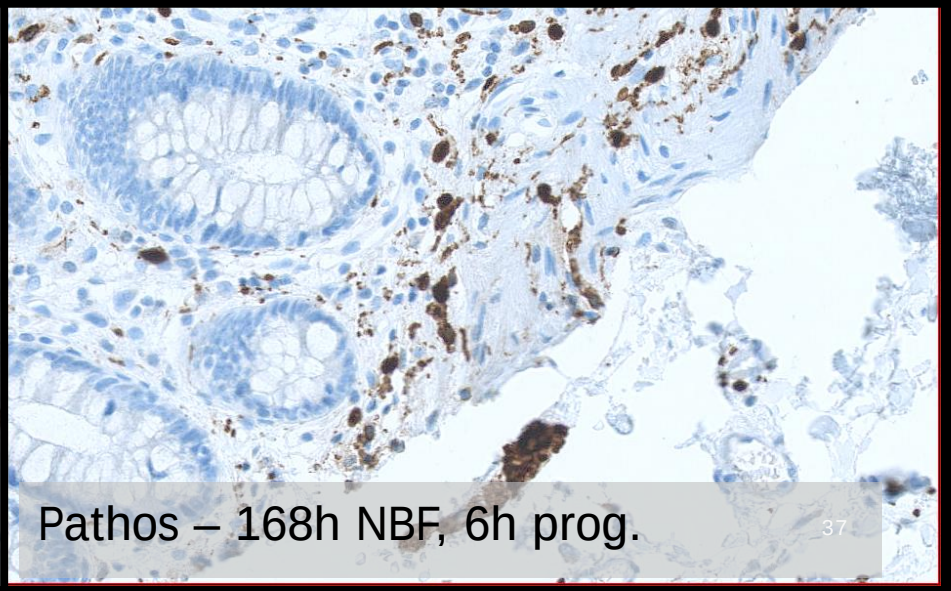
Pathos – 3h NBF, 6h prog.



Pathos – 24h NBF, 6h prog.



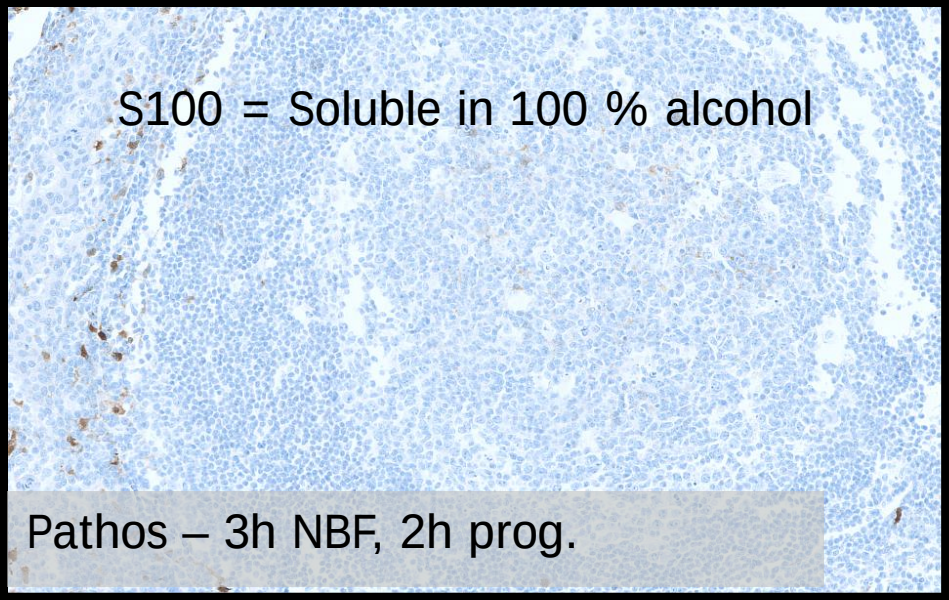
Pathos – 48h NBF, 6h prog.



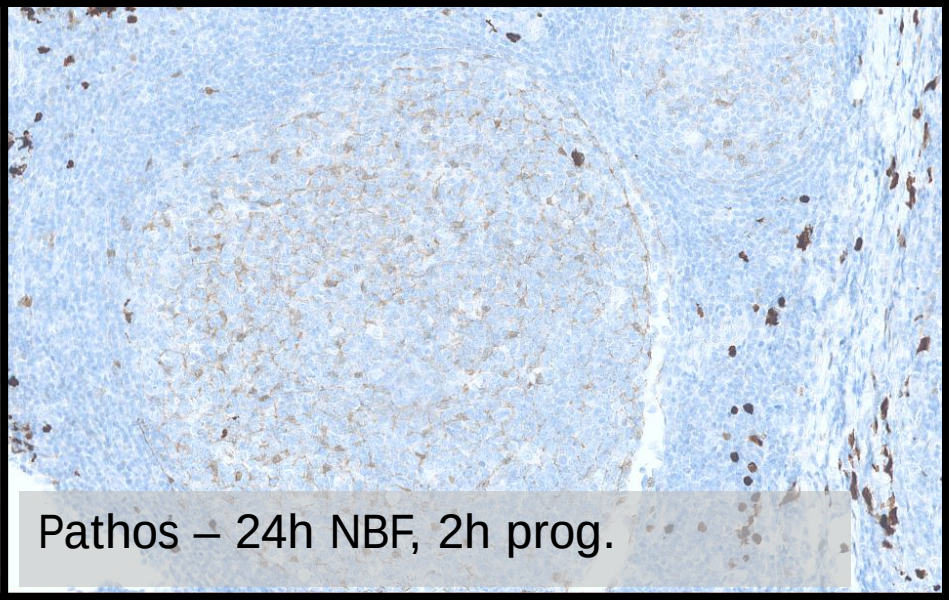
Pathos – 168h NBF, 6h prog.

IHC – The Technical Test Approach

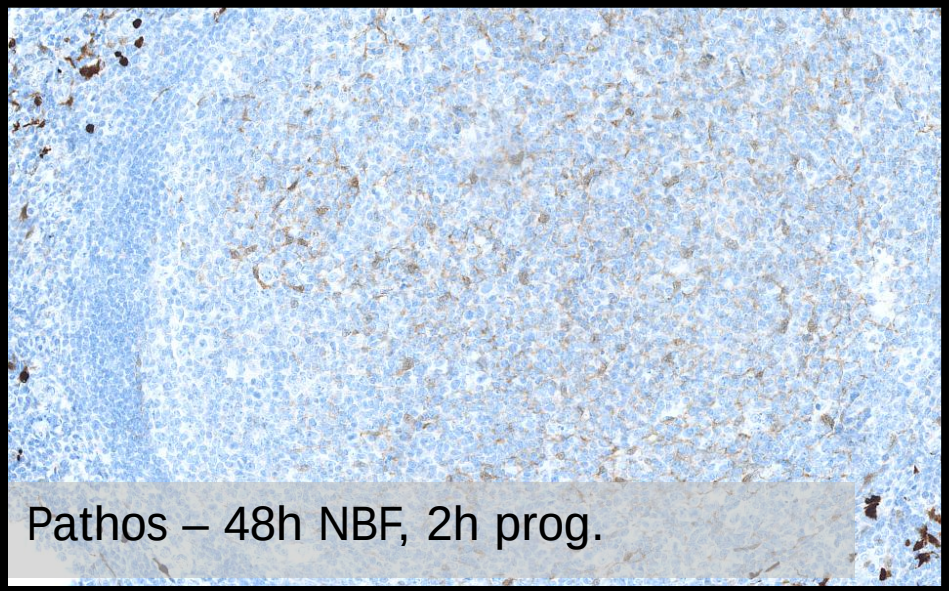
Tonsil: S100, polyclonal



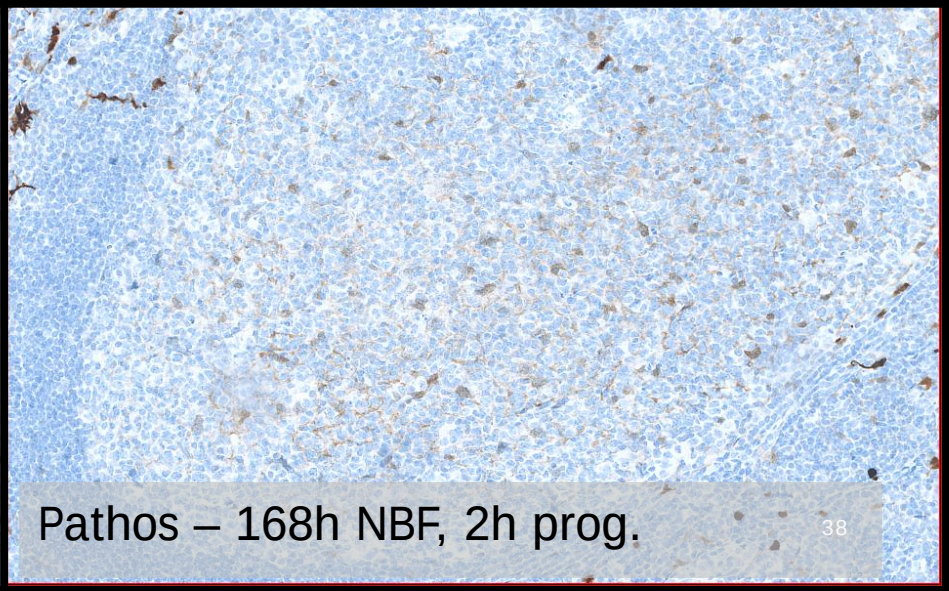
Pathos – 3h NBF, 2h prog.



Pathos – 24h NBF, 2h prog.



Pathos – 48h NBF, 2h prog.



Pathos – 168h NBF, 2h prog.

IHC – The Technical Test Approach

(*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, MLT,† and Anna Marie Mulligan, MB, MSc, FRCPath*†*

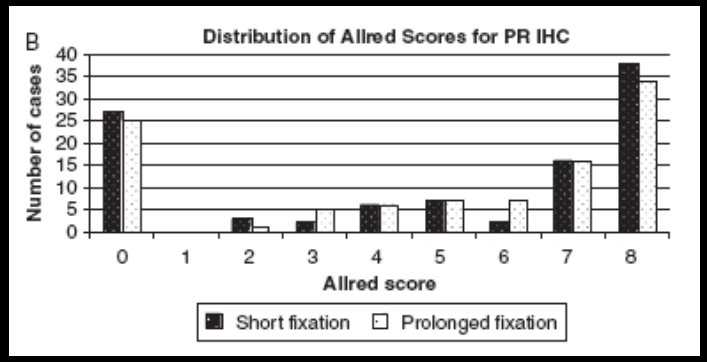
13 hours versus 79 hours in 10% NBF (the week-end dilemma.....)

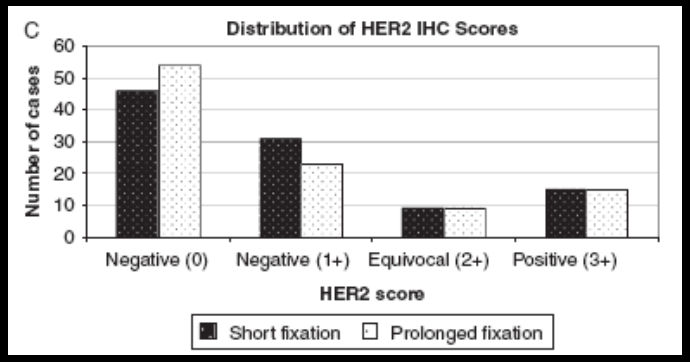
101 breast carcinomas:

99 % Concordance between short fixation and long fixation for ER (SP1)

95 % Concordance between short fixation and long fixation for PR (1E2)

98 % Concordance between short fixation and long fixation for HER2 (A0485)





IHC – The Technical Test Approach

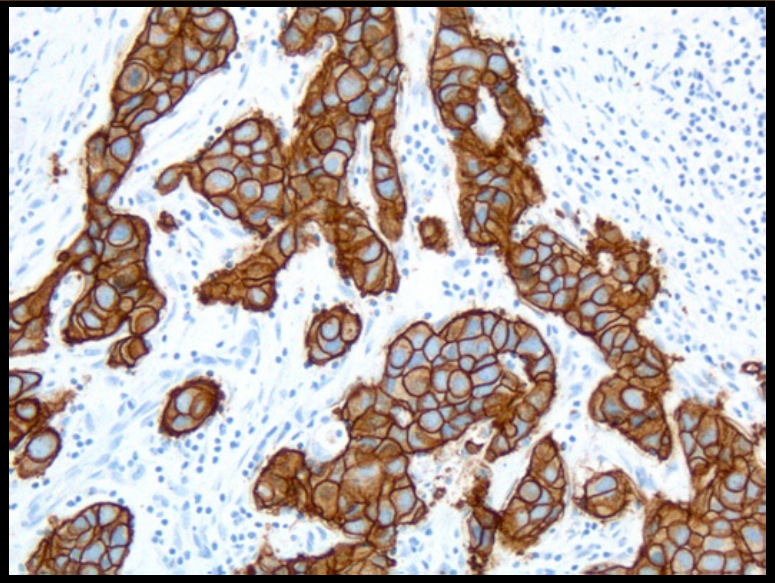
Internal IHC validation	4 h. NBF	24 h. NBF	48 h. NBF	168 h. NBF
Tumour 1	1+	1+	1+	1+
Tumour 2	3+	3+	3+	3+
Tumour 3	0	0	0	0
Tumour 4	1+	1+	1+	1+
Tumour 5	0	0	0	0
Tumour 6	3+	3+	3+	3+
Tumour 7	0	0	0	0
Tumour 8	0	0	0	0
Tumour 9	0	0	0	0

Breast carcinomas, HER-2 PATHWAY, rmAb 4B5

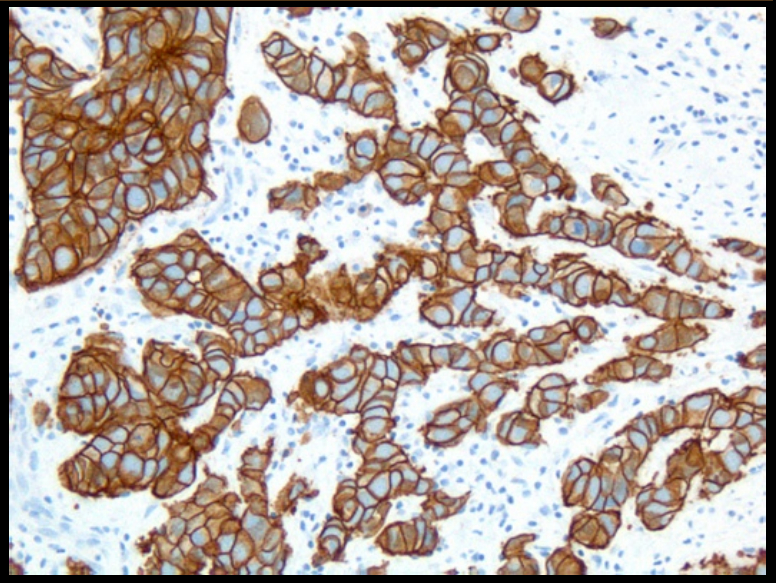
(CC1 Mild, Ab inc. 20 min. 36°C, UltraView DAB)

IHC – The Technical Test Approach

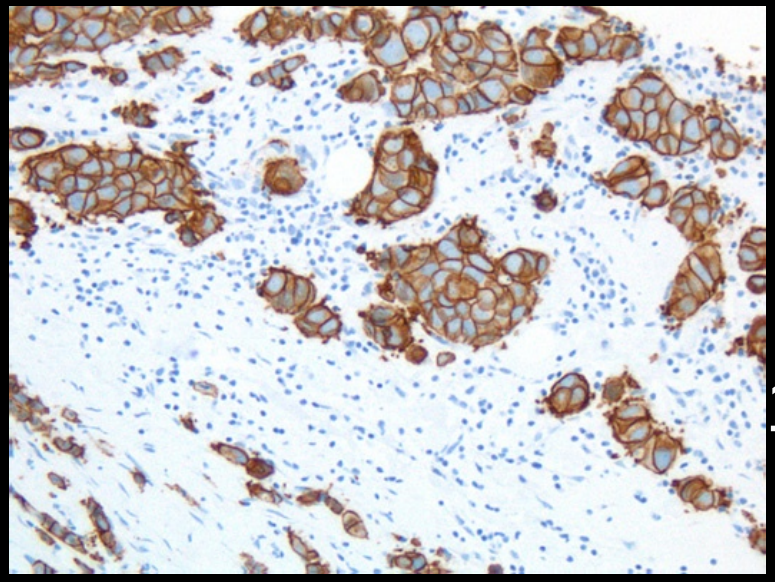
4 h



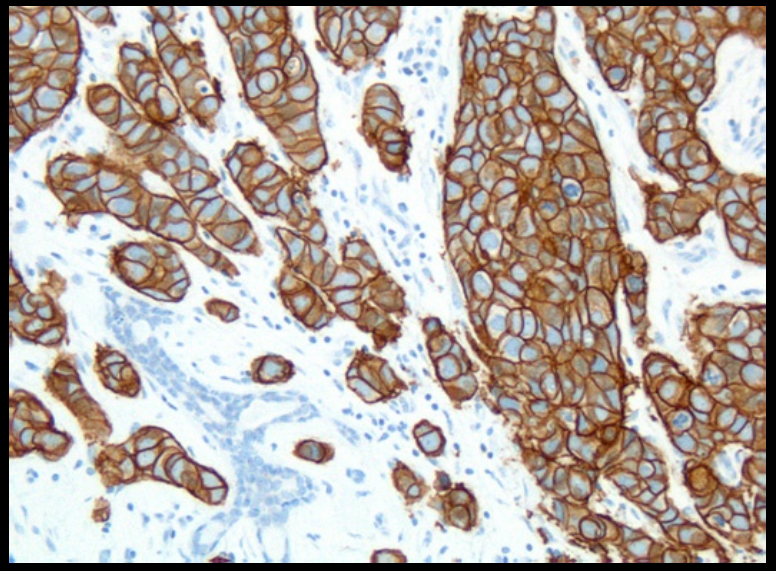
24 h



48 h



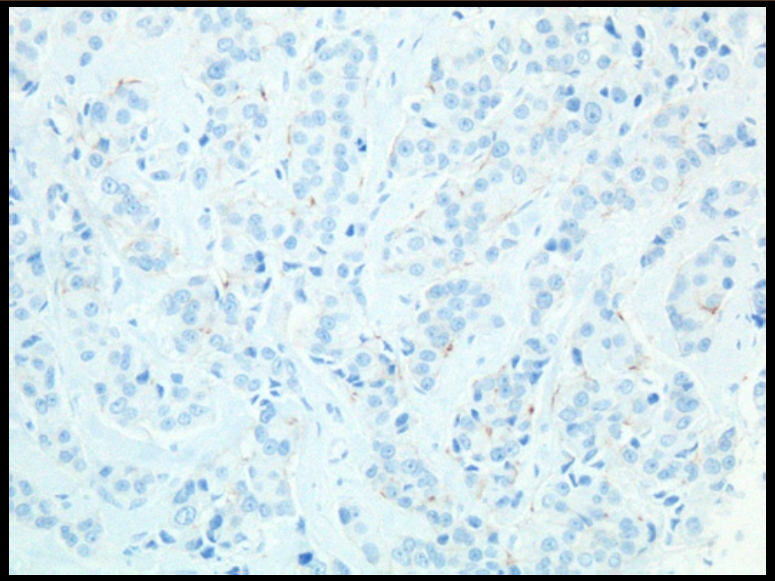
168 h



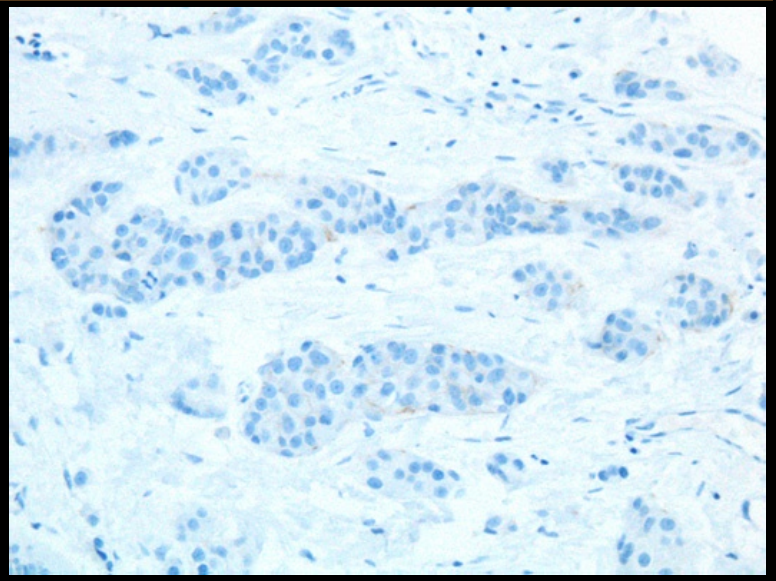
Breast carcinoma 3+, HER-2 PATHWAY, rmAb 4B5

IHC – The Technical Test Approach

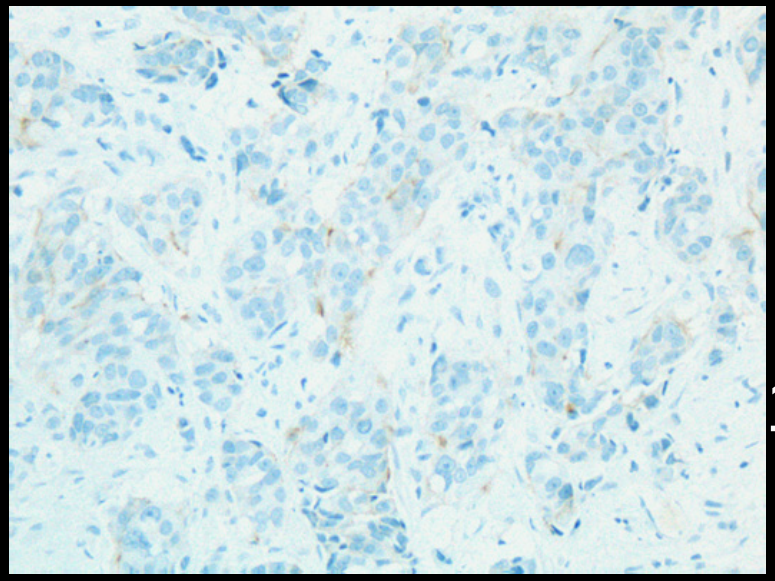
4 h



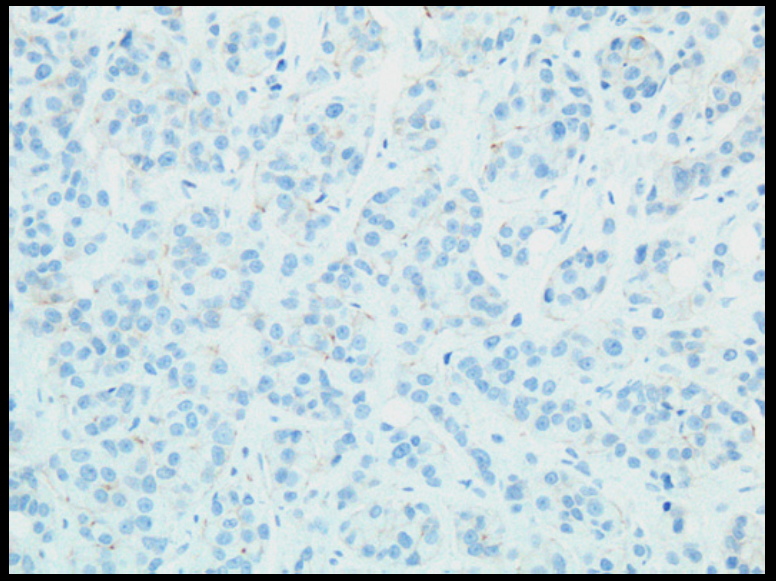
24 h



48 h



168 h



Breast carcinoma 1+, HER-2 PATHWAY, rmAb 4B5

IHC – The Technical Test Approach

HER2 rmAb 4B5

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

ER rmAb SP1

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

PR rmAb 1E2

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

ECAD mAb NCH-36

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

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

IHC – The Technical Test Approach

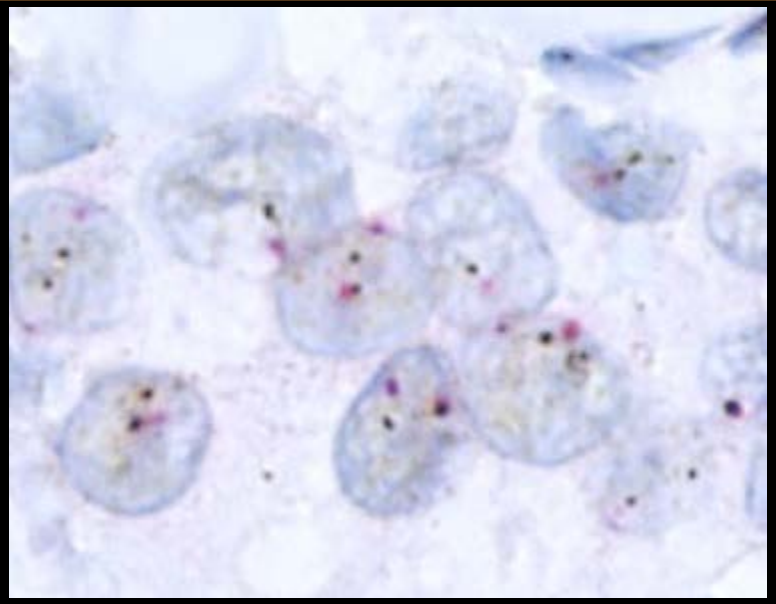
Internal SISH validation	4 h. NBF	24 h. NBF	48 h. NBF	168 h. NBF
Tumour 1	-	-	-	FN
Tumour 2 Amp	+	+	+	+
Tumour 3	(?)	-	FN	FN
Tumour 4	-	-	FN	FN
Tumour 5	-	-	-	-
Tumour 6 Amp	+	+	+	+
Tumour 7	-	-	-	FN
Tumour 8 poly.	-	-	-	FN
Tumour 9 poly.	-	-	-	FN

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

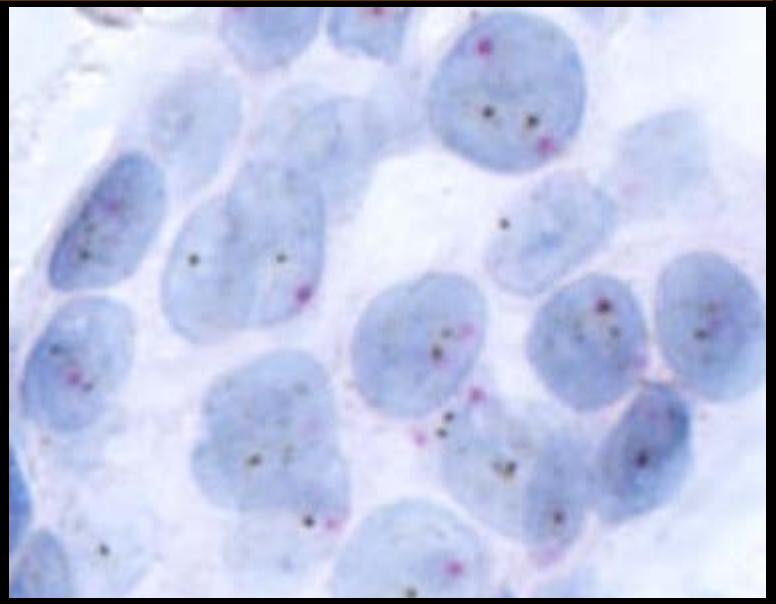
Breast carcinomas, Dual SISH CCrb ext, P3. 8 m

IHC – The Technical Test Approach

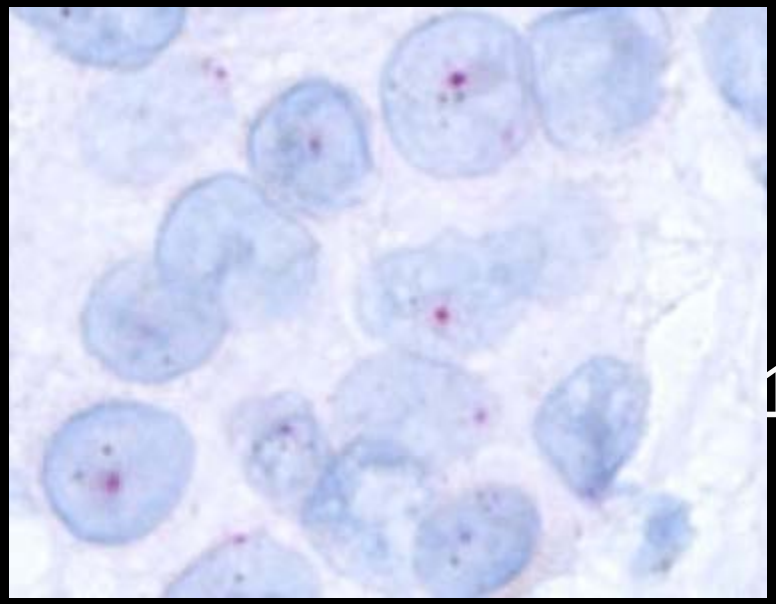
4 h



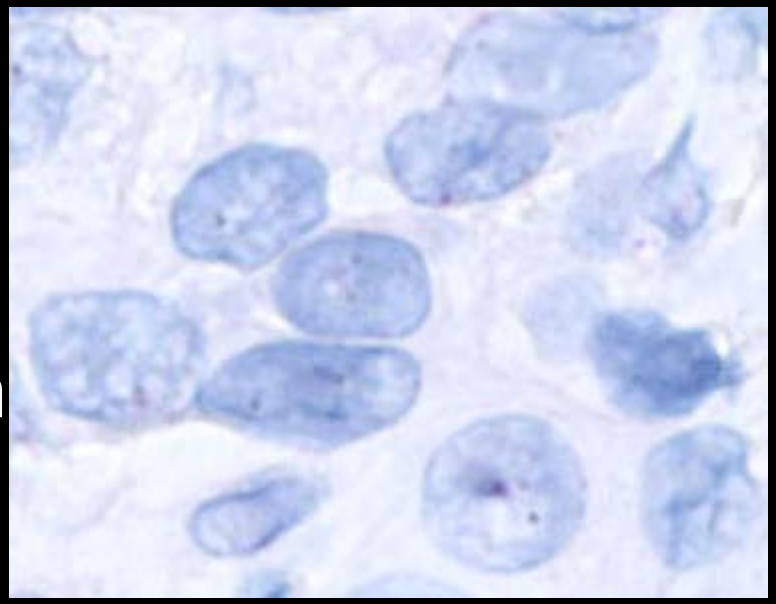
24 h



48 h



168 h



Breast carcinoma, 1+ Dual SISH CCrb ext, P3. 8 m

IHC – The Technical Test Approach

Impact / Change of fixation time:

1. Use present standard times(s) as reference

2. Evaluate all biomarkers on material with the full diagnostic range of expression levels

3. Evaluate all different methods applied as diagnostic tools – IHC / ISH / view-RNA etc

IHC – The Technical Test Approach

Alternatives to Formalin

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

IHC – The Technical Test Approach

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

Fixative with focus on

Morphology
Molecular biology
IHC

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

PreAnalytiX
A QIAGEN / BD Company

IHC – The Technical Test Approach

Experimental and Molecular Pathology 94 (2013) 188–194

Contents lists available at SciVerse ScienceDirect

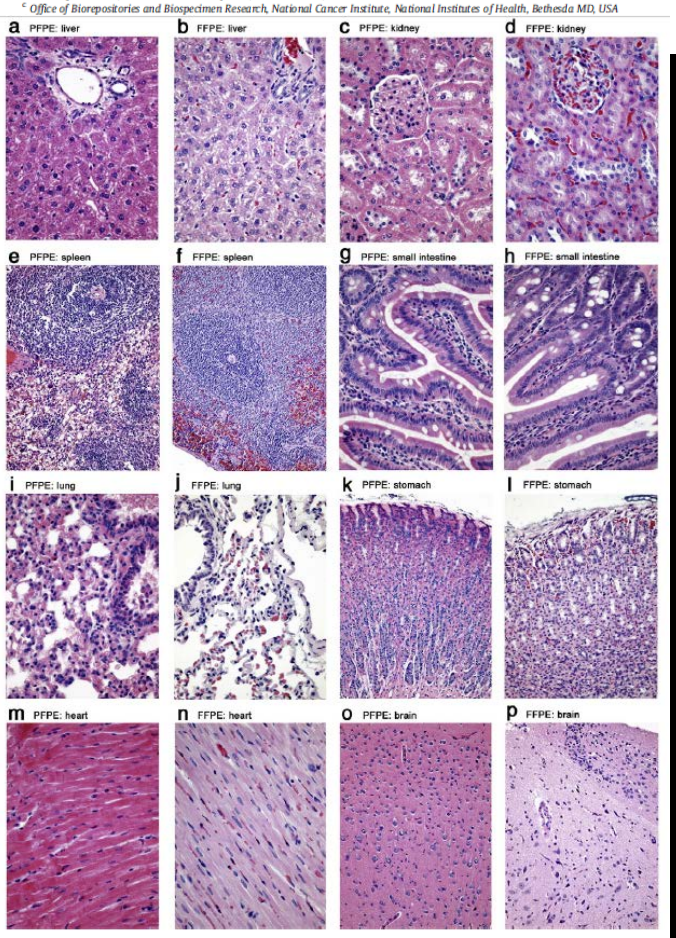
Experimental and Molecular Pathology

journal homepage: www.elsevier.com/locate/yexmp

Non-formalin fixative versus formalin-fixed tissue: A comparison of histology and RNA quality

Daniel Groelz [a,*], Leslie Sobin [d], Philip Branton [c], Carolyn Compton [c,e], Ralf Wyrich [a], Lynne Rainen [b]

[a] Qiagen GmbH, Research and Development, Hilden, Germany
[b] PreAnalytiX GmbH, Research and Development, Franklin Lakes, USA
[c] Office of Biorepositories and Biospecimen Research, National Cancer Institute, National Institutes of Health, Bethesda MD, USA



In conclusion, tissue fixed with the PAXgene Tissue System is morphologically comparable to formalin-fixed tissue but yields RNA which performs as well as RNA from fresh frozen tissue in RT PCR assays. For RNA from PFPE tissue, reverse transcription was not inhibited, and RNA purified from PFPE samples performed similarly to RNA isolated fromfresh frozen tissue regardless of amplicon length.

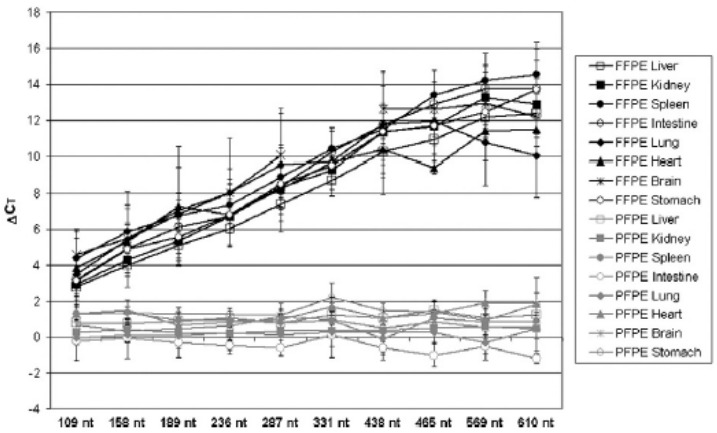


Fig. 4. Reverse transcription and amplification using 10 ng RNA each from rat tissue fresh frozen (FF), PAXgene fixed paraffin-embedded (PFPE) or formalin-fixed, paraffin-embedded (FFPE), in ten different SYBR-Green real time one step RT PCR assays. Amplicons of the rat beta-actin gene ranged from 109 to 610 nucleotide (nt). Average delta-CT values ($\Delta C_T = C_{T|FFPE|} - C_{T|FF|}$ or $\Delta C_T = C_{T|PFPE|} - C_{T|FF|}$) are shown for triplicate extractions from two different tissue samples amplified in duplicates for each type of tissue and fixation method.

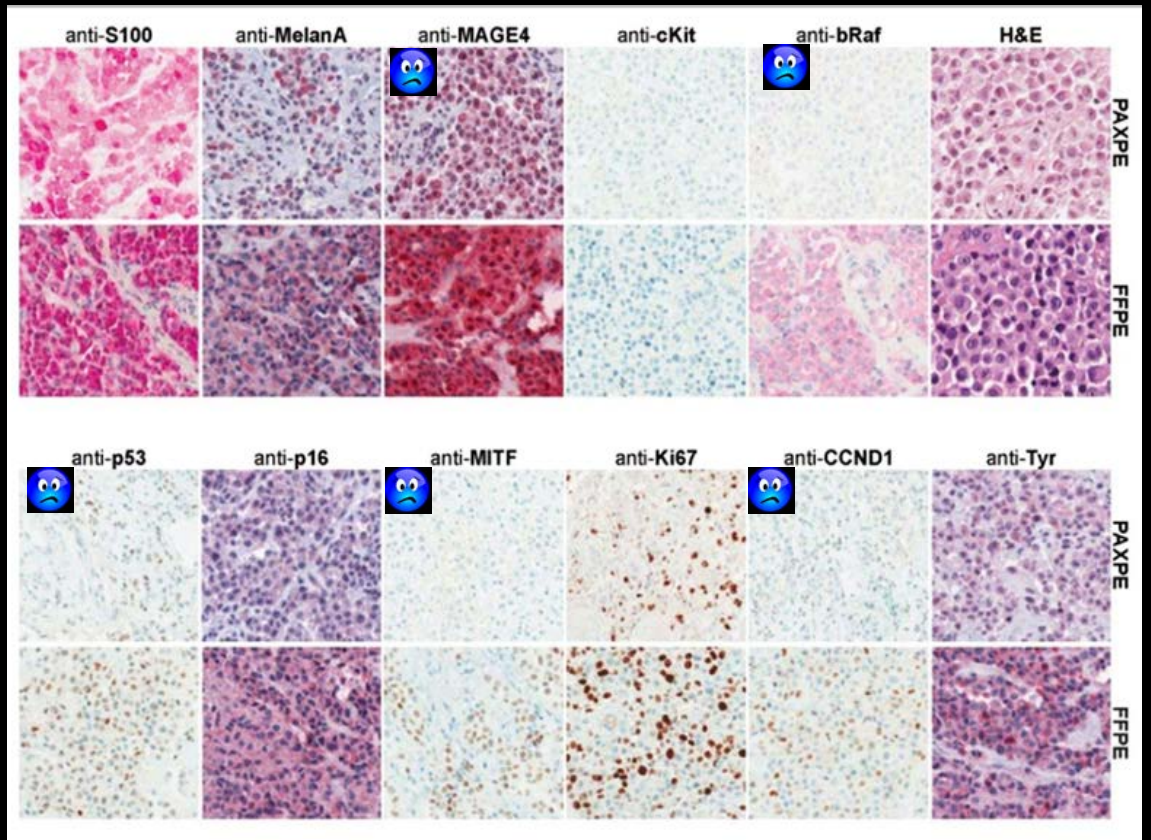
IHC – The Technical Test Approach

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

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.



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.

IHC – The Technical Test Approach

A Critical Evaluation of the PAXgene Tissue Fixation System

Morphology, Immunohistochemistry, Molecular Biology, and Proteomics

William Mathieson, PhD,[1,2] Nathalie Marcon, MD,[1] Laurent Antunes, MD,[1]

Am J Clin Pathol July 2016;146:25-40

Description of Antibodies, Protocols, and Immunohistochemistry Outcome

Antibody	Clone/Company	Ref	Dilution	FFPE Protocol[a]	Equivalent Staining Intensity FFPE vs PFPE[b]
Lung tissue					
TTF1	8G7G37/1 Ventana	790-4398	PD	CC1S - 16'	Yes (suboptimal)
TTF1	8G7G37/1 Eurobio	CM087B	1:100	CC1M - 32'	Yes (suboptimal)
p63	4A4 Ventana	790-4509	PD	CC1S - 16'	Yes (suboptimal)
p63	BC4A4 Eurobio	PM163AA	PD	CC1S - 32'	Yes (suboptimal)
p40	Polyclonal Diag Biosystem	RP163-05	1:100	CC2M - 32'	Yes (suboptimal)
p40	BC28 Eurobio	ACI3066C	1:100	CC1S - 32'	Yes
Napsin A	Polyclonal Ventana	760-4446	PD	CC1M - 16'	Yes (suboptimal)
Napsin A	TMU-Ad 02 Eurobio	CM388CK	1:100	CC1M - 32'	Yes (suboptimal)
CK5/6	D5/16 B4 Ventana	790-4554	PD	CC1S - 16'	Yes
CK5/6	D5/16 B4 Dako	M7237	1:100	CC1M - 32'	Yes
CD56	MRQ-42 Ventana	760-4596	PD	CC1M - 16'	Yes (suboptimal)
CD56	123C3 Dako	M7304	1:100	CC1M - 32'	Yes (suboptimal)
Colon tissue					
CK7	SP52 Ventana	790-4262	PD	CC1S - 16'	No
CK7	OV-TL12/30 Dako	M7018	1:200	CC1M - 32'	No
CK20	SP33 Ventana	790-4431	PD	CC1S - 16'	Yes
CK20	Ks20.8 Dako	M7019	1:50	CC1M - 32'	Yes
Collagen IV	CIV22 Ventana	760-2632	PD	Protéase 1 - 32'	Yes (suboptimal)
Collagen IV	CIV22 Dako	M0785	1:50	CC1M - 32'	Yes
Ki67	30-9 Ventana	790-4286	PD	CC1S - 16'	No
Ki67	Mib-1 Dako	M7240	1:100	CC2M - 32'	Yes
MLH1	M1 Ventana	790-4535	PD	CC1S - 16'	No
MLH1	ES05 Dako	M3640	1:50	CC1M - 20'	No
MSH2	G219-1129 Ventana	760-4265	PD	CC1M - 16'	No
MSH2	FE11 Dako	M3639	1:50	CC1M - 20'	No
MSH6	44 Ventana	790-4455	PD	CC1S - 16'	No
MSH6	EP49 Dako	M3646	1:50	CC1M - 20'	No
PMS2	EPR3947 Ventana	760-4531	PD	CC1S - 32'	No
PMS2	EP51 Dako	M3647	1:40	CC1M - 20'	No

FFPE, formalin-fixed paraffin-embedded; PD, prediluted; PFPE, PAXgene-fixed paraffin-embedded.
[a]CC1S: pH 8.4; 60 min AR; Ab IT: 16, 20 or 32 min or 1 h. CC2S: pH 6.0; 60 min AR; Ab IT: 16, 20 or 32 min or 1 h. CC1M: pH 8.4; 30 min AR; Ab IT: 16, 20 or 32 min or 1 h. CC2M: pH 6.0; 36 min AR; Ab IT: 16, 20 or 32 min or 1 h. Protease 1 – 32 min: protease 8 min; Ab IT: 32 min. CC1S optiview 32': pH 8.4; 56 min AR; Ab IT: 32 min. CC2M optiview 1 h: pH 6.0; 32 min AR; Ab IT: 1 h. CC2S optiview 1 h: pH 6.0; 56 min AR; Ab IT: 1 h. CC2s optiview 1 h: pH 6.0; 8 min AR; Ab IT: 1 h.
[b]Yes = no significant difference in immunoreactivity; Yes (suboptimal) = staining interpretable but suboptimal in PFPE compared to FFPE; No = PFPE staining insufficient for

FFPE vs PFPE

PFPE = FFPE (7/28)

PFPE = Suboptimal (10/28)

PFPE = Insufficient (11/28)

Conclusion

…Although IHC is compromised in PFPE sections compared to FFPE sections when FFPE IHC protocols are used, this can usually be addressed through protocol-optimization.

IHC – The Technical Test Approach

Pre-analytical variable	Published guidelines ASCO/CAP - CLSI	Litterature based guidelines*
Fixative	10 % NBF**	10 % NBF
Fixation time	8 – 72 hours	*24 hours*
Fixative – tissue ratio	1:10	1:1 – 1:20
MW assisted fixation	Pre-fixation 6 hours	Pre-fixation 0 – 24 h.

* Engel and. Moore (*2011*) Effects of Preanalytical Variables on the Detection of Proteins by Immunohistochemistry in Formalin-Fixed, Paraffin-Embedded Tissue. Archives of Pathology & Laboratory Medicine: May 2011, Vol. 135, No. 5, pp. 537-543.

** 10 % Neutral buffered formalin = 4 % Neutral buffered formaldehyde

Decalcification

Impact on IHC

- 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

Decalcification - Results: Courtesy Ole Nielsen

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
Decalc™ (HCl)	159	23	1	8	2

Reference/No decalcification: +++

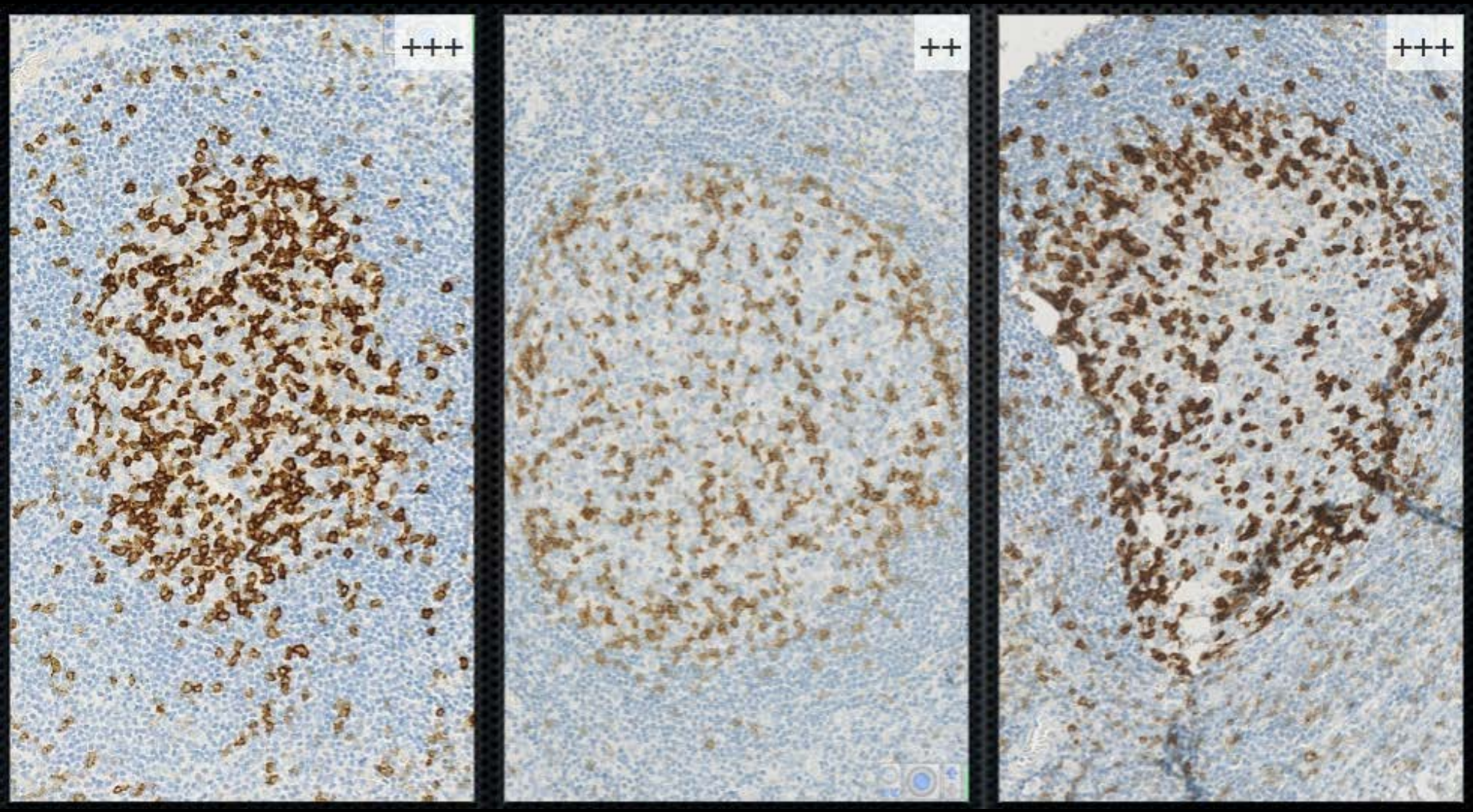
Buffet formic acid (BFA): (4M formic acid + 0.5M Na-formiat)

IHC – The Technical Test Approach

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	+++	++(+)	++(+)	+++

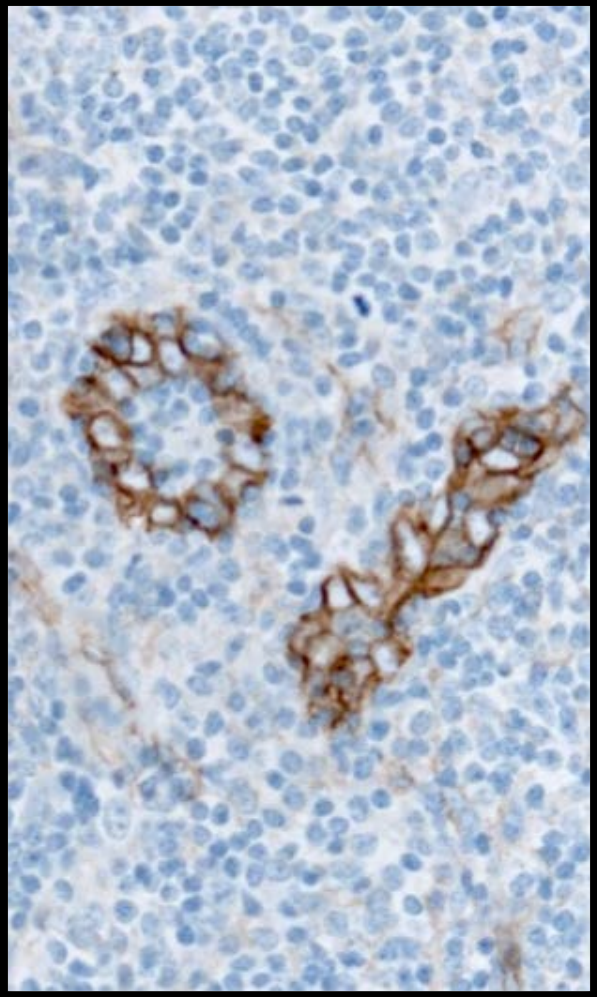
Many Haemato-lymphoid markers affected

CD279 (PD-1), mAb clone NAT105

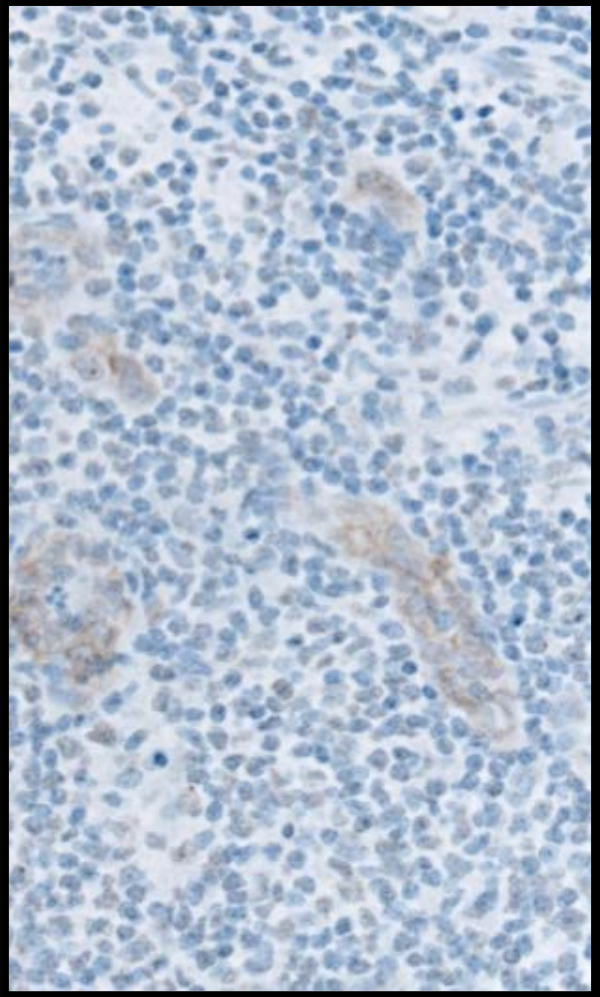


No decalcification | Formic acid 16 hours | 10% EDTA 96 hours

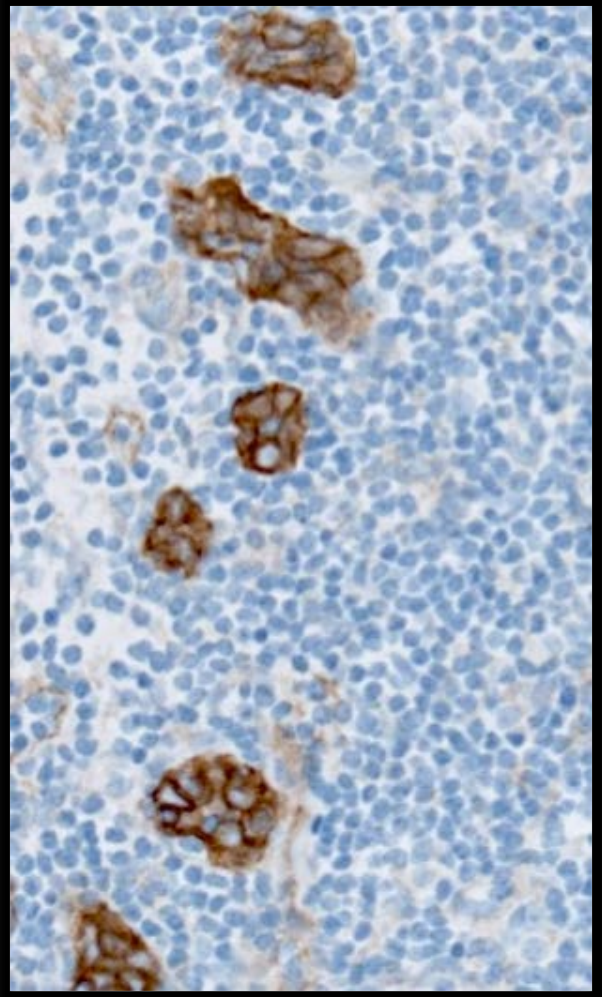
CD105, mAb clone SN6h



No decalcification

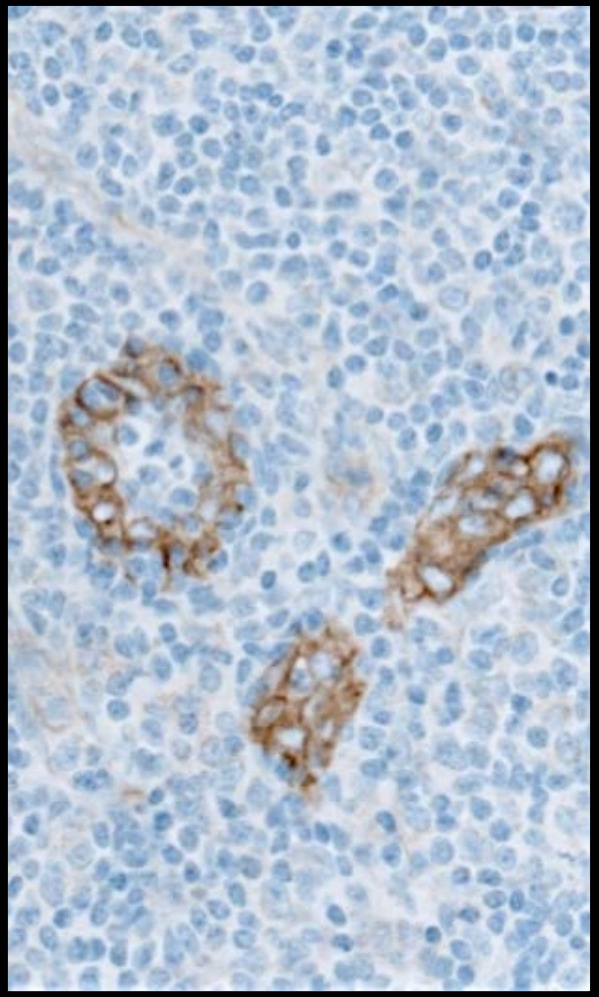


Formic acid 16 hours

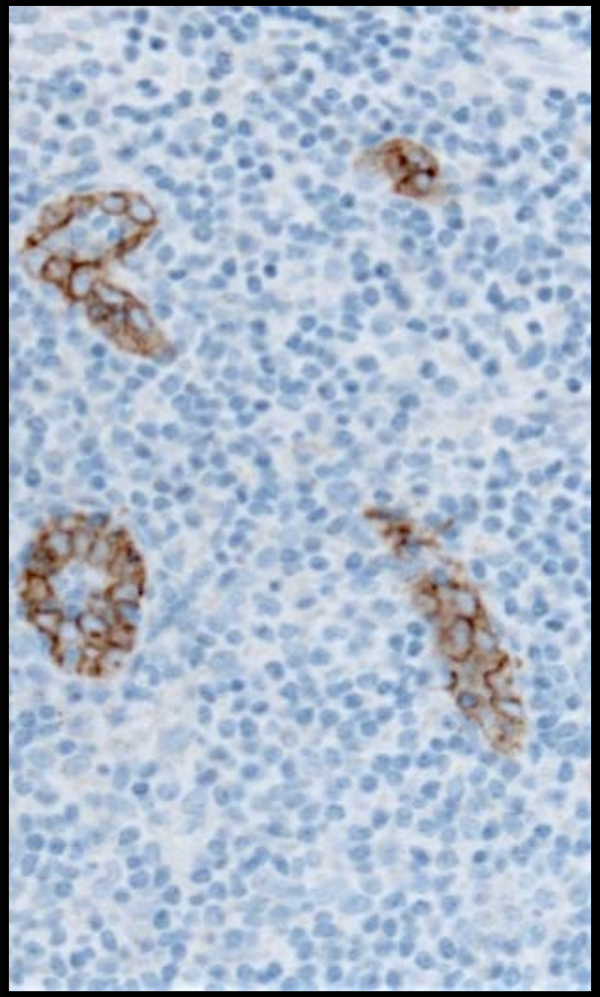


10% EDTA 96 hours

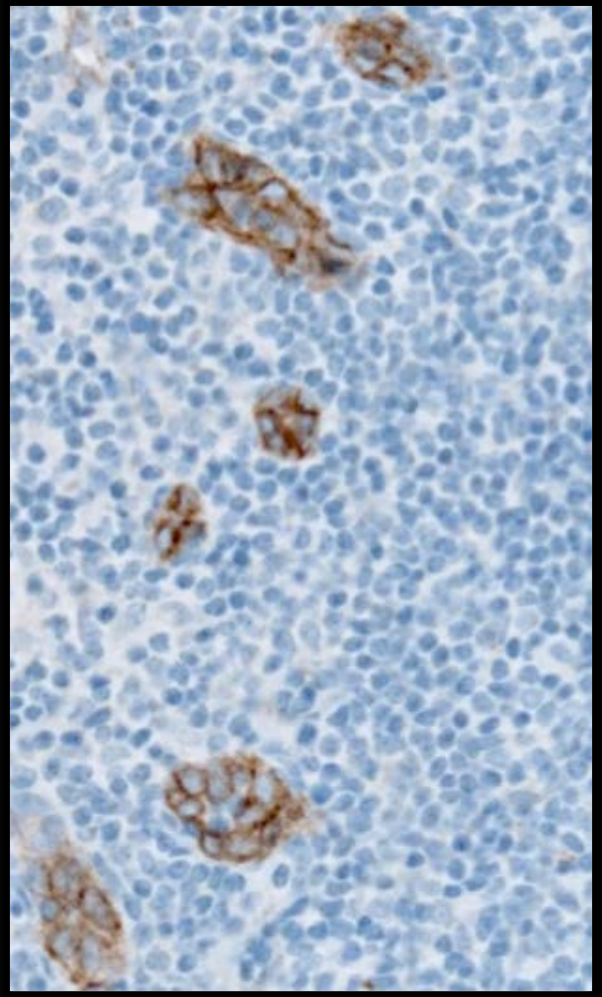
CD105, mAb clone 4G11



No decalcification

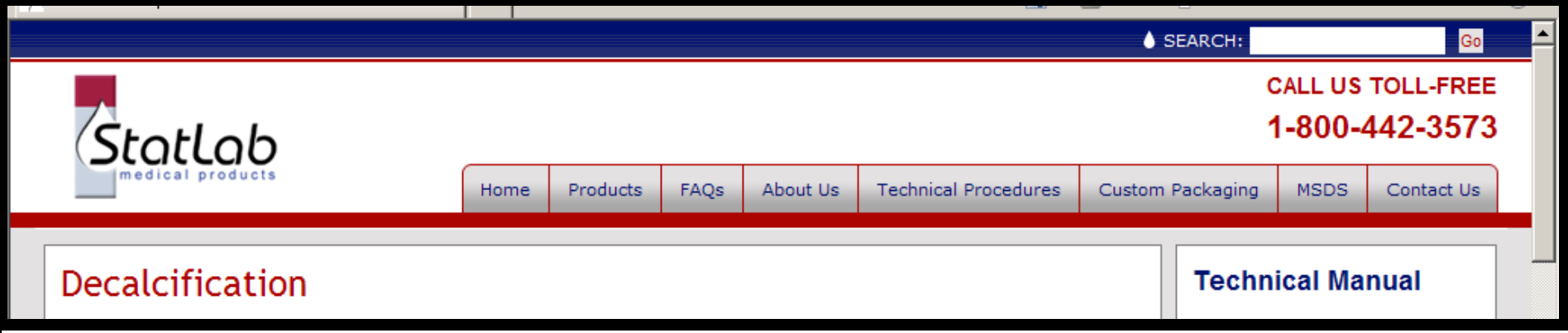


Formic acid 16 hours



10% EDTA 96 hours

IHC – The Technical Test Approach

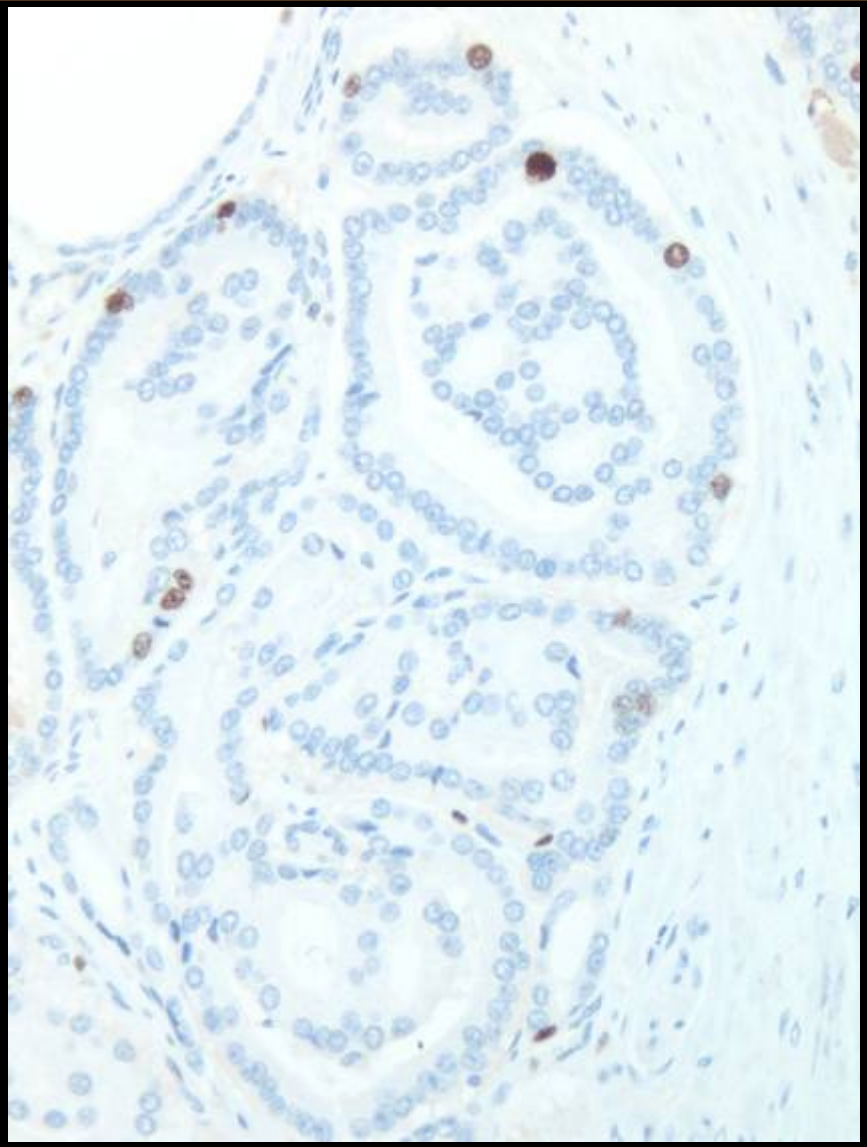


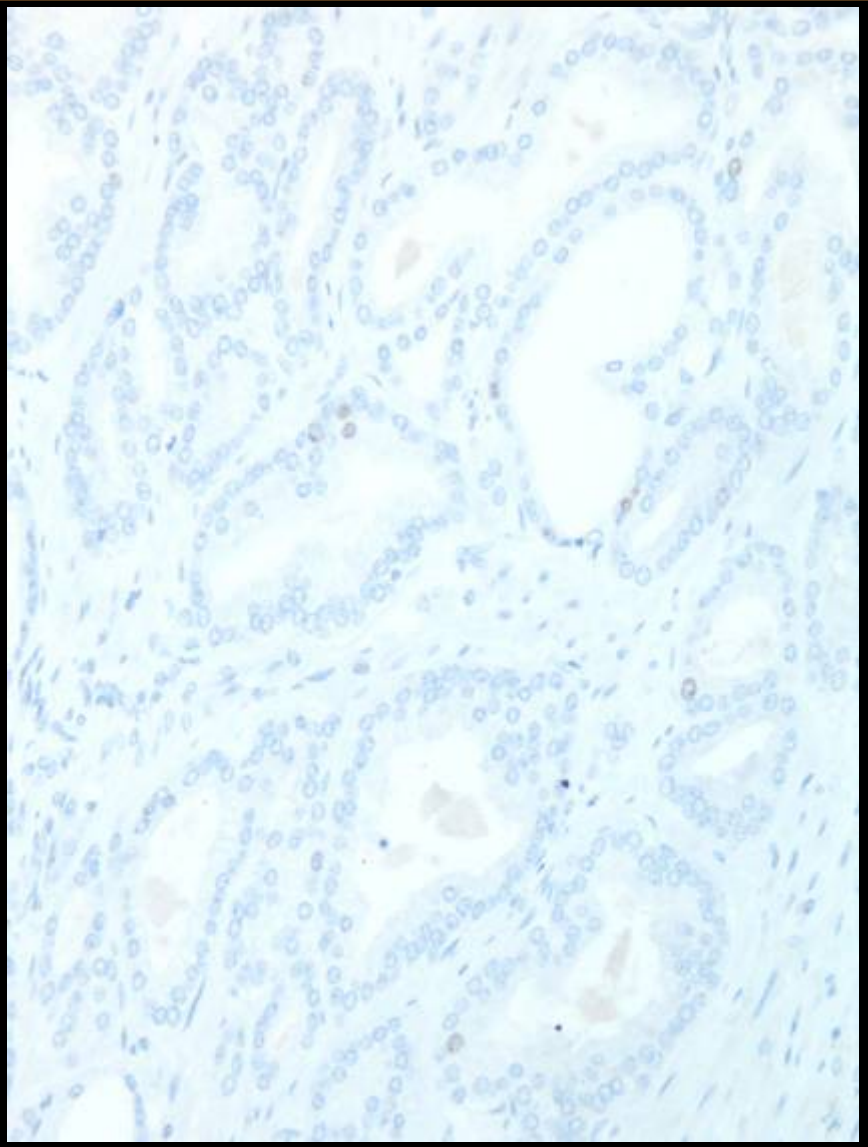
EDF™, *Enhanced Decalcification Formulation*, (CAT# SL85-32) is a combination formic acid/formalin solution designed to enhance fixation and gently decalcify bone specimens. Nuclear staining is excellent, even after prolonged exposures. EDF™ is available in 32 oz. bottles.

Strong acid:	Mild acid:	Calcium chelate:
10% HCL (Decal™)	10% Formic acid	5 - 10% EDTA
Fast (1-2h)	Intermediate (6-24h)	Slow (24-96h)
IHC+	IHC+++*	IHC+++
DNA(+)	DNA+/++	DNA++

* e.g. CD79a, clone JCB117 reduced, Elastase, clone NP57 extracted

IHC – The Technical Test Approach





Prostate – Ki67, rmAb clone 30.9

10 % NBF 24h → 24h 10 % form. acid

10 % NBF + 10 % form. acid 24h

IHC – The Technical Test Approach

MY APPROACH

Optimal processing of bone marrow trephine biopsy: the Hammersmith Protocol

K N Naresh, I Lampert, R Hasserjian, D Lykidis, K Elderfield, D Horncastle, N Smith, W Murray-Brown, G W Stamp

J Clin Pathol 2006;**59**:903–911. doi: 10.1136/jcp.2004.020610

Table 2 Turnaround time for bone marrow trephine biopsy specimens including first panel of immunohistochemistry

	Procedure	Time (h)	Procedure completion on day
1	Fixation	20–24	1
2	Decalcification	6	1
3	Processing and embedding	12–16	2
4	Sectioning for haematoxylin and eosin, special stains and first panel of immunostains	1	2
5	Staining procedures including first panel of immunostains	6	3

1. AZF (or 10 % NBF)
2. 10% formic acid + 5% NBF

Take-home messages

- The Hammersmith Protocol provides a comprehensive approach to handle and investigate bone marrow trephine biopsy samples with an intent to aid and affect management of haematological conditions, especially haematological malignancies.
- The protocol provides excellent morphology, optimal antigen preservation for a complete array of immunohistochemistry and nucleic acid preservation for PCR and mRNA in situ hybridisation-based studies.
- Morphology is comparable to plastic sections as 1-µm-thin sections can be made.
- The protocol can be used in a routine histopathology setup and does not require any specialised equipment.
- The protocol provides a fast turnaround time with results of haematoxylin and eosin and other histochemical stains being available in 48 h and those of other immunostains being available in 72 h of carrying out the biopsy.

IHC – The Technical Test Approach

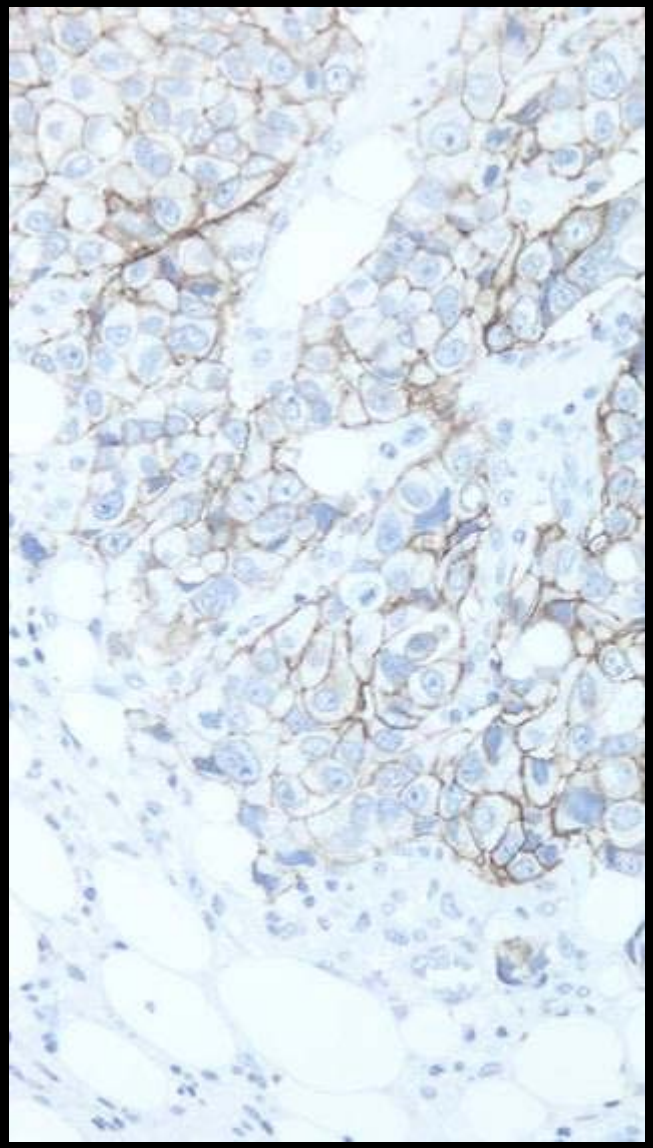
- 3 - 4 um sections mounted on Superfrost +, TOMO or Dako FLEX slides
  - RTU systems based on 4 um sections
  - Level adequate for morphology, signal-to-noise and interpretation

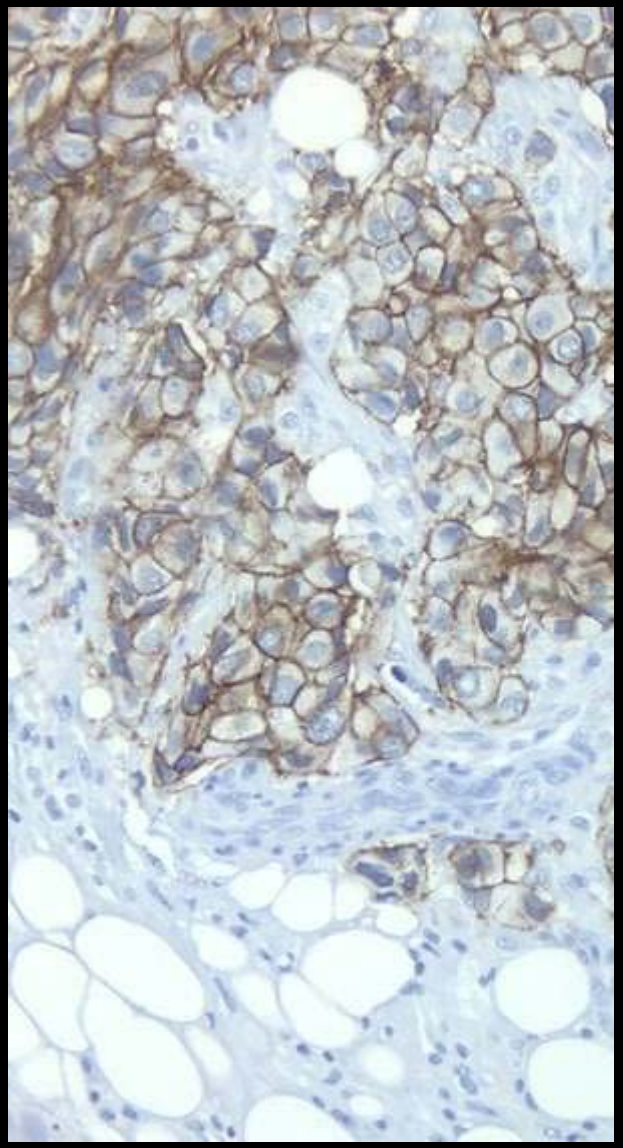
- 1 to 2 hours at 60°C
  Alternatively night over at 37 °C followed by 1 hour at 60°C

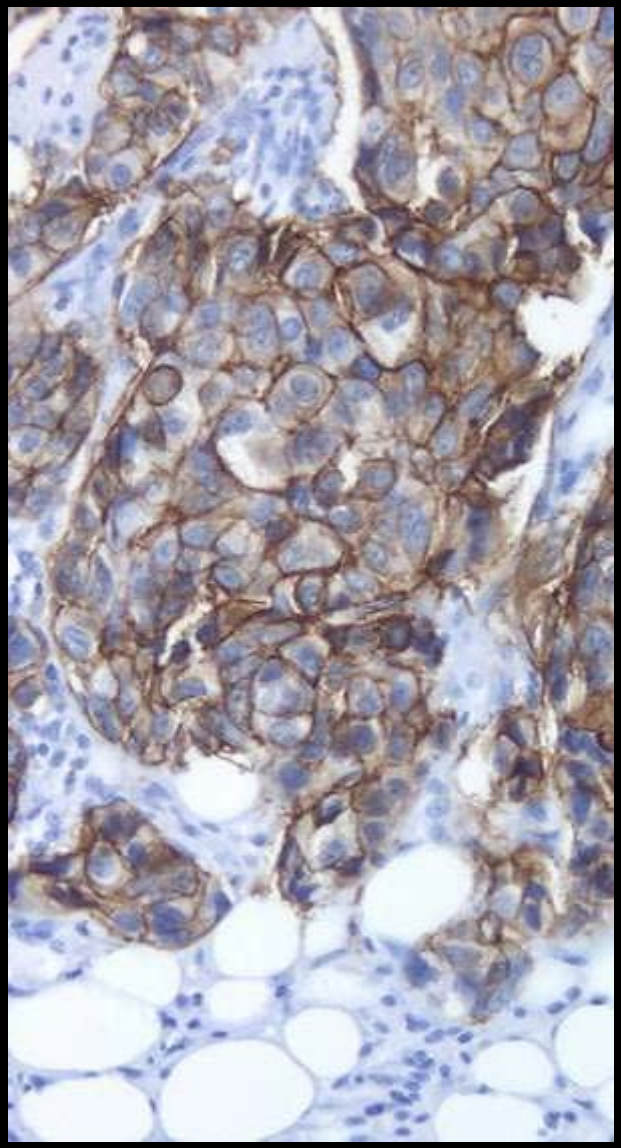
- Having problems with attachment of sections

  - Try change of slides (TOMO and Dako Flex superior)
  - Verify formalin fixation time is adequate –
    - Too short time and long alcohol time hardens tissue
  - Verify quality of reagents for tissue processing
  - Verify section thickness
  - Optimize HIER....

IHC – The Technical Test Approach







HER-2; FDA approved kit

1-2 µm

3-4 µm

6-7 µm

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60°C 1h. Ref. score	60°C 16 h.		80°C 16 h.		95°C 1.5 h.	
3+	3+	15/15	3+	1/3	3+	4/25
2+	2+	10/22	2+	0/5	2+	4/34

Immunocytochemistry Volume 6 Issue 3 (Run 76)

Immunocytochemistry 2008; Volume 6 Issue 3

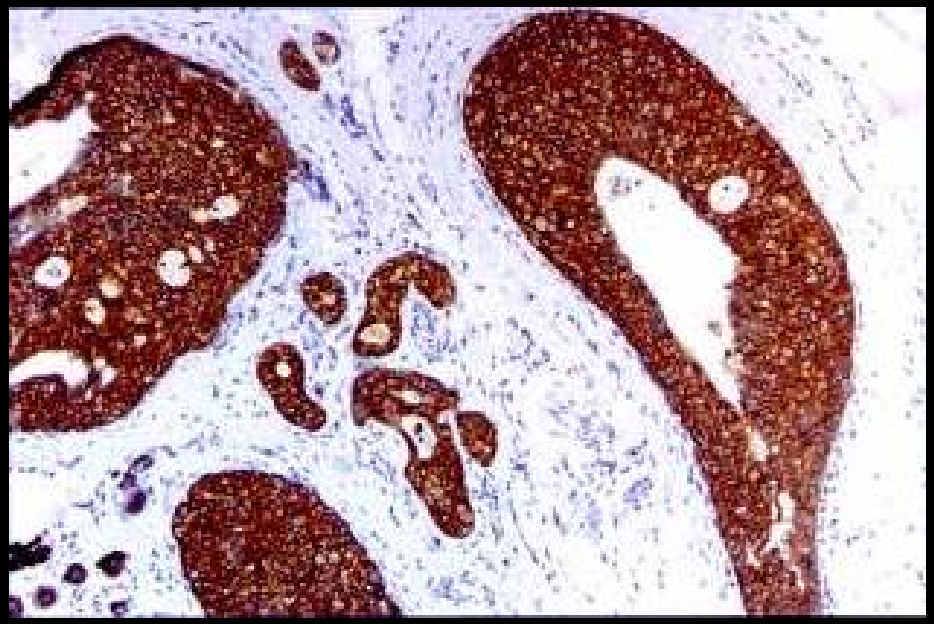
TECHNICAL ARTICLE

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

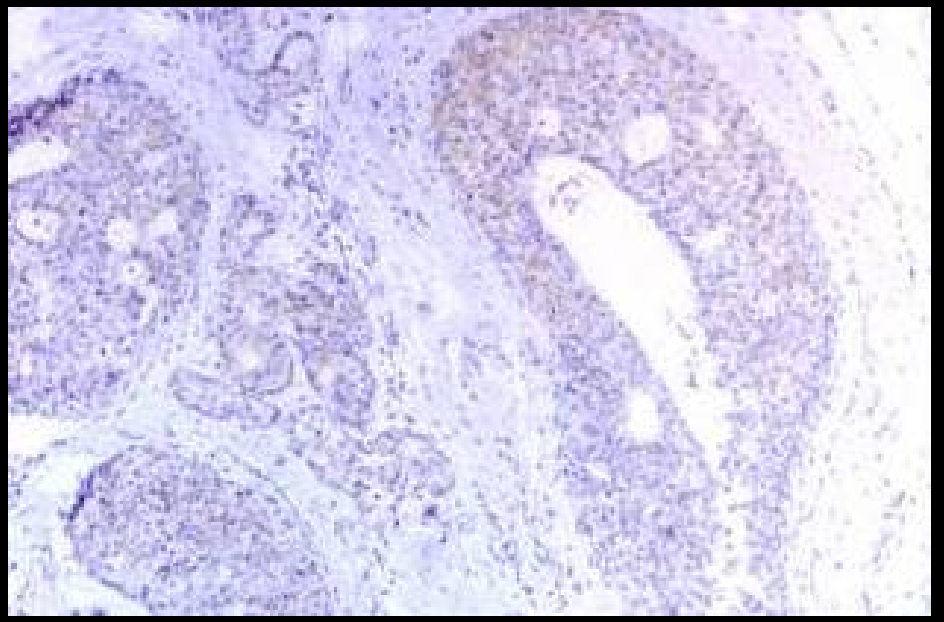
Bent Lundgaard Hansen, Henrik Winther and Kristian Moller

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

Correspondence: Kristian.Moller@dako.com



60°C 1h. HER-2: 3+



80°C 16h. HER-2: 1+

IHC – The Technical Test Approach

Paraffin Section Storage and Immunohistochemistry: Effects of Time, Temperature, Fixation, and Retrieval Protocol with Emphasis on p53 Protein and MIB1 Antigen

Wester, Kenneth Ph.D.; Wahlund, Eva B.L.T.; Sundström, Christer M.D., Ph.D.; Ranefall, Petter Ph.D.; Bengtsson, Ewert Ph.D.; Russell, Pamela J. Ph.D.; Ow, Kim T. M. Sc.; Malmström, Per-Uno M.D., Ph.D.; Busch, Christer M.D., Ph.D.

AIMM : Volume 8(1), March 2000, pp 61-70

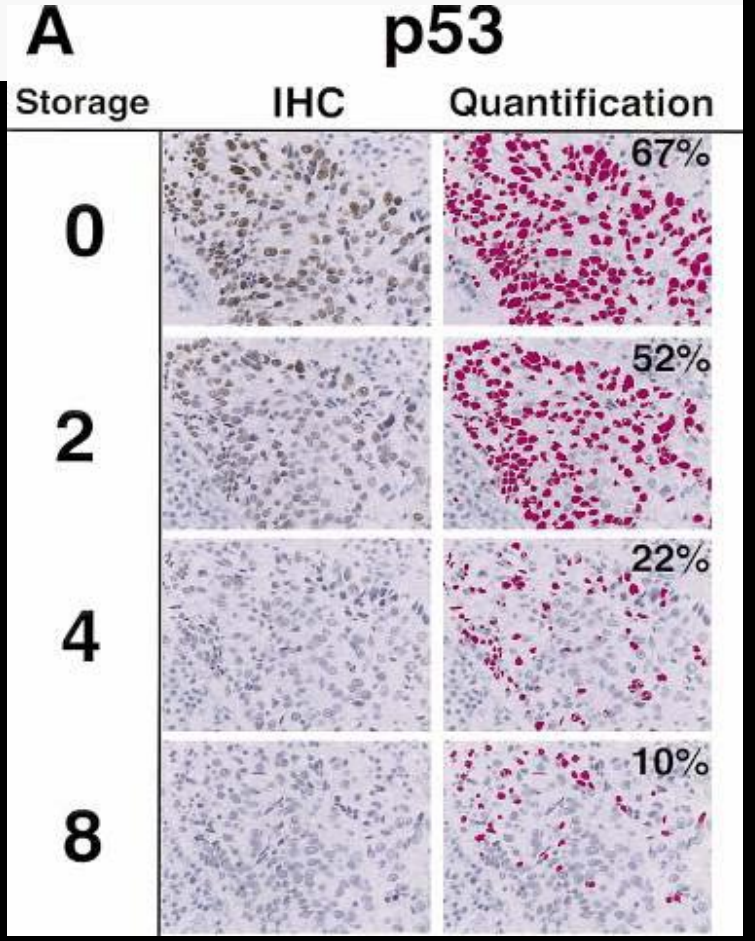
1 - 2 weeks at room-temp.

Otherwise -20 / -80°C

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

Baking just before use.

Coating with paraffin is not confirmed to be beneficial



IHC – The Technical Test Approach

General settings for tissue processing for IHC

- Time to Fixation; 1 hour – optimally at temp controlled conditions at 4C
- Use 10% Neutral Buffered Fomalin (same as 4% formaldehyde)
- Time in Fixation (10% NBF); 8-72 hours
- Gentle decalcification must be performed on appropriate fixed material
- 3-4 um sections applied on slides with high quality adhesiveness
- Store slides at maximum 1 week at room temp before IHC

IHC – The Technical Test Approach

Questions ...???