

**Advanced course on surgical pathology:
the “next generation step” in immunohistochemistry.
*A systemic evaluation of a rapidly evolving diagnostic procedure***

Tissue preservation and fixation protocols.

Gianni Bussolati
University of Torino

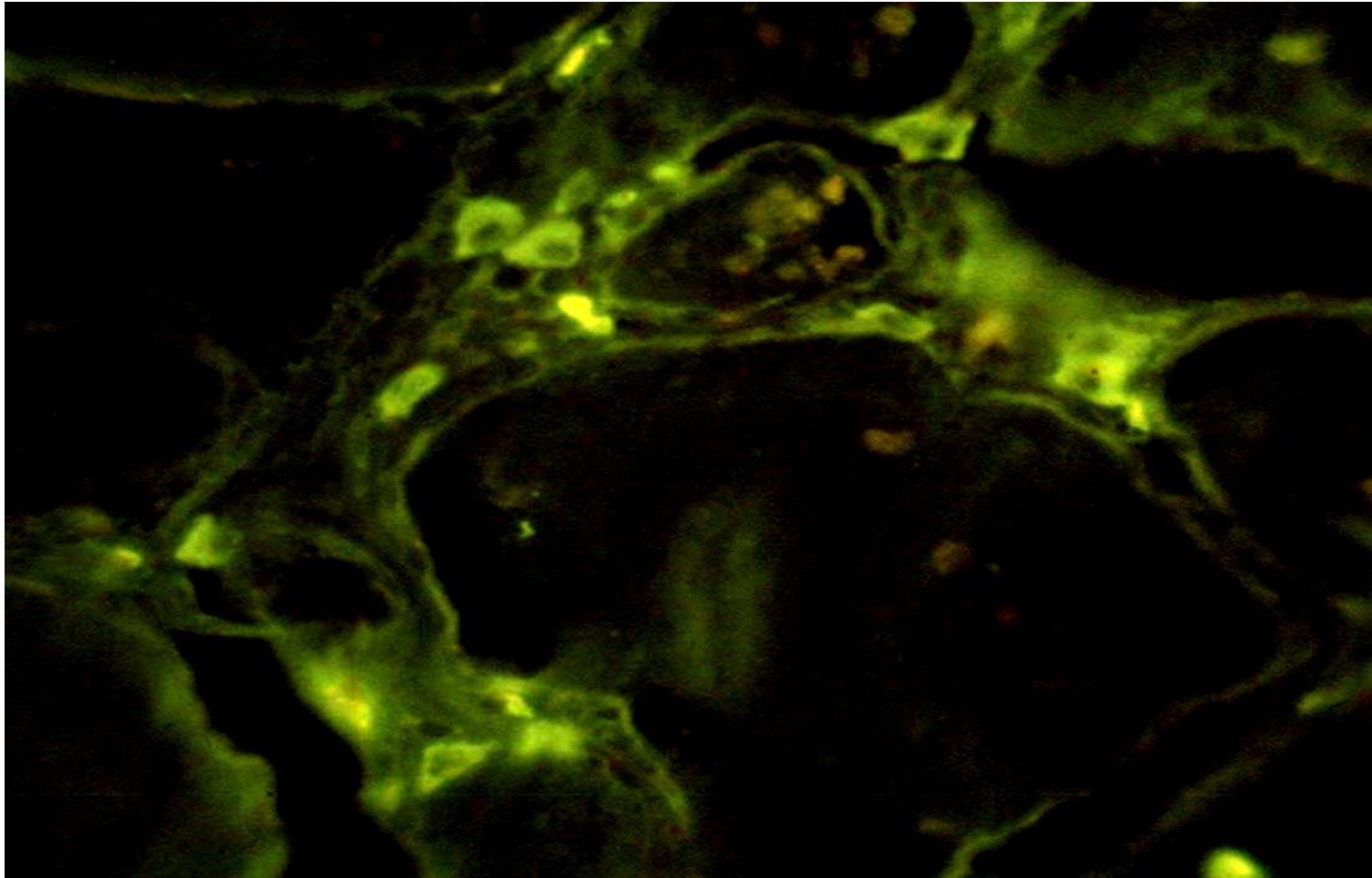
June 8, 2015 Trento

Paradoxical Pre-requisite for Immunohistochemistry

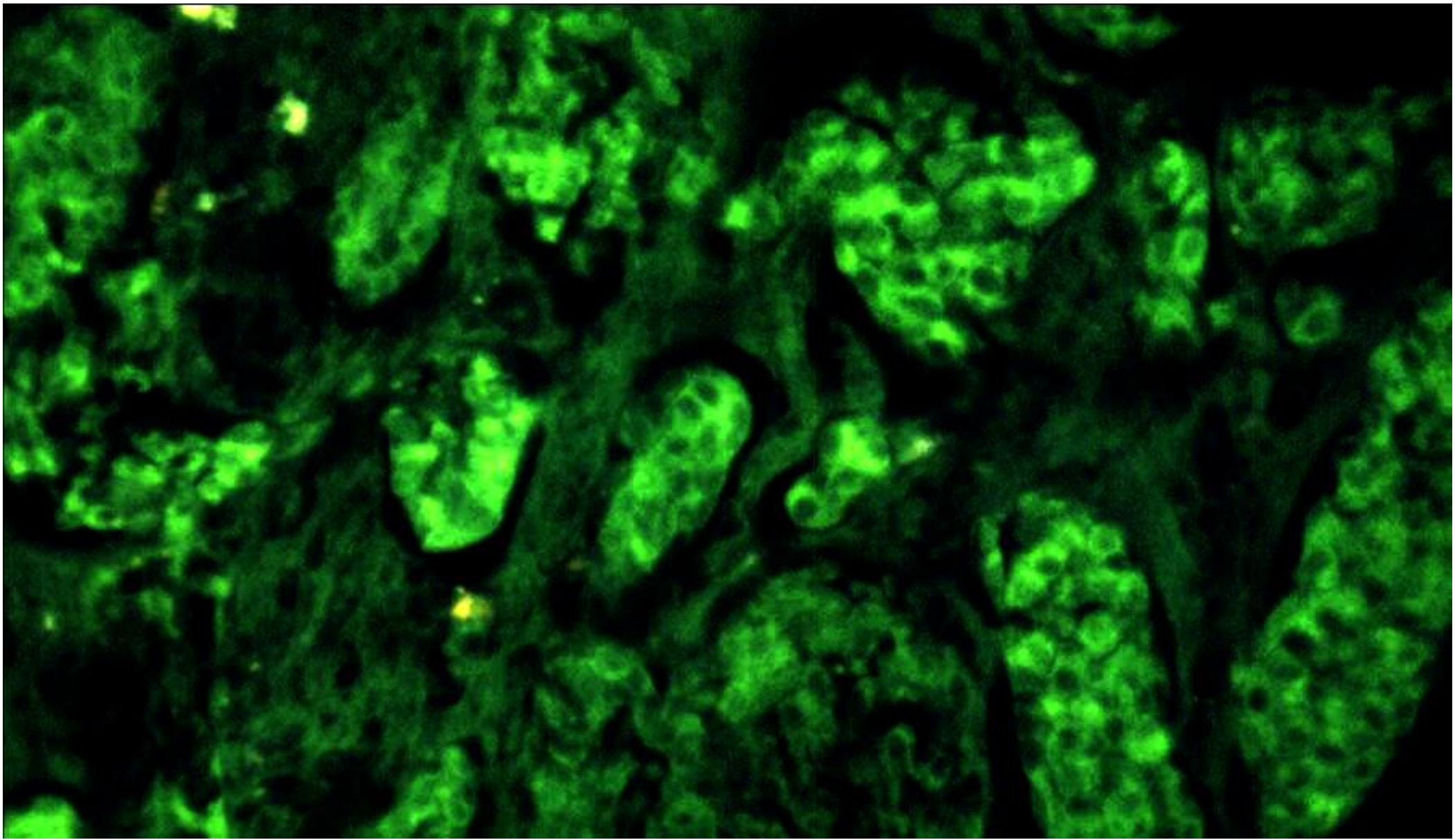
Antigens should be:

- Reactive

- Insoluble



Bussolati G., Pearse AGE. J Endocrinol. 37:205-209, 1967



Bussolati et al. Virchows Arch B Cell Pathol. 2:234-238, 1969

Antigen(s) fixation in Immuno-histochemistry

De-hydratation (denaturation)

Drying

Alcohol

Cross-linking

[J Histochem Cytochem.](#) 2011 59(1):13-32..

**Antigen retrieval immunohistochemistry:
review and future prospects
in research and diagnosis over two decades.**

[Shi SR,](#) [Shi Y,](#) [Taylor CR.](#)

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

Pre-analytical procedures in histopathology

Surgical
theatres

Pathological
Anatomy

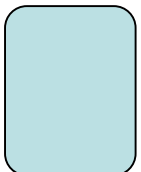
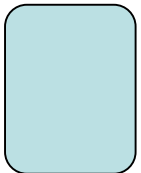
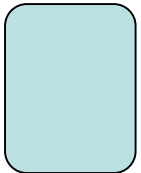
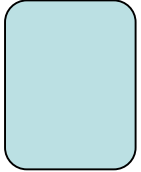
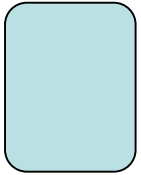
Grossing

Fixation

Embedding

Sectioning

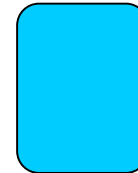
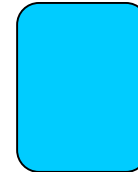
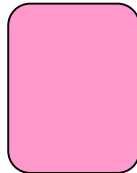
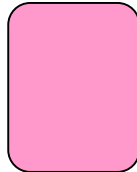
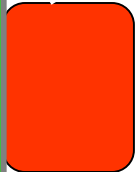
Staining



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Interval : From the surgical table to the pathology lab

Alternatives :

a) Tissues left fresh

What Are the Preanalytic, Analytic, and Postanalytic Variables That Must Be Controlled to Ensure That the Assays Reflect the Tumor ER and PgR Status?

- The time from tumor removal to fixation should be kept to ≤ 1 hour

PRE-REQUISITES:

- Pathology Lab within walking distance
- Committment of Surgeons, Staff, Pathologists ...

In Future, same for Colon, Lung cancers...

Interval : From the surgical table to the pathology lab

Alternatives :

a) Tissues left fresh

b) Tissues immersed in formalin

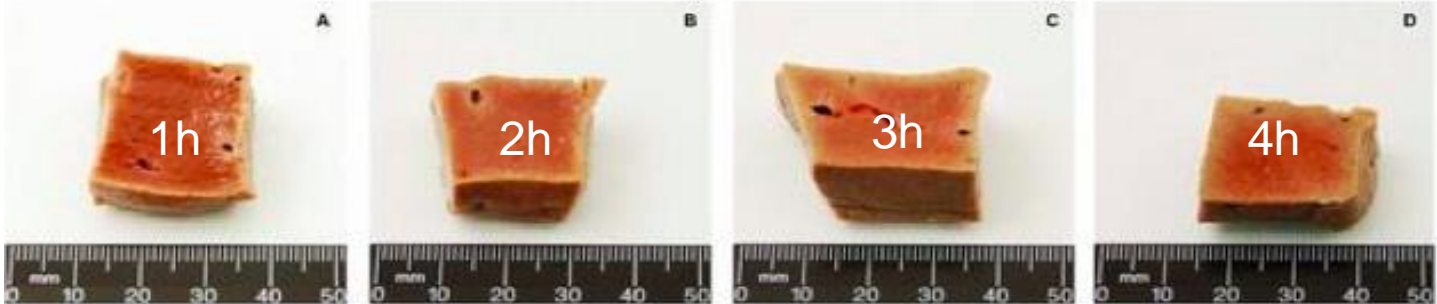
Cold Ischemia Time

Alternative: b) Tissues immersed in formalin

Drawbacks (in large specimens):

- Degradation continues in deep areas
- Tissue banking is hampered
- Formalin containing vessels heavy to carry
- Spilling of formalin may occur
- Fumes dispersed while grossing
- Nurses refuse to handle this “carcinogen” in surgical theatre (and without hoods)
- Tissue forgotten by the surgeon because “already safe in formalin”

Formalin penetration time

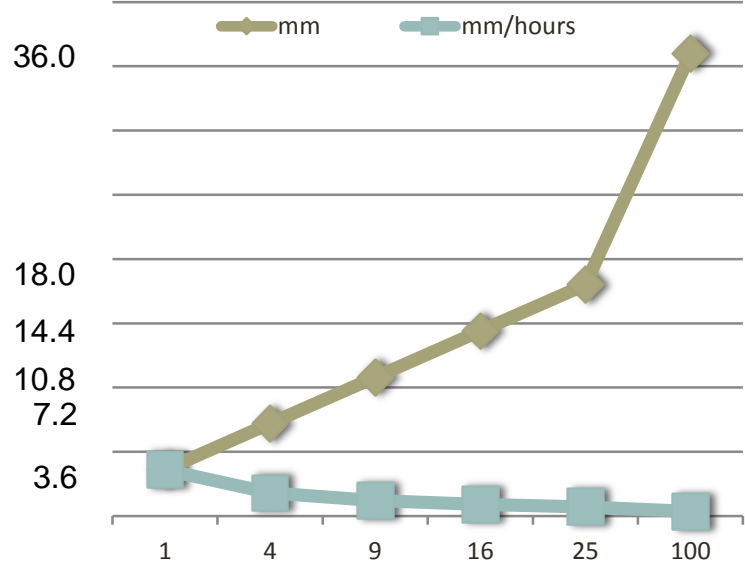


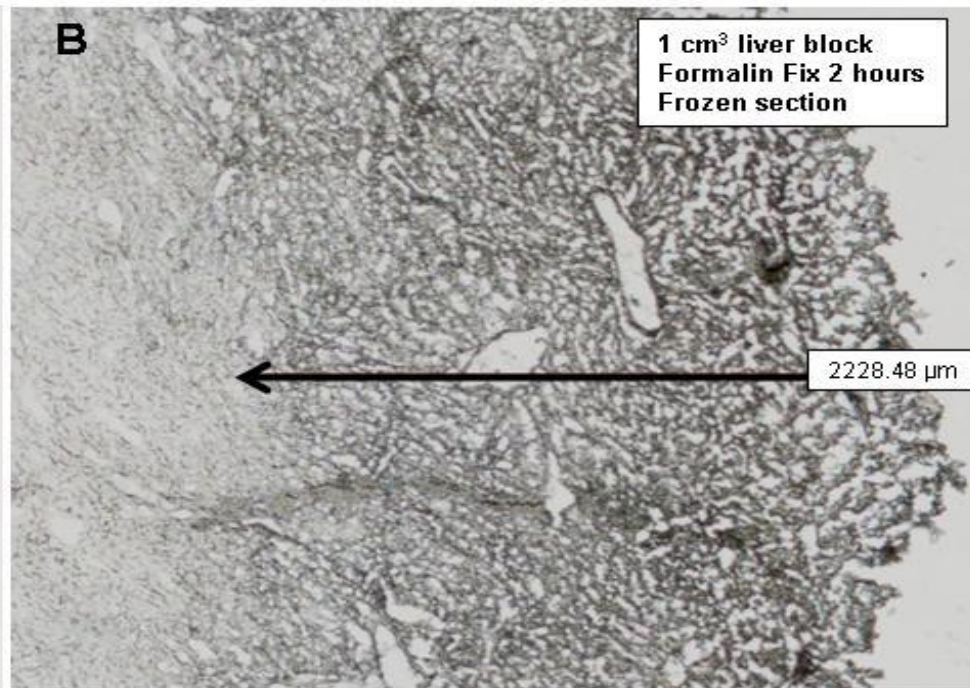
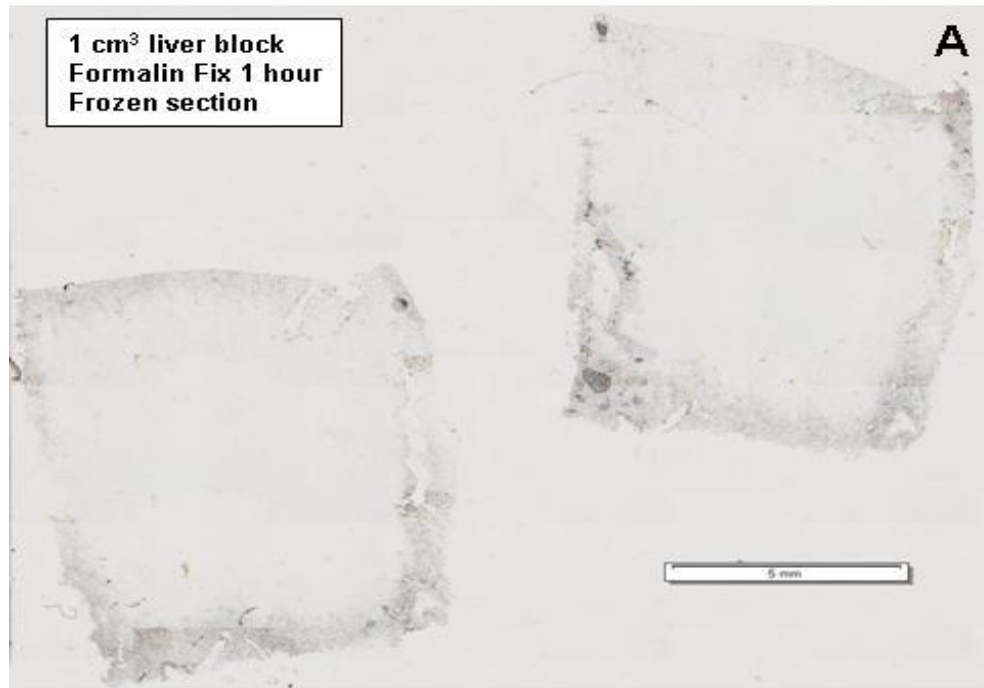
Author: Geoffrey Rolls
 Institute: Leica Biosystems, Wetzlar,
 Germany 06. March 2012

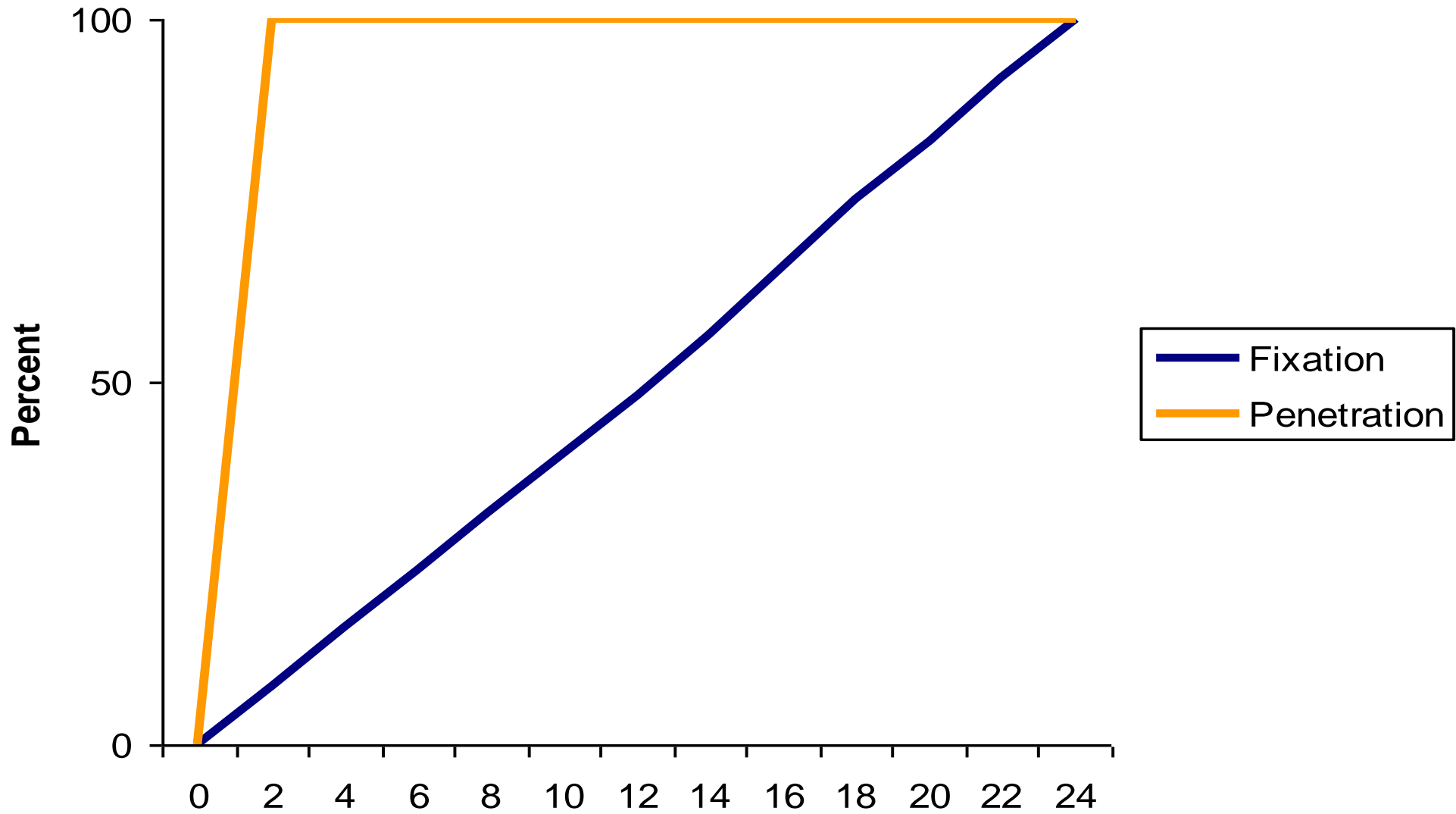
The first layer of cells (20 microns) takes a second. (Bakers $K = 3.6$) depth of penetration, $(d) = K\sqrt{t}$ (K is the coefficient of diffusion), (T is time)

Bryan R. Hewlett
 Technical Specialist
 Anatomical Pathology
 Hamilton Regional Laboratory Medicine Program
 Ontario, Canada

hours	mm/hours	mm
1	3.6	3.6
4	1.8	7.2
9	1.2	10.8
16	0.9	14.4
25	0.72	18
100 (4 days)	0.36	36



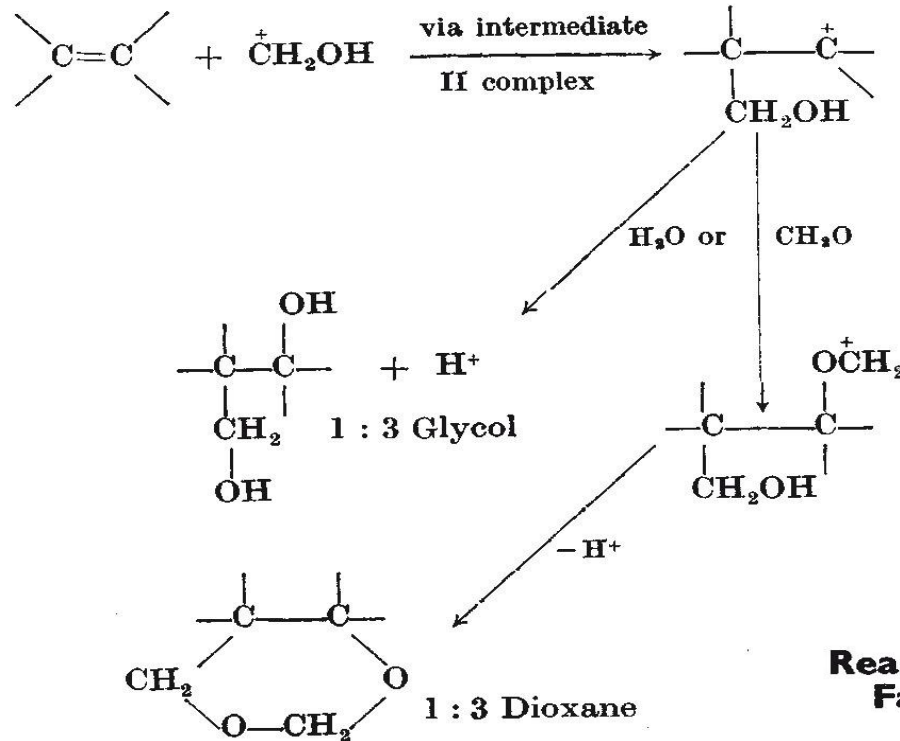




(a) Formation of electrophilic species:



(b) Reaction with double bond:



Reaction of Formaldehyde with Unsaturated Fatty Acids during Histological Fixation

DENNIS JONES
G. AUSTIN GRESHAM

Department of Pathology,
University of Cambridge.

Tissues immersed in

Phosphate – buffered Formalin:

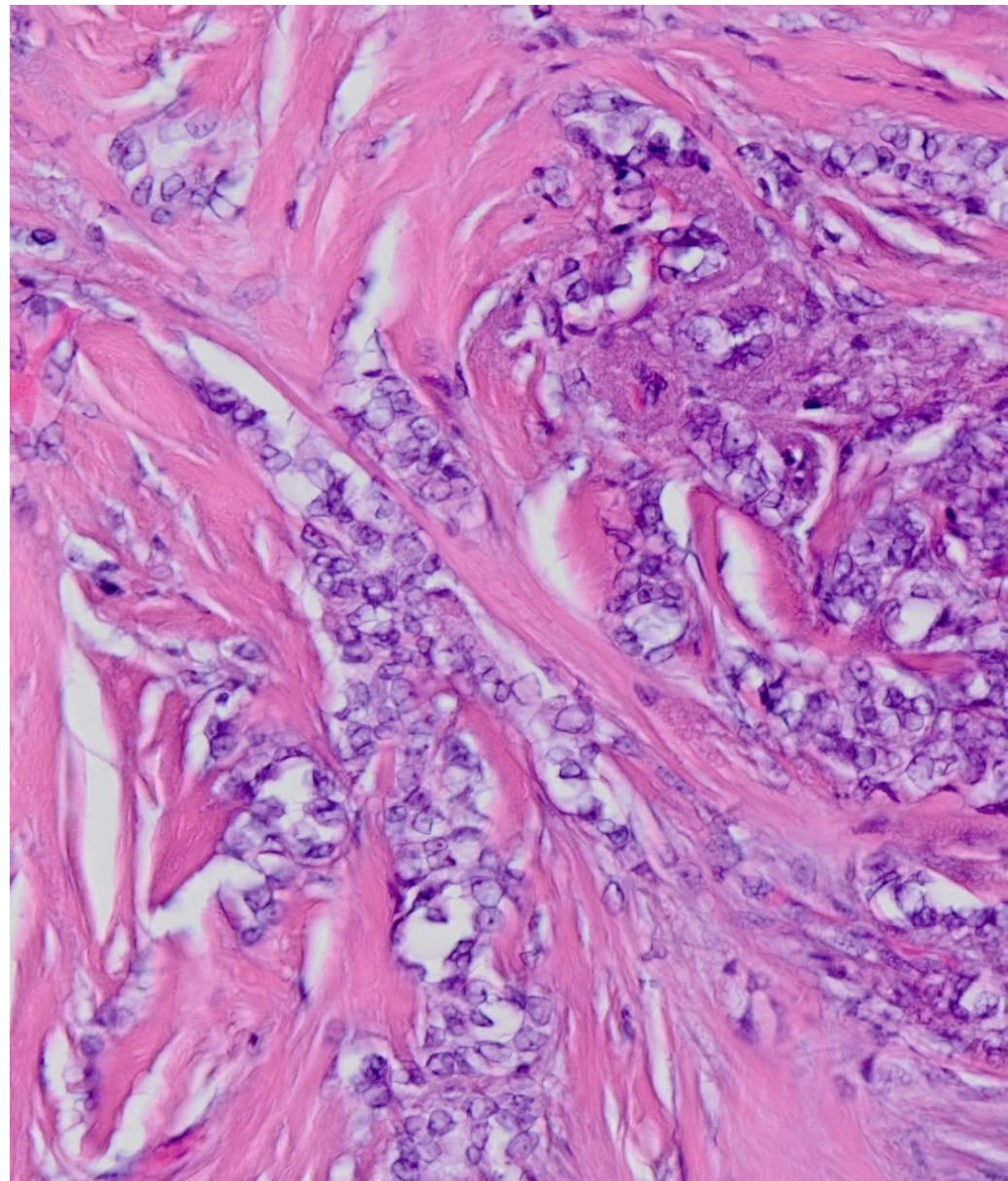
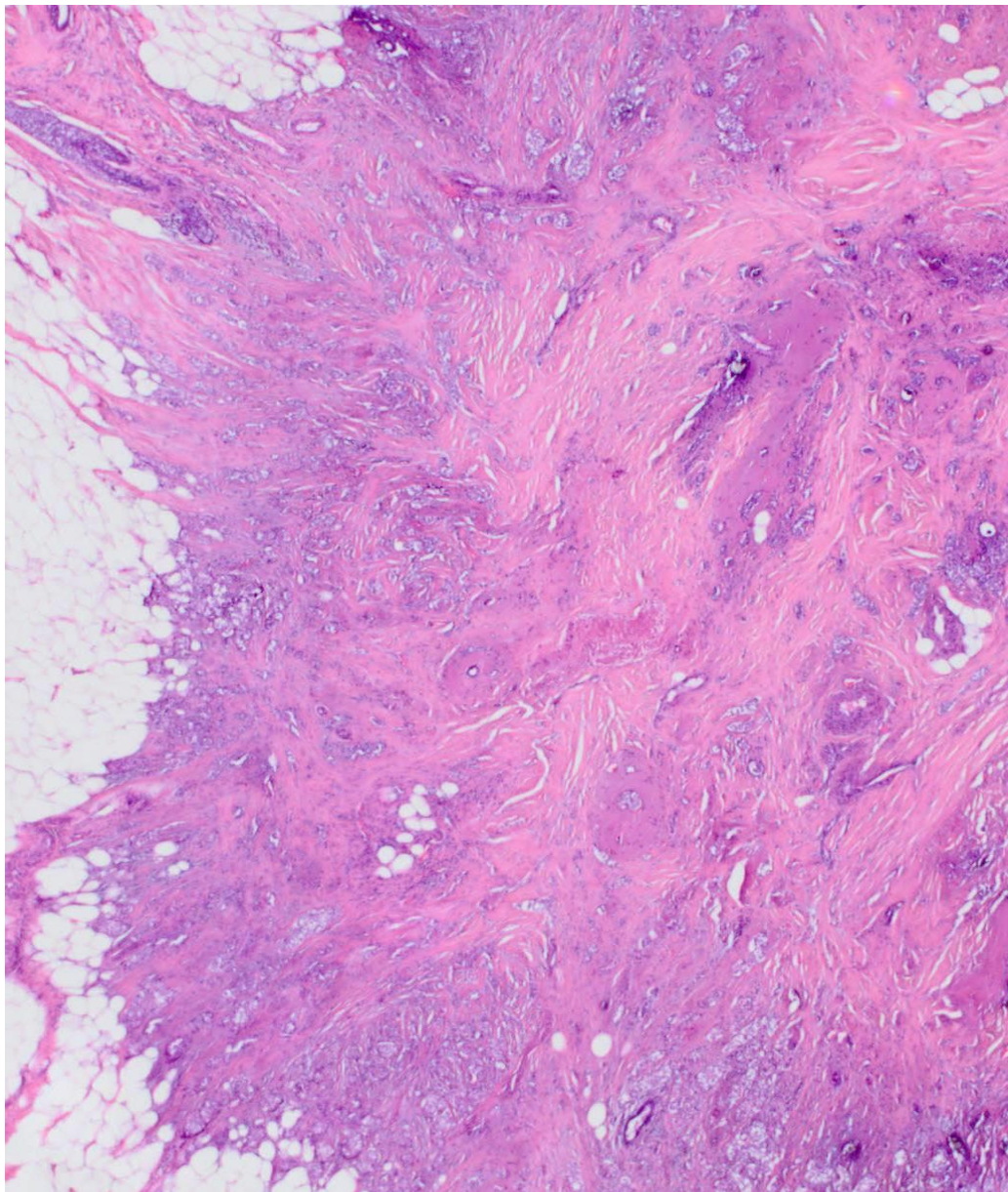
Small Biopsies (<1 cm) = Uniform fixation

Large specimen (> 2 cm)

- **Outside = Fixed**
- **Inside = Autolysis**

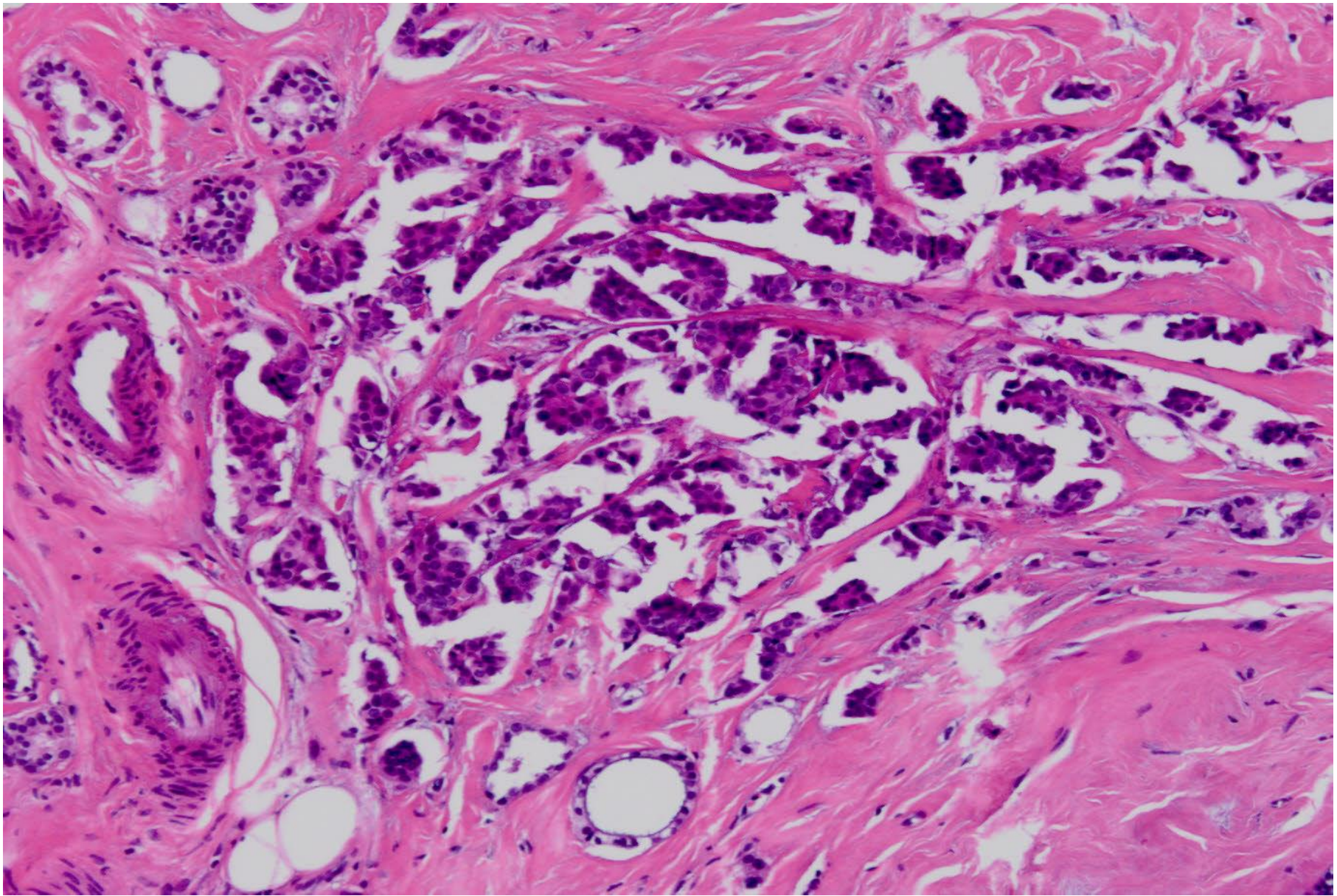
Jan. 2006:

**Breast Surgeons start to
operate on Friday afternoon.**



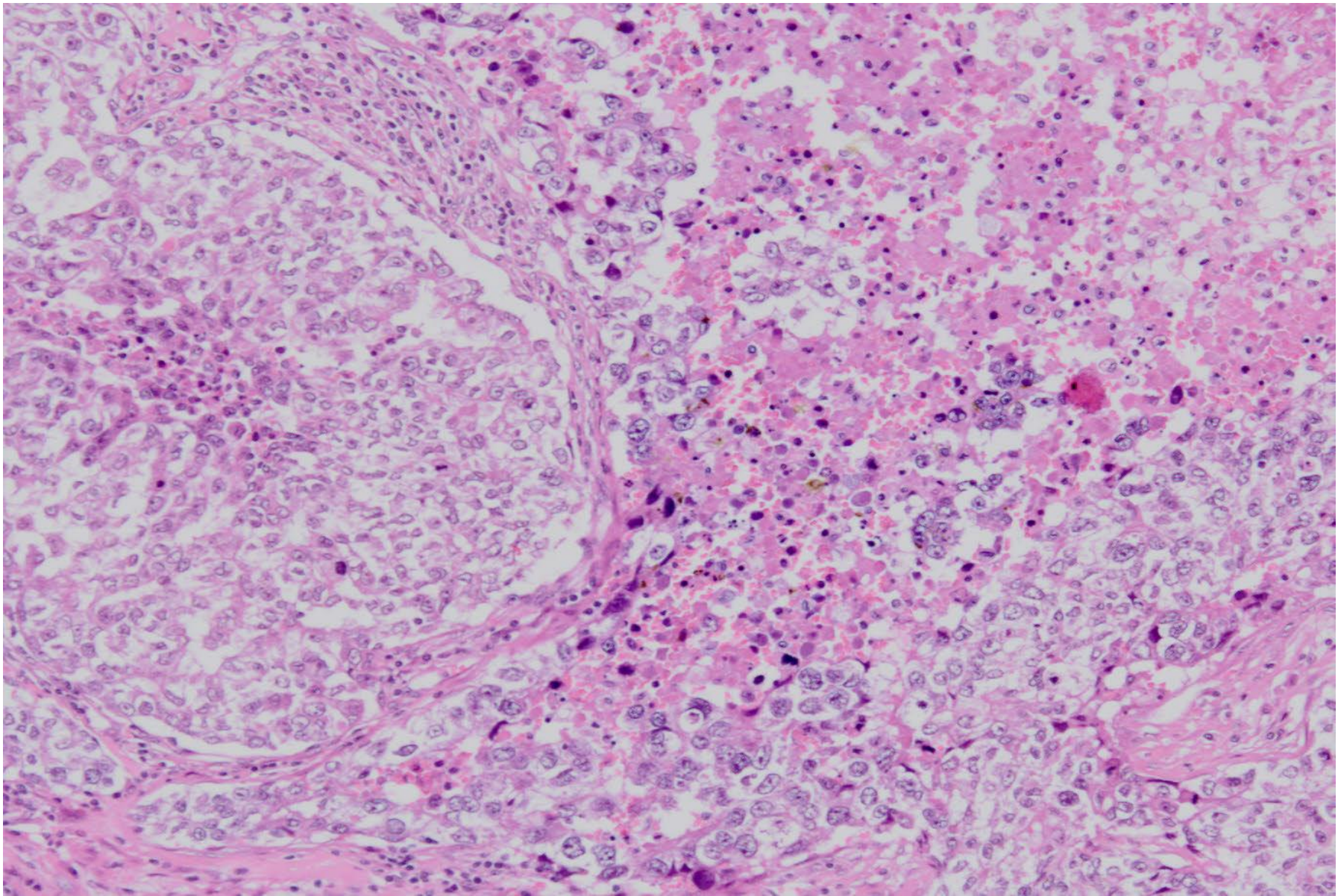
Breast Cancer. Operated on Friday; Immersed in Formalin; Arrived on Monday.

Jan. 2006



Breast Cancer. Operated on Friday; Immersed in Formalin; Arrived on Monday.

Jan. 2006



Breast Cancer. Operated on Friday; Immersed in Formalin; Arrived on Monday.

Jan. 2006

VACUUM PACKING and COOLING PROCEDURE:

1. Immediately after excision, specimens are transferred to an adjacent room where the TissueSAFE is installed.
2. The surgical specimen is placed into sterilized specimen bags and sealed under vacuum.
3. Case information are written directly on the printed note field. Starting of vacuum (cold ischemic time) is printed.



SPECIMEN RECEPTION



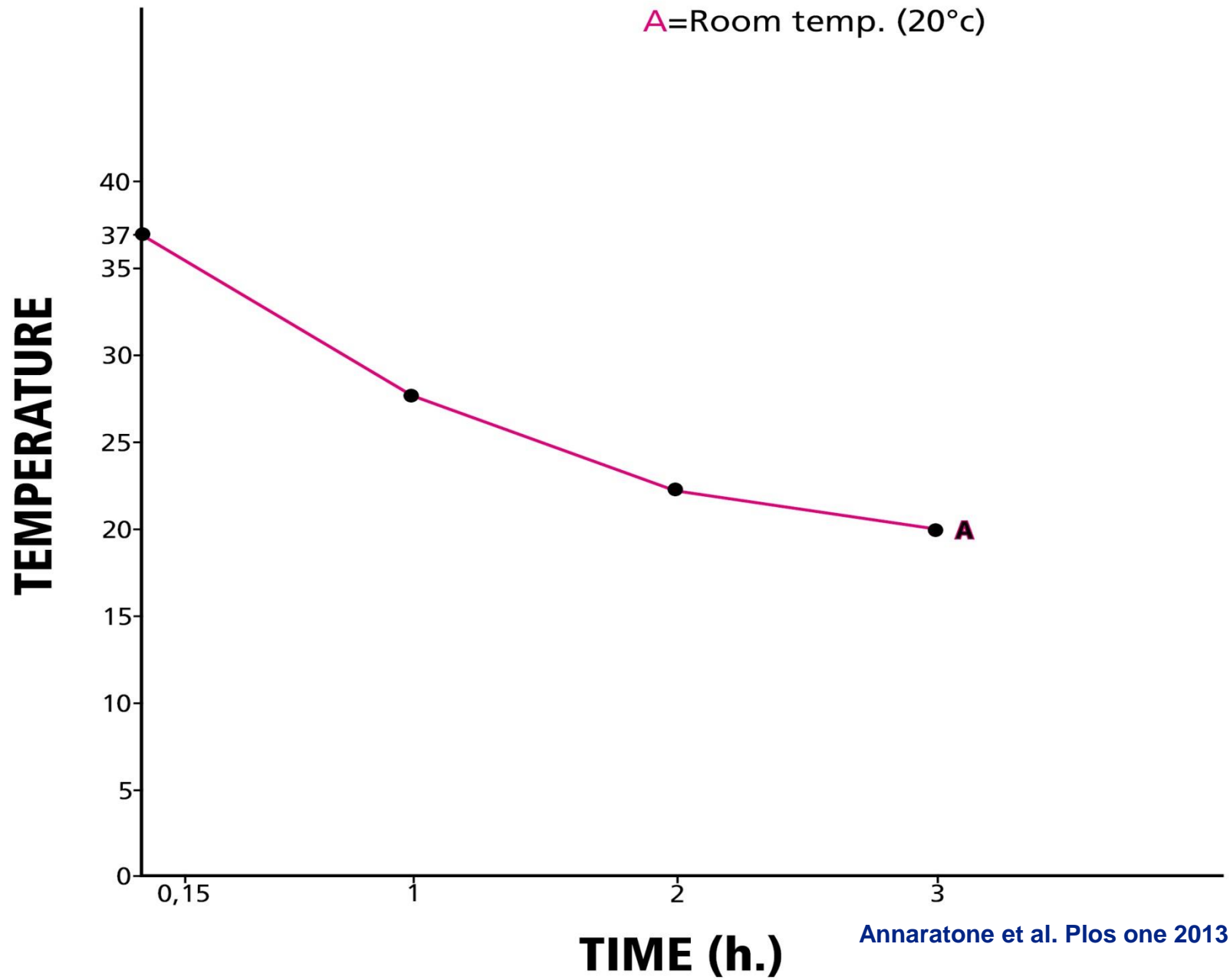
SPECIMEN STOCKING AT 4° C

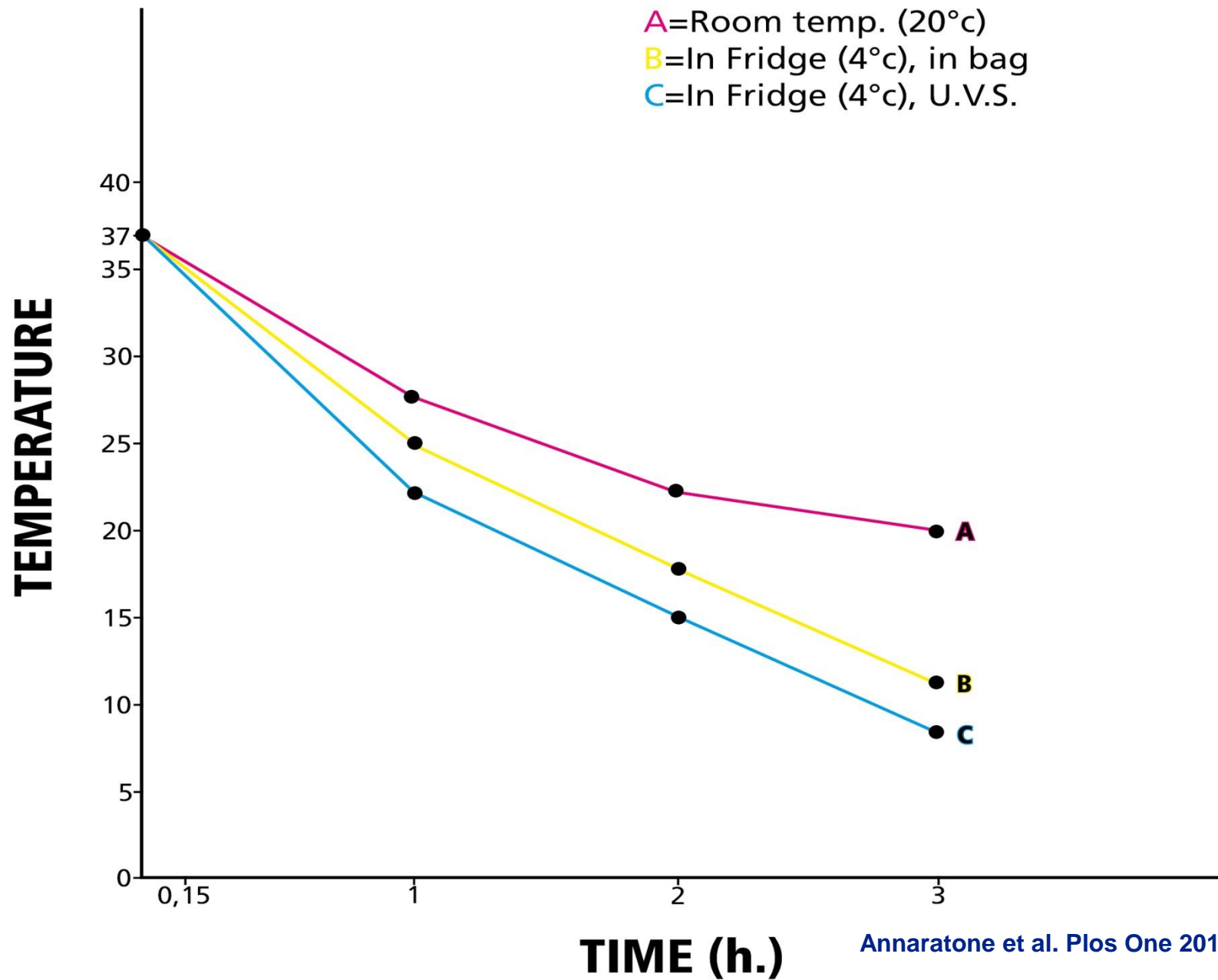


How cold is cold ischemia?



Annaratone et al. Plos one 2013





Science of the Total Environment 408 (2010) 3092–3095



Contents lists available at [ScienceDirect](#)

Science of the Total Environment

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



Vacuum-based preservation of surgical specimens: An environmentally-safe step towards a formalin-free hospital

Cinzia Di Novi ^a, Davide Minniti ^b, Silvana Barbaro ^b, Maria Gabriella Zampirolo ^b, Antonio Cimino ^c, Gianni Bussolati ^{c,*}

Staff satisfaction

<i>Level of Satisfaction</i>	<i>Freq.</i>		<i>Percent</i>	
	<i>formalin</i>	<i>under-vacuum</i>	<i>formalin</i>	<i>under-vacuum</i>
Low	42	6	39.25	8.57
average	44	17	41.12	24.29
high	21	47	19.63	67.14
Total	107	70	100.00	100.00

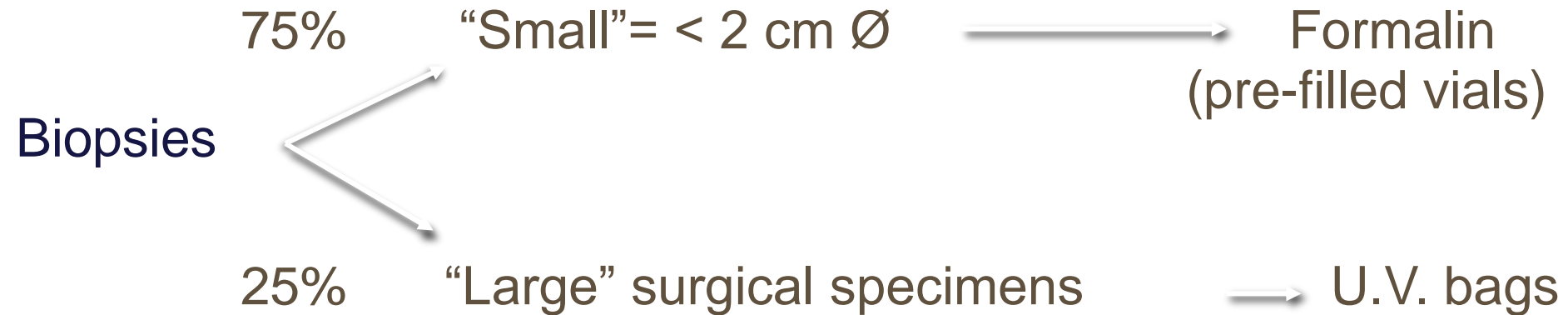
Result of Survey among Staff Operators (October 2008 – April 2009)

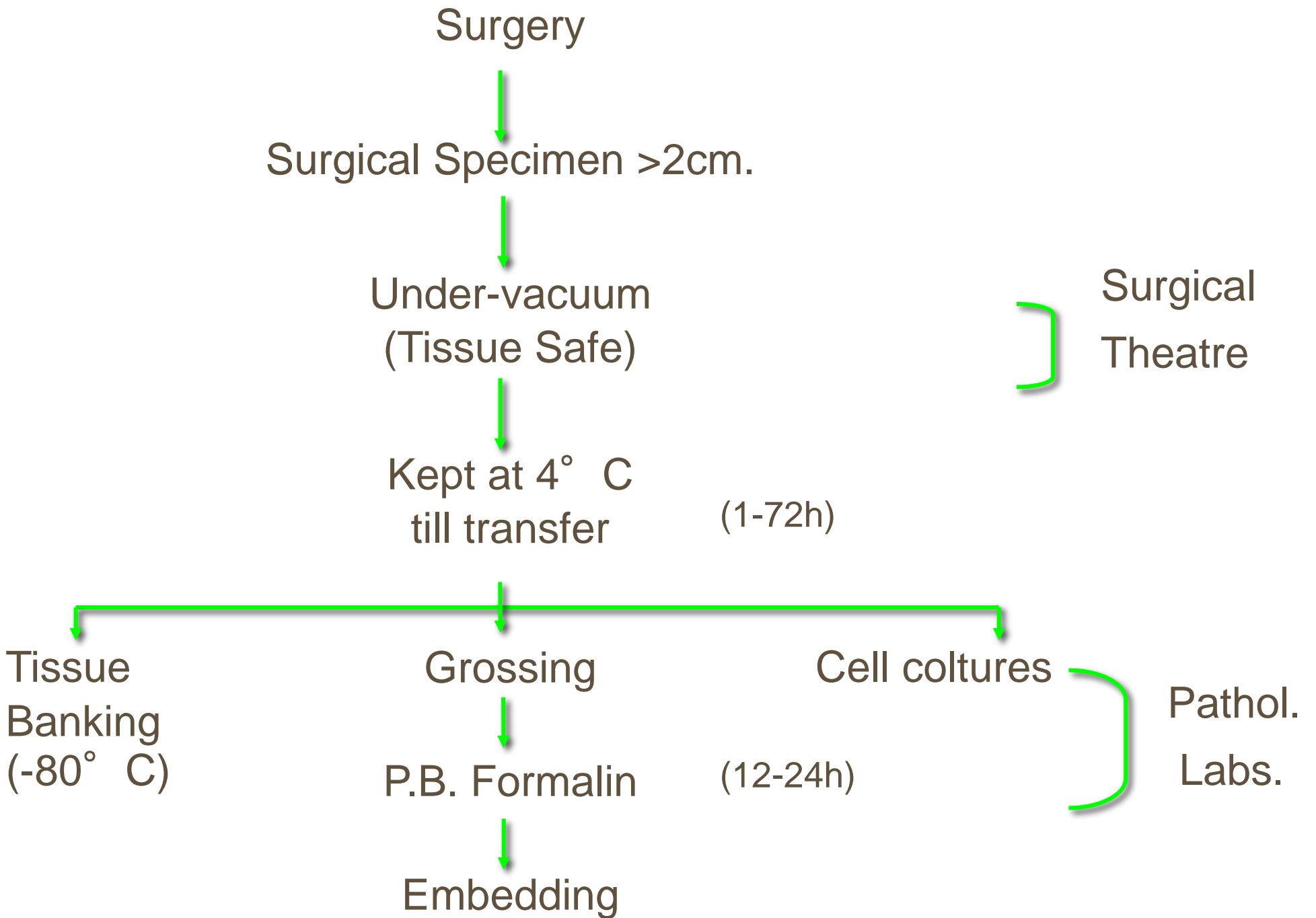
- **Satisfaction:**
 - Low for Formalin
 - High for U.V.
- **Handling & Gross Anatomy**
Histopathol. + ICC - U.V. = no drawbacks

Project: “....Towards a Formalin-free Hospital”

“Molinette” Hospital, Turin

- 1162 Beds; >54.000 yearly admissions; > 40.000 histopathological exams. (2008)





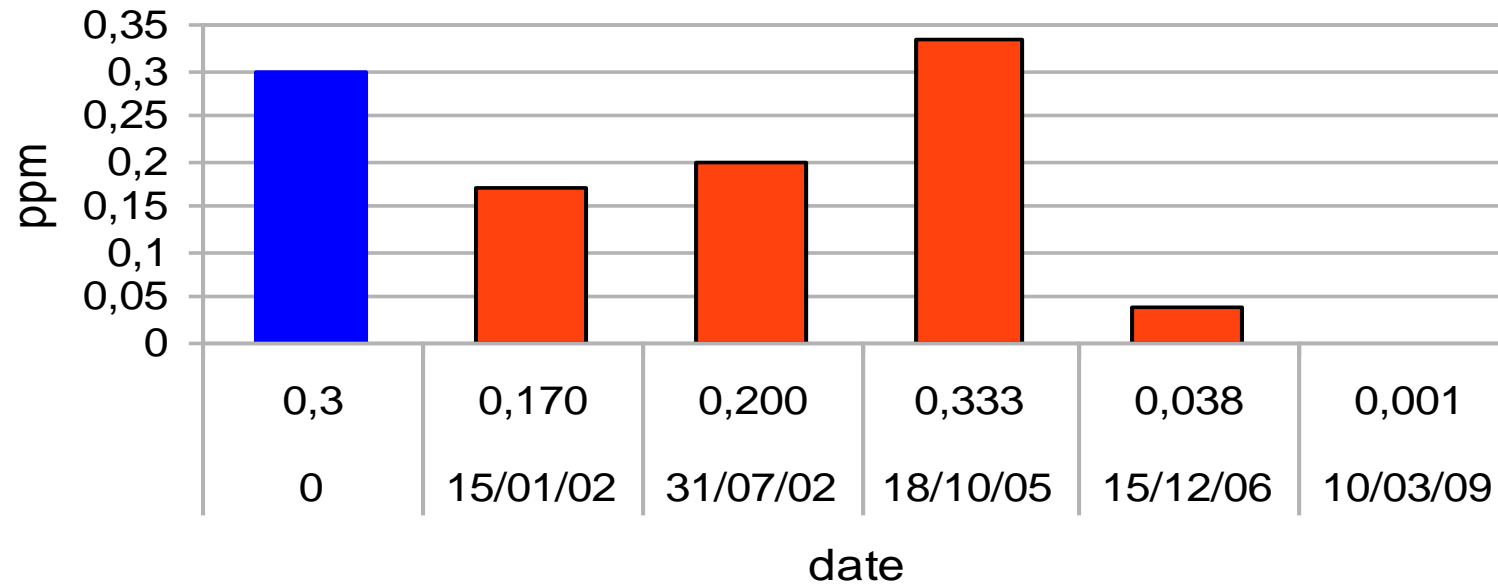
Interval : From the surgical table to the pathology lab

Alternative: c) Tissues preserved under vacuum

Merits:

- **No more formalin in surgical theatre (except for small specimens, where pre-filled tubes are employed)**
- **No spilling**
- **No fumes**
- **No drying of tissues**
- **Colours preserved**
- **Lack of insulating air around tissues allows fast cooling**
- **Tissues (bags) light and easy to carry**
- **Structure (RNA, Antigens) preserved up to days**
- **Banking (selective) allowed**
- **Demonstrating of operated tissues is convincing for students and surgeons**

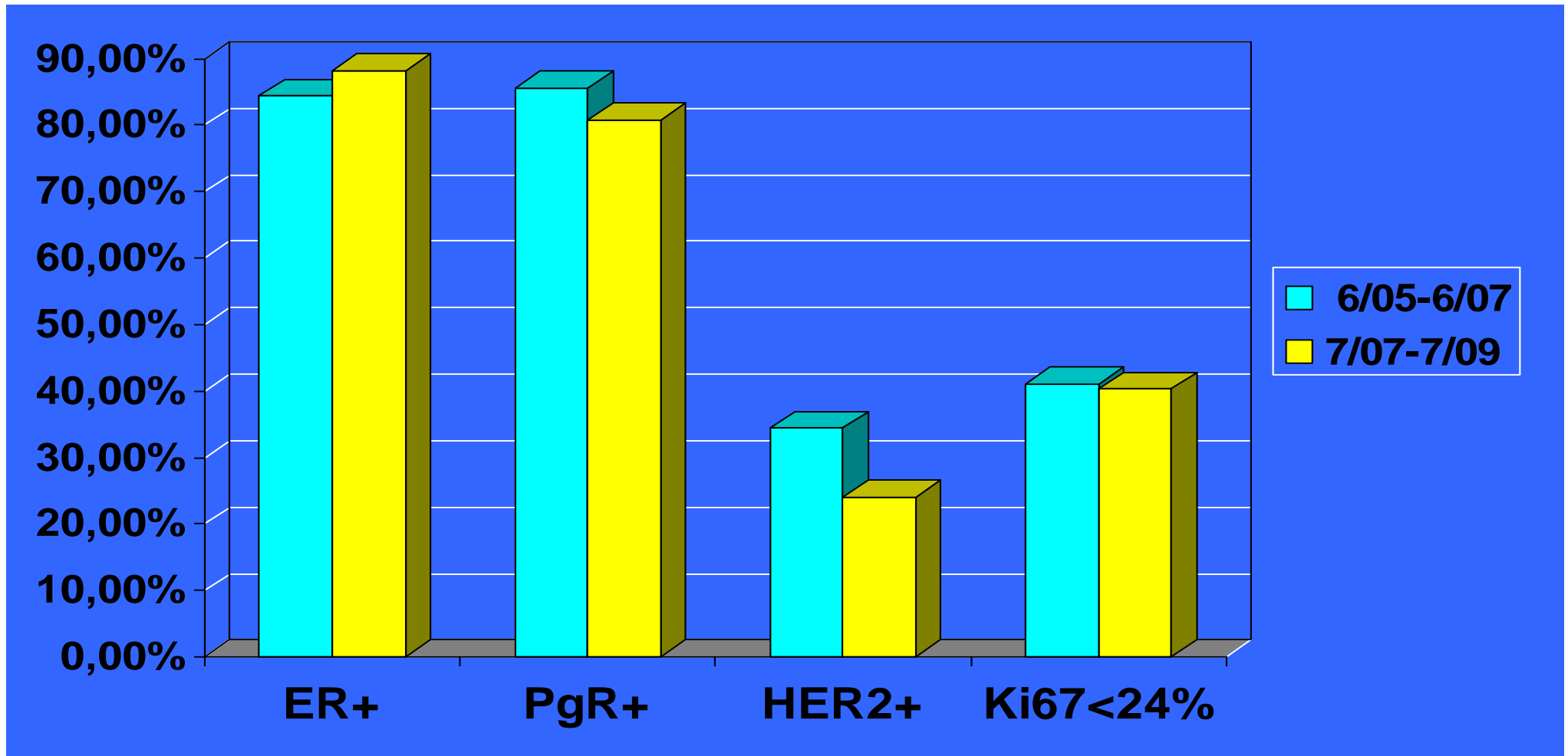
Formaldehyde values in Grossing Room



Threshold Limit Value (TLV)

ICC evaluation of therapeutic/prognostic parameters in a continuous series of breast cancers

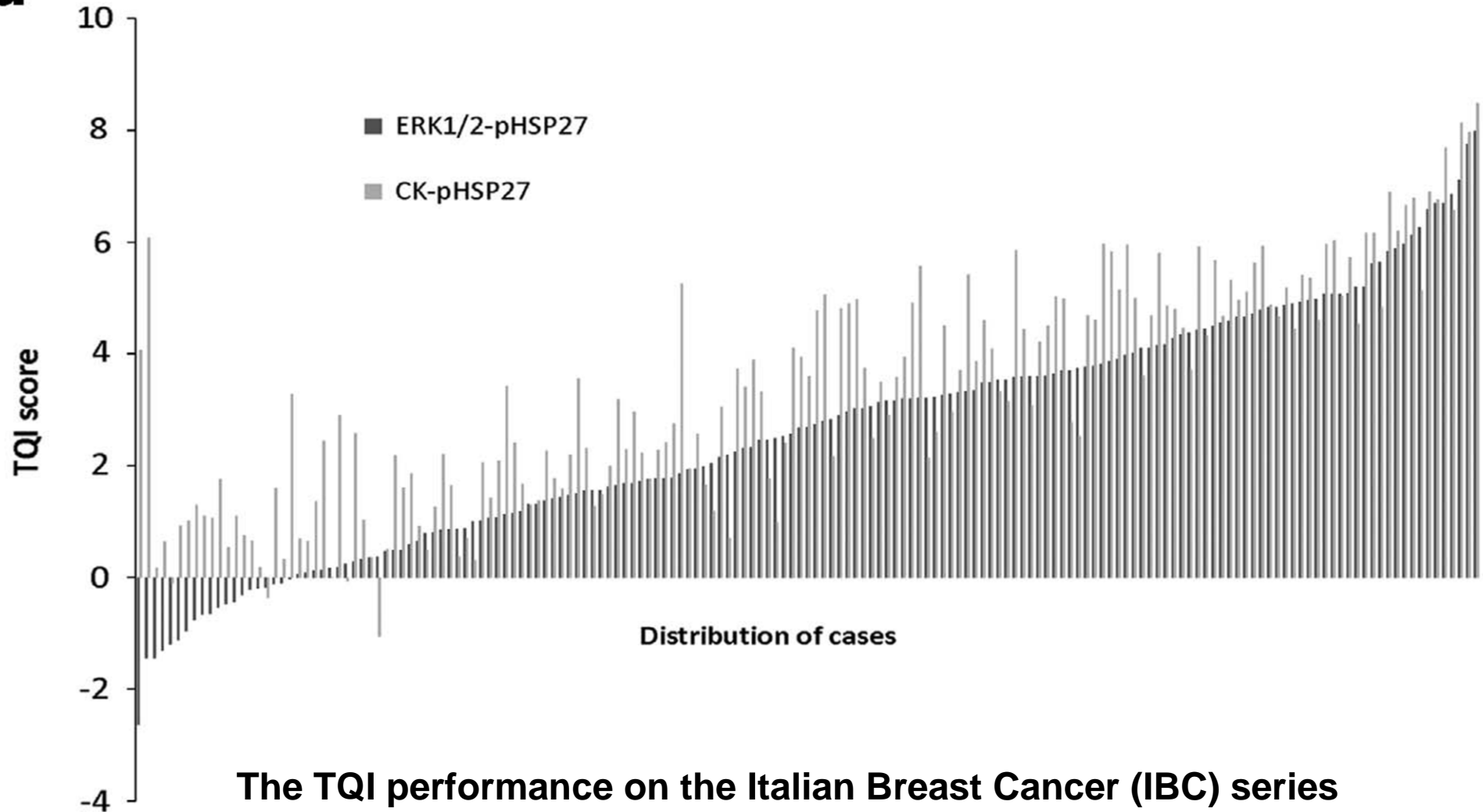
Years 6/05-6/07 vs. 7/07-7/09. N=375



- **A Tissue Quality Index – an Intrinsic Control for Measurement of Effects of Preanalytical Variables on FFPE Tissue**
- Neumeister V.M., Parisi F, England A.M., Siddiqui S., Anagnostou V, Zarrella E, Vassilakopoulou M, Bai Y, Saylor S., Sapino A., Kluger Y., Hicks D.G., Bussolati G., Kwei S.and Rimm D.L.
- Department of Pathology and Surgery, Yale University School of Medicine
- Department of Pathology, University of Rochester School of Medicine
- Department of Biomedical Sciences and Human Oncology, University of Turin, Turin, Italy

- The TQI represents a proof of concept for the use of epitope expression to provide a mechanism for monitoring tissue quality.
- Association of negative TQI values (an indicator for loss of tissue quality) with increasing cold ischemic time..

d



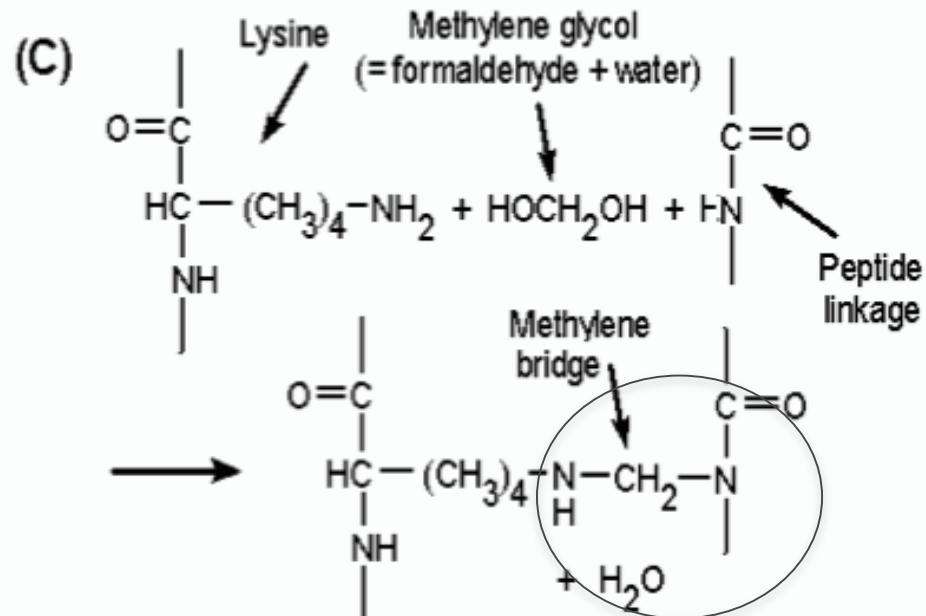
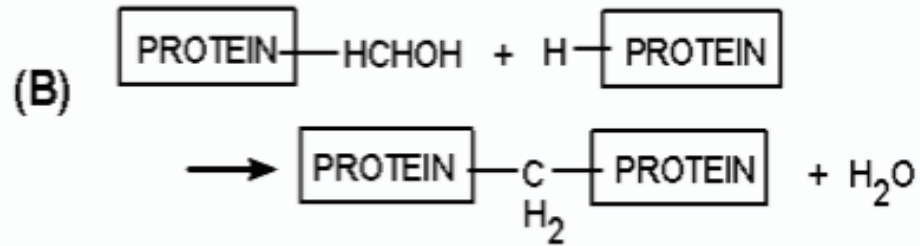
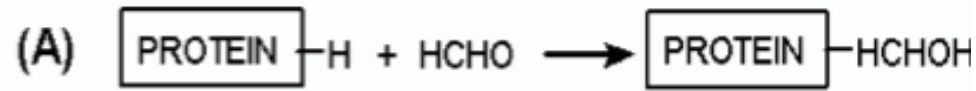
**The TQI performance on the Italian Breast Cancer (IBC) series
..special handling conditions appear to result
in significantly less epitope degradation.**

The IBC series consists of patient samples which were vacuum sealed and stored at 4° C (UVSC) to prevent degradation.

The quantification of pHSP27, Cytokeratin and pERK1/2 and calculation of the TQI revealed mainly positive TQI scores (147 out of 169 readable samples have a positive TQI) suggesting better preservation of the tissue through vacuum sealing and storage at 4° C

FORMALDEHYDE

CHEMICAL REACTION



The main effect of formaldehyde in tissues is linked to the **formation of methylol groups on amino groups** first, followed by the **establishment of cross-linking methylene groups** that lead to proper fixation

Initial binding of formaldehyde to protein is

- largely completed in 24 hours but the formation of methylene bridges proceeds much more slowly.

- still relatively weak and reversible; stronger cross-linking continues to occur over time.

Complete fixation is thought to take at least 7 days. Even after this time cross-links continue to form slowly.

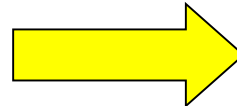
Formalin

- Which Formalin?
- How much
- Time / Temperature
- Goals: Preservation of

Morphology

Nucleic Acids

Antigens (IHC)



Formalin

- **Which Formalin?**
- **How much**
- **Time**
- **Goals: Preservation of**

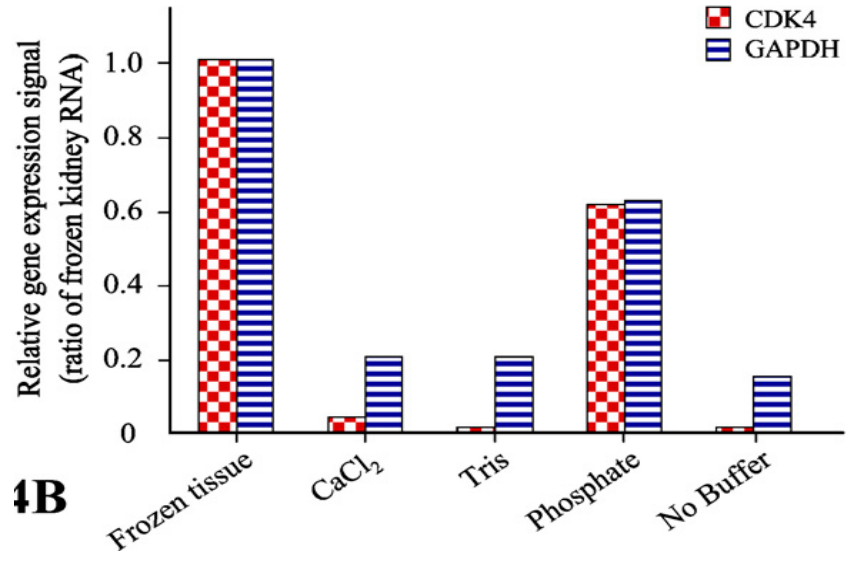
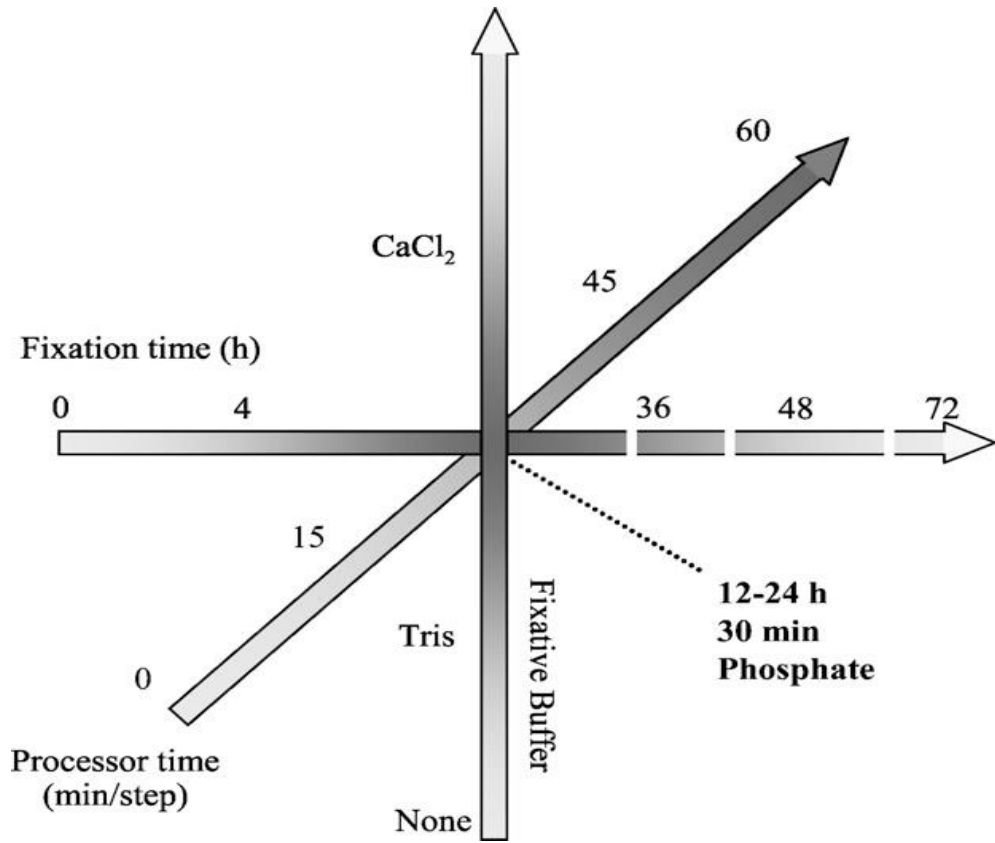
Critical factors

- Time to fixation
- Type of fixative
- Time of fixation
- Temp. of fixation
- Size of specimen

Morphology

Nucleic Acids

Antigens (IHC)



Chung et al. J.
Histochem. Cytochem.
2008

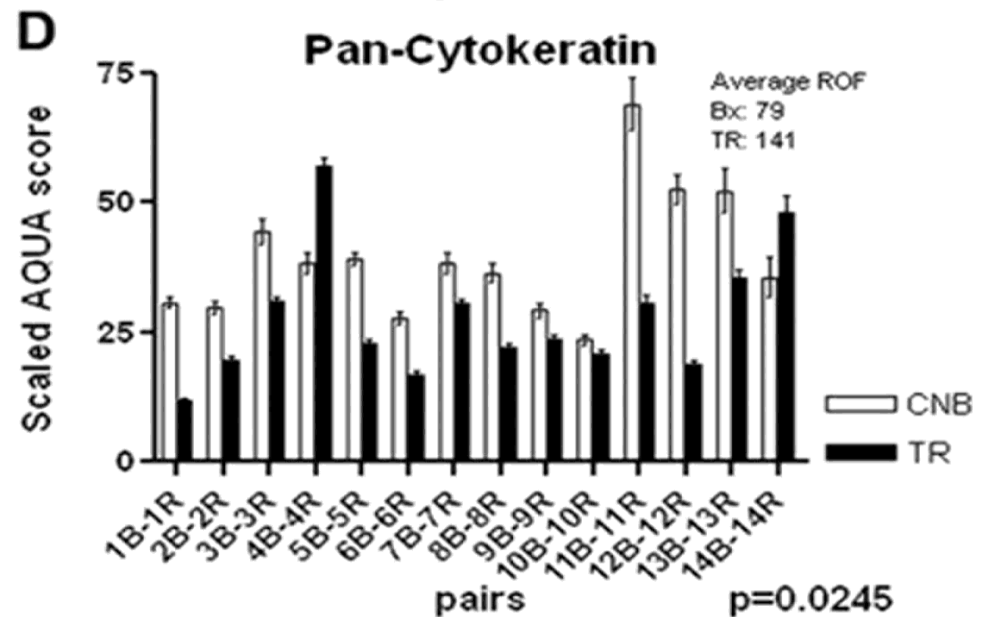
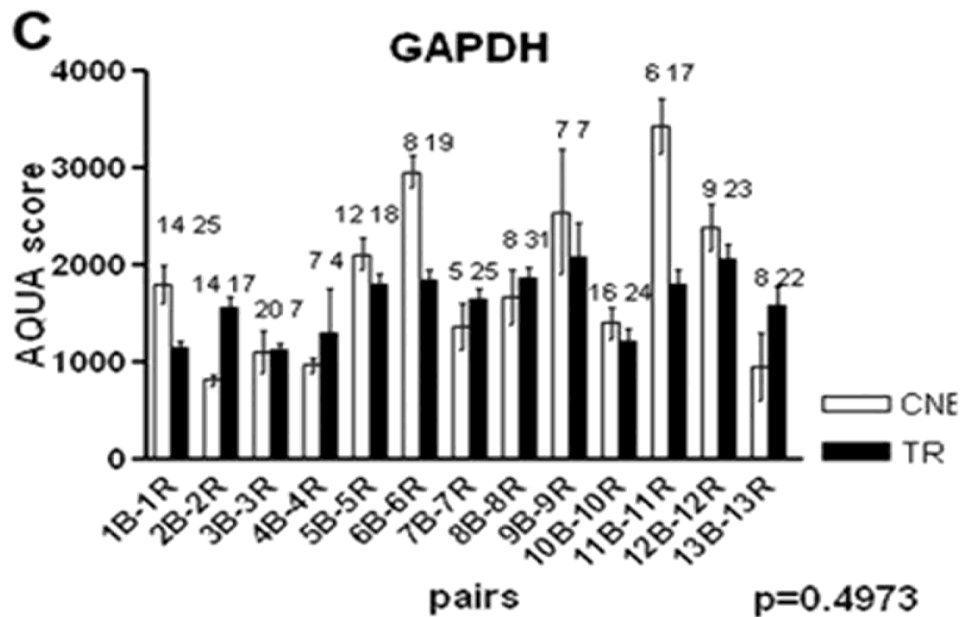
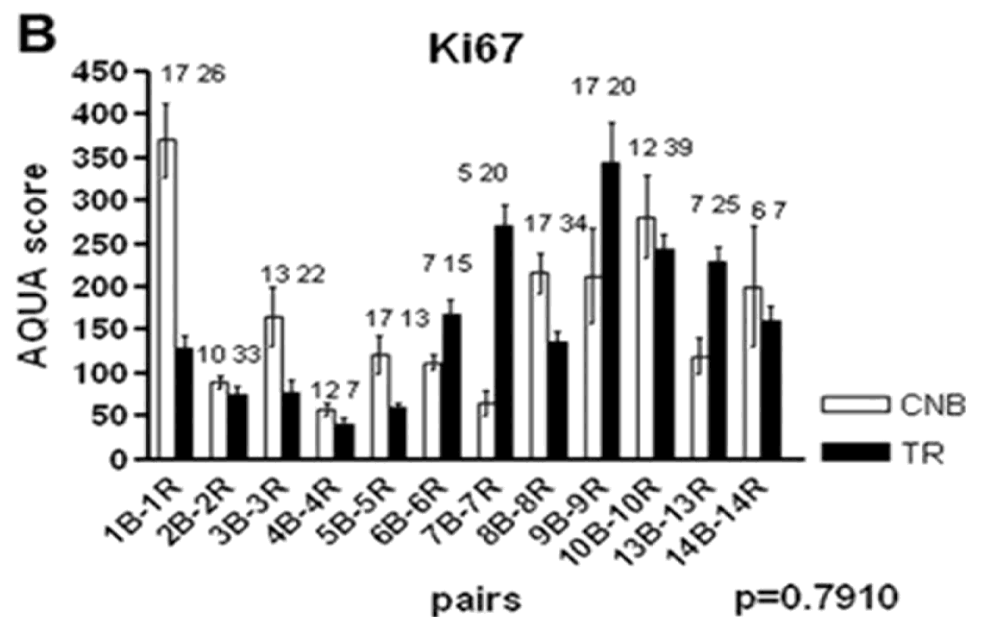
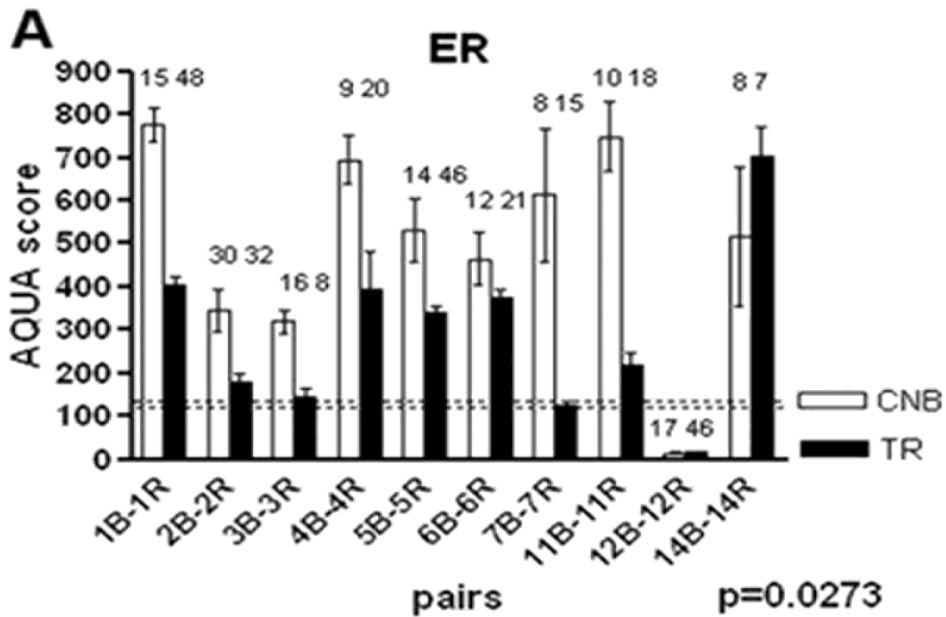
Table 4. Formal and Literature-Based Recommendations for Specimen Fixation and Processing Variables for Optimal Staining for Most Antigens Via Immunohistochemistry

Preanalytic Variable	Published Guidelines and Recommendations	Literature-Based Recommendations
Fixation delay	<1 h ⁸	<12 h ¹⁻⁴
Fixation		
Fixative formula ^a	10% NBF, pH not specified ^{8,10}	10% NBF, pH 5–7 ^{1,2,6,12}
Time in fixative ^a	24 h ⁸⁻¹⁰	24 h ^{1,2,6,11,13,16-18,20-21}
Tissue to fixative ratio	1:10 ^{8,10}	1:1 to 1:20 ²
Dehydration (total duration)	1.25–15 h ¹⁰	10 h ^{1,2}
Paraffin impregnation		
Type of paraffin	Low-melting-point paraffin (55°C–58°C) ¹⁰	Low-melting-point paraffin (45°C) ¹
Total duration	0.5–4.5 h ¹⁰	1–2 h ^{1,28} or 8 h ²
Slide drying	24 h at room temperature or 1 h at 50°C–60°C ¹⁰	24 h at room temperature ¹ or overnight at 37°C ²
Storage duration		
Paraffin block ^a	Indefinitely ¹⁹	≤25 y ^{16,33-35}
Slide-mounted sections ^a	7 d ⁹ or <6 wk ⁸	<6 d ³⁸

.....brief fixation of very highly expressing breast cancers does not seem to alter ER, PR, and HER2 status.

The Effects of Under 6 Hours of Formalin Fixation on Hormone Receptor and HER2 Expression in Invasive Breast Cancer

Kalkman et al., *Am J Clin Pathol* 2014;142:16-22



Core biopsies (CNB) vs. Tumor Res. (TR)

Bai et al., 2011

- the levels of ER in tumor resection specimens was significantly lower than in core needle biopsy specimens. ...none of the cases in this study changed from a designation of ER-positive to a designation of ERnegative between the core needle biopsy and the tumor resection,

Pre-analytic variables and phospho-specific antibodies: the Achilles heel of immunohistochemistry

Summar Siddiqui and David L Rimm

Breast Cancer Research 2010, **12**:113

Critical roles of specimen type and temperature before and during fixation in the detection of phosphoproteins in breast cancer tissues

Sibylle Gündisch, Laura Annaratone, Christian Beese,
Enken Drecol, Caterina Marchiò, Elena Quaglino,
Anna Sapino, Karl-Friedrich Becker, Gianni Bussolati

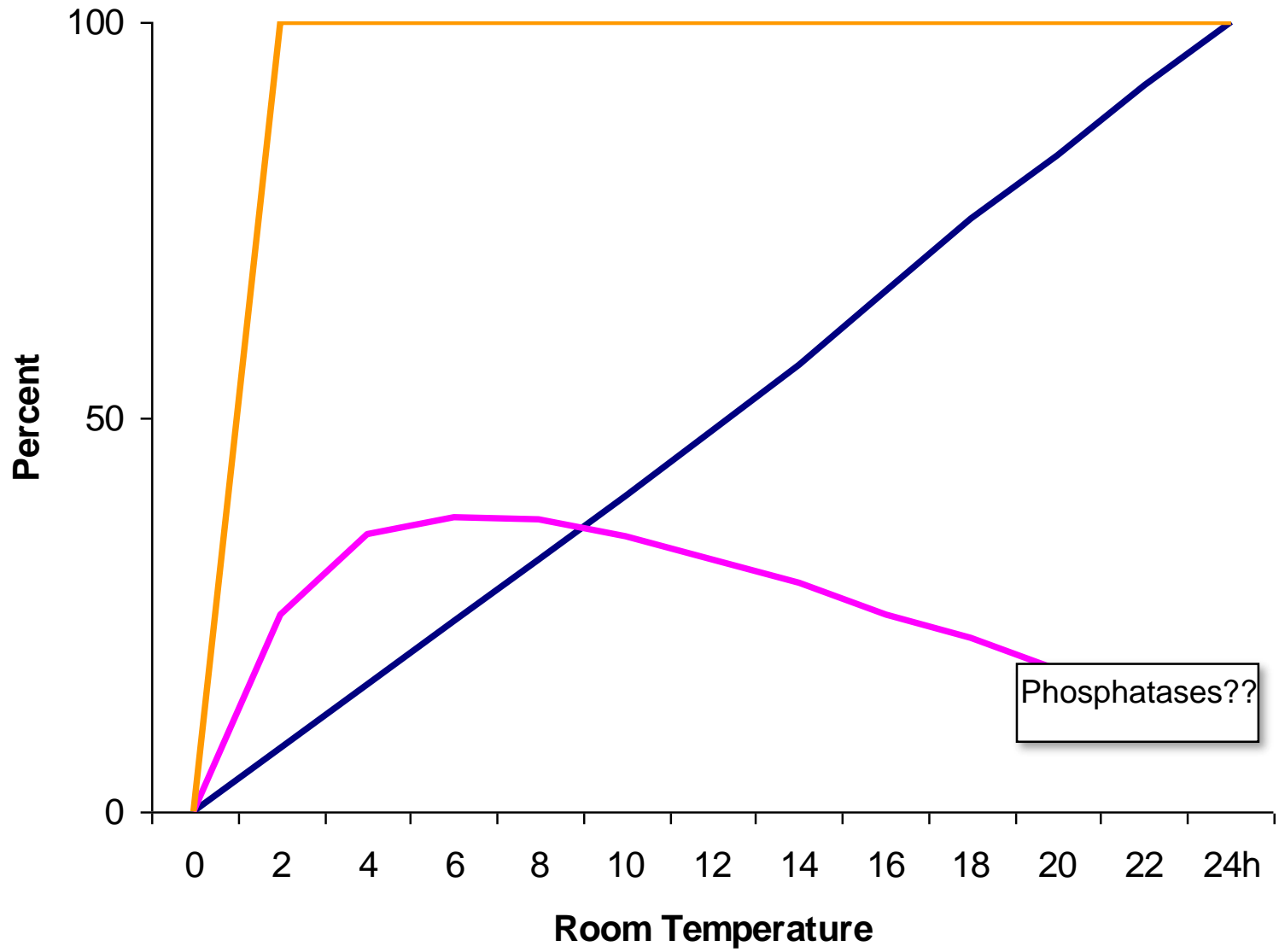
PLoS One. 2011;6(6):e21043. Epub Jun 15

A formalin fixation procedure preserving nucleic acid integrity

**Gianni Bussolati, Laura Annaratone, Enzo Medico,
Giuseppe D' Armento and Anna Sapino.**

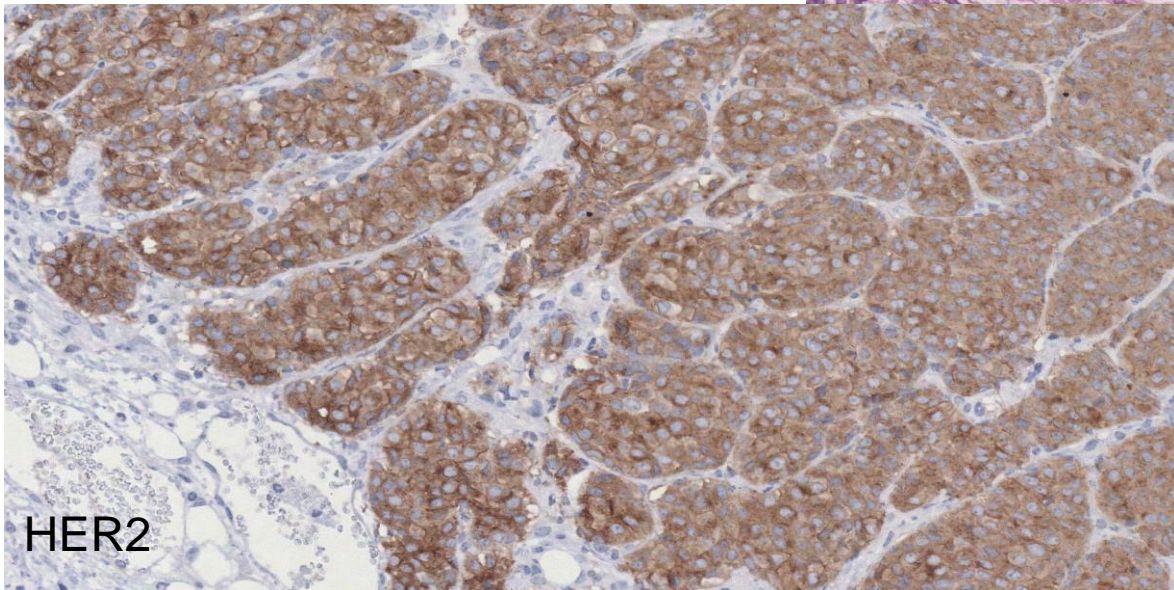
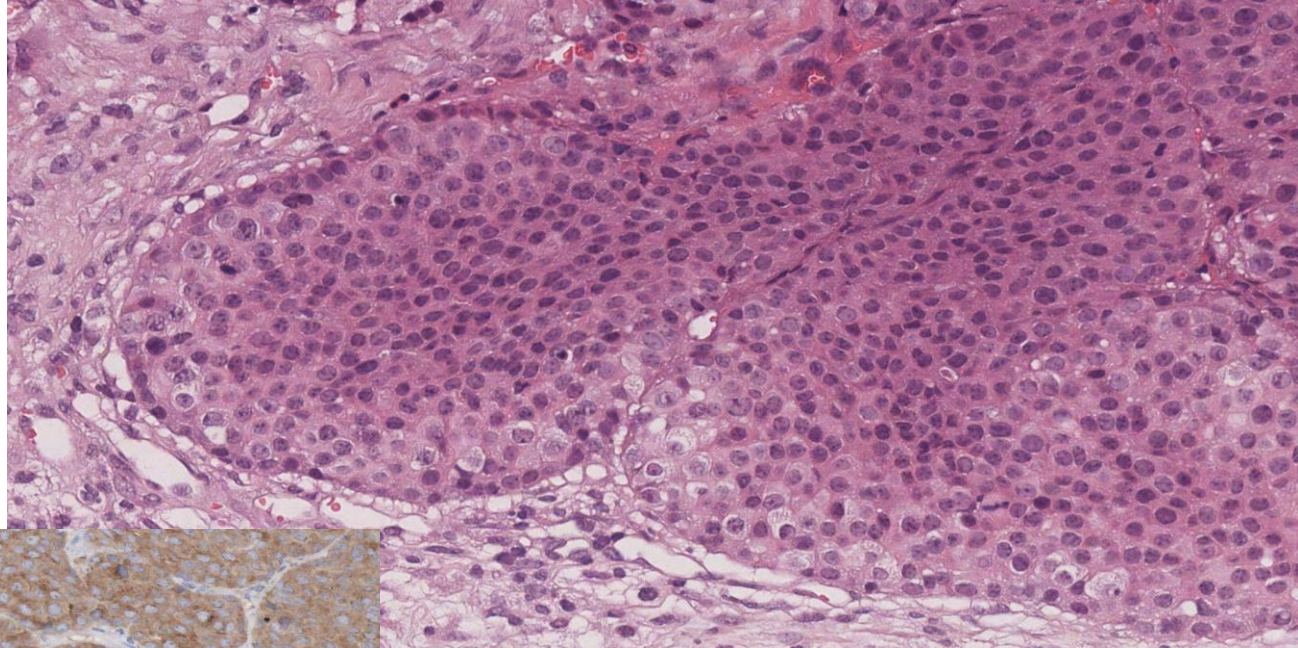
Experimental protocols for Cold Fixation (CF):

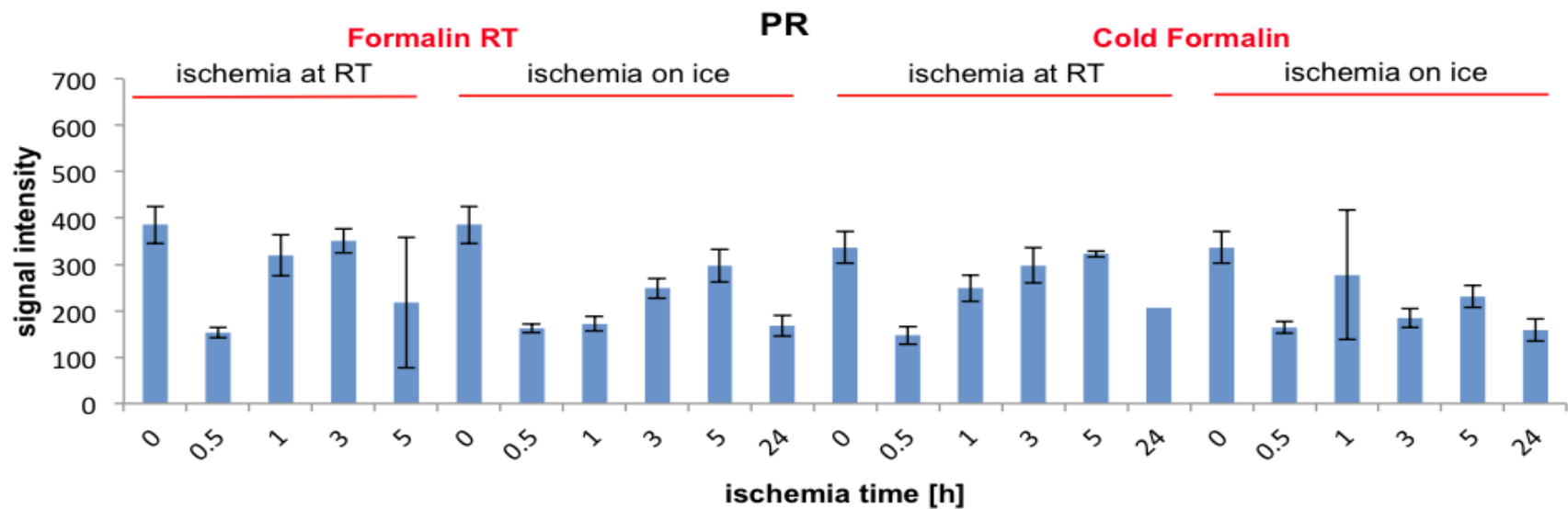
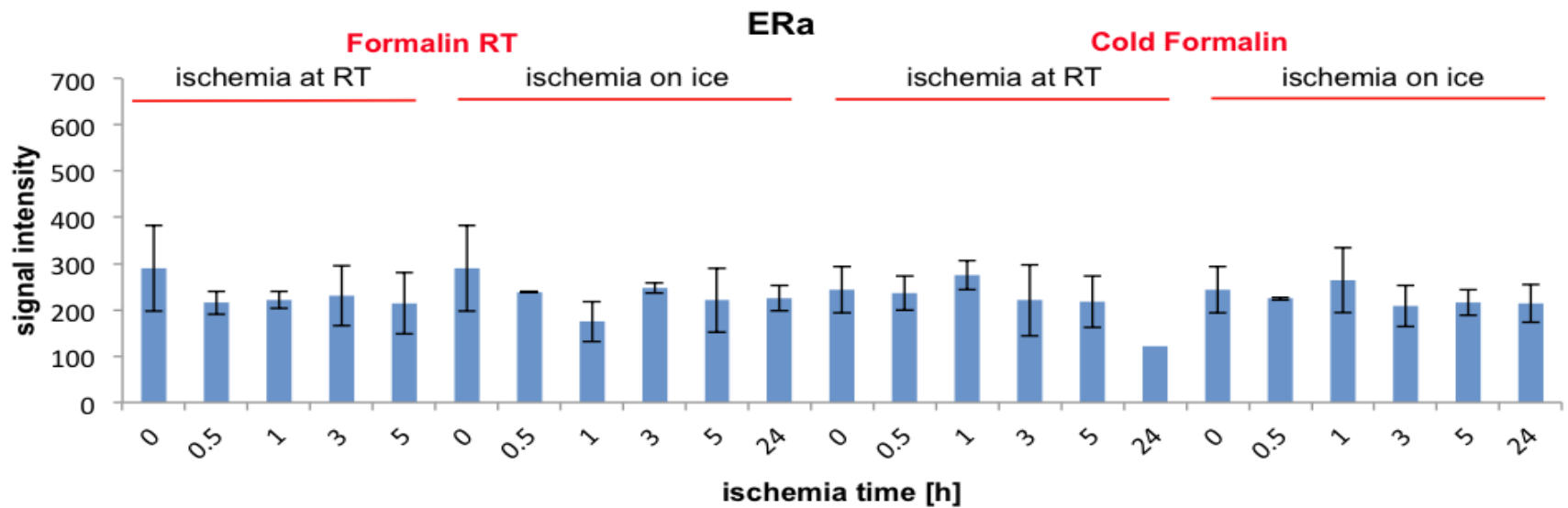
- 1) Fixation in PBF at 4° C for 24 h, followed by heating at 50° C for 20 min using MW, followed by Ethanol dehydration and Paraffin embedding.
- 2) Fixation in PBF at 4° C for 24 h, followed by heating at 90° C for 5 min, followed by Ethanol dehydration and Paraffin embedding.
- 3) Fixation in PBF at 4° C for 24 h, followed by 95%Ethanol at 4° C for 2 h, then standard Ethanol dehydration and Paraffin embedding.



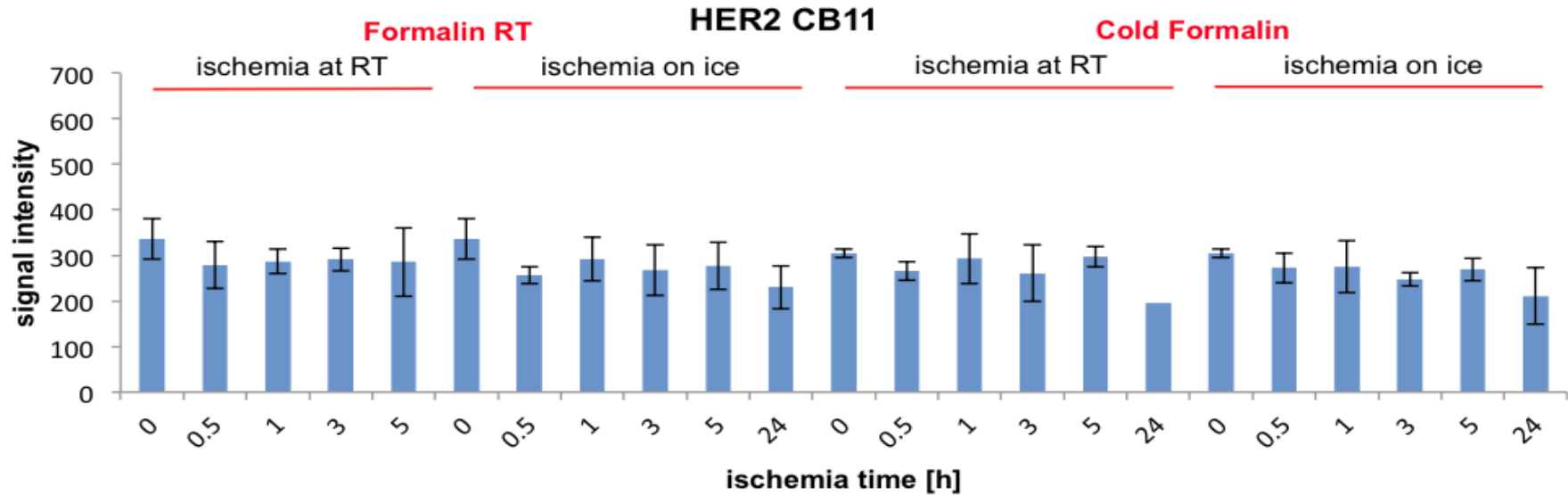
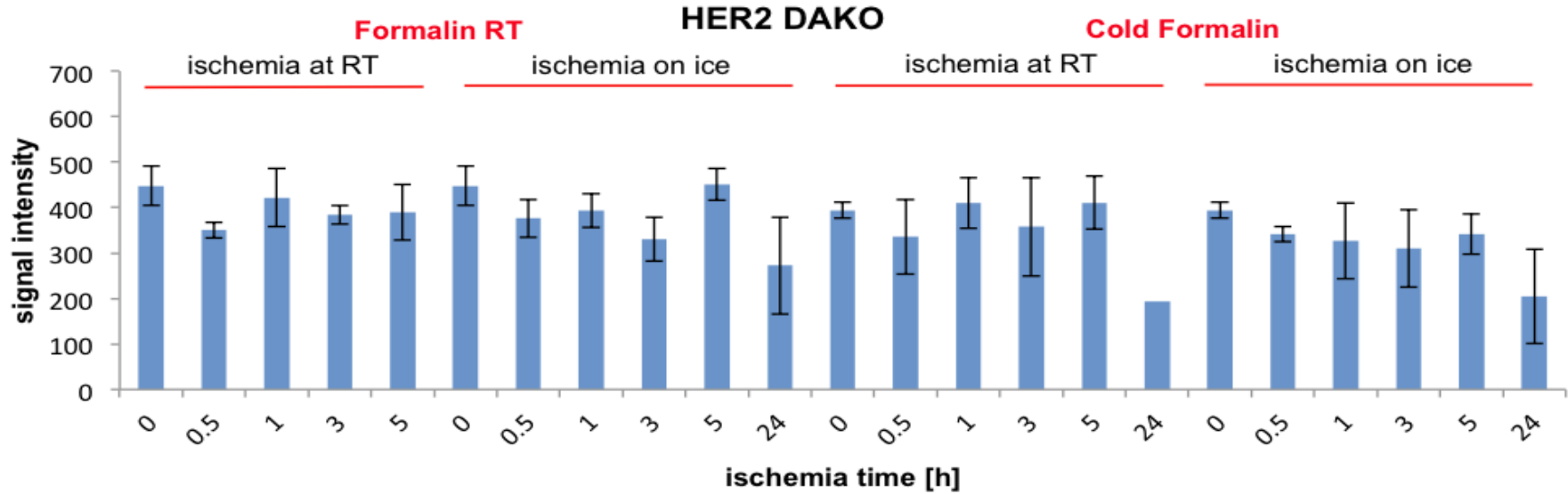
Phosphatases??

HER2 over-expr. Breast Carcinoma in Balb/CneuT mice

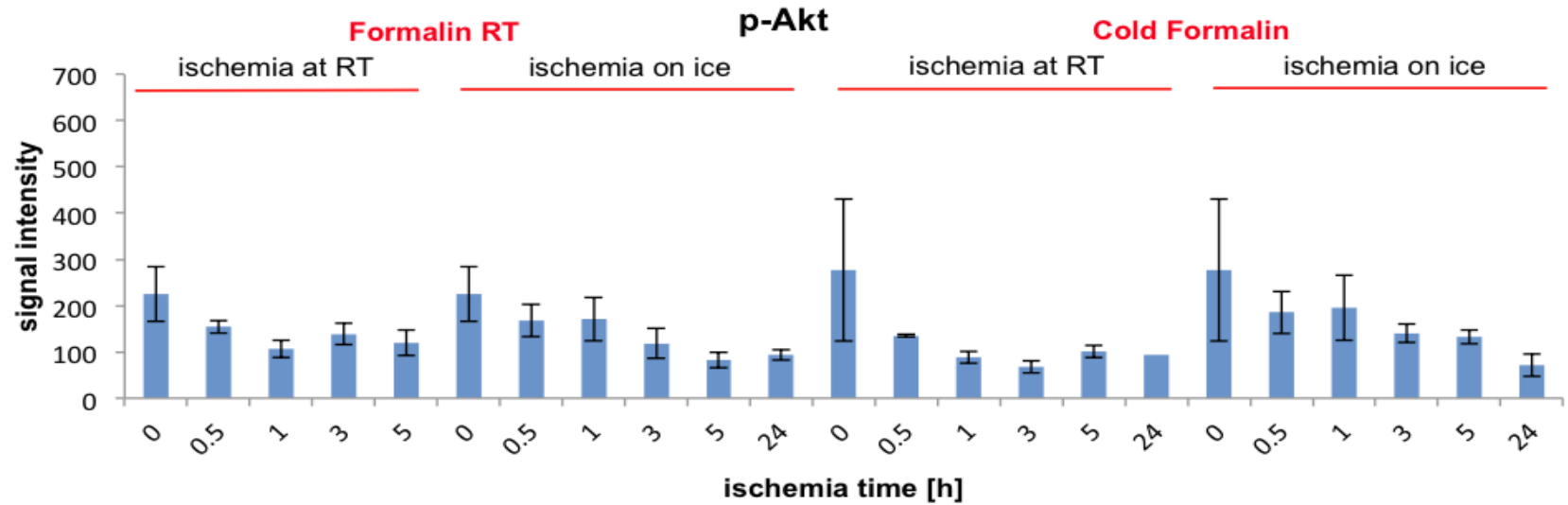
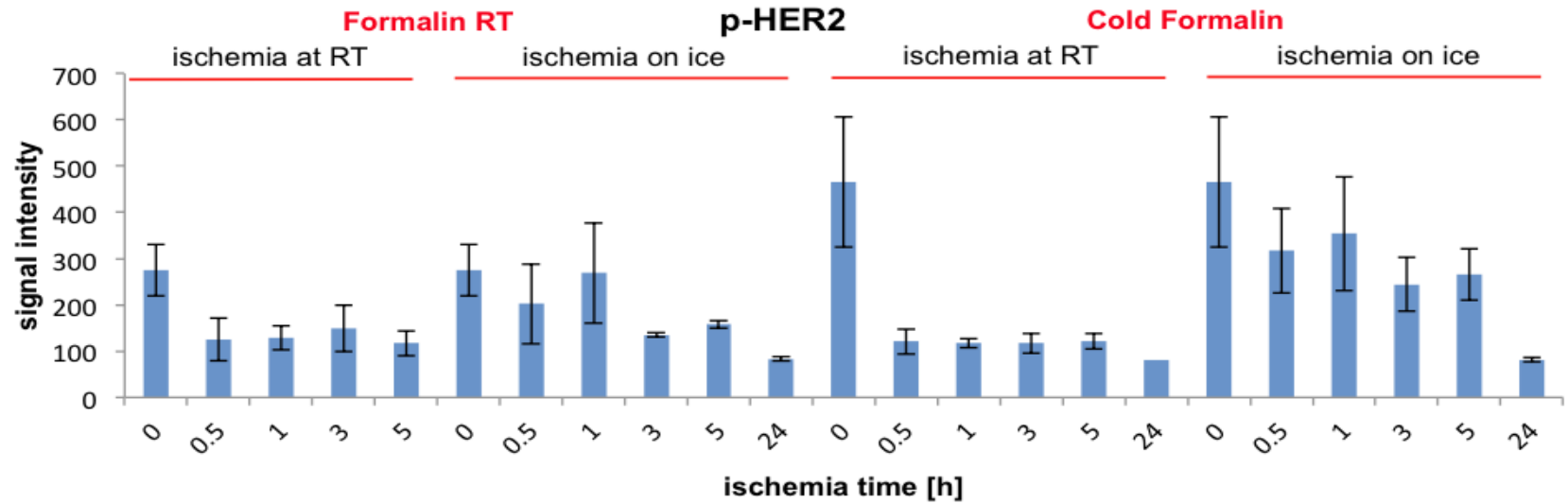


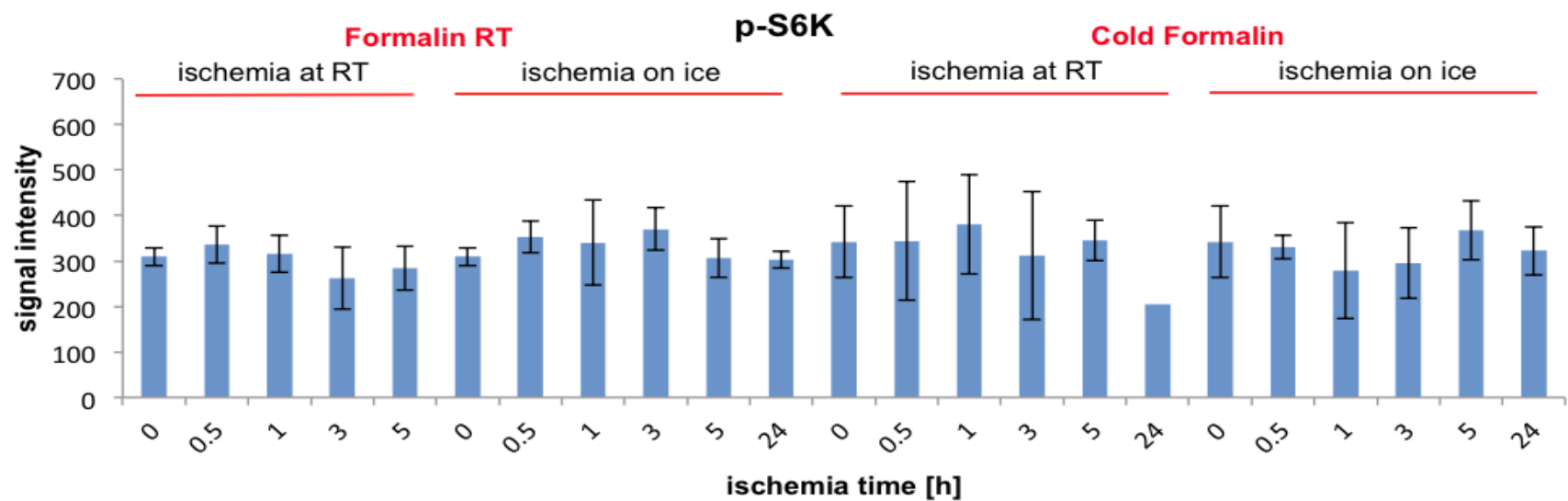
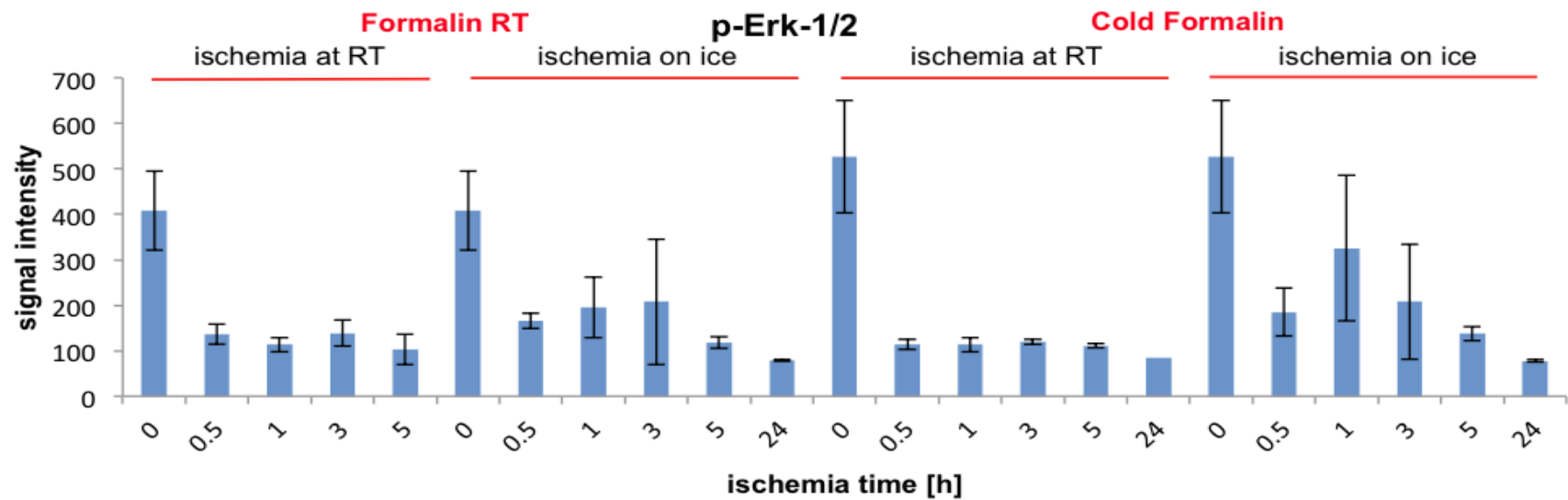


Mouse samples, FFPE, total proteins:

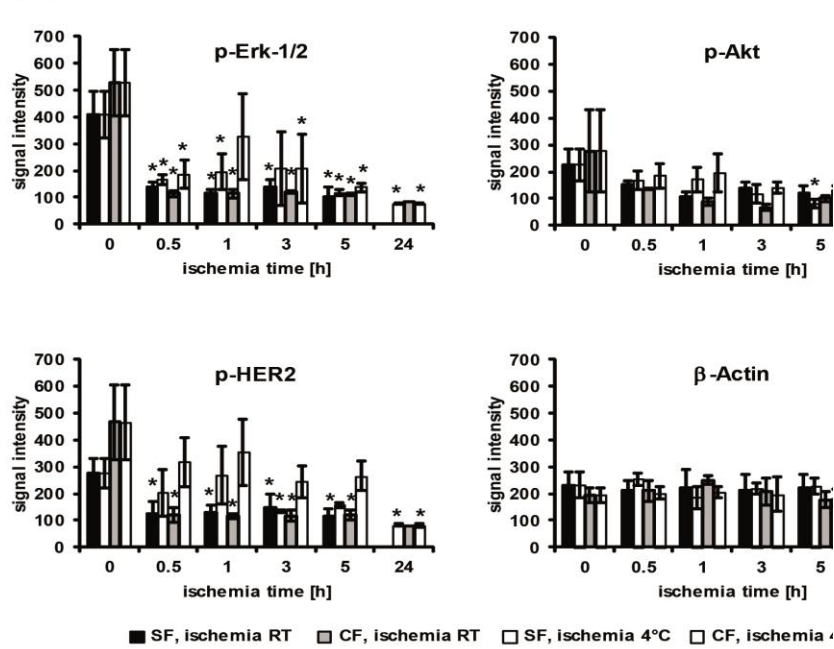


Mouse samples, FFPE, phosphoproteins:

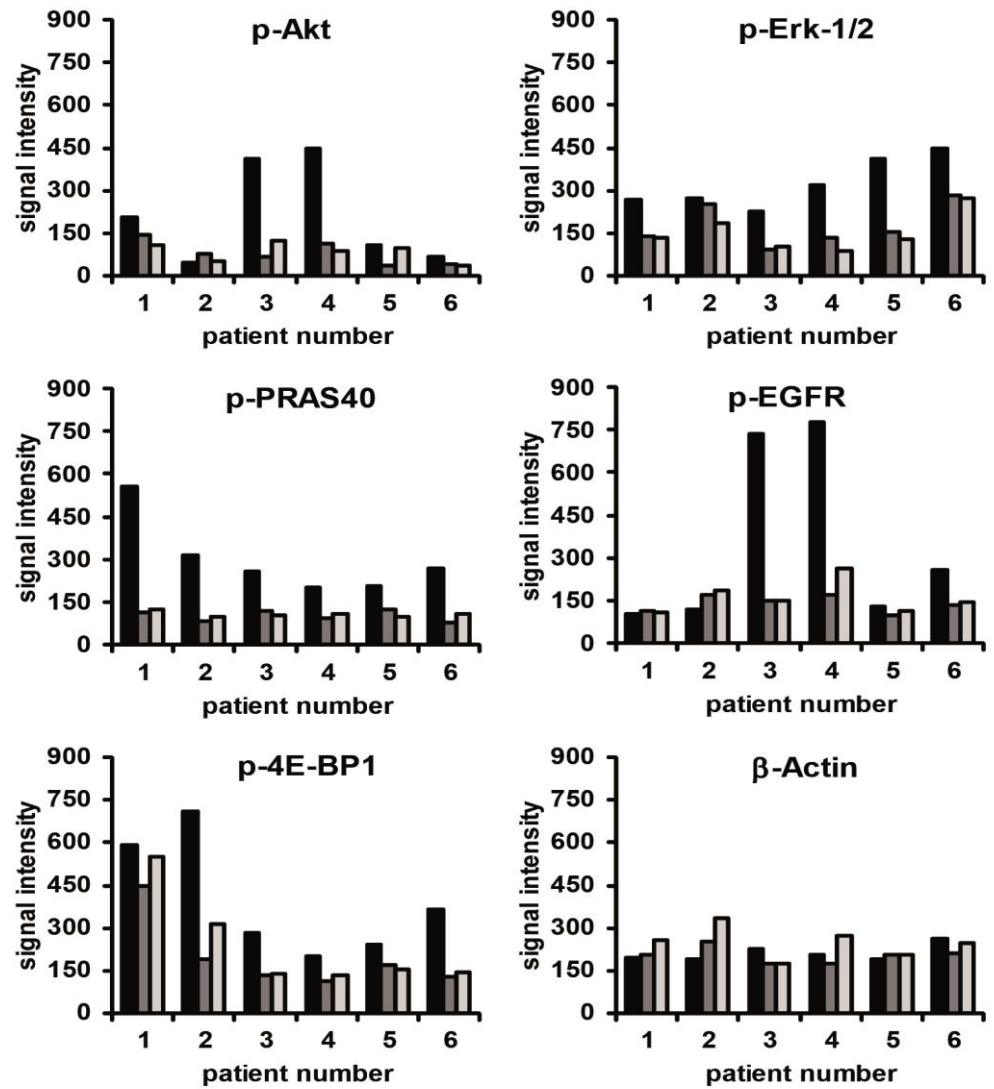




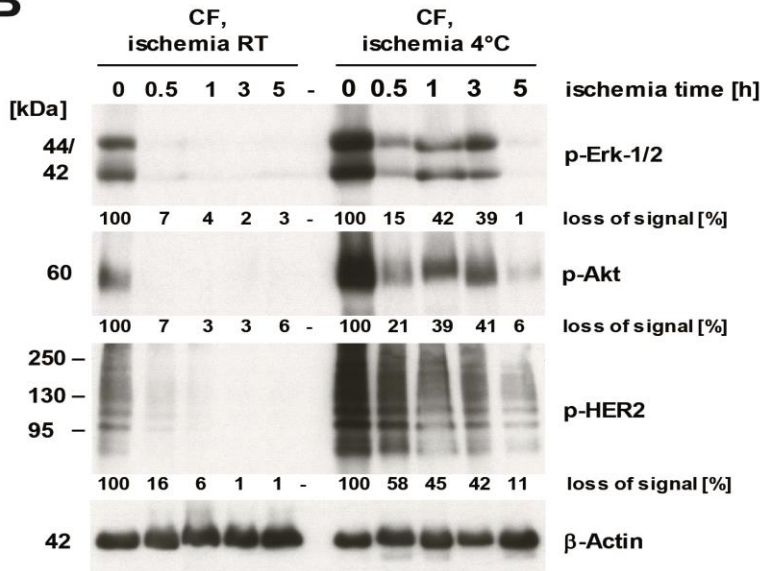
A



■ SF, biopsy ■ CF, resection specimen □ SF, resection specimen



B



Guendisch S. et. al, 2015

- 1) cold conditions are superior to conventional fixation at room temperature: fixation in cold formalin (4° C) improves the preservation of proteins and phosphoproteins in breast cancer tissues .
- 2) evaluation of phosphoproteins seems to be reliable on core biopsy specimens and may be misleading in resection specimens due to sample processing.

Conclusions

- Antigenic reactivity /
/antibody Affinity-Avidity
- Structural preservation
- Insolubility /Stability
- Formalin / Alcohol

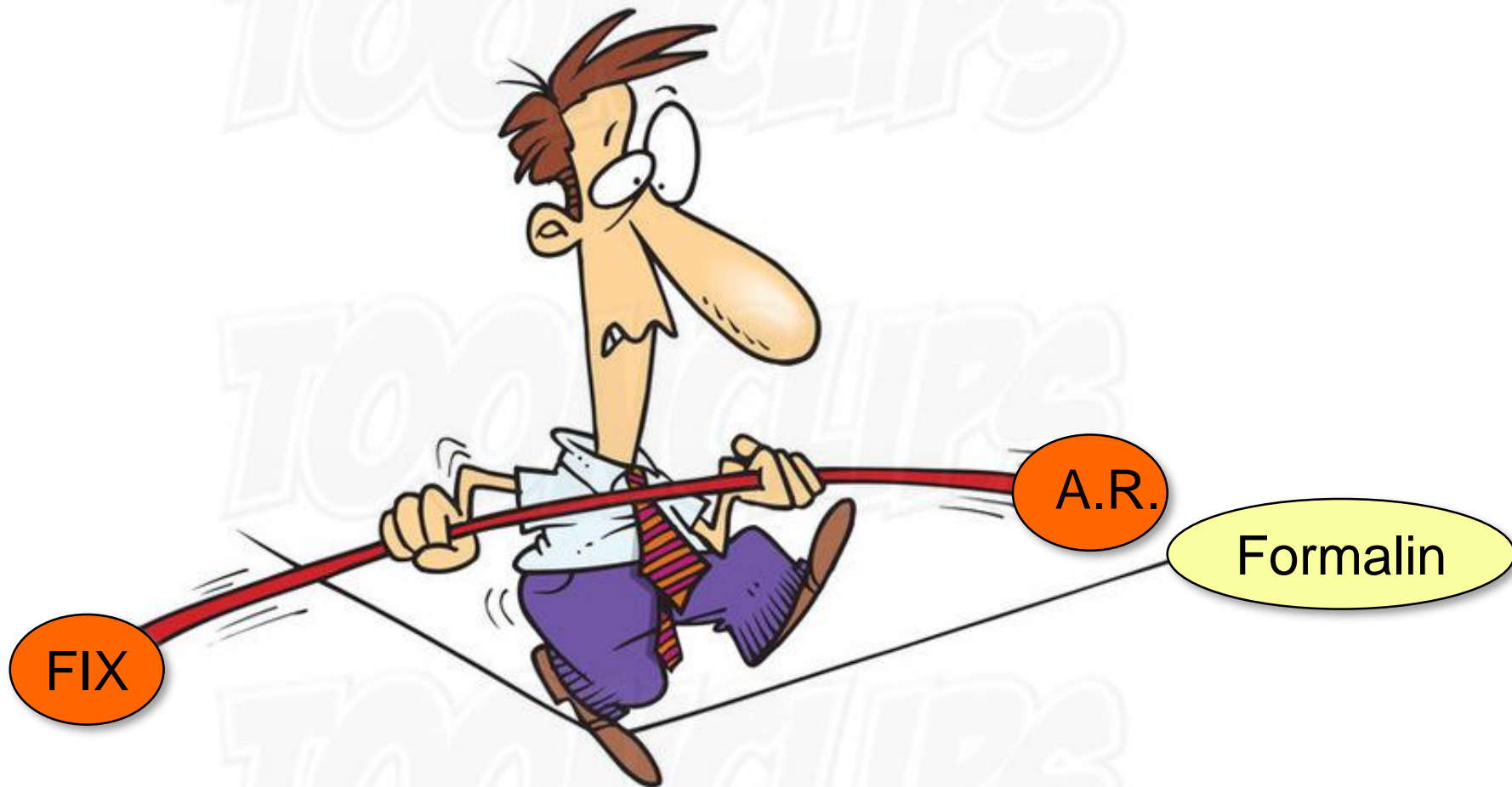
Compromise

Practice

Impact on quantitation



..keeping balance on a tight rope



..keeping balance on an unsafe rope

COMMISSION REGULATION (EU) No 895/2014**of 14 August 2014****amending Annex XIV to Regulation (EC) No 1907/2006 of the European Parliament and of the Council concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH)**

(Text with EEA relevance)

THE EUROPEAN COMMISSION,

Having regard to the Treaty on the Functioning of the European Union,

Having regard to Regulation (EC) No 1907/2006 of the European Parliament and of the Council of 18 December 2006 concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), establishing a European Chemicals Agency, amending Directive 1999/45/EC and repealing Council Regulation (EEC) No 793/93 and Commission Regulation (EC) No 1488/94 as well as Council Directive 76/769/EEC and Commission Directives 91/155/EEC, 93/67/EEC, 93/105/EC and 2000/21/EC ⁽¹⁾, and in particular Articles 58 and 131 thereof,

Whereas:

- (1) Formaldehyde, oligomeric reaction products with aniline (technical MDA) meets the criteria for classification as carcinogenic (category 1B) in accordance with Regulation (EC) No 1272/2008 of the European Parliament and the Council ⁽²⁾ and therefore meets the criteria for inclusion in Annex XIV to Regulation (EC) No 1907/2006 set out in Article 57(a) of that Regulation.

Entry Nr	Substance	Intrinsic property(ies) referred to in Article 57	Transitional arrangements		Exempted (categories of) uses	Review periods
			Latest application date (*)	Sunset date (**)		
'23.	Formaldehyde, oligomeric reaction products with aniline (technical MDA) EC No: 500-036-1 CAS No: 25214-70-4	Carcinogenic (category 1B)	22 February 2016	22 August 2017	—	—

Requirements of a (potential) substitute of Formalin

Non Toxic

Cheap

Cross-linking (Aldehyde)

Preservation of Morphology & Antigenicity.
(Nucleic Acids)