The Importance of Tissue Quality for Molecular Techniques

- September 2019

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Outline

Best practices for optimal sample quality

- Tissue sample collection
- Tissue processing
- Embedding and microtomy
- Biomolecule extraction
- Quality control testing

Comparative results of molecular assays

- Nucleic Acids: NGS
- Proteins: LCMS and IHC

Goals

- Understand the tissue quality requirements for optimal molecular pathology test results
- Differentiate steps needed in the sample preparation workflow for better results
- Implement quality control procedures into your lab

Tissue sample collection

Garbage in, garbage out

One man's trash is another man's treasure

Histopathology and molecular pathology – clash of two worlds

Histo-Pathology	Molecular
Visual – a good Pathologist can make a correct diagnosis on a poor H&E or IHC slide	Quantitative – issues such as fixation, processing, DNA extraction methods … all impact the accurate outcome of molecular tests.[a]
Specimen and handling procedures well established	Many times tissue is byproduct of Histology
Once tissue is fixed it is relatively stable	Nucleic Acids (NA) should be regularly checked for degradation • Quantity • Quality

Cree IA, et al. *J. of Clin. Path.* 2014
Espina V, et al. *Prot. Clin. Appl.* 2009
Han van Krieken J, et al. *Virch. Archiv.* 2012
[a] Hewitt SM, et al. *Clin. Cancer Res.* 2012

Changing times – FFPE block preparation is more critical than ever

Traditional tissue blocks meant for H&E staining are now being used for clinical techniques:

- Special Stains
- IHC
- In Situ
- Molecular Testing
- Proteomics

"It is impossible to underplay the importance of fixation in histopathology. Whether the scientist is interested in the extraction of information on lipids, proteins, RNA or DNA, fixation is critical to this extraction." William J.Howat, Beverley A.Wilson

Impact of Pre-Analytical Conditions on the Antigenicityof Lung Markers: ALK and MET, Rachel Miller, MS,* Trish Thorne-Nuzzo, BS,* Isabell Loftin, PhD,† Abigail McElhinny, PhD,†Penny Towne, MBA,* and June Clements, MD, Appl Immunohistochem Mol Morphol Volume 00, Number 00, '' 2019

Effect of Fixatives and Tissue Processing on the Content and Integrity of Nucleic Acids Mythily Srinivasan Daniel Sedmak Scott Jewell, The American Journal of Pathology Volume 161, Issue 6, December 2002, Pages 1961-1971

Tissue fixation and the effect of molecular fixatives on downstream staining procedures William J.HowatBeverley A.Wilson Methods Volume 70, Issue 1, November 2014, Pages 12-19

Pitfalls throughout the workflow process

Homogenous Handling of Tissue

OR → Grossing → Processing → Embedding → Microtomy

Important variables

- Ischemic Time
- Fixation Type
- Fixation Duration

Important variables

- Heat
- Reagents

Important variables

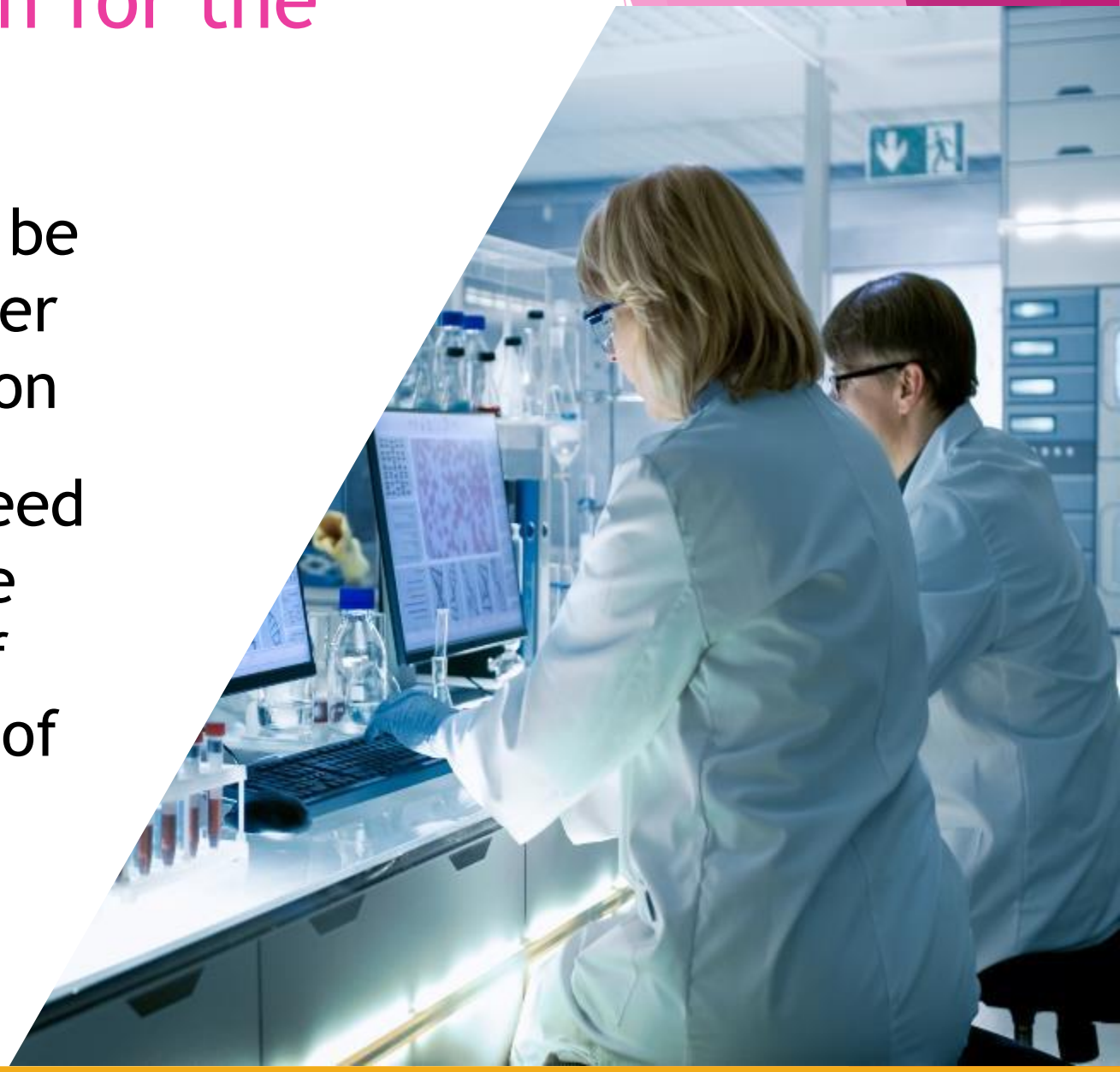
- Cleanliness
- Consistent Handling

For Diverse End Applications

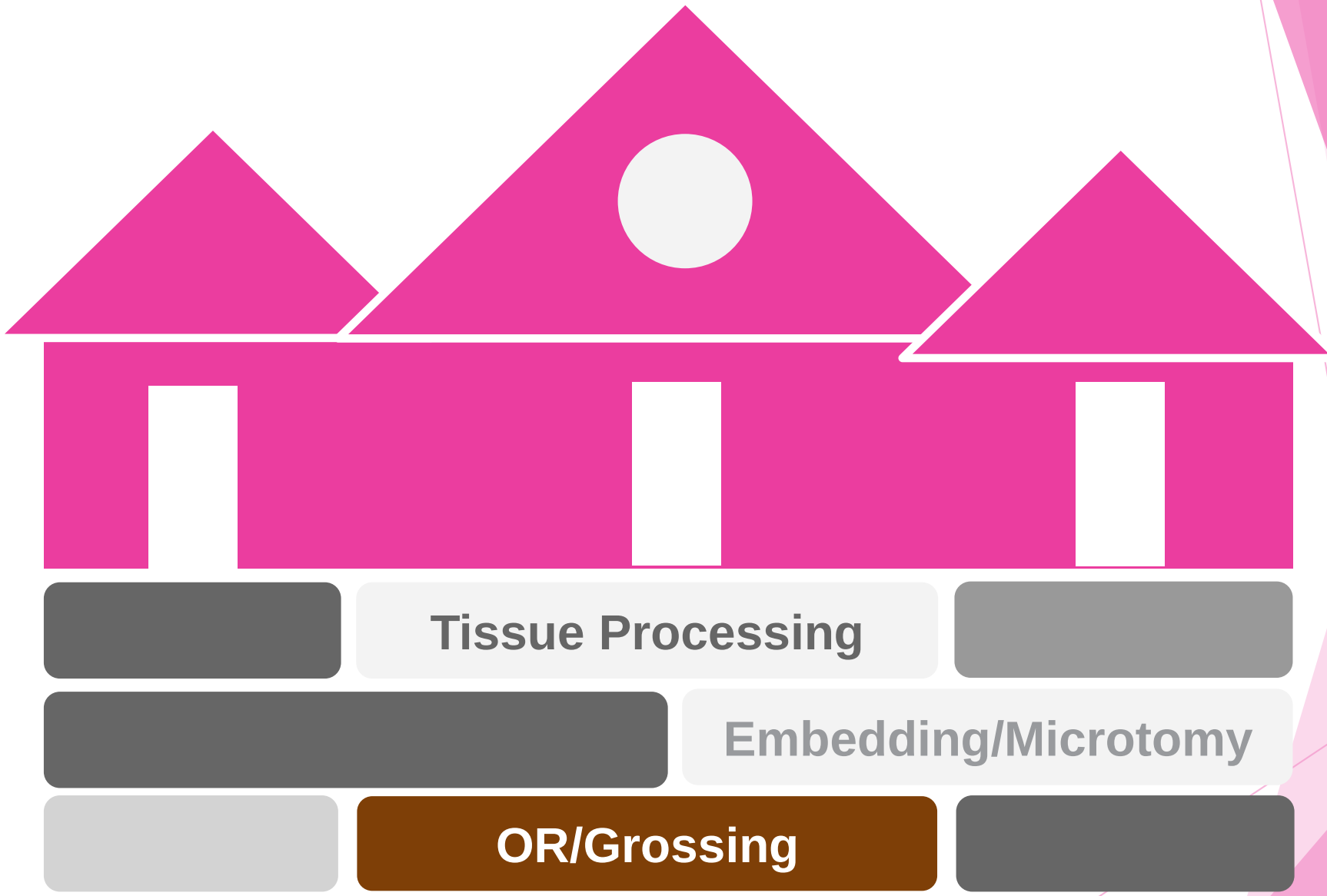
- H&E
- Special Stains
- IHC
- Molecular

What do these changes mean for the histology laboratory?

- Pre-analytical processes need to be reviewed to ensure all stakeholder needs are taken into consideration
- New procedures and practices need to be developed that incorporate the procurement and handling of tissue the incorporates all types of testing



A house built on quality



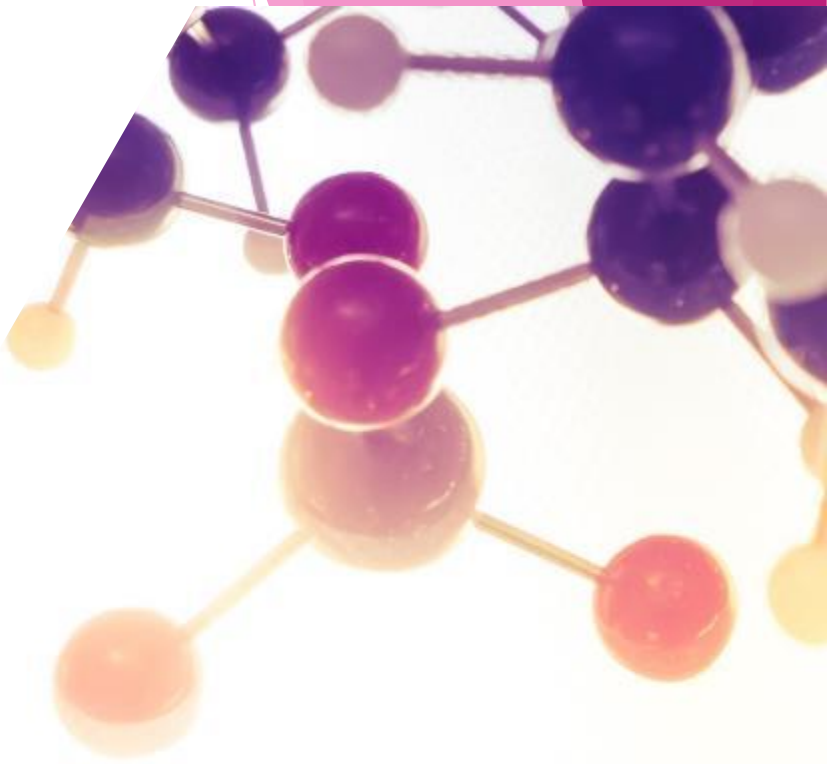
Integrity

Integrity: the state of being whole and undivided

“Not long ago, ‘tissue integrity’ merely meant determining that a patient’s tissue was not so degraded its use in rendering a histologic diagnosis was impossible…”

Now, Histology is seeing an increase of quantifiable testing, which means tissue integrity must now consider:

- Antigenicity
- Stain intensity
- DNA/RNA preservation



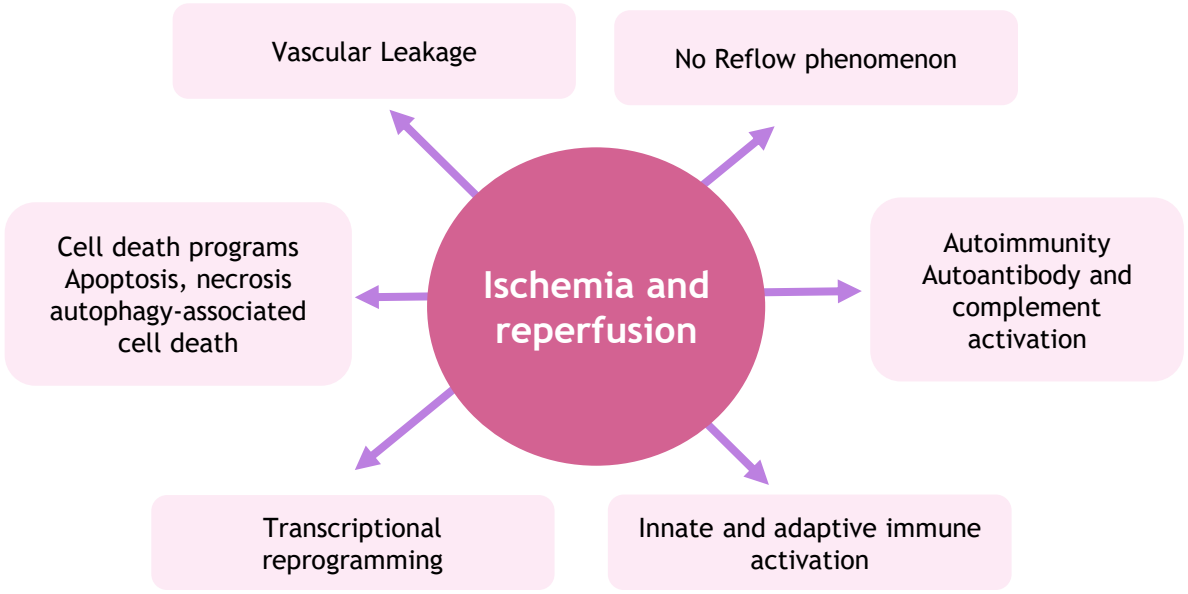
“Recognition that tissue is alive and reactive following procurement”

Allen CA., *Arch of Path. and Lab. Med.* 2014

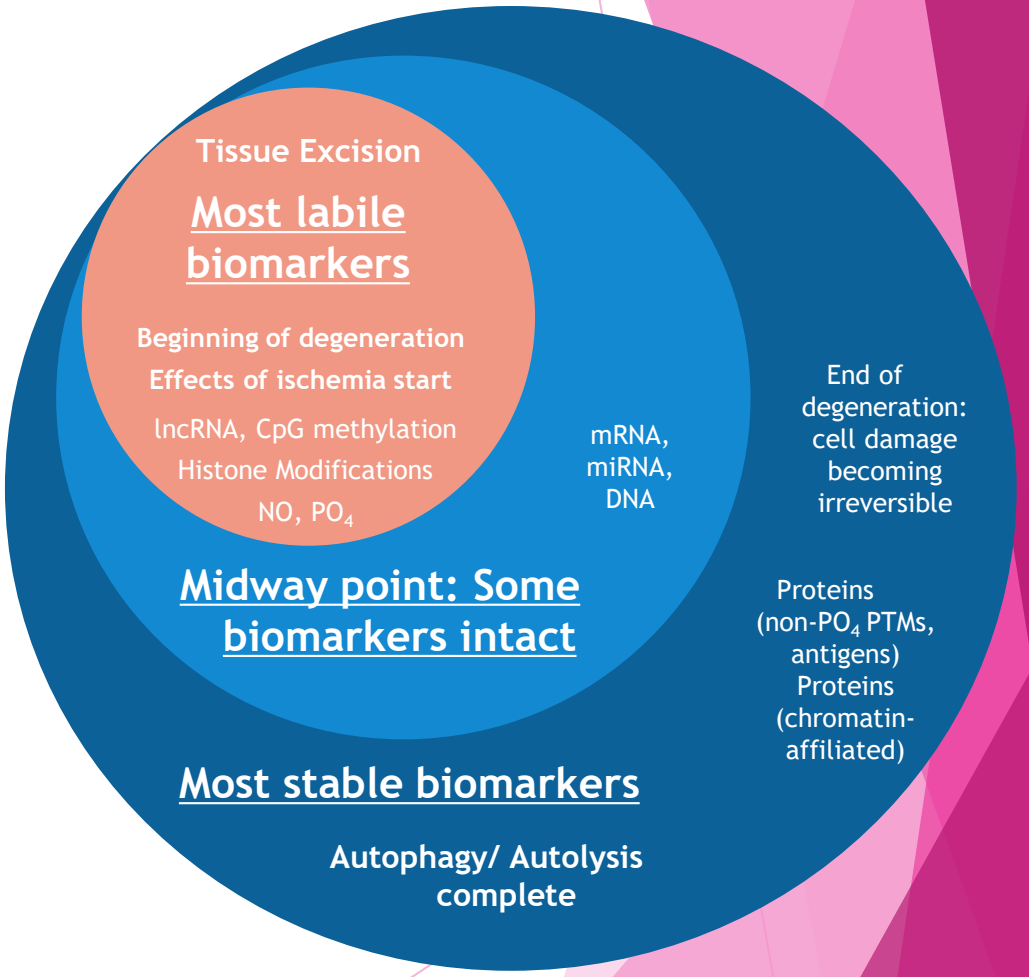
Ischemic time degradation of tissue and biomarkers

Ischemia and Reperfusion – from Mechanism to Translation

Holger K Eltzschig & Tobias Eckle



Biomarker availability over time *ex vivo*



2007 - Clinicians are demanding biospecimen processing standardization for IHC

	Target Antigen			
	Her2	ER/PgR	Her2	Her2
Guideline Publication Date	**2007**	**2010**	**2014**	**2018**
Ischemia time	As short as possible	As short as possible	Less than 1 hour	No change
Fixative	10% NBF	10% NBF	10% NBF	10% NBF
Min Fix Time	6 hrs	6 hrs	6 hrs	6 hrs
Max Fix Time	48 hrs	72 hrs	72 hrs	72 hrs

ASCO GUIDELINES

CAP

ASCO/CAP Guidelines – original, holistic attempt to address preanalytical variability started in 2007

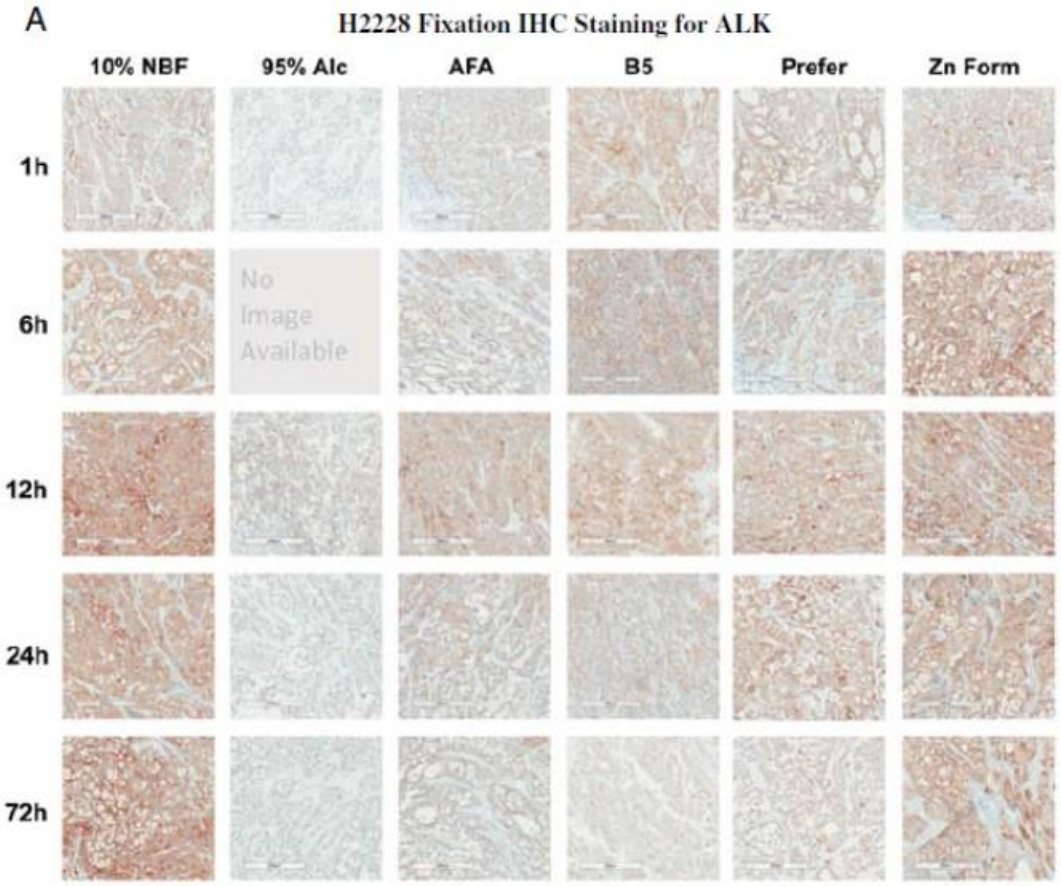
- Recommendations for maximum time between surgical removal and time into fixative (ischemia time), especially for tissues which will be tested for Her2, ER, PR
- Recommendations for total fixation time for certain tissues which will be tested for Her2, ER, PR
- To standardize and to improve quality of biospecimens
  - So as to improve reliability & repeatability of IHC assays

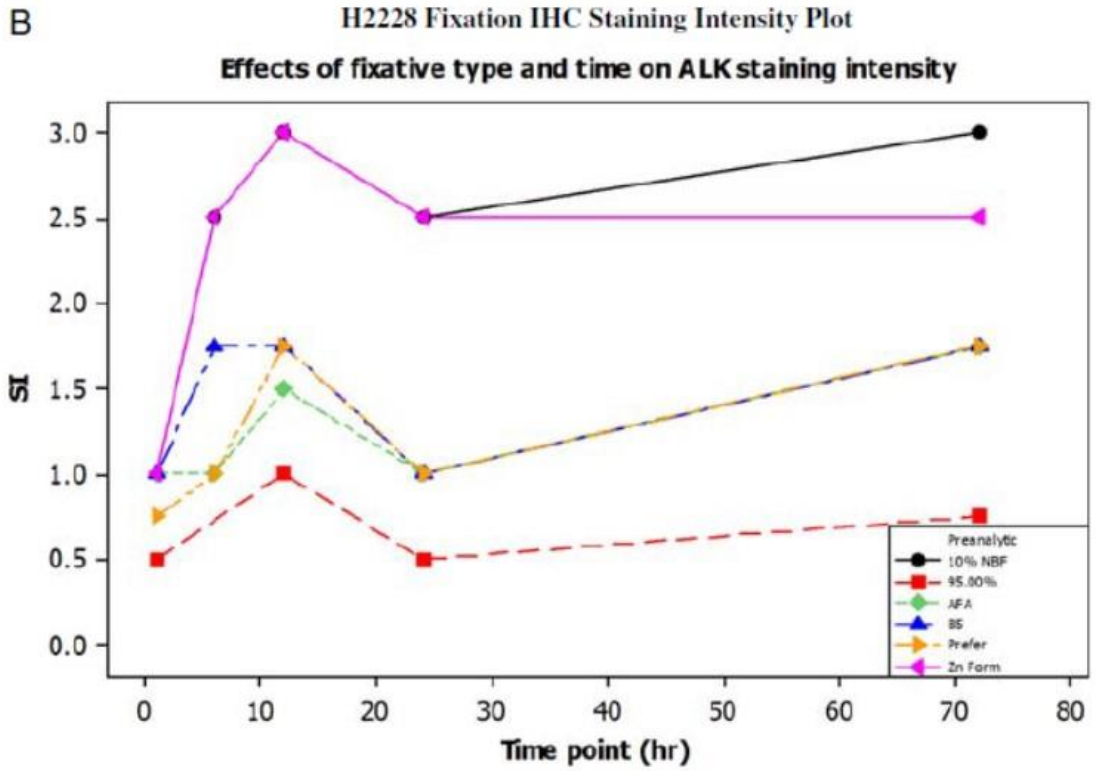
Hammond MEH, et al. *J. of Oncol. Prac.* 2010
Wolff AC, et al. *Arch. of Path. and Lab. Med.* 2007
Wolff AC, et al. *Arch. of Path. and Lab. Med.* 2014
Wolff AC, et al. *Arch. of Path. and Lab. Med.* 2018

Preanalytic Variables – Lung Carcinoma, IHC

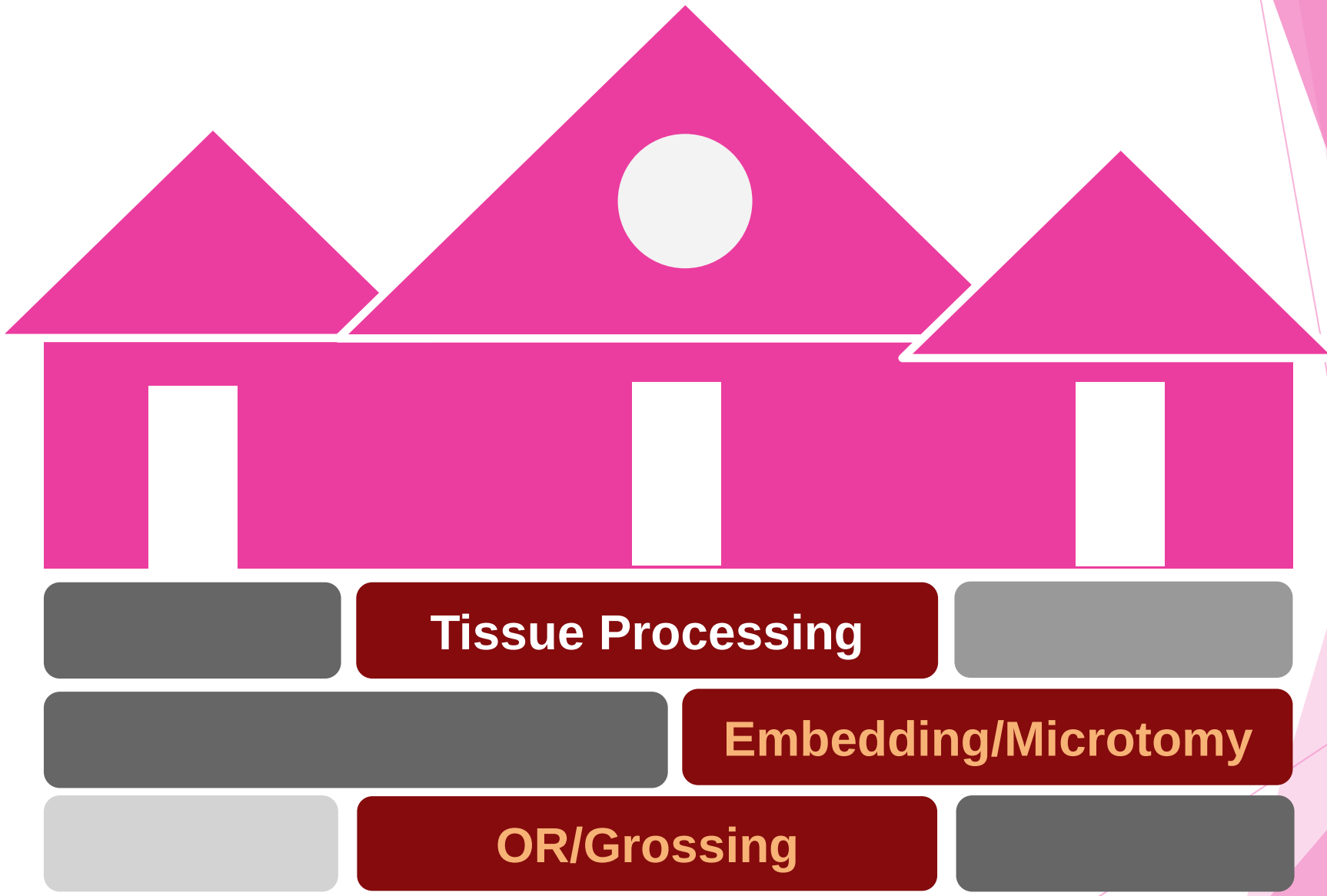
Impact of Pre-Analytical Conditions on the Antigenicity of Lung Markers: ALK and MET

Rachel Miller, MS, Trish Thorne-Nuzzo, BS,* Isabell Loftin, PhD,† Abigail McElhinny, PhD,† Penny Towne, MBA,* and June Clements, MD**





A House Built on Quality



Brief history of tissue processors

1960 — 2020

Technicon (circa 1960)

Shandon Hypercentre

Shandon Pathcentre

Thermo Scientific™ Excelsior™

Thermo Scientific™ Revos™

Visually, there has been limited change to tissue processors since the 1970s

What has changed?

Quality
- Addition of heat, pressure and vacuum
- Agitation methods
- Reagent management systems

Safety
- Open to closed reduced exposure to reagents
- Inclusion of fume filtration

Efficiency
- Multiple retorts
- Better user interface
- New reagents

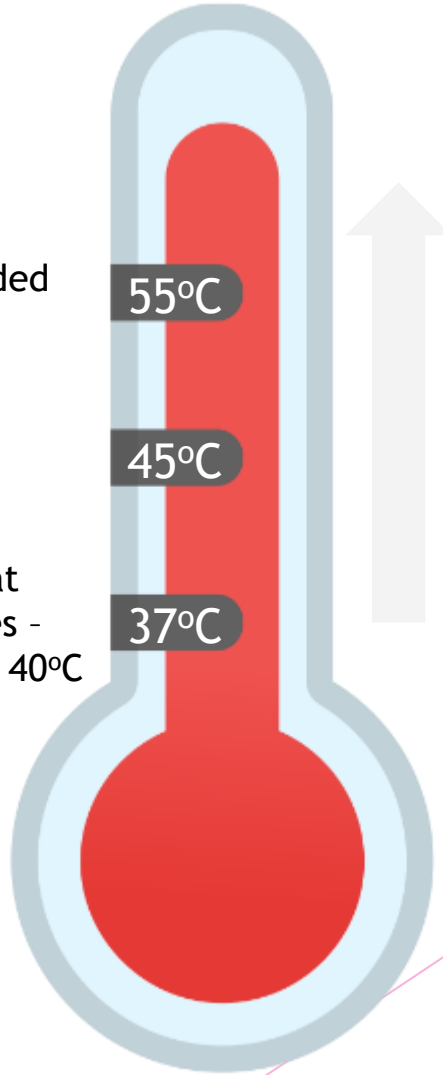
Traditional fixation & processing - heat

Heat is by traditional convection (solution is heated by the heated chamber walls)

Maximum recommended temperature

Heat can be added, recommended at low range: 37 to 45°C

Traditionally occurs at ambient temperatures – room temperature to 40°C



As heat increases:

- Rate of fixation is accelerated (to a point)
- Dehydration is accelerated
- Clearing is accelerated

Microwave Technology – The Emergence of Rapid Processing

Benefits of Microwaves

- Time reduction
- Improved workflow options
- Elimination of Xylene
- Healthier environment
- Generation of new hybrid processors
- New technology and choice

How Microwaves Work

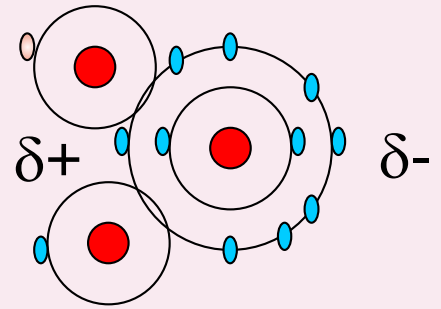


Diagram based on Wolfe. Molecular and Cellular Biology. 1993.

Magnetron – generates electromagnetic waves called microwaves

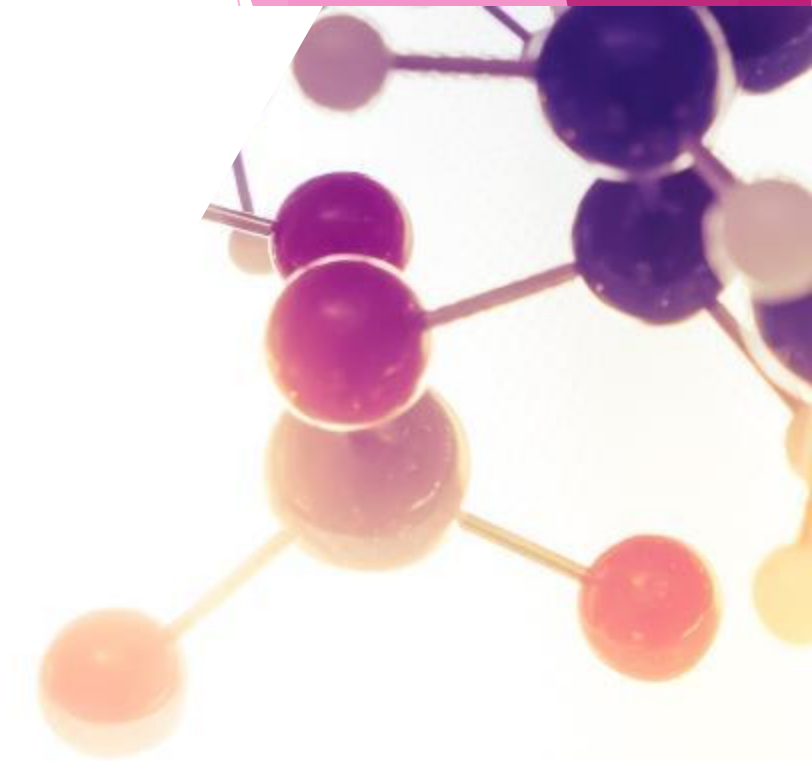
Energy of microwave transformed into *heat* when absorbed by water

Collision of molecules adds energy

Polarized/dipolar molecules oscillate back & forth at 2.5 billion times per second – causes friction that produces *heat*

- Permanent dipoles – such as water
- Induced dipoles – take on polar character when in an electric field
- Neutral particles – non-polar molecules – such as xylene, paraffin

Potential Challenges of Microwave Technology



Fixation

- It is very important that optimal tissue fixation is achieved prior to introduction of microwaves to generate *heat*, or only the outer periphery of the tissue will fix
  - "…for formaldehyde fixation the effect of microwave exposure can only be optimal when methylene glycol is present throughout the tissue when temperature increase starts." *(Kok & Boon, page 132)*

Clearants

- Non-polar molecules do not produce heat
  - Xylene isomers have small to zero dipole moment
    - IPA is polar – has a dipole moment close to water
    - JFC by Milestone is a specially developed clearant for microwave processing – made of long chain hydrocarbon, organic solvent, alcohol blend

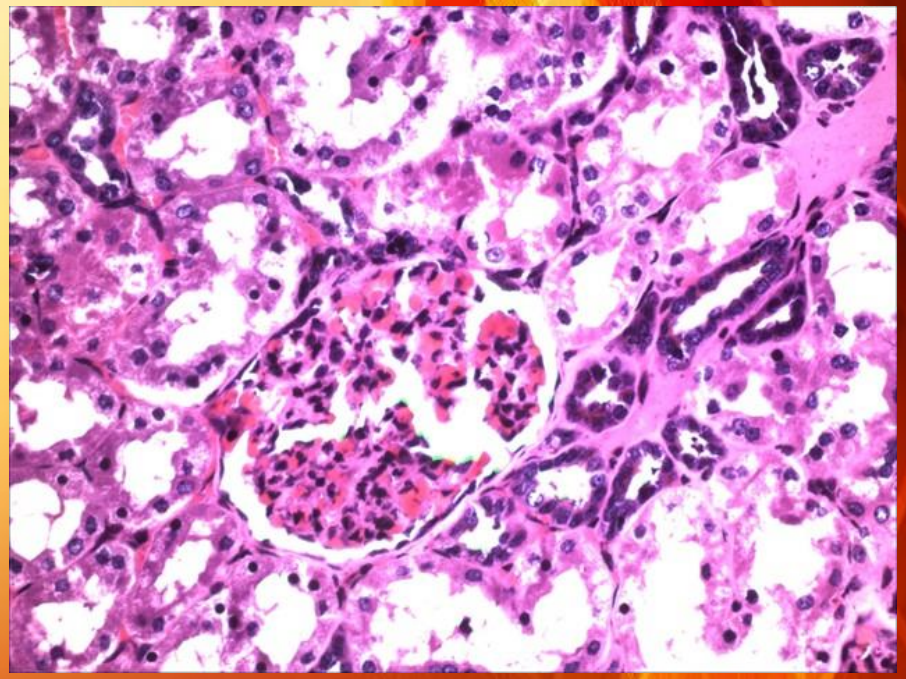
Paraffin has no dipole moment

- Impregnation occurs either by flash technique with one or two baths
- Or several baths with progressively higher temperatures

Rapid Tissue Processing – High Heat Impact on Processing

Horobin states that tissue processing temperatures above 55°C many of the following conditions could occur[a]

- Reagents may be lost or altered.
- Reagents are more reactive at higher temperatures (more toxic and flammable).
- Alteration of tissue proteins are a morphological risk and can negatively impact diagnosis – can arise directly from higher heating or by heating reagents.
- Protein antigenicity (for immunohistochemistry staining) can be affected; if important, keep temperatures below 60°C.
- Potential red blood cell lysis



Kidney, H&E, over-dehydration.
JHF 2016.

a.Horobin R.W, Problems and artifacts of microwave accelerated procedures in neurohistotechnology and resolutions. *Methods: A Companion to Methods in Enzymology*. 15: 101-106, 1998.

Rotational Agitation

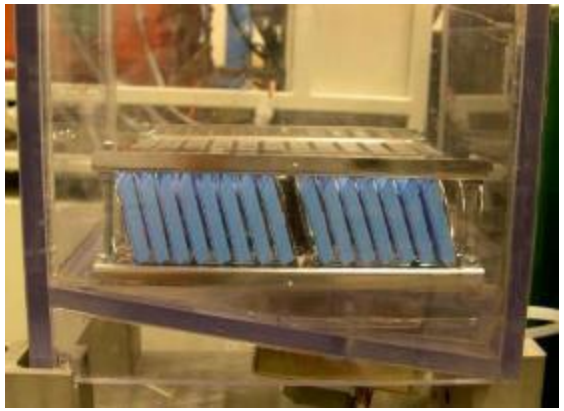
What is Rotary Agitation?

- Canting the processing chamber (tilting)
- Partially filling the chamber to allow the tissue to leave the reagent, be briefly suspended out of the reagent and re-submersed into the reagent again (similar to agitation on an automated stainer)
- Smart basket design that allows for easy fluid movement in and out of the cassettes

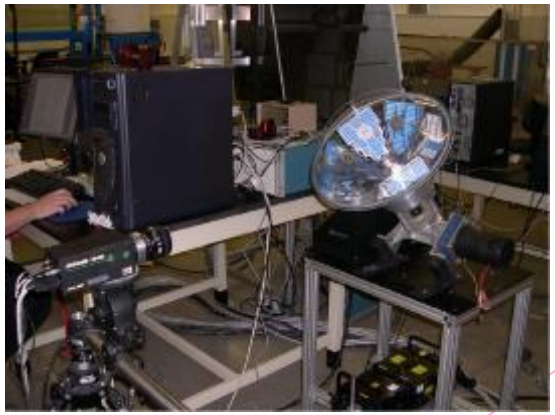
Rotational Agitation vs. Tidal Agitation – Experimental Design

PIV: Particle Image Velocimetry
PLIF: Planar Laser-Induced Fluorescence

Instrumentation & assembly to measure **tidal agitation**



Instrumentation & assembly to measure **rotational agitation**

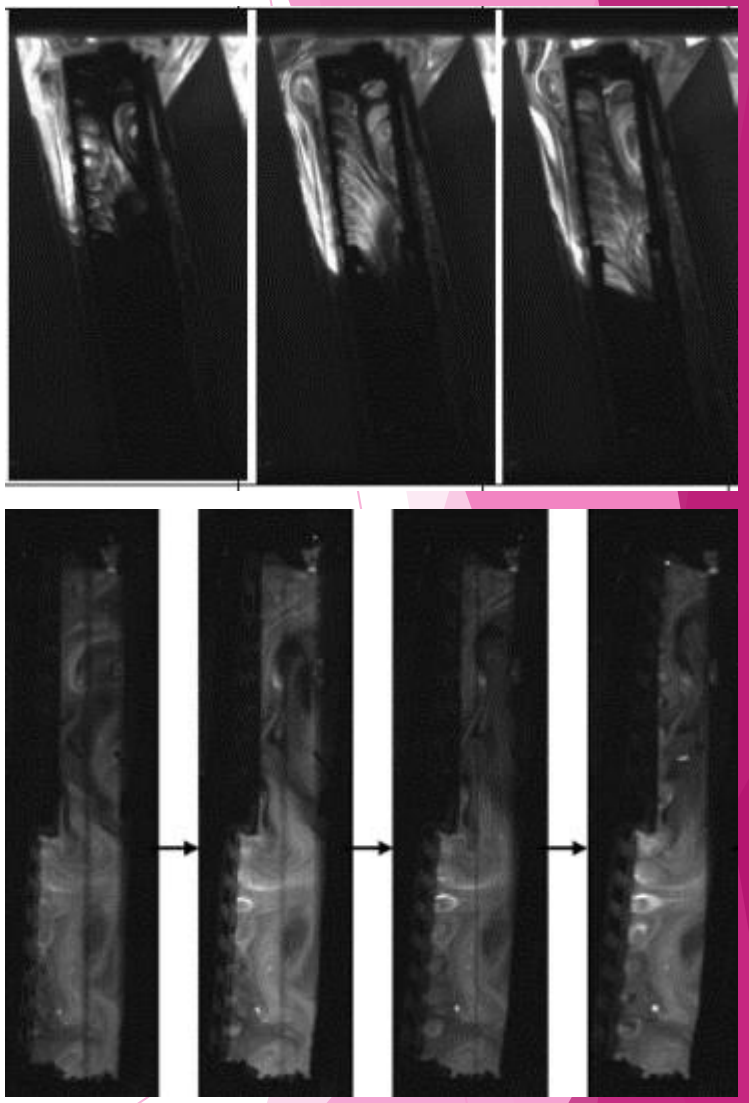


All photos: Parviz Merati, et al. 2011

Rotational Agitation “Flow Rate is Almost 7 Times Larger”

The major difference between the mean velocity fields between the two processors, are the magnitudes and direction of the velocity vectors. The overall magnitude of the velocity vectors are approximately one order of magnitude smaller in the tidal agitation processor compared with the RTP processor. In addition, ***the tidal agitation processor fluid direction is along the cassette length***, rather than being perpendicular to it like the RTP processor. ***Both of these differences would help to reduce the processing time in the RTP model***.

Benefit: Up to a 60% reduction in processing time versus traditional processing protocols

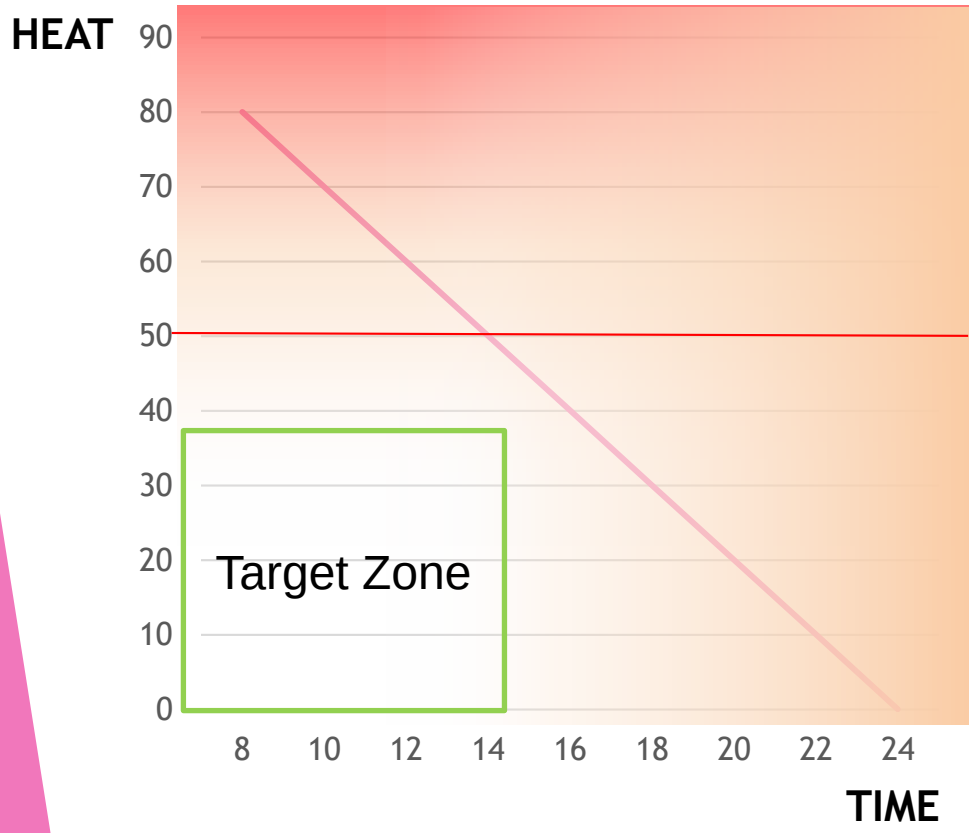


•Merati, P et al. Comparison of tidal agitation and rotary agitation utilizing particle image velocimetry (PIV) and planar laser induced fluorescence (PLIF) methods. Western Michigan University College of Engineering and Applied Sciences Project Report. 2011

All photos and diagrams: Parviz Merati, et al. 2011

Positively impacting tissue processing quality

HEAT VS. TIME



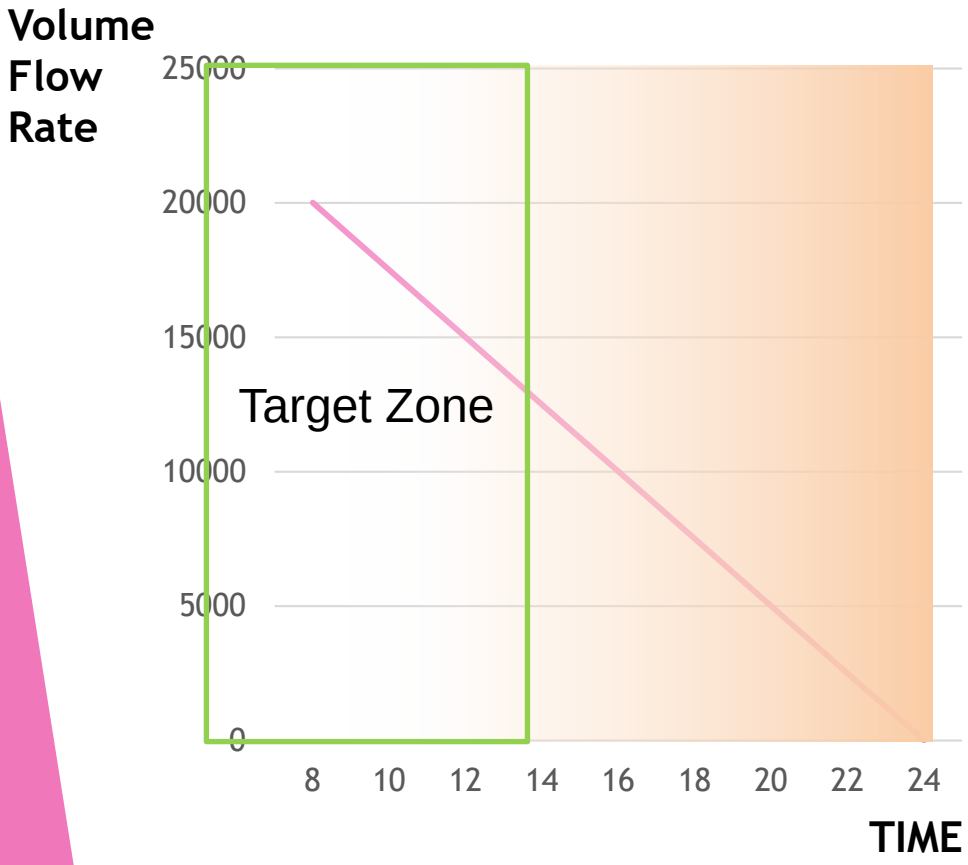
- Evidence suggests that tissue damage begins to become significant at 50°C
- Current guidelines recommend a minimum of 8 hours but less than 72. Evidence suggests damage to RNA is immediate and increases but not significantly from 12-24 hours.[a]
- *Conclusion: To produce high-quality results for all downstream testing reducing heat and time is beneficial*

Total processing time includes fixation tine off the processor

a. Factors in Tissue Handling and Processing That Impact RNA Obtained From Formalin-fixed, Paraffin-embedded Tissue
Joon-Yong Chung, Till Braunschweig, Reginald Williams, Natalie Guerrero, Karl M. Hoffmann, Mijung Kwon, Young K. Song, Steven K. Libutti, and Stephen M. Hewitt

Positively impacting tissue processing quality

AGITATION VS. TIME



- Traditional agitation types provide minimal fluid velocity.
- Advanced agitation and smart design can mean agitation methods can be used instead of heat to reduce processing time[A]
- *Conclusion: Rapid processing no longer needs to be dependent on heat*

Total processing time includes fixation tine off the processor

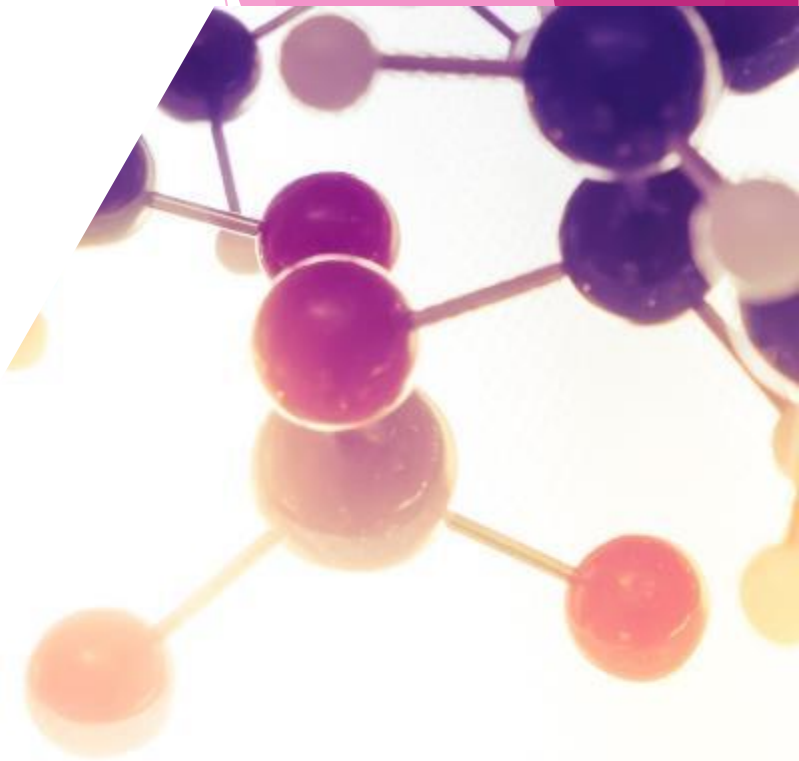
[A] Rotational and Tidal Agitation Fluid Exchange in Comparison of Tissue Processors *Conducted by: Western Michigan University (WMU) Fluids Laboratory Research Period: September 1, 2005 to December 30, 2005 Authored By: Dr. Parviz Merati, Jared Boyd, Hueng Leong*

Three Supporting Studies

Study 1: To test processing heat & time

Study 2: To test fixative type & time

Study 3: To test formalin performance on NGS, IHC, LC-MS



Study 1: High Heat and Time

Sample Collection and Fixation

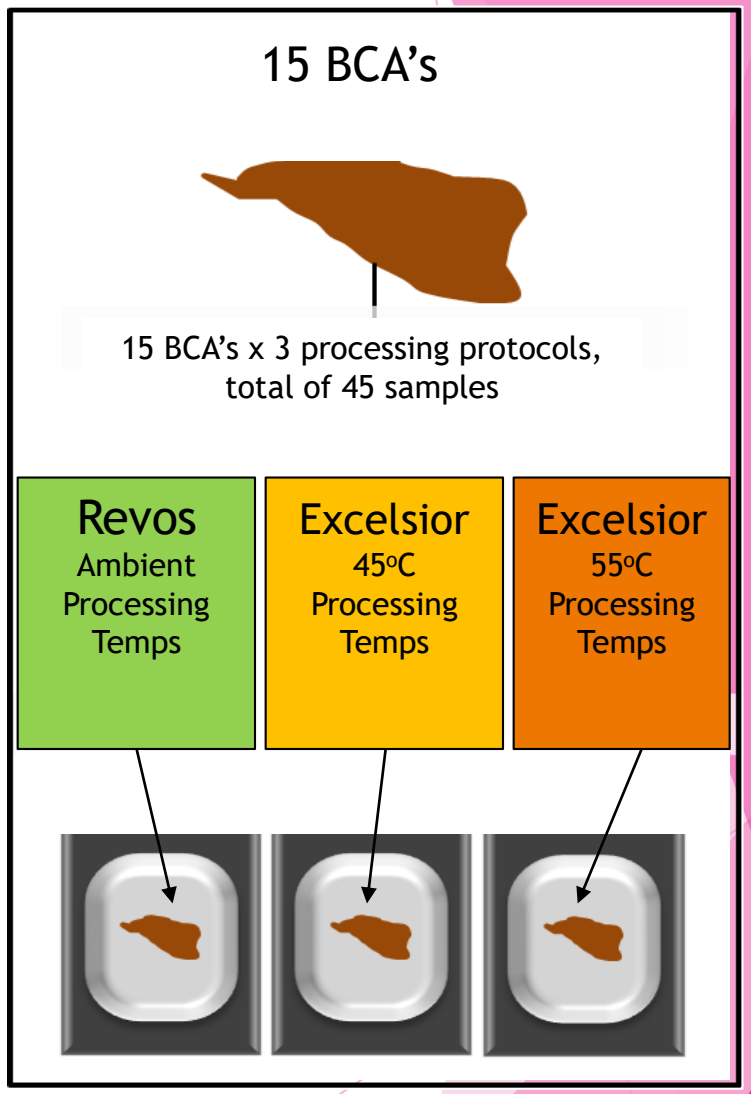
- It is required that the protocol be reviewed by an IRB (Institutional Review Board). The IRB at Asterand Biosciences (a division of BioIVT) will review the test protocol prior to test commencement.
- Fifteen (15) different breast cancer cases will be obtained from Asterand Biosciences (a division of BioIVT). The breast cancer cases will have a variety of known but different protein and DNA biomarker signatures (ER, PR, Ki67, BRCA, Her2, etc)
- The cold ischemia time will be minimized to be less than 60 minutes whenever possible.
- To control for beginning tissue nucleic acid quality, the RIN value of the tissues will be at least 7 or better. This is will be included as part of the Asterand tissue annotation.
- These tissues will be grossed using RNAse-minimized conditions. The tissues will be grossed into three equal portions, to a size of approximately 10 x 10 x 3 mm.
- The tissues will be placed in pre-labeled, RNAse-minimized tissue cassettes and fixed in 10% neutral buffered formalin for 24-72 hours, according to the ASCO-CAP Guidelines.

Preparation of fixed paraffin embedded Tissue

- Each case will be grossed into three equal portions such that each portion has approximately the same amount of tumor; the tissue size is to be approximately 10 x 10 x 3 mm. The tissues will be submitted in tissue cassettes.
  - The first tissue portion will be processed using the REVOS rapid tissue process protocol and standard reagents (10% neutral buffered formalin, reagent grade alcohols, xylene, and histology grade paraffin). The protocol utilizes reagents at ambient temperatures and at 35^{o}C. The paraffin wax steps are programmed at 62^{o}C.
  - The second tissue portion will be processed using the standard 6-hour xylene protocol and standard reagents (10% neutral buffered formalin, reagent grade alcohols, xylene, and histology grade paraffin). on the Excelsior AS tissue processor. The protocol utilizes reagents at temperatures of 45^{o}C. The paraffin wax steps are programmed at 65^{o}C.
  - The third tissue portion will be processed using the standard 6-hour xylene protocol and standard reagents (10% neutral buffered formalin, reagent grade alcohols, xylene, and histology grade paraffin). This protocol will be placed on the Excelsior AS tissue processor. The protocol utilizes reagents at temperatures of 55^{o}C. The paraffin wax steps are programmed at 65^{o}C.
- Each tissue will be embedded in paraffin according to laboratory standard operating procedure.

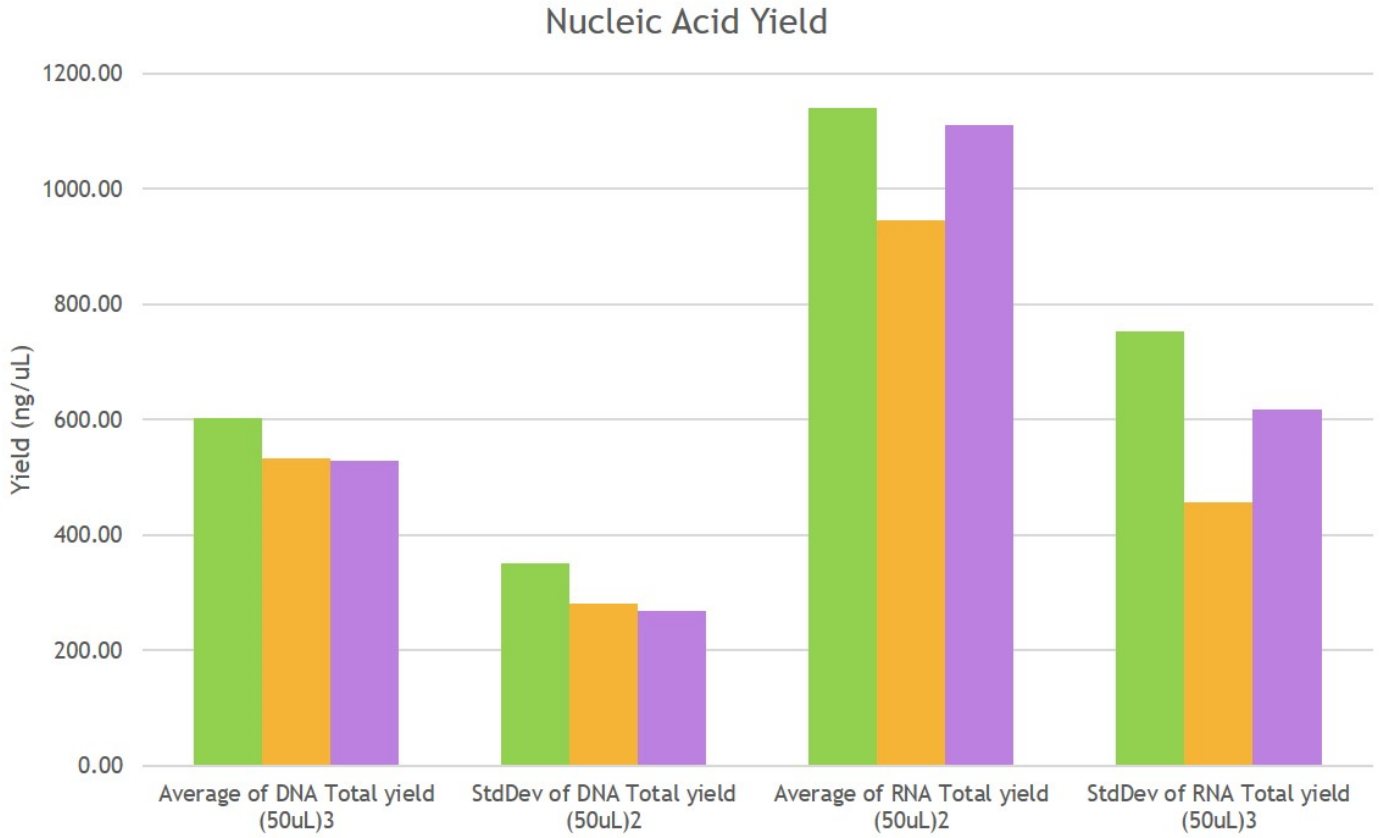
Excelsior and Revos Test Plan

Sample Name	Block Number	Process
Breast Cancer Case 1	Breast 1A	Revos Rapid Tissue
Breast Cancer Case 1	Breast 1B	Excelsior Xylene 6 hour 45C
Breast Cancer Case 1	Breast 1C	Excelsior Xylene 6 hour 55C
Breast Cancer Case 2	Breast 2A	Revos Rapid Tissue
Breast Cancer Case 2	Breast 2B	Excelsior Xylene 6 hour 45C
Breast Cancer Case 2	Breast 2C	Excelsior Xylene 6 hour 55C
Breast Cancer Case 3	Breast 3A	Revos Rapid Tissue
Breast Cancer Case 3	Breast 3B	Excelsior Xylene 6 hour 45C
Breast Cancer Case 3	Breast 3C	Excelsior Xylene 6 hour 55C
Breast Cancer Case 4	Breast 4A	Revos Rapid Tissue
Breast Cancer Case 4	Breast 4B	Excelsior Xylene 6 hour 45C
Breast Cancer Case 4	Breast 4C	Excelsior Xylene 6 hour 55C
Breast Cancer Case 5	Breast 5A	Revos Rapid Tissue
Breast Cancer Case 5	Breast 5B	Excelsior Xylene 6 hour 45C
Breast Cancer Case 5	Breast 5C	Excelsior Xylene 6 hour 55C
Breast Cancer Case 6	Breast 6A	Revos Rapid Tissue
Breast Cancer Case 6	Breast 6B	Excelsior Xylene 6 hour 45C
Breast Cancer Case 6	Breast 6C	Excelsior Xylene 6 hour 55C
Breast Cancer Case 7	Breast 7A	Revos Rapid Tissue
Breast Cancer Case 7	Breast 7B	Excelsior Xylene 6 hour 45C
Breast Cancer Case 7	Breast 7C	Excelsior Xylene 6 hour 55C
Breast Cancer Case 8	Breast 8A	Revos Rapid Tissue
Breast Cancer Case 8	Breast 8B	Excelsior Xylene 6 hour 45C
Breast Cancer Case 8	Breast 8C	Excelsior Xylene 6 hour 55C
Breast Cancer Case 9	Breast 9A	Revos Rapid Tissue
Breast Cancer Case 9	Breast 9B	Excelsior Xylene 6 hour 45C
Breast Cancer Case 9	Breast 9C	Excelsior Xylene 6 hour 55C
Breast Cancer Case 10	Breast 10A	Revos Rapid Tissue
Breast Cancer Case 10	Breast 10B	Excelsior Xylene 6 hour 45C
Breast Cancer Case 10	Breast 10C	Excelsior Xylene 6 hour 55C
Breast Cancer Case 11	Breast 11A	Revos Rapid Tissue
Breast Cancer Case 11	Breast 11B	Excelsior Xylene 6 hour 45C
Breast Cancer Case 11	Breast 11C	Excelsior Xylene 6 hour 55C
Breast Cancer Case 12	Breast 12A	Revos Rapid Tissue
Breast Cancer Case 12	Breast 12B	Excelsior Xylene 6 hour 45C
Breast Cancer Case 12	Breast 12C	Excelsior Xylene 6 hour 55C
Breast Cancer Case 13	Breast 13A	Revos Rapid Tissue
Breast Cancer Case 13	Breast 13B	Excelsior Xylene 6 hour 45C
Breast Cancer Case 13	Breast 13C	Excelsior Xylene 6 hour 55C
Breast Cancer Case 14	Breast 14A	Revos Rapid Tissue
Breast Cancer Case 14	Breast 14B	Excelsior Xylene 6 hour 45C
Breast Cancer Case 14	Breast 14C	Excelsior Xylene 6 hour 55C
Breast Cancer Case 15	Breast 15A	Revos Rapid Tissue
Breast Cancer Case 15	Breast 15B	Excelsior Xylene 6 hour 45C
Breast Cancer Case 15	Breast 15C	Excelsior Xylene 6 hour 55C



Comparative Results

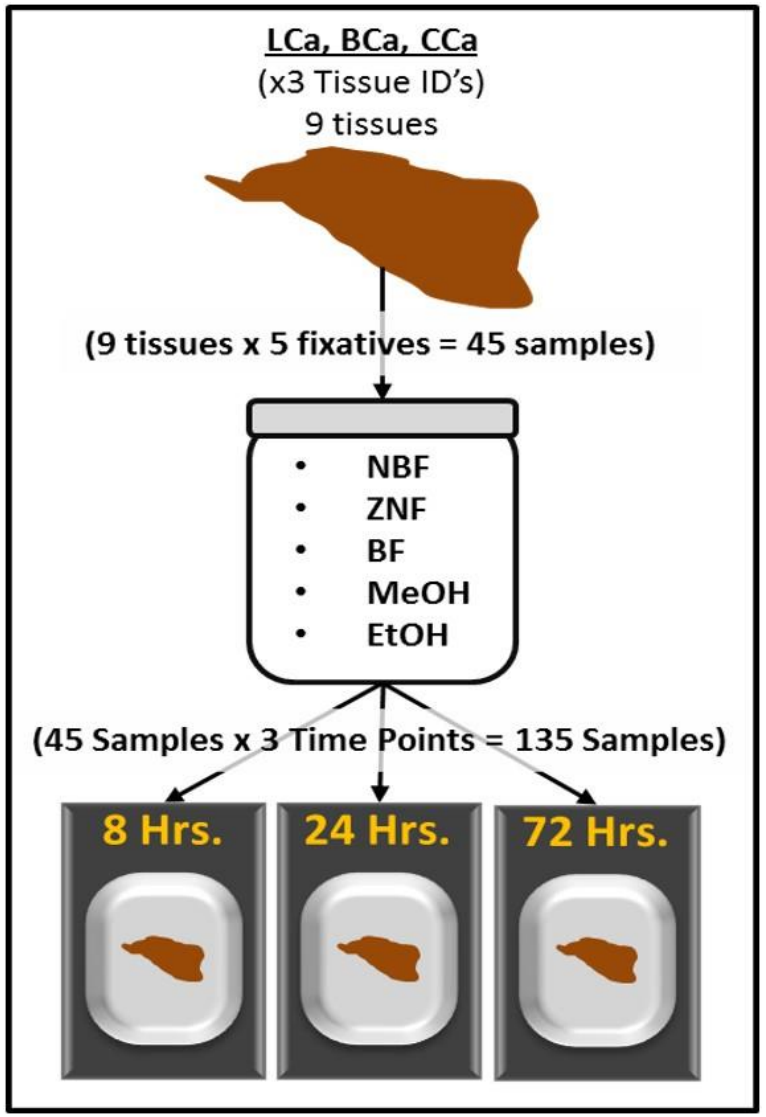
Quality Control Results: Qubit



A combination of heat and length of reagent incubation time contribute to nucleic acid degradation

Condition Details
Revos Protocol (5 hours)
45C Protocol (10 hours)
55C Protocol (10 hours)

Study 2: Fixative Type, Time Test Plan



- Tissues were grossed & fixed according to the schematic (left)
- All tissues were processed using the same tissue processing protocol
  - Excelsior AS
  - Fixation offline
- Tissues were sectioned for H&E, IHC, and NGS

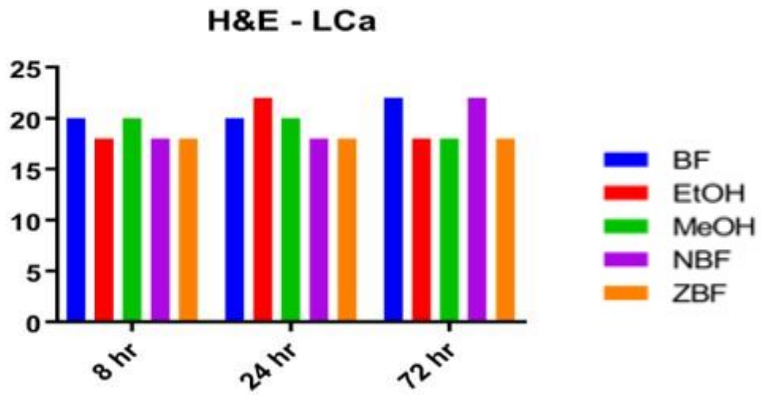
Isaac, J et al. Poster presentation. ASMS, 2016

Fixation Type, Time: H&E of Lung Cancer

1. Formalin & Zinc Formalin produce excellent morphology by H&E at all time points

2. Bouin's produces excellent morphology by H&E at all time points

3. How do they compare for IHC & molecular?



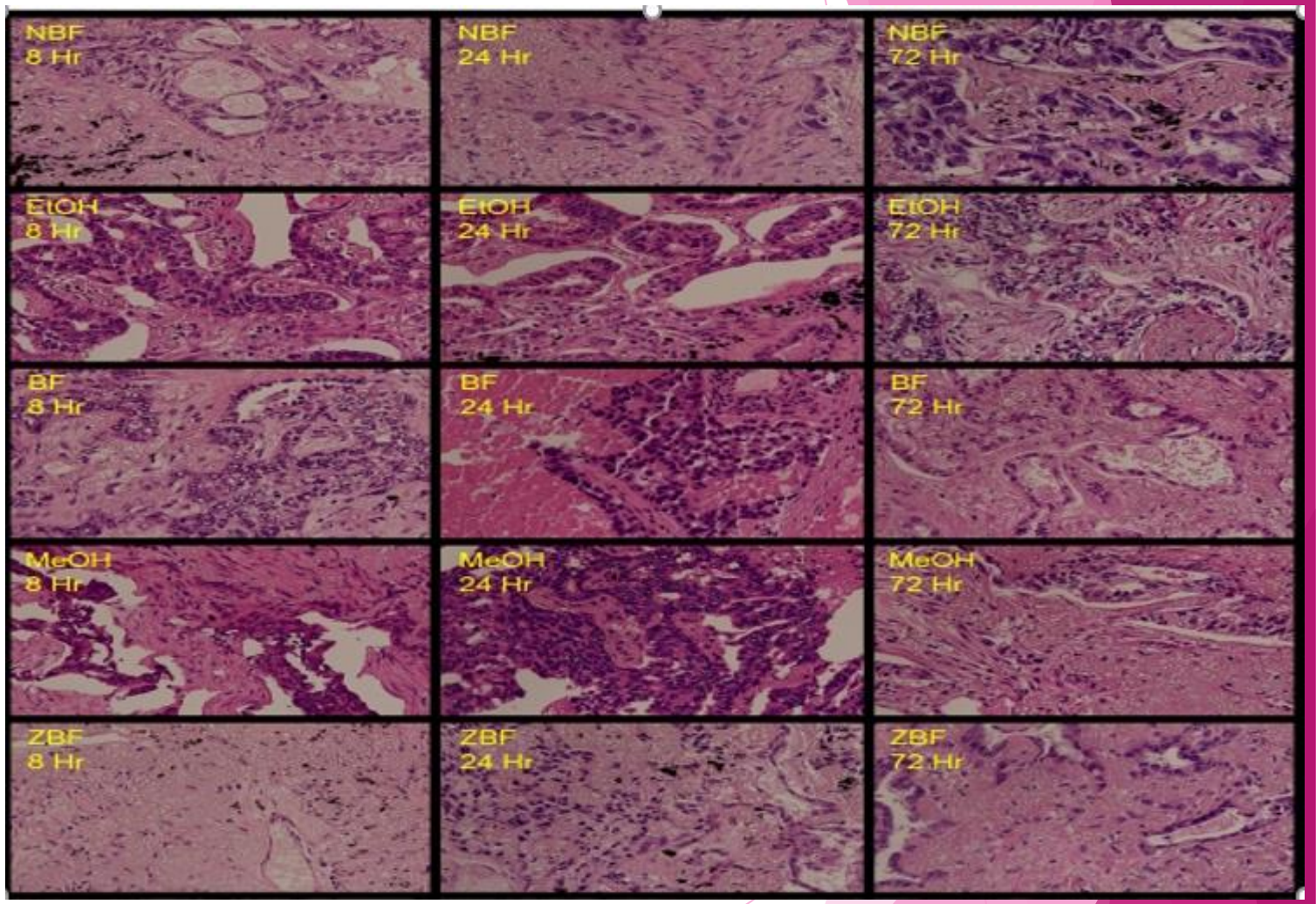
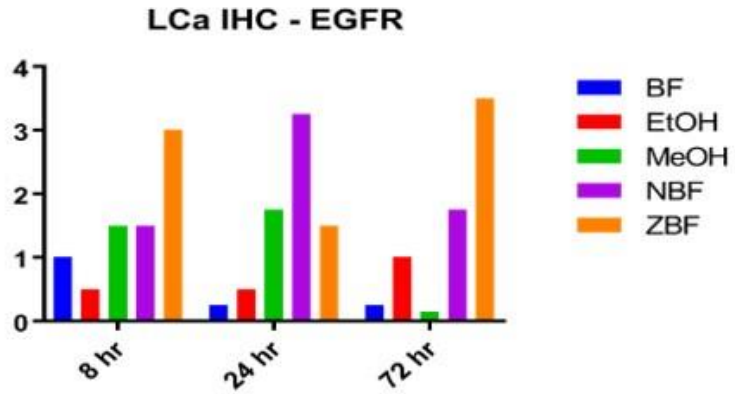


Figure 1A

Isaac, J et al. *J Mol Diag*. 2016

Fixation Type, Time: IHC EGFR – Lung Cancer

1. Formalin produces excellent IHC signal at 24 hours
2. Zinc Formalin produces excellent IHC signal at 8 and 72 hours
3. Bouin's produces poor IHC staining at all time points
4. How do they compare for molecular?



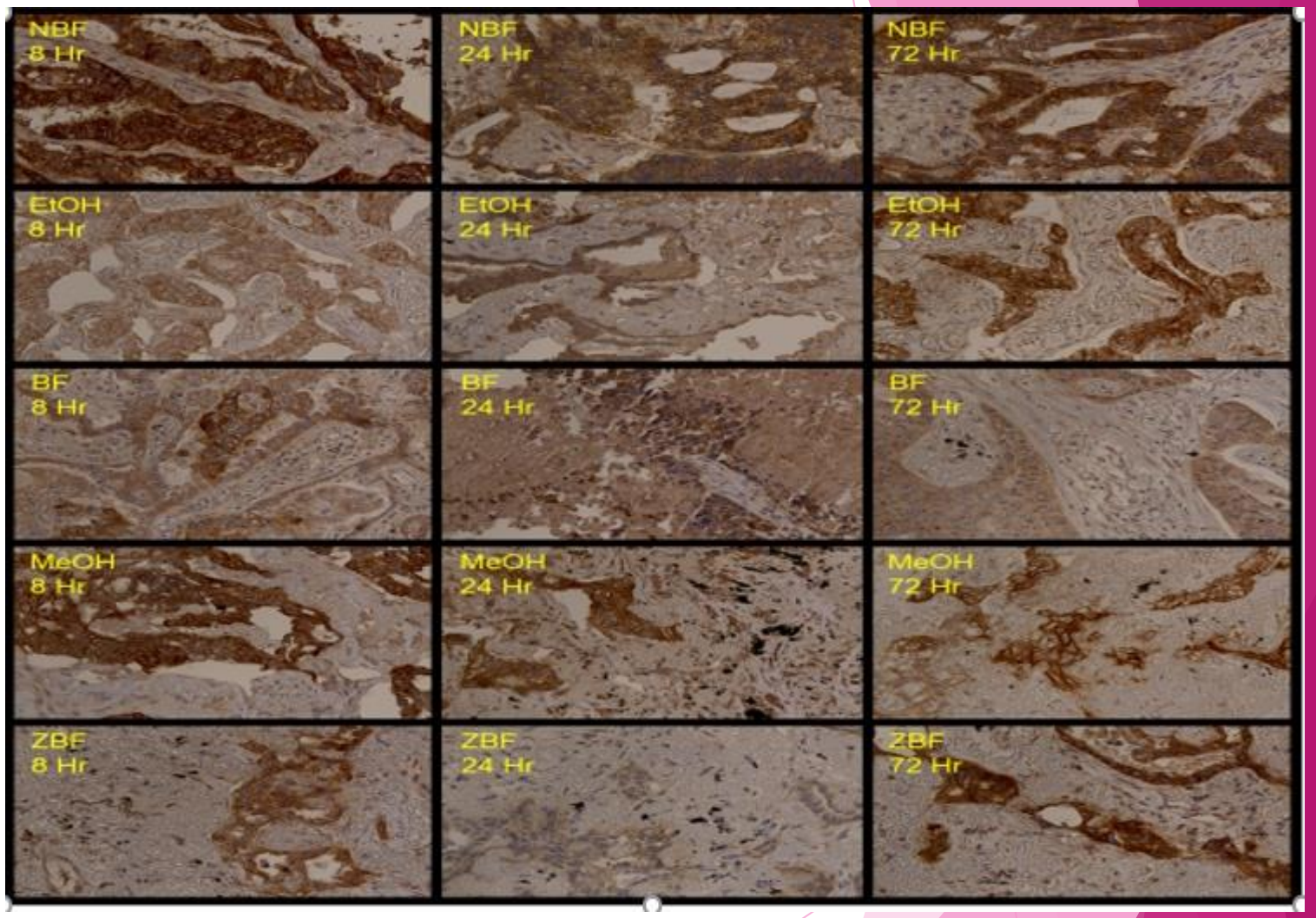


Figure 1B

Isaac, J et al. *J Mol Diag*. 2016

Fixative Type, Time - NGS Results

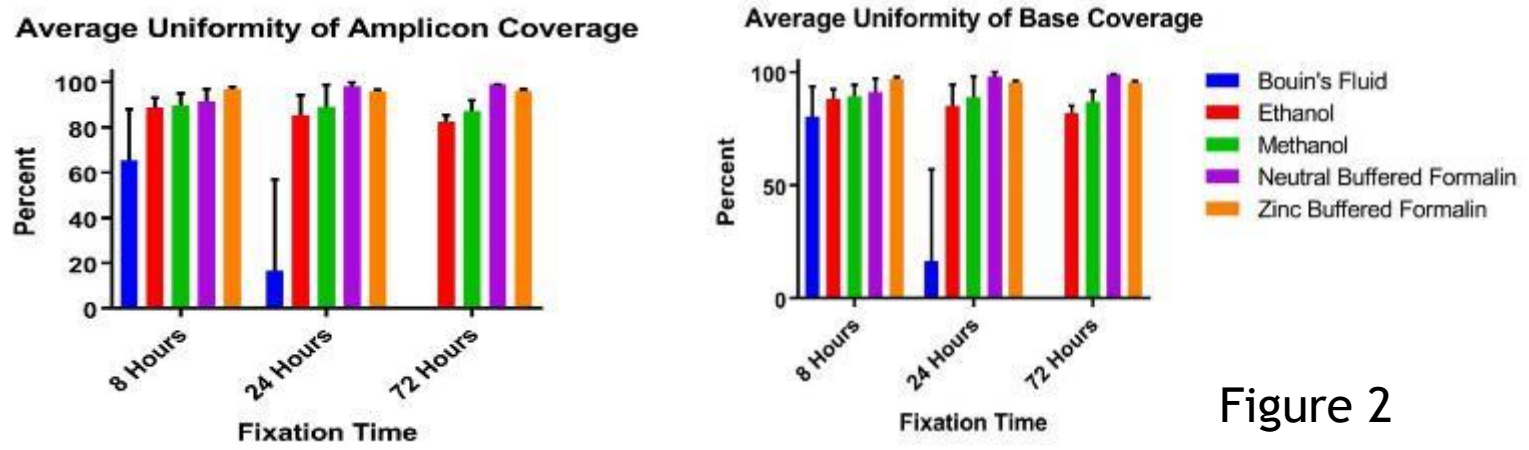


Figure 2

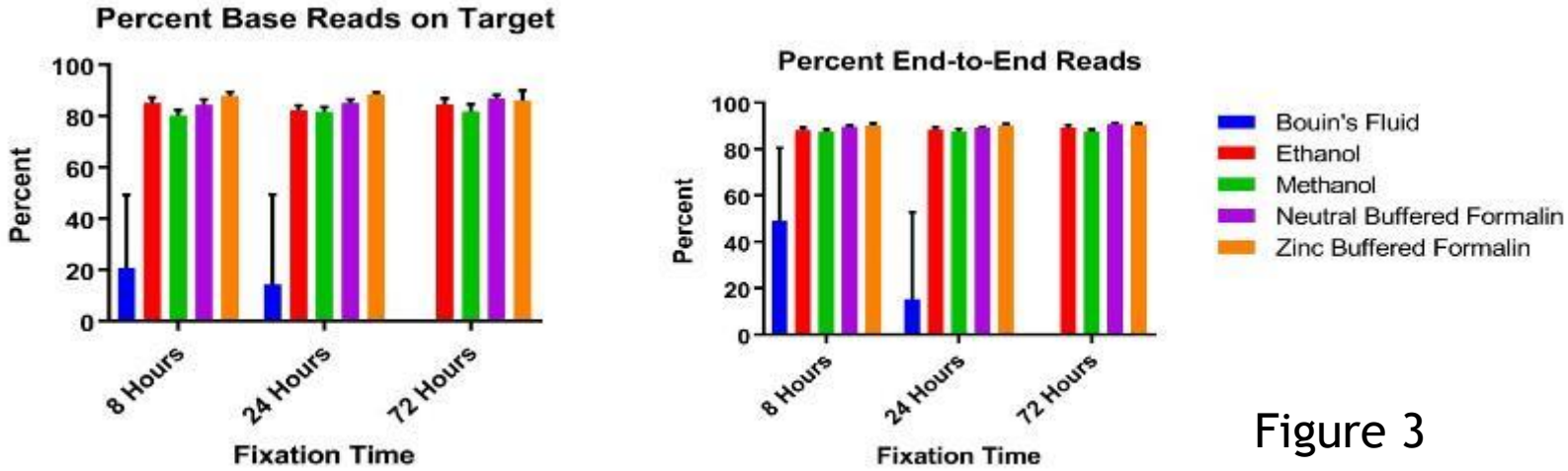


Figure 3

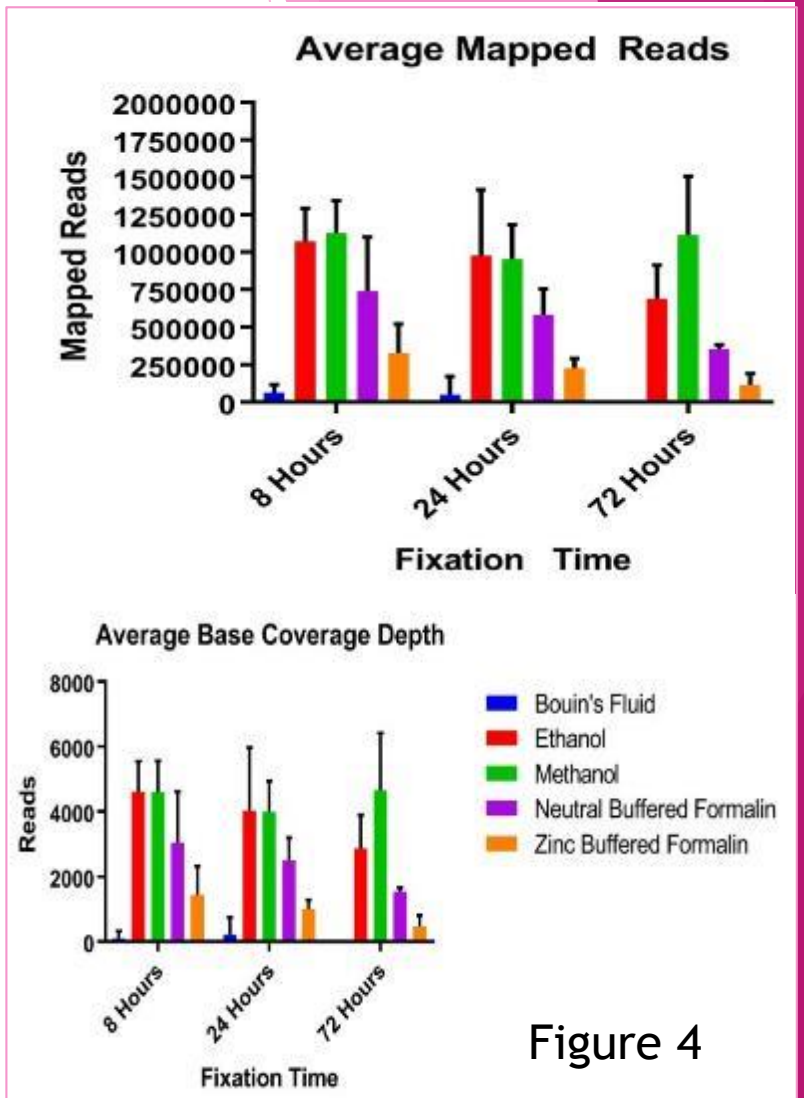


Figure 4

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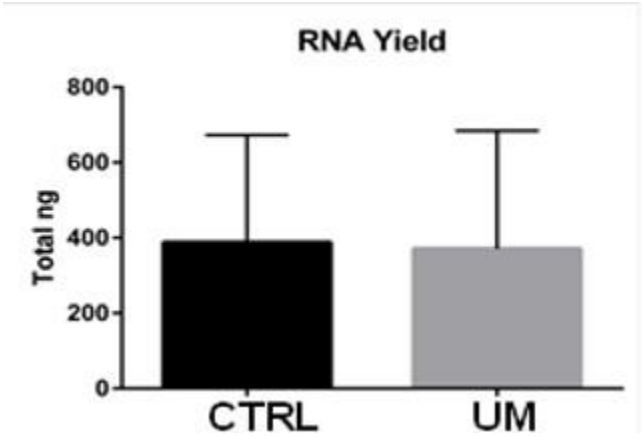
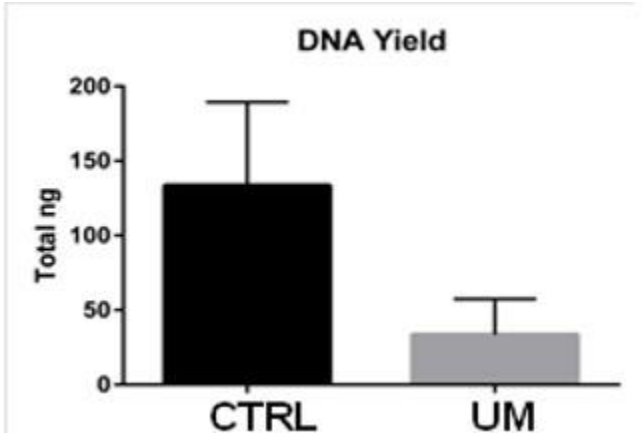
Study 3 - Optimized Combined Tissue Method

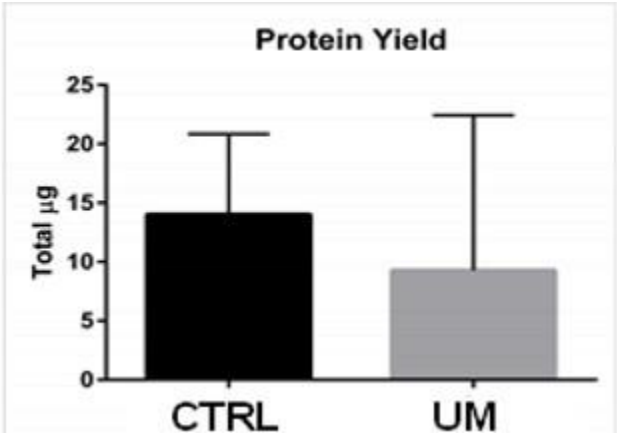
Test Plan:

Goal: To determine the feasibility of isolation of RNA, DNA and protein from a single formaldehyde-fixed, paraffin-embedded (FFPE) section to detect lung cancer biomarkers

- Correlation of RNA expression and DNA mutations by Next Generation Sequencing (NGS)
- Protein expression by liquid chromatography mass spectrometry (LC-MS)





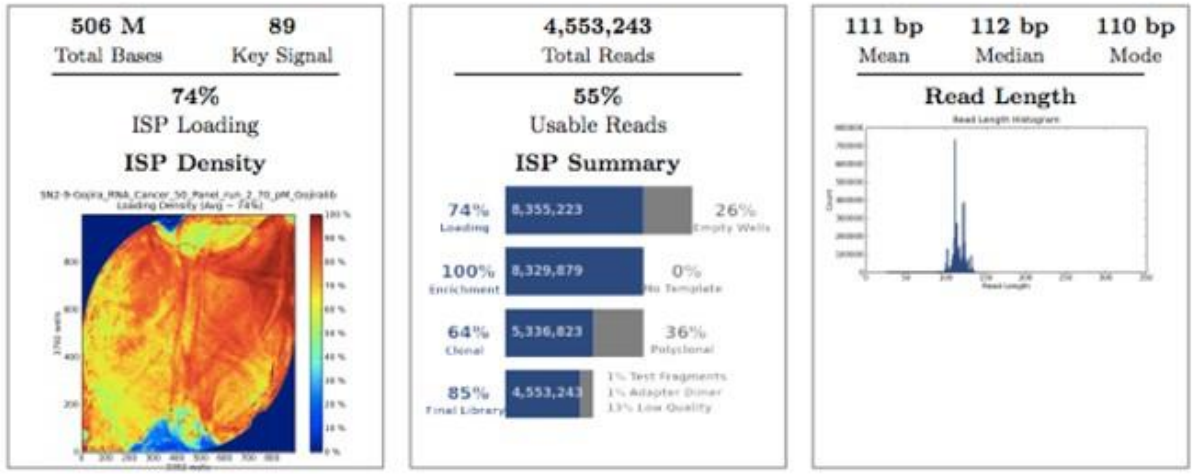


Isaac, J et al. Poster presentation. ASMS, 2016

Optimized Combined Tissue Method

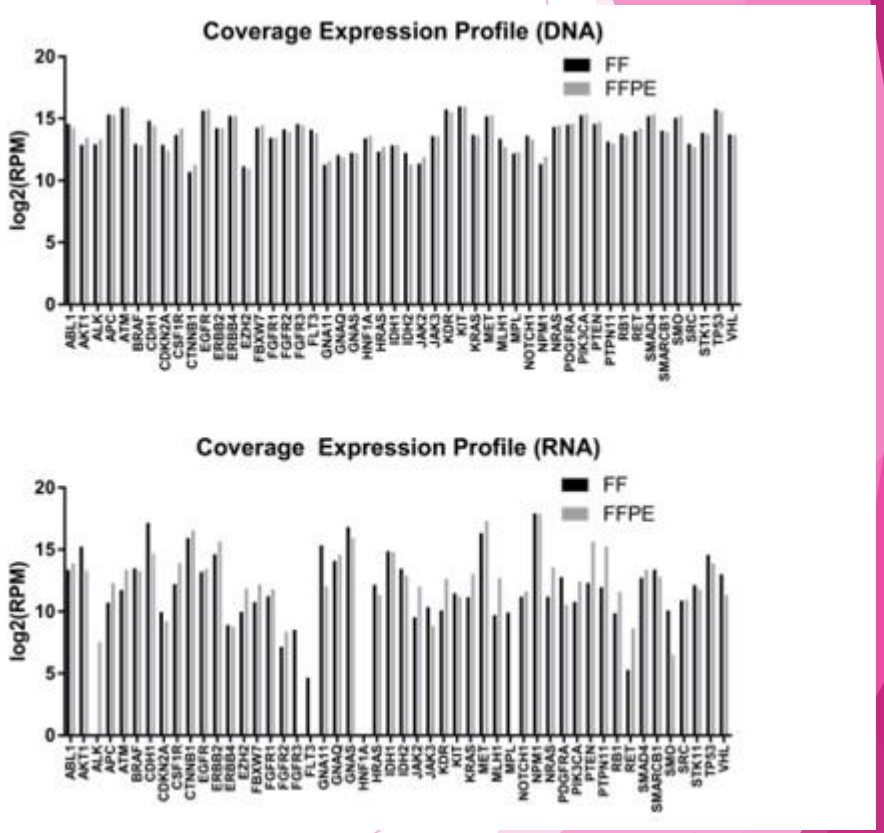
For Next Generation Sequencing, formalin is a good fixative for both RNA and DNA
Fresh-frozen (FF) tissue is highly concordant with FFPE

Figure 2. RNA NGS QC Parameters



AmpliSeq RNA Cancer 50 gene Targeted Panel (NGS)	% reads on Target	Targets detected at 100X
UM_FF	99.39%	48/50
UM_FFPE	99.20%	46/50

Figure 3: RNA & DNA Coverage



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Optimized Combined Tissue Method

- For IHC, formalin is the gold standard
- For LC-MS, formalin reduces the ability to ID target peptides

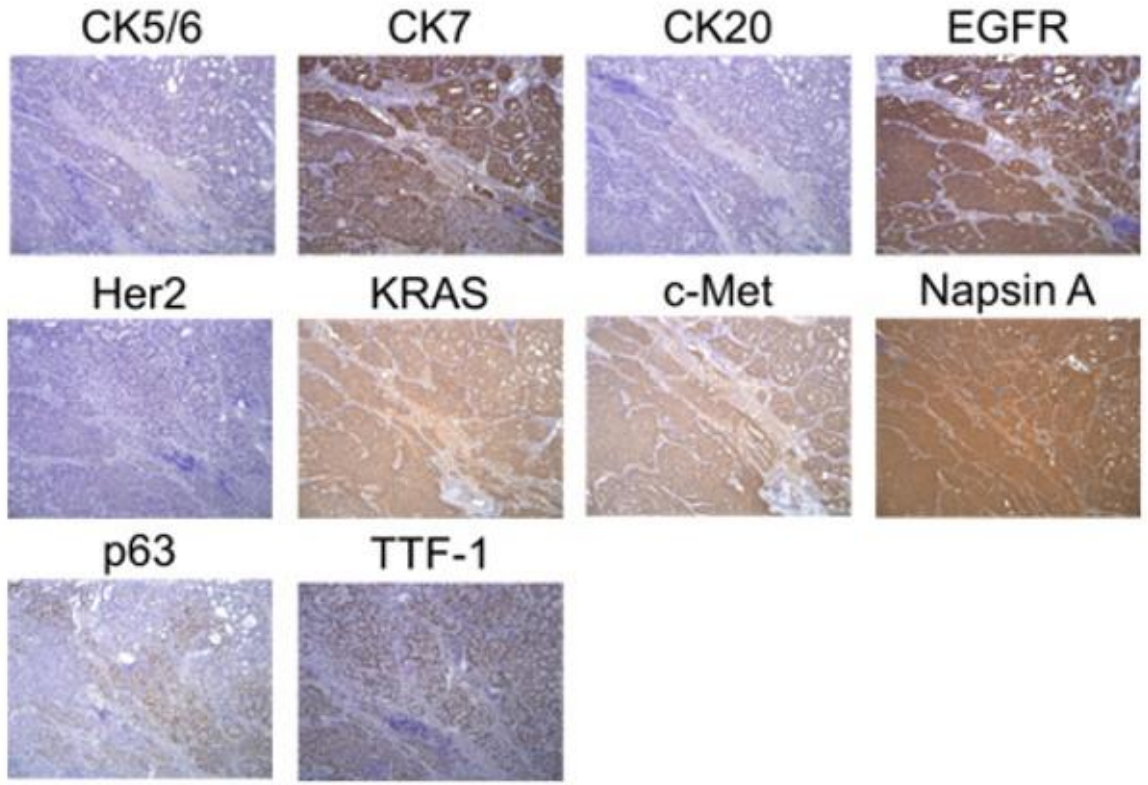

Figure 4: IHC for Lung Cancer

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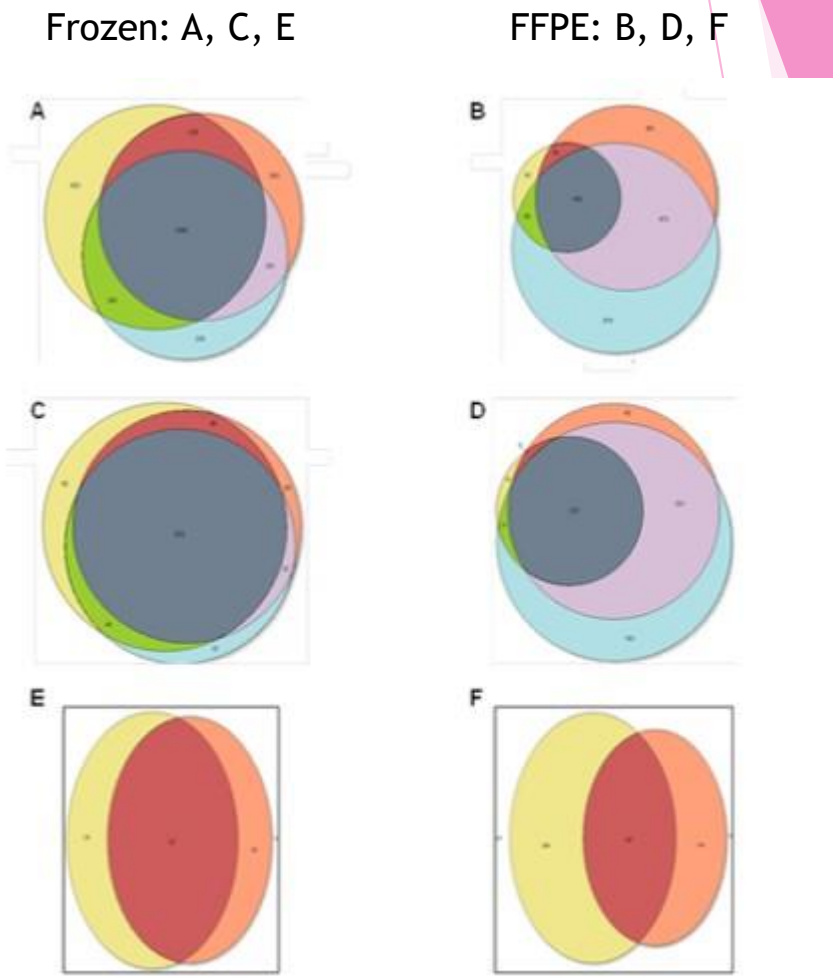
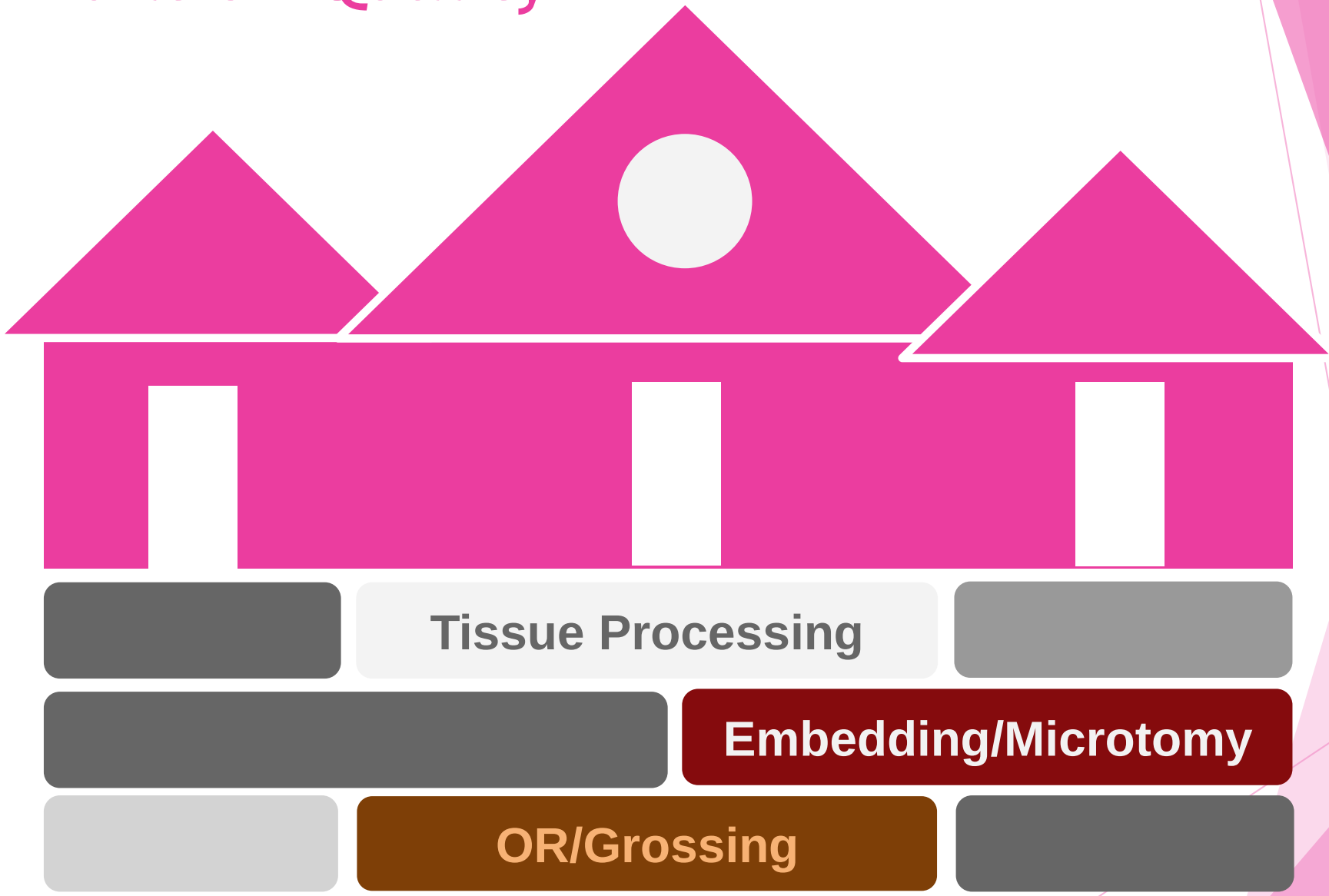


Figure 5: Concordance of frozen and FFPE peptides by Discovery LC-MS

A House Built on Quality



Cleanliness is Key!

Challenge: Minimize exogenous DNA and RNA during embedding and microtomy

- Don’t breathe on samples
- If you have a cold wear a protective mask
- Clean, gloved hands, spritzed with RNAse-*Away*™ and dried
- Preclean all surfaces with RNAse-*Away*™
- Clean between each specimen
- Change out microtome blades with each sample

Nuclease-Minimized Embedding and Microtomy Protocol

Embedding:

- Directly after processing (not allowing the tissue to sit at 62°C for extended periods of time), a Rnase-Free designated (RFD) tissue embedding center will be wiped down with RNase-Away (Thermo Scientific™ HistoStar™ embedding workstation).
- The samples will then be embedded with HPLP individually at the HistoStar.
- RFD forceps and embedding molds (pre-treated with RNase-Away) will be used to embed tissue.
- A RFD Shandon ParaTrimmer will be used to cut away excess paraffin from tissue in the block prior to microtoming.
- After the block has solidified, it will be stored at 4°C in an RFD container.

Microtomy:

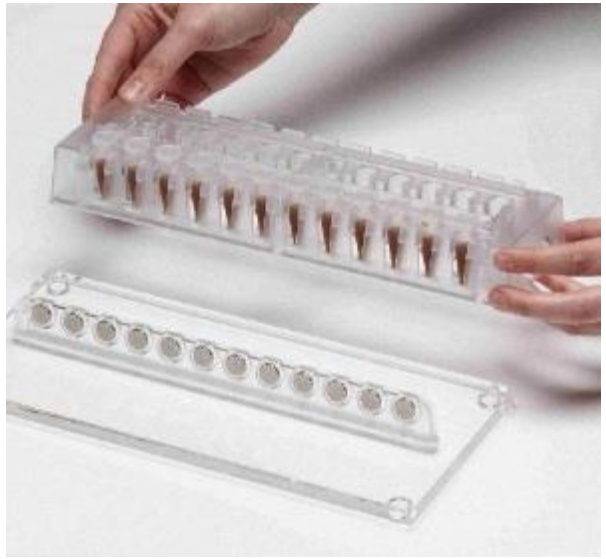
- A RFD microtome, forceps and brush will be wiped down with RNase-Away prior to microtoming. RFD 2.0 ml microcentrifuge tubes and / or slides will be precleaned and prelabeled.
- Sections will be cut from blocks while wearing gloves.
- Blocks will be retrieved from 4°C RFD container and placed onto ice block of RNase-free H_2O.
- Microtome blades will be changed in between blocks.
- Sections will be kept at -20˚C in the 1.5 mL tube until nucleic acid (NA) extraction.
- Sectioned blocks will be stored at 4˚C in a labeled container for reference.

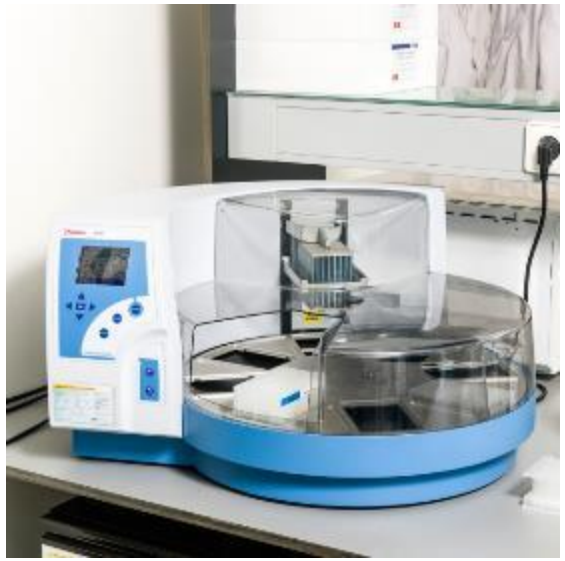
Biomolecule Extraction from FFPE

Variability in methods can lead to differences in final results

Standardization is key

Automation is helpful





Biomolecule Extraction from FFPE

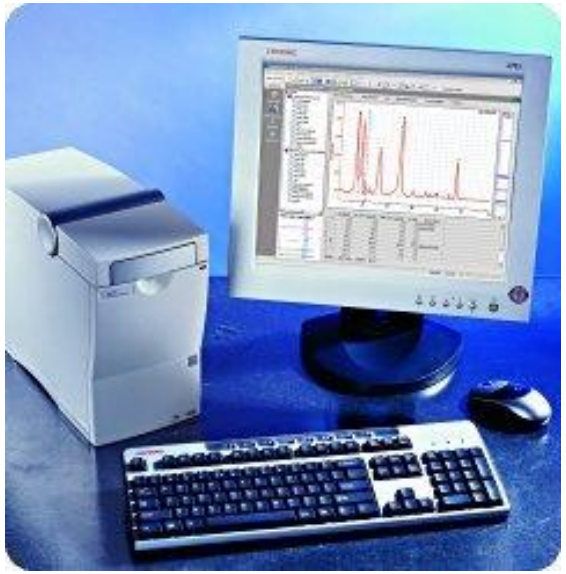
Biomolecule Extraction: magnetic bead technology to automate nucleic acid and protein purification.

- Magnetic particle separation

Row	Row Name	Content	Reagent/Sample Volume per Well
A	DNAse I	1x DNase I Reaction Buffer	200 µL
B	Tip	12-tip comb	EMPTY
C	Sample	Lysed sample Kingfisher Magnetic Beads Ethanol	450 µL 40 µL 400 µL
D	EMPTY	EMPTY	EMPTY
E	EMPTY	EMPTY	EMPTY
F	Wash 1	Wash Buffer 1	900 µL
G	Wash 2_1	Wash Buffer 2	700 µL
H	Wash 2_2	Wash Buffer 2	700 µL

- *96-well plate and elution strip preparation*
  - While samples are incubating, begin preparation of the 96 well plate
    - Pipette all wash buffers as above.
    - Pipette ethanol, magnetic beads and 1x DNase I reaction buffer as above: 20 µL (260 µL)10x DNAse I reaction buffer and 180 µL (2340) nuclease free dH_2O. (large volumes are 13x individual well volume. One for each of 12 columns, 1 extra to ensure sufficient volume for pipetting)
    - Do NOT prepare the elution strip at this time.
    - FLEX 12 well tips to ensure attachment to magnetic pin head

Quality Control – Bioanalyzer



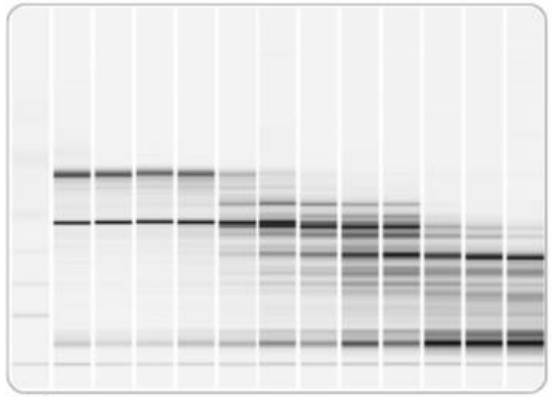


Figure 1
A total RNA sample was degraded for varying times and the resulting samples were analyzed on the Agilent 2100 Bioanalyzer System using the Eukaryote Total RNA Nano assay. A shift towards shorter fragment sizes can be observed with progressing degradation.

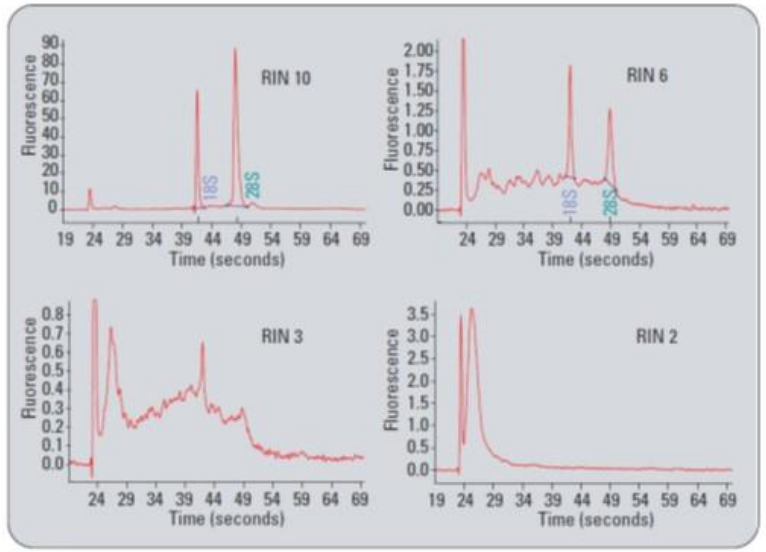


Figure 2
Sample electropherograms used to train the RNA Integrity Number (RIN) software. Samples range from intact (RIN 10), to degraded (RIN 2).

Bioanalyzer: an automated electrophoresis solution for the sample quality control of biomolecules.

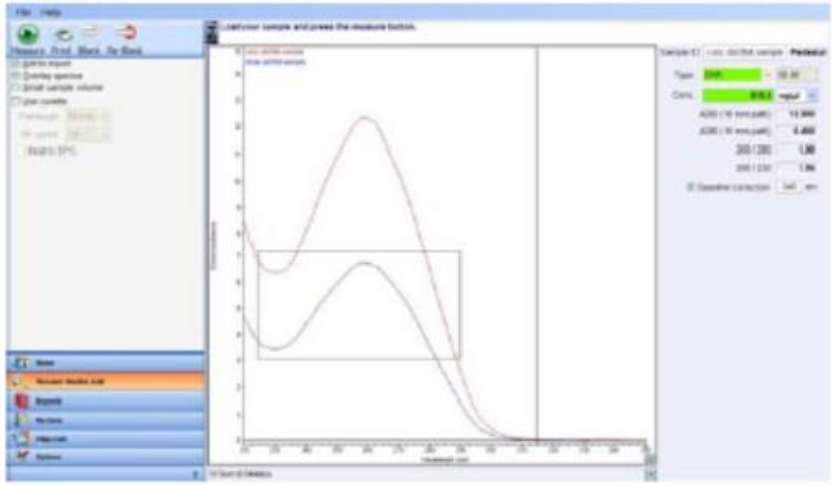
- Measure of RNA degradation
- RNA degrades over time
- RIN Value Range 0-10
- High quality RNA is 8 or better
- RIN values of 6-7 can be acceptable

Agilent Technologies. Application Guide. RNA Integrity Number (RIN) – Standardization of RNA Quality Control. www.agilent.com 2016.

Quality Control: Spectrophotometer

Spectrophotometer technology: a device for measuring wavelengths of light over a wide range of the electromagnetic spectrum

- Nucleic acids:
  - 260/280 ratio assess DNA and RNA purity
    - 1.8 or better is pure for DNA
    - 2.0 or better is pure for RNA
  - 260/230 ratio a secondary measure of purity
    - Presence of co-purified contaminants
- Proteins:
  - A280 application
  - Trypsin, tyrosine, cysteine disulfide bonds absorb at 280 nm
  - Concentration of protein measured in mg/ml
- UV/Vis capability



NanoDrop 2000/200c Spectrophotometer V1.0 User Manual. www.thermoscientific.com 2009.

Quality Control – Fluorometer

Fluorometer technology uses fluorescent dyes to determine the concentration of either nucleic acids or proteins in a sample

Uses:

- DNA
- RNA
- Protein

Reagent/Assay	Assay range	Sample starting concentration range
Qubit dsDNA HS Assay	0.2-100 ng	10 pg/µl-100 ng/µl
Qubit dsDNA BR Assay	2-1,000 ng	100 pg/µl-1 µg/µl
Qubit ssDNA Assay	1-200 ng	50 pg/µL-200 ng/µL
Qubit RNA Assay	5-100 ng	250 pg/µl-100 ng/µl
Qubit RNA BR Assay	20-1,000 ng	1 ng/µ-1 µg/µL
Qubit Protein Assay*	0.25-5 µg	12.5 µg/ml-5 mg/ml

Invitrogen™. User Guide: Qubit 3.0 Fluorometer. www.thermoscientific.com 2014.

Quality Control

Instrument	Result Provided	Pros	Cons
Bioanalyzer	RIN Value	Provides RIN Value Biobanks often include RIN value as part of the sample annotation	Can be technically challenging when first starting to use the instrument Requires use of consumables such as chips and reagents
Nanodrop Spectrophotometer	Protein Concentration, Purity	Quick result; direct measurement through spectrophotometry Provides insight into contaminants such as ethanol	Not as accurate as Qubit for sample concentratons below 40 ng/ml
Qubit Fluorometer	Protein Concentration; RNA Concentration; DNA Concentration	More accurate than Nanodrop for concentrations less than 40 ng/ml An important QC test ahead of NGS, since concentrations are often low	Takes longer to get a result, requires use of fluorophores From a practicality standpoint, you can really only prep and test 10-12 samples at a time

Summary

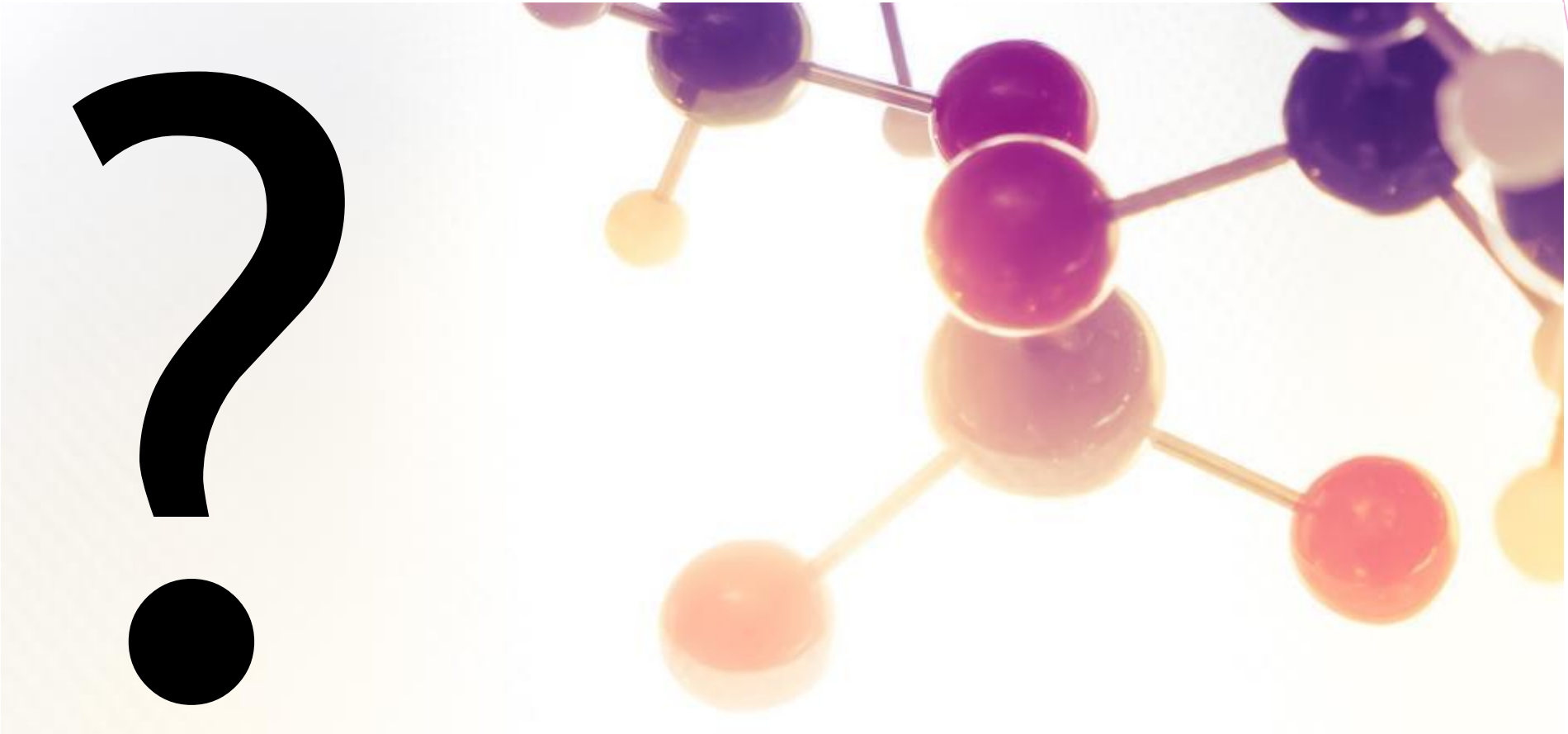
Molecular assays are here

Molecular testing is forcing us to rethink

- Preanalytic parameters
- Fixation
- Processing
- Histology techniques
- Quality controls

Is your lab ready?

Questions & Comments



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Wolff AC, et al. Recommendations for human epidermal growth factor receptor 2 testing in breast cancer. Archives of Pathology and Laboratory Medicine. 138:241-256. 2014

Wolff AC, et al. Recommendations for human epidermal growth factor receptor 2 testing in breast cancer. Archives of Pathology and Laboratory Medicine. 142:1364-1382. 2018