

Unlocking microRNA and
mRNA Expression Data
from FFPE Samples:
A Whole Transcriptome
Approach

Genisphere[®]

SIGNAL + SAMPLE AMPLIFICATION PRODUCTS



→ **Gene Expression**

→ microRNA Expression

- **Affymetrix Gene Expression Arrays**
- **Genisphere Sample Prep kit**

sensation™

Sensation: Procedure Overview

First Strand cDNA Synthesis

1 hour, then 1 hour for purification

Total RNA is reverse transcribed using random 9mer primer and dT12V primer, dNTP mix, and Reverse Transcriptase. The cDNA is purified with RNAClean® XP.

Promoter Synthesis

1 hour

The cDNA is poly d(A) tailed on the 3' end using dATP and Terminal Deoxynucleotidyl Transferase. A T7 dT oligo with a 3' blocking group is hybridized to the 3' dA tail of the cDNA. Klenow and dNTP mix generate a double stranded T7 promoter region on the single stranded cDNA.

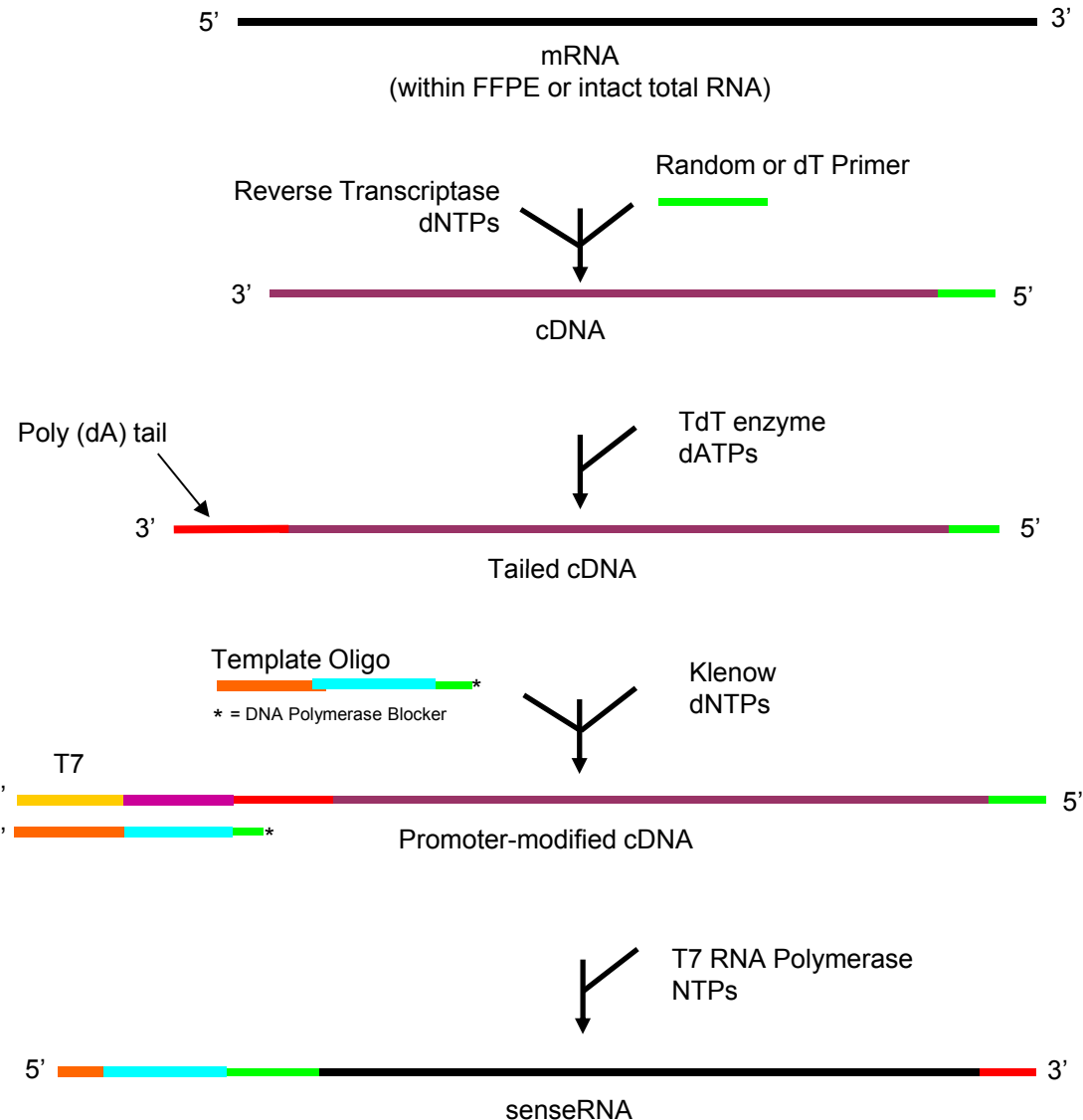
In Vitro Transcription

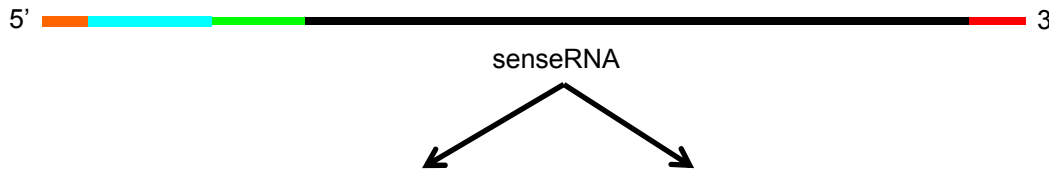
Overnight (16-18 hours), then 1 hour for purification

The promoter-modified cDNA is in vitro transcribed using T7 RNA Polymerase and NTP Mix. The senseRNA is purified with RNAClean XP.

Analysis of senseRNA

The senseRNA is ready for gene expression applications such as qRT-PCR or biotin-labeling for Affymetrix® Arrays (see next page).



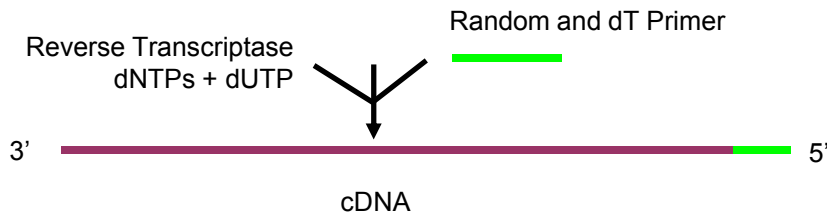


Biotin Labeling Kit for 3' Arrays

cDNA Synthesis

2.5 hours, then 1 hour for purification

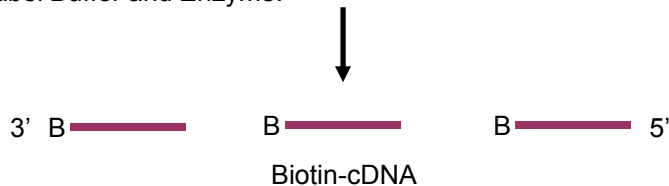
SenseRNA is reverse transcribed using random and dT primer, dNTP mix + biotin-dUTP, and Reverse Transcriptase. The cDNA is purified with RNAClean XP.



Terminal Labeling of cDNA

1 hour

cDNA is fragmented and end-labeled with 1-Step Fragment and Label Buffer and Enzyme.



Analysis of Biotin-cDNA

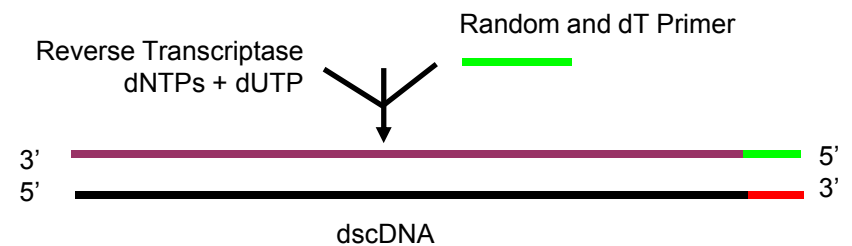
The biotin-cDNA is ready for hybridization to Affymetrix 3' IVT Expression Arrays.

Biotin Labeling Kit for ST/Exon Arrays

Double Stranded cDNA Synthesis

3.5 hours, then 1 hour for purification

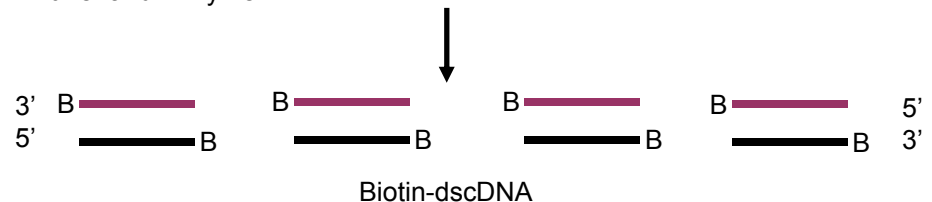
SenseRNA is reverse transcribed using random and dT primer, dNTP mix + dUTP, and Reverse Transcriptase. Second Strand cDNA is made with DNA Polymerase I and RNase H. The ds-cDNA is purified with RNAClean XP.



Terminal Labeling of double stranded cDNA

1 hour

dscDNA is fragmented and end-labeled with 1-Step Fragment and Label Buffer and Enzyme.



Analysis of Biotin-dscDNA

The biotin-dscDNA is ready for hybridization to Affymetrix Whole-Transcript Expression Arrays.

- Random and dT primers used in first step, reverse transcription (RT)
 - Whole Transcriptome amplification
 - Same procedure whether RNA is intact or FFPE
- No 5' extension on primers
 - No bias during RT; true random primers
 - No Template (no RNA) produces minimal amplification products
- RT from 3' end then amplify from 5' end
 - No bias to either 3' or 5' end of mRNAs
 - Accurate sense-strand amplification products
- Labeling options for 3' arrays and ST/exon arrays

Pillai R, et al. Validation and Reproducibility of a Microarray-Based Gene Expression Test for Tumor Identification in Formalin-Fixed, Paraffin-Embedded Specimens. *Journal of Molecular Diagnostics* January 2011, Vol. 13, No. 1.

Roberts L, et al. Identification of methods for use of formalin-fixed, paraffin-embedded tissue samples in RNA expression profiling. *Genomics* 2009, 94(5):341-8.

Koh SS, et al. Molecular classification of melanomas and nevi using gene expression microarray signatures and formalin-fixed and paraffin-embedded tissue. *Mod Pathol.* 2009 Apr;22(4):538-46.

Goff LA, et al. Evaluation of Sense-Strand mRNA Amplification by Comparative Quantitative PCR. *BMC Genomics*, 5:76, 2004.

1. Acquire Matched FFPE and Fresh Frozen Samples from **Colon**
2. Purify FFPE samples using **Ambion RecoverAll™ kit** and FF samples using **Qiagen® miRNeasy Mini kit**
3. Follow target preparation protocols from Genisphere
100ng input FFPE total RNA
50ng input FF total RNA
4. Hybridize biotin-labeled targets to Affymetrix **U133plus2.0** and **GeneST** Arrays

Colon Samples from 2005

| | | |
|------|--------|-----------|
| FF | 3 Reps | RIN = 8.5 |
| FFPE | 3 Reps | RIN = 2.2 |

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U133plus2.0 Array Metrics

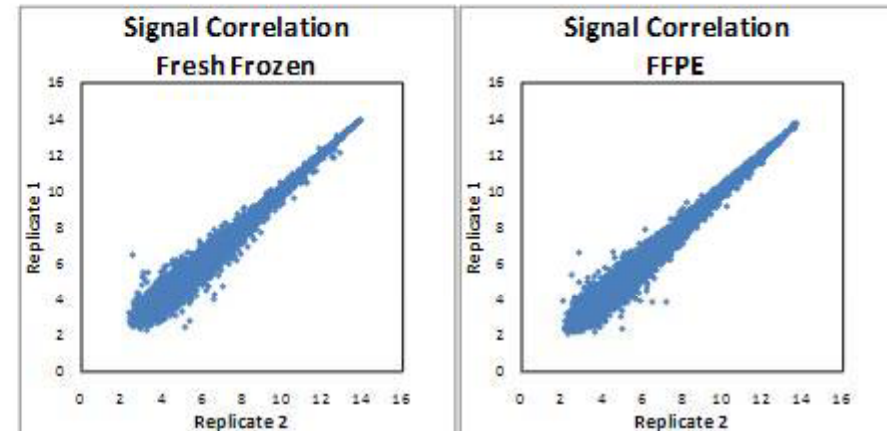
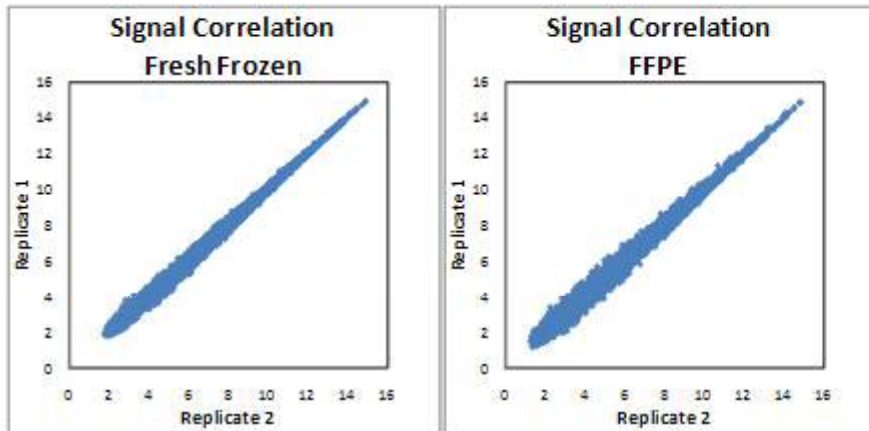
| Sample | SF | %P | Actin 3'/5' | GAPDH 3'/5' |
|----------------|-----|------|-------------|-------------|
| FFPE Colon -1 | 5.9 | 53.4 | 1.2 | 1.6 |
| FFPE Colon-2 | 6.7 | 52.0 | 1.2 | 1.5 |
| FFPE Colon -3 | 8.3 | 52.2 | 1.2 | 1.5 |
| Frozen Colon-1 | 4.2 | 64.1 | 1.2 | 1.1 |
| Frozen Colon-2 | 4.3 | 63.9 | 1.2 | 1.0 |
| Frozen Colon-3 | 4.4 | 64.0 | 1.2 | 1.0 |

HG U133 Plus 2.0 Arrays

HG 1.0 ST Arrays

| | Fresh Frozen | FFPE |
|-----------------------|--------------|-------|
| Median CV, Gene Level | 4.71% | 7.30% |
| Probesets Used | 38202 | 38202 |

| | Fresh Frozen | FFPE |
|-----------------------|--------------|-------|
| Median CV, Gene Level | 5.46% | 6.61% |
| Probesets Used | 33297 | 33297 |



Pearson = 0.997

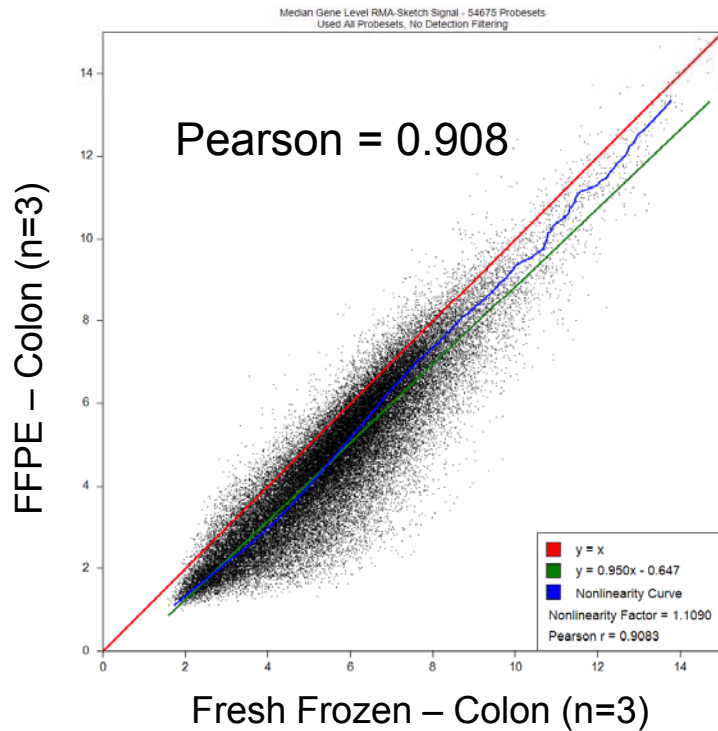
Pearson = 0.995

Pearson = 0.994

Pearson = 0.992

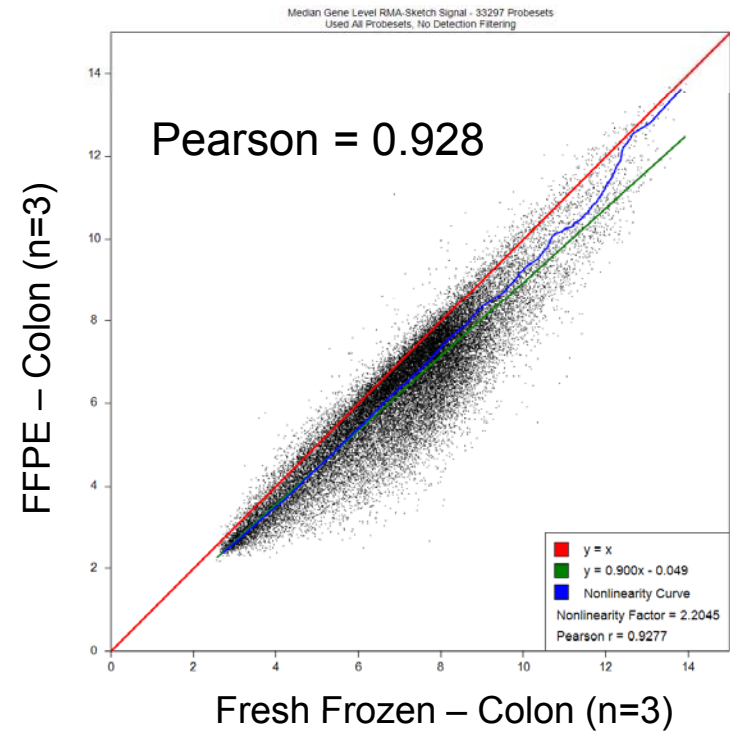
HG U133 Plus 2.0 Arrays

Signal Correlation - Fresh Frozen vs. FFPE



HG 1.0 ST Arrays

Signal Correlation - Fresh Frozen vs. FFPE



1. Acquire FFPE Samples from a **variety of tissues**
2. Purify FFPE samples using **Agencourt® FormaPure kit**
3. Follow target preparation protocols from Genisphere:
30ng input FFPE total RNA
4. Hybridize biotin-labeled targets to Affymetrix **U133A 2.0** Arrays

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| FFPE Sample | SF | BG Avg | %P | Signal(P) | Actin 3'/5' | GAPDH 3'/5' |
|--------------------|-----------|---------------|-----------|------------------|------------------------|------------------------|
| Pancreas -1 | 4.2 | 34.8 | 62.1 | 1130.7 | 1.30 | 2.01 |
| Pancreas -2 | 3.5 | 34.4 | 67.3 | 1025.6 | 1.31 | 2.08 |
| Lymph | 3.8 | 35.0 | 58.5 | 1185.3 | 1.31 | 1.65 |
| Bladder | 2.8 | 40.1 | 63.3 | 1117.6 | 1.26 | 1.66 |
| Liver | 9.3 | 33.7 | 48.3 | 1513.4 | 1.76 | 1.85 |
| Lung -1 | 5.6 | 34.6 | 53.4 | 1374.3 | 1.46 | 1.68 |
| Lung -2 | 1.8 | 38.4 | 68.5 | 970.7 | 1.21 | 1.53 |
| Kidney -1 | 2.4 | 36.7 | 65.5 | 1081.4 | 1.20 | 1.52 |
| Kidney -2 | 5.1 | 34.1 | 49.3 | 1432.6 | 1.24 | 1.52 |
| Ovary -1 | 8.2 | 33.9 | 48.3 | 1497.2 | 1.33 | 1.77 |
| Ovary -2 | 2.6 | 37.9 | 68.0 | 989.8 | 1.38 | 1.74 |

1. Acquire Matched FFPE and Fresh Frozen Samples from **Breast**
2. Purify FFPE samples using **Ambion RecoverAll™ kit** and FF samples using **Qiagen® miRNeasy Mini kit**
3. Follow target preparation protocols from Genisphere
10ng and 20ng input FFPE total RNA
10ng and 20ng input FF total RNA
4. Hybridize biotin-labeled targets to Affymetrix **GeneST** Arrays

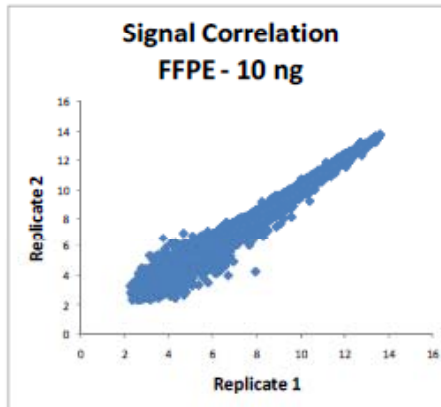
The logo for 'sensation™' features the word 'sensation' in a lowercase, sans-serif font. Above the 'a' and 'i' are three blue circles of varying sizes, and a blue dot is positioned above the 's'. A trademark symbol (TM) is located at the top right of the word.

HG 1.0 ST Arrays – 10 ng

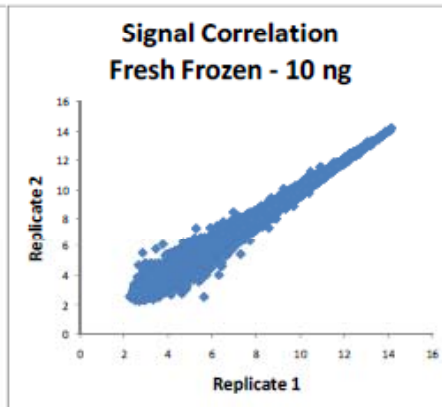
| | Fresh Frozen | FFPE |
|-----------------------|--------------|-------|
| Median CV, Gene Level | 6.67% | 7.09% |
| Probesets Used | 33297 | 33297 |

HG 1.0 ST Arrays – 20 ng

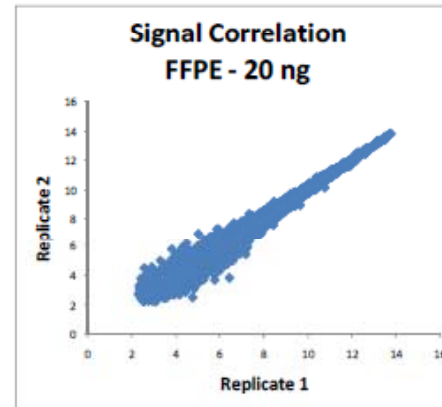
| | Fresh Frozen | FFPE |
|-----------------------|--------------|-------|
| Median CV, Gene Level | 6.24% | 5.85% |
| Probesets Used | 33297 | 33297 |



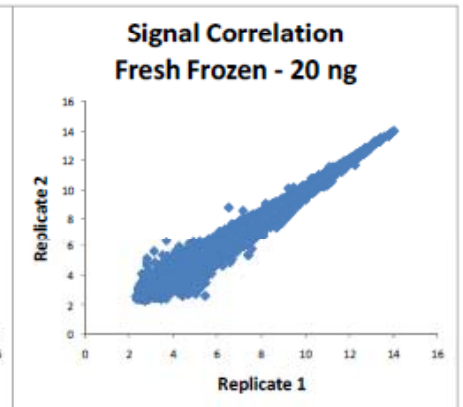
Pearson = 0.992



Pearson = 0.993



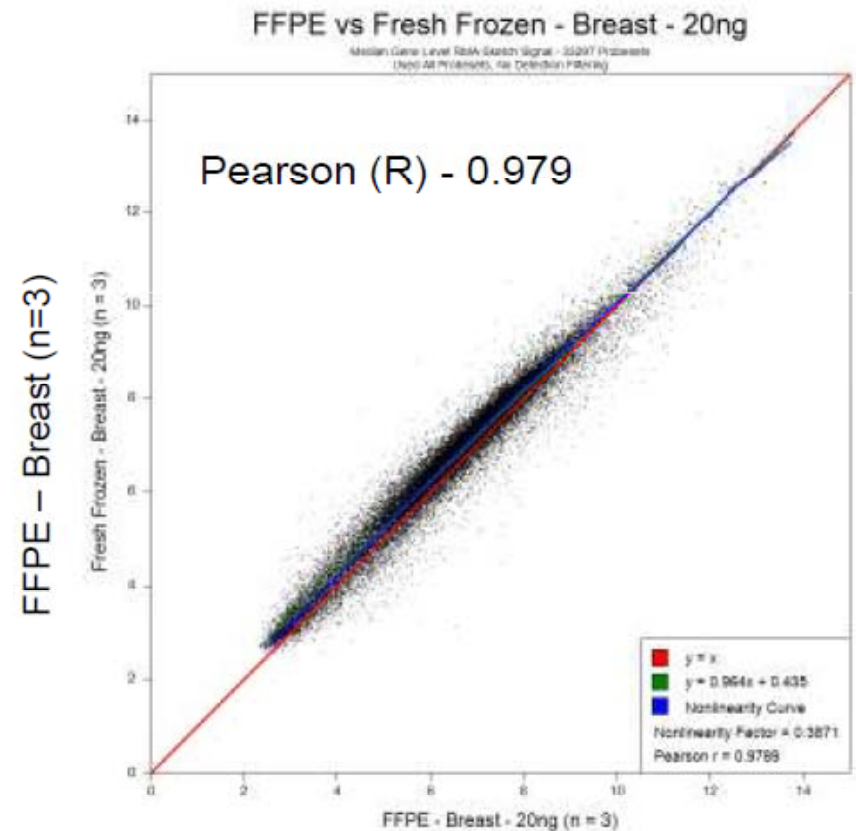
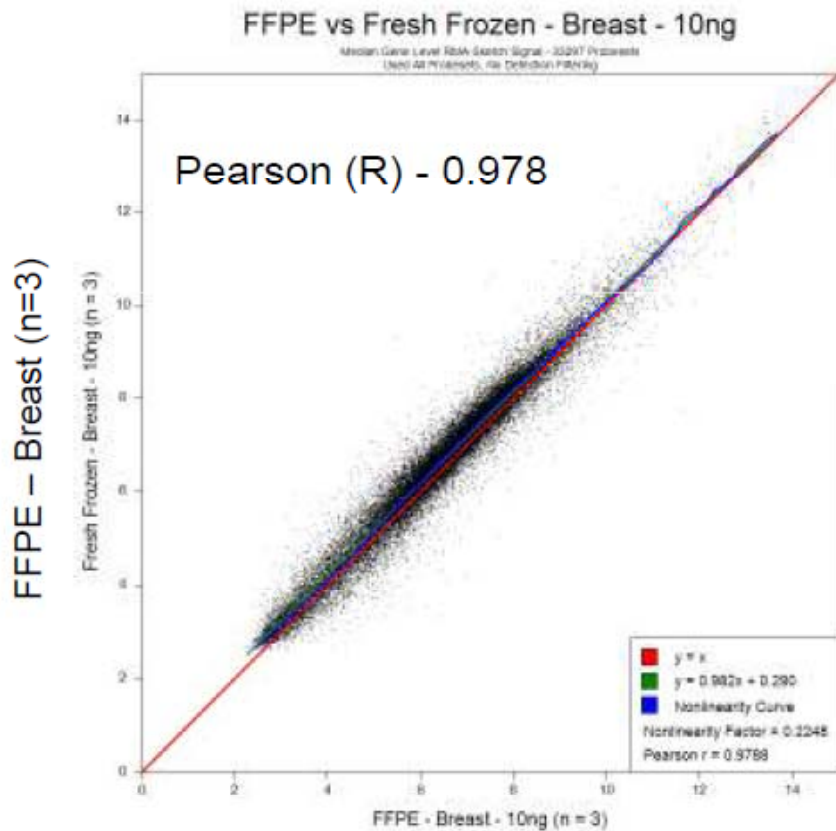
Pearson = 0.994



Pearson = 0.993

HG 1.0 ST Arrays – (10ng)

HG 1.0 ST Arrays – (20ng)



Fresh Frozen – Breast (n=3)

Fresh Frozen – Breast (n=3)



Gene Expression

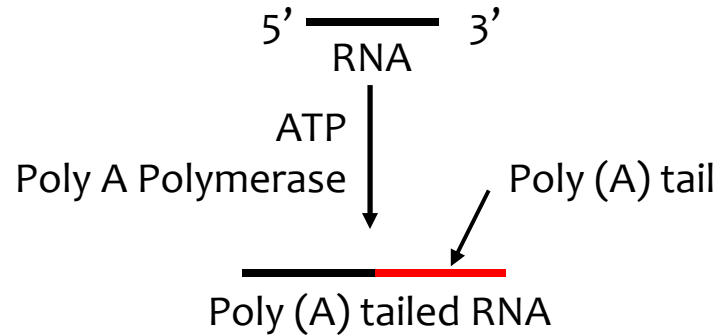
microRNA Expression

- Affymetrix MicroRNA Arrays
- Genisphere Sample Prep kit

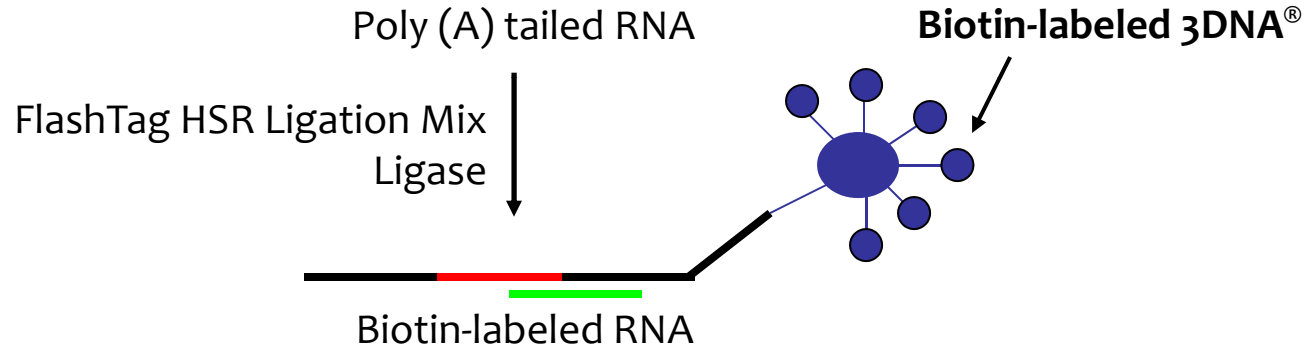
*flash*tag™

- Extract total RNA (which includes microRNA) with Applied Biosystems RecoverAll™ Total Nucleic Acid Isolation Kit for FFPE
- Label 100-1000ng FFPE total RNA with FlashTag HSR
- Same procedure whether RNA is intact or FFPE

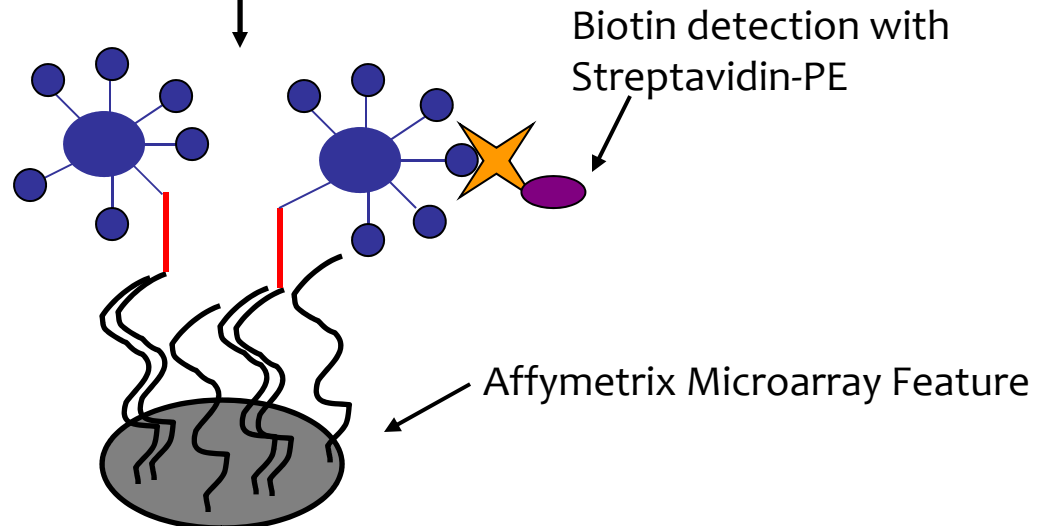
1 Poly (A) Tailing
(15 minutes)



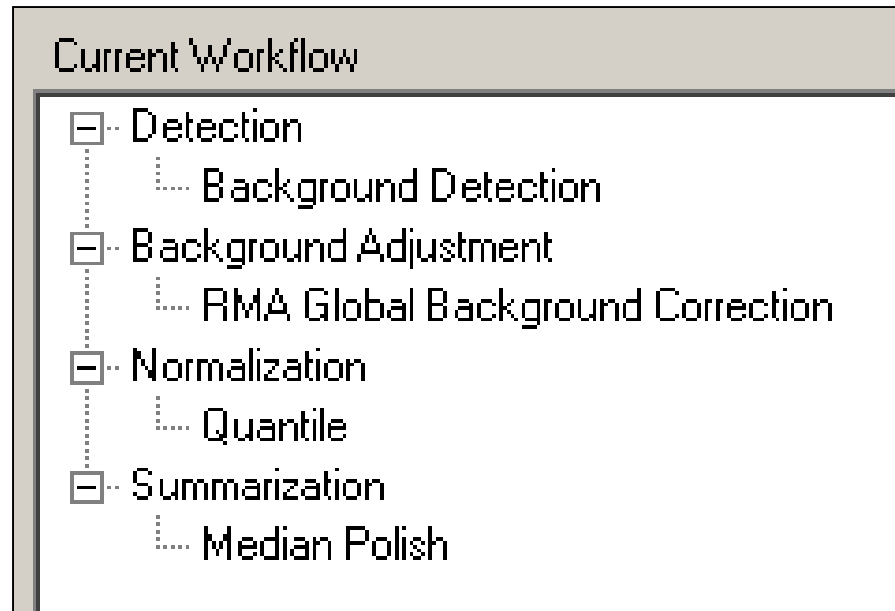
2 Ligation
(30 minutes)



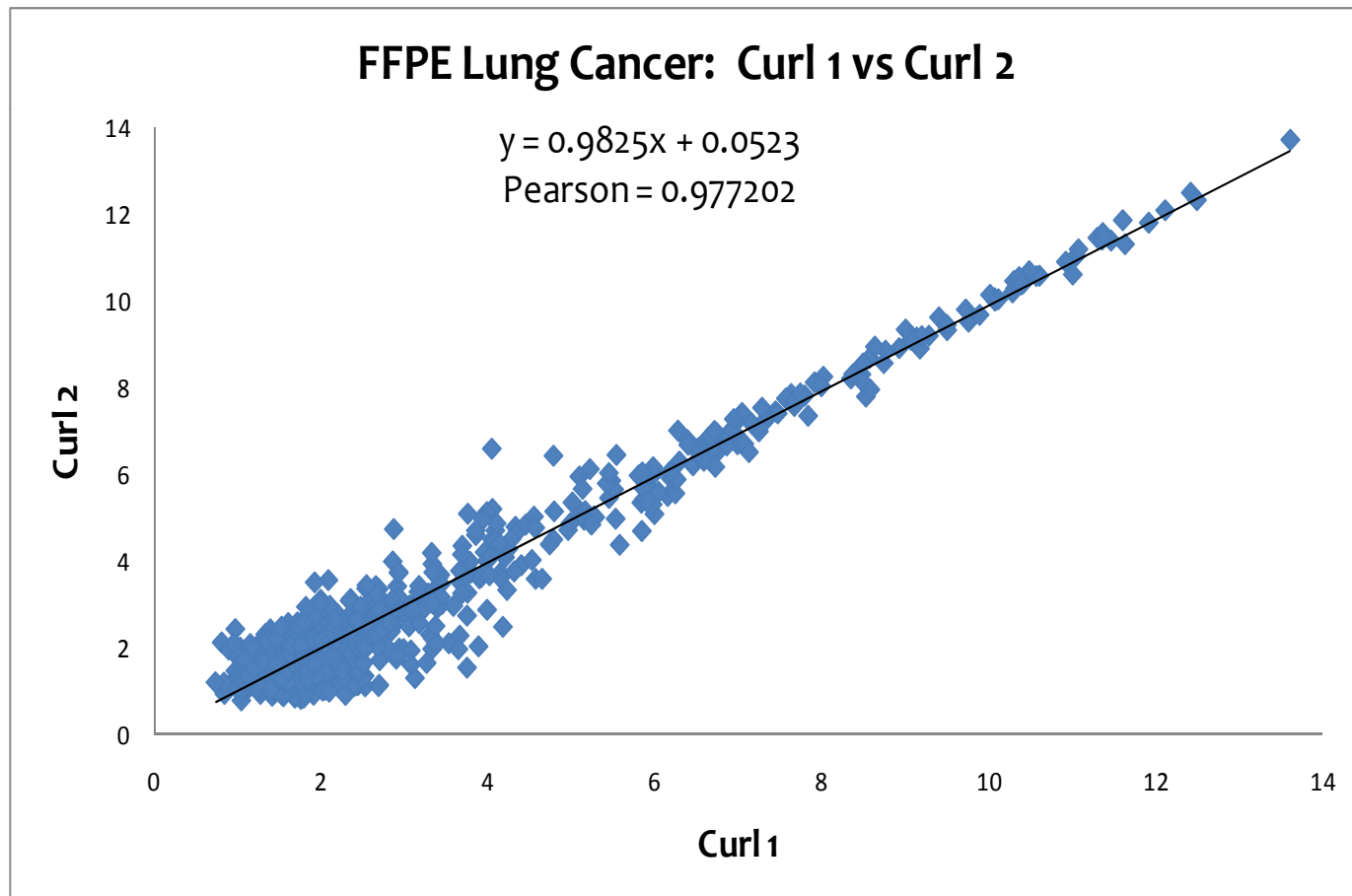
3 Analysis



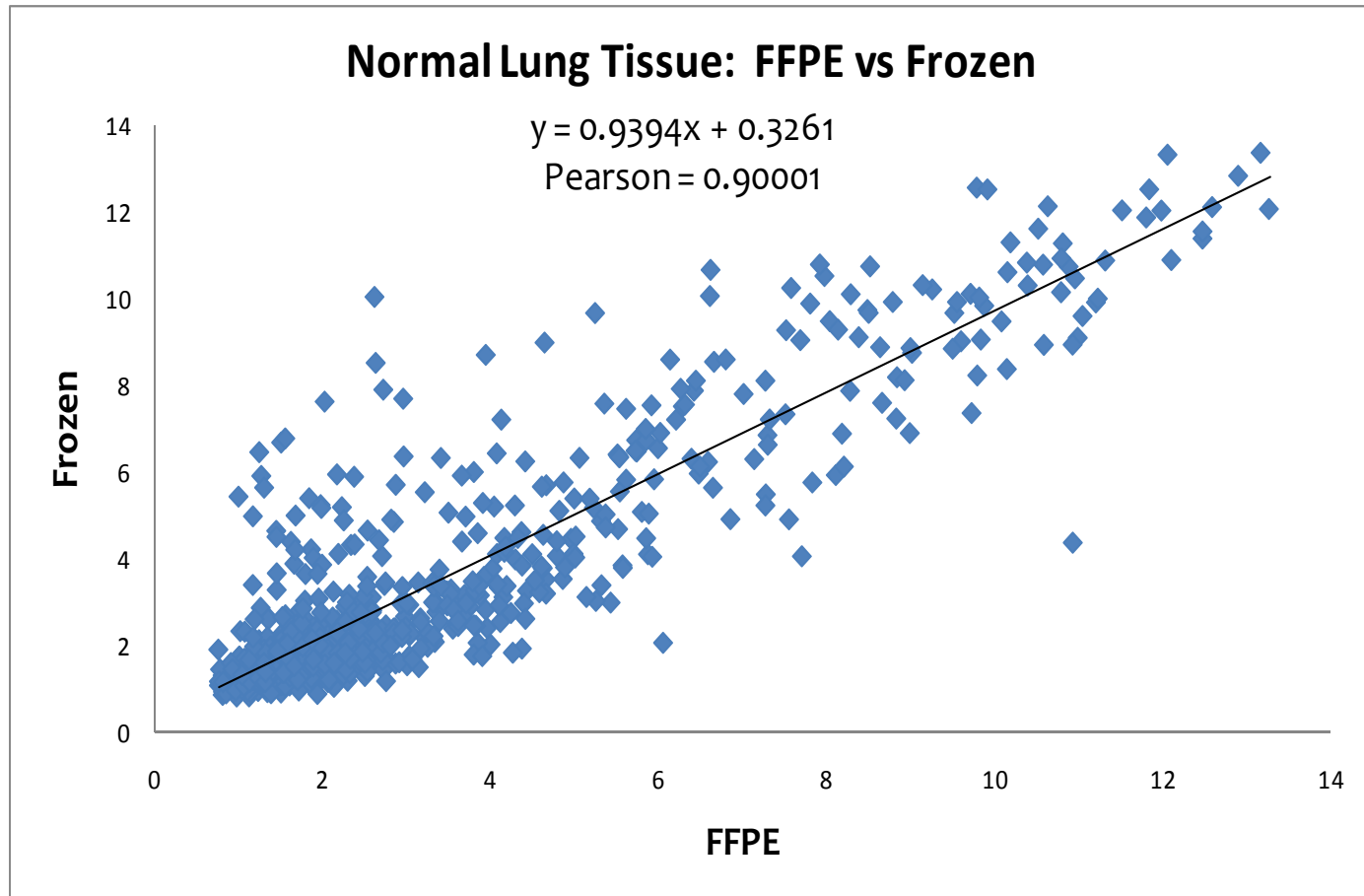
- miRNA QC Tool Software
 - Free, from Affymetrix
 - Easy to use
 - Recommended workflow:

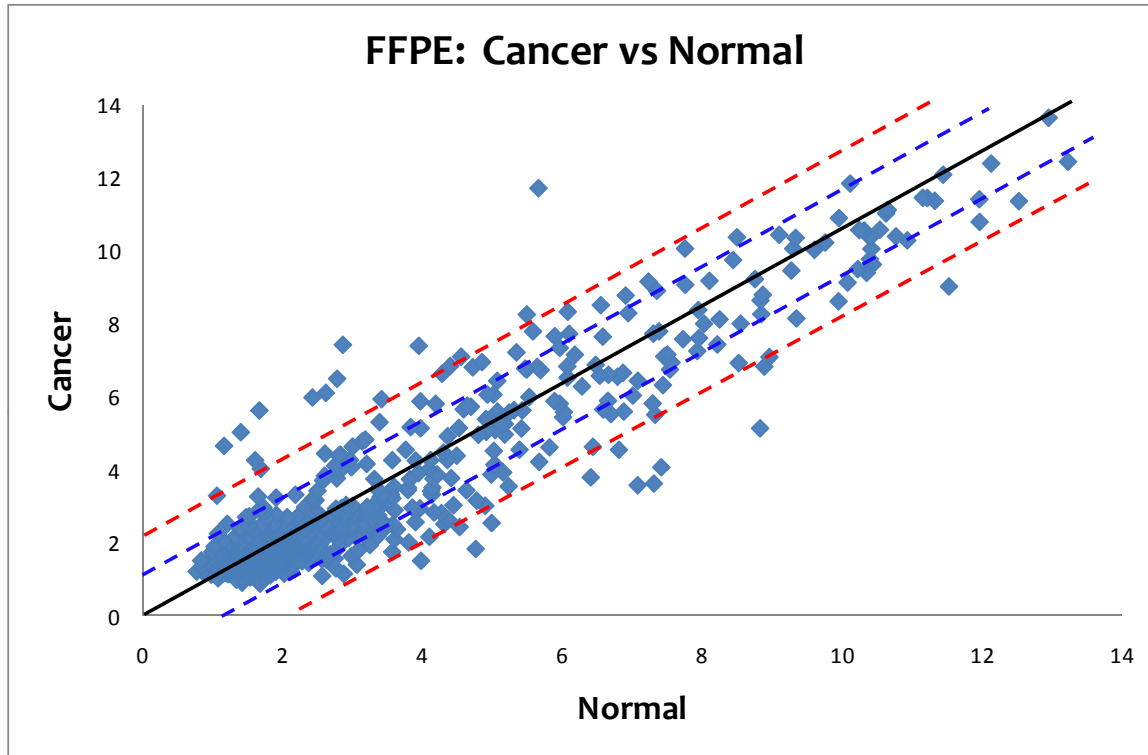


- Duplicate FFPE RNA extractions and FlashTag HSR labeling reactions (of 100ng FFPE total RNA) show excellent reproducibility



- 100ng FFPE total RNA has similar range of array signal intensities, and number of detected probesets, compared to 100ng frozen total RNA





| Fold-Change | Number of microRNAs |
|-----------------------------------|---------------------|
| $\geq 4\text{-fold (+)}$ ----- | 22 |
| $\geq 2\text{-fold (+)}$ ----- | 81 |
| $\geq 4\text{-fold (-)}$ ----- | 12 |
| $\geq 2\text{-fold (-)}$ ----- | 56 |

• Interesting microRNAs:

- miR-210
- miR-146a
- miR-34c
- miR-17

Journal of Carcinogenesis 2010, 9:8
MicroRNAs and lung cancer: Biology and applications in diagnosis and prognosis

Unlocking microRNA and mRNA Expression Data from FFPE Samples: A Whole Transcriptome Approach

sensation™

- intact or degraded total RNA
- One round of amplification
- Same procedure whether RNA is intact or FFPE
- Affymetrix Gene Expression arrays

*flash*tag™

- intact or degraded total RNA
- microRNA labeling
- Same procedure whether RNA is intact or FFPE
- Affymetrix miRNA arrays

- Technical Support
 - Email: techsupport@genisphere.com
 - Phone: 1-877-888-3362 or 215-996-3040

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