Formalin fixation at low temperature better preserves nucleic acid integrity

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Disclosure of interests:

G.B. was originally responsible for the invention of the Cold Fix procedure, but is not the owner of the patents and does not receive Royalties.
RNA yield from FF-PET

Fragmented

Result of RNAse activity (?)
formaldehyde does not inhibit RNAses.

Fixation

RNAse

Penetration

Room Temperature

Percent

0 2 4 6 8 10 12 14 16 18 20 22 24h
Figure 4. The relationship between RNase activity in whole lysates and temperature.

The graph shows the relationship between temperature and fixation, RNAse and penetration over 24 hours at 4°C. The x-axis represents temperature in degrees Celsius (°C) and the y-axis represents percent. The graph includes three different lines for fixation, RNAse, and penetration, each marked with different colors: blue for fixation, pink for RNAse, and orange for penetration.
Experimental protocols for Cold Fixation:

1) Fixation in PBF at 4°C for 24 h, followed by heating at 50°C for 20 min using MW, followed by Ethanol dehydration and Paraffin embedding.

2) Fixation in PBF at 4°C for 24 h, followed by heating at 90°C for 5 min, followed by Ethanol dehydration and Paraffin embedding.

3) Fixation in PBF at 4°C for 24 h, followed by 95% Ethanol at 4°C for 2 h, then standard Ethanol dehydration and Paraffin embedding.
Cases of:
Breast Cancer
Colon Cancer
Stomach cancer
Pancreatic cancer

Study flowchart. Flowchart illustrating the design of the study.
Colon cancer processed either routinely (c, d) or following the CF procedure (a, b)
Breast cancer processed either routinely (c, d) or following the CF procedure (a, b)
Stomach cancer processed either routinely (c, d) or following the CF procedure (a, b)
Standard fixation (SF) procedure vs Cold fixation (CF) procedure

**G6PD**

- MW: Molecular Weight
- SF1: Standard Fixation Sample 1
- SF2: Standard Fixation Sample 2
- CF1: Cold Fixation Sample 1
- CF2: Cold Fixation Sample 2
- FZ1: Frozen Sample 1

MW     SF1     SF2     CF1     CF2

- 660 bp

**CK-20**

- MW: Molecular Weight
- SF1: Standard Fixation Sample 1
- SF2: Standard Fixation Sample 2
- CF1: Cold Fixation Sample 1
- CF2: Cold Fixation Sample 2
- FZ1: Frozen Sample 1

MW SF1 SF2 CF1 CF2 FZ1 SF1 SF2 CF1 CF2 FZ1 SF1 SF2 CF1 CF2 FZ1

- 329 bp
- 500 bp
- 716 bp
Bussolati et al., Figure 3. Messenger RNAs from Cold-Fixed samples are detected by microarray probes hybridizing more than 500b upstream from the reverse transcription start site. Graph showing the fraction of probes with detectable signal (y-axis) for each bin of distance of target sequence from the mRNA poly(A) site from which RT is initiated (x-axis).
The plots show probes of less than 200 bases (A, E) and 200-500 bases (B, F).
The plots show probes of 500–700 bases (C, G), more than 700 bases (D, H)
Venn diagram comparing in parallel the genes detected in 2 fresh samples (red) and 2 cold fixed (CFFPE) samples (blue) from the same cases of breast cancer. Using the software Agilent Genespring 11.5.1 we generated lists of genes detected in cold fixed and fresh sample. The number of genes detected in the fresh samples was 29726 and in the cold fixed samples was 28501 genes. The number of genes detected in the fresh samples was only slightly (4.12%) higher than in the cold-fixed ones.
A formalin fixation procedure preserving nucleic acid integrity.

Gianni Bussolati, Laura Annaratone, Enzo Medico, Giuseppe D’Armento and Anna Sapino.
The Cold Fix Apparatus
Assessment of RNA conservation in Cold-Formalin Fixed Paraffin Embedded Tissue blocks after two years

RT-PCR for detection of CK-20 in colorectal cancer (500bp).

Samples:
A- 0  RNA extraction year 2010
A- 2  RNA extraction year 2012
B- 0  RNA extraction year 2010
B- 2  RNA extraction year 2012
C- 0  RNA extraction year 2010
C- 2  RNA extraction year 2012
Surgery

Surgical Specimen >2cm.

Under-vacuum (Tissue Safe)

Kept at 4°C till transfer (1-72h)

Tissue Gene Expr. Banking Profiling (-80°C) (12-24h)

Grossing

P.B. Formalin

Embedding

Surgical Theatre

Pathol. Labs.
(a) Formation of electrophilic species:

\[ \text{CH}_2\text{=O} + \text{H}^+ \rightarrow \text{CH}_2\text{=O}^+ \text{H} \leftrightarrow \text{CH}_2\text{OH} \]

(b) Reaction with double bond:

\[ \text{C=CH} + \cdot \text{CH}_2\text{OH} \xrightarrow{\text{via intermediate} \ \text{Hf complex}} \text{C} \cdot \text{CH}_2\text{OH} \]

\[ \text{OH} \]

\[ \text{C} \cdot \text{C} + \text{H}^+ \]

\[ \text{CH}_2 \text{OH} \]

\[ \text{OH} \]

\[ \text{1:3 Glycol} \]

\[ \text{C} \cdot \text{C} \]

\[ \text{CH}_2\text{OH} \]

\[ \text{C} \cdot \text{C} \]

\[ \text{CH}_2\text{OH} \]

\[ \text{1:3 Dioxane} \]

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Reaction of Formaldehyde with Unsaturated Fatty Acids during Histological Fixation

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The scatter plot puts in comparison $2^\Delta\text{Ct}$ values obtained from a fixed sample with $2^\Delta\text{Ct}$ values obtained from the same sample, but frozen, both evaluated with Breast Cancer RT2 Profiler PCR Array (QIAGEN) and analysed with QIAGEN Web-Based PCR Array Data Analysis Software.