Measuring Biomarker Stability in Frozen Tissue
Post-acquisition variables:

- Time at room temperature
- Temperature of room
- Type of fixative
- Time in fixative
- Rate of freezing
- Freeze / Thaw
- Size of aliquots
- Storage temperature
- Storage duration
- Storage in vacuum
- Processing methods
NCI Best Practices for Biospecimen Resources

• Technical Operations Best Practices
  – Collection, processing, storage, retrieval, use
  – Collection and managing clinical data
  – QA/QC
    – Biosafety
    – Informatics, inventory control and tracking

• Ethical, Legal and Policy
  – Custodianship
  – Informed consent
  – Privacy protection
  – Access to biospecimens and data
  – Intellectual property and resource sharing
Principals of Evidence Based Biospecimen Research

• What practices affect the quality of a specimen?
• Can they be measured?
• Do the measurements have meaning to data acquired in research testing?
Frozen Tissue

• Still the pristine specimen carrying intact non-chemically altered DNA, RNA, and proteins.
• Will be needed for biomarker discover.
• May be needed for future clinical information that can’t be resolved by robust assays.
Sample Packaging and Storage

• Can the quality of frozen specimens be protected with more stringent packaging?

• What is the duration of protection?
Questions

- Oxidation - RNA, DNA, & proteins
- Dehydration
- Morphological deformation
- Residual and active enzyme activity
- Storage container, temperature, length of storage
Operational Variables

- Sample size
- Freezing rates
- Packaging analysis
  - Sealer
  - Pouch
- Tissue container
Polymer Laminate Diagrams

Typical Vapor Barrier Laminations

- Aluminum
- Polyethylene (heat seal)
- Polyethylene (oriented)
- Nylon or polypropylene (oriented)
- Adlar (PCTFE)
- Polyethylene (heat seal)
- PET (Mylar) (oriented)

6mil aluminized film
6mil transparent film
Proposed Solution

1. Foil laminate (high barrier packaging)
2. Embedding cassettes: protect tissue from distortion
3. Modified atmosphere packaging: vacuum or inert gas, i.e., argon
### Vessel Freezing Periods

Based on the exhaustion of LN2 boiling

<table>
<thead>
<tr>
<th>Container</th>
<th>Time to Freeze (sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRE tray in standard bag</td>
<td>50</td>
</tr>
<tr>
<td>Standard 5 ml tube</td>
<td>70</td>
</tr>
<tr>
<td>20 mil clamshell</td>
<td>23</td>
</tr>
<tr>
<td>30 mil clamshell</td>
<td>25</td>
</tr>
<tr>
<td>30 mil clamshell tray in bag (vacuum)</td>
<td>30</td>
</tr>
<tr>
<td>Barrier bag (vacuum)</td>
<td>26</td>
</tr>
<tr>
<td>Barrier bag (no vacuum)</td>
<td>30</td>
</tr>
<tr>
<td>Embedding tray in bag (vacuum)</td>
<td>30</td>
</tr>
</tbody>
</table>
Sample Dissection for a Single Case

mucosal tissue sample

Baseline  Air (cryovial)  Argon  Vacuum

(-10C  -20C  -80C)(-10C  -20C  -80C)(-10C  -20C  -80C)

200 mg pieces  200mg pieces  200mg pieces

Months:
- Baseline: 1, 2, 3, 6, 12
- Air (cryovial): 1, 2, 3, 6, 12
- Argon: 1, 2, 3, 6, 12
- Vacuum: 1, 2, 3, 6, 12
Sample Dissection

3 gram tissue sample

200 mg frozen

100 mg
Total mRNA
RIN#
qRT-PCR
Biomarkers

100 mg
Histology
Immunohistochemistry
Digital Imaging & Analysis

formalin fixation
Biomarkers

GAPDH - Glyceraldehyde 3-phosphate dehydrogenase (message)
- implicated in non-metabolic processes, transcription activation, initiation of apoptosis, and ER to Golgi vesicle shuttling – house keeping gene

CEA – glycoprotein-cell adhesion (protein & message)
- CEA is a tumor marker to identify recurrences
- In humans, the carcinoembryonic antigen family consists of 29 genes, 18 of which are normally expressed.

CDX2 (protein and message)
- CDX2 expression is maintained in the adult small and large intestinal epithelia
- CDX2 expression is induced or up-regulated in pathologic states

CK20 (protein)
- cytokeratin, major cellular protein of mature enterocytes and goblet cells and is specifically expressed in the gastric and intestinal mucosa.
Elongation ratio
(Epithelial cells: red; Stromal cells: yellow)

Courtesy of Sanford Barsky, M.D., Ph.D.
Degree of change between different packaging methods versus storage temperature for CDX2, CEA, and CK20
Measurement of Change in mRNA

qRT-PCR Baselines

Cycle Threshold vs. Subjects

- CDX2
- CEA
- GAPDH

# Cycles / Time point of storage - Baseline # cycles
RIN Degradation Varying Temperature at Month 3

RNA Integrity Number

Temperature

AIR
ARGON
Vacuum
Changes in Biomarker qRT-PCR

Storage [-10C] : Packaging [Air]

Storage [-20C] : Packaging [Air]

Storage [-80C] : Packaging [Air]

Changes in Biomarker qRT-PCR
Biomarker Measurement Varying Temperature at Month Three

![Graph showing cycle threshold vs. temperature for different conditions: AIR GAPDH, ARGON GAPDH, Vacuum GAPDH, AIR CEA, ARGON CEA, and Vacuum CEA. The graph demonstrates how cycle threshold changes with varying temperatures for each condition.]
GAP-DH Cycle Threshold in Air

\[ y = 17.345e^{0.054x} \]
\[ R^2 = 0.845 \]

\[ y = 16.462e^{0.018x} \]
\[ R^2 = 0.402 \]

\[ y = 16.667e^{0.102x} \]
\[ R^2 = 0.868 \]
Air (cryovial) Packaging
(-80C)

Percent Change

GAPDH
CEA
CDX2
Conclusions / Future Studies

• Degradation occurs at about 5% in the first 12 months according to percent change in the original quantity of message
• No difference in packaging seen at this time
• Continue to measure the duration of time and packaging for affects in tissue quality
• Varying types of biomarkers need to be tested
• Compare fixation methods to the frozen tissue
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