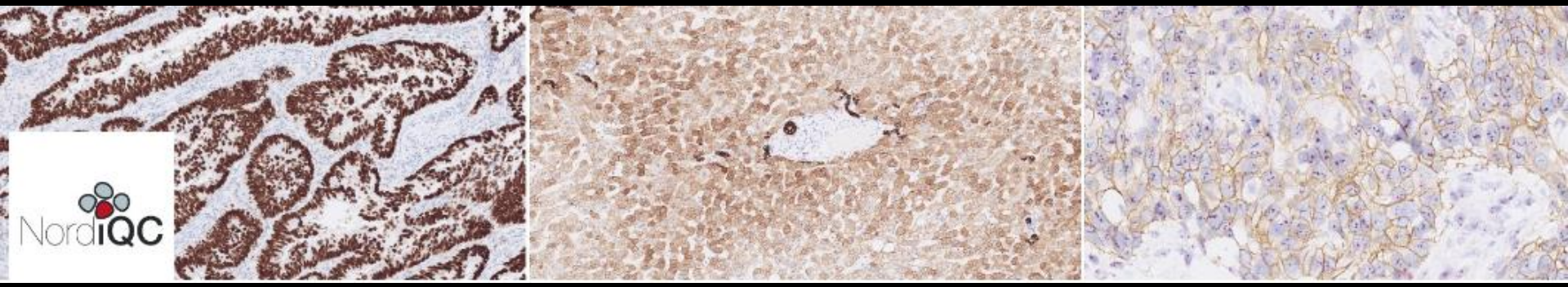
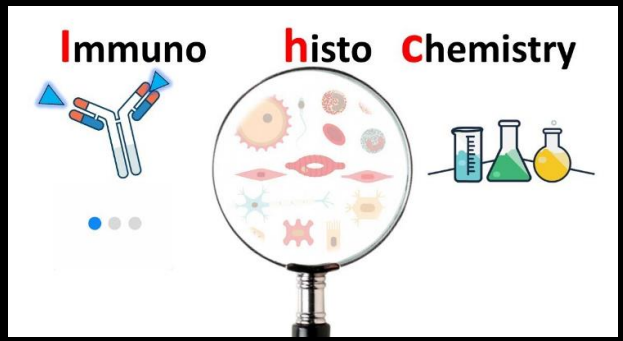


Immunohistochemical principles

-

The total IHC technical test approach Pre-analytics

Søren Nielsen
Director, NordiQC
Aalborg University Hospital, Denmark



IHC – The Technical Test Approach

Agenda:

1. Examples on main critical pre-analytical steps
2. How to make best practice choices
3. Open forum to discuss own experiences

IHC – The Technical Test Approach

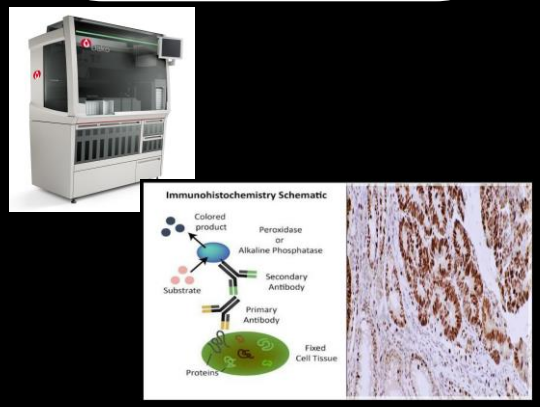
"Immunohistochemistry is technically complex, and no aspect of this complexity can be ignored, from the moment of collecting the specimen to issuance of the final report "
Taylor CR. Arch Pathol Lab Med 2000; 124:945

Pre-Analytical

Ischemia
Fixation process
(Decalcification)
Tissue processing
Paraffin embedding
Sectioning
Storage

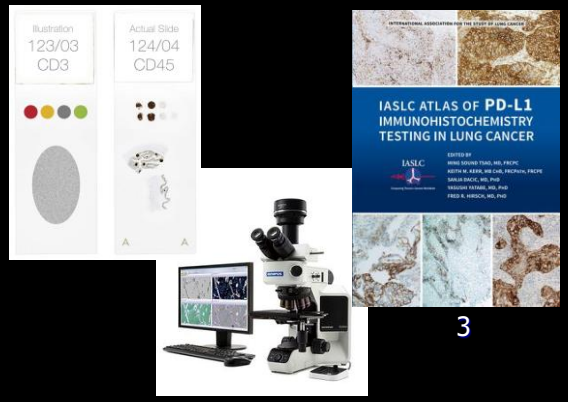
Analytical

IHC platform
Epitope retrival
Primary antibody
Detection system
Chromogen
Counterstaining
Mounting



Post-Analytical

Usage of controls
Positive controls
Negative controls
"Critical controls"
Scoring / read-out
Interpretation
Reporting



IHC – The Technical Test Approach

Arch Pathol Lab Med. 2019;143:1346–1363;

Preanalytics and Precision Pathology

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

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

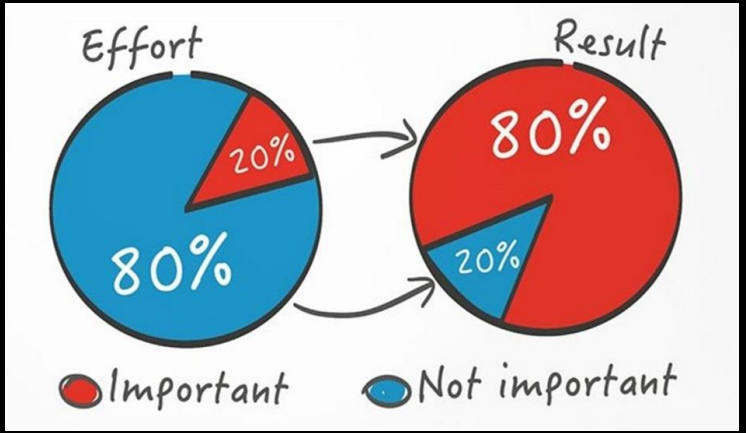
DIAGNOSTICS |
Analytical science, Laboratory management, Quality assurance and quality control, Biochemistry and molecular biology

Garbage In, Garbage Out

The hidden reason laboratory test results may not be as reliable as they seem

Carolyn Compton | 03/16/2018

Pareto's principle;



60-80% of errors in pathology estimated to be related to pre-analytics…..

5 main parametres identified to represent 80% of the errors

IHC – The Technical Test Approach

1. Cold ischemia time (time from removal to fixative)
2. Method of processing (section thickness, temperature, fixative volume to tissue mass ratio)
3. Type and quality of fixative
4. Total time in formalin
5. Storage conditions (blocks and cut slides)

IHC – The Technical Test Approach

1. Cold ischemia time (time from removal to fixative)
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4. Total time in formalin
5. Storage conditions

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

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

1098 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

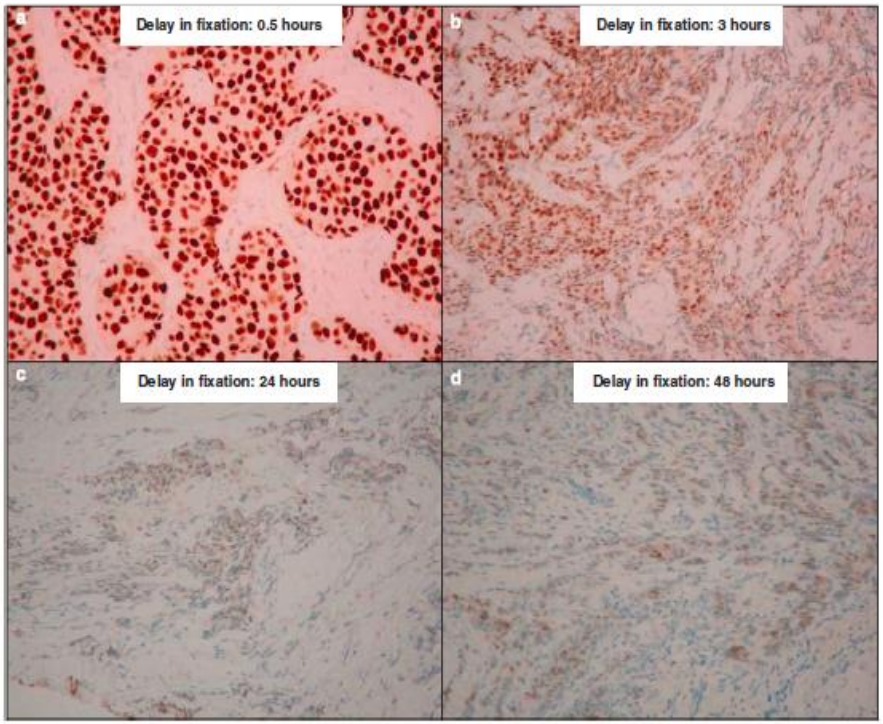


Figure 1 An example of a non-refrigerated case. The tumor was strongly positive for estrogen receptor (similarly to core biopsy) at 0.5 h of delayed fixation (**a**) but demonstrated significant reduction at 3 h (**b**), 24 h (**c**), and 48 h (**d**). All photomicrographs were taken at × 200.

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

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

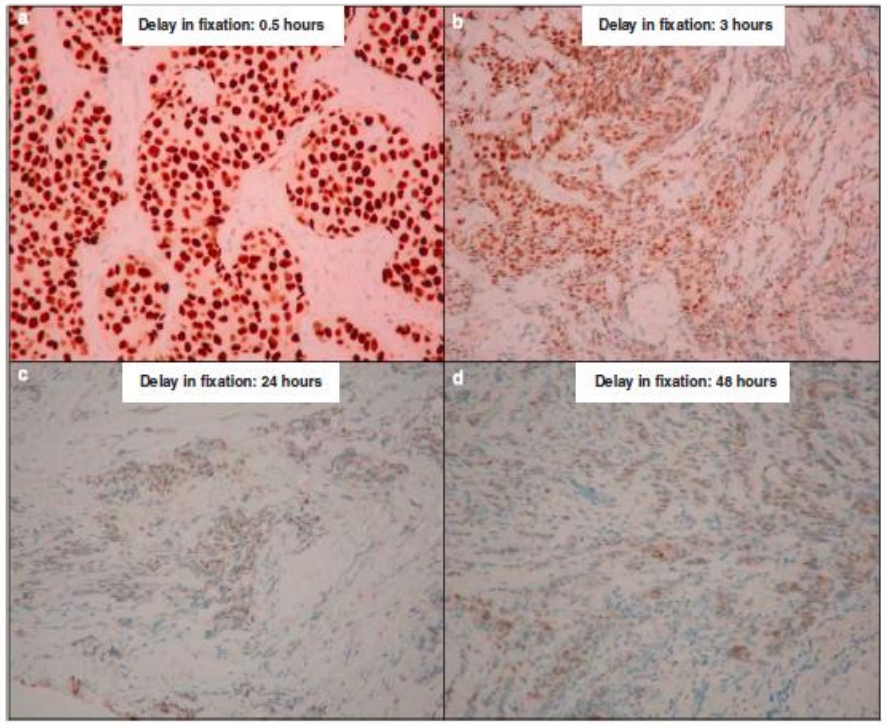


Figure 1 An example of a non-refrigerated case. The tumor was strongly positive for estrogen receptor (similarly to core biopsy) at 0.5 h of delayed fixation (**a**) but demonstrated significant reduction at 3 h (**b**), 24 h (**c**), and 48 h (**d**). All photomicrographs were taken at × 200.

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

Virchows Archiv (2019) 475:191–199
https://doi.org/10.1007/s00428-019-02595-9

ORIGINAL ARTICLE

Check for updates

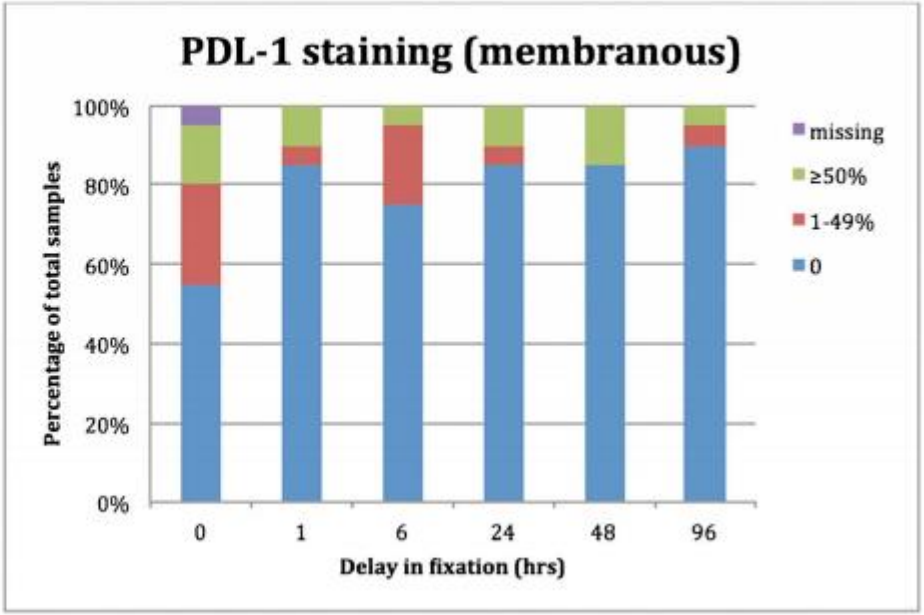
Impact of delayed and prolonged fixation on the evaluation of immunohistochemical staining on lung carcinoma resection specimen

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

Received: 5 January 2019 / Revised: 14 May 2019 / Accepted: 3 June 2019 / Published online: 1 July 2019

"Samples with delay in fixation showed deterioration of tissue quality leading to reduction in the expression of CK 7, Keratin MNF116, CAM 5.2, CK 5/6, TTF-1, CMET, Napsin A, D2-40, and PD-L1. Prolonged fixation had no influence on the performance of immunohistochemical stains. Delay of fixation negatively affects the expression of different immuno-histochemical markers, influencing diagnostic (cytokeratins) and predictive (PD-L1) testing."

Fig. 2 The distribution of PD-L1 (E1L3N (XP)) staining divided in 4 categories is shown for samples with delay in fixation. Of note, the number of cases with positive PD-L1 staining (1–49% and ≥ 50%) is lower after delay in fixation



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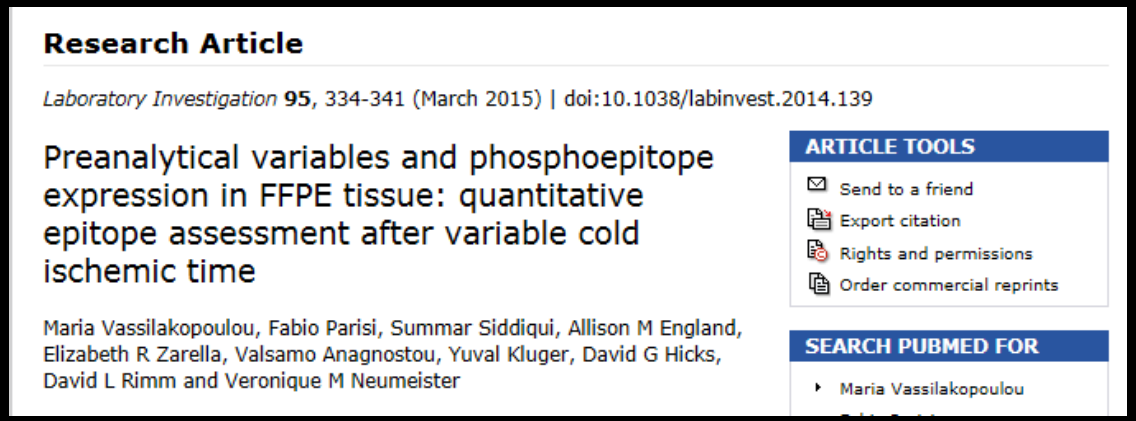

Research Article

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…☺

IHC – The Technical Test Approach

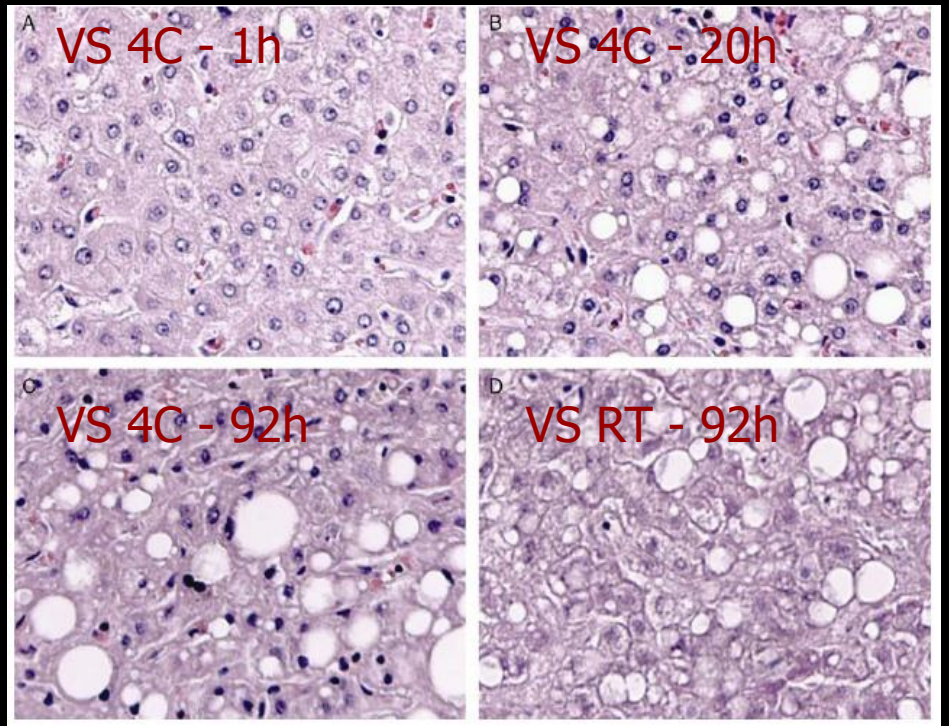
Concl.: Cooling preserved specimens, whereas vacuum sealing added no effect

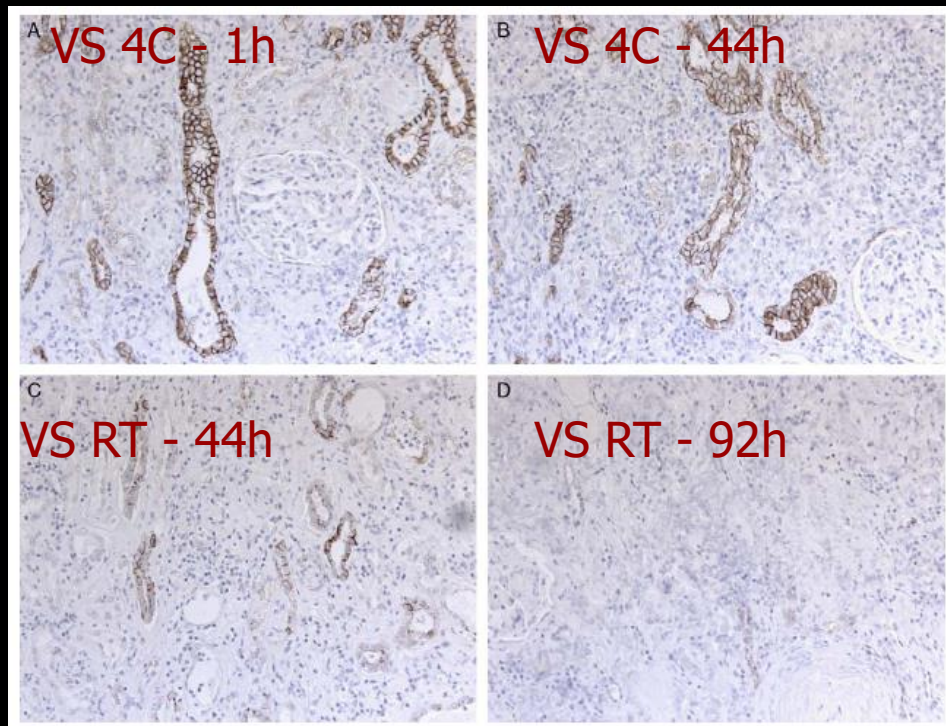
TECHNICAL ARTICLE

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

1. Cold ischemia time (time from removal to fixative)
2. Method of processing (section thickness, temperature, fixative volume to tissue mass ratio)
3. Type and quality of fixative
4. Total time in formalin
5. Storage conditions (blocks and cut slides)

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

How fast is NBF?

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?

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

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

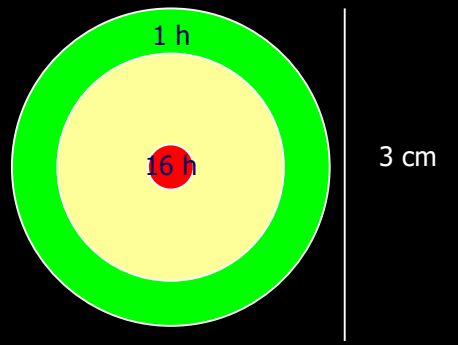
1 hour = 3.6 mm

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)



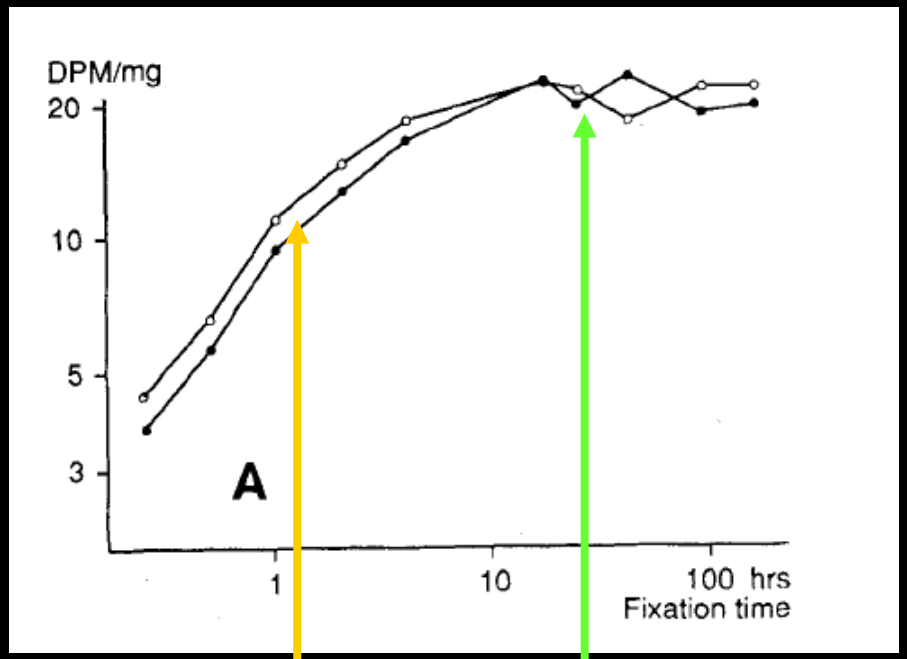
(to double the depth; 4x the time)

IHC – The Technical Test Approach

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

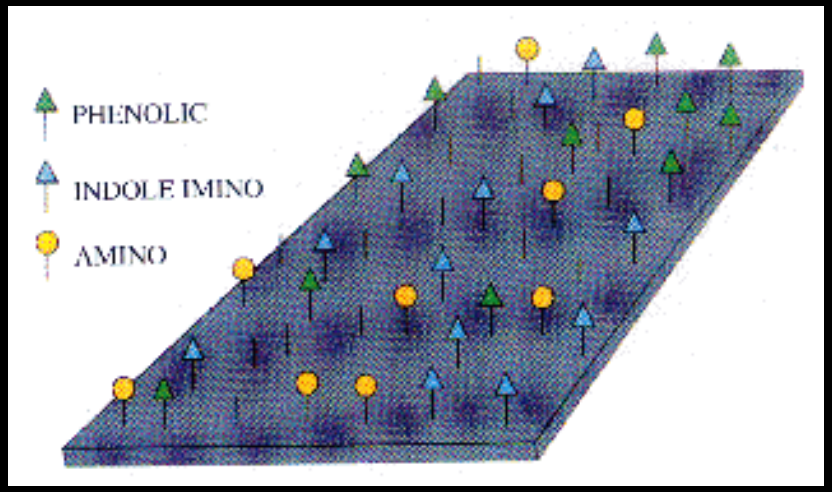
100 % binding of formaldehyde after 24 hours at 25°C

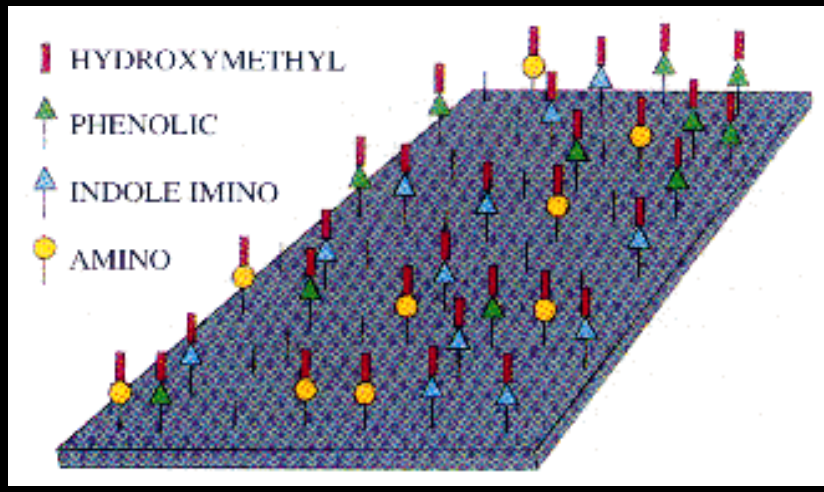
50 % binding of formaldehyde after 100 min. at 25°C

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

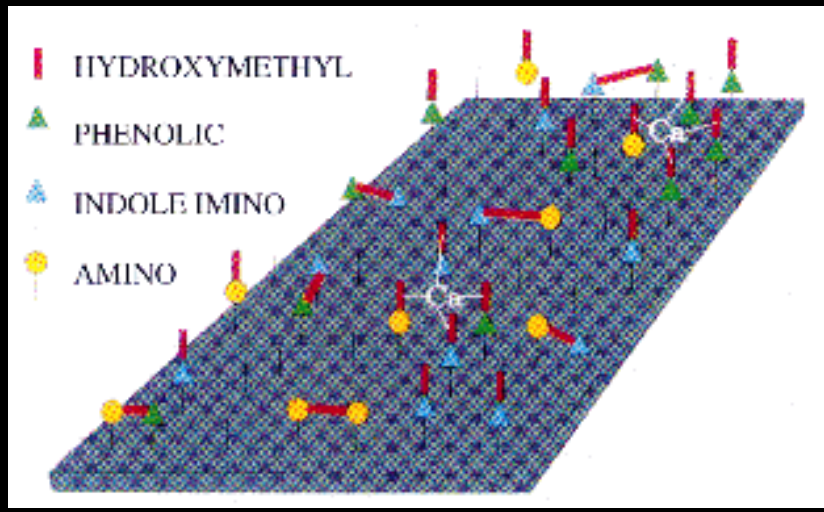
IHC – The Technical Test Approach

< 24 h

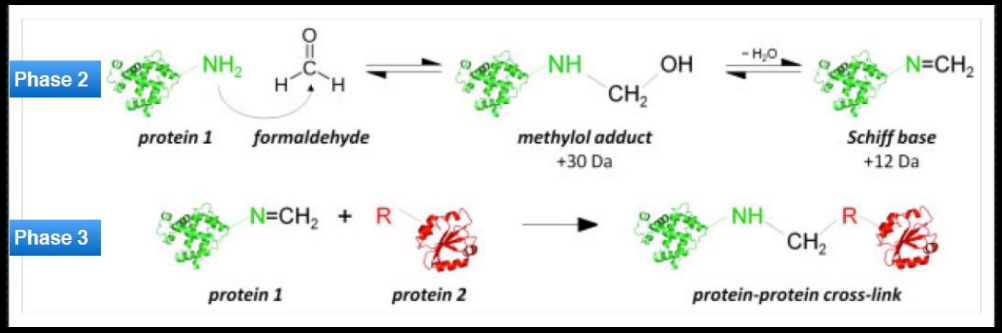




Formaldehyde is a cross linking protein fixing agent, reacting ” clock-wise” - the longer the more effective !



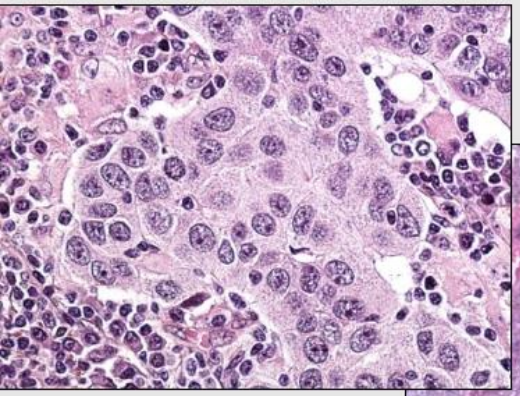
> 24 h

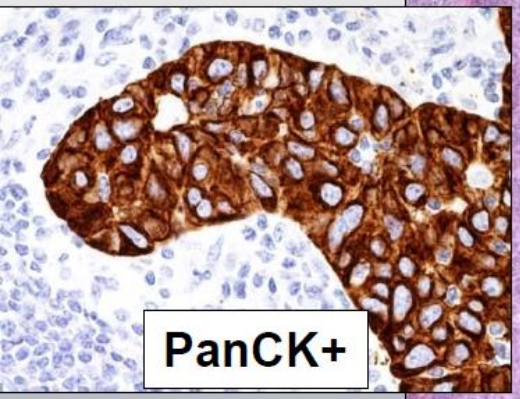


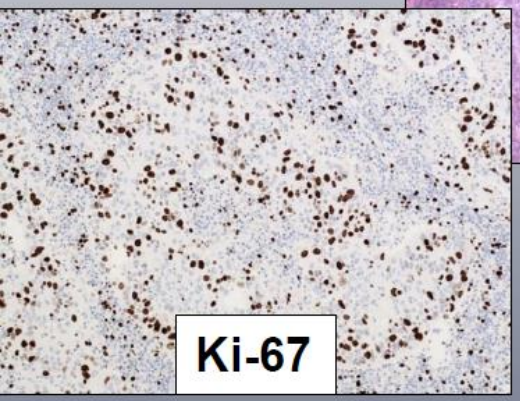
Methylene glycol + free aldehyde = NBF

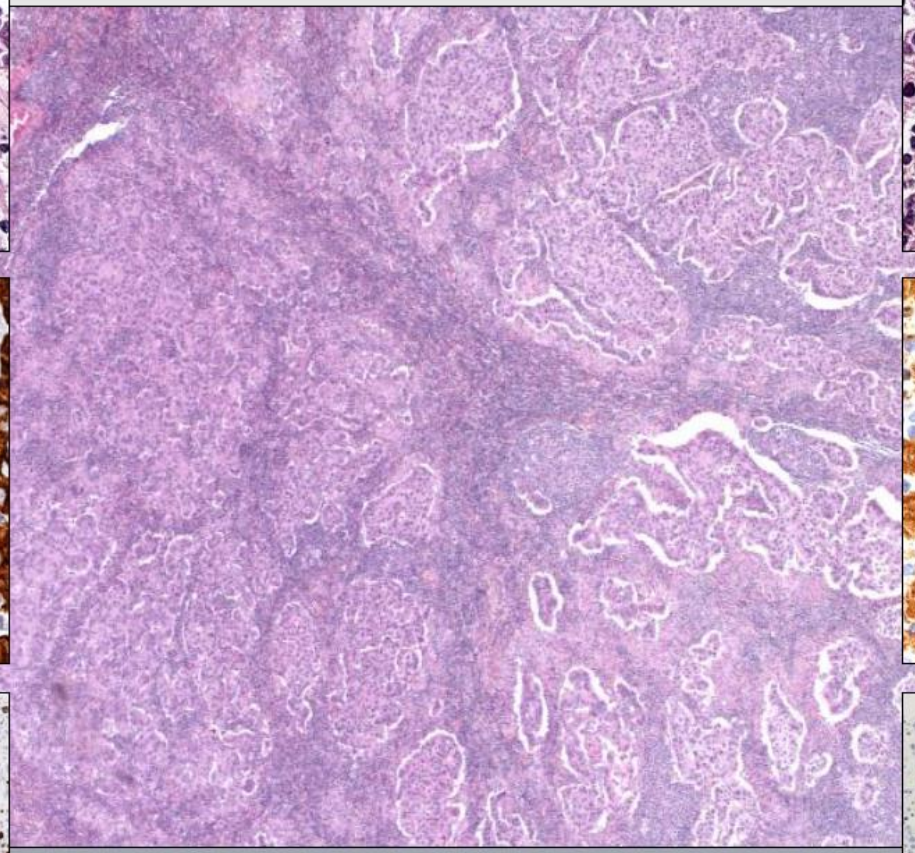
IHC – The Technical Test Approach

Delayed / short fixation…….

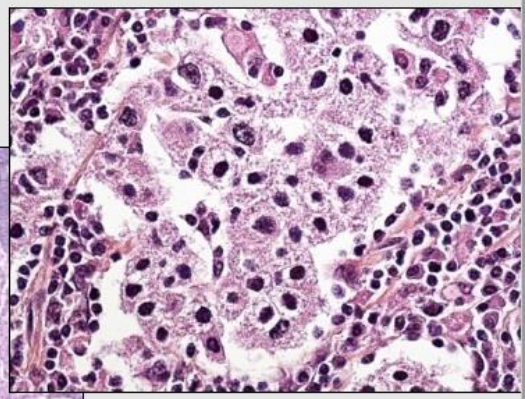


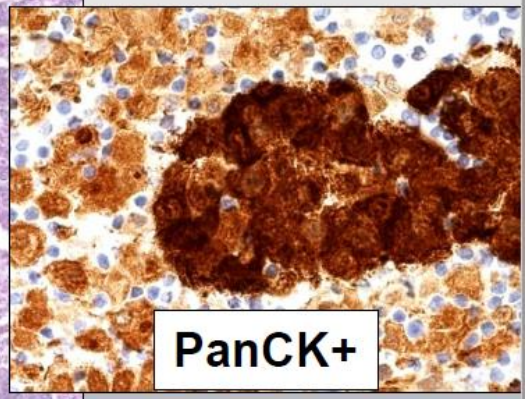


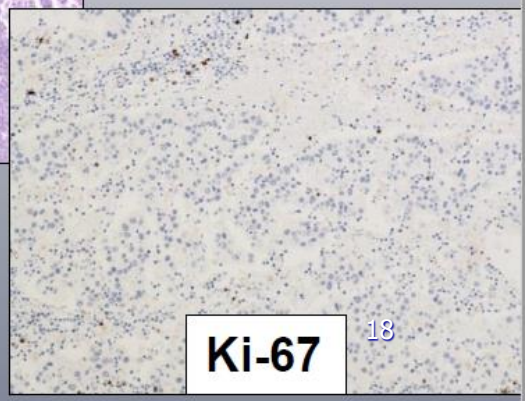




By courtesy, Jan Klos

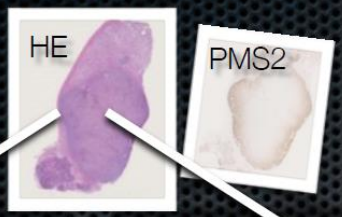




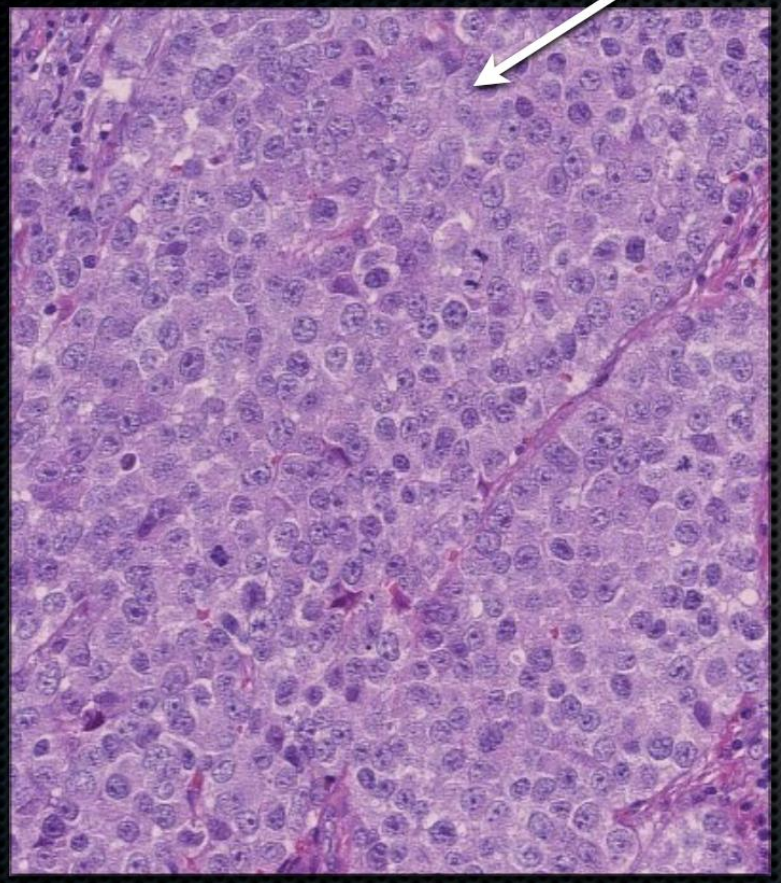


IHC – The Technical Test Approach

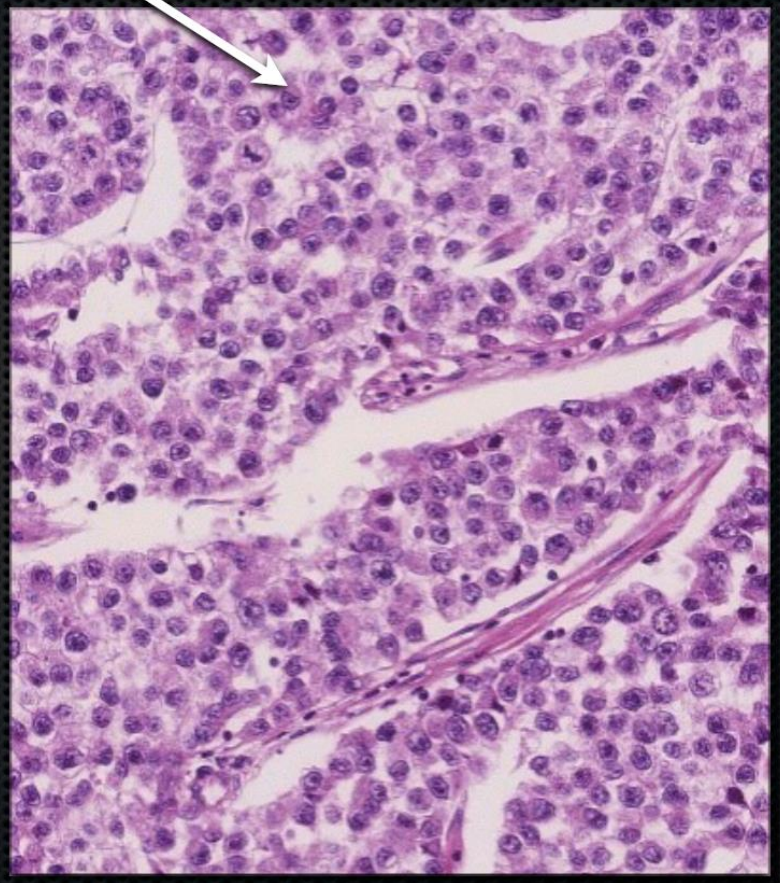
Seminoma



Poor tissue handling



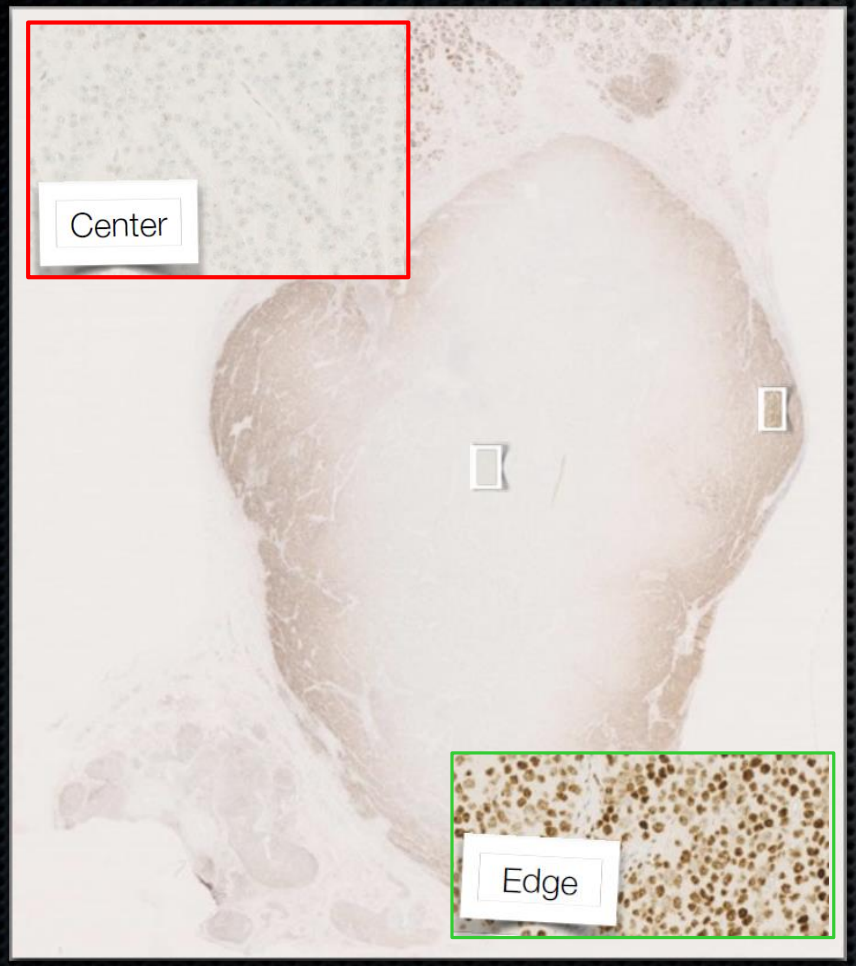
Edge



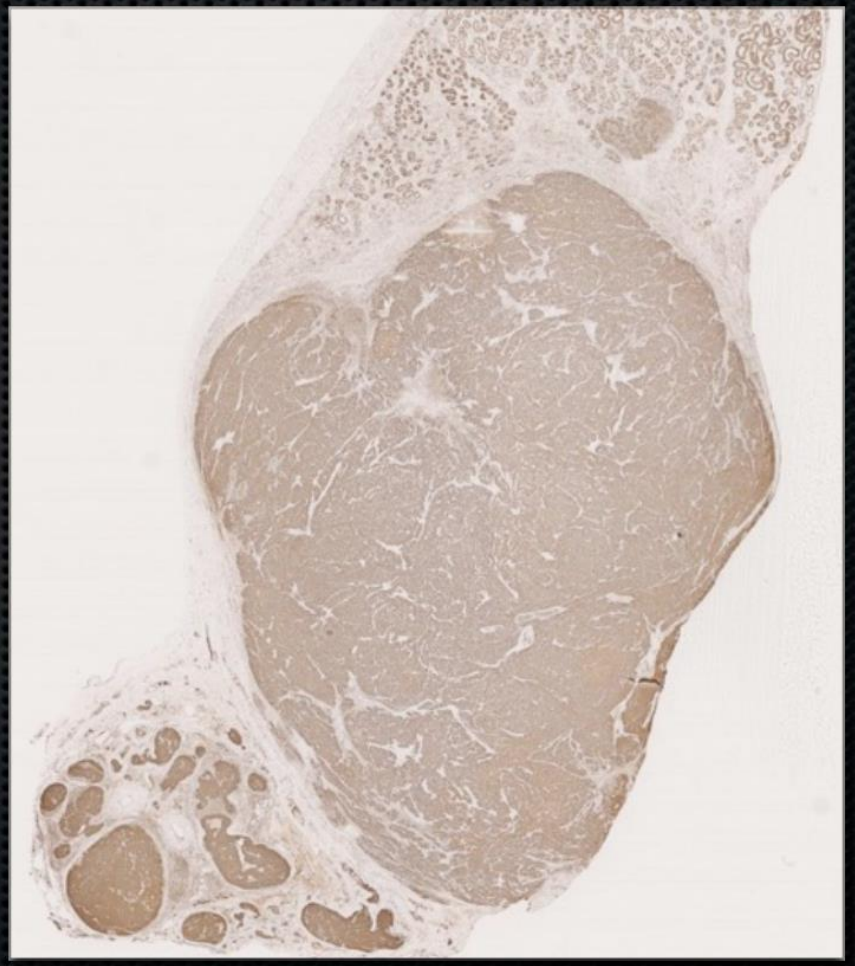
Center

By courtesy, Ole Nielsen

IHC – The Technical Test Approach



PMS2, EPR3947



MSH6, EP49

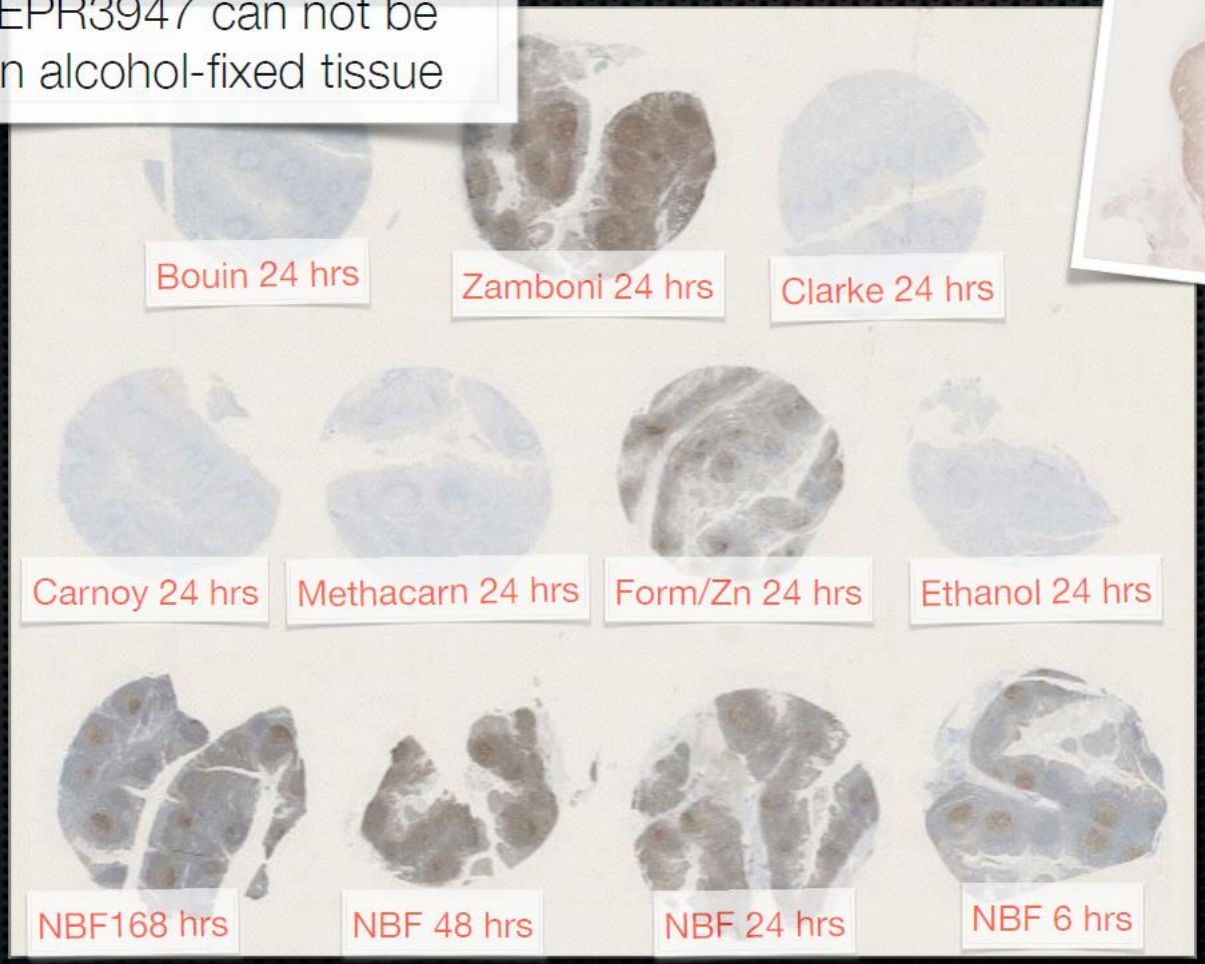
By courtesy, Ole Nielsen

IHC – The Technical Test Approach

Poor tissue handling

PMS2, EPR3947 and fixatives

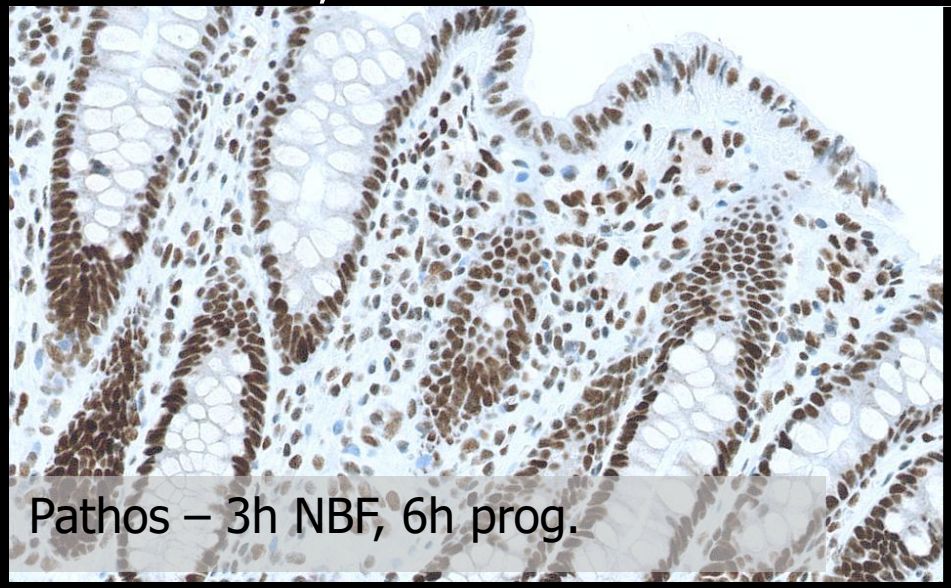
Clone EPR3947 can not be used on alcohol-fixed tissue



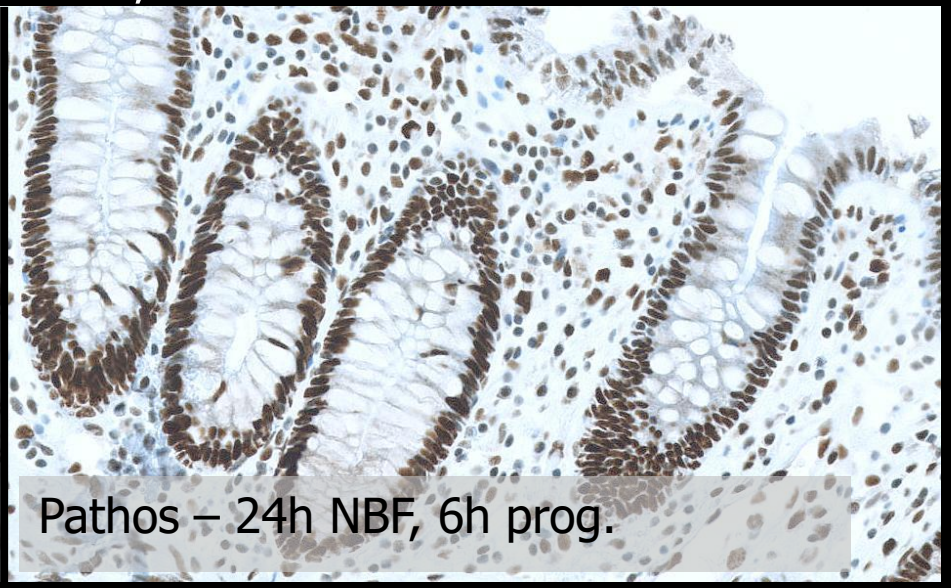
By courtesy, Ole Nielsen

IHC – The Technical Test Approach

Colon: MSH2, mAb clone G219-1129 & MSH6, clone EP49

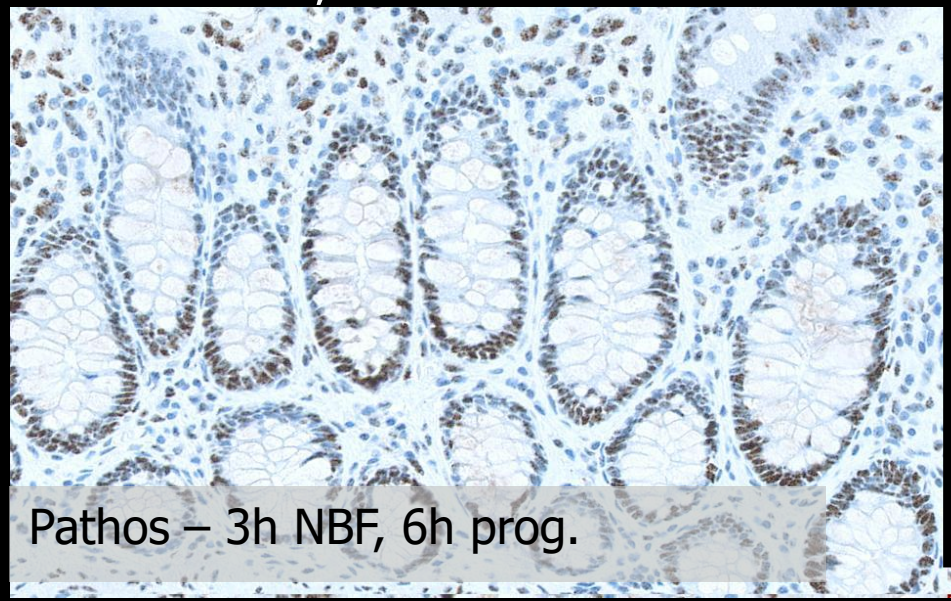


Pathos – 3h NBF, 6h prog.

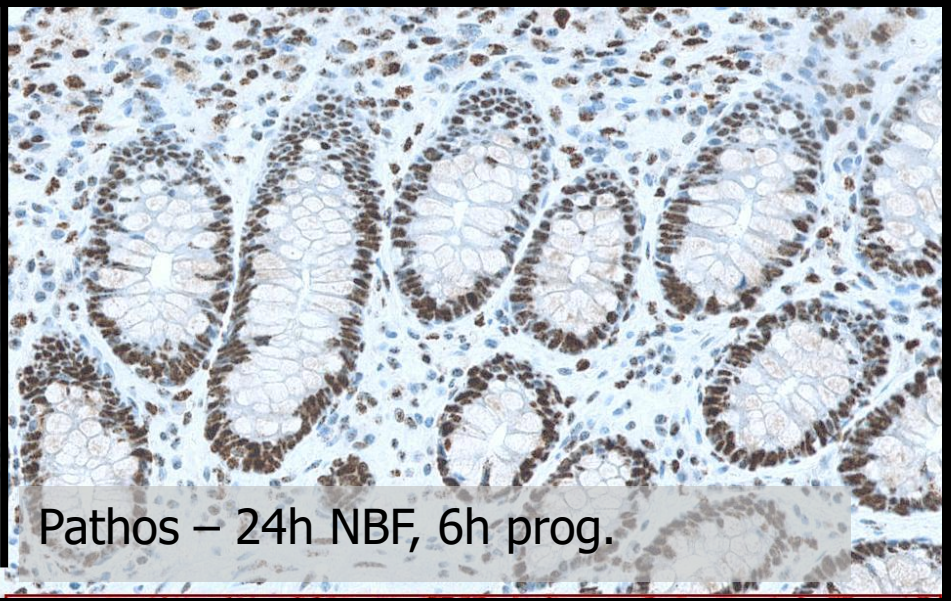


Pathos – 24h NBF, 6h prog.

Colon: MLH1, mAb clone ES05 & PMS2 clone EP51

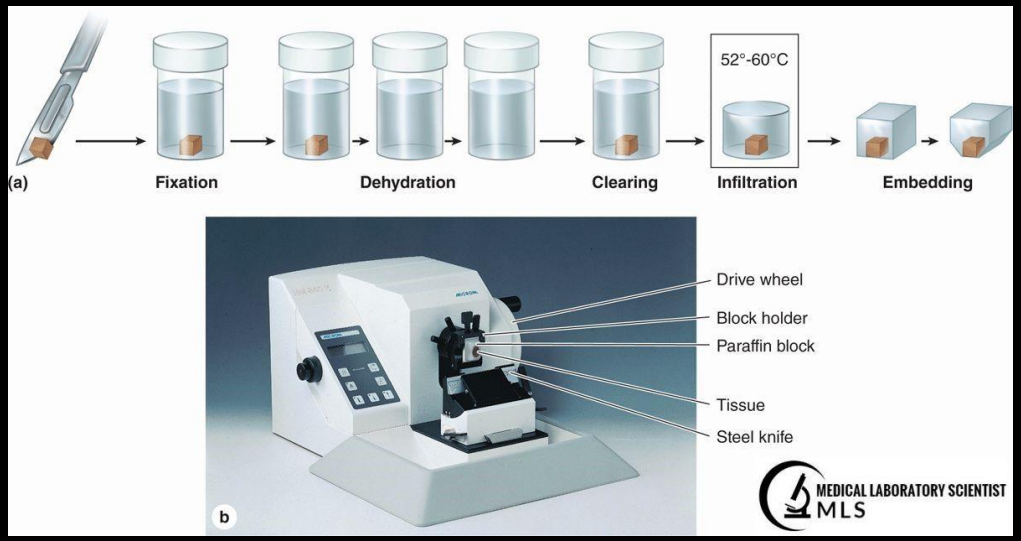


Pathos – 3h NBF, 6h prog.

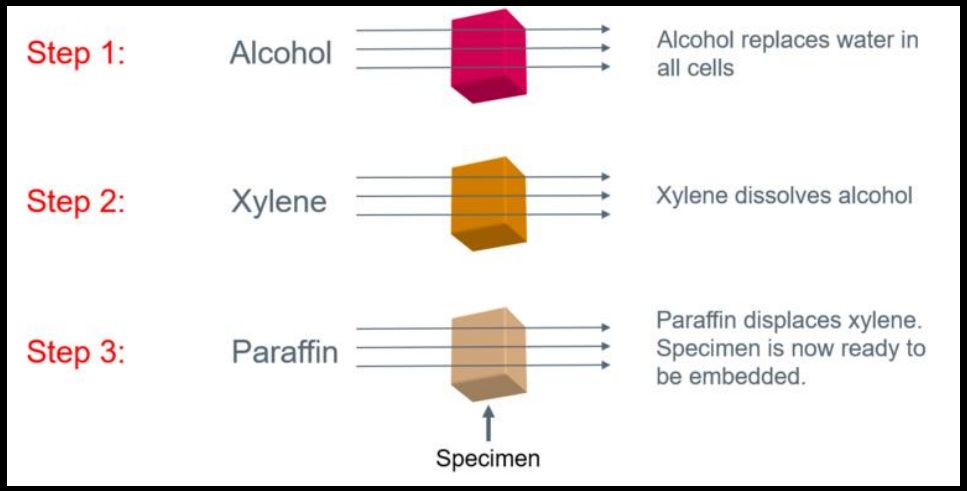


Pathos – 24h NBF, 6h prog.

IHC – The Technical Test Approach



Sequence	Reagent	For small biopsy specimens		For large surgical specimens	
		Time (min)	°C	Time (min)	°C
Fixation	Formalin	44	45	44	45
Dehydration	Ethanol (30%)	30	45	30	45
	Ethanol (50%)	30	45	30	45
	Ethanol (70%)	30	45	30	45
	Ethanol (80%)	30	45	30	45
	Ethanol (95%)	30	45	60	45
	Ethanol (100%)	30	45	90	45
Clearing	Xylene	45	45	45	45
	Xylene	45	45	45	45
	Xylene	90	45	90	45
Impregnation	Paraffin	60	65	60	65
	Paraffin	60	65	60	65
	Paraffin	80	65	80	65



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

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

Arch Pathol Lab Med. 2018;142:1364–1382

Human Epidermal Growth Factor Receptor 2 Testing in Breast Cancer

American Society of Clinical Oncology/College of American Pathologists Clinical Practice Guideline Focused Update

Antonio C. Wolff, M. Elizabeth Hale Hammond, Kimberly H. Allison, Brittany E. Harvey, Pamela B. Mangu, John M.S. Bartlett, Michael Bilous, Ian O. Ellis, Patrick Fitzgibbons, Wedad Hanna, Robert B. Jenkins, Michael F. Press, Patricia A. Spears, Gail H. Vance, Giuseppe Viale, Lisa M. McShane, Mitchell Dowsett

6 - 72h

ASCO special articles

Estrogen and Progesterone Receptor Testing in Breast Cancer: ASCO/CAP Guideline Update

Kimberly H. Allison, MD[1]; M. Elizabeth H. Hammond, MD[2]; Mitchell Dowsett, PhD[3]; Shannon E. McKernin[4]; Lisa A. Carey, MD[5]; Patrick L. Fitzgibbons, MD[6]; Daniel F. Hayes, MD[7]; Sunil R. Lakhani, MD[8,9]; Mariana Chavez-MacGregor, MSc[10]; Jane Perlmutter, PhD[11]; Charles M. Perou, PhD[5]; Meredith M. Regan, ScD[12]; David L. Rimm, MD, PhD[13]; W. Fraser Symmans, MD[10]; Emina E. Torlakovic, MD, PhD[14,15]; Leticia Varella, MD[16]; Giuseppe Viale, MD[17,18]; Tracey F. Weisberg, MD[19]; Lisa M. McShane, PhD[20]; and Antonio C. Wolff, MD[21]

J Clin Oncol 38:1346-1366.

6 - 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

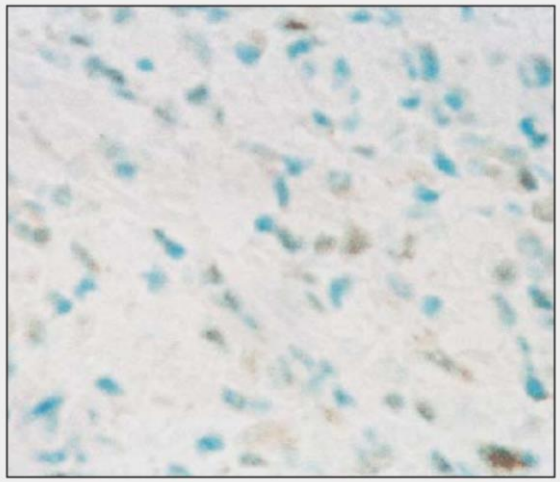


Image 1 Fixation, 3 h; antigen retrieval, 40 min.

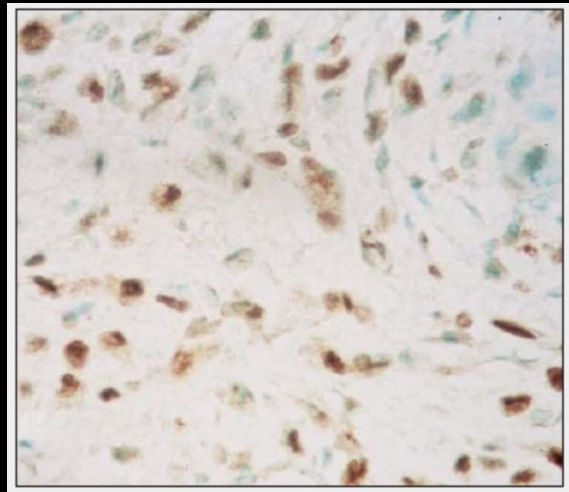


Image 2 Fixation, 6 h; antigen retrieval, 40 min.

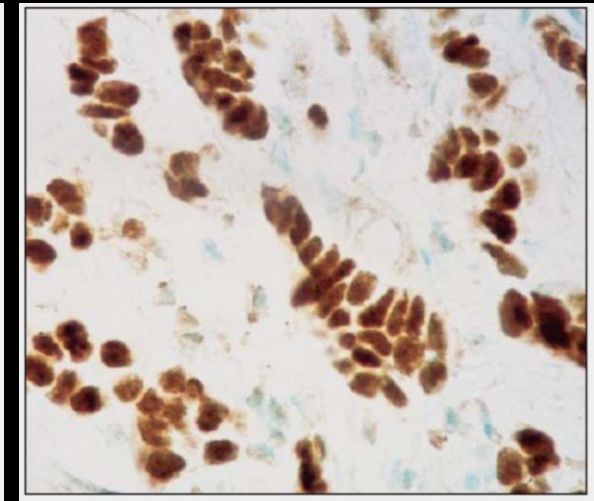


Image 3 Fixation, 8 h; antigen retrieval, 40 min.

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

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.: 6-72 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 high expressing tumours**
Each biomarker/antibody must be evaluated!!!

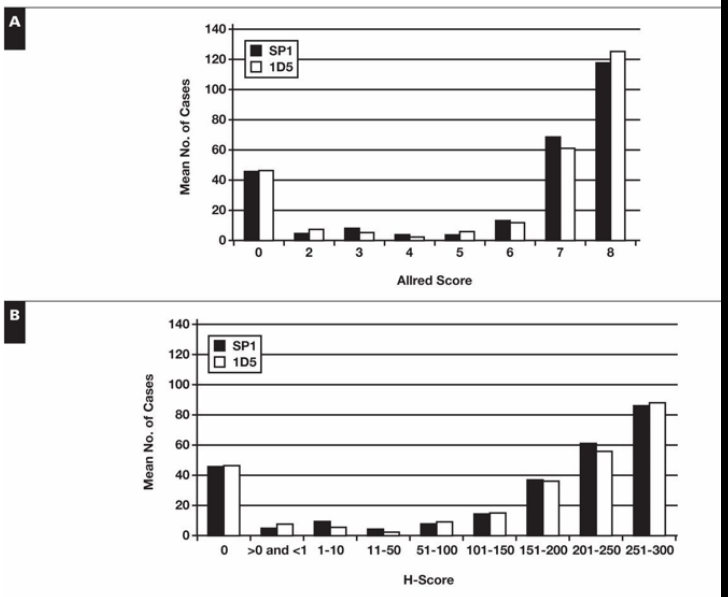
IHC – The Technical Test Approach

Classification	IHC result
Positive for ER or PgR	1% to 100% of tumor nuclei are positive for ER or PgR
Low ER-positive[a]	1% to 10% of tumor cell nuclei are immunoreactive
Negative for ER or PgR	<1% or 0% of tumor cell nuclei are immunoreactive
Uninterpretable for ER or PgR	Sample is inadequate, external and internal controls do not stain appropriately, or preanalytic variables interfere with the IHC assay's accuracy

ASCO/CAP 2020 classification

ER indicates estrogen receptor; IHC, immunohistochemistry, PgR, progesterone receptor.

[a]Only applies to reporting of ER, not PgR.



Am J Clin Pathol, Volume 140, Issue 4, October 2013, Pages 487–494, https://doi.org/10.1309/AJCP1RF9FUIZRDPI

cancers Cancers 2021, 13, 2262 MDPI

Guidelines

Breast-Gynaecological & Immuno-Oncology International Cancer Conference (BGICC) Consensus and Recommendations for the Management of Triple-Negative Breast Cancer

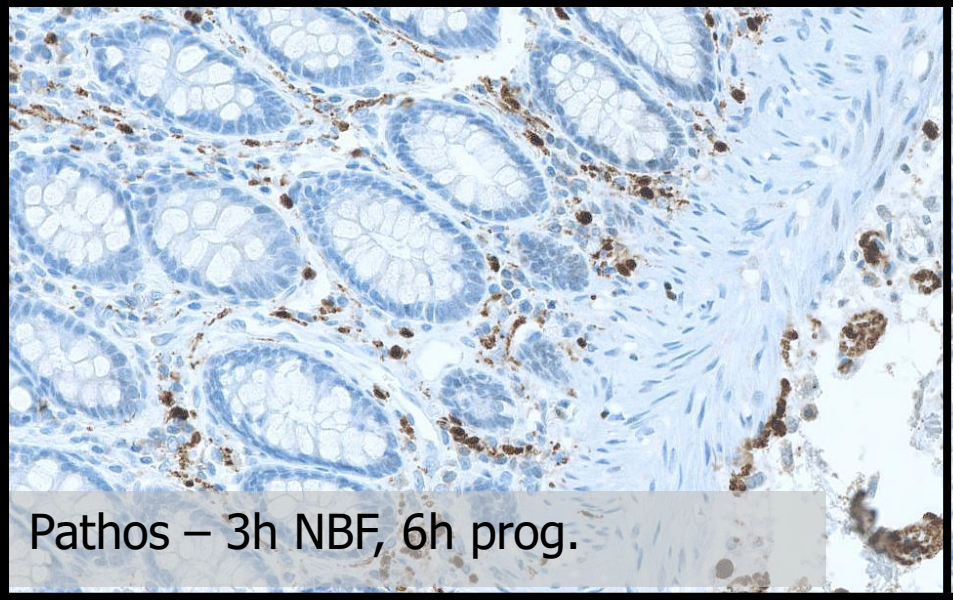
Hesham Elghazaly [1,*], Hope S. Rugo [2,*], Hamdy A. Azim [3], Sandra M. Swain [4], Banu Arun [5], Matti Aapro [6], Edith A. Perez [7], Benjamin O. Anderson [8], Frederique Penault-Llorca [9], Pierfranco Conte [10], Nagi S. El Saghir [11], Cheng-Har Yip [12], Marwan Ghosn [13], Philip Poortmans [14], Mohamed A. Shehata [15], Armando E. Giuliano [16], Jessica W. T. Leung [17], Valentina Guarneri [10], Joseph Gligorov [18], Bahadir M. Gulluoglu [19], Hany Abdel Aziz [1], Mona Frolova [20], Mohamed Sabry [1], Charles M. Balch [21], Roberto Orecchia [22], Heba M. El-Zawahry [3], Sana Al-Sukhun [23], Khaled Abdel Karim [1], Alaa Kandil [24], Ruslan M. Paltuev [25], Meteb Foheidi [26], Mohamed El-Shinawi [27,28], Manal ElMahdy [29], Omalkhair Abulkhair [30], Wentao Yang [31], Adel T. Aref [32], Joaira Bakkach [33], Nermean Bahie Eldin [1] and Hagar Elghazawy [1]

"Estrogen receptor low-positive tumours (ERLP), defined as 1–10% ER positivity by immunohisto-chemistry (IHC), are relatively uncommon, accounting for 2–6% of all BCs. Although the updated ASCO/CAP 2020 guidelines still consider these tumours as ER-positive tumours, they recommended classifying them differently as ERLP."

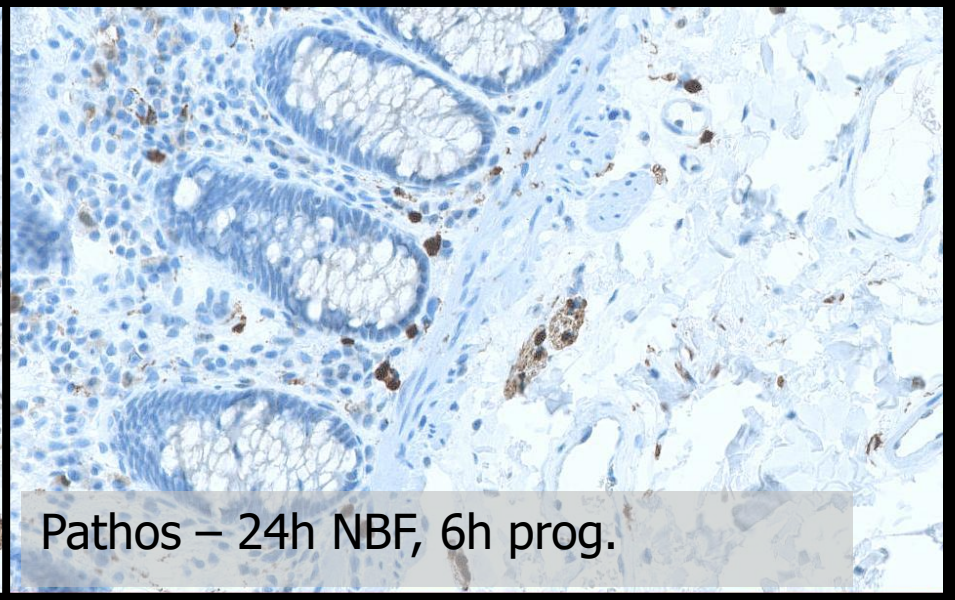
..the panel agreed that these tumours would be treated clinically as TNBC, being not eligible to receive endocrine therapy as a monotherapy."

IHC – The Technical Test Approach

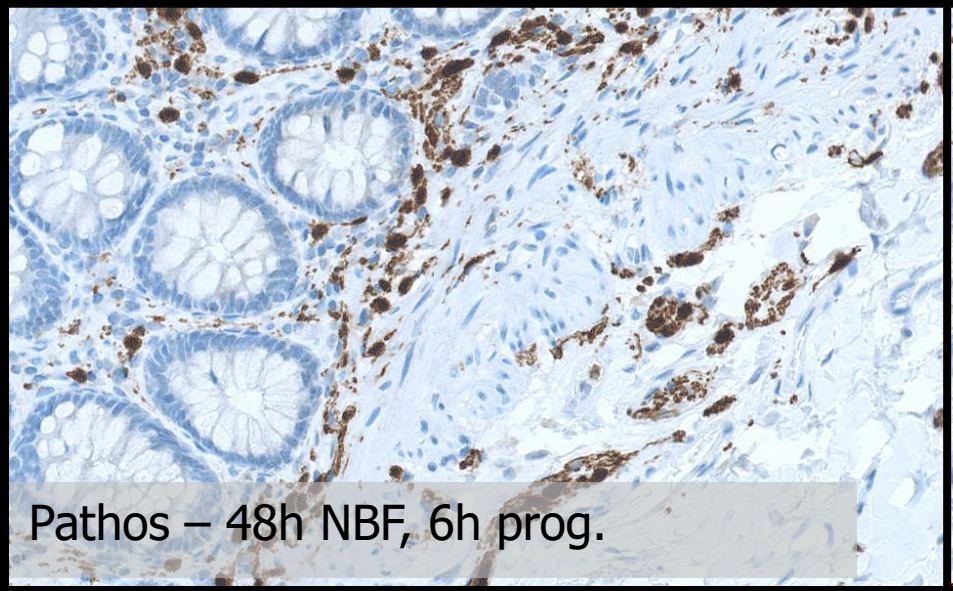
Colon: S100, polyclonal



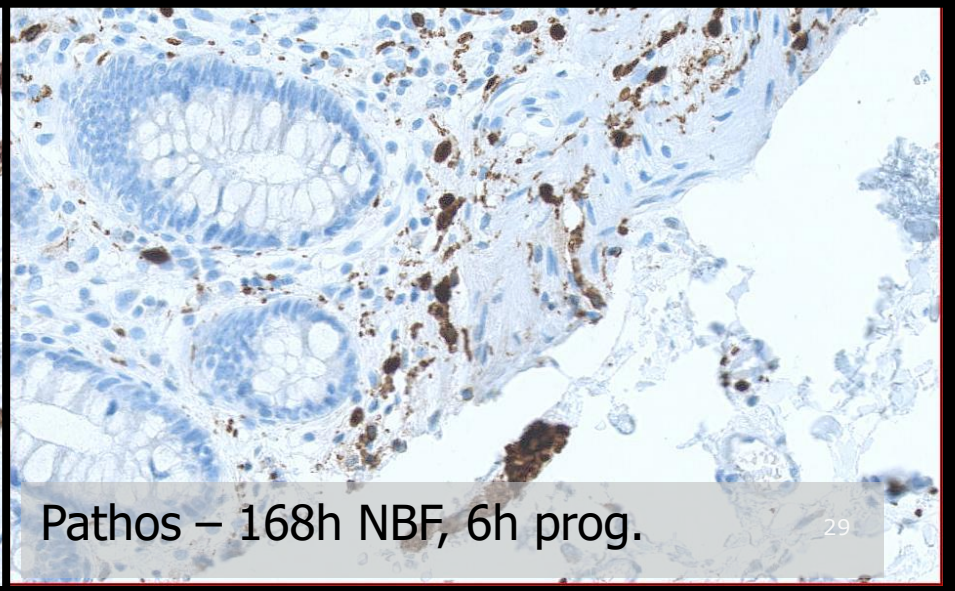
Pathos – 3h NBF, 6h prog.



Pathos – 24h NBF, 6h prog.



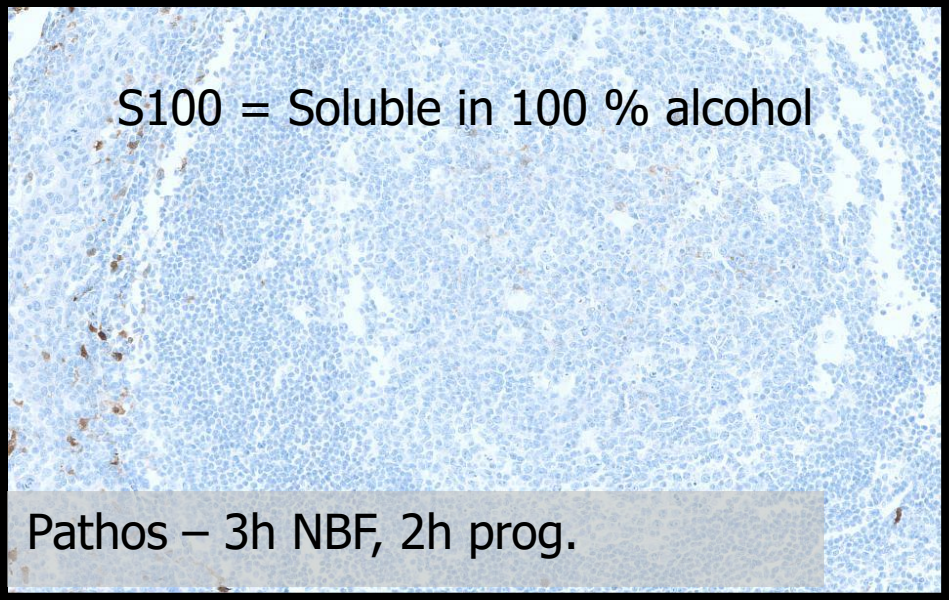
Pathos – 48h NBF, 6h prog.



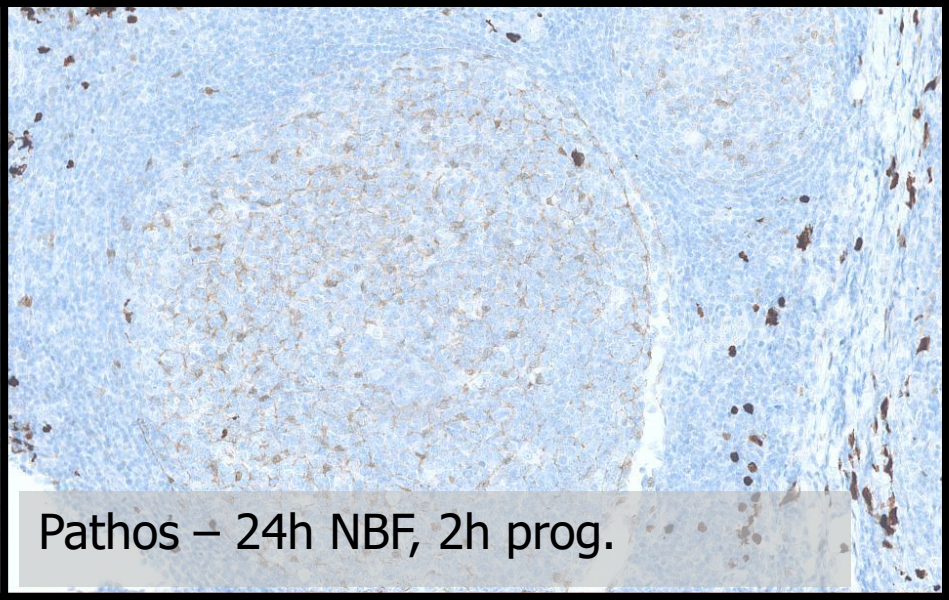
Pathos – 168h NBF, 6h prog.

IHC – The Technical Test Approach

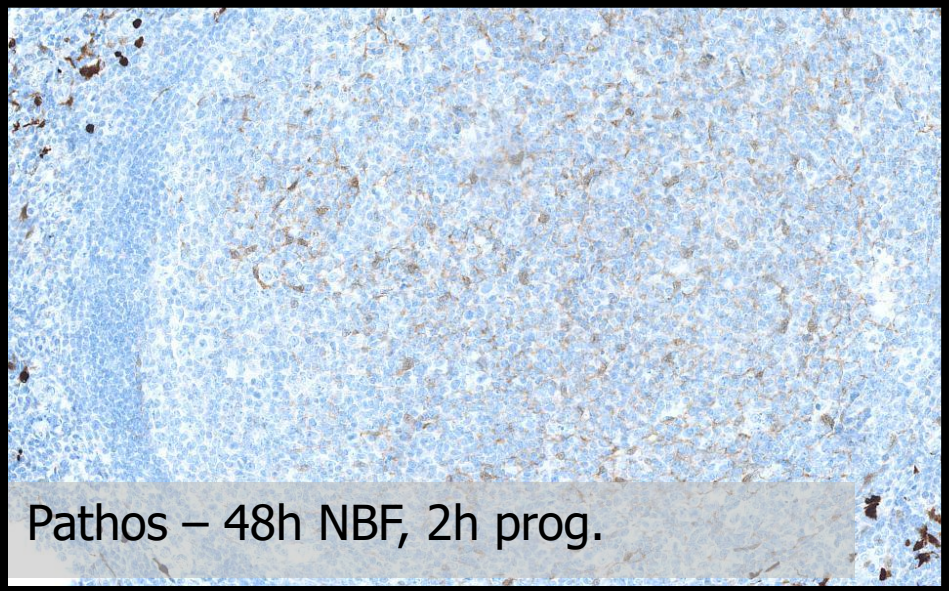
Tonsil: S100, polyclonal



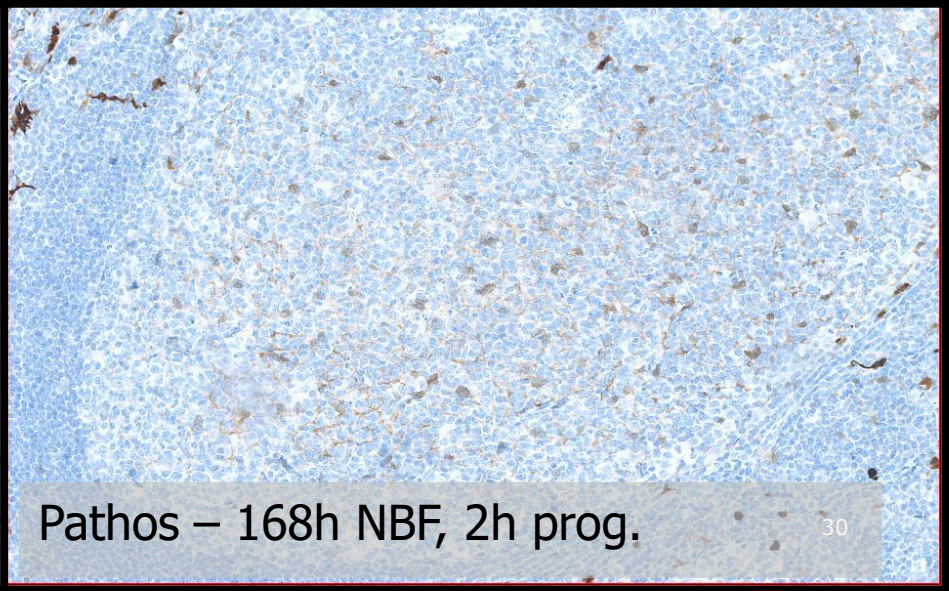
Pathos – 3h NBF, 2h prog.



Pathos – 24h NBF, 2h prog.



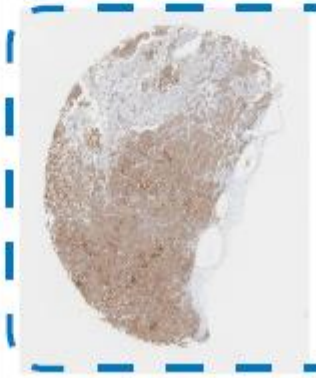
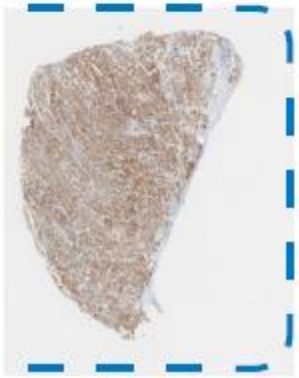
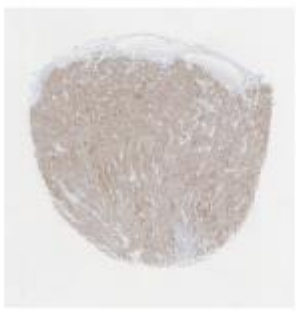
Pathos – 48h NBF, 2h prog.



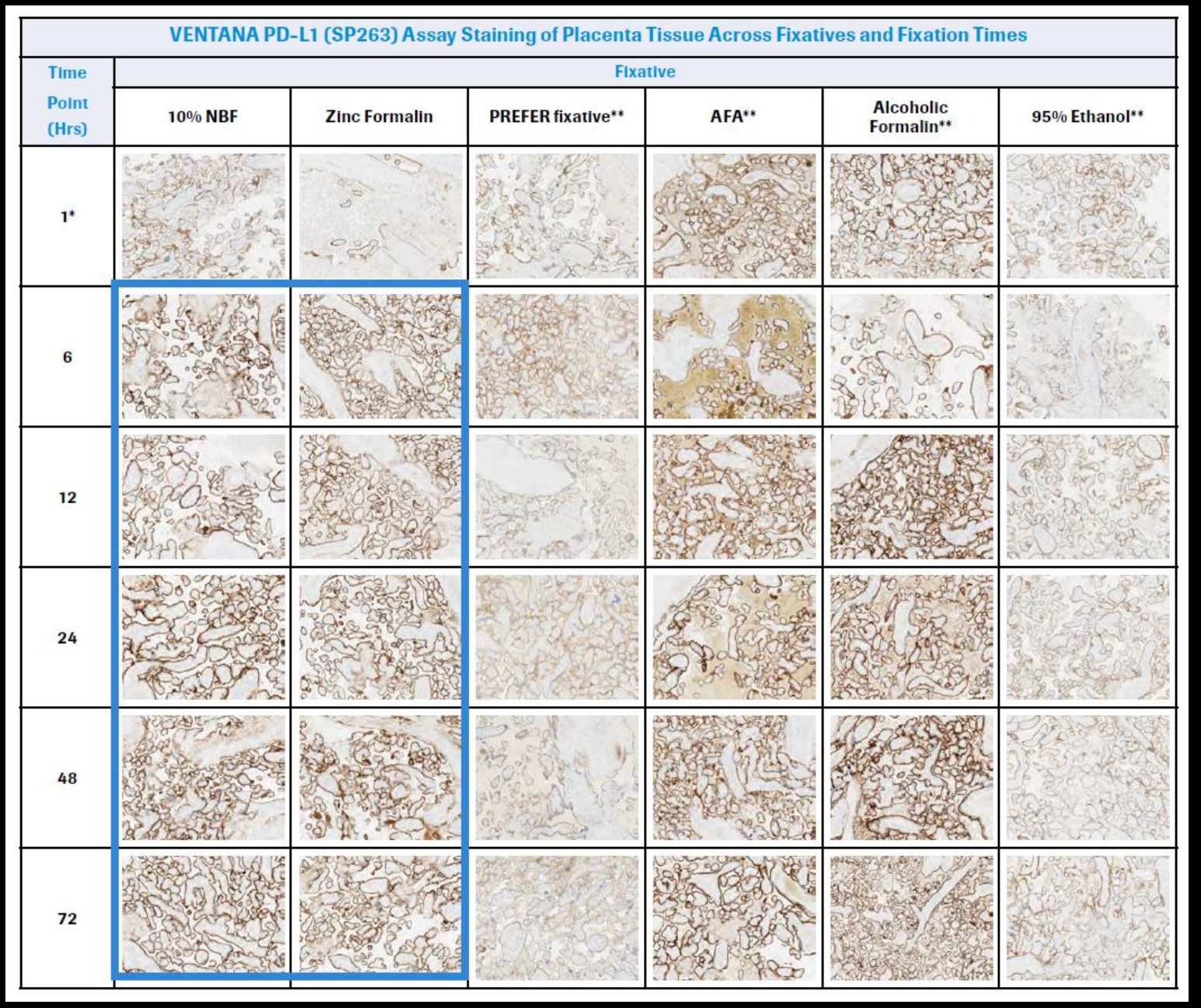
Pathos – 168h NBF, 2h prog.

IHC – The Technical Test Approach

Examples of the Impact of Fixation Conditions with VENTANA ALK (D5F3) CDx Assay

Fixation Time (Hours)	Fixative			
	10% NBF	Zinc Formalin	AFA	95% Ethanol
1				
12				

IHC – The Technical Test Approach



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*†*

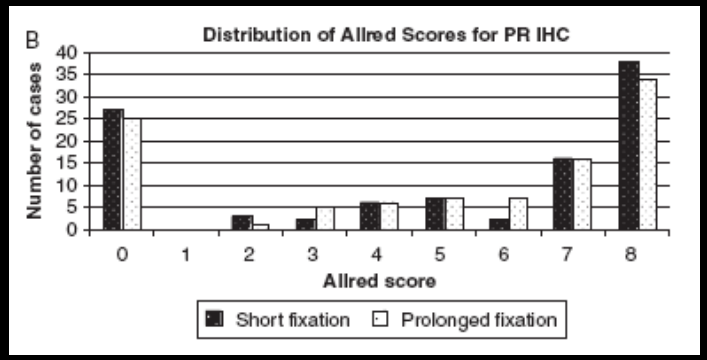
13 hours versus 79 hours in 10% NBF (the week-end dilemma…..)

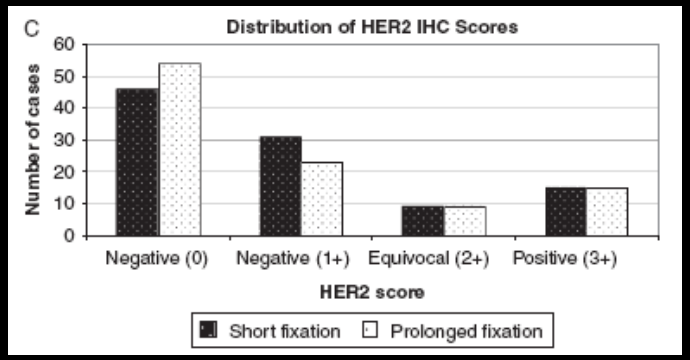
101 breast carcinomas:

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

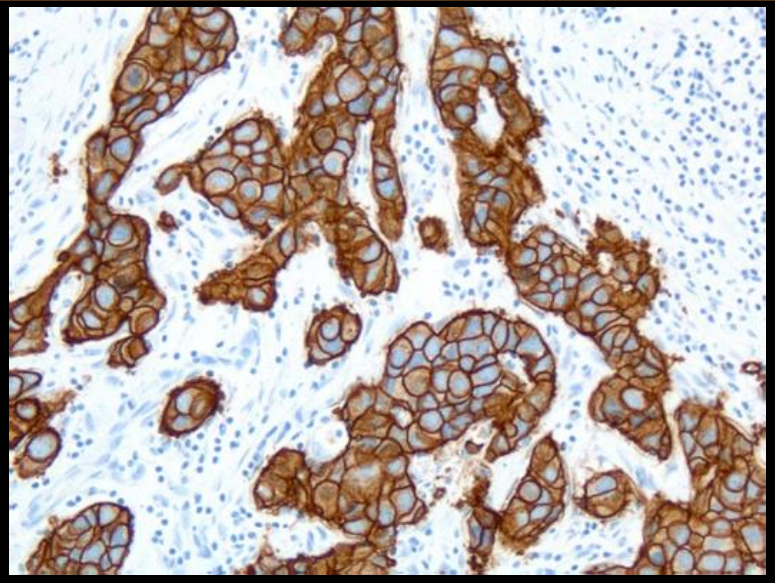
Internal IHC validation	4 h. NBF	24 h. NBF	48 h. NBF	168 h. NBF
Tumour 1	1+	1+	1+	1+
Tumour 2	3+	3+	3+	3+
Tumour 3	0	0	0	0
Tumour 4	1+	1+	1+	1+
Tumour 5	0	0	0	0
Tumour 6	3+	3+	3+	3+
Tumour 7	0	0	0	0
Tumour 8	0	0	0	0
Tumour 9	0	0	0	0

Breast carcinomas, HER-2 PATHWAY, rmAb 4B5

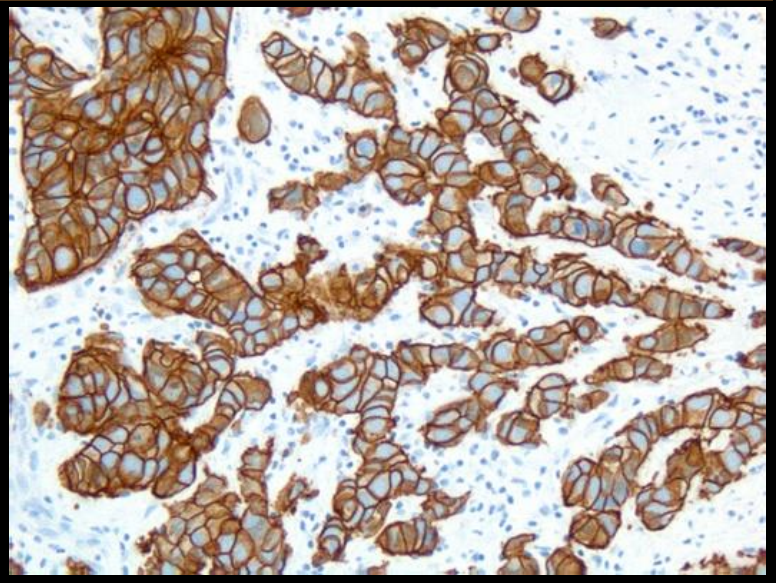
(CC1 Mild, Ab inc. 20 min. 36°C, UltraView DAB)

IHC – The Technical Test Approach

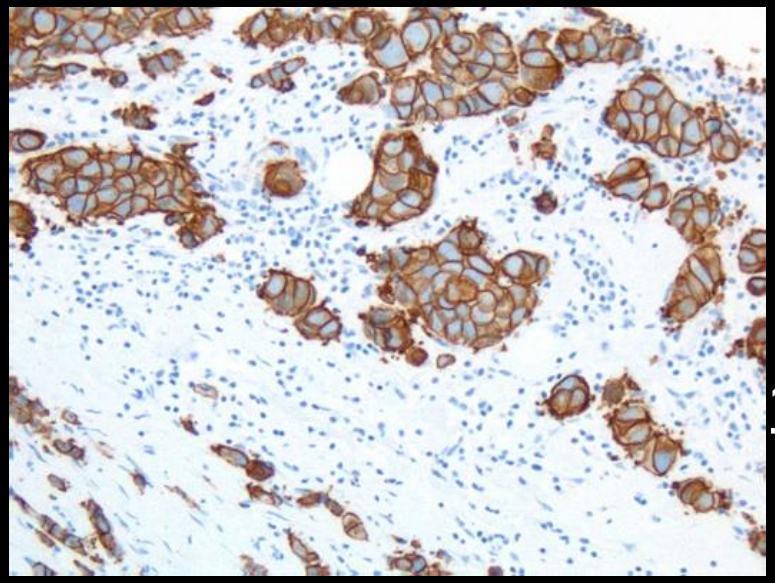
4 h



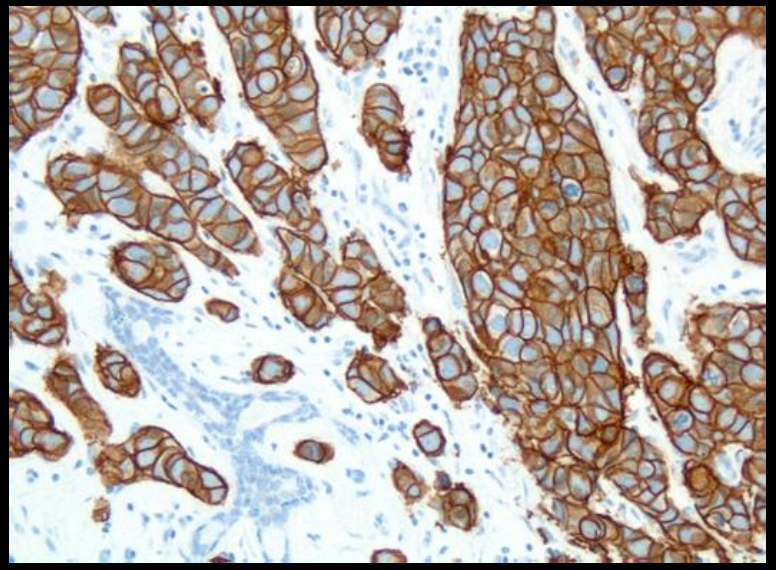
24 h



48 h



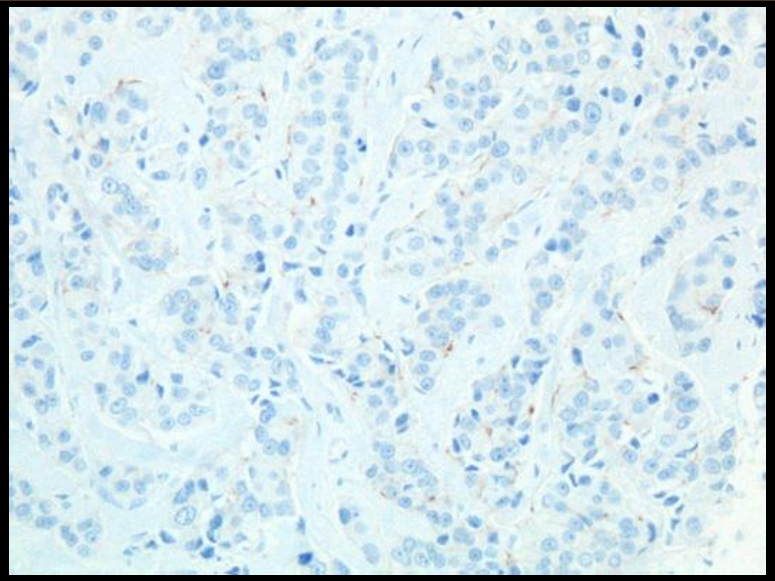
168 h



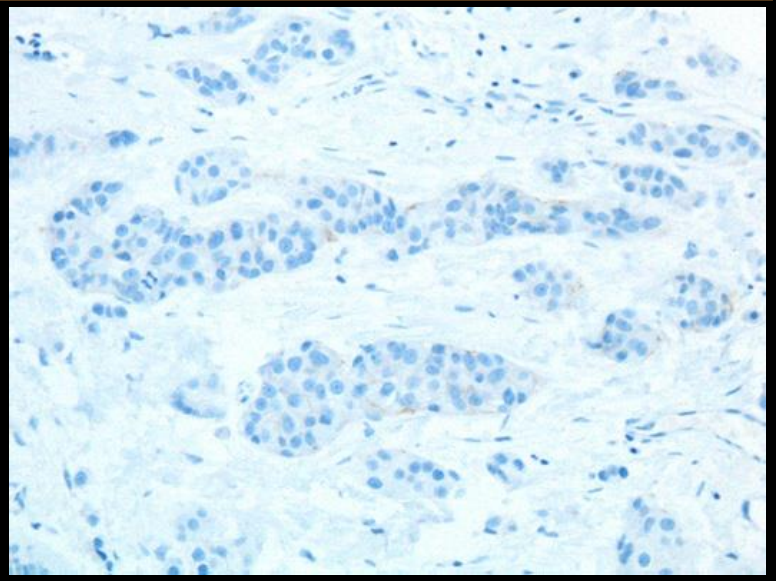
Breast carcinoma 3+, HER-2 PATHWAY, rmAb 4B5

IHC – The Technical Test Approach

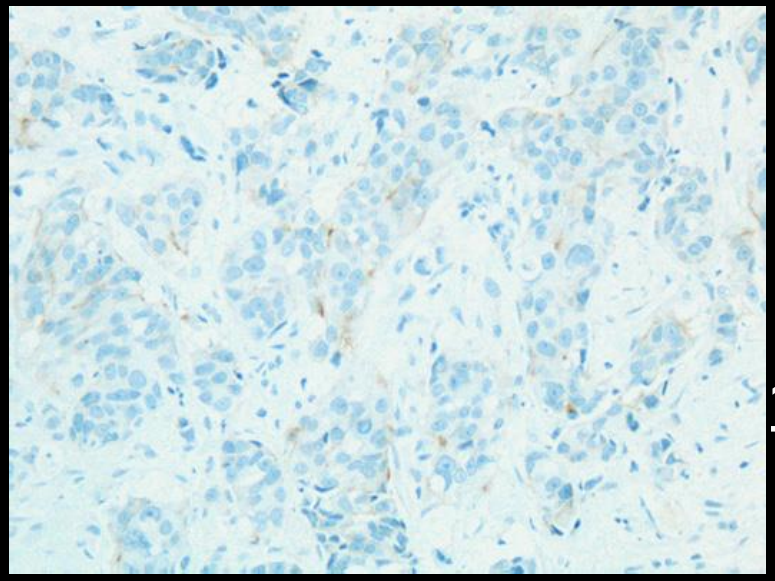
4 h



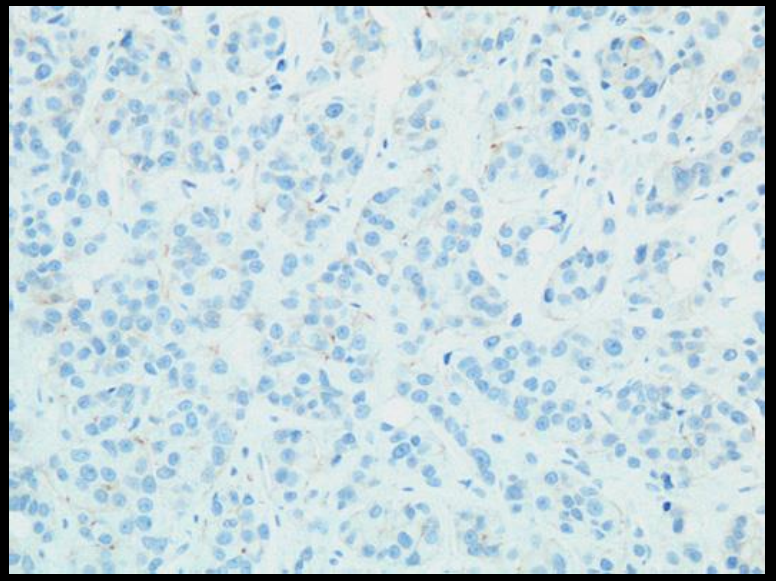
24 h



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

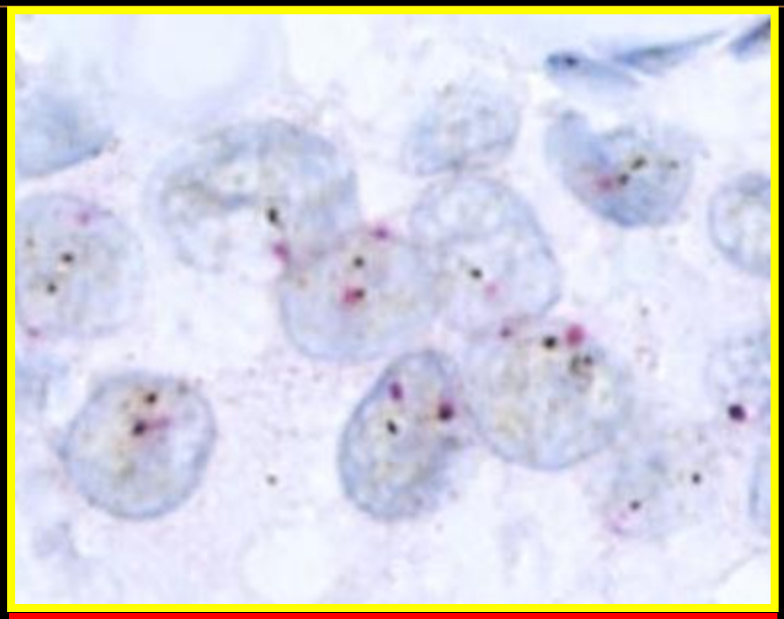
Internal SISH validation	4 h. NBF	24 h. NBF	48 h. NBF	168 h. NBF
Tumour 1	-	-	-	FN
Tumour 2 Amp	+	+	+	+
Tumour 3	(?)	-	FN	FN
Tumour 4	-	-	FN	FN
Tumour 5	-	-	-	-
Tumour 6 Amp	+	+	+	+
Tumour 7	-	-	-	FN
Tumour 8 poly.	-	-	-	FN
Tumour 9 poly.	-	-	-	FN

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

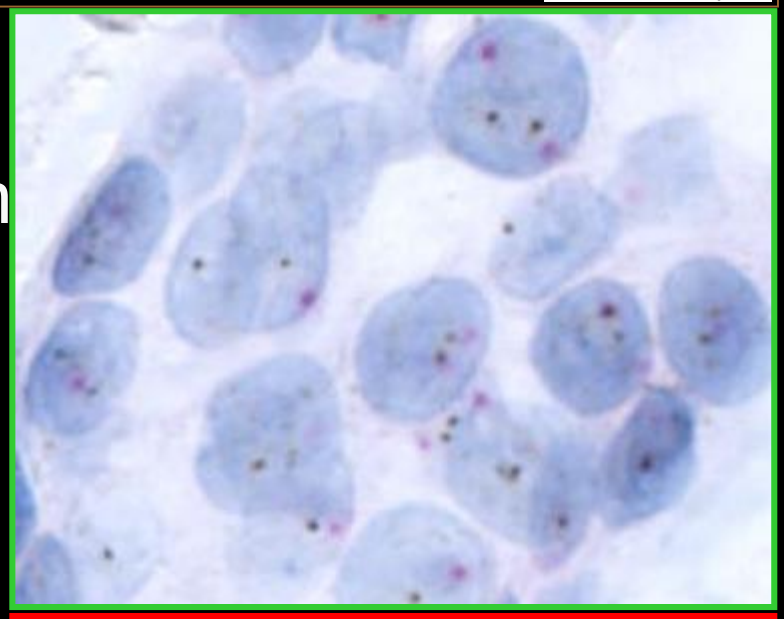
Breast carcinomas, Dual SISH CCrb ext, P3. 8 m

IHC – The Technical Test Approach

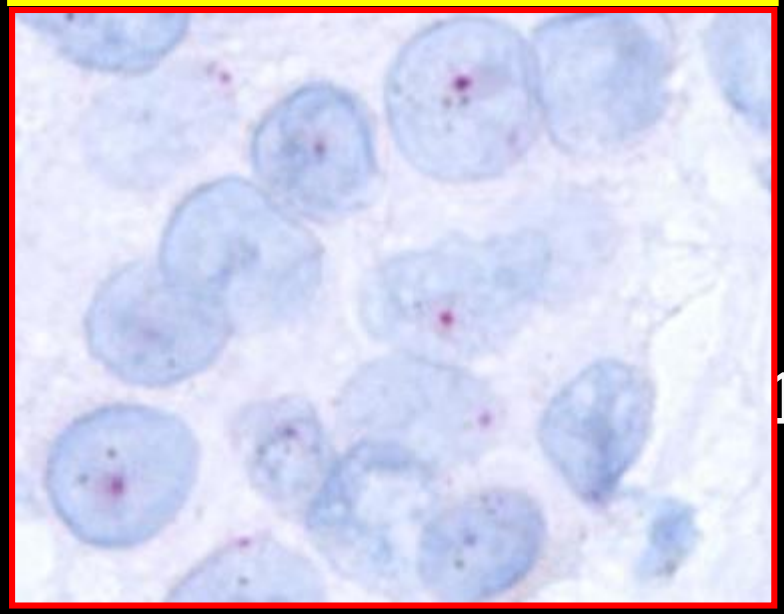
4 h



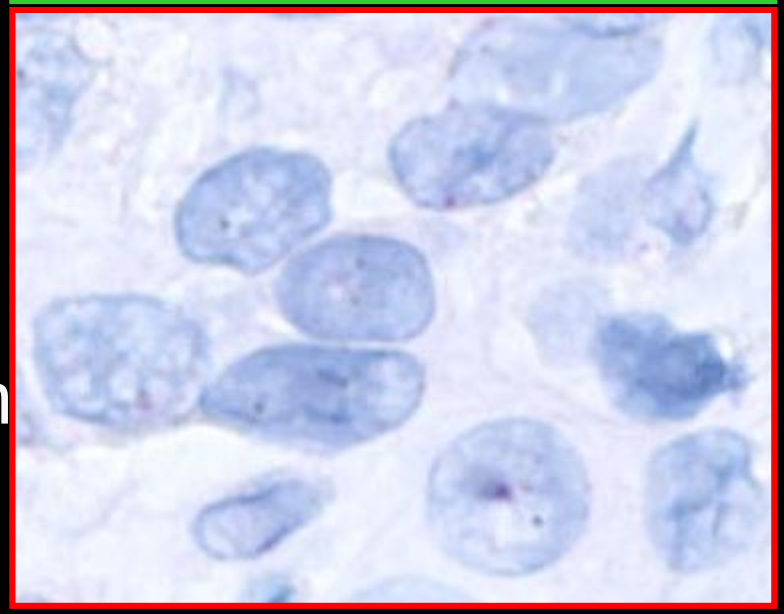
24 h



48 h



168 h



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

IHC – The Technical Test Approach

1. Cold ischemia time (time from removal to fixative)
2. Method of processing (section thickness, temperature, fixative volume to tissue mass ratio)
3. Type and quality of fixative
4. Total time in formalin
5. Storage conditions (blocks and cut slides)

IHC – Alternatives to 10% 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

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

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

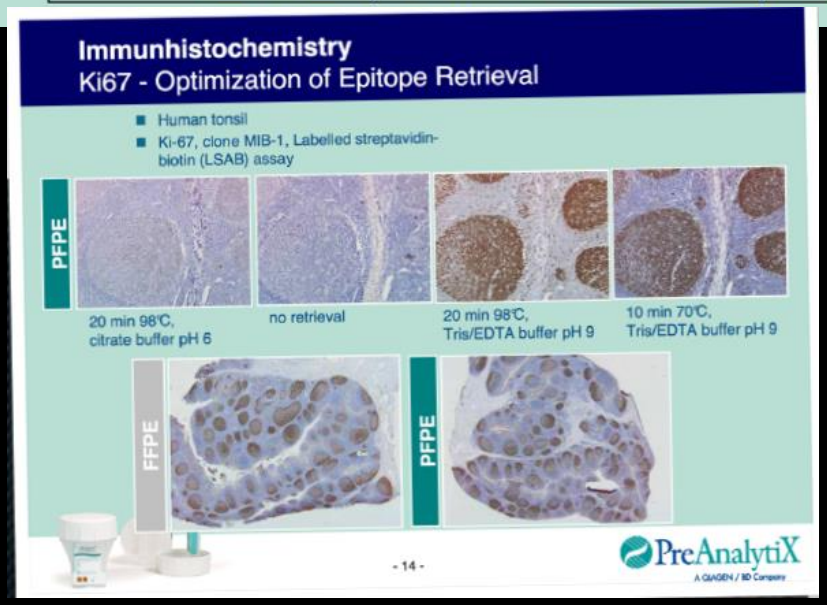
PreAnalytiX
A QIAGEN / BD Company

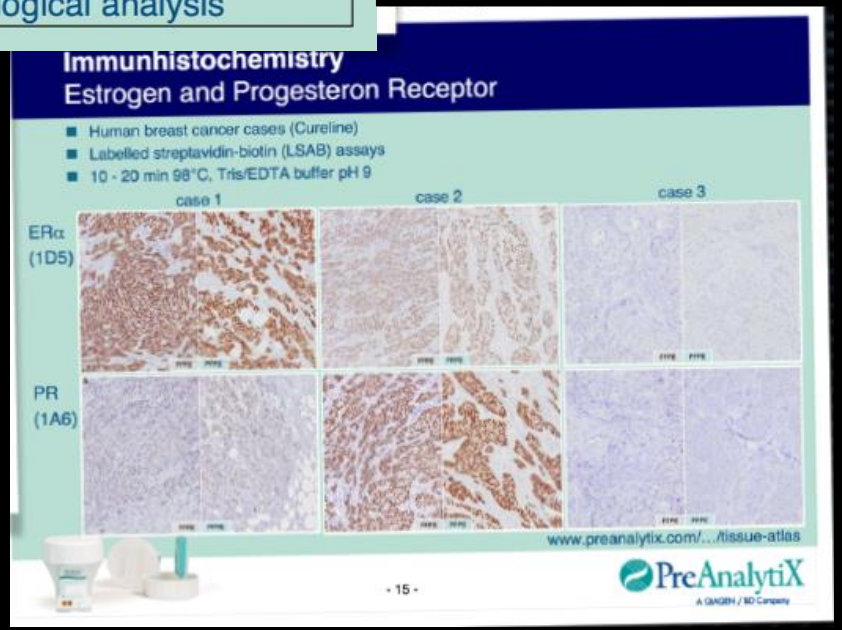
IHC – The Technical Test Approach

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





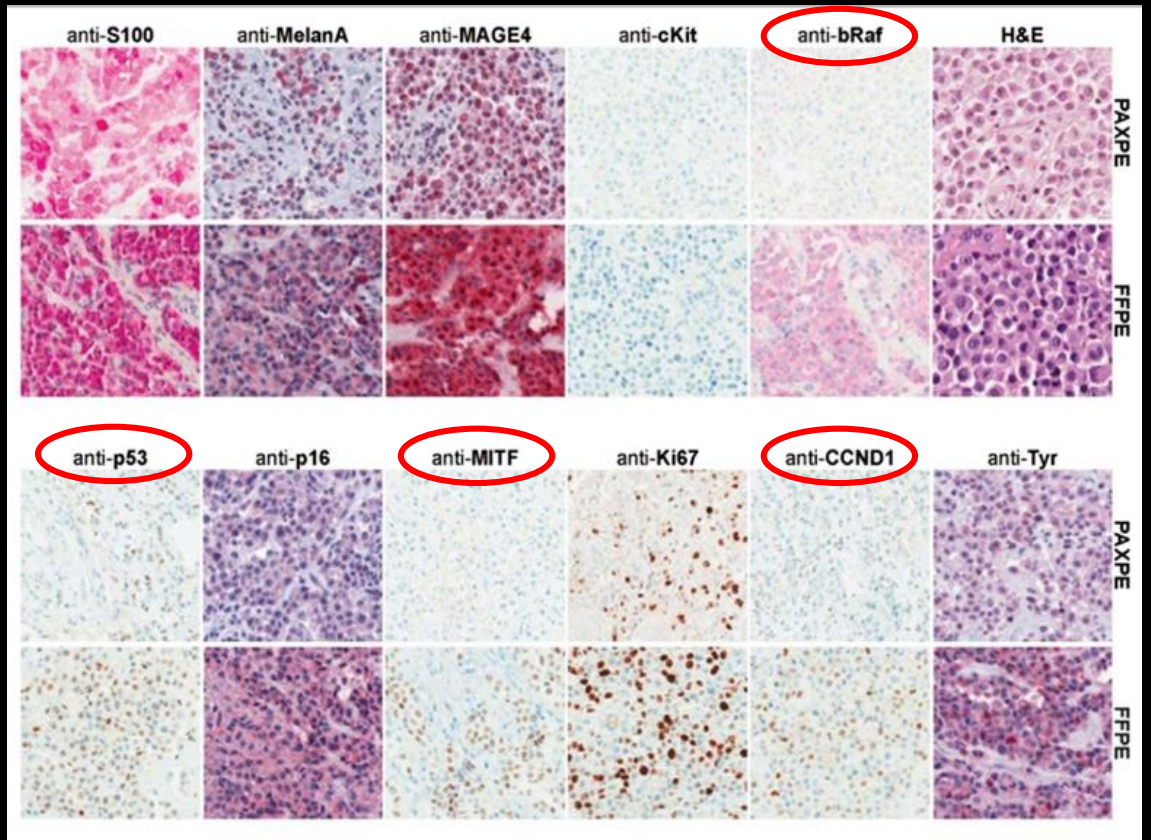
IHC – The Technical Test Approach

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]

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	Protease 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

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

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

Change of fixation time / fixative:

1. Use present standard fixative and time ranges as reference

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

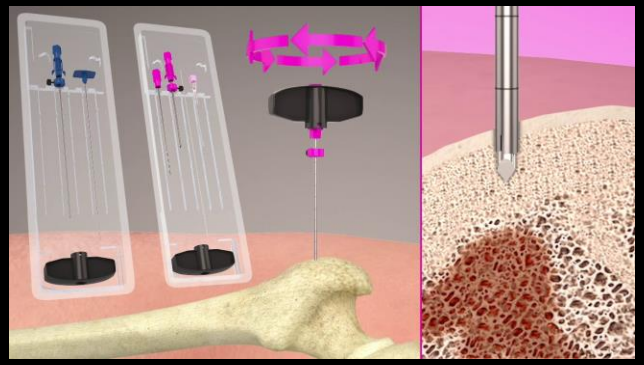
3. Evaluate all different methods applied as diagnostic tools – IHC / ISH / PCR / NGS

IHC – The Technical Test Approach

Decalcification and impact on IHC;

Types

- Strong acid – HCL
- Mild acid – Formic acid
- Chelating agent - EDTA



Time, Temperature

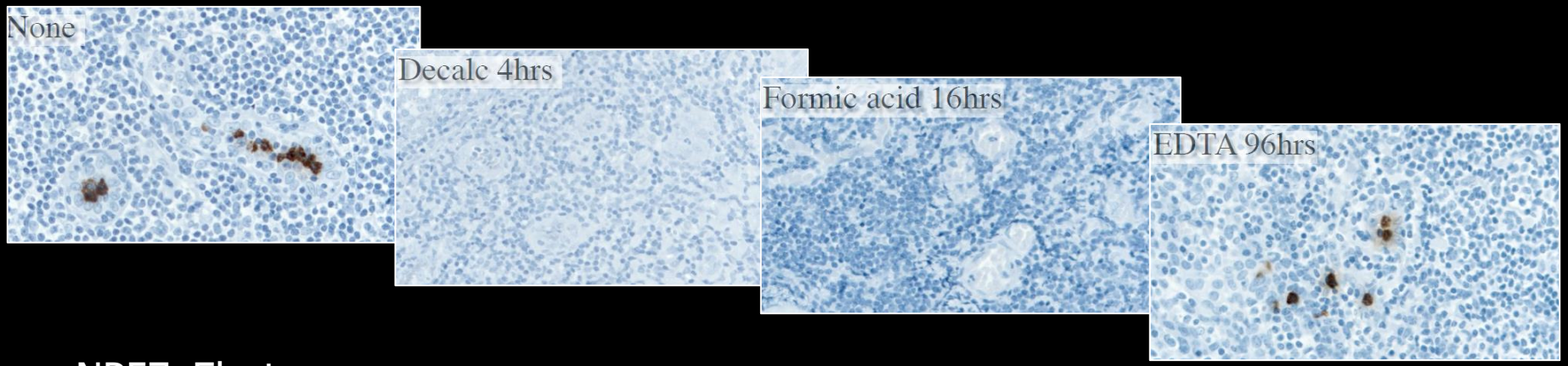
Time in NBF before decalcification

IHC – The Technical Test Approach

193 Abs. Fixed for 24 h in 10% NBF

Intensity / Method	0/+	++	++(+)	+++	++++
Decalc (HCL)	159	23	1	8	2
Formic acid*	1	15	8	163	6
10% EDTA**	0	0	5	185	3

* 4M formic acid + 0,5M sodium formiate, ** pH 7



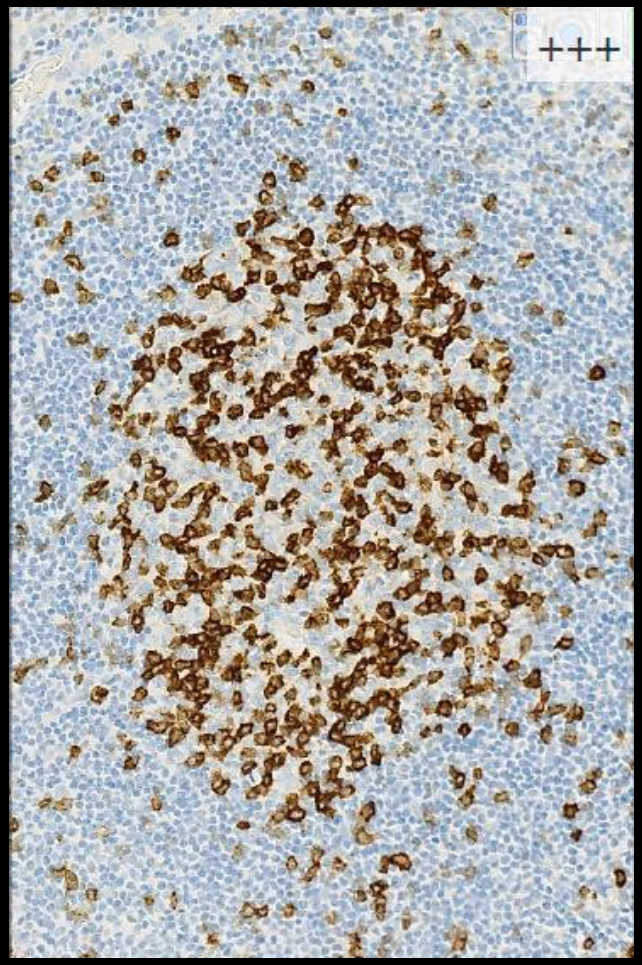
NP57, Elastase

Data; Ole Nielsen, OUH, Denmark

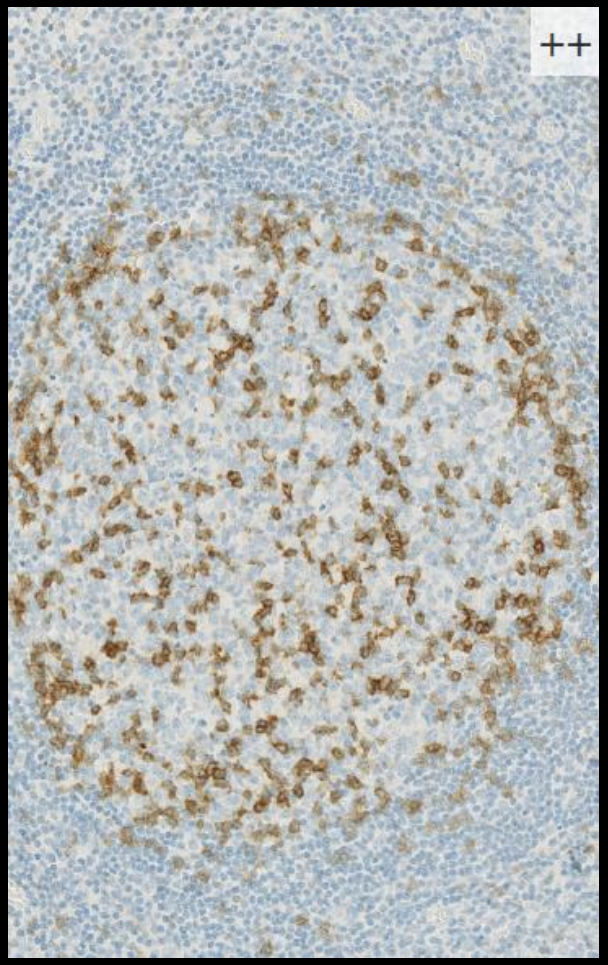
IHC – The Technical Test Approach

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

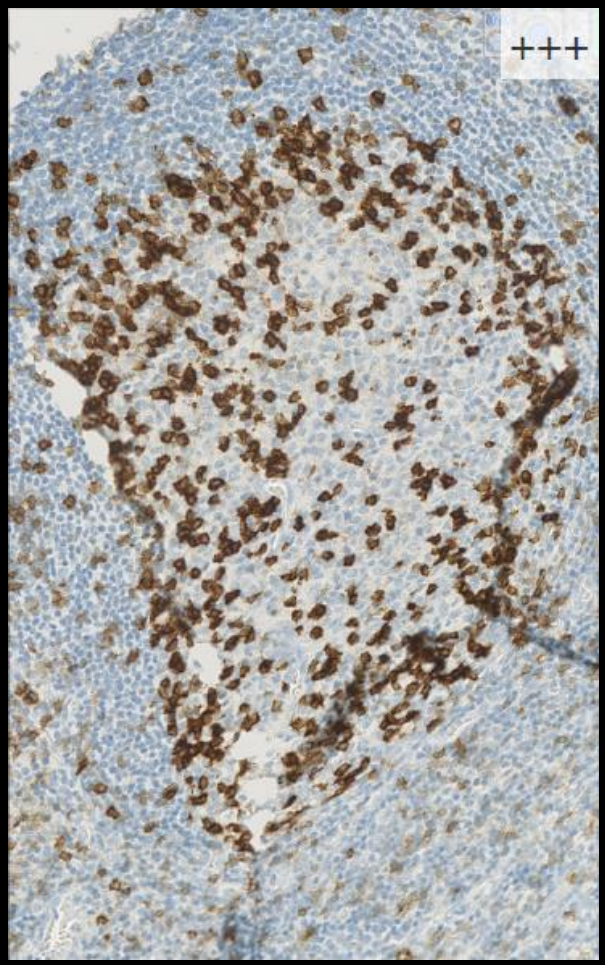
IHC – The Technical Test Approach



No declacification



Formic acid

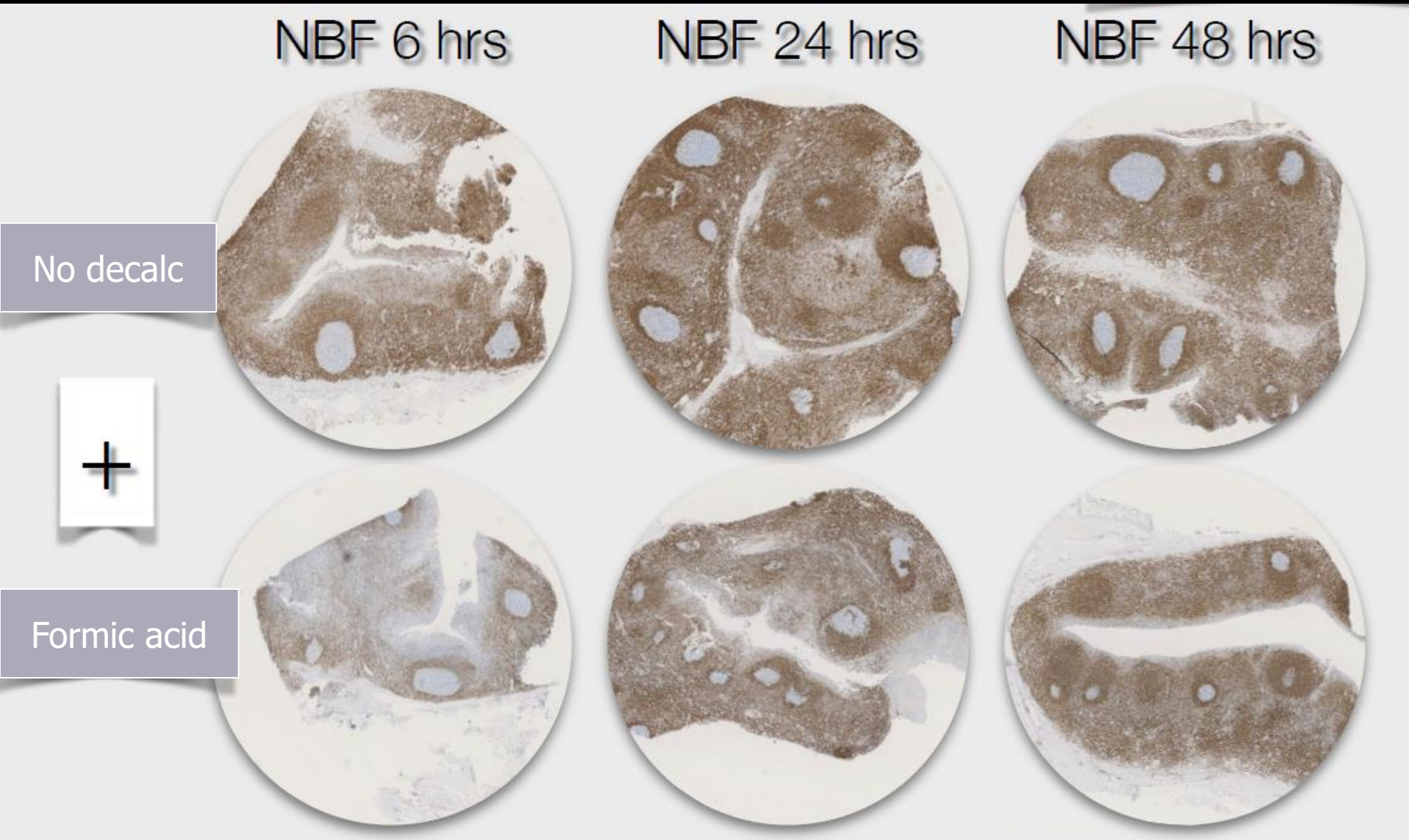


10 % EDTA

PD-1 (CD279) – mAb clone NAT105

IHC – The Technical Test Approach

Fixation time and decalcification in Formic acid; mAb clone 124, Bcl2



IHC – The Technical Test Approach

1. Cold ischemia time (time from removal to fixative)
2. Method of processing (section thickness, temperature, fixative volume to tissue mass ratio)
3. Type and quality of fixative
4. Total time in formalin
5. Storage conditions (blocks and cut slides)

IHC – The Technical Test Approach

Antigen stability in cut sections and blocks – what is up and down…???

Decline in Antigenicity of Tumor Markers by Storage Time Using Pathology Sections Cut From Tissue Microarrays

Fiona M. Blows, MSc, Hamid R. Ali, PhD,†‡ Sarah-J. Dawson, PhD,* John Le Quesne, PhD,§ Elena Provenzano, MB,‖ Carlos Caldas, MD,* and Paul D.P. Pharoah, PhD*¶*

Appl Immunohistochem Mol Morphol 2016;24:221–226

"Biomarker antigenicity shows a small decline over time that is unlikely to have an important effect on studies of prognostic biomarkers".

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

Modern Pathology (2004) 17, 1414–1420

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

Institute for Pathology, University of Basel, Basel, Switzerland

"In summary, the data of this study confirm a major impact of the age of tissue sections on the outcome of IHC analyses".

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 | Volume 96 March 2016

"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".

IHC – The Technical Test Approach

Modern Pathology (2004) 17, 1414–1420

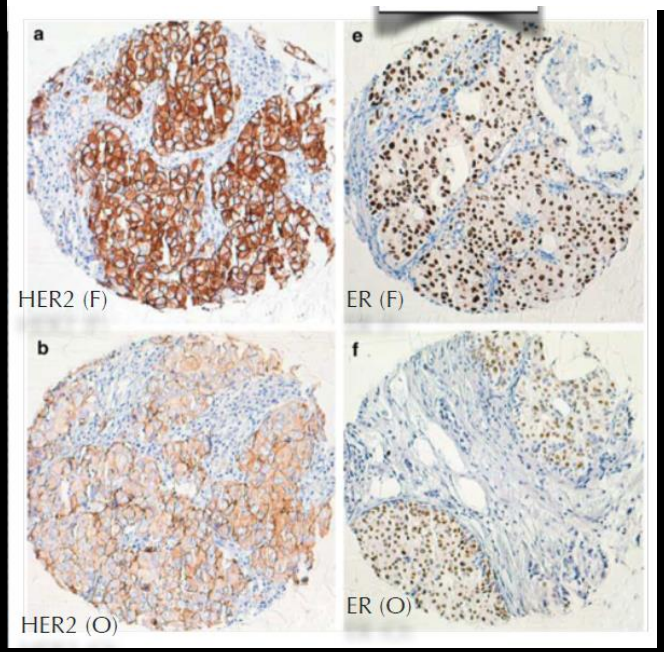
www.modernpathology.org

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

Institute for Pathology, University of Basel, Basel, Switzerland

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



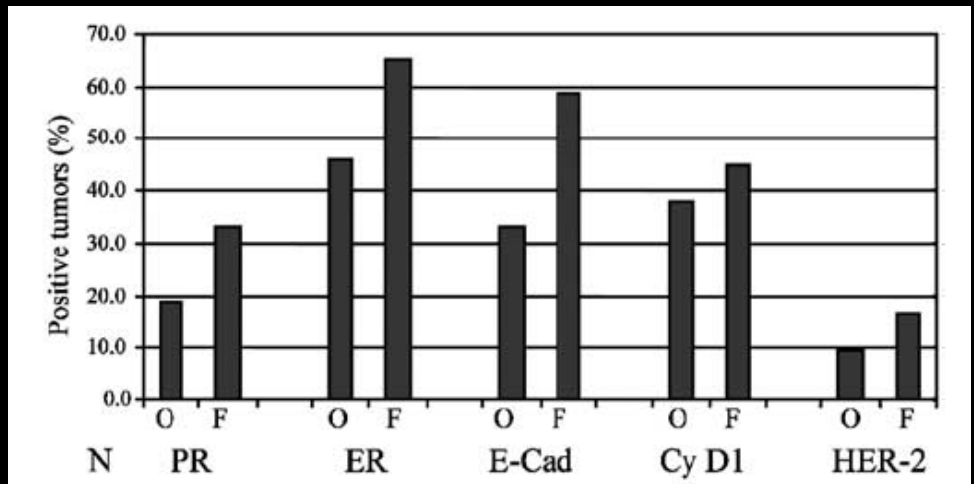


Figure 2 Influence of slide aging on the fraction of positive cases. For each antibody, the frequency of positive cases is shown as separate bars for old (O) and fresh (F) sections.

IHC – The Technical Test Approach

Factors influencing antigen preservation in cut sections;

Time

Temperature

Water amount in slide

Moist / humidity in room

Light

All with negative effects

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

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

IHC – The Technical Test Approach

Is there an expiry date for tissue blocks…???

We use archive tissue for the entire IHC lifecycle…

Development
Validation
QC

&

CLINICAL DIAGNOSTICS

IHC – The Technical Test Approach

Is there an expiry date for tissue blocks…???

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 | Volume 96 March 2016

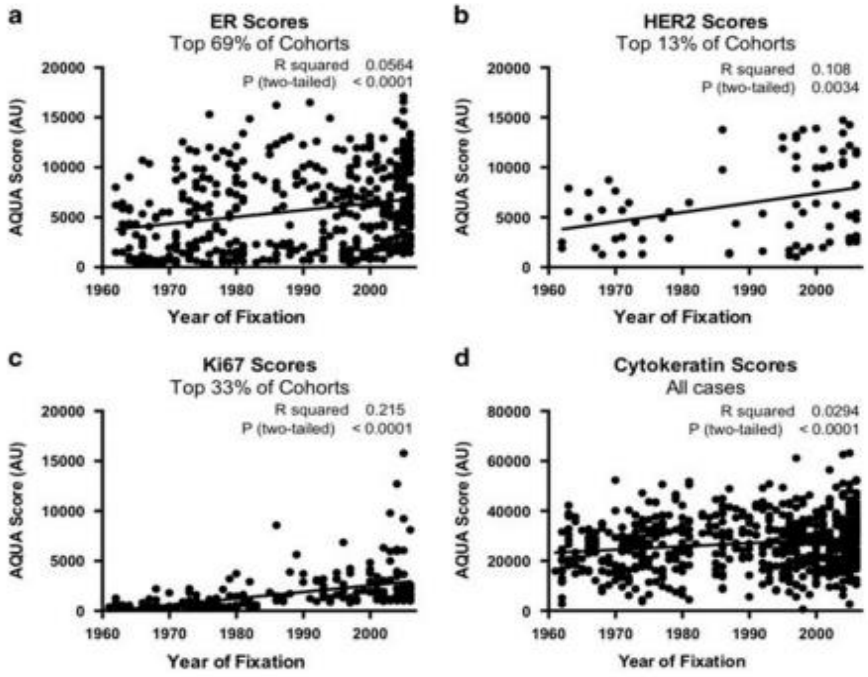


Figure 2 The distribution of scores for each biomarker as a function of tissue age after omitting the fraction of expected negative cases. (**a**) ER, (**b**) HER2, (**c**) Ki67 and (**d**) cytokeratin. The fraction of positive cases is shown by percentage beneath the biomarker in the title. The regression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

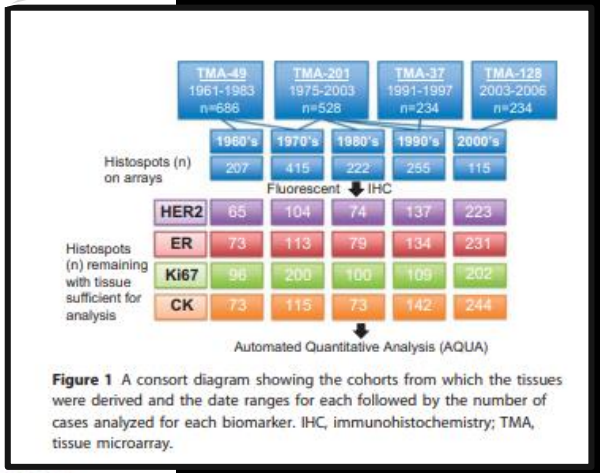


Figure 1 A consort diagram showing the cohorts from which the tissues were derived and the date ranges for each followed by the number of cases analyzed for each biomarker. IHC, immunohistochemistry; TMA, tissue microarray.

IHC – The Technical Test Approach

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

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

Yan Jin[1,2], Xuxia Shen[1,2], Yunjian Pan[2,3], Qiang Zheng[1,2], Haiquan Chen[2,3], Hong Hu[2,3#], Yuan Li[1,2#]

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

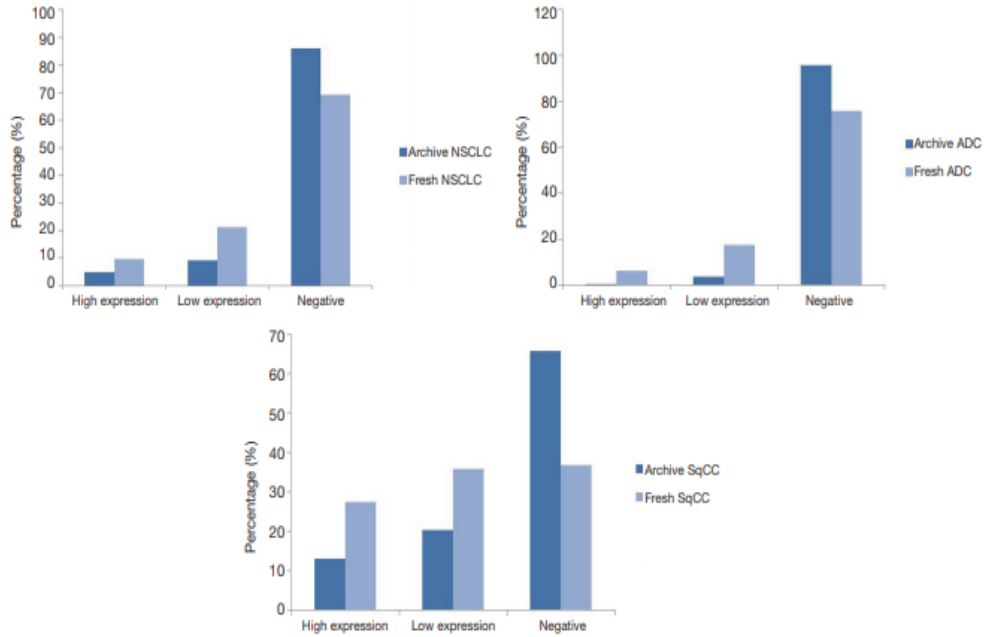
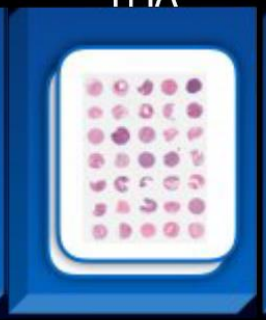
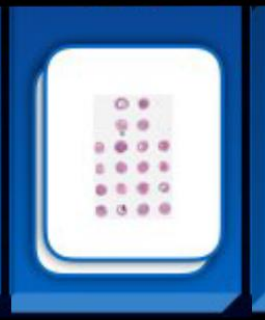
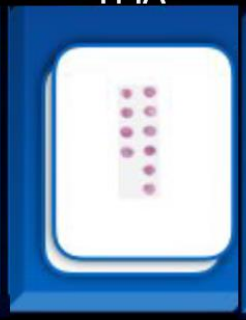


Figure 2 Comparison of PD-L1 expression in recently acquired samples and archived NSCLC samples. PD-L1, programmed death ligand-1; NSCLC, non-small cell lung cancer; ADC, adenocarcinoma; SqCC, squamous cell carcinoma.

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

IHC – The Technical Test Approach

External tissue control tool-box:

Calibration TMA's			Analytical "Validation" TMA's		Lab QC TMA
iCAPC TMA	Specificity TMA	Pre-analyt. TMA	Accuracy TMA	Index TMA	"Daily QC" TMA
					
"Gold standard" tissue controls	"Normal" tissues	iCAPCs processed as lab procedures	"Lesional" tissues	"Lesional" tissues	iCAPCs + selected tissues

Take home message

No general problem to use archive tissue for most IHC markers and different purposes in the lifecycle of IHC.

However both for diagnostic purposes and IHC development blocks < 3-5 years preferable.

TMA's and QC blocks might show reduced expression overtime.

IHC – The Technical Test Approach

1. Cold ischemia time (time from removal to fixative)
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IHC – The Technical Test Approach

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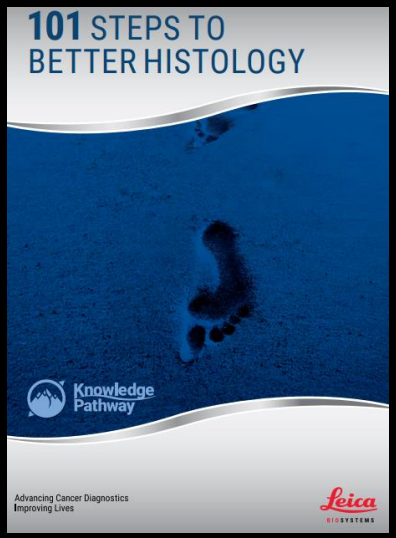
5. Storage conditions (blocks and cut slides)

IHC – The Technical Test Approach

Not much data on tissue processing impact on IHC…… !!!!!



General best practice recommendations;

- Tissue to formalin ratio; 1:10
- Tissue thickness of max 3-4 mm
- Use quality products for etanol, clearing and paraffin
- Maintain processor and exchange reagents on regular basis
- Avoid overloading tissue in cassettes
- Avoid overloading of cassettes in tissue processor containers

https://www.leicabiosystems.com/knowledge-pathway/an-introduction-to-specimen-processing/

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Appl Immunohistochem Mol Morphol • Volume 21, Number 4, July 2013

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

HE
ER, PR, Ki67 &
HER2 IHC/ISH

A total of 233 consecutive needle core breast biopsy specimens were included in this study.
The fixation time was strictly standardized, ranging from 18 to 24h.
After fixation, half of the core specimens from each case were randomly assigned to the Leica ASP 300S conventional processor (a total of 14 hours) and the other half in the Sakura Tissue-Tek Xpress 120 (1 h program).

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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.

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

TABLE 4. Progesterone Receptor Status in the Conventionally Processed and in the Matched MW-assisted Processed NCBs of Breast Carcinomas

	PR Status (Conventional)		
PR Status (MW)	**Positive**	**Negative**	**Total**
Positive	48	0	48
Negative	0	31	31
Total	48	31	79

TABLE 5. Ki-67–Labeling Index in the Conventionally Processed and in the Matched MW-assisted Processed NCBs of Breast Carcinomas

	Ki-67–Labeling Index (Conventional)		
Ki-67–Labeling Index (MW)	**Low**	**High**	**Total**
Low	36	3	39
High	0	31	31
Total	36	34	70

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

HE
ER, PR, Ki67 &
HER2 IHC/ISH

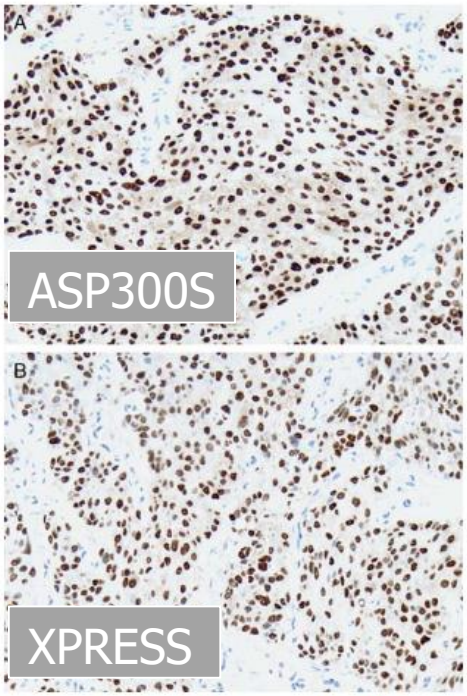

FIGURE 2. Needle core biopsy: invasive ductal carcinoma. Immunohistochemical reaction for estrogen receptor in the nuclei of tumor cells. The reaction is the same in the specimens prepared using the conventional processing method (A) and the microwave-assisted processing method (B) (A and B, immunoperoxydase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

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Understanding Preanalytical Variables and their Effects on Clinical Biomarkers of Oncology and Immunotherapy

Lokesh Agrawal[1], **Kelly B. Engel**[1], **Sarah R. Greytak**[1], and **Helen M. Moore**[1,*]

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A Review of Preanalytical Factors Affecting Molecular, Protein, and Morphological Analysis of Formalin-Fixed, Paraffin-Embedded (FFPE) Tissue

How Well Do You Know Your FFPE Specimen?

B. Paige Bass, PhD; Kelly B. Engel, PhD; Sarah R. Greytak, PhD; Helen M. Moore, PhD

• *Context.*—Formalin fixation and paraffin embedding is a timeless, cost-efficient, and widely adopted method of preserving human tissue biospecimens that has resulted in a substantial reservoir of formalin-fixed, paraffin-embedded blocks that represent both the pathology and preanalytical handling of the biospecimen. This reservoir of specimens is increasingly being used for DNA, RNA, and proteomic analyses.

Objective.—To evaluate the impact of preanalytical factors associated with the formalin fixation and paraffin embedding process on downstream morphological and molecular endpoints.

Data Sources.—We surveyed the existing literature using the National Cancer Institute's Biospecimen Research Database for published reports investigating the potential influence of preanalytical factors associated with the formalin fixation and paraffin embedding process on DNA, RNA, protein, and morphological endpoints.

Conclusions.—Based on the literature evidence, the molecular, proteomic, and morphological endpoints can be altered in formalin-fixed, paraffin-embedded specimens by suboptimal processing conditions. While the direction and magnitude of effects associated with a given preanalytical factor were dependent on the analyte (DNA, RNA, protein, and morphology) and analytical platform, acceptable conditions are highlighted, and a summary of conditions that could preclude analysis is provided.

(*Arch Pathol Lab Med.* 2014;138:1520–1530; doi: 10.5858/arpa.2013-0691-RA)

IHC
FISH
PCR
NGS

Meta analysis –
252 publications

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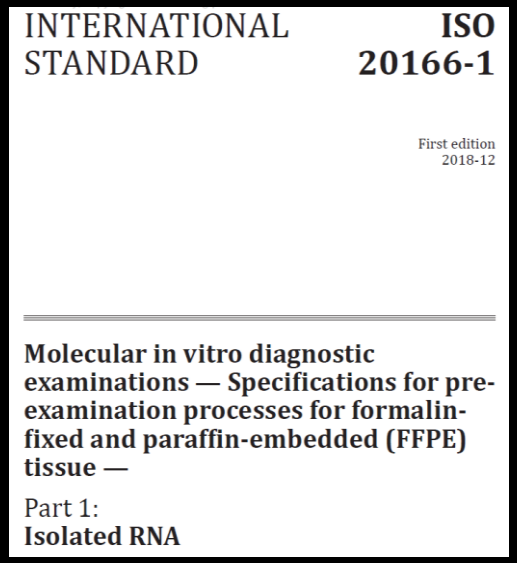

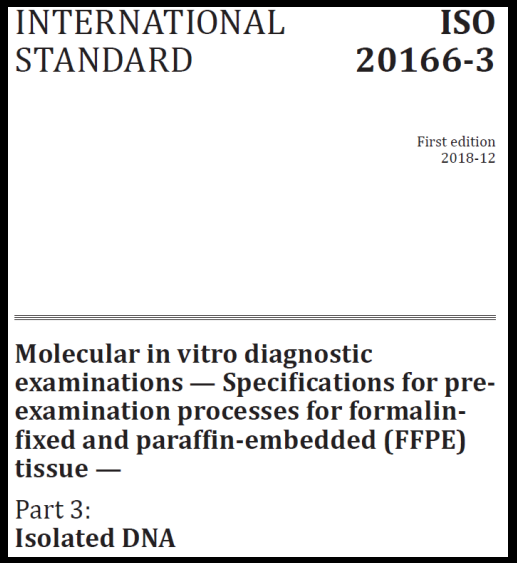

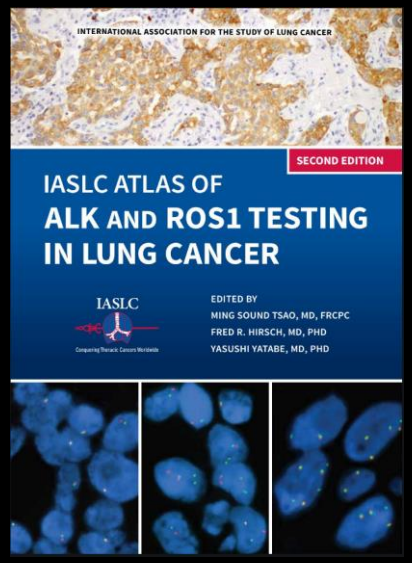

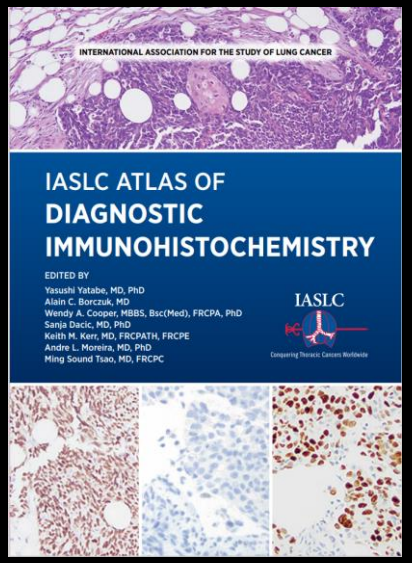

Table 2. Guidelines for core pre-analytical procedures for tissue from international and national authorities

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

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

Huss et al; S Nielsen Stain Quality Management & Biomarker Analysis
In press

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Conclusions;

Pre-analytics are the fundament for optimal IHC
Up to 80% of errors in pathology related to pre-analytics

- Time to and time in Formalin documented essential !!!
- Storage conditions of slides/blocks can affect IHC
- Decalcification in EDTA preferable for IHC and other assays
- Use good laboratory practice for tissue handling/processing

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