



Working with Fixatives, Fixation, and Tissue Processing to Improve Morphology and RNA Quality

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Morphology and RNA Quality

Goal

- Optimize tissue morphology, RNA integrity, and RNA utility

Problem

- Goals often seem mutually exclusive
 - Well fixed sample (formalin) = compromised RNA
 - Good RNA (OCT) = poor morphology

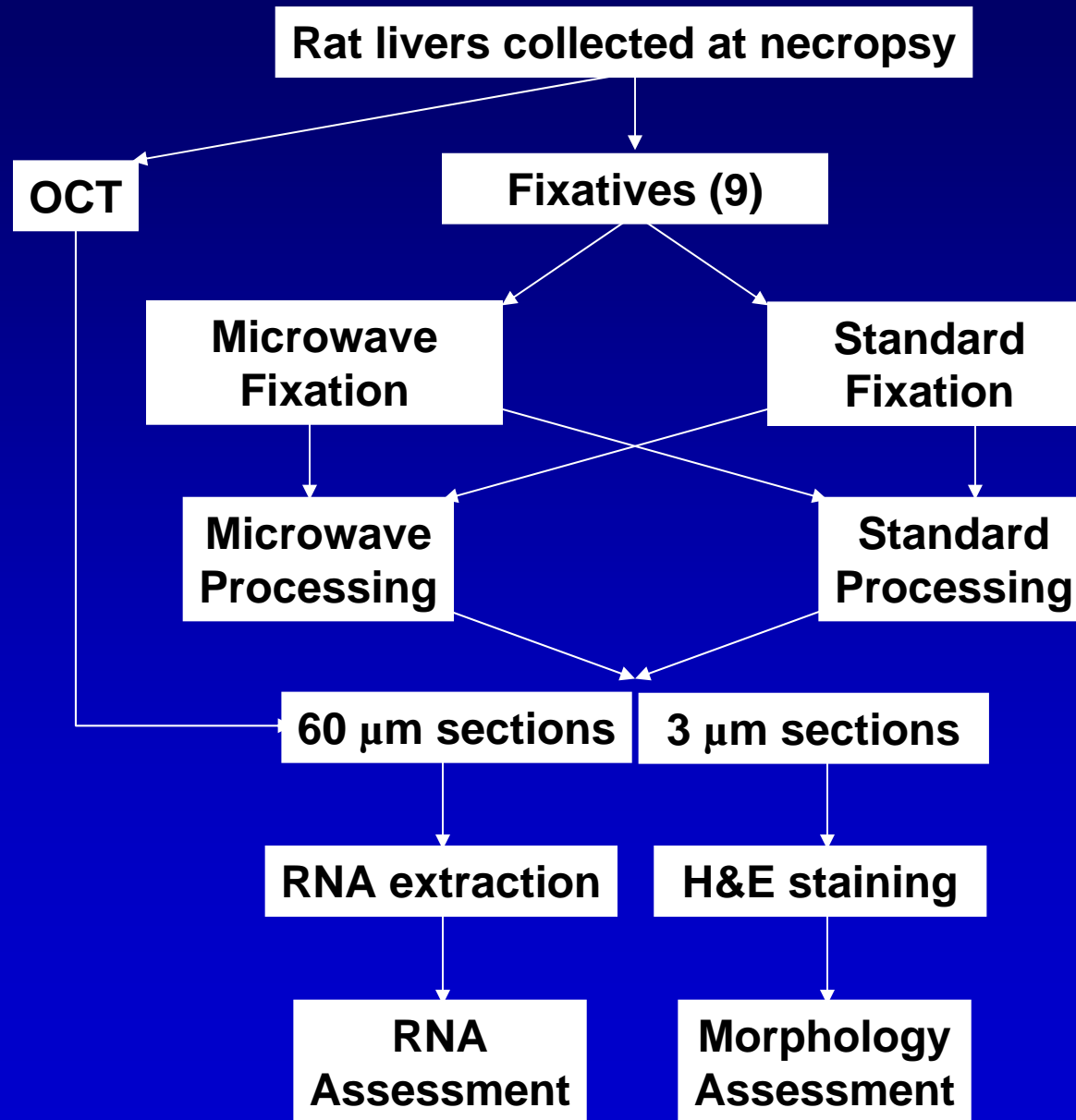
Experimental Design

- Test tissue: rat liver
- 9 fixatives
- Fixation and processing methods:
 - Microwave and standard
- Assessment:
 - Morphology: subjective by a pathologist
- RNA quality:
 - Agilent Bioanalyzer
 - LCM (laser capture microdissection)
 - qRT-PCR (Taqman quantitative RT-PCR)
 - Microarray analysis

Fixatives

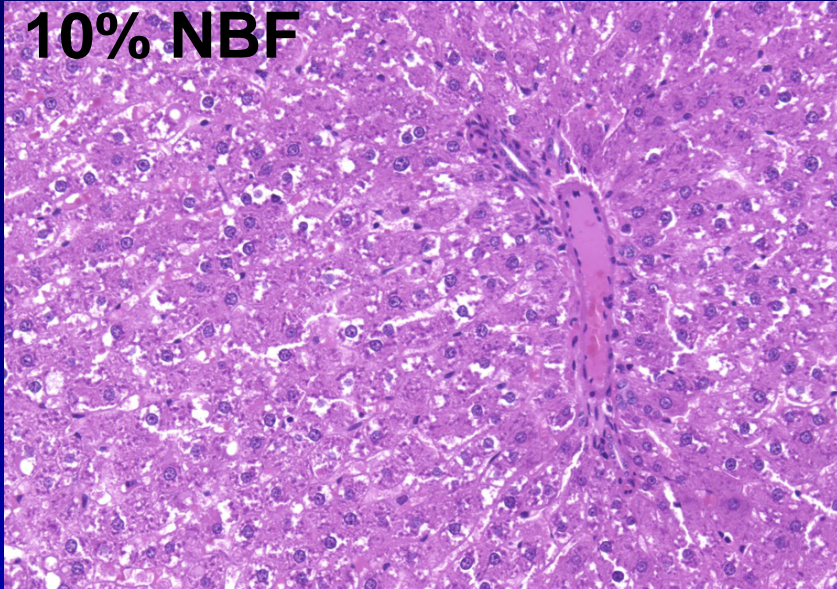
- Aldehyde-based
 - 10% NBF
 - modified Davidson's II
- Alcohol-based
 - 70% Ethanol
 - Modified Carnoy's (no chloroform)
 - Modified Methacarn (8 methanol: 1 glacial acetic acid)
 - Universal Molecular Fixative (UMFIX)
- Picrate
 - Bouin's
- Holding Solution
 - 30% Sucrose
 - PBS

Flow Chart of Sample Handling and Evaluation

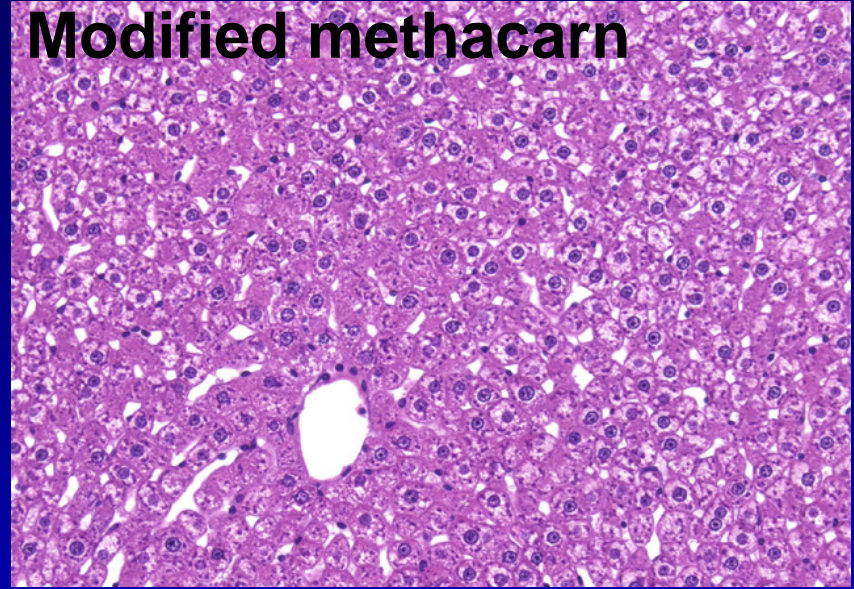


Rat Liver (40X)

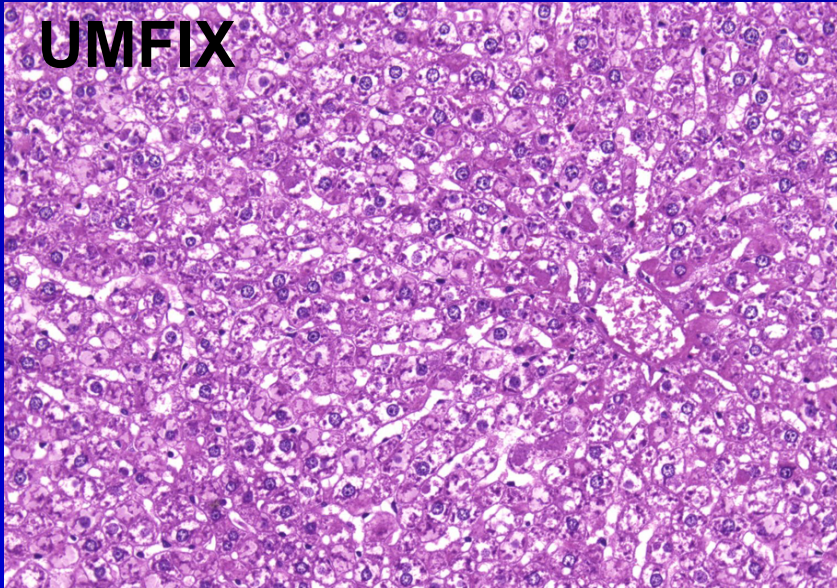
10% NBF



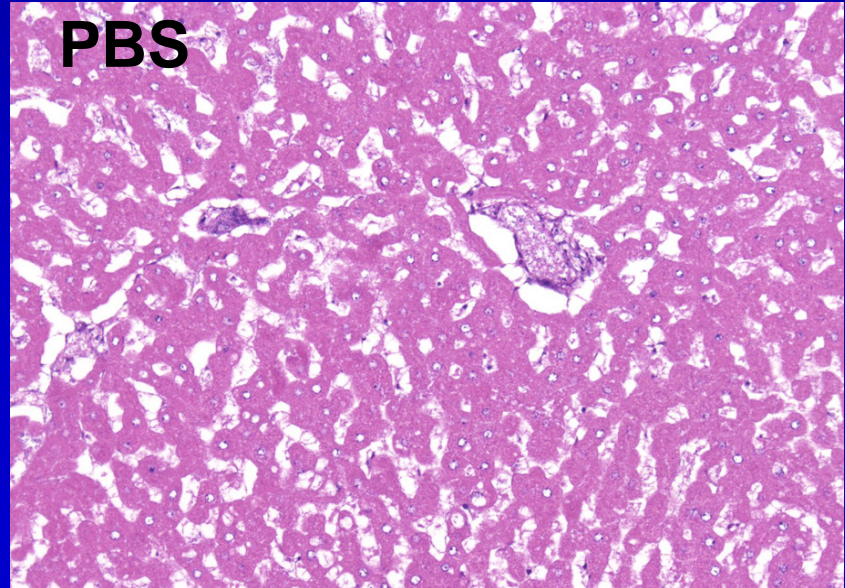
Modified methacarn



UMFIX



PBS

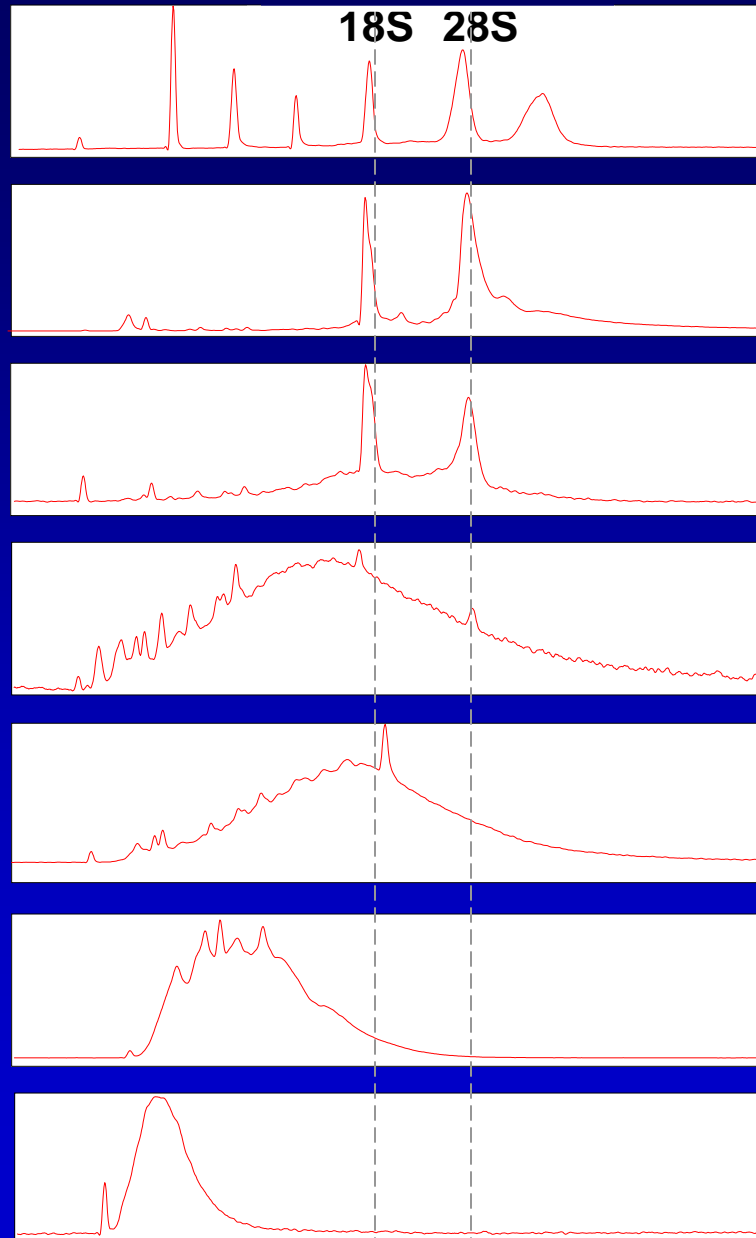


Morphology Results

- Grading scheme:
 - Nuclear, cytoplasmic, and cell membrane detail
- Top score:
 - Modified methacarn
- Next best:
 - 70% ethanol, 10% NBF, modified Carnoy's
- Microwave fixation and processing:
 - Moderately improved morphology for most fixatives
- Optimization required for microwave methods
 - Avoid “cooking” of tissues

RNA Quality Assay: Agilent Bioanalyzer

Evaluated 18S
and 28S rRNA
integrity



Ladder:
0.2, 0.5, 1, 2, 4, 6 kb

Control:
Rat liver total RNA
(Ambion)

Category 1
OCT

Category 2
Modified methacarn
UMFIX

Category 3
70% ethanol
Modified Carnoy's
PBS (microwave fixation)

Category 4
30% sucrose

Category 5
10% NBF
Modified Davidson's II
PBS (standard fixation)

Conclusions for RNA Integrity

- Most important factor: *The Fixative*
- Best RNA quality:
 - Modified methacarn
 - UMFIX
- Microwave methods are irrelevant to RNA quality

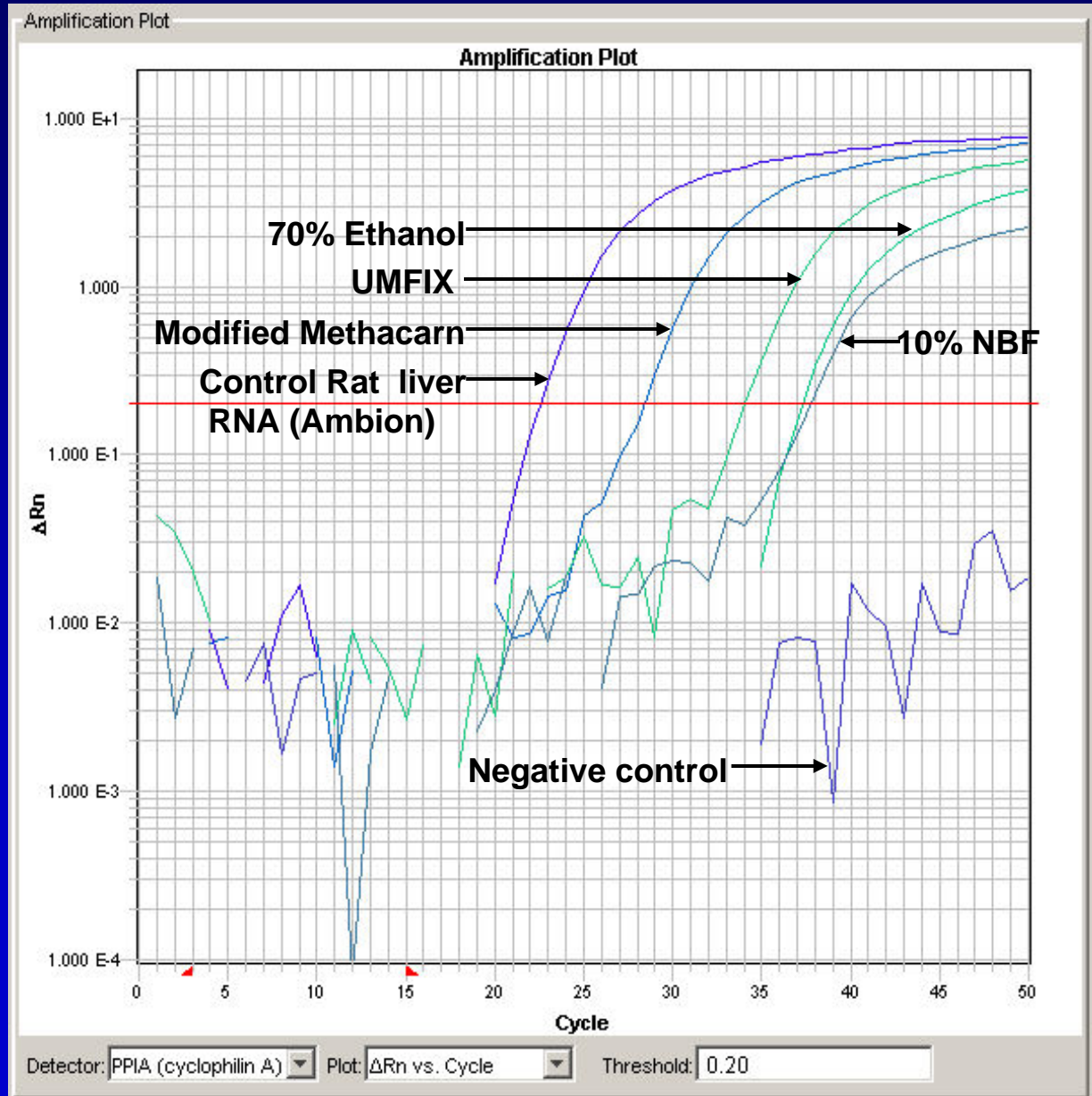
Practical Application: Taqman qRT-PCR

Cycle number:

- Point where slope crosses the line

Fewer cycles:

- More robust amplification, better mRNA integrity

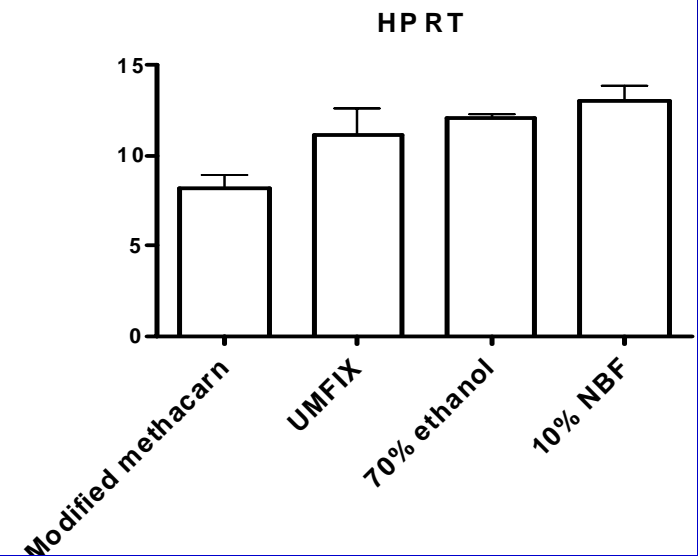
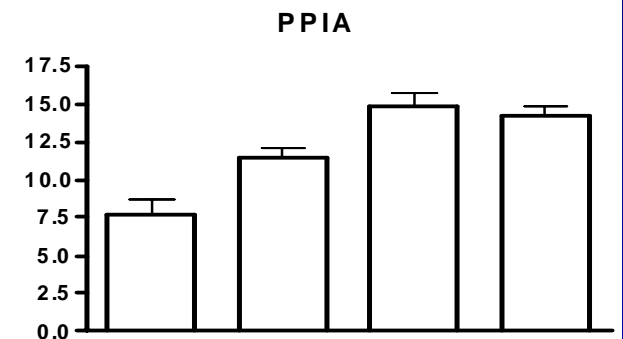
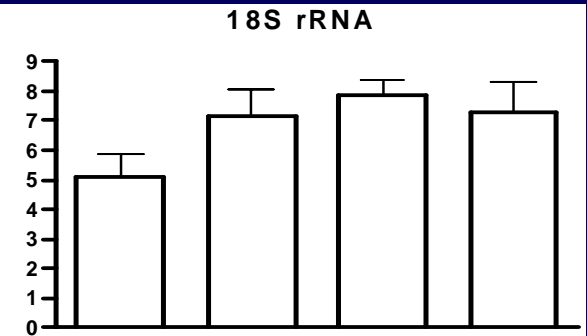


Taqman qRT-PCR

Average Ct of each fixative-derived RNA above the control mean for three specific transcripts

Fixative	Average Ct above the control		
Modified methacarn		7	
UMFIX		10	
10% NBF		12	
70% Ethanol		12	

- 18S rRNA
- PPIA (peptidylprolyl isomerase A / cyclophilin A)
- HPRT (hypoxanthine guanine phosphoribosyl transferase)

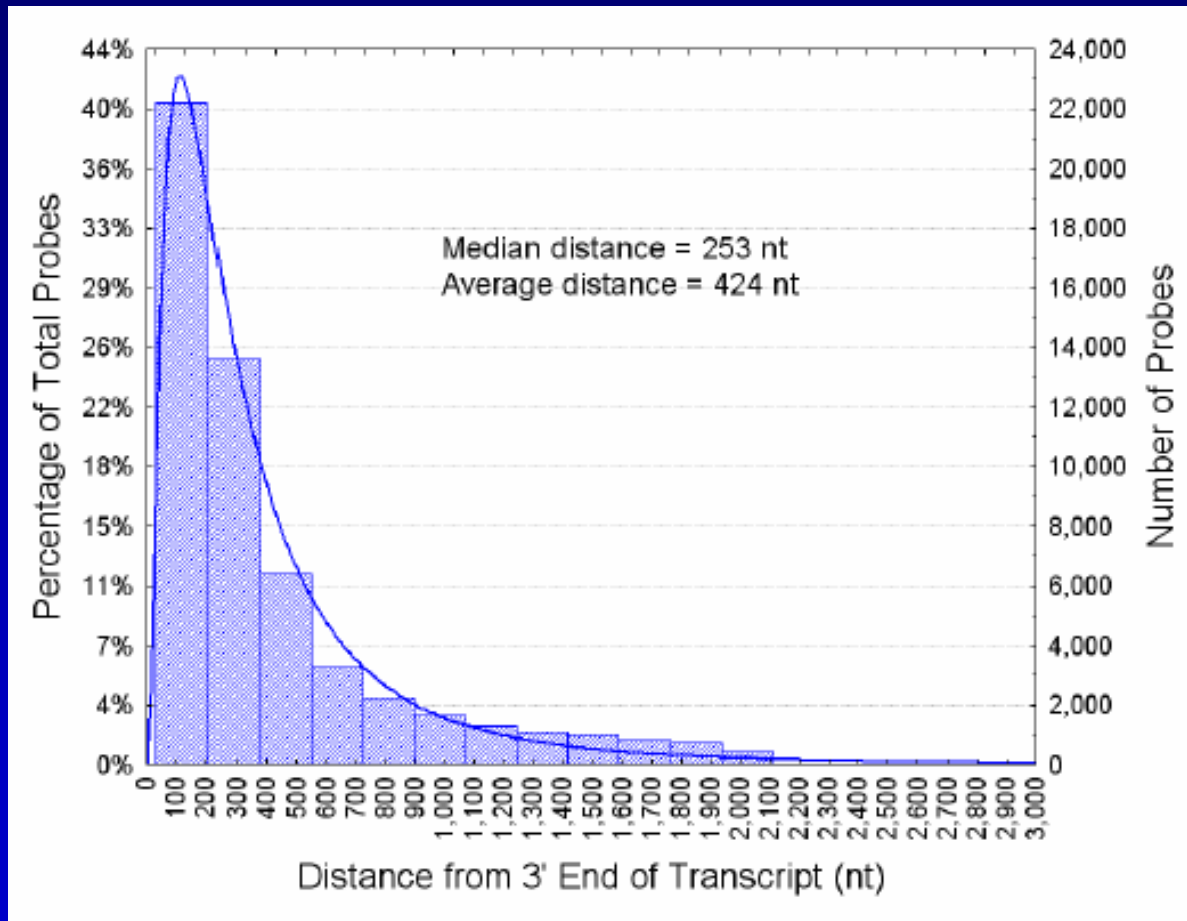




Microarray analysis

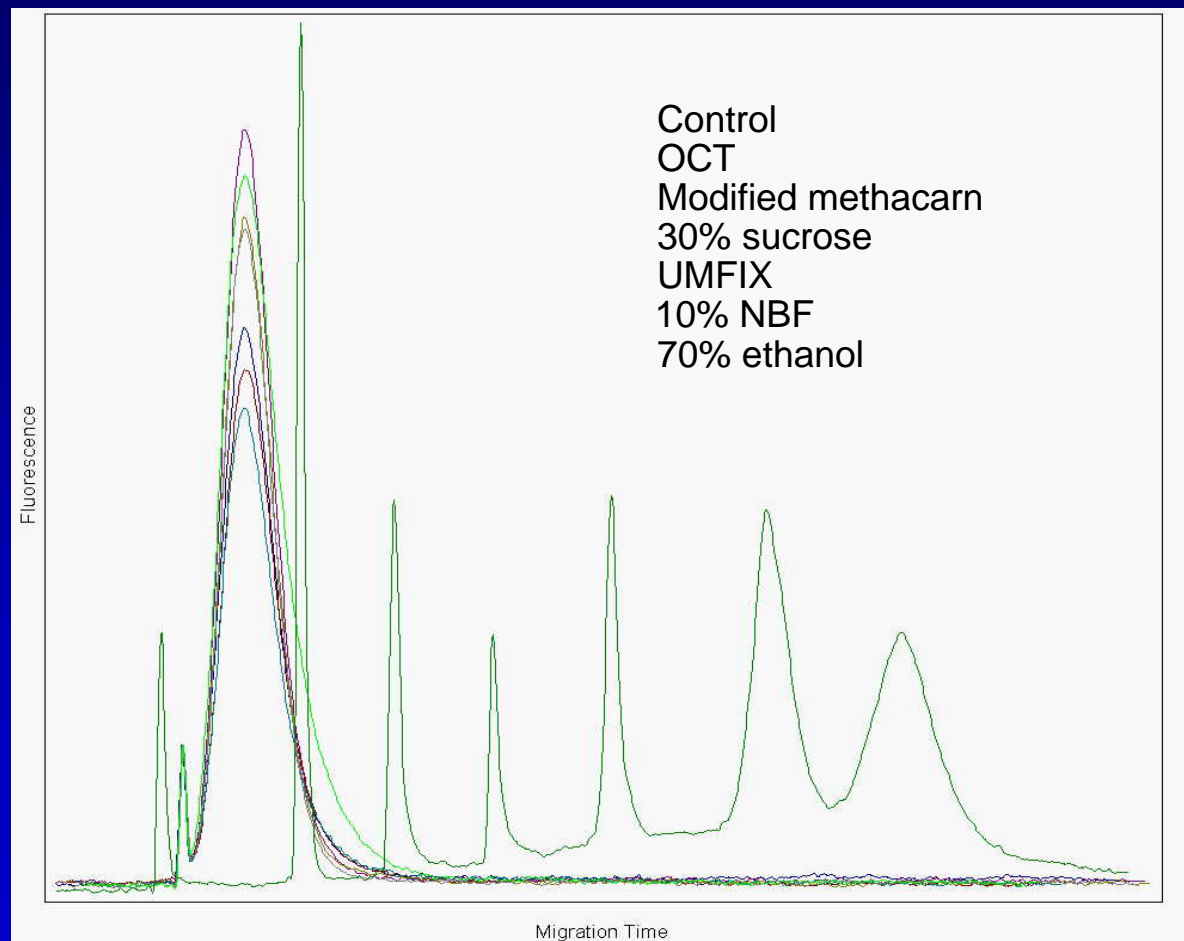
- Goal:
 - Assess the concordance of RNA isolated from fixed tissues to that isolated from snap frozen tissue
 - Assess changes in fixed RNA over time
- Microarray system:
 - GE Amersham CodeLink Rat Whole Genome Bioarray
 - 34,000 gene targets
 - Uses oligo(dT) primers
 - 30mer probes

Probe Design Distance from 3' end of Transcripts



GE Amersham Codelink

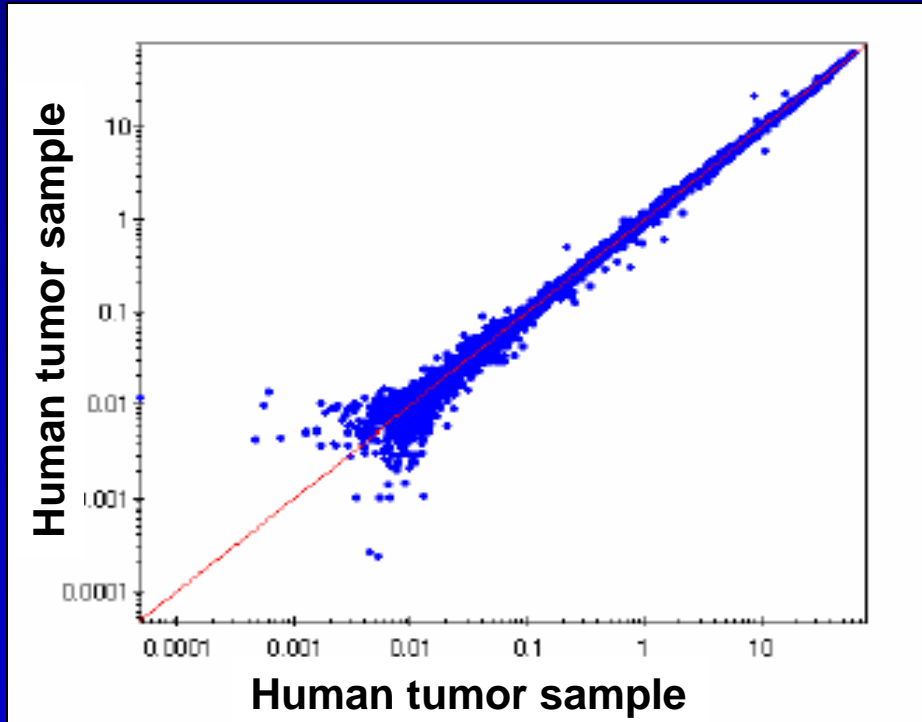
Fragmented cRNA Assessment: Agilent Bioanalyzer Profile



- Chemical fragmentation is random
- cRNA: 25-200 bases in length

Array-to-array Signal Intensity Reproducibility

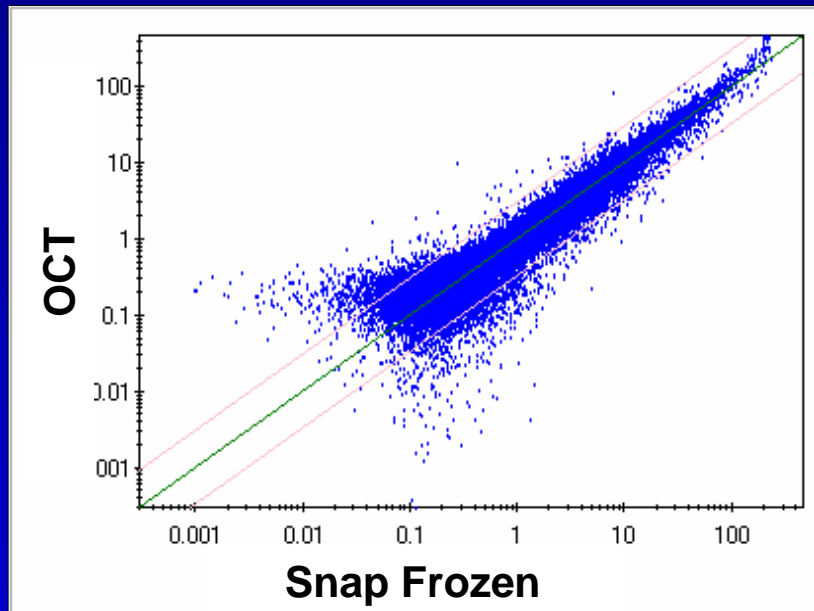
GE Amersham Codelink Array-to-Array Comparison



- All genes are depicted as dots
- Shape of plot is evaluated
 - Scatter plot should look like a “rocket”
 - “Skew” = genes that appear to be differentially regulated
- What is an acceptable “background” level?
 - Snap frozen vs. OCT

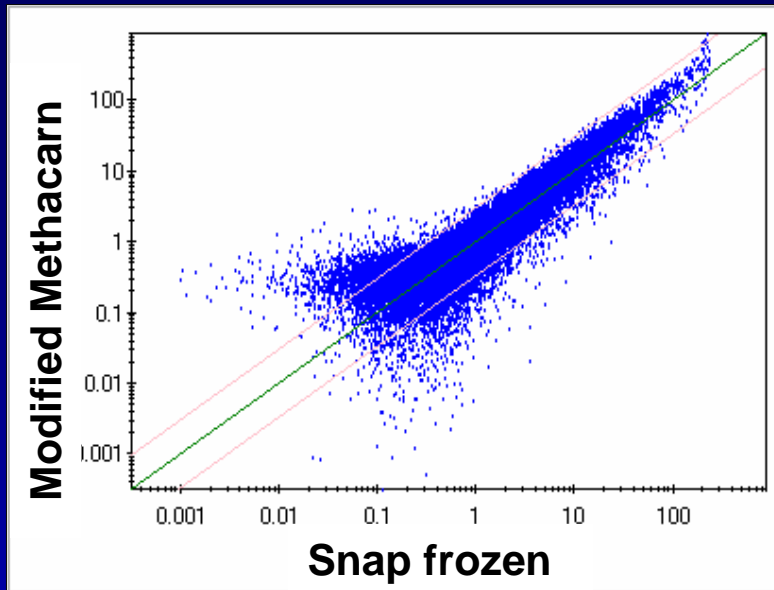
Signal Intensity Reproducibility

Snap Frozen vs. OCT

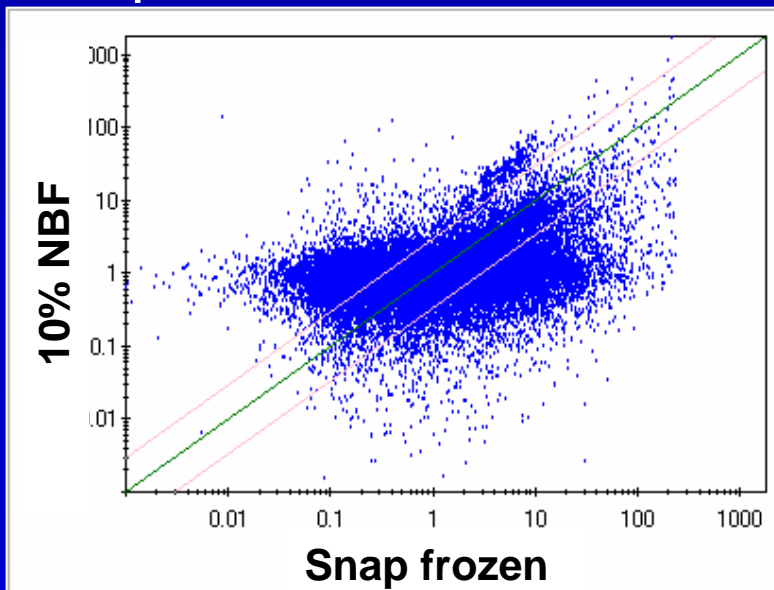


- All probes shown
- Red lines show 3-fold boundary
- Some deviation of OCT from snap frozen is expected, due to a longer freezing time and cryosectioning

Snap Frozen vs. Modified Methacarn



Snap Frozen vs. 10% NBF



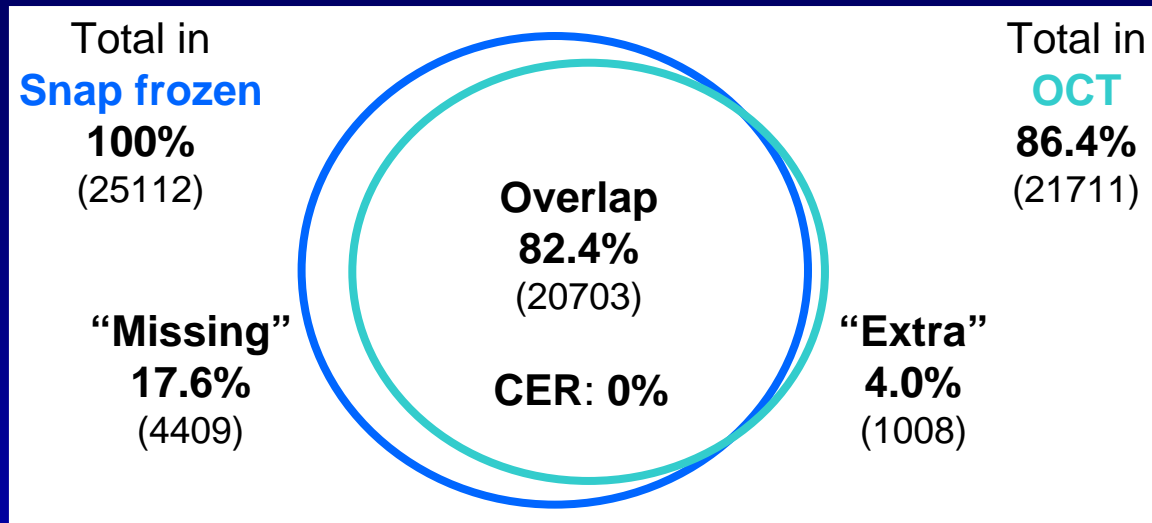
Scatter plots

- Modified methacarn has a scatter plot similar to that of OCT
- 10% formalin is very skewed and has very little similarity to snap frozen

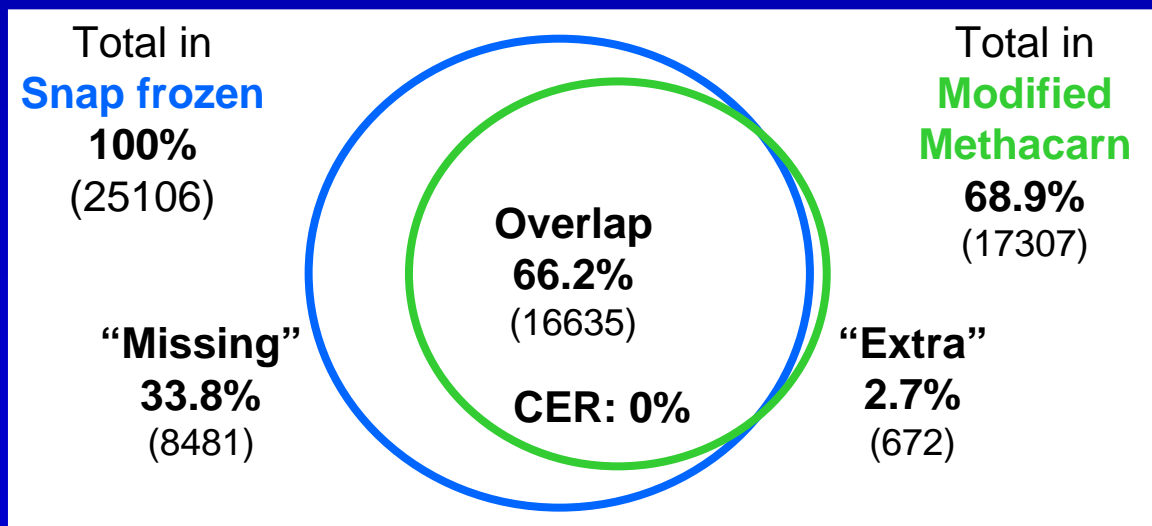
Definitions

- Overlap
 - genes detected in both samples
- “Missing”
 - genes detected in the control sample that are not in the experimental
- “Extra”
 - genes detected in the experimental sample that are not in the control
- Corrected Error Rate (CER)
 - “false positives” – overlapping transcripts that would be considered differentially expressed (3-fold or more)

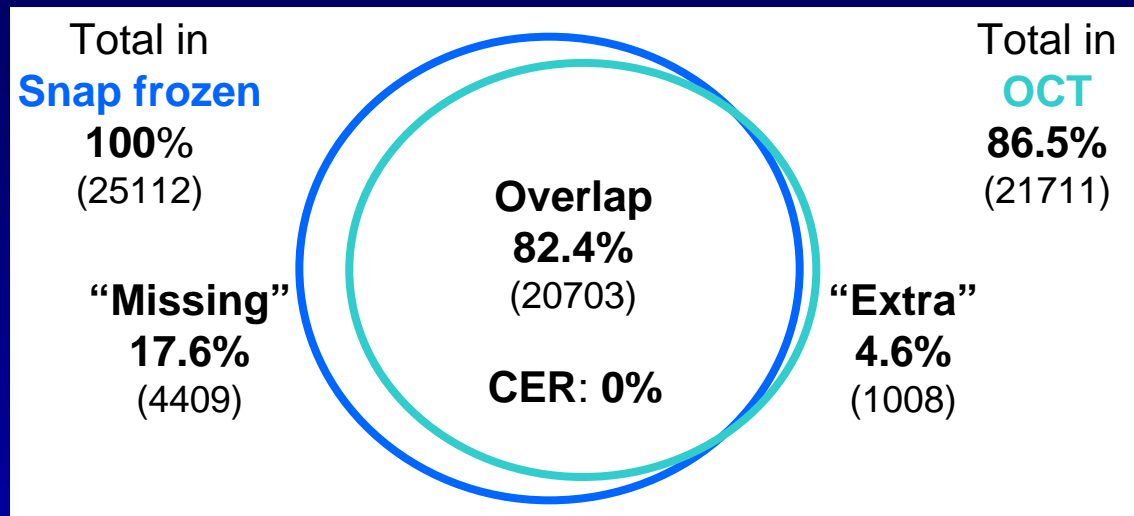
Snap Frozen vs. OCT



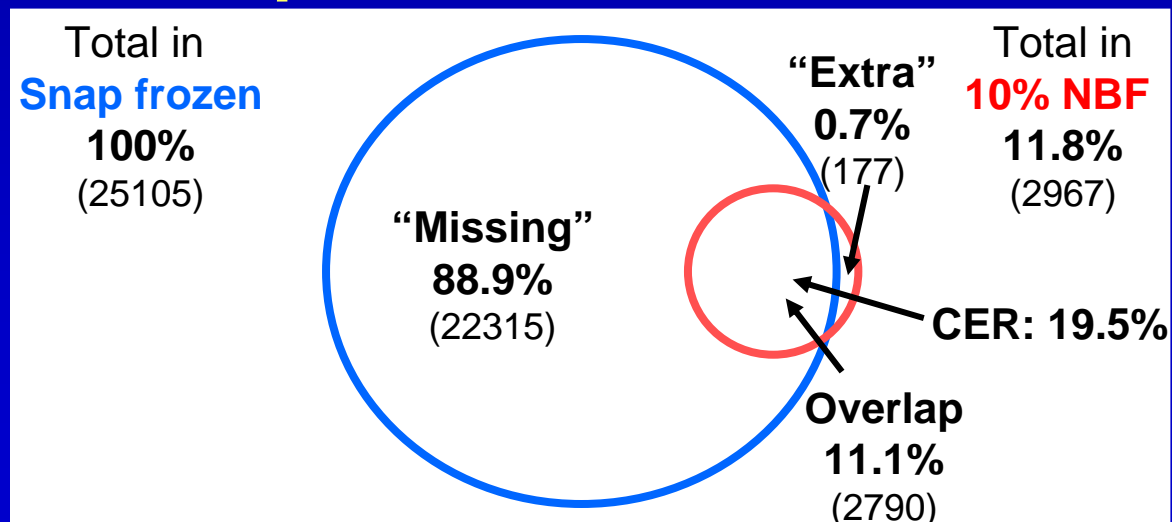
Snap Frozen vs. Modified Methacarn



Snap Frozen vs. OCT



Snap Frozen vs. 10% NBF



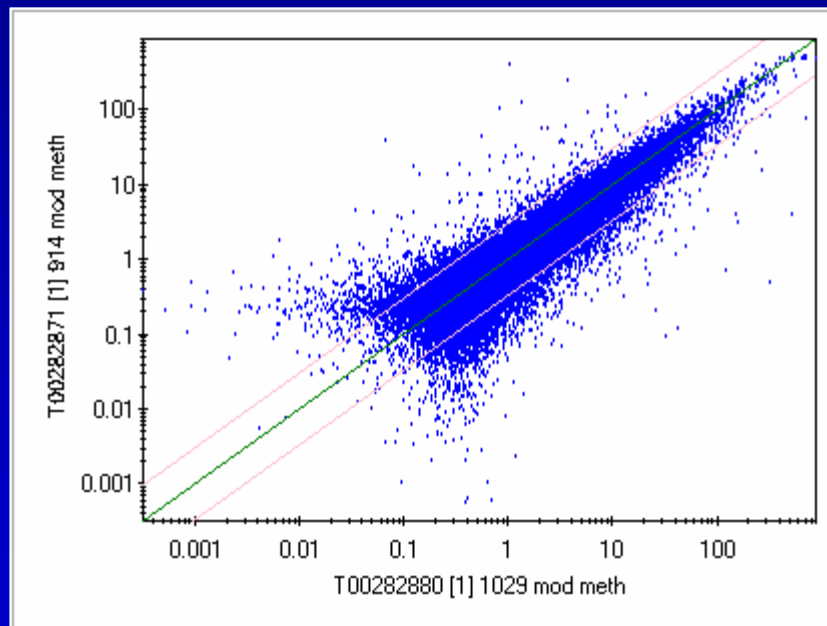
Summary: Snap Frozen vs. Fixed Samples

Snap frozen vs.	Overlap	CER
OCT	82.4%	0%
Modified methacarn	66.2%	0%
10% NBF	11.1%	19.5%

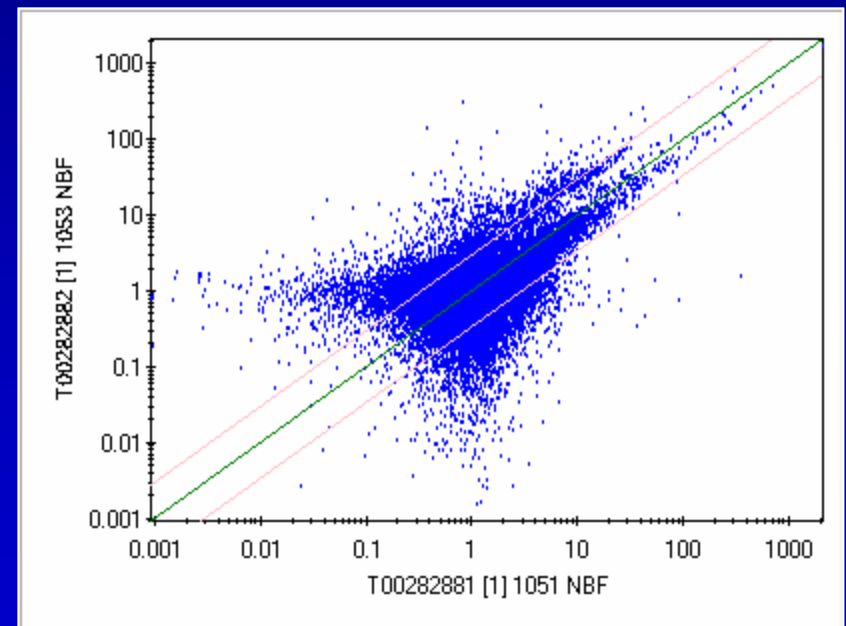
- Acceptable background: the difference between snap frozen and OCT
- Modified methacarn contains 66% of the transcripts found in snap frozen; the false positive rate is negligible.
- 10% NBF contains 11% of the transcripts found in snap frozen; nearly 20% of those are false positives

Does RNA in Fixed Tissues Degrade Further Over Time?

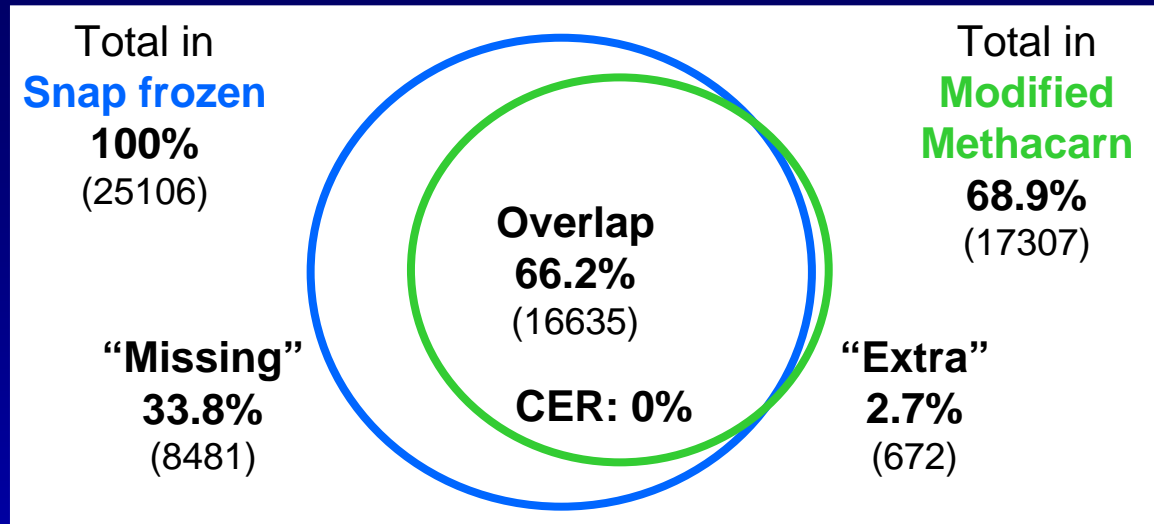
Modified Methacarn vs.
Aged Modified Methacarn



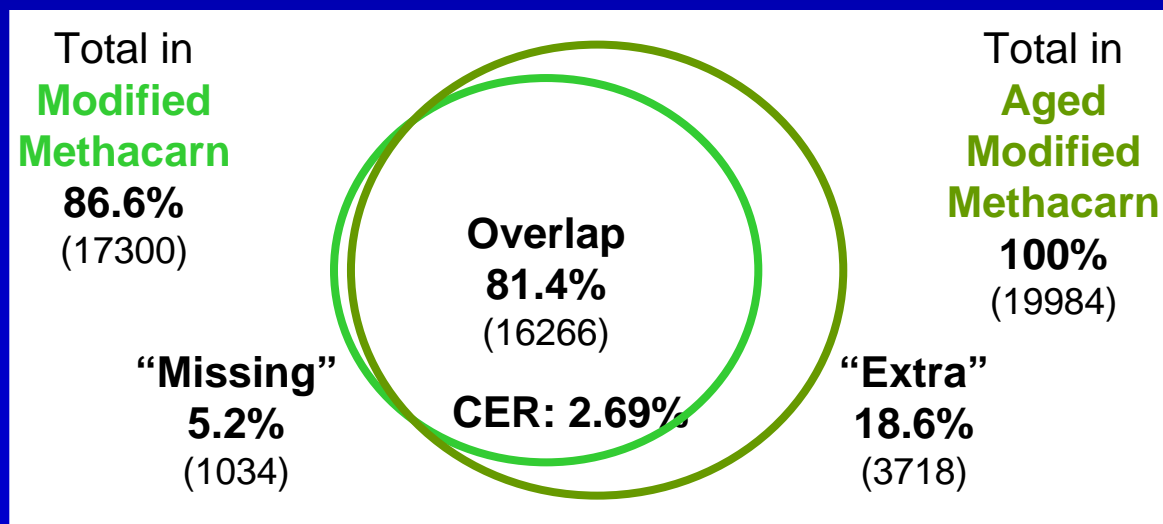
10% NBF vs. Aged 10% NBF



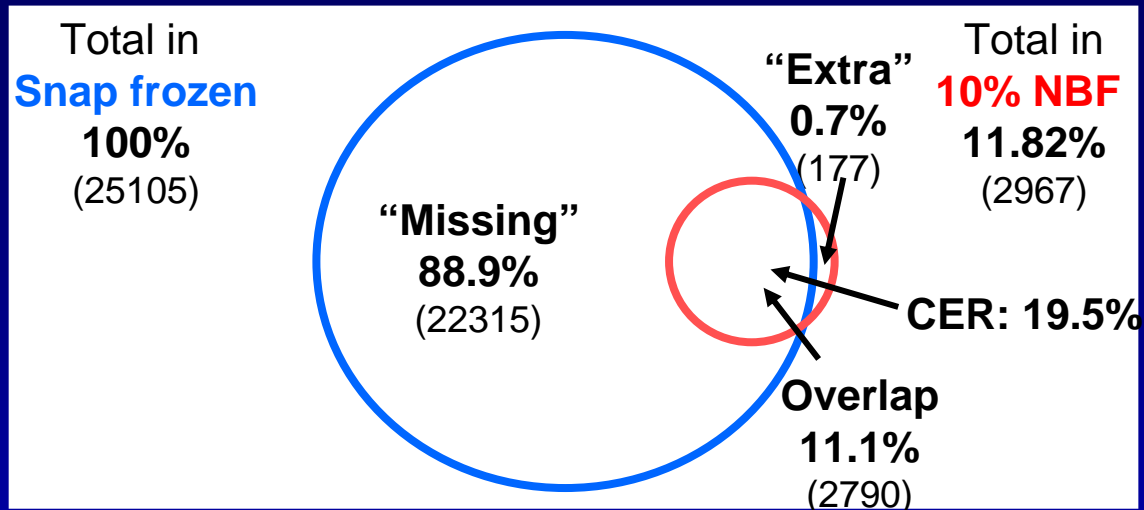
Snap frozen vs. Modified Methacarn



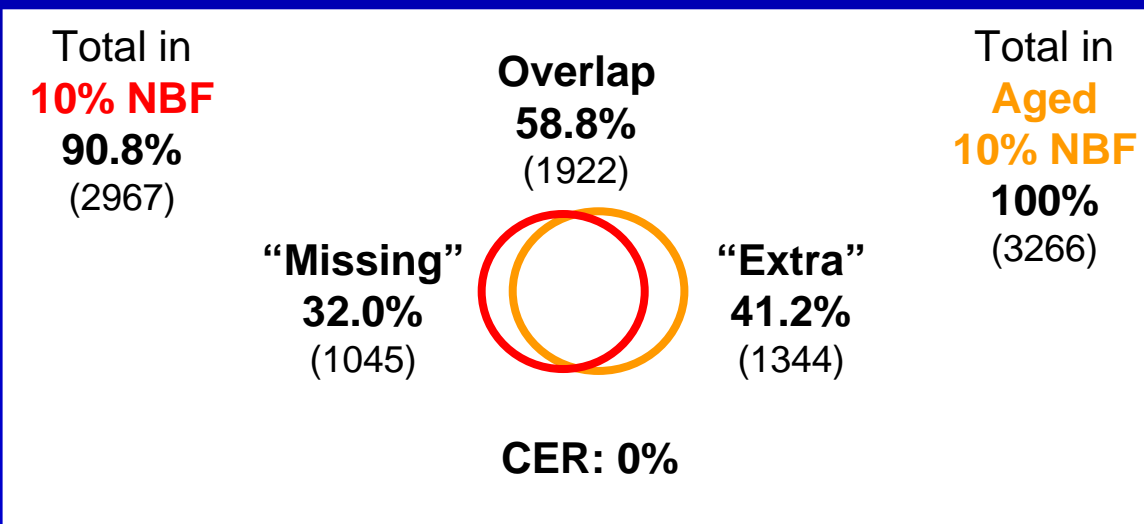
Modified Methacarn vs. Aged Modified Methacarn



Snap Frozen vs. 10% NBF



10% NBF vs. Aged 10% NBF



Summary: Changes in Fixatives in One Year

Comparison	Overlap	CER
Modified methacarn vs. Aged Modified methacarn	81.4%	2.69%
10% NBF vs. Aged 10% NBF	58.8%	0%*

* Only had 1922 overlapping probes

- The change in modified methacarn over time is similar to the “background” difference between snap frozen and OCT.
- 10% NBF changes somewhat over time, but there are very few transcripts detected at either time point

Implications

- The 28S:18S rRNA profile should not be the sole determinant of RNA quality
 - It does not necessarily predict RNA utility
 - It does not predict how well the RNA can be modified by enzymes (eg., reverse transcriptase)
- Very fragmented RNA can yield small amplicons for qRT-PCR
- New definition for RNA quality
 - How it looks AND how it performs

Conclusions

- Determine what quality of RNA is fit for your purpose
 - Most fixatives yield RNA useable for qRT-PCR
 - Archival samples: 10% NBF may work for qRT-PCR, but only extremely abundant transcripts would be detected on microarrays
- Samples preserved in different fixatives **cannot** be compared quantitatively

Acknowledgements

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