Defining Quality
In Tissue Biospecimens

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**Speaker Disclosure(s)**

<table>
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<tr>
<th>Conflict of Interest Disclosure 1</th>
<th>Stephen Hewitt has no financial interest or other relationship with the manufacturers of the products or providers of the services that will be discussed that could, in any way, influence the study to be presented.</th>
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<td>Conflict of Interest Disclosure 2</td>
<td>I am disclosing that I have no financial and/or business interests in or am I a consultant to or receive funding from external companies.</td>
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<td>The speaker, is a Captain in the US Public Health Service. This presentation and the conclusions are my own views and opinions and should not be construed as an official statement or advice from the NCI, the NIH, , the PHS, DHHS or the US federal government.</td>
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Disclosure

“Employee” Of The US Federal Government

Editor-in-Chief, *Journal of Histochemistry & Cytochemistry*

Associate Editor, *FASEB Journal*

Editorial Board, *Biopreservation And Biobanking*

Consultant & Collaborator, Center For Device & Radiological Health, Food & Drug Administration

Chair, Consensus Committee On Immunology and Ligand Assays, Clinical and Laboratory Standards Institute
Quality Is Everything

Quality Remains Subjective

• Tissue Quality
  – Histology
  – Proteins
  – Nucleic Acids

• Clinical Data
  – Complete
  – Detailed
  – Defined
Old Technology / New Science

• Histopathology has been evolving since the 1870s.
  – H&E, Formaldehyde, IHC.....

• Molecular Biology evolution gained momentum in the 1980s.
  – Cancer genetics took off with PCR, in 1987.

• Convergence of the two started in early 2000’s
  – Earliest use of FFPE in molecular genetics is 1980s, with routine use not occurring till 200’s.
Throughput Drives Quality, Reproducibility & Standardization

- Henry Ford and the Model-T Production Line.

- Prior to the Microarray, which offered multiplex evaluation of genes at low cost, and drove studies to large sample sizes, standardization and quality was lost in the complexity of the studies.
Models & Mechanisms

• Prior To The 2004 Affymetrix Meeting There Were No Integrated Models

• Efficiency of RNA recovery from fixed tissue
  – No Data until Chung et al (Diag Mole Path 2006)

• Ischemia and Fixation
  – Chemical models of Fox et al (JHC 1985) incomplete
  – No biologic model until Chung et al (JHC 2008)

• Impregnation
  – No model until Chung et al (JHC 2008)

• Storage
  – No model until Xie et al (JHC 2011) and Chung et al (JHC2018)
RNA Recovery From Tissue

RNA In Frozen Tissue

RNA In FFPE - Anticipated

RNA In FFPE - Revised

Results Of RT-PCR

- Frozen
- Anticipated
- Model 1
- Model 2
- Model 3

- 5' UTR
- Exon 1
- Exon 2
- Exon 3
- 3' UTR
- poly A

RNA Recovery From Tissue
RNA Recovery – Quantity

- First Study To Address RNA Recovery Based On Equal Volumes Of Starting Material
- FFPE Demonstrates A 30% Recovery
- Ethanol–fixed, PE Has A Recovery Of 80%
  - Formalin Contributes To the Majority Of The Damage
Quantitative Amplification Based On RNA Source & Primer Location

Relative expression level of GAPDH gene (relative to 3'UTR region of fresh rat kidney, log$_2$)

Region of GAPDH gene

Same Quantity of Starting RNA

Random Prime cDNA

<table>
<thead>
<tr>
<th>Region of GAPDH gene</th>
<th>Fresh</th>
<th>RT</th>
<th>37oC</th>
<th>RNAlater</th>
<th>FF</th>
<th>FFPE</th>
</tr>
</thead>
<tbody>
<tr>
<td>5' ORF</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Middle of ORF</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3' UTR</td>
<td></td>
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Revised Model Of Chemical Fixation

• Tissue Hypoxia & Switching To Glycolysis - “Drowning”
  — RNA stores consumed, Alterations in Phospho-Proteome

• Infiltration & Inhibition Of Glycolysis & Oxidative Phosphorylation
  — Halting Of Most Biologic Process

• Chemical Reactions Crosslinking Proteins and Nucleic Acids
  — Halting Of Remaining Enzymatic Activity
Effective demonstration of differences, but entirely qualitative.
RNA Quality
Same Two Variables

No More Informative
Calculating PERM

\[ \text{PERM} = F_U_{25} + (2 \cdot F_U_{30}) + (3 \cdot F_U_{35}) + (4 \cdot F_U_{40}) + 5 \cdot F_U_{45} \\
+ (6 \cdot F_U_{50}) + (7 \cdot F_U_{55}) + (8 \cdot F_U_{60}) + (9 \cdot F_U_{65}) \]
## PERM Evaluation Of RNA Quality

<table>
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<tr>
<th>Processing Time</th>
<th>Fixative Buffer</th>
<th>PERM</th>
<th>Relative Quality</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 minutes</td>
<td>Phosphate</td>
<td>14.2</td>
<td>0.24</td>
</tr>
<tr>
<td>15 minutes</td>
<td>Phosphate</td>
<td>50.2</td>
<td>0.84</td>
</tr>
<tr>
<td>30 minutes</td>
<td>Phosphate</td>
<td>55</td>
<td>0.93</td>
</tr>
<tr>
<td>45 minutes</td>
<td>Phosphate</td>
<td>74.3</td>
<td>1.25 (highlighted)</td>
</tr>
<tr>
<td>30 minutes</td>
<td>CaCl2</td>
<td>30.9</td>
<td>0.52</td>
</tr>
<tr>
<td>30 minutes</td>
<td>Tris</td>
<td>12.2</td>
<td>0.21</td>
</tr>
<tr>
<td>30 minutes</td>
<td>Phosphate</td>
<td>63.9</td>
<td>1.07</td>
</tr>
<tr>
<td>30 minutes</td>
<td>None</td>
<td>17</td>
<td>0.06 (highlighted)</td>
</tr>
</tbody>
</table>
PERM PERFORMANCE

Graphs showing correlations:
A. Relative GAPDH expression signal (ratio of frozen kidney RNA) vs. PERM
   - $r = 0.963$, $p < 0.001$, $n = 20$
B. Relative CDK4 expression signal (ratio of frozen kidney RNA) vs. PERM
   - $r = 0.869$, $p < 0.001$, $n = 20$
C. Relative ACTB expression signal (ratio of frozen kidney RNA) vs. PERM
   - $r = 0.974$, $p < 0.001$, $n = 20$
D. Relative average expression signal (ratio of frozen kidney RNA) vs. PERM
   - $r = 0.974$, $p < 0.001$, $n = 20$
E. Quantitation cycle of HPR1 vs. PERM
   - $r = -0.900$, $p < 0.001$, $n = 18$
PERM

Paraffin Embedded RNA Metric

• Applicable to:
  – Fixed Tissues
    • All Fixatives
• Validation:
  – Extensive retrospective and prospective validation in our pre-analytic model systems
  – Real differences in RNA quality are detected by a difference in 5 QM units
• Limitations:
  • Intra-laboratory
    – Lacks External Calibrators
    – Intra-species
      • Can not directly compare data from mice, fish and snails
PERM

• Can Be Applied To Any Platform That Separates RNA By Mass
• 5 Units Is Considered A Real Different In RNA Quality
• Fit-For-Purpose Approach To Quality
  – RT-PCR Assays
  – Micro-array
  – RNAseq – Somewhat Insensitive To RNA Quality
Biomolecule Degradation In Formalin Fixed, Paraffin Embedded Tissue

• Slide Oxidation
  – Decreased Staining Of Some Antigens When Applied To Previously Cut Sections

• RNA Degradation
  – Decreased Recovery & Quality Of RNA Isolated In Older Tissue Blocks

• Theory – “Oxidation” Prevention – Barriers
  – Paraffin
  – Gasses

• Data - NONE
Quantitative Measures Of Quality

• Protein
  – Quantity
  – Integrity
    • No good current assays

• DNA
  – Fragment Length

• RNA
  – More Is Always Better
  – Measures Of Quality
    • RIN
      – Poor correlation with gene specific measurements
    • PERM (AKA QM)
      – Weighted area under the curve approach
Fixation Time: Differences Between Coagulative & Crosslinking Fixatives

- Is the 24hr Fixation Time of NBF generalizable to other fixatives?
- What is the impact on other biomolecules?
- Evaluated from 4 hours to 6 months
Immunohistochemistry
How Bad Is Overfixation?
Proteins Extracted From Paraffin Blocks

Western Blot

Protein Array
Protein

• Discordant Between Techniques
  – Western Blot Ratios Less Stable Than Protein Array Data

• Western Blot Is Sensitive To Non-Full Length Peptide, While The Protein Array Technology Detects Any Intact Epitope
  – Protein Array Technology Demonstrates That Progressive Degradation Results In Loss Of Detection Of Protein
RNA

- Replicates Prior Data For Crosslinking Fixatives, But Demonstrates The Potential Of Coagulative Fixatives
DNA

- Proves One Thing: DNA Is Durable
Fit-For-Purpose Biospecimens

• What Assays Are The Material Appropriate For?

• Are There Objective & Quantitative Measures Of Quality?

• Nothing Is Forever, Biospecimens May ”Age-out” For Some Applications
Frontiers Of Biospecimen Science

• Acquisition To Stabilization
  – Golden Five Minutes
• Improving Long Term Storage
  – Frozen & Chemically Preserved Specimens
• Economic & Environmental Concerns Of Biobanking
  – Carcinogens & Cost Considerations
• Preservation Of Biohazardous Specimens
  – Inactivation Verses Biomolecular Quality