FONDAZIONE BRUNO KESSLER

PROVINCIA AUTONOMA DI TRENTO
Assessorato alla salute e solidarietà sociale

Azienda Provinciale per i Servizi Sanitari
Provincia Autonoma di Trento

Ordine dei Medici Chirurghi e degli Odontoiatri della provincia di Trento

High precision data production and integration: the “next generation step” in immunohistochemistry

June 8th, 2015

New techniques, antibodies and introduction to multiplexing

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Introduction to multiplexing

	Serial sectioning	Double/multiple staining (no stripping)	Multiplexing (stripping)
N. of sections	As many as the N. of stains	About half as the N. of stains	One
“Custom” IHC/IF	none	Color contrast double stains / multiple IF	Erasable, directly conjugated Abs / Ab stripping / Spectral deconvolution
Imaging	any	WSI*, confocal	WSI**
Image analysis	Stereology reconstruction sw	Image cytometry	Image cytometry
Limits	Lesion representation in tissue	Species / colours / fluorochromes pairing	Tissue wear & tear

* WSI = whole slide image; ** the use of WSI is compulsory

REF	First name	Method	Application
J Histochem Cytochem **26,** 322–324 (1978)	Tramu G	$KMnO_4$ – H_2SO_4 -> $Na_2S_2O_5$	Double IHC
J Histochem Cytochem **34,** 1725–1729 (1986)	Kolodziejczyk E	PBS-Glycerin @ 130 C x 4 min	IHC and IF
Cytometry **47,** 32–41 (2002)	Wählby C	Eution in 0.1M lysine buffer pH 2 -> MWO boiling	IF
PNAS USA **110,** 11982–11987 (2013)	Gerdes MJ	Alkaline oxidation chemistry on cyanine dyes	IF
J Histochem Cytochem **57,** 567–575 (2009)	Pirici D	25 mM glycine-HCl, 10% SDS, pH 2	IF, IHC
J Histochem Cytochem **57,** 899–905 (2009)	Glass P	0.15 M $KMnO_4$/0.01 M H_2SO_4 x 2 min	IHC
J Histochem Cytochem **62,** 519–531 (2014)	Gendusa R	62 mM Tris, 2% SDS, 100 mM b-mercaptoethanol, pH 6.75	IF, IHC

PRIOR ART: problems

Antigen is lost

Antibodies do not detach

Antibodies do not denaturate

Sensitivity is increased with repeated staining

Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4

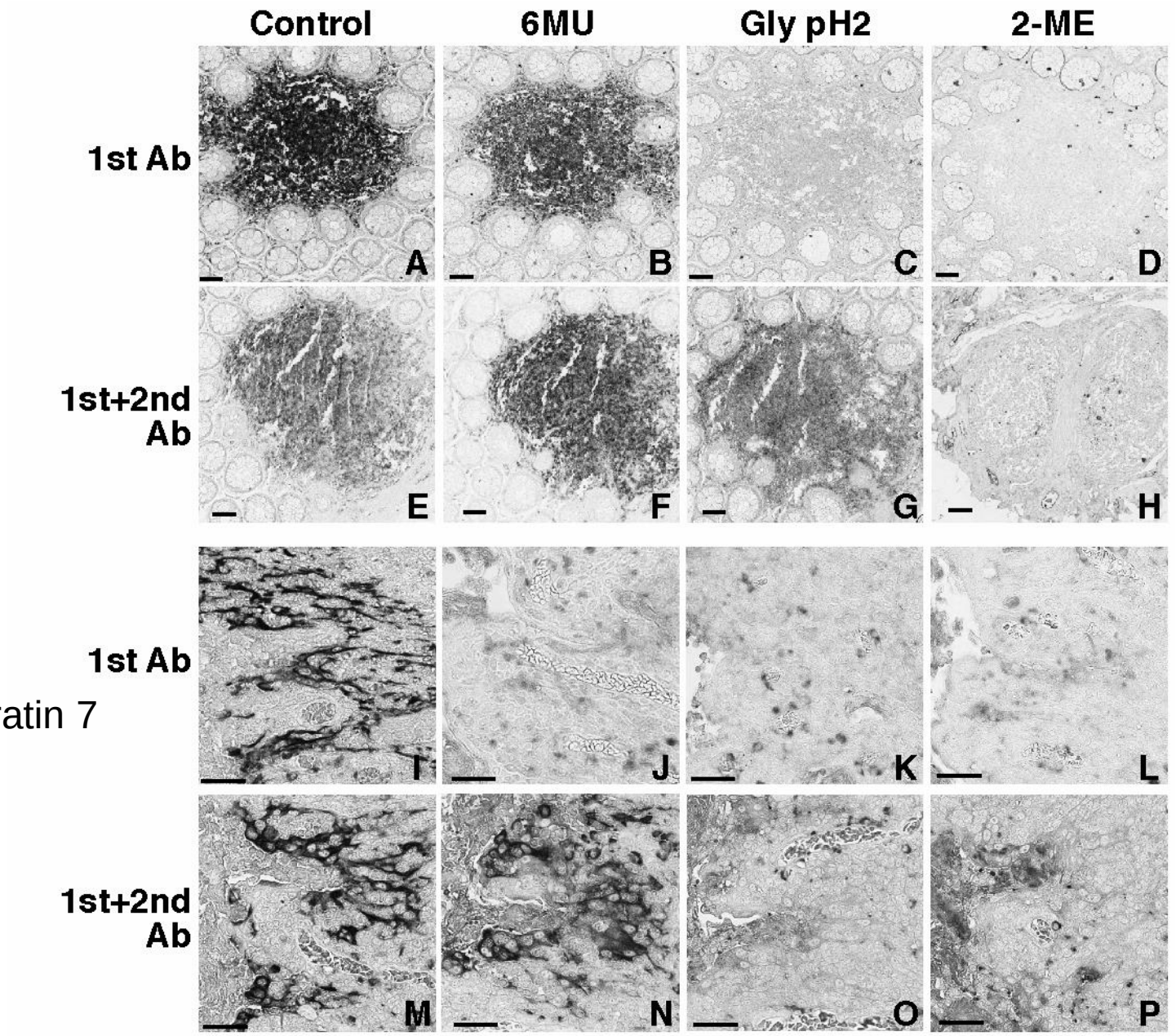
by
U. K. LAEMMLI
MRC Laboratory of Molecular Biology,
Hills Road, Cambridge

Using an improved method of gel electrophoresis, many hitherto unknown proteins have been found in bacteriophage T4 and some of these have been identified with specific gene products. Four major components of the head are cleaved during the process of assembly, apparently after the precursor proteins have assembled into some large intermediate structure.

Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227,** 680–685 (1970).

CD45 Rabbit
Monoclonal
EP322Y
3.60×10^{-11}

Anti Cytocheratin 7
Rabbit
Monoclonal
EPR1619Y
2.10×10^{-10}



SOME UNCOMFORTABLE TRUTHS

- Antibodies survive “denaturing” conditions such as 6MU and/or heath >90C
- Antibodies remain attached to the antigen in extreme conditions
- The higher the affinity, the less probable an antibody is eluted
- The affinity of the secondary/tertiary antibody is transferred to the first, antigen-binding antibody

ANTIBODIES R FOREVER

Histopathology

Histopathology 2013, **63**, 869–876. DOI: 10.1111/his.12225

Antibodies are forever: a study using 12–26-year-old expired antibodies

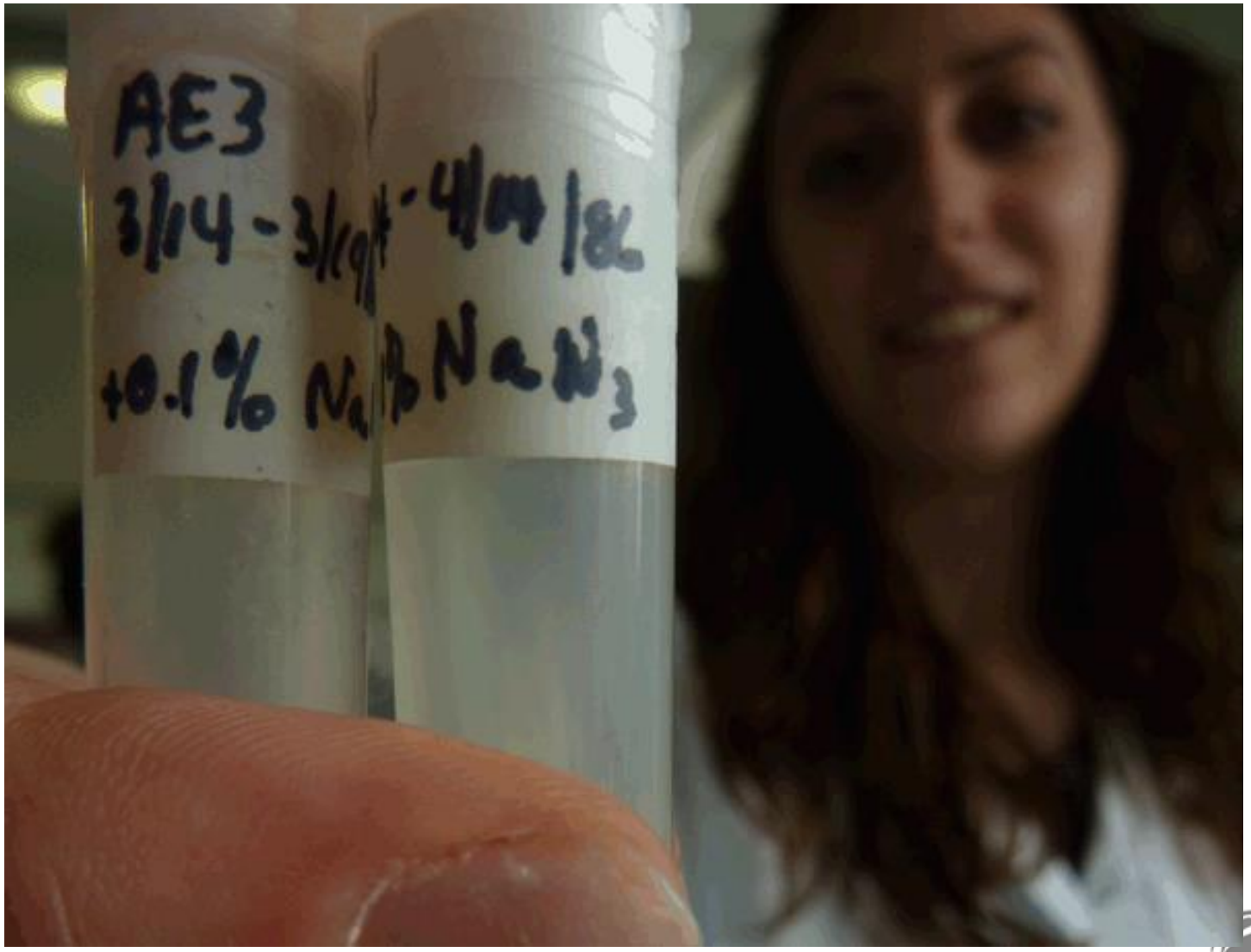
Maria C Argentieri,[1] Daniela Pilla,[1] Alice Vanzati,[1,2] Silvia Lonardi,[3] Fabio Facchetti,[3] Claudio Doglioni,[4] Carlo Parravicini[5] & Giorgio Cattoretti[1,2]

Aims: The aim of this study was to investigate whether the shelf-life of diagnostic antibodies is longer than the expiry date on the label.

Methods and results: Four independent laboratories tested a small number of diagnostic antibodies kept at +4°C for 12–26 years, and found them to work perfectly on routine histology sections.

Conclusions: Diagnostic antibodies may have a workable half-life in excess of 10 years, and the emphasis on performance should shift to the preservation of antigenic targets in the tissue.

ANTIBODIES R FOREVER



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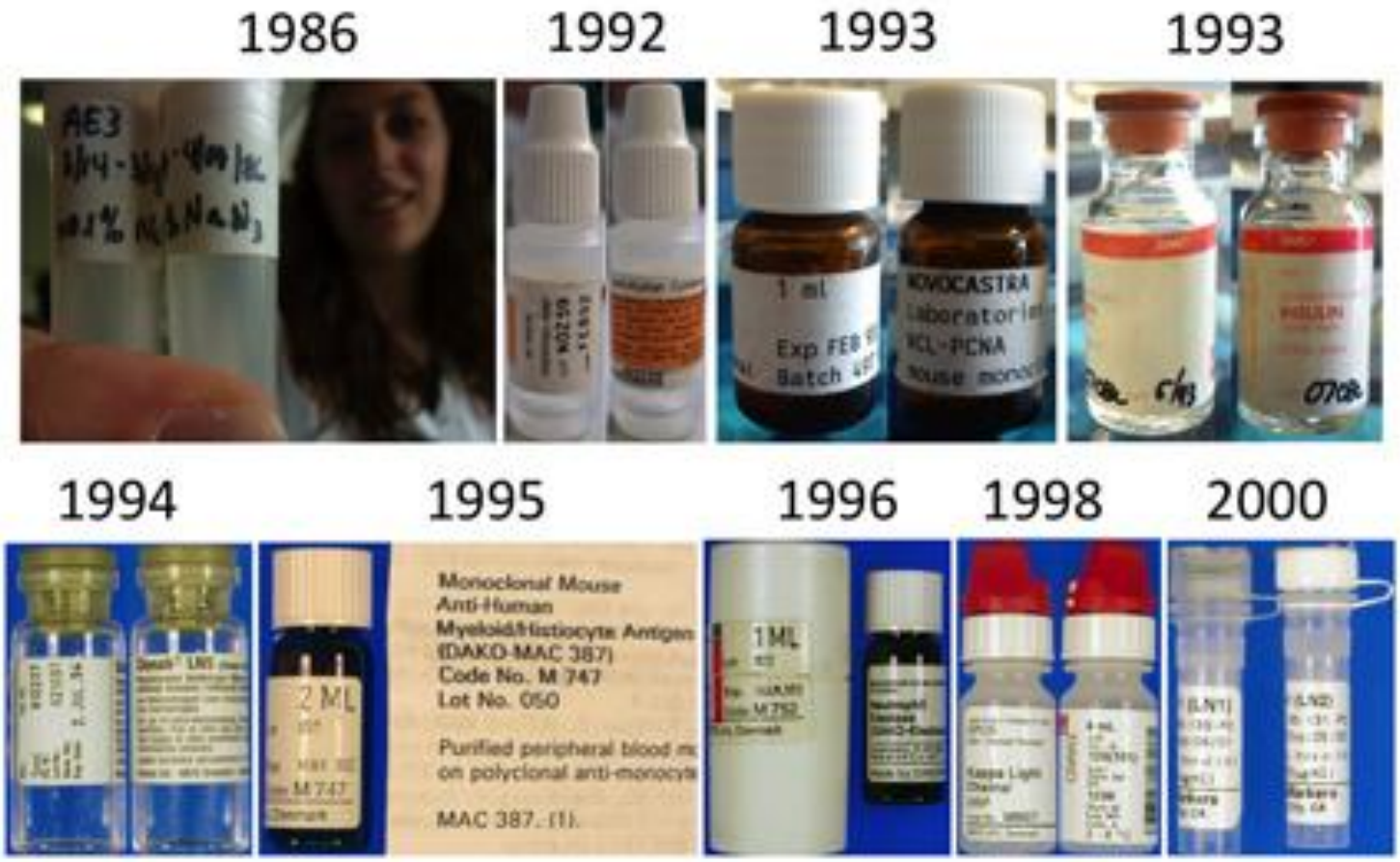

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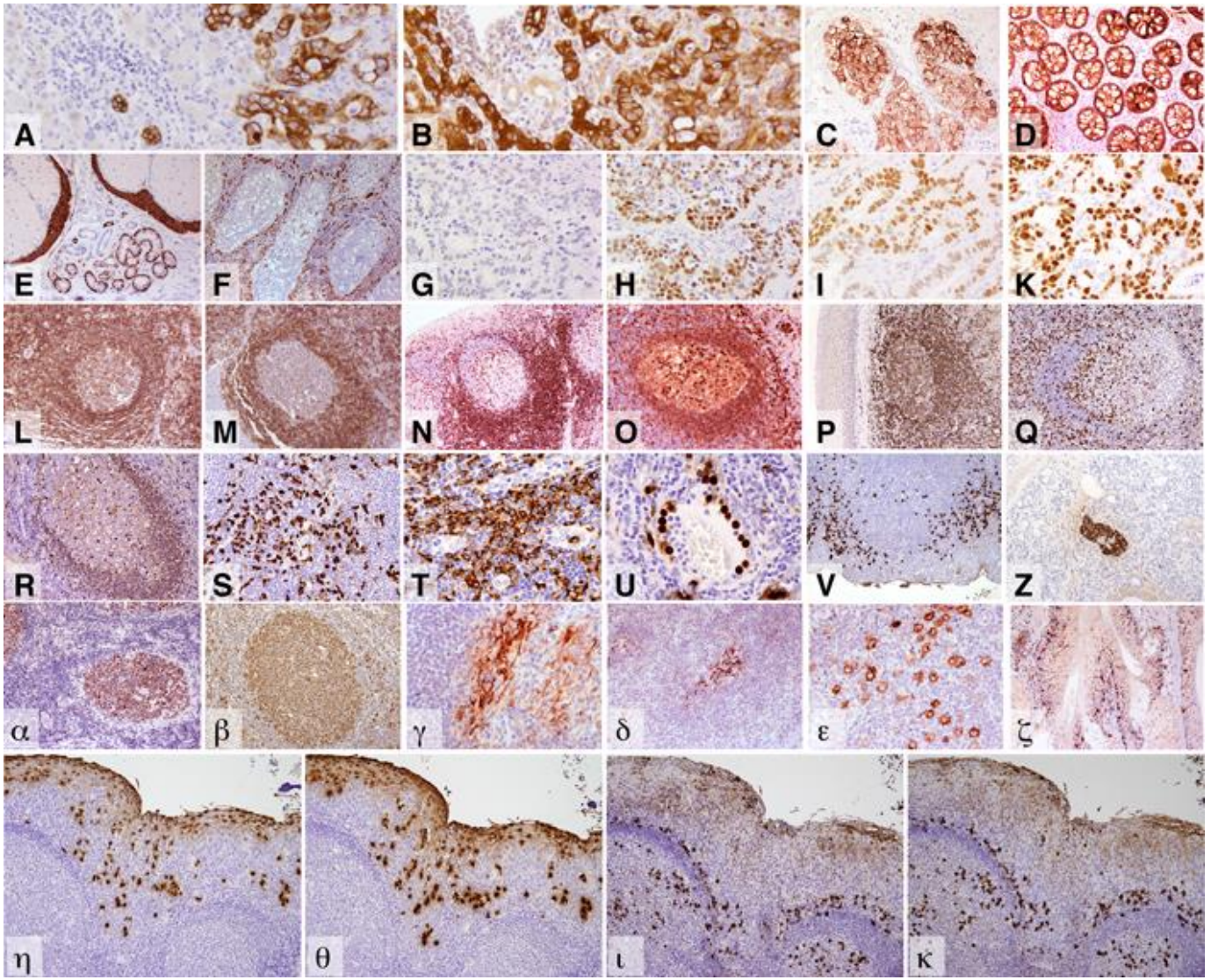
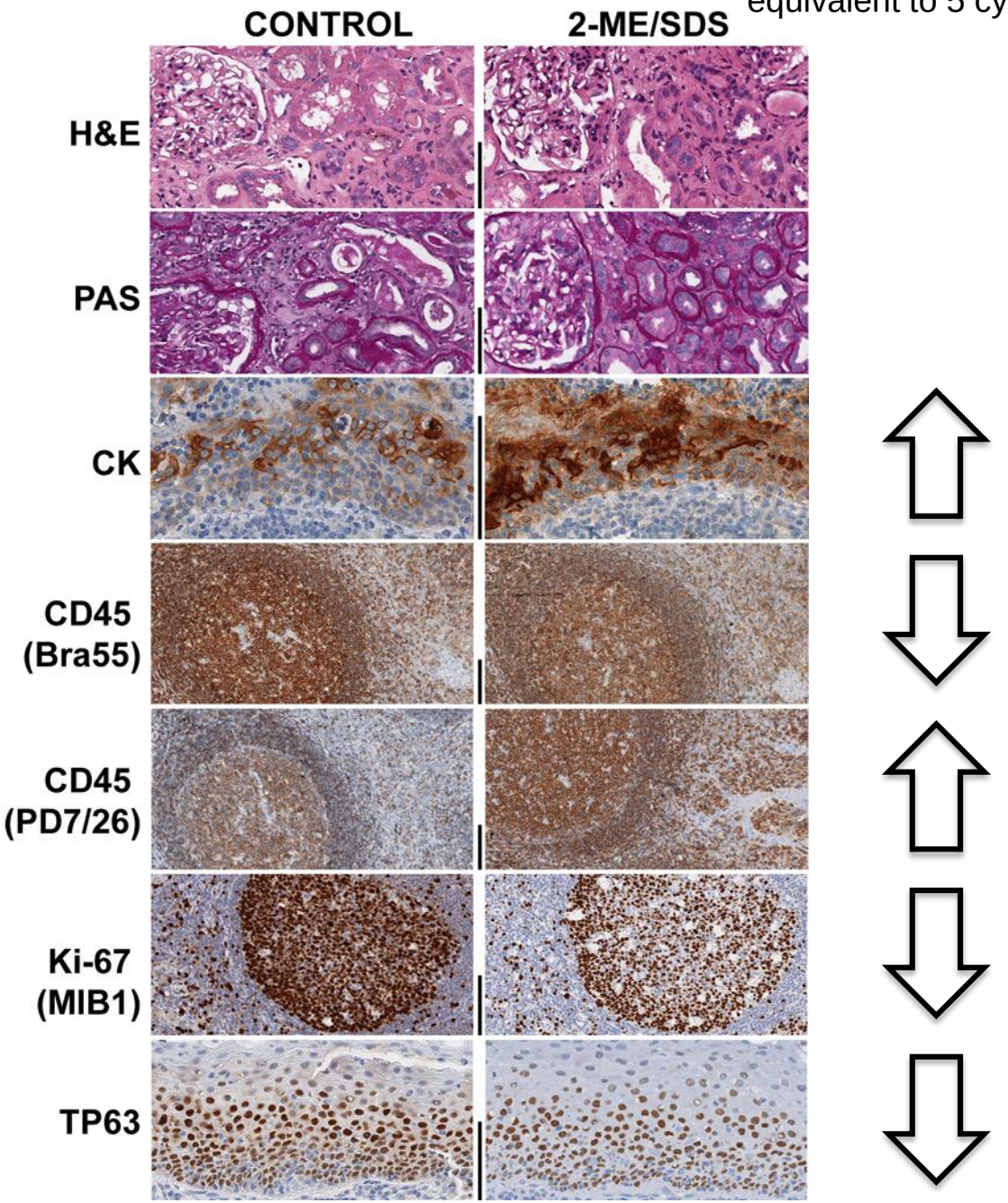


2-ME/SDS affects antigenicity?

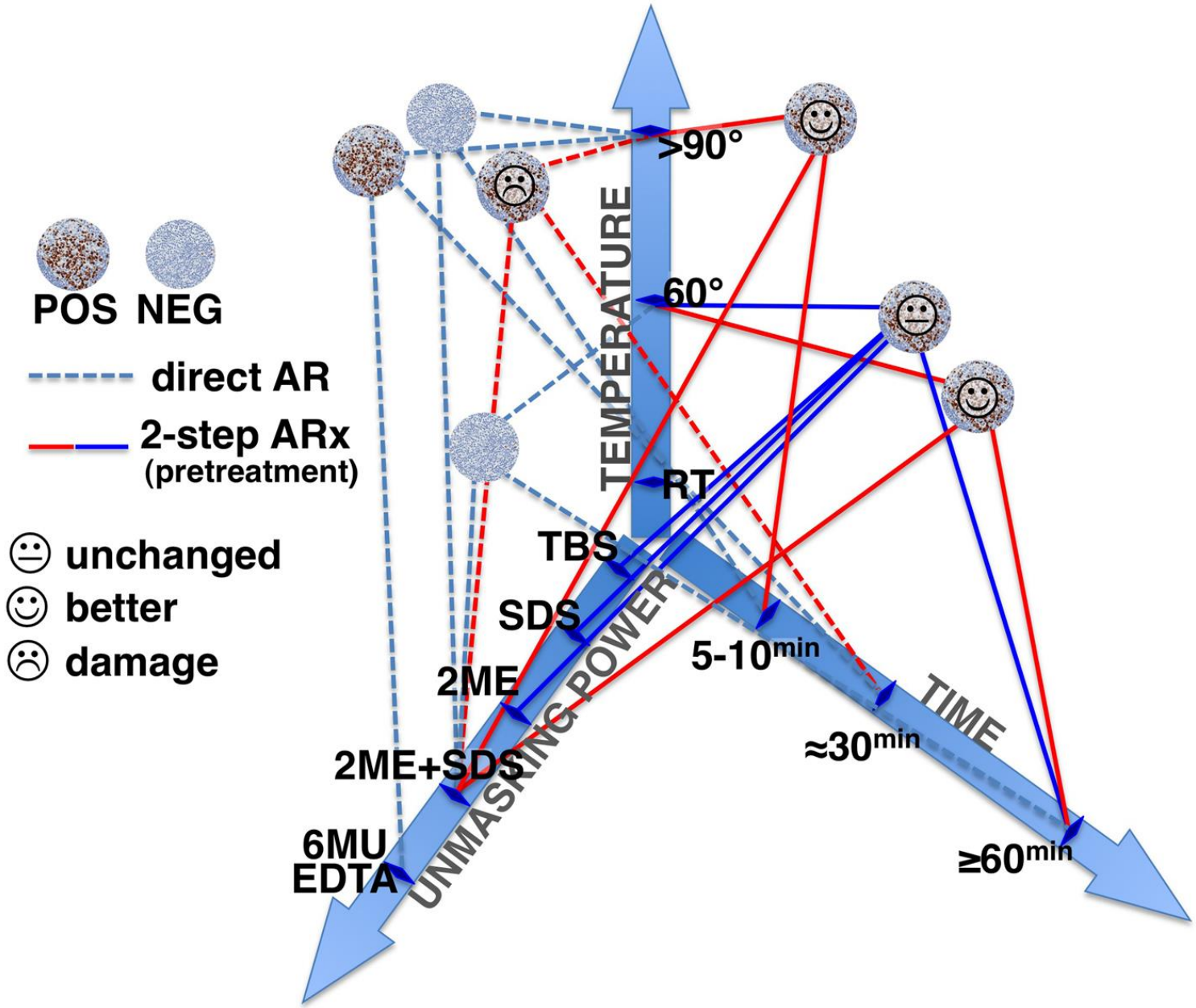
equivalent to 5 cycles



Journal of Histochemistry & Cytochemistry **62,** 519–531 (2014).

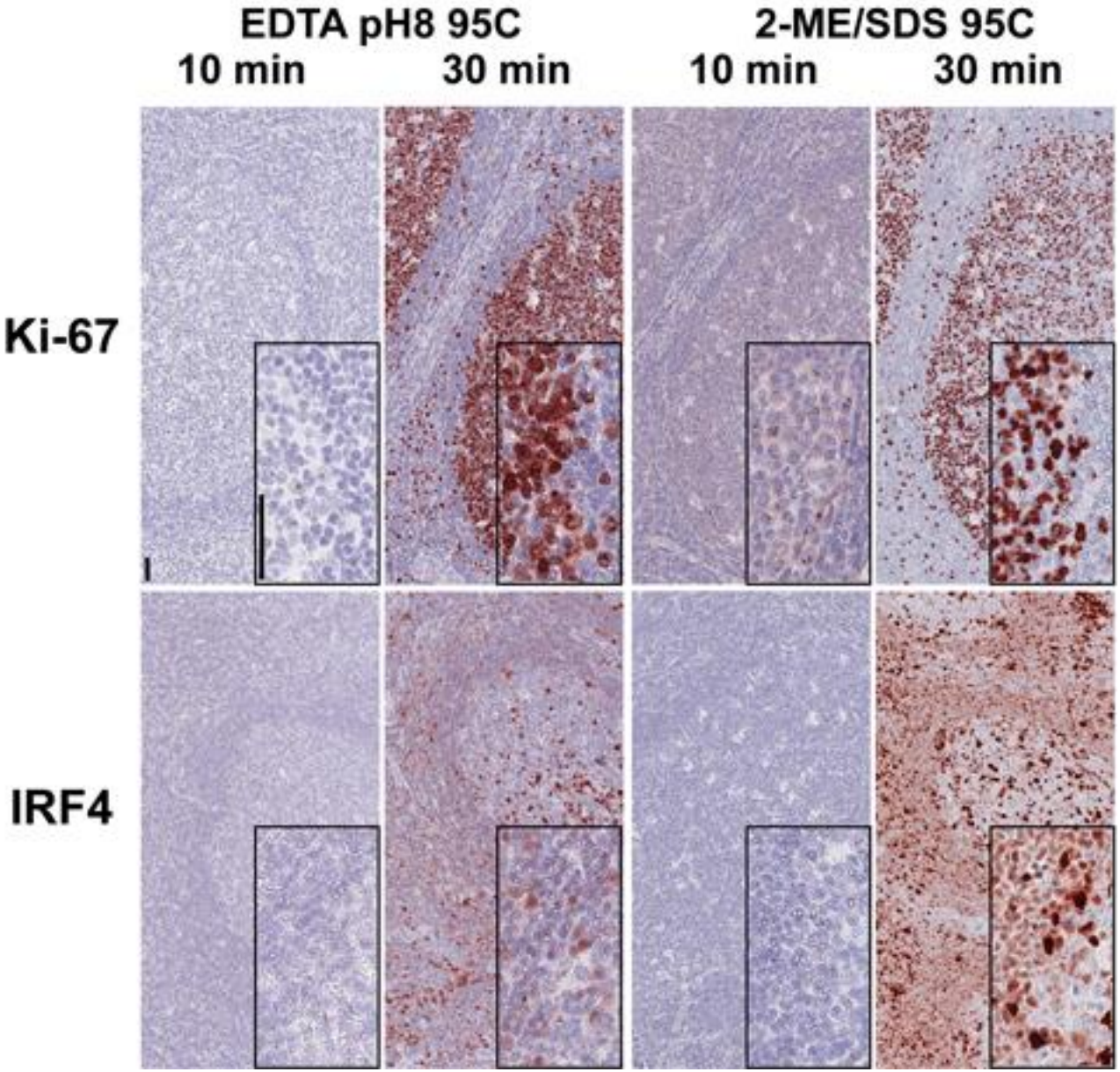
2-ME/SDS is a retrieval agent?

2-ME/SDS plus heat is the core of a protein extraction method from FFPE.
Where is the balance btw retrieval and extraction?



A 2-Step Laemmli and Antigen Retrieval Method Improves Immunodetection
Carla R. Scalia, Rossella Gendusa, and Giorgio Cattoretti, *AIIM, in press, 2015*

2-ME/SDS as AR



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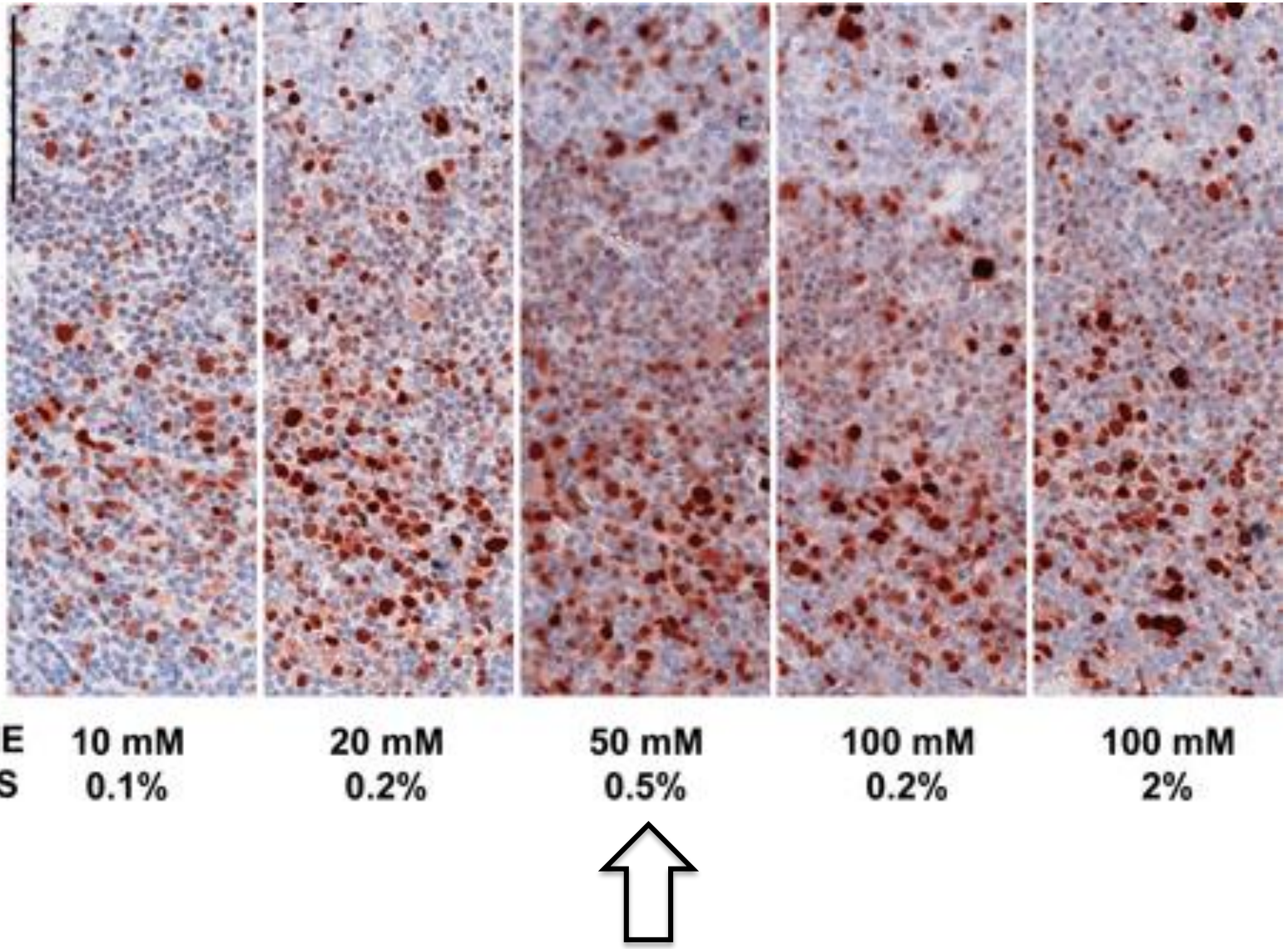
2-ME/SDS as AR

IRF4



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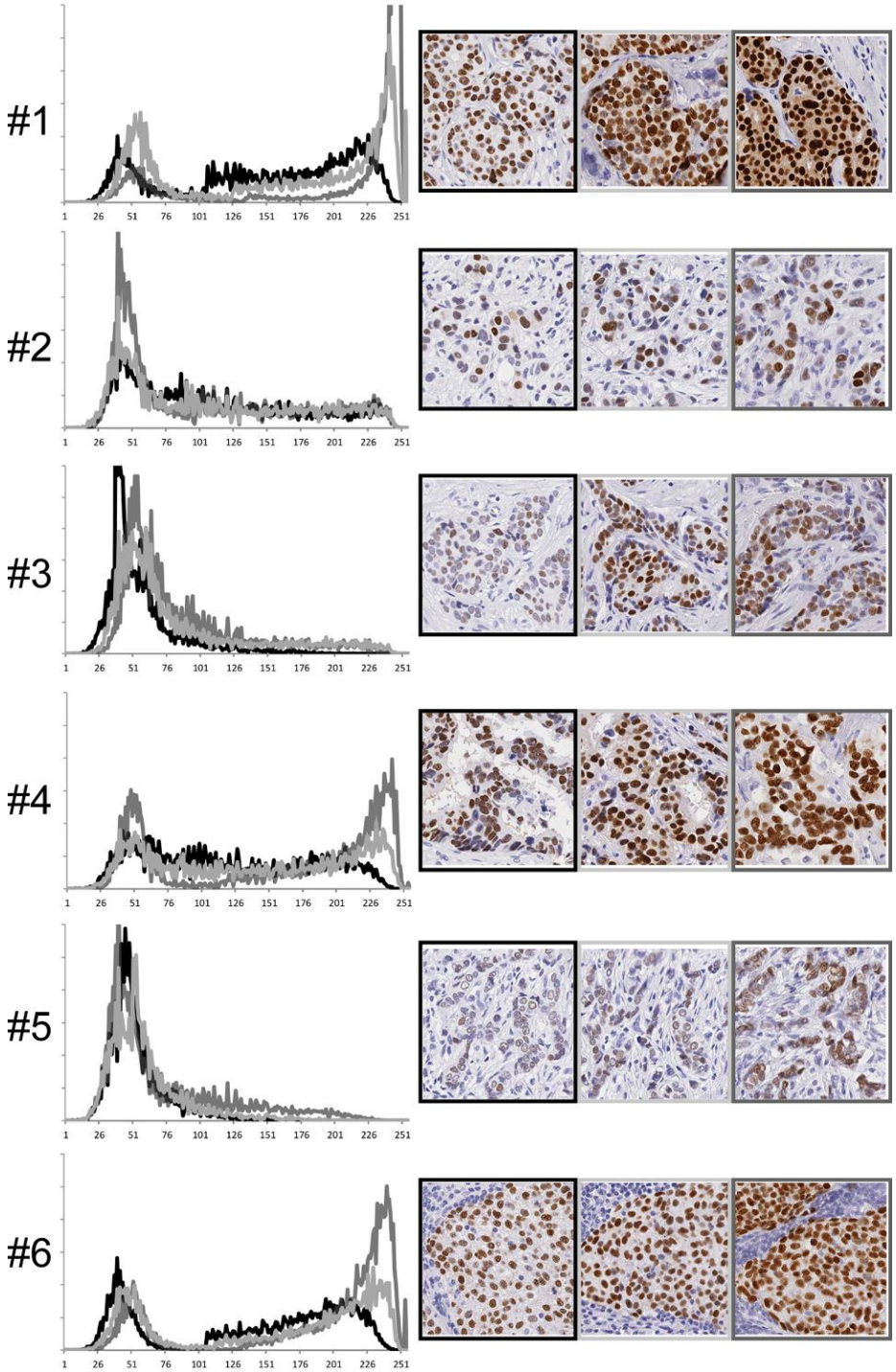

Breast cancer:
ER

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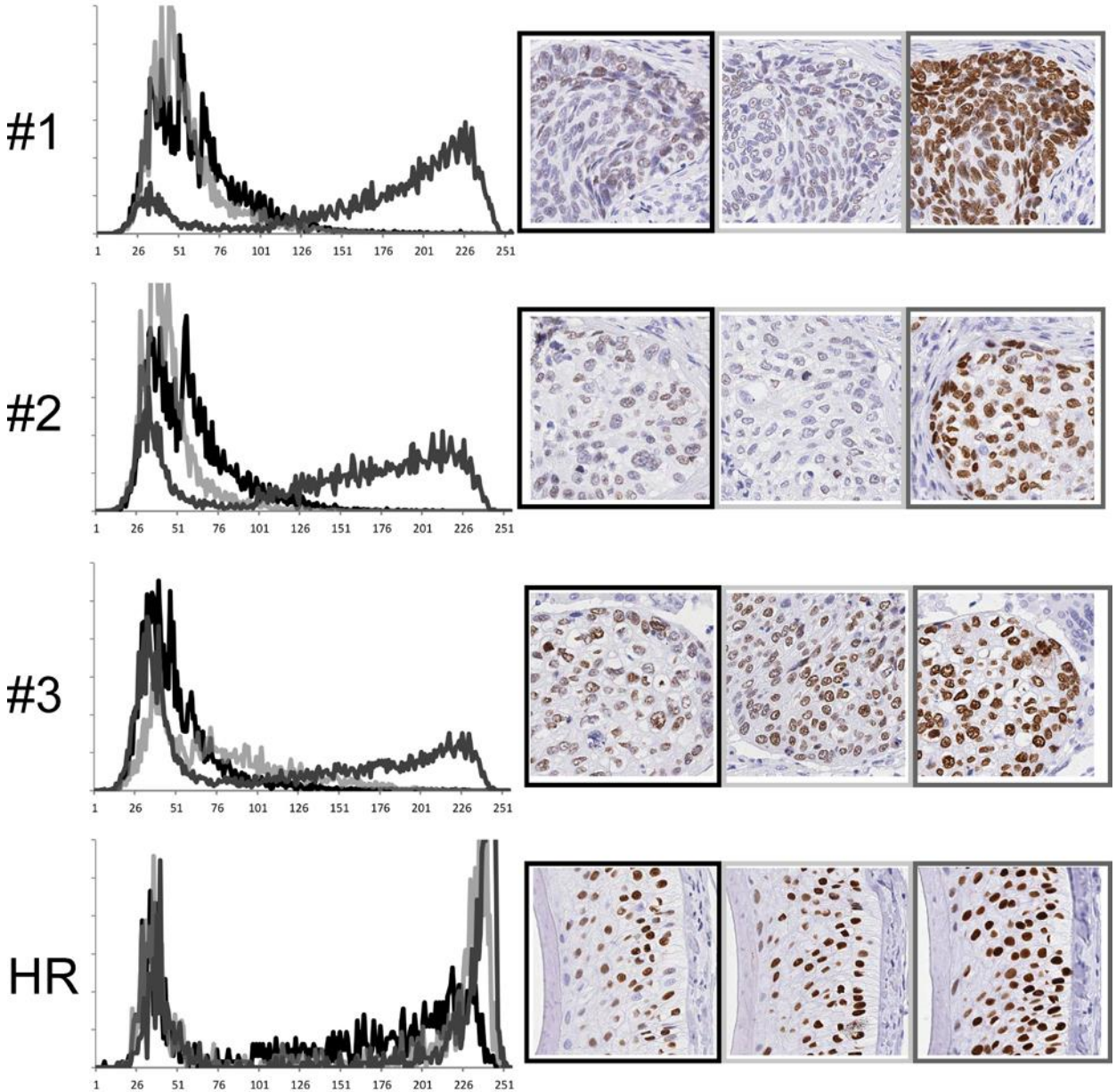

Lung cancer:
TP63

CONCLUSION

- 2-ME/SDS pretreatment OR AR temperatures >105C improve significantly immunoavailability over extraction
- The formaldehyde-induced bonds are unmodified by a 2-ME/SDS pretreatment and are of the same order of strength as the silane-glass-tissue bonds
- The first 2-ME/SDS treatment may be best performed as a split Laemmli + AR procedure
- Any subsequent 2-ME/SDS treatment will affect only native proteins (antibodies)
- When you start to disintegrate the section, you may be extracting too much

MULTIPLEXING (BY SEQUENTIAL STAINING-DESTAINING) IS PROBLEM-FREE?

Subtle negative changes in the hydration of the section and the media covering it (e.g. glycerin gelatin, water-based mounting medium) may

A- causing reversible re-masking of tissue antigens

B- causing irreversible folding of both fixed and native proteins (e.g. antibodies)

C- impair stripping by the 2-ME/SDS buffer

USPTO provisional patent N. 62062750