Technical aspects of molecular pathology, the importance of the pre-analytical phase

Jacques Van Huysse, AZ Sint-Jan Brugge

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Overview

- Guidelines
- Examples
- Decalcification
- Tumour %
- Conclusions

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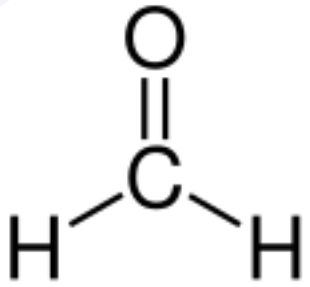
Table 3. Essential Preanalytical Factors Affecting Molecular Testing and Recommendations for Pathology Practice

Top 6 Preanalytical Factors for Tissue for the Maintenance of Nucleic Acid and Protein Quality and Integrity	Top 6 Preanalytical Factors for Blood/Serum for the Maintenance of Nucleic Acid and Protein Quality and Integrity
Time to stabilization (cold ischemia time) • 1 h or less	Time to first processing step • <60 min (unless EDTA or specialty cell-stabilization tube used) • 4–6 h for EDTA tube • ≤48 h for cell-stabilization tube
Method of stabilization • Fixative: 10% phosphate-buffered formalin, pH 7.0 • Total time in formalin: at least 6 h, not more than 24–36 h (tissue with high fat content may require 48 h) • Acid decalcification, before or during stabilization, is contraindicated for nucleic acid analyses	Method of acquisition • Tube type: specialized for a specific molecule species versus not • If processing time is >2–3 h, use ACD tube • EDTA tube for nucleic acid studies, proteomics studies, or circulating cell-free nucleic acid studies (cell-free nucleic acid analysis requires rapid stabilization or potentially specialty cell-stabilization tubes) • Volume of tube fill • Complete fill per manufacturer's recommendation • Tube inversions per manufacturer's recommendations (typically 10) • Draw order • Culture tube • Nonadditive tube • Coagulation tube (sodium citrate) • Clot activator tube • Clot activator and serum separator tube (clot activator plus separator gel) • Heparin tube (lithium or sodium heparin) • EDTA tube • Tubes with other additives (eg, sodium fluoride; ACD)
Method of processing • Specimen thickness not to exceed 4–5 mm • Volume to mass ratio 4:1 at a minimum, preferably 10:1, with tissue completely submerged	Method of stabilization • Tube inversions per manufacturer's recommendations
Tissue processor variables • Processor maintenance daily per manufacturer's recommendations • Quality of processing fluids rigorously maintained • Maintenance of formalin purity and pH • Attention to water (ie, formalin) contamination of alcohol baths • Type of paraffin • Low-melt paraffin (melts at <60°C)	Method of processing • Centrifugation speed/time per validated protocol and biomolecule being studied • Temperature: room temperature (defined as 18°C–25°C) unless validated protocol dictates otherwise
Storage conditions • Dry, pest-free conditions at room temperature (defined as 18°C–25°C)	Storage conditions • ≤1 freeze-thaw cycle: for nucleic acids and proteins use aliquots
Documentation data for the above factors and/or deviations from the recommendations	Documentation data for the above factors and/or deviations from the recommendations Documentation should be included in the laboratory's standard operating procedures and quality control logs
Note: Tissue specimens considered unacceptable for molecular testing include desiccated tissues or those known to have been improperly collected or stored	Note: Blood specimens considered unacceptable for molecular testing include hemolyzed samples or those known to have been improperly collected or stored

Abbreviations: ACD, acid-citrate-dextrose; EDTA, ethylenediaminetetraacetic acid.

Fixation

- Buffered formalin 10%
- (4% formaldehyde)
- pH 6,8-7,2
- Crosslinks between proteins and proteins and between proteins and nucleic acids
- Preservation of the secondary protein structure



- Carcinogen

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Fixation

- Influenced by
  - The type of tissue
  - The amount of tissue versus the amount of fixative
  - Temperature
- Delayed or to short fixation
  - Autolysis
  - Insufficient cross linking
  - Alcoholic (coagulative) fixation at dehydration
- Over fixation
  - Too much cross linking

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Fixation

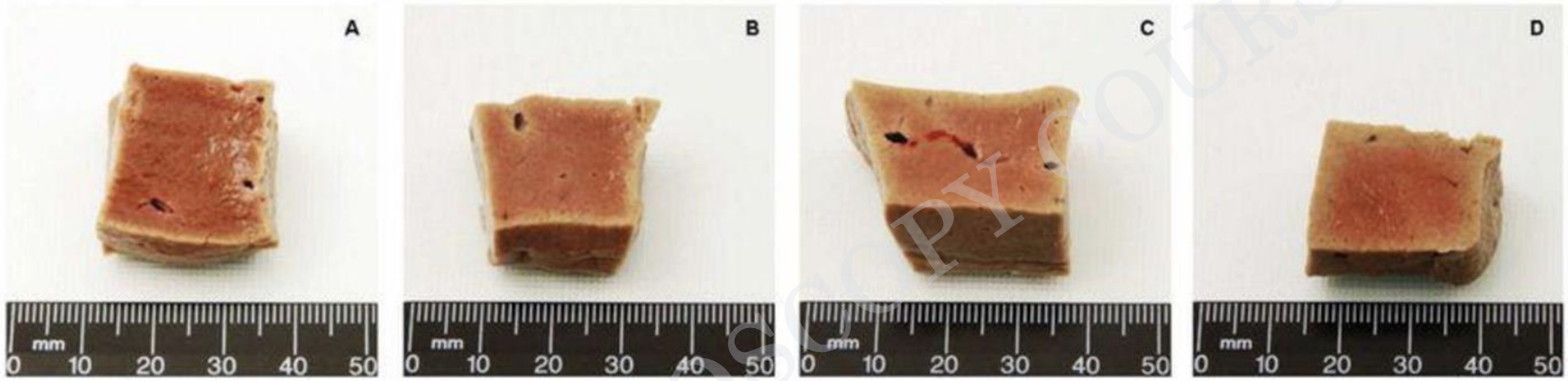

Figure 1: A composite photograph showing the rate at which 10% neutral buffered formalin penetrates into 25 mm cubes of liver. At the end of each time period a cube has been sliced to reveal the advancing fixation front. A: one hour (approximately 0.8 mm penetration), B: two hours (approximately 1.2 mm penetration), C: four hours (approximately 1.6 mm penetration) and D: eight hours (approximately 2.2 mm penetration). Note that after eight hours the centre of the specimen remains unfixed.

https://www.leicabiosystems.com/en-be/knowledge-pathway/fixation-and-fixatives-2-factors-influencing-chemical-fixation-formaldehyde-and-glutaraldehyde/

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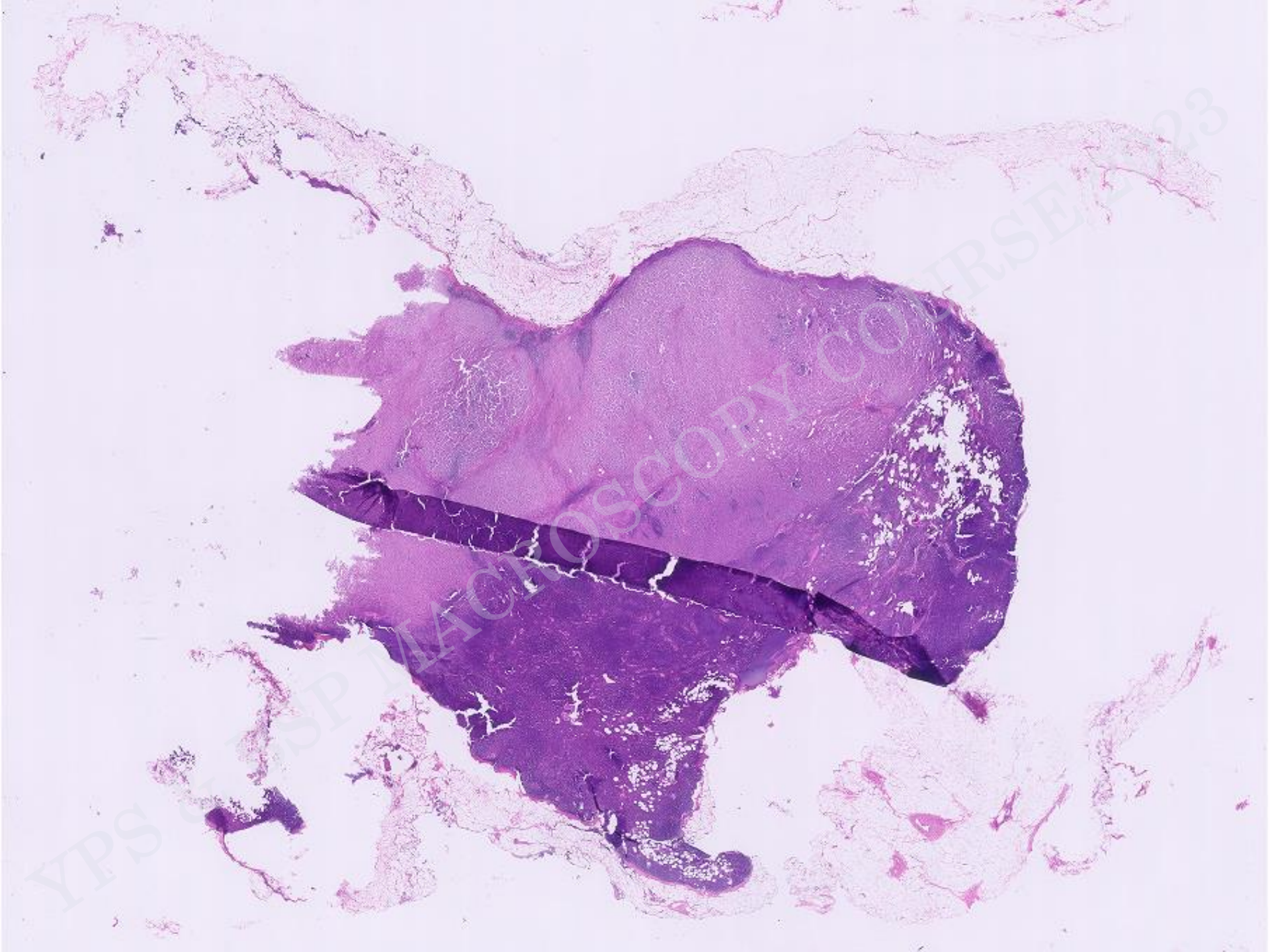
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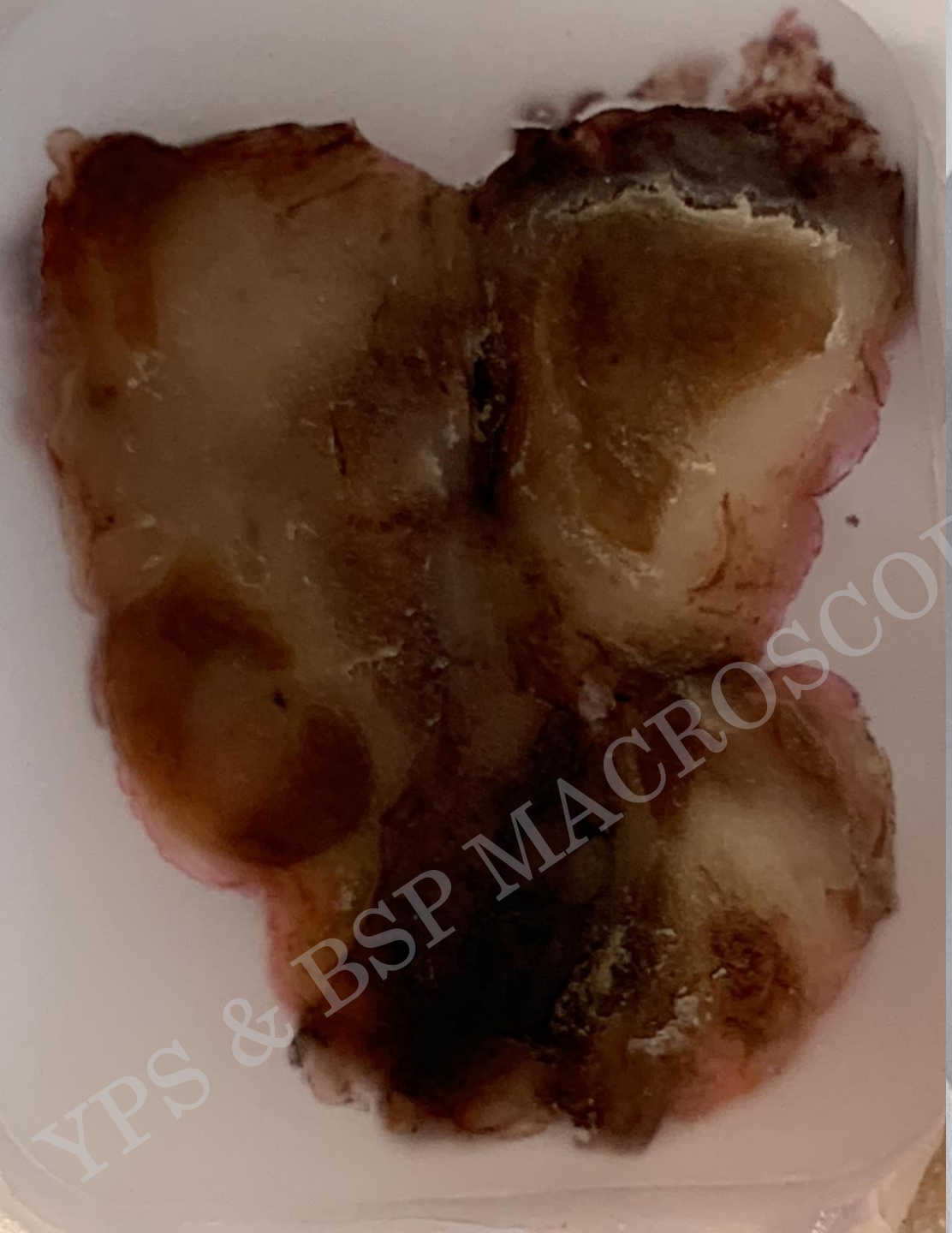

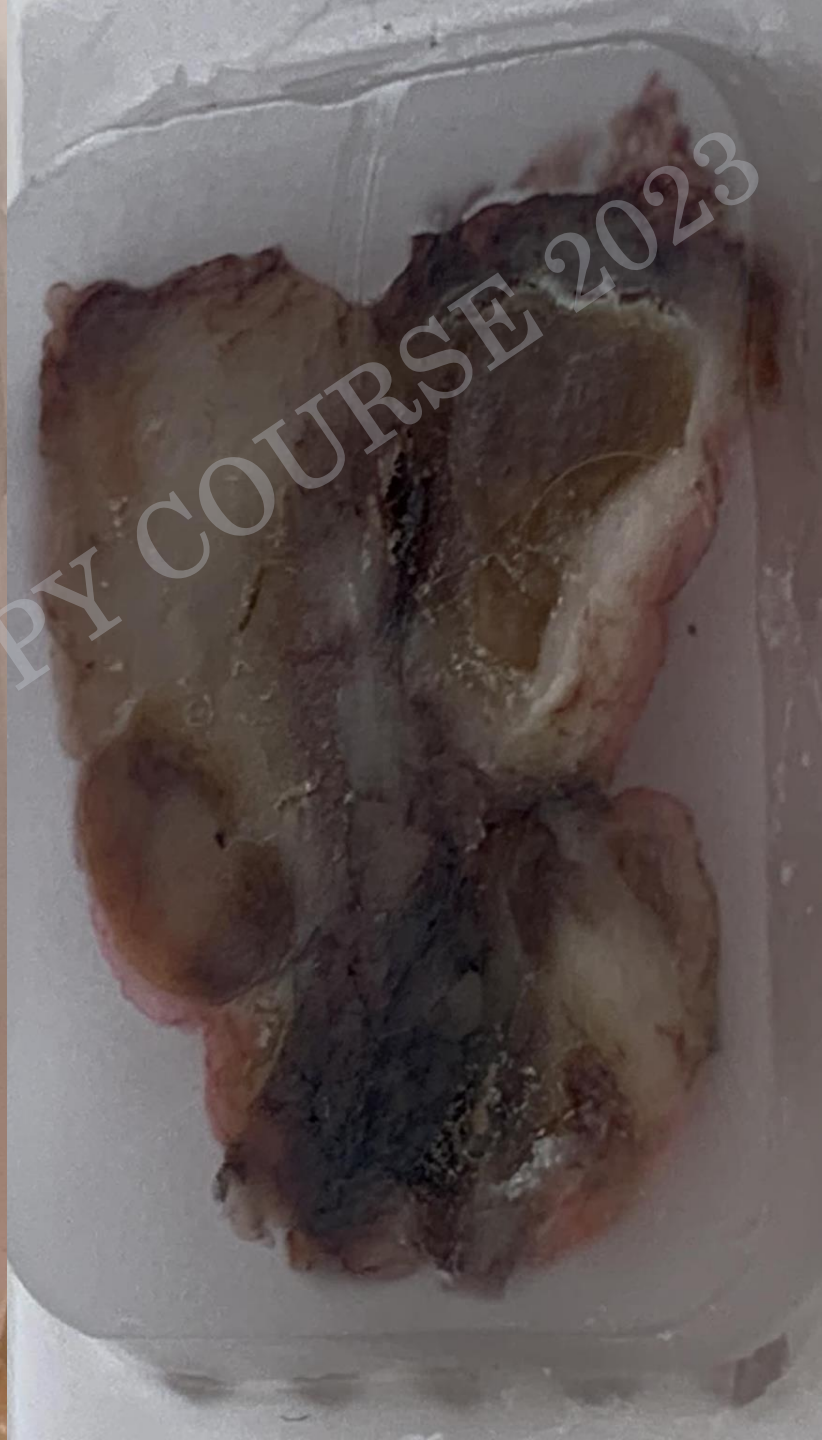

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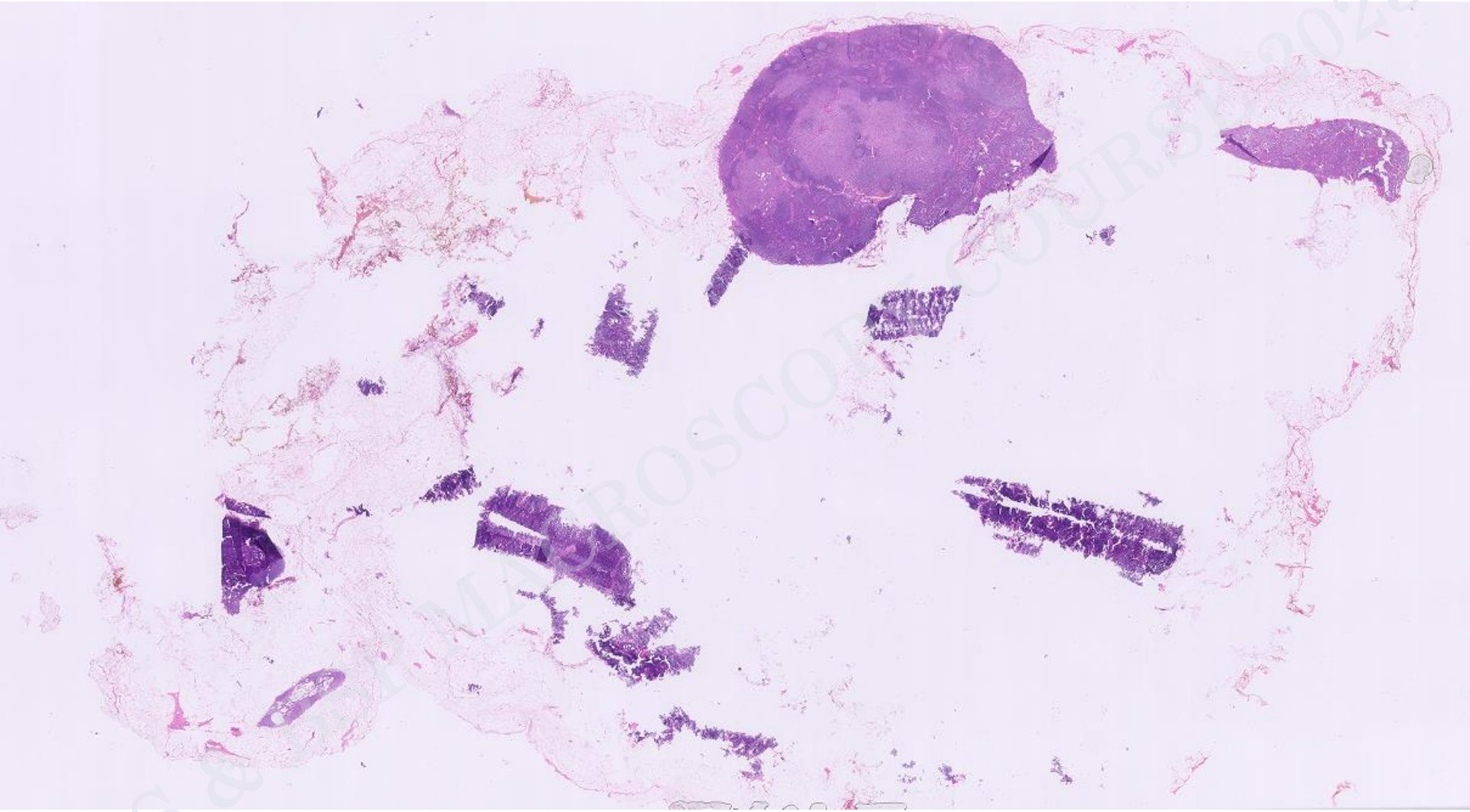

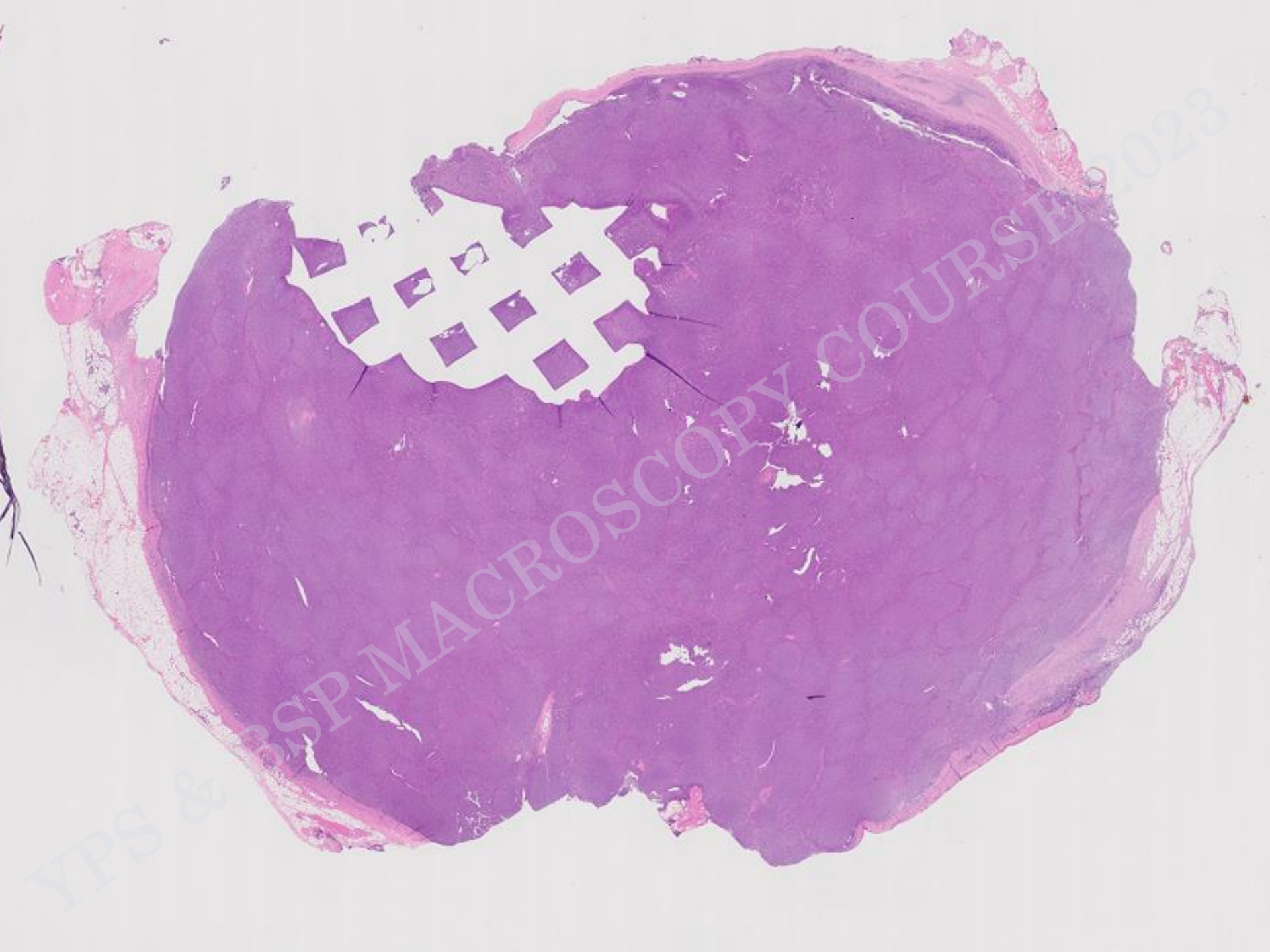
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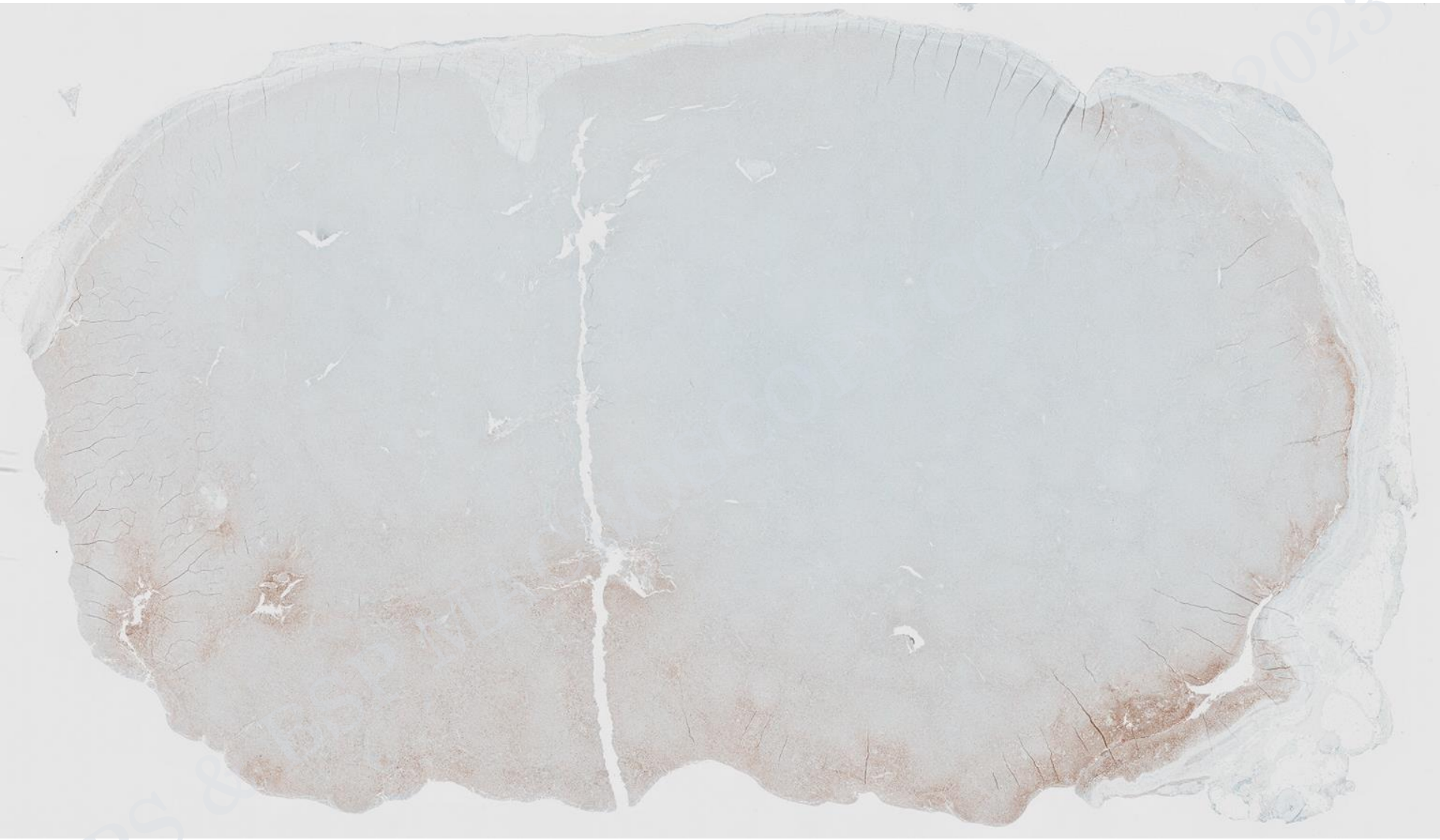
CD10



ER IHC

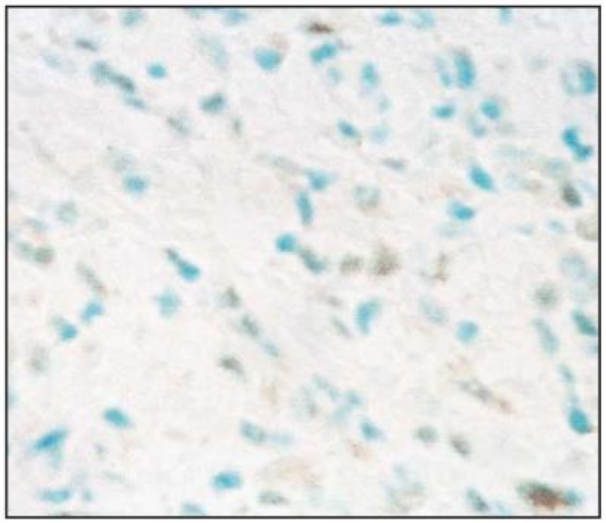


Image 1 Fixation, 3 h; antigen retrieval, 40 min.

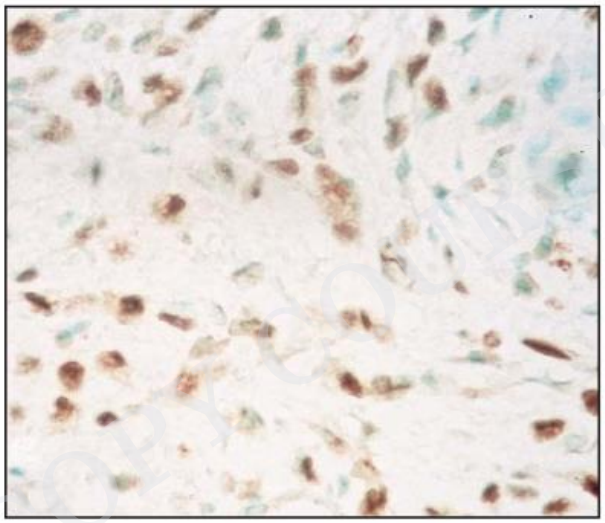


Image 2 Fixation, 6 h; antigen retrieval, 40 min.

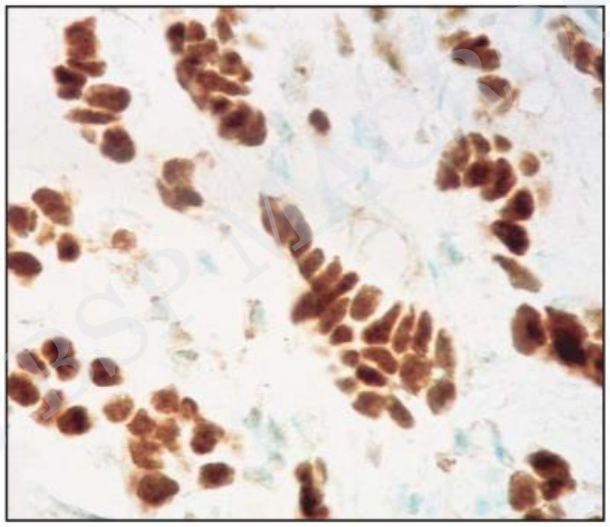


Image 3 Fixation, 8 h; antigen retrieval, 40 min.

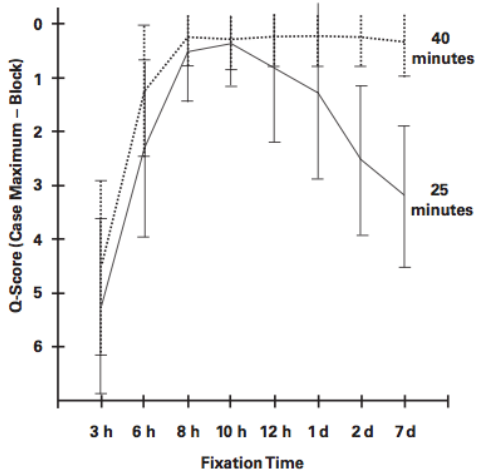
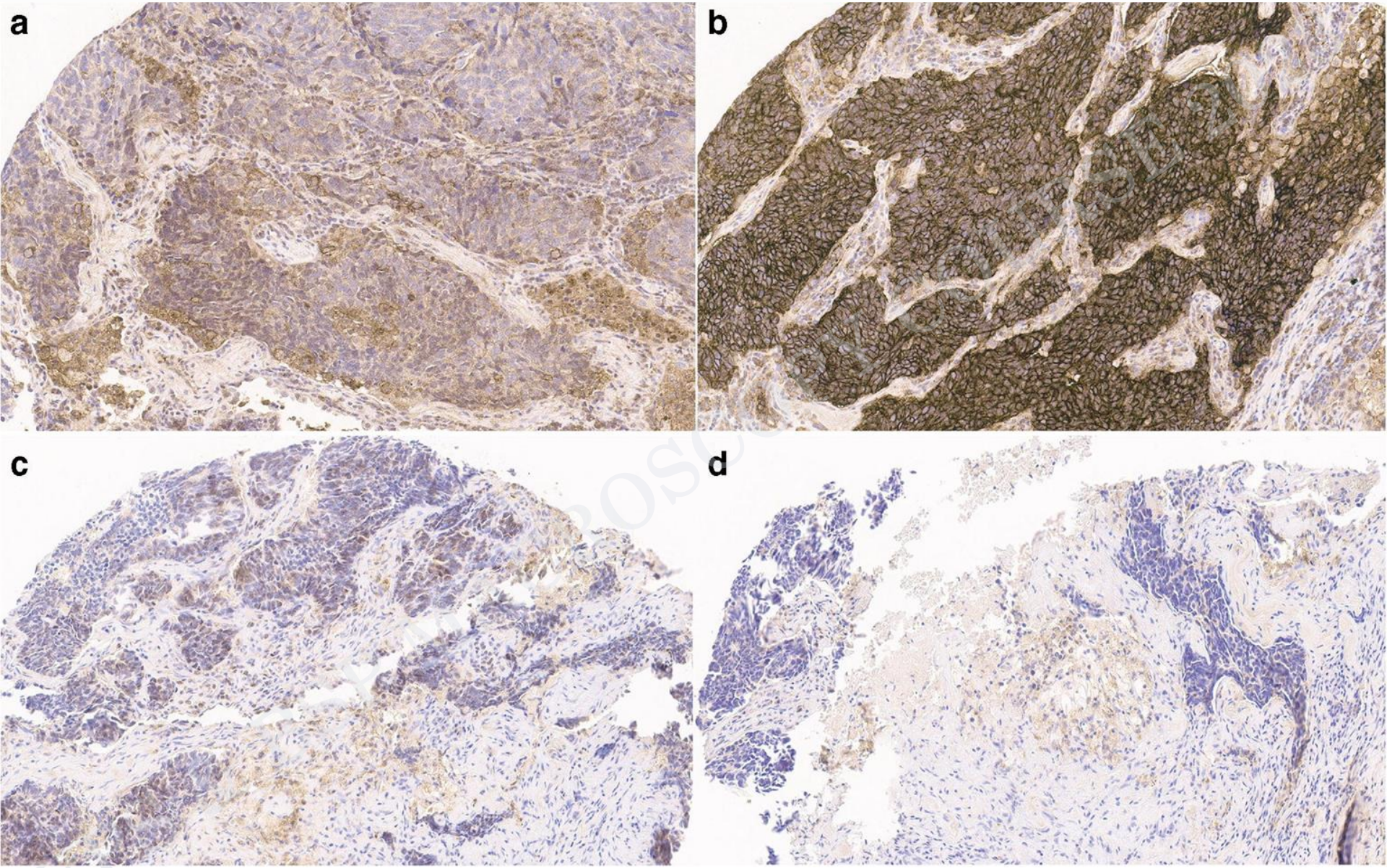


Figure 1 Plateau, case maximum (max) estrogen receptor (ER) scores occurred at 6-8 hours. Standard immunohistochemical method used 40 minutes of antigen retrieval.

Goldstein et al, Am J Clin Pathol, 2003

Presentation of PD-L1 staining in a tumor sample after normal fixation (**a**), after 6 h (**b**), 48 h (**c**), and 96 h (**d**) of delay in fixation (for all objective × 20). Note: deterioration of membrane staining in 48+ h delayed fixation and increase of non-specific staining



van Seijen, M., Brcic, L., Gonzales, A.N. *et al.* Impact of delayed and prolonged fixation on the evaluation of immunohistochemical staining on lung carcinoma resection specimen. *Virchows Arch* **475,** 191–199 (2019). https://doi.org/10.1007/s00428-019-02595-9

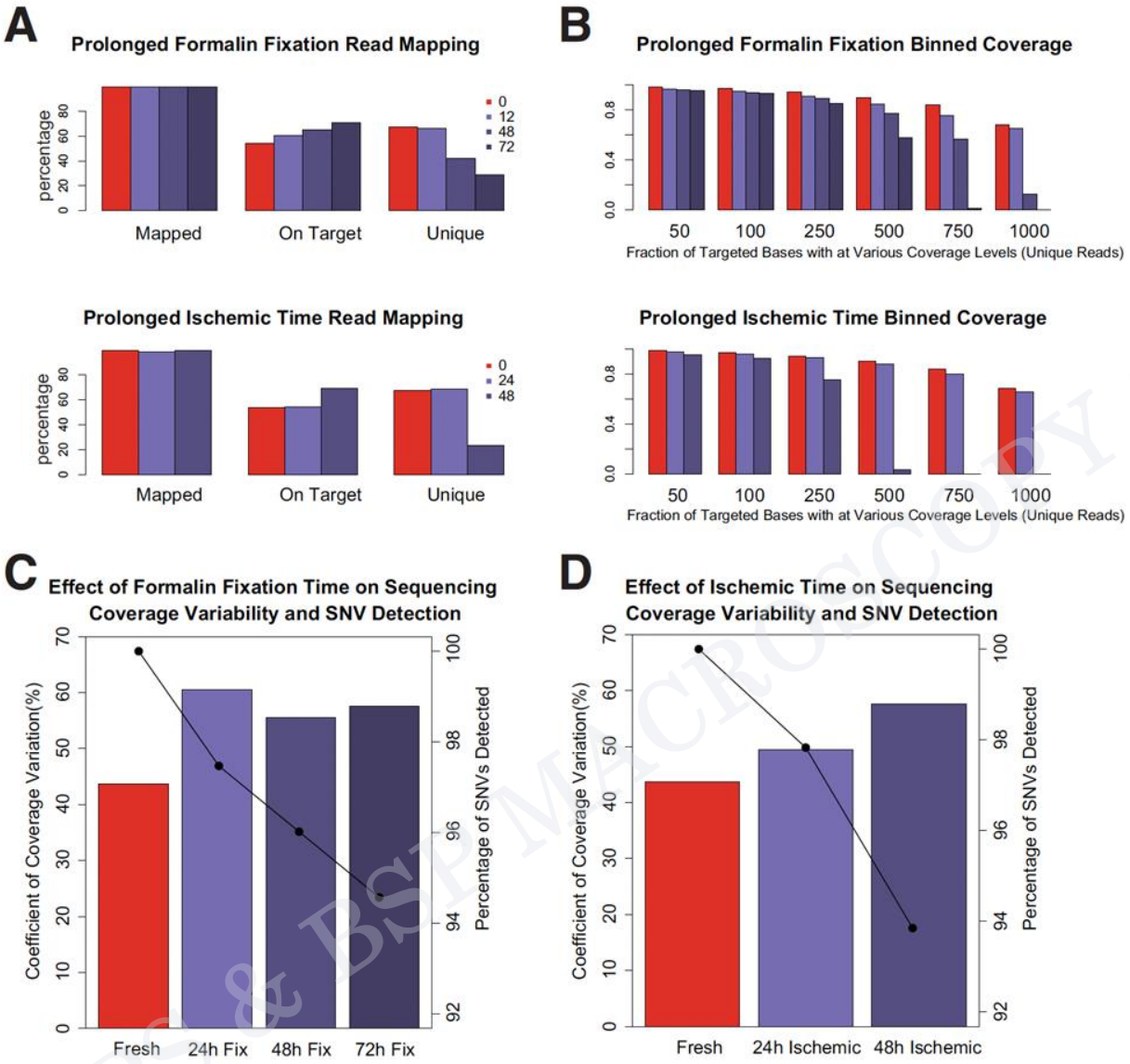


Figure 7 Effect of preanalytic factors on NGS. **A**: Percentage of mapped, on-target, and unique reads for a single surgical resection specimen that was snap frozen (red) or subjected to 24-hour (light purple), 48-hour (medium purple), or 72-hour (dark purple) formalin fixation. Percentage of mapped, on-target, and unique reads for a single surgical resection specimen that was snap frozen (red) or subjected to 24 (light purple) or 48 (medium purple) hours of ischemia followed by routine processing. **B**: The fraction of positions at unique on-target coverage levels between $\times 50$ and $\times 1000$ for the frozen specimen (red) or after formalin fixation for 24 (light purple), 48 (medium purple), or 72 (dark purple) hours. The fraction of positions at unique on-target coverage levels between $\times 50$ and $\times 1000$ for the frozen specimen (red) or after a 24-hour (light purple) or 48-hour (medium purple) ischemic time. **C**: The coverage coefficient of variation (bar plot, left scale) for snap frozen (red) compared with tissue after 24, 48, or 72 hours of formalin fixation. The superimposed plot (right scale) shows the percentage of SNVs detected in each sample. **D**: The coverage coefficient of variation (bar plot, left scale) for snap frozen (red) compared with tissue after a 24- or 48-hour ischemic time. The superimposed plot (right scale) shows the percentage of SNVs detected in each sample.

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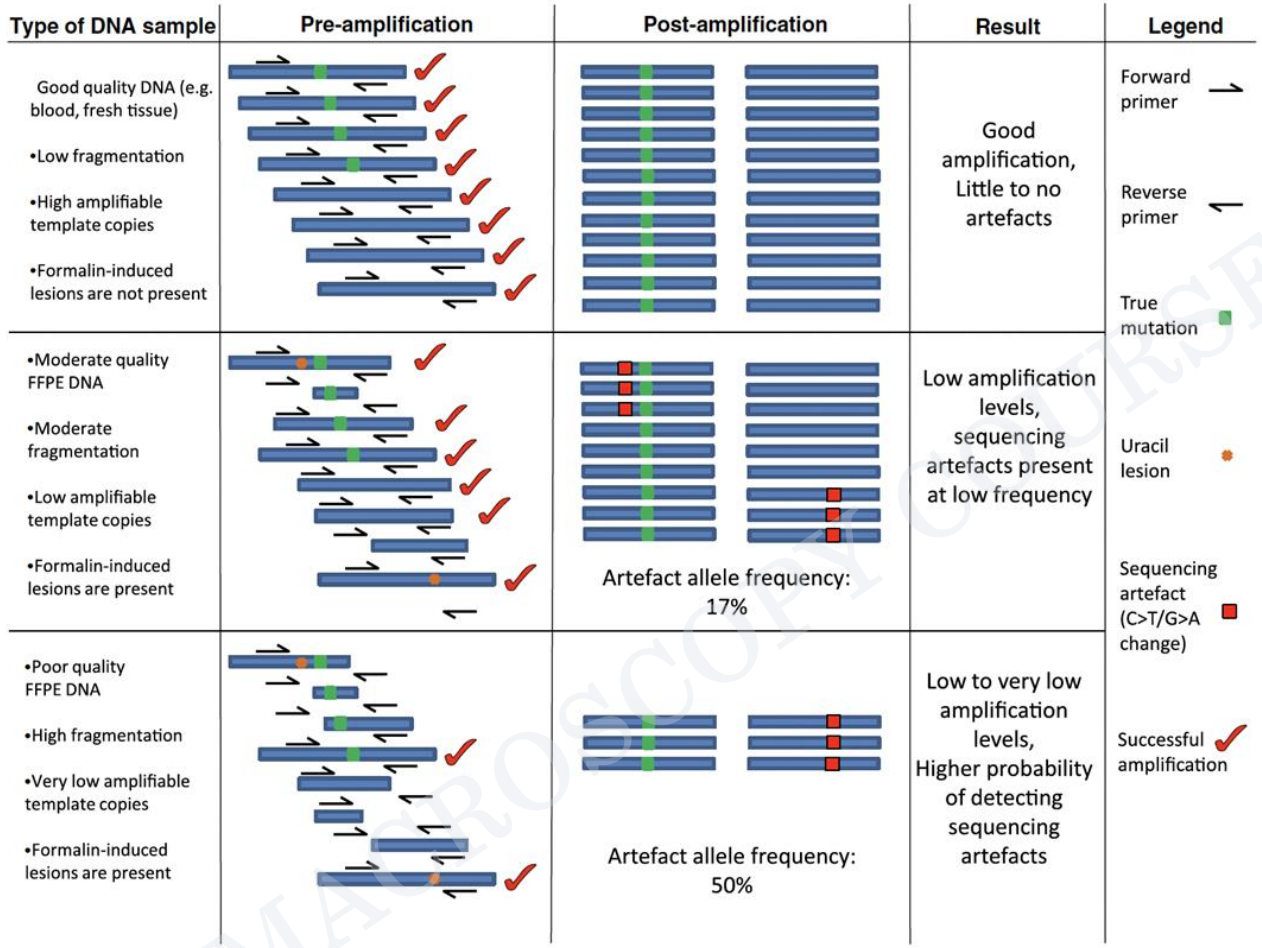

Figure 5 Low template copies are associated with higher probability of sequencing artefacts post-PCR amplification. In good quality DNA from sources such as blood and fresh frozen tissue, fragmentation and uracil lesions are present at very low levels. In this circumstance, high amounts of amplifiable template increase the likelihood of accurately identifying mutations due to high sequencing coverage with little or no stochastic enrichment of sequencing artefacts. In FFPE DNA with moderate fragmentation, the number of amplifiable templates is reduced, with some formalin-induced uracil lesions being present in template DNA. Subsequently PCR amplification results in lower coverage due to less amplifiable template numbers. Uracil lesions are also amplified, and due to the lower copy numbers, can appear as non-reproducible sequencing artefacts (C>T/G>A changes). These artefacts will be low in frequency. In the case of FFPE with high amounts of fragmentation, the numbers of amplifiable template are severely limited. An artefact in one of these templates can then appear as a moderate to high frequency sequencing variant. These can subsequently be interpreted as real mutations.

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Wong et al. BMC Medical Genomics 2014, 7:23

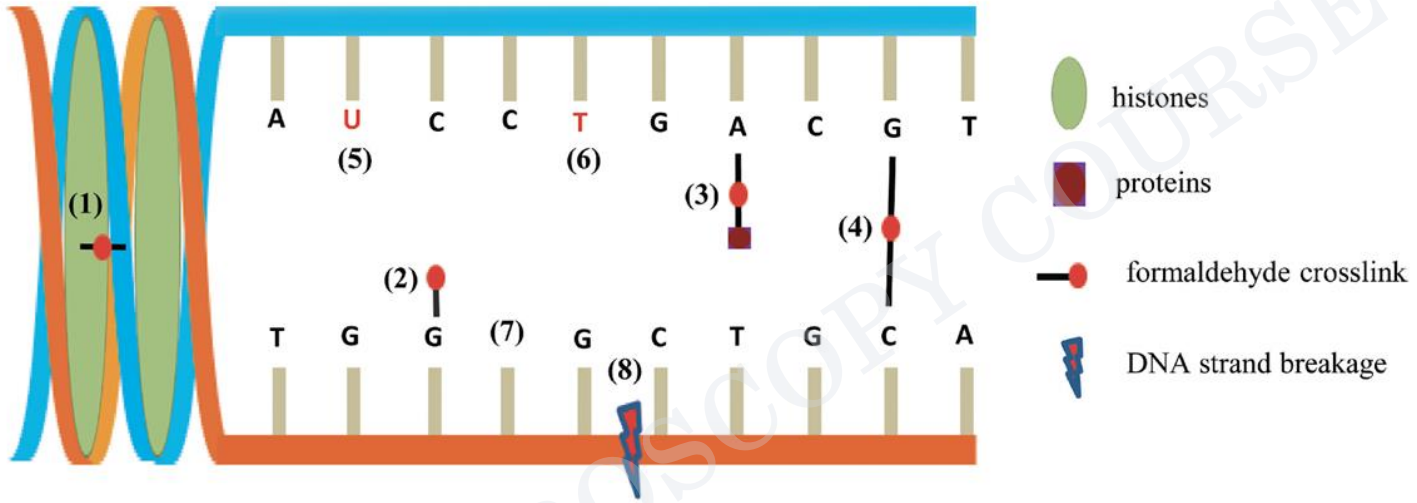

Fig. 1. DNA damage present in formalin-fixed tissues.

DNA extracted from formalin-fixed tissues contains various types of damage. Formaldehyde, the main component of formalin, is highly reactive with DNA bases and proteins, generating histone–DNA crosslinks (1), formaldehyde–DNA adducts (2), DNA–protein crosslinks (3), and DNA–DNA crosslinks (4). Uracil (5) and thymine (6), which result from deamination of cytosine and 5-mC, respectively, are also present in FFPE DNA. DNA bases are also lost, resulting in abasic sites (7), and DNA strands are broken, leading to fragmentation of DNA (8).

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Clinical Chemistry 61:1 (2015)

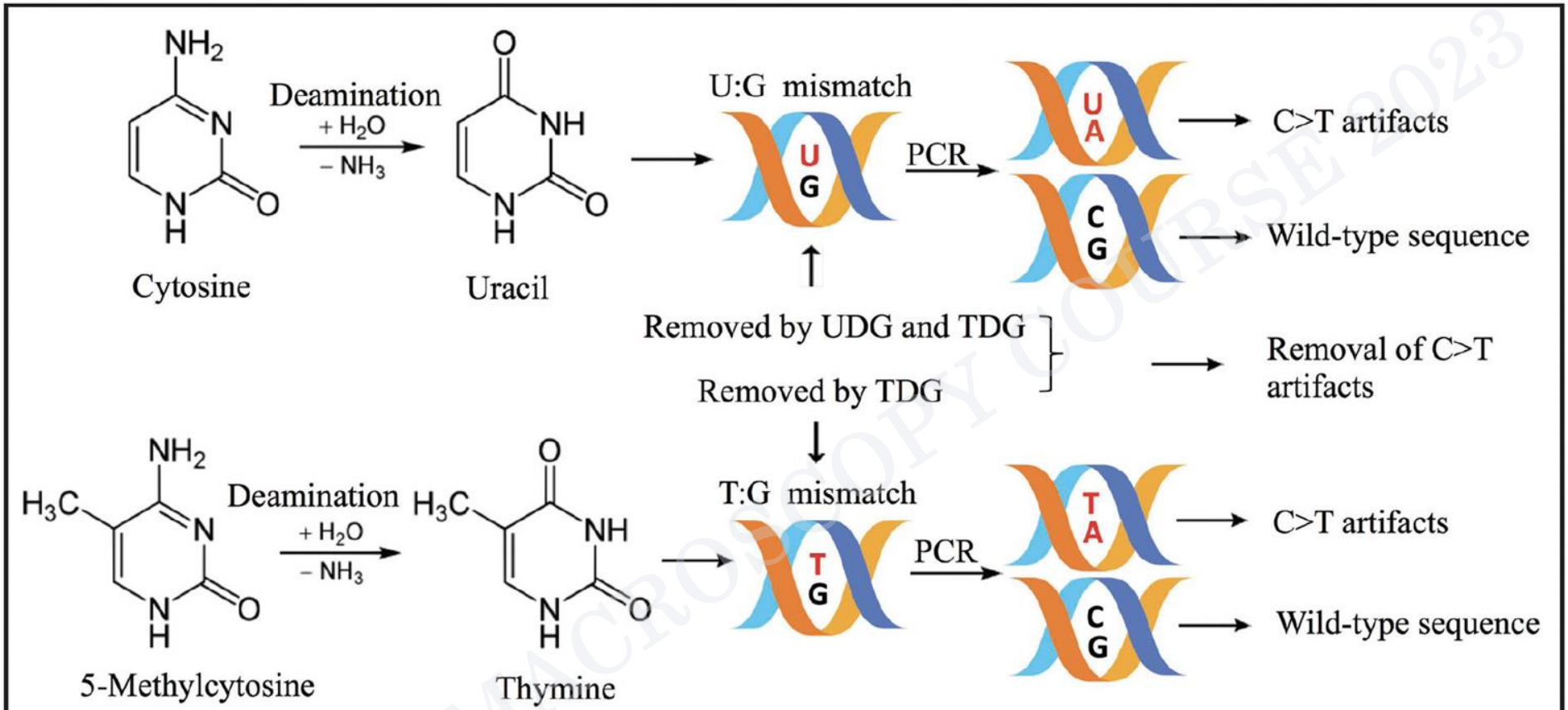

Fig. 4. Deamination of cytosine and 5-mC as the sources of false-positive *EGFR* T790M mutations.

Cytosine and 5-mC are deaminated to uracil and thymine, respectively. When amplified by PCR, the resultant U:G and T:G mismatches become the sources of C>T sequence artifacts. UDG removes the uracil from U:G mismatches. TDG removes the uracil from U:G and thymine from T:G mismatches. Thus, pretreatment of formalin-fixed DNA with UDGs or TDGs enables artifactual C>T (and G>A) sequence artifacts to be minimized.

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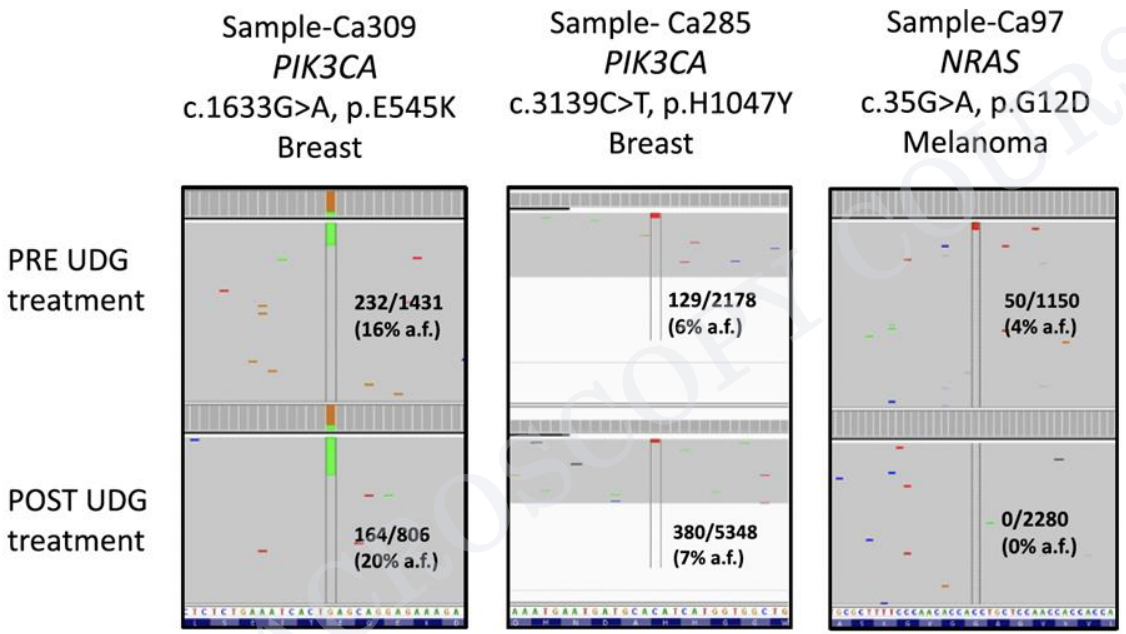

Figure 4 Uracil-DNA glycosylase treatment of FFPE DNA samples distinguishes true and false positive clinical relevant mutations. Integrative Genomic Viewer (IGV) screenshots of two breast cancers and one melanoma sample pre- and post- uracil-DNA glycosylase (UDG) treatment samples. The two breast cancer samples have confirmed *PIK3CA* mutations (E545K for Ca309 and H1047Y for Ca285) as these mutations were still detected after UDG treatment. The *NRAS* G12D mutation identified in the pre-UDG sample (Ca97) was a false positive as it was not present after UDG treatment. The variant reads over the total reads and overall allele frequency (a.f.) are shown for each case.

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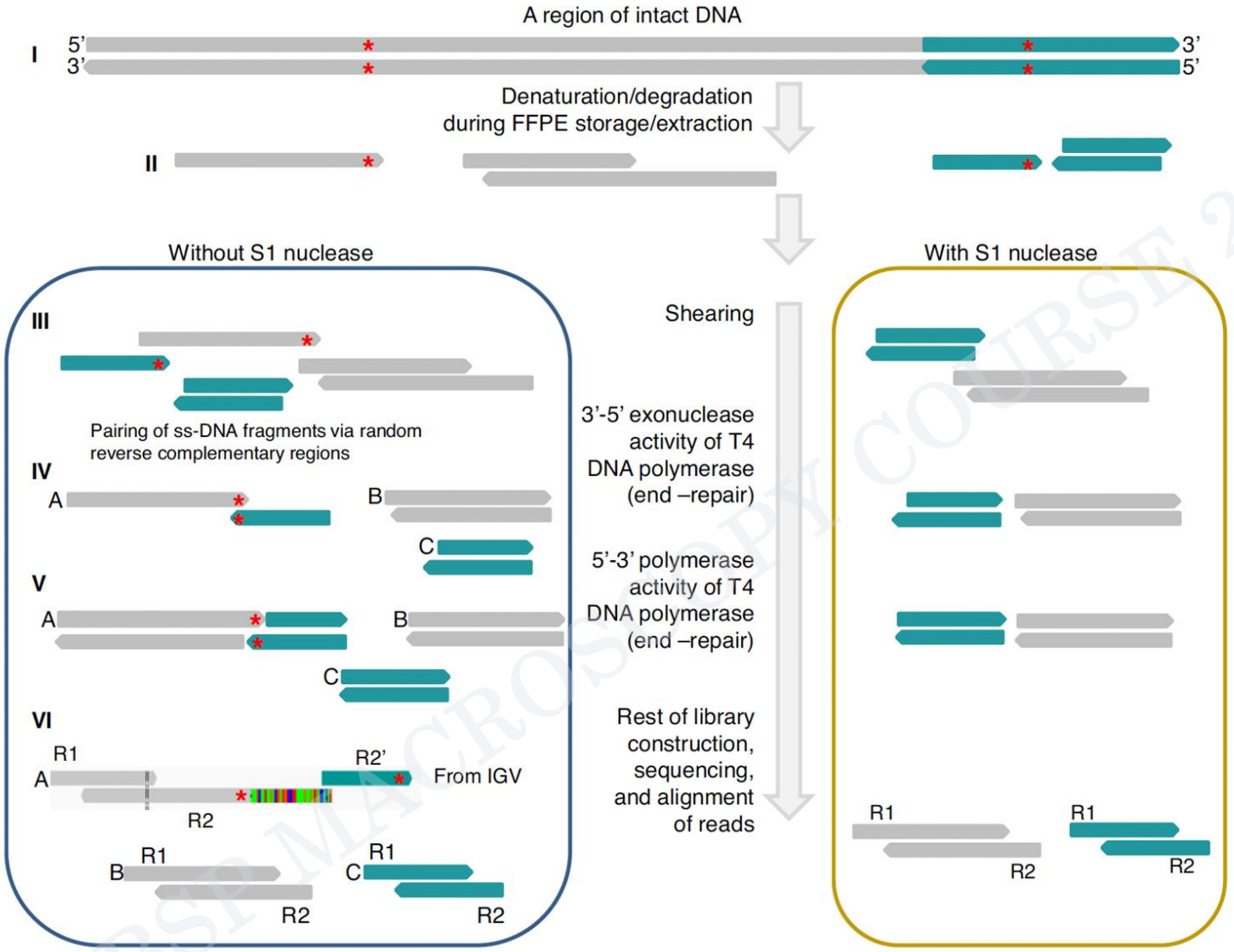

Figure 2. SSAR mapping and diagrammatic depiction of the proposed mechanism. The SSAR example shown is a screen shot of an actual IGV image. At the top (I) we depict a ds-DNA region of intact gDNA. In the process of FFPE preparation, storage and extraction (II), gDNA is fragmented and denatured. In the absence of S1 nuclease (III left), ss-DNA fragments from non-contiguous regions of the genome anneal via short complementary repetitive sequences (red asterisks). In contrast, ss-DNA fragments and overhangs are removed upon treatment with S1 nuclease (III right). During the end-repair step of library construction, T4 DNA polymerase removes overhangs (IV) and fills ends (V), resulting in the formation of double-stranded chimeric fragments ('A' in V). One class of such chimeric fragments yield SSARs ('A' in VI). R1 = read; R2 = read 2. For SSARs, part of Read 2 aligns in the expected paired-end orientation while the distal end of Read 2 does not match the reference at that position and instead aligns to a nearby region of the reference genome in the opposite orientation (denoted as R2′).

YPSEB BSP MACROSCOPY COURSE 2023

Nucleic Acids Research, 2019, Vol. 47, No. 2

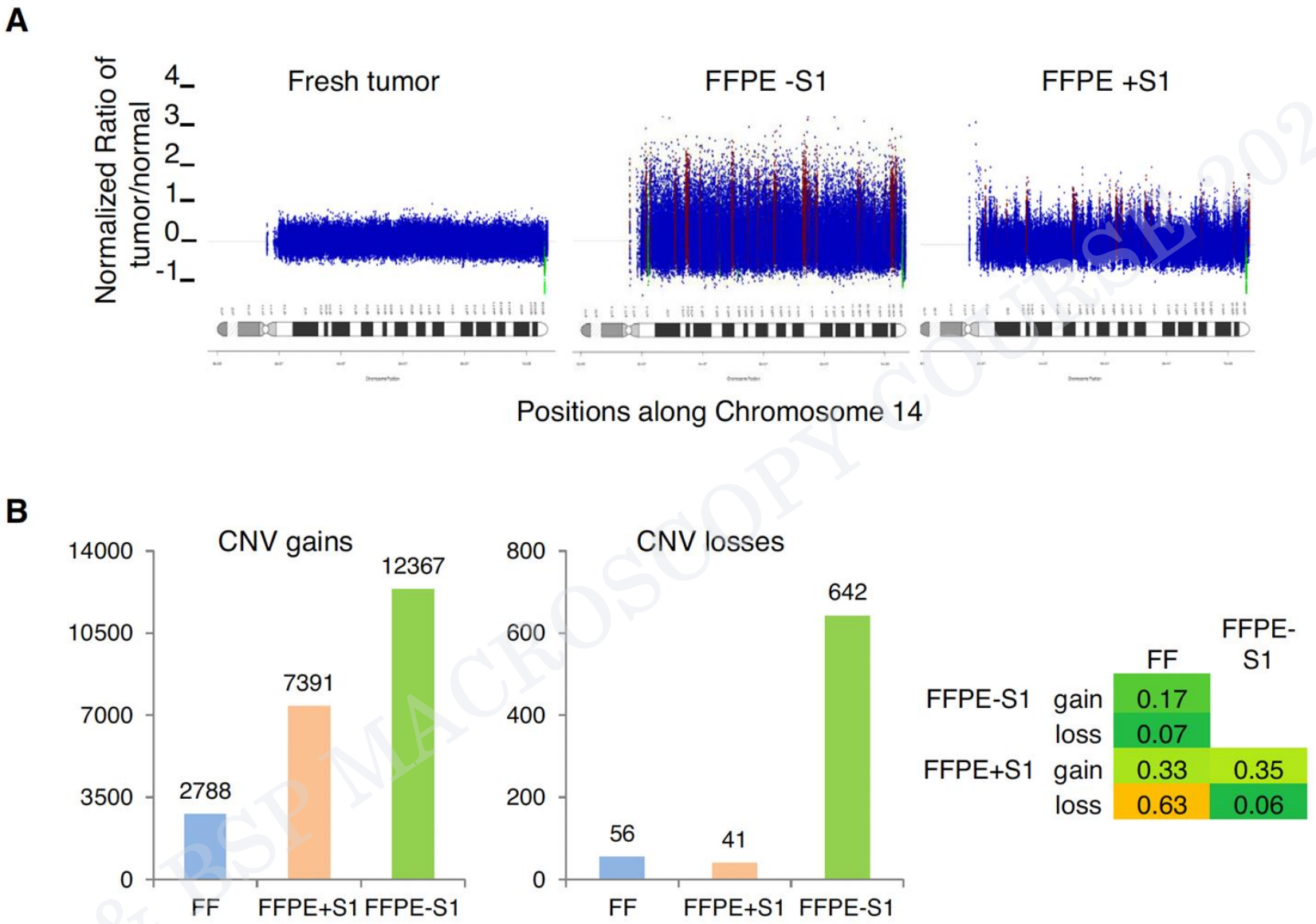

Figure 6. Effects of S1 nuclease treatment on FFPE-associated CNV noise. (**A**) Example illustrating CNV noise. Samples are the same as in Figure 5. Using a bin size of 200 reads, CNV segments were calculated in the tumor samples relative to the normal blood sample and the resulting profile is shown for chromosome 14. (**B**) CNV counts at the gene level. Gains are shown on the left and losses are shown in the middle panel. Jaccard's intersection index (Materials and Methods) is shown in the right panel as a measure of overlap of gene-level CNVs between the three samples.

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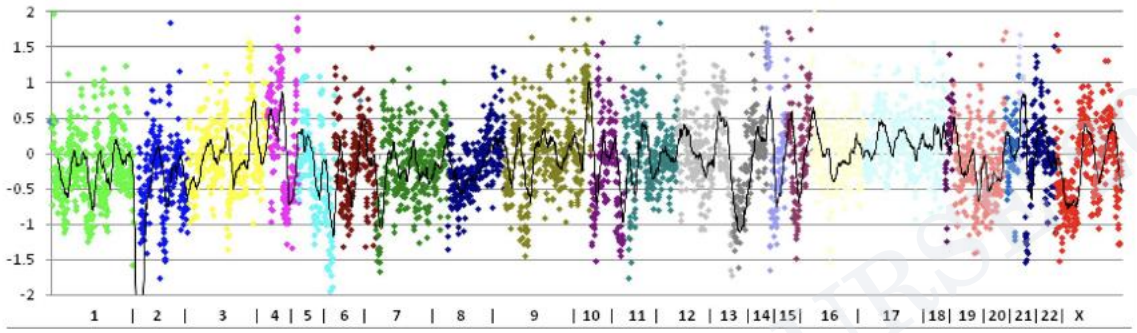
Table 2. Strategies for minimization of sequence artifacts from FFPE DNA.

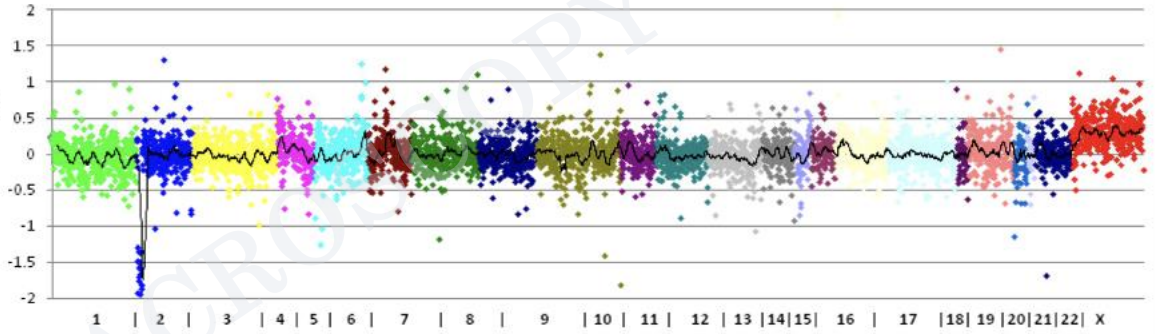
Step	Strategy
DNA extraction	Assessment of tumor purity and identification of tumor-enriched areas by a pathologist
	Macrodissection or coring of the tumor-enriched areas
	Use of sufficient tissue, whenever possible, to ensure that a sufficient quantity of DNA is isolated for subsequent molecular testing
	Heat treatment to remove formaldehyde-induced crosslinks and to facilitate subsequent tissue digestion with proteinase
	Extended proteinase K treatment to digest tissue and to remove proteins cross-linked to DNA
DNA assessment	Assessment of double-stranded DNA quantity using fluorometry
	Quantification of amplifiable templates using qPCR or digital PCR, especially for massively parallel sequencing. Use amplicons sizes that correspond to the mean amplicon size of the sequencing assay
Sample library preparation	In vitro removal of uracil prior to PCR amplification of FFPE DNA
	Using assays generating short amplicons to increase the number of templates for PCR
	Capture-based target enrichment allowing the recognition of the initial templates in sequence reads using their unique start and end sites
	Using primers specific for each strand of the DNA template in amplicon-based target enrichment approach
	Molecularly tagging DNA templates for identification of sequence artifacts
PCR amplification	Use of specific DNA polymerases (e.g. Pfu and KAPA) that have low bypass efficiency over DNA lesions such as uracil and abasic sites
	Use a high-fidelity DNA polymerase to reduce polymerase errors
Validation of sequence variants from amplicon-based MPS	Running each test in duplicate so that separate pools of templates are used
	Using orthogonal methods for clinically actionable mutations

Clinical Chemistry 61:1 (2015)

Primary thymic carcinoma
9 vear old archival FFPE block



Recurrent/metastatic disease,
1 month old FFPE block



2000	2006	2011	2014	2015	2016	2017	2018	2019	2020	2021	2022
0/1	1/2	1/1	0/1	1/1	1/1	1/3	2/3	6/6	14/15	38/38	480/492

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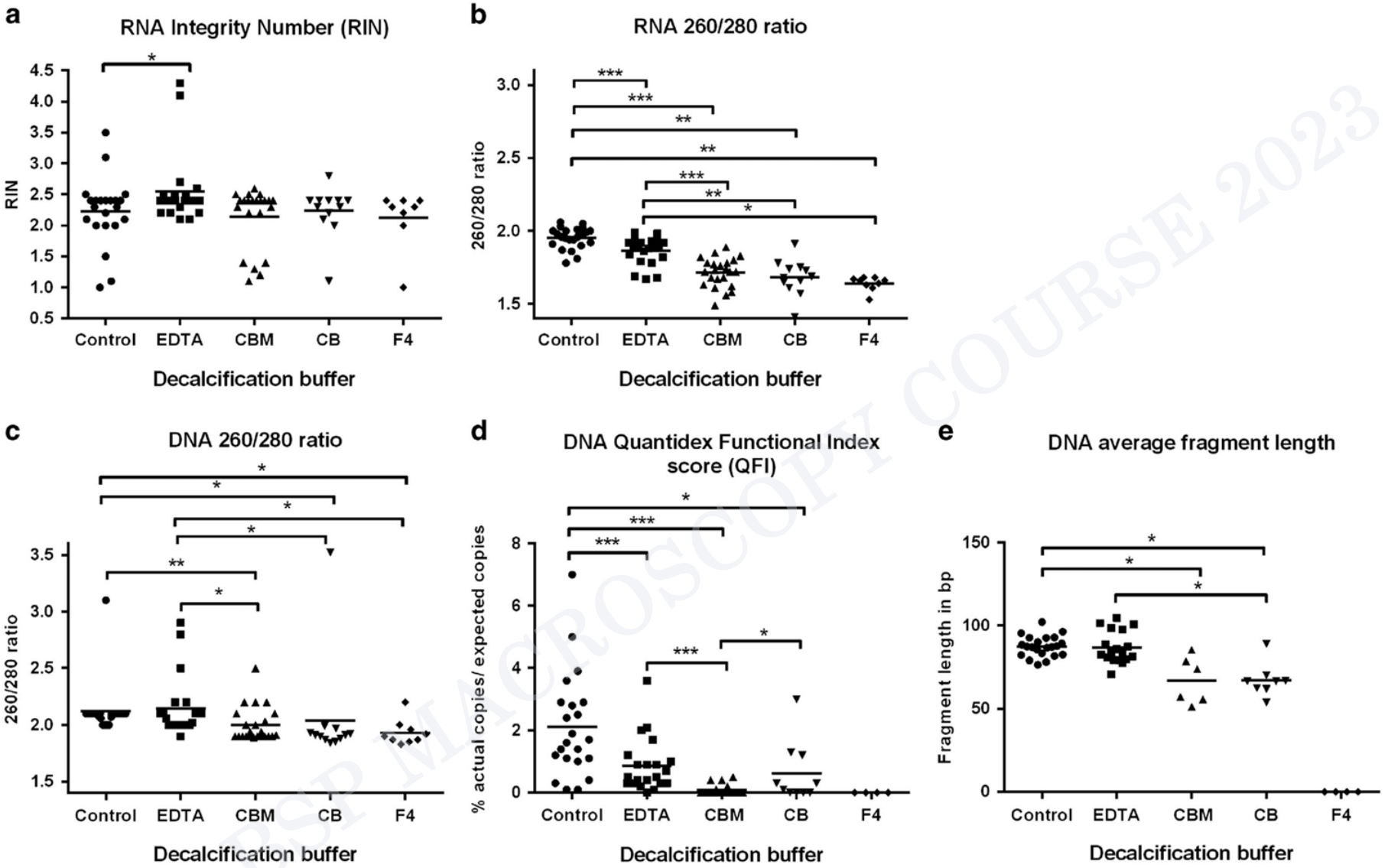

Figure 4 Quality and quantity of RNA and DNA isolated from tissue pretreated with EDTA, Christensen's buffer with (CBM) and without microwave (CB) and Formical-4 (F4; $n = 23$). (**a**) 260/280 ratios for RNA, measured by Nanodrop. (**b**) Bioanalyzer RNA Integrity Number (RIN) values. (**c**) 260/280 ratios for DNA, measured by Nanodrop. (**d**) QFI (Quantitative Functional Index) in percentages (actual copy number/expected copy number, based on quantity of DNA measured by Nanodrop). (**e**) Average DNA fragment length in base pairs, measured with size ladder PCR. DNA input is corrected for amplifiable copy number, measured with the QuantideX qPCR assay. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

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Decalcification in breast cancer pathology, Schrijver *et al* Modern Pathology (2016) 00, 1–11

blood®
Volume 134, Supplement 1, 13 November 2019, Page 4659

803.Emerging Diagnostic Tools and Techniques

Acid-Based Decalcification Methods Compromise Genomic Profiling from DNA and RNA

1711 consecutive formalin-fixed paraffin embedded samples were evaluated by CGP during routine clinical care via DNA and RNA sequencing, using a hybrid-capture next-generation sequencing assay (FoundationOne®Heme).

Acid based decalcification was associated with significantly higher failure rates than non-decalcified samples for both DNA (29.1% vs 3.7%) and RNA (67.4% vs 30.8%)

If decalcification is required for processing, EDTA based decalcification methods and/or minimizing decalcification times is recommended.

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The estimation of tumor cell percentage for molecular testing by pathologists is not accurate

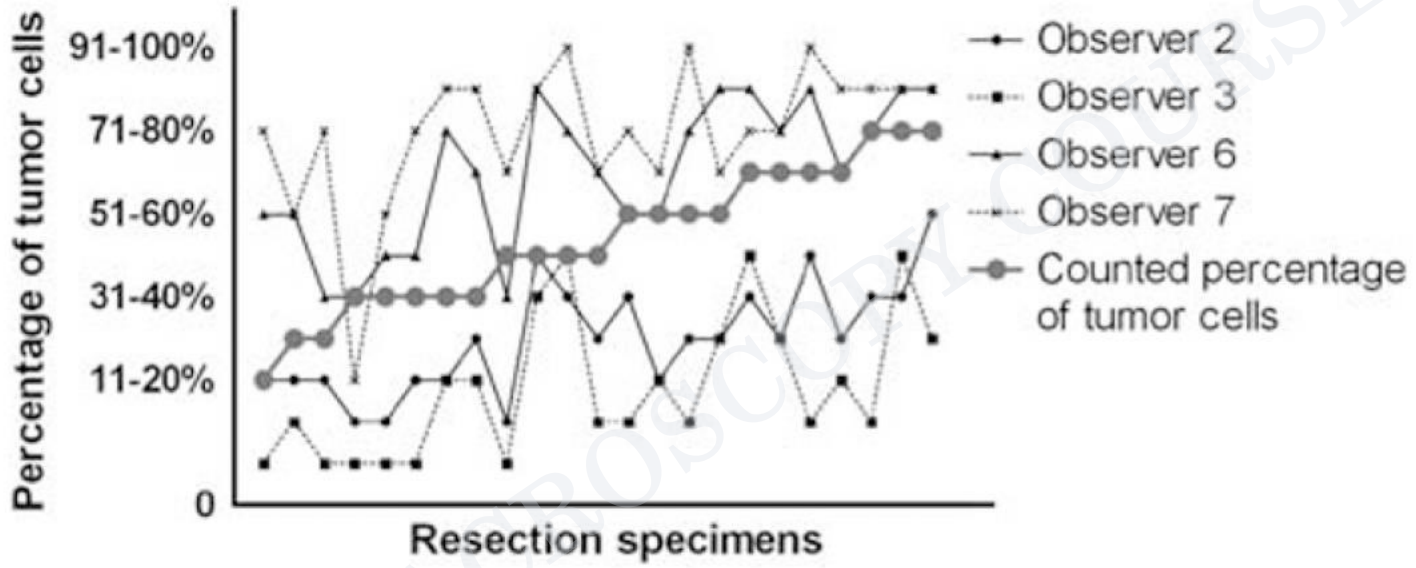

Figure 4 Performance of the observers with the largest systematic errors, compared with the counted percentage of tumor cells, in the resection specimens. The results of two observers with systematic overestimation and of two observers with systematic underestimation are shown. The specimens were ordered according to the ascending percentage of tumor cells.

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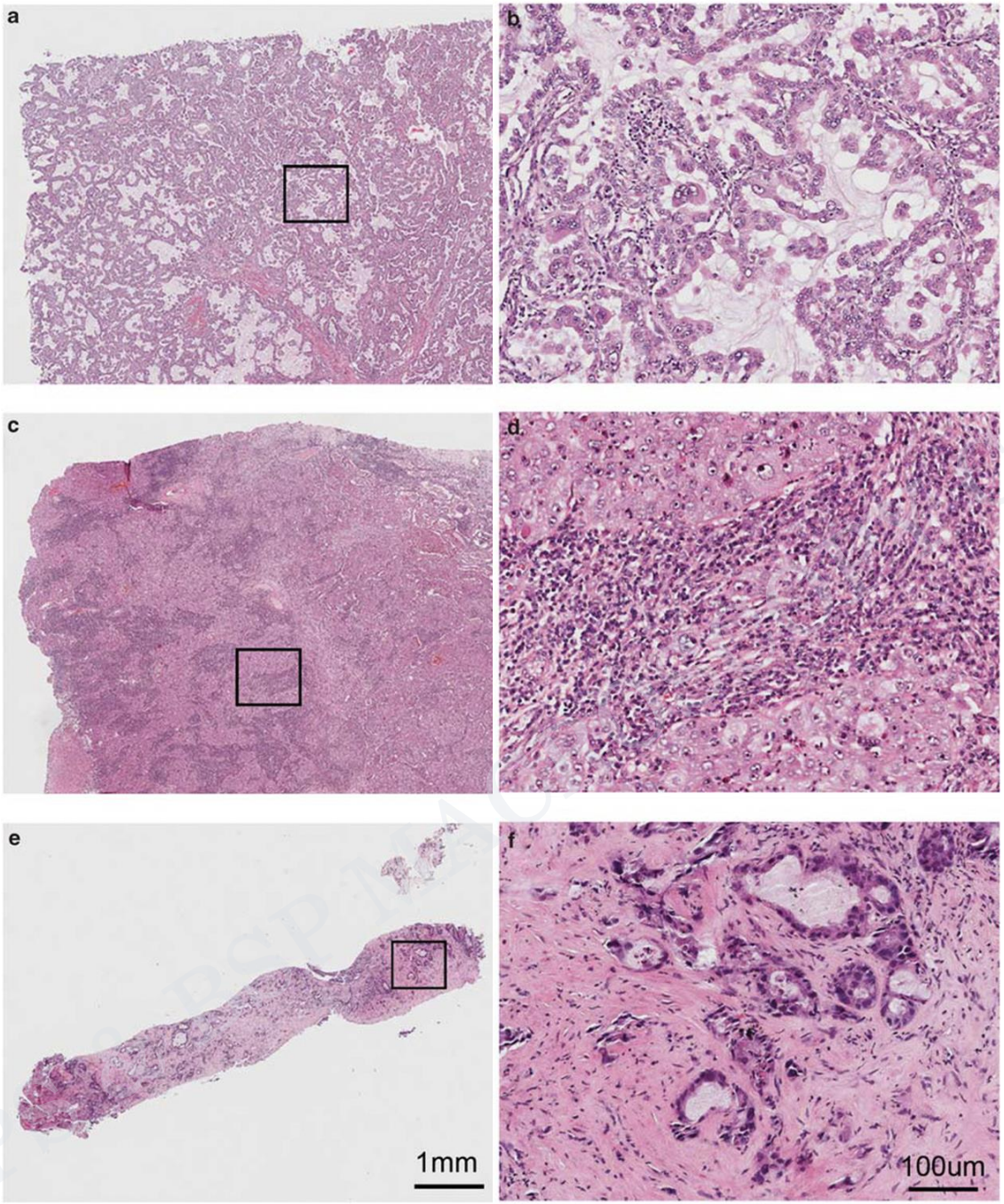
Smits *et al* Modern Pathology (2014) 27, 168–174



2 Individual estimates per slide, corresponding to the shown in Figure 1 (**a**, **c**, **e**, respectively). Arrows in the (**a**–**c**) represent the counted percentages of tumor cells.

YPATHOLOGY COURSE 2023

Smits *et al* Modern Pathology (2014) 27, 168–174

Virchows Arch. 2017 Jan;470(1):21-27. doi: 10.1007/s00428-016-2042-6. Epub 2016 Nov 16.

Adequately defining tumor cell proportion in tissue samples for molecular testing improves interobserver reproducibility of its assessment

- The average difference between lowest and highest estimated percentage was 66%
- The widest range of interobserver variation was observed for samples with dense or scattered lymphocytic infiltrates or with mucinous stroma.

- Estimations were more accurate in cases with a low percentage of tumor cells.
- Macrodissection of the most homogeneous area in the tissue reduced inter-laboratory variation.

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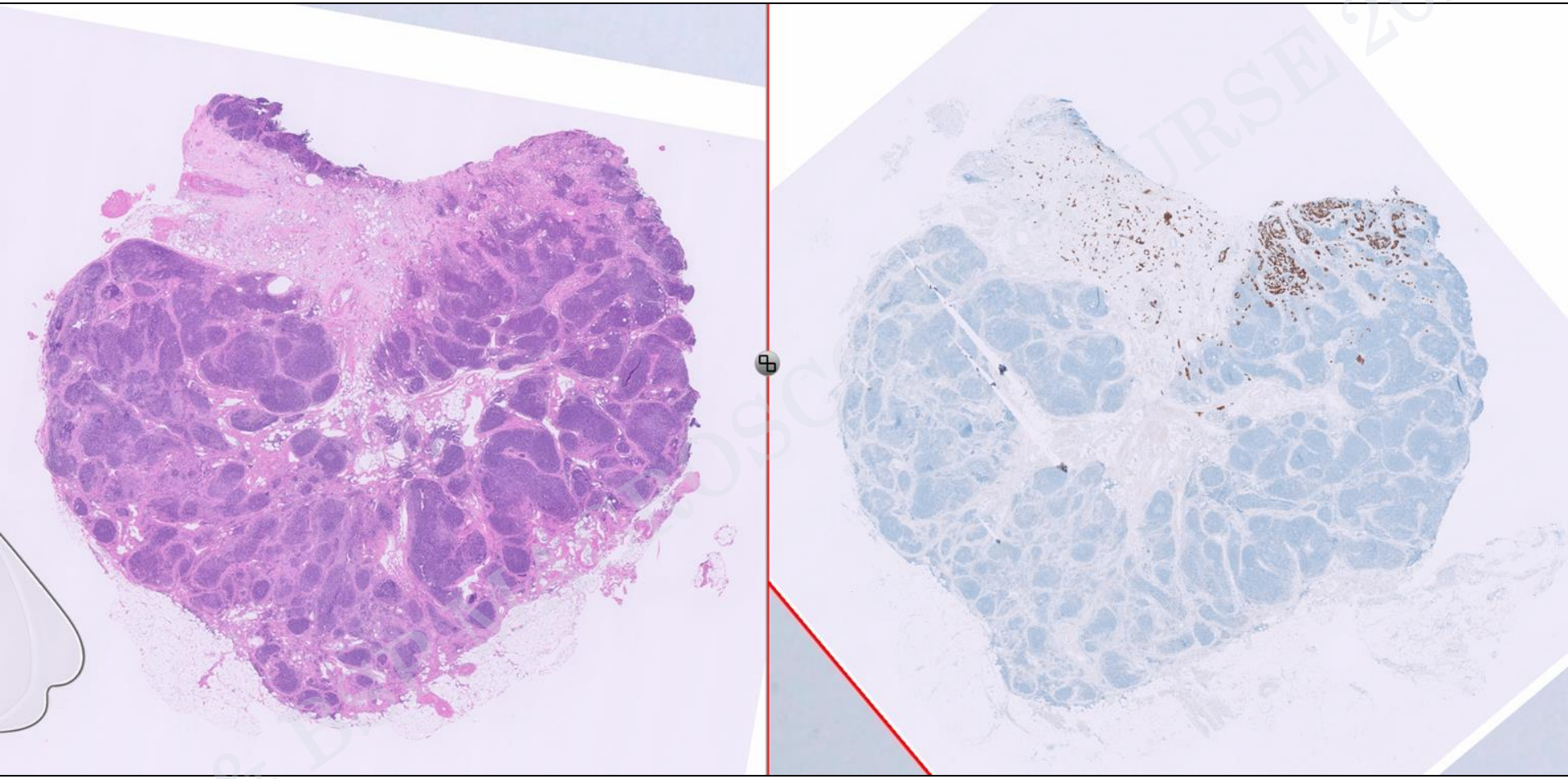

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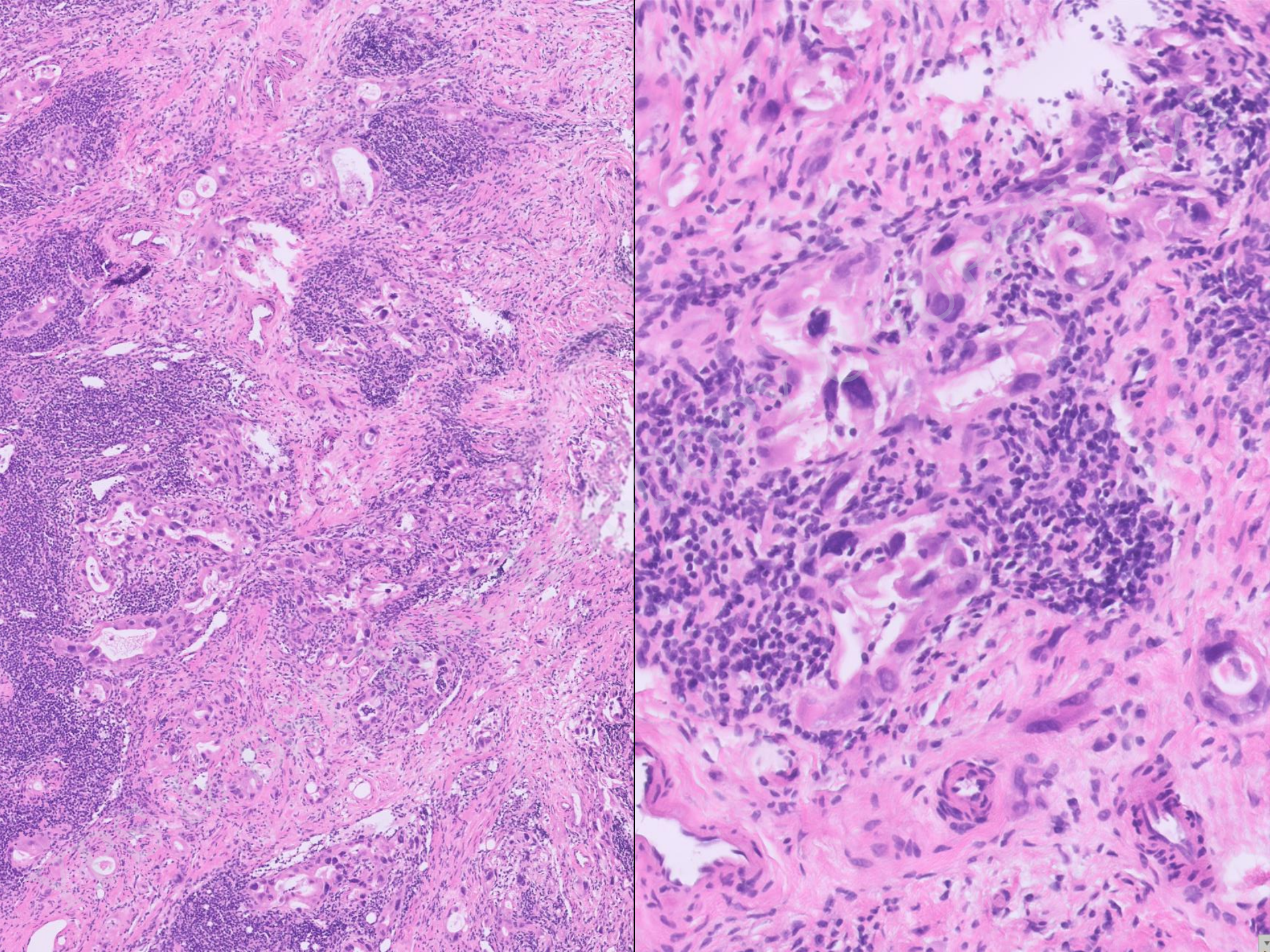

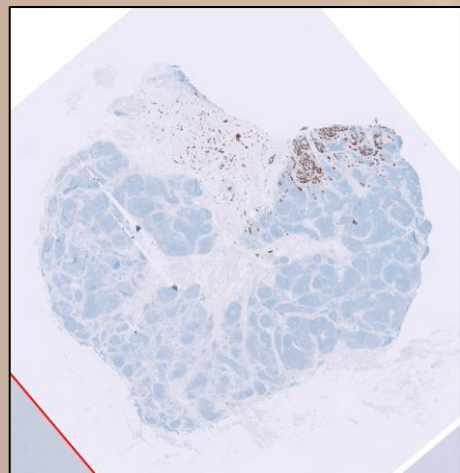

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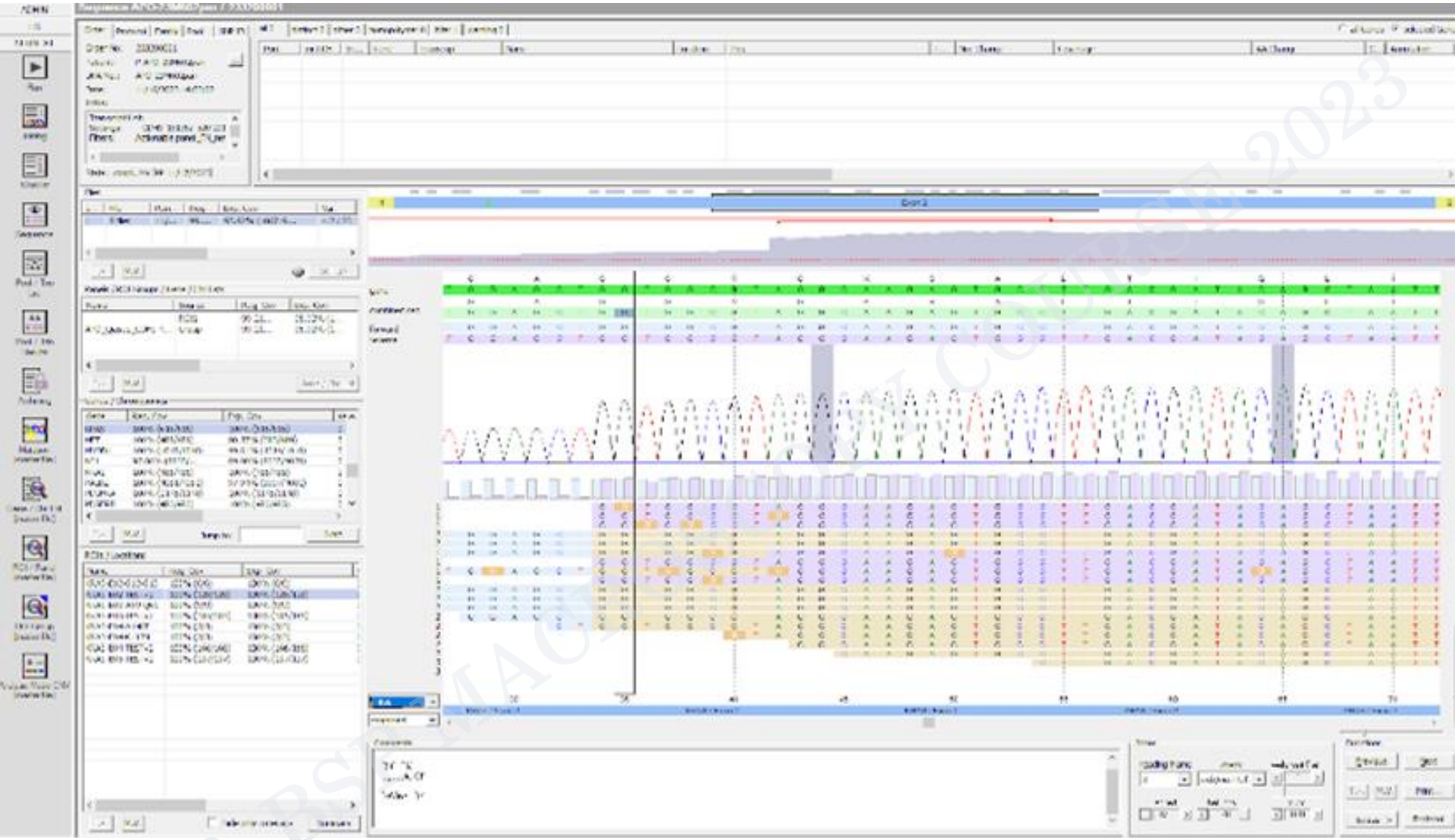

HRAS (NM_005343)	WT	/	/
KRAS (NM_004985.4)	c.35G>A, p.(Gly12Asp)	Pathogeen	0.87%(920)
PIK3CA (NM_006218.3)	WT	/	/

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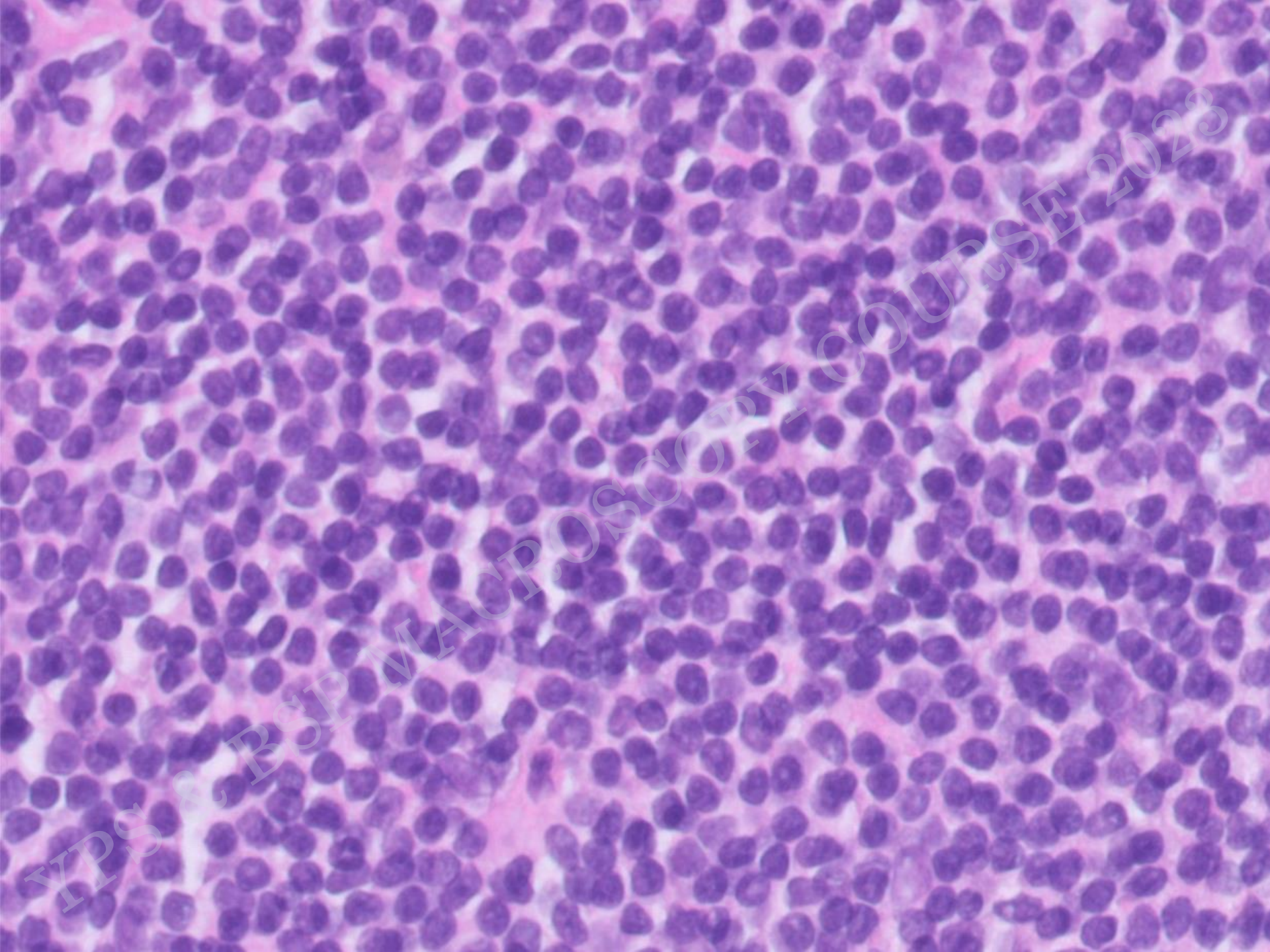
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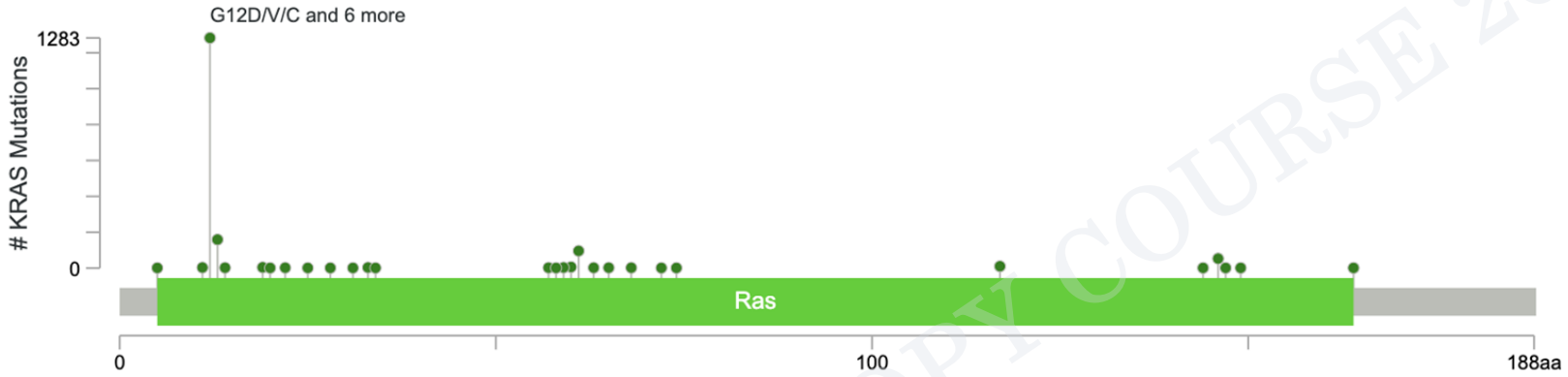

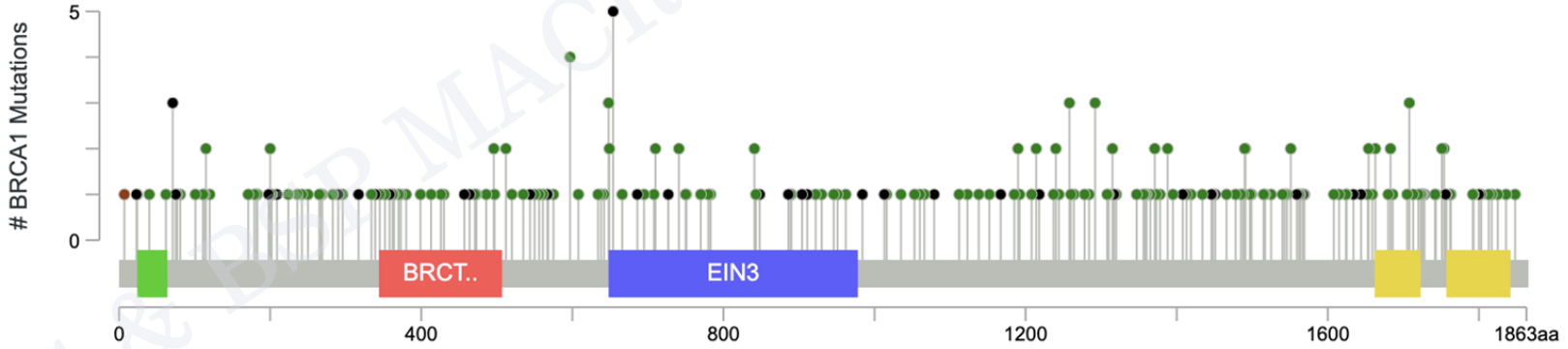

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Take home message

- Tissue is the issue (still)
- Everything starts with a good HE
- Proper handling of the primary sample is crucial
  - Respect cold ischemia time and duration of fixation
  - Use buffered formalin only
  - If decalcification is necessary, EDTA based decalcification is recommended
  - Use standardized and validated methods

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