FlashTag™ Biotin HSR RNA Labeling Kit

for GeneChip® miRNA Arrays

"For Research Use Only. Not for use in diagnostic procedures."

Slide deck information provided courtesy of Genisphere, LLC.

Contents

- **FlashTag Biotin HSR RNA Labeling Kit**

  Features, procedure, workflow, performance, controls, and ELOSA QC Assay

- **FFPE Sample Labeling**

- **Plasma and Serum Sample Labeling**

- **miRNA QC Tool Software**

  (Applicable to miRNA 1.0 and 2.0 Arrays only)

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Key Features and Benefits

- **Easy-to-use**

  Two step, 45-minute assay – from RNA sample to labeled target – without any purification steps

- **Low sample input**

  Requires as little as 130 ng total RNA

- **Variety of samples**

  Works with any intact, degraded, or FFPE RNA sample

- **Built-in controls**

  Contains RNA spike control oligos for GeneChip miRNA Arrays

- **Identical to reagents previously available from Genisphere**

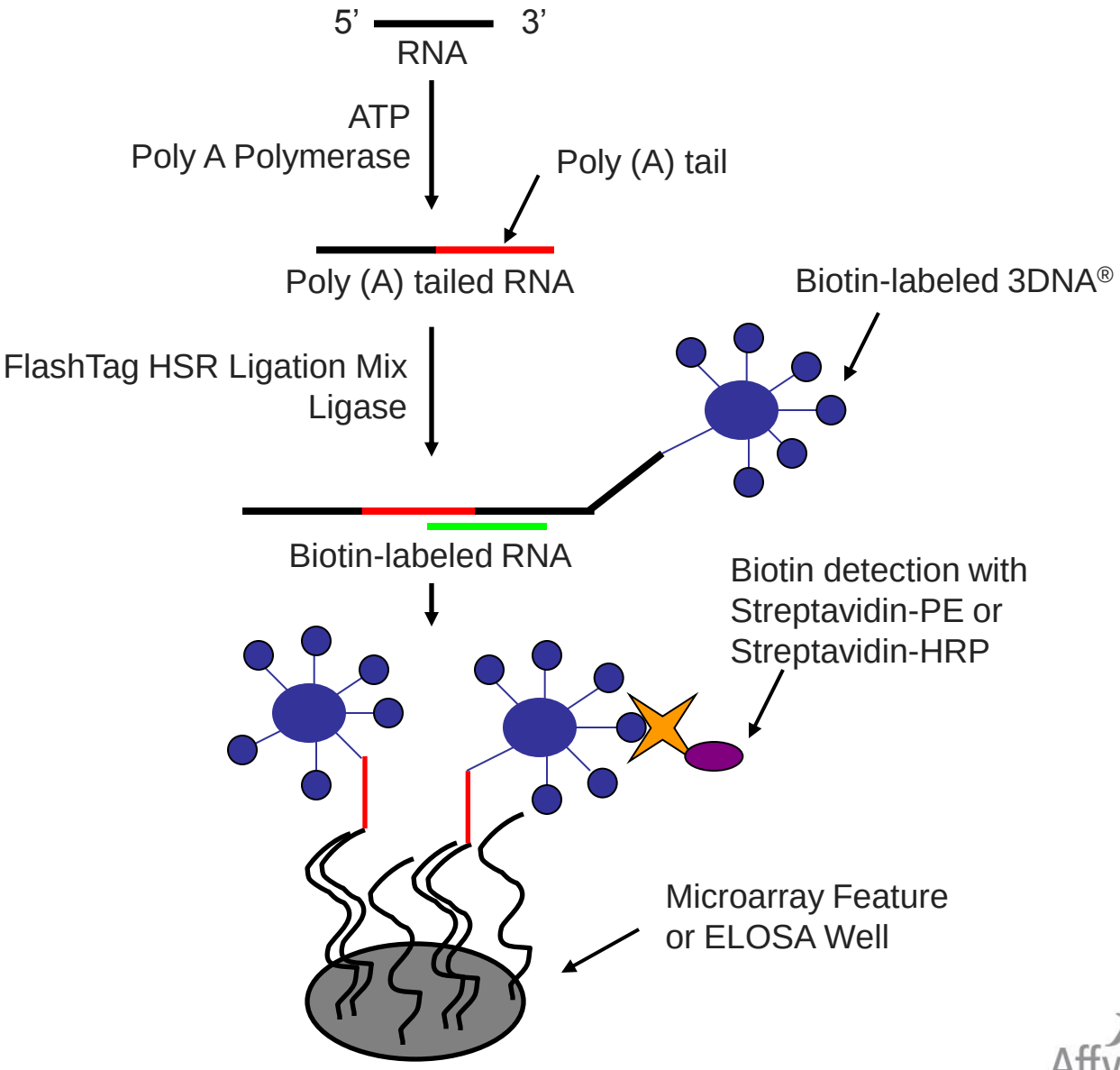
FlashTag Biotin HSR RNA Labeling Procedure

Two steps, 45-minutes, no purification

❶ **Poly (A) Tailing (15 minutes)**

❷ **Ligation (30 minutes)**

❸ **Analysis**



Assay Workflow

	FlashTag HSR RNA Labeling and Hybridization	ELOSA QC Assay
Prior to Assay	Sample Preparation RNA Purification and Quantification	Coat Wells with ELOSA Spotting Oligos (Vial 9), overnight incubation
Day 1	Poly (A) Tailing, 15 minutes FlashTag Ligation, 30 minutes →	Washing and Blocking plate, 1 hour Sample Hybridization SA-HRP Binding Signal Development } 2 hours
	miRNA GeneChip Hybridization, 16 hours ←	Successful QC
Day 2	Array Washing, Staining, and Scanning	

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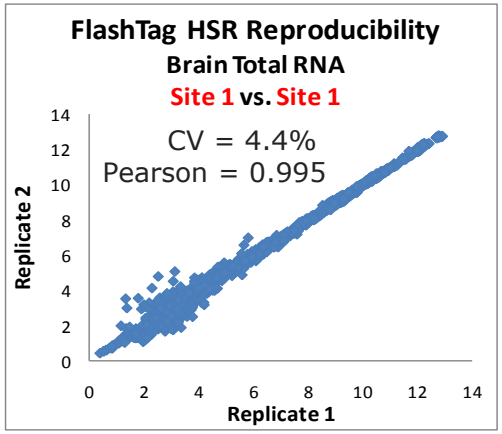
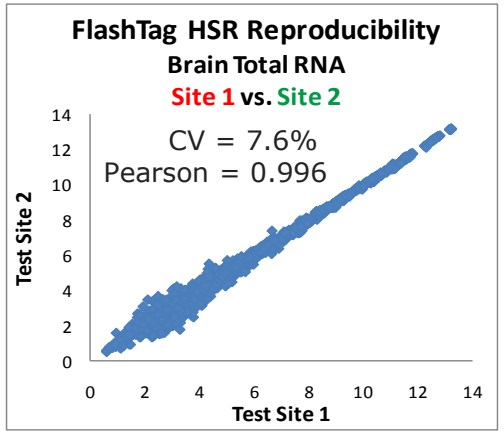
Performance Summary

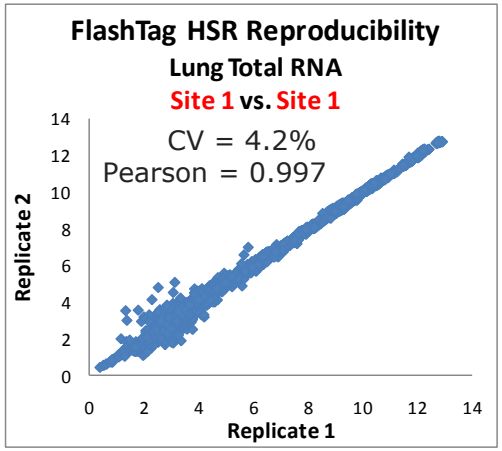
- **Reproducibility:** inter- and intra-lot signal correlation is typically R>0.95
- **Sensitivity:** detects 94% of miRNA transcripts at 1.0 attomole
- **Dynamic Range:** >3 logs
- **Specificity:** 1 nucleotide discrimination
- **Correlation to qRT-PCR:** $R^2 = 0.7957$

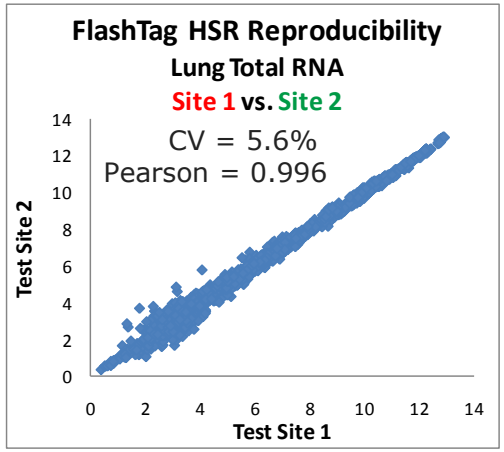
Reproducibility

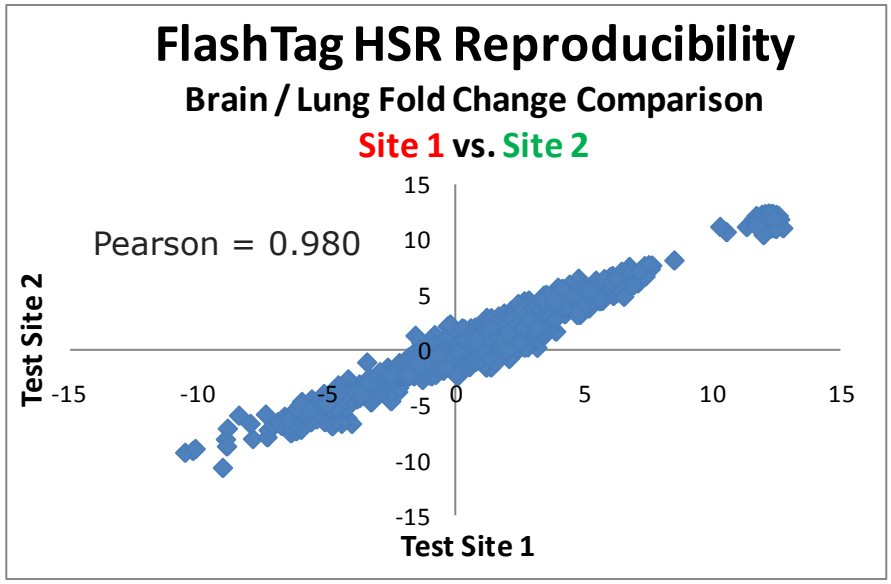
To determine reproducibility, 200ng of Ambion FirstChoice® human brain and lung total RNAs were FlashTag labeled and hybridized to GeneChip miRNA Arrays in duplicate at **Site 1** and in singlet at **Site 2**. The % CVs and Pearson correlations were calculated using present probesets.





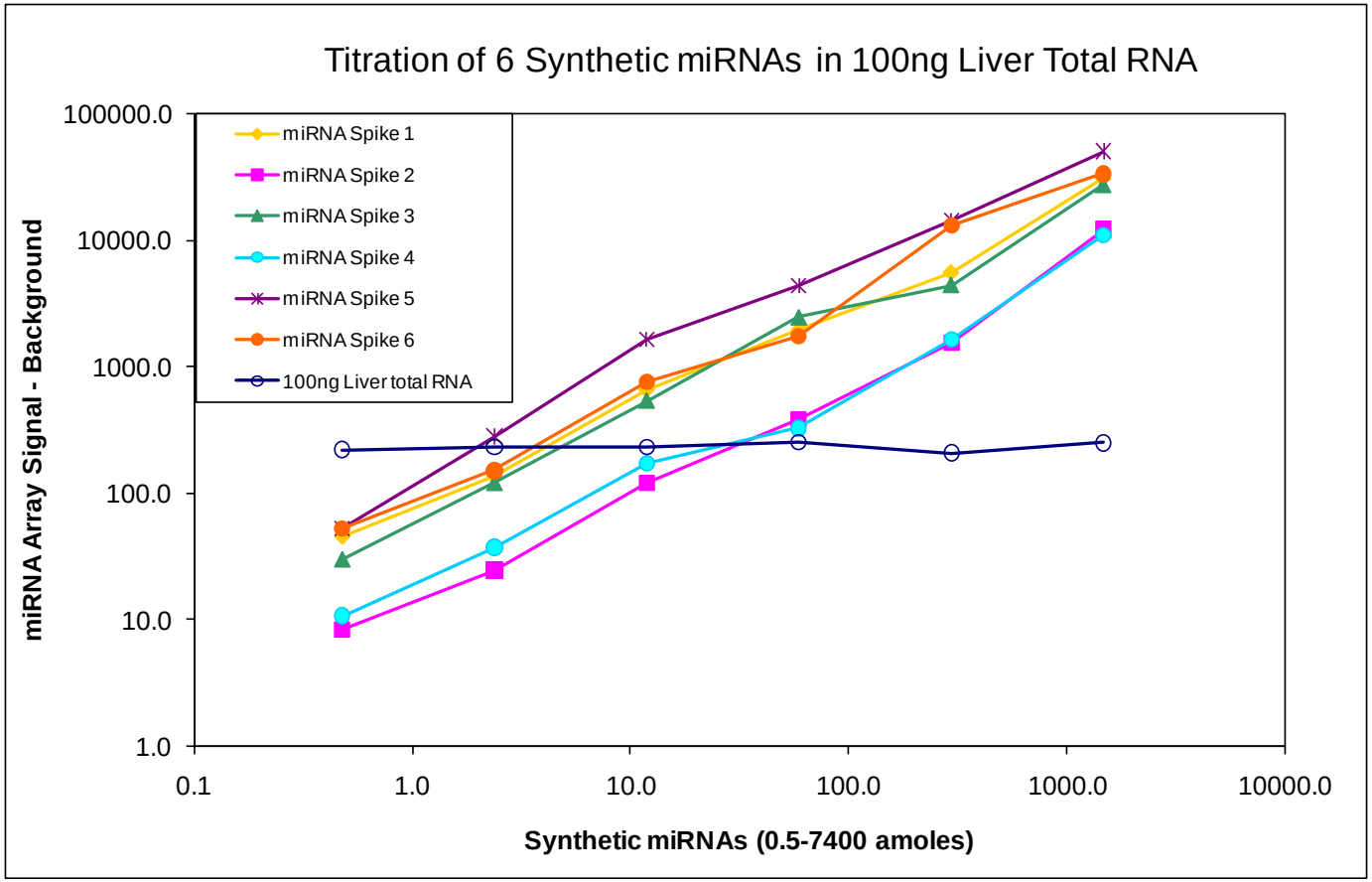






Sensitivity and Dynamic Range

To determine sensitivity, 0.5-7400 amoles of 6 synthetic RNA 22mer oligos, complementary to features present on the GeneChip miRNA Array, were titrated in a background of 100ng human liver total RNA (Ambion FirstChoice®), FlashTag HSR labeled, and hybridized in duplicate. Average signal-background intensities were plotted for each miRNA spike input.



Specificity

To determine specificity, synthetic hsa-let7a, 7b, 7c, and 7f (0.02ng each) were individually FlashTag HSR labeled in a background of 20 different synthetic miRNAs (0.4ng each) and hybridized to GeneChip miRNA Arrays. The 20 synthetic miRNAs are known to have no cross-reactivity with the let7 family arrayed probes. For analysis, array signals were normalized to the perfect match probe-target for each array.

		Labeled MicroRNA			
		Let 7a	Let 7b	Let 7c	Let 7f
Array Feature	7a	**100%**	**43%**	**37%**	**4%**
	7b	**2%**	**100%**	**9%**	**0%**
	7c	**8%**	**26%**	**100%**	**0%**
	7d	**9%**	**5%**	**6%**	**0%**
	7e	**1%**	**1%**	**1%**	**0%**
	7f	**1%**	**2%**	**2%**	**100%**
	7g	**0%**	**0%**	**0%**	**0%**
	7i	**0%**	**0%**	**0%**	**0%**

miRNA	Sequence
let-7a	UGAGGUAGUAGGUUGUAUAGUU
let-7b	UGAGGUAGUAGGUUGUGUGGUU
let-7c	UGAGGUAGUAGGUUGUAUGGUU
let-7d	AGAGGUAGUAGGUUGCAUAGUU
let-7e	UGAGGUAGGAGGUUGUAUAGUU
let-7f	UGAGGUAGUAGAUUGUAUAGUU
let-7g	UGAGGUAGUAGUUUGUACAGUU
let-7i	UGAGGUAGUAGUUUGUGCUGUU

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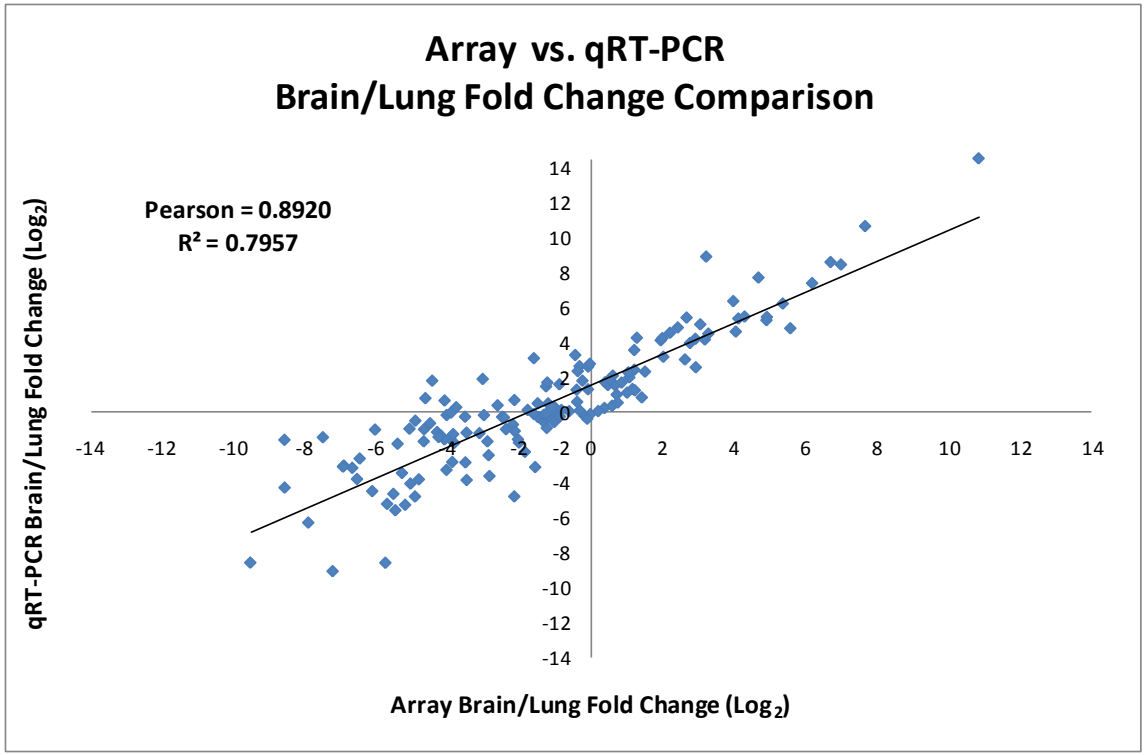
Comparison to qRT-PCR

Array: 200ng human brain and lung total RNAs (Ambion FirstChoice®) were labeled with FlashTag HSR in duplicate and hybridized to GeneChip miRNA Arrays. Replicate Log_2 signal intensities were averaged. To determine Fold Change, lung average Log_2 signal was subtracted from brain average Log_2 signal.

qRT-PCR: Ct values were downloaded from Ambion TechNotes 14(2) – May 2007: miRNA Expression in FirstChoice® Human Brain Reference RNA (http://www.ambion.com/techlib/tn/142/5.html). To determine Fold Change, brain Ct values were subtracted from lung Ct values.

Brain/Lung Fold Changes were plotted for 149 miRs in common. Since the RNAs are from very different tissues, a broad range of fold changes was observed in both Array and qRT-PCR data.



Controls Provided in Kit

FlashTag Biotin HSR

- Vial 1 - 10X Reaction Buffer
- Vial 2 - 25mM $MnCl_2$
- Vial 3 - ATP Mix
- Vial 4 - PAP Enzyme
- Vial 5 - 5X FlashTag Ligation Mix Biotin
- Vial 6 - T4 DNA Ligase
- Vial 7 - Stop Solution
- **Vial 8 - RNA Spike Control Oligos**
- Vial 9 - ELOSA Spotting Oligos
- Vial 10 - ELOSA Positive Control
- Vial 11 - Nuclease-Free Water
- Vial 12 - 27.5% Formamide

- Vial 8 consists of five oligos which are spiked into the RNA sample prior to FlashTag labeling. These oligos contain controls for the GeneChip miRNA Array and the ELOSA QC Assay.
  - Oligos 2, 23, and 29 are RNA and confirm poly(A) tailing and ligation.
  - Oligo 31 is poly(A) RNA and confirms ligation.
  - Oligo 36 is poly(dA) DNA and confirms ligation and lack of RNAses in the RNA sample.
- The Affymetrix library file lists the following names for these probe sets:
  - spike in-control-2 st
  - spike in-control-23 st
  - spike in-control-29 st
  - spike in-control-31 st
  - spike in-control-36 st
- Each probe set should show >1000 units (signal-background).

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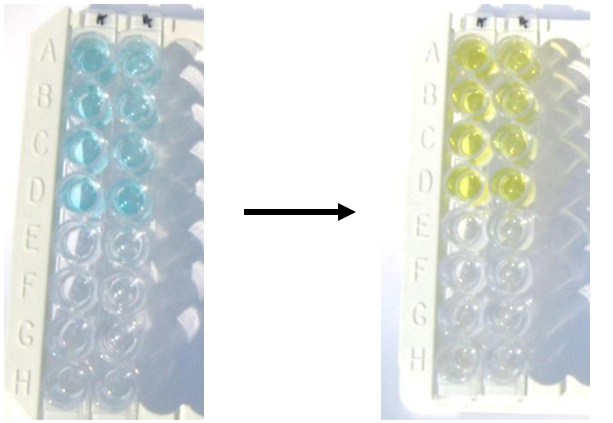
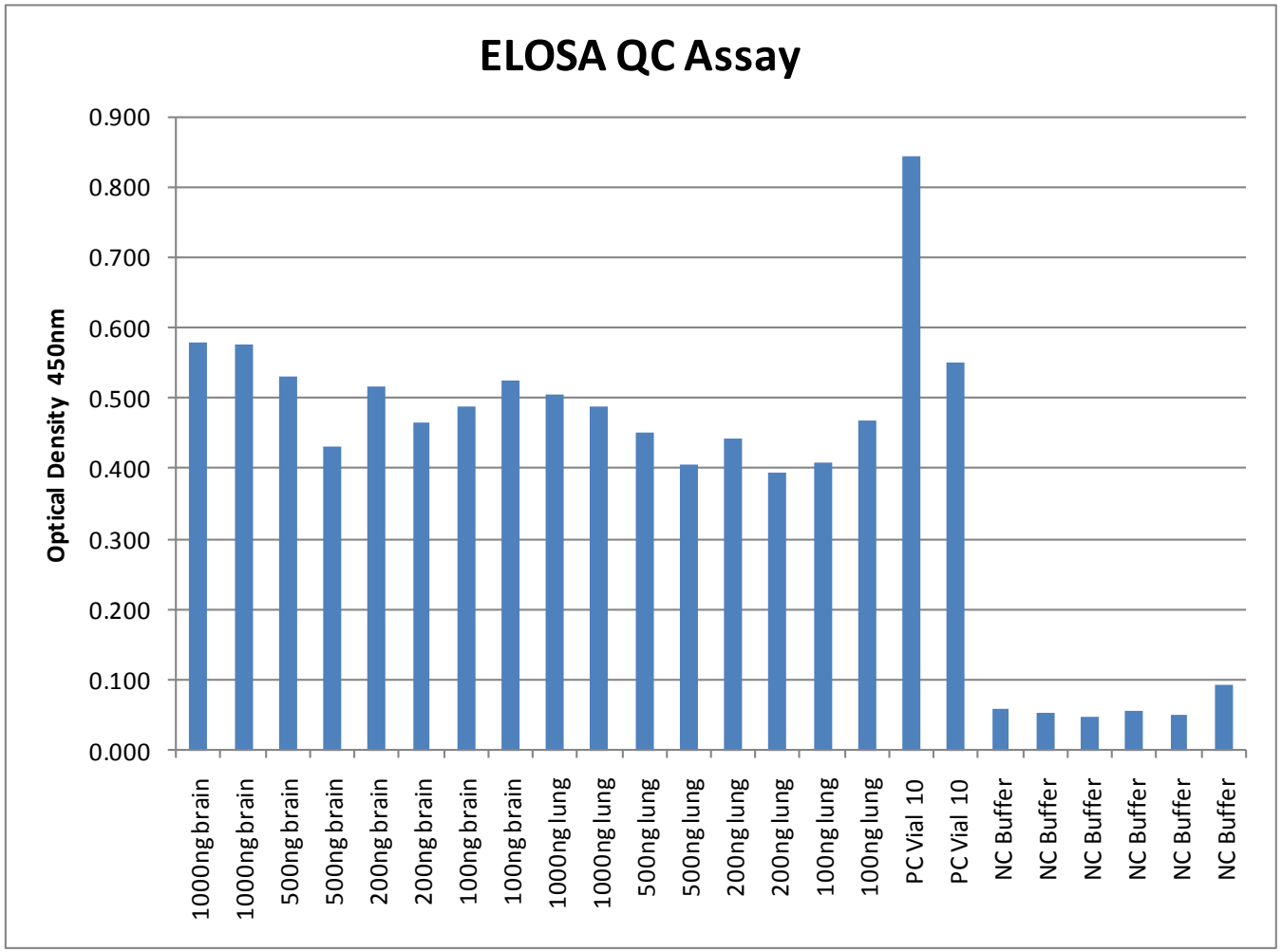
ELOSA QC Assay

- Suggested but optional
- ELOSA QC Assay confirms FlashTag Biotin HSR labeling
  - RNA Spike Control Oligos (Vial 8) are added to RNA sample and biotinylated during the FlashTag Biotin HSR labeling process
  - Biotinylated RNA Spike Control Oligos (Vial 8) hybridize to ELOSA Spotting Oligos (Vial 9) immobilized in a microwell format
  - ELOSA Positive Control (Vial 10) is already biotinylated and provides a control for the ELOSA assay
  - Results can be read visually (enzymatic color reactions indicate a positive result), or quantitated with a plate reader reading absorbance at 450nm



Before Stop Solution **After Stop Solution**

Example of an ELOSA QC Assay



- 16 Sample Reactions, 2 Positive Control (Vial 10), 6 Negative Control (Buffer)
- Criteria: Readings of greater than 0.10 OD (450nm) over a negative control should be considered positive. Proceed with Array Hybridization.

FlashTag™ Biotin HSR RNA Labeling Kit

for GeneChip® miRNA Arrays

FFPE Sample Labeling

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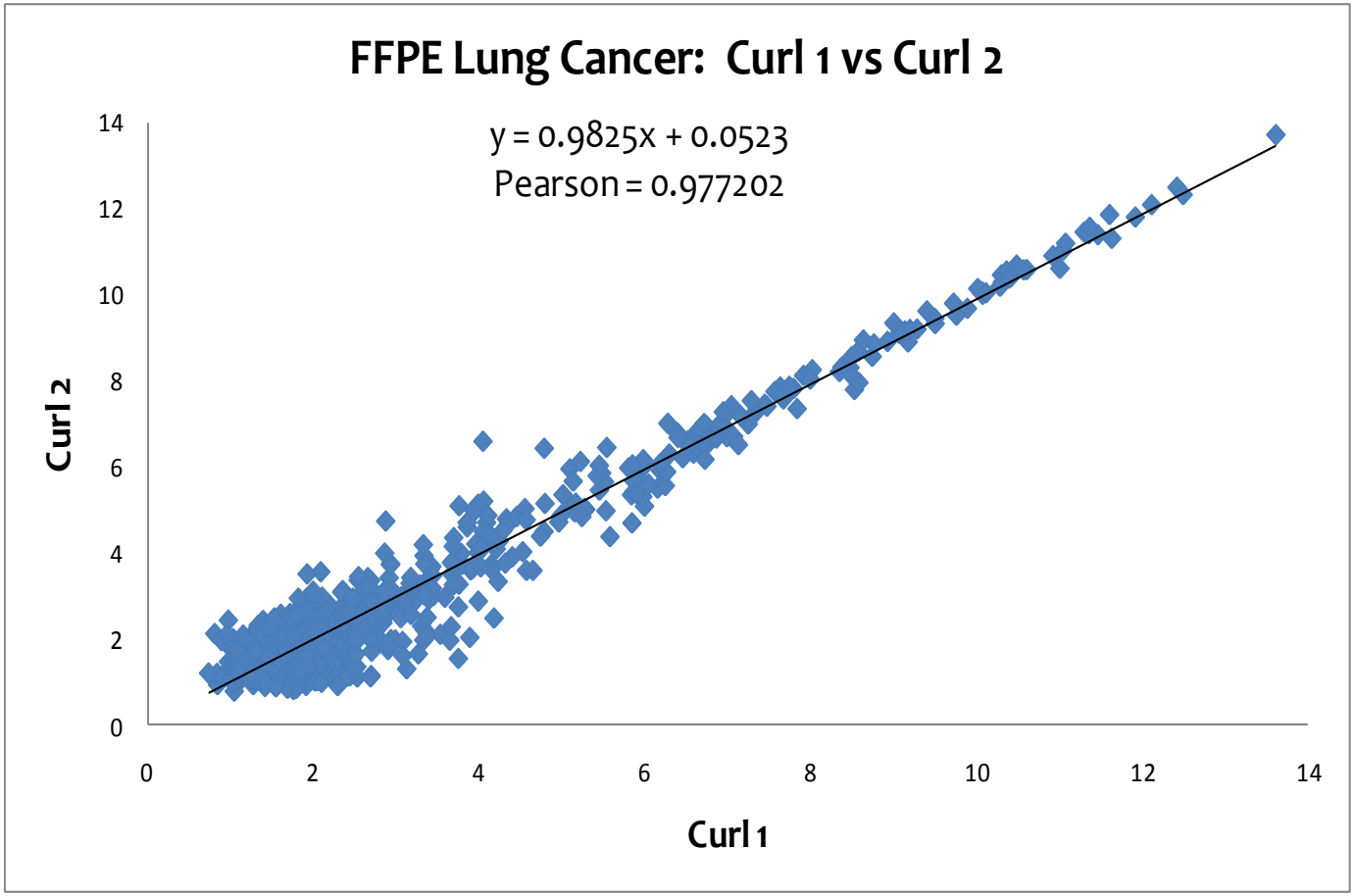
RNA Isolation and Labeling

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

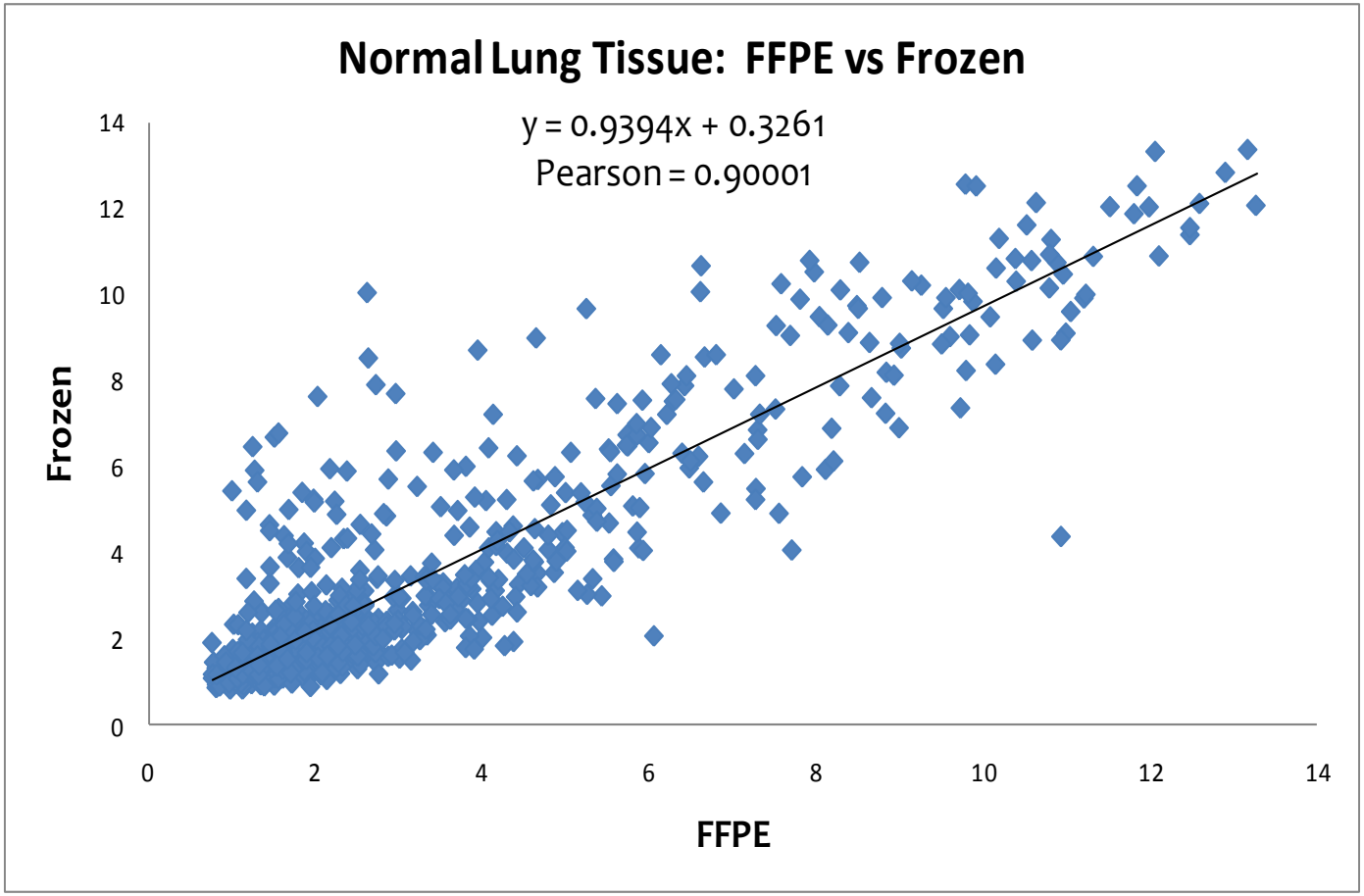
FlashTag HSR FFPE

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

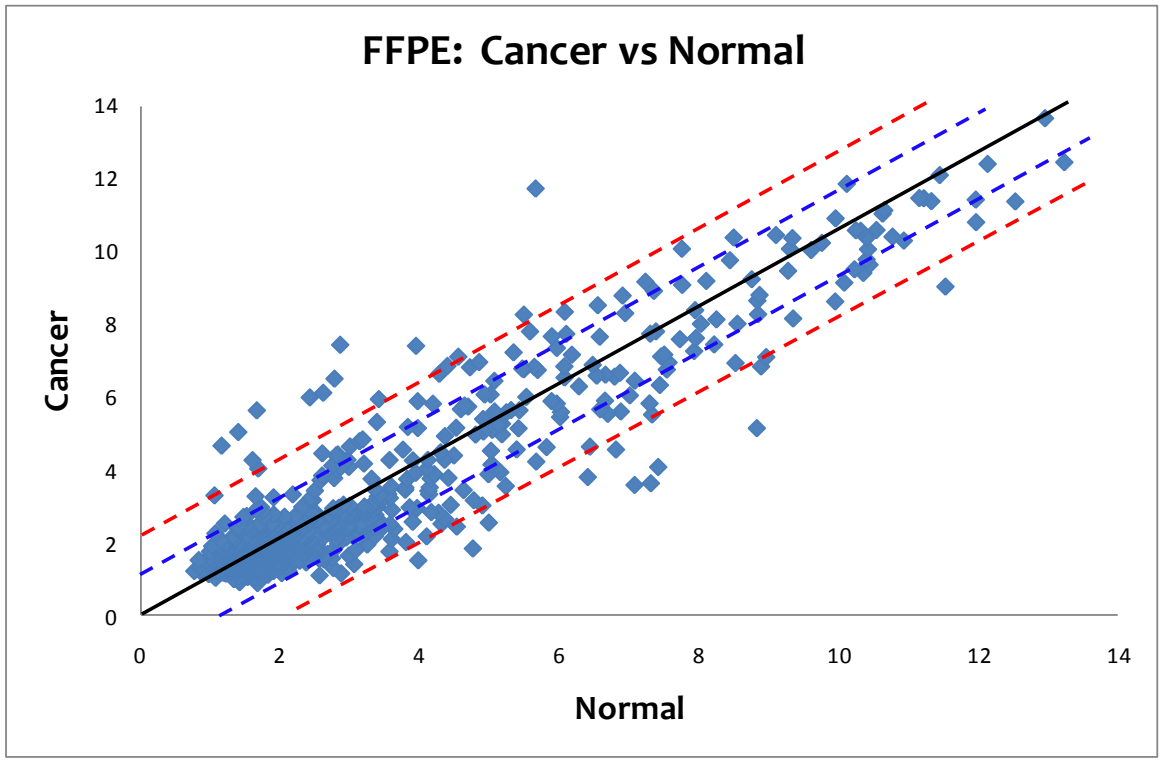


FlashTag HSR FFPE *vs.* Fresh Frozen

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



FlashTag HSR in FFPE Samples



Fold-Change	Number of microRNAs
≥ 4-fold (+)	22
≥ 2-fold (+)	81
≥ 4-fold (-)	12
≥ 2-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

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Summary and Reference

- miRNA expression profiling can be accurately achieved from FFPE archival specimens

- Micro RNA Expression Profiles as Adjunctive Data to Assess the Risk of Hepatocellular Carcinoma Recurrence After Liver Transplantation

  doi: 10.1111/j.1600-6143.2011.03788.x

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FlashTag™ Biotin HSR RNA Labeling Kit

for GeneChip® miRNA Arrays

Serum and Plasma Sample Labeling

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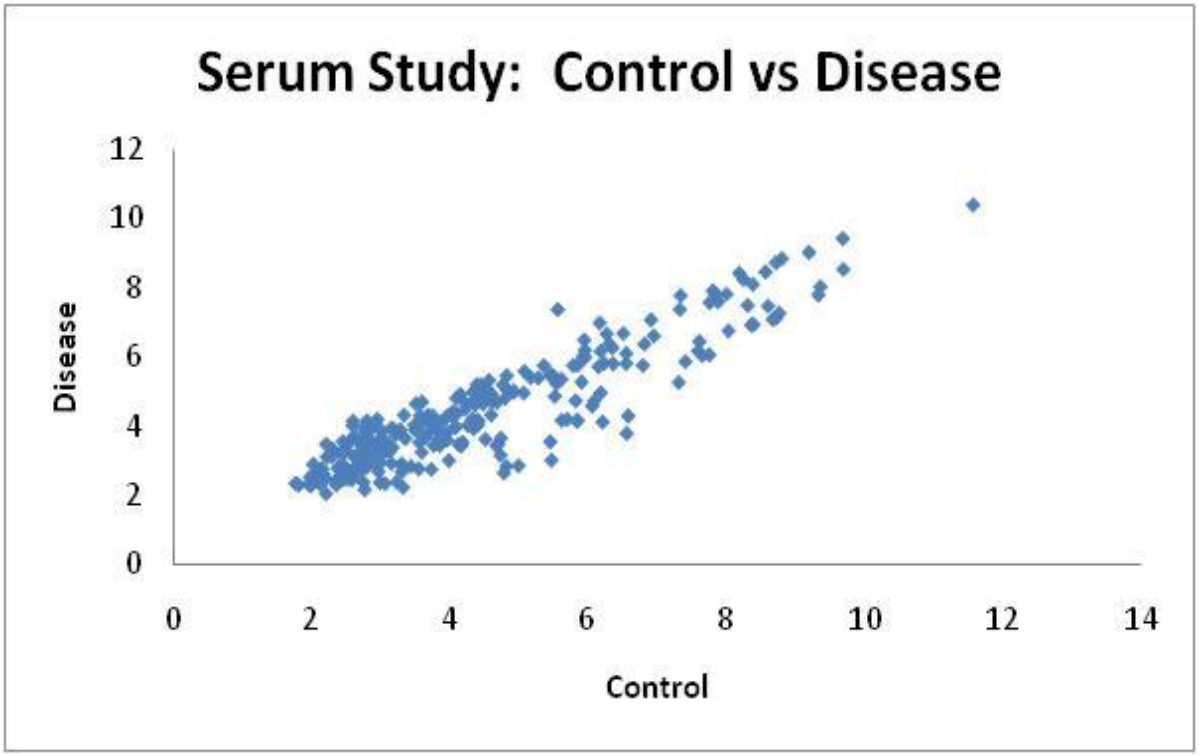
RNA Isolation and Labeling

- Total RNA (including microRNA) may be extracted from serum or plasma with a variety of RNA isolation kits – usually with a modified procedure (check literature)
- Label as much total RNA as possible with FlashTag HSR
- Same procedure whether RNA is intact or degraded

FlashTag HSR in Serum Samples

- Differential miRNA profiles observed in control (n=3) vs disease (n=3) serum samples



Fold-Change	Number of microRNAs
≥ 4-fold (+)	7
≥ 2-fold (+)	39
≥ 4-fold (-)	1
≥ 2-fold (-)	18

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Summary and References

- miRNA expression profiling can be accurately achieved from plasma or serum specimens – serving potentially as biomarkers for diseases

- Impact of Cellular miRNAs on Circulating miRNA Biomarker Signatures
  doi:10.1371/journal.pone.0020769

- Genome-Wide Maps of Circulating miRNA Biomarkers for Ulcerative Colitis
  doi:10.1371/journal.pone.0031241

FlashTag™ Biotin HSR RNA Labeling Kit

for GeneChip® miRNA Arrays

miRNA QC Tool Software (for miRNA 1.0 and 2.0 Arrays only)

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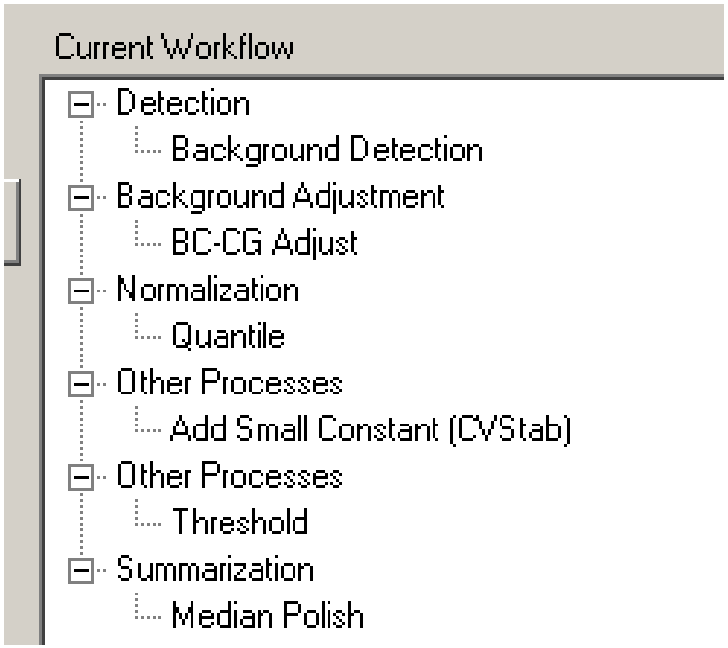
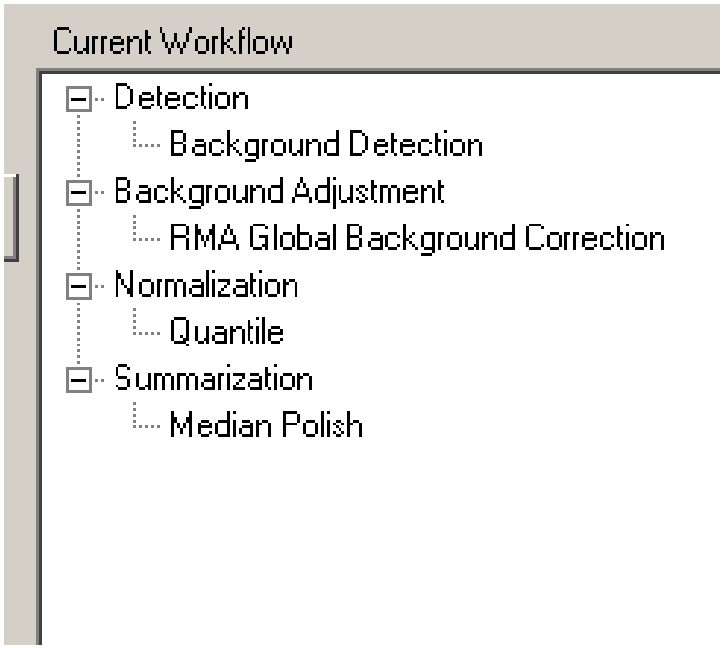
Experimental Design

200ng human brain and lung total RNAs (Ambion FirstChoice®) were labeled with FlashTag HSR in duplicate and hybridized to GeneChip miRNA Arrays. Replicate CEL files were loaded into miRNA QC Tool and run with both the Default workflow and the miRNA Analysis workflow. Replicate Log_2 signal intensities were averaged. To determine Fold Change, lung average Log_2 signal was subtracted from brain average Log_2 signal.

Default



miRNA Analysis



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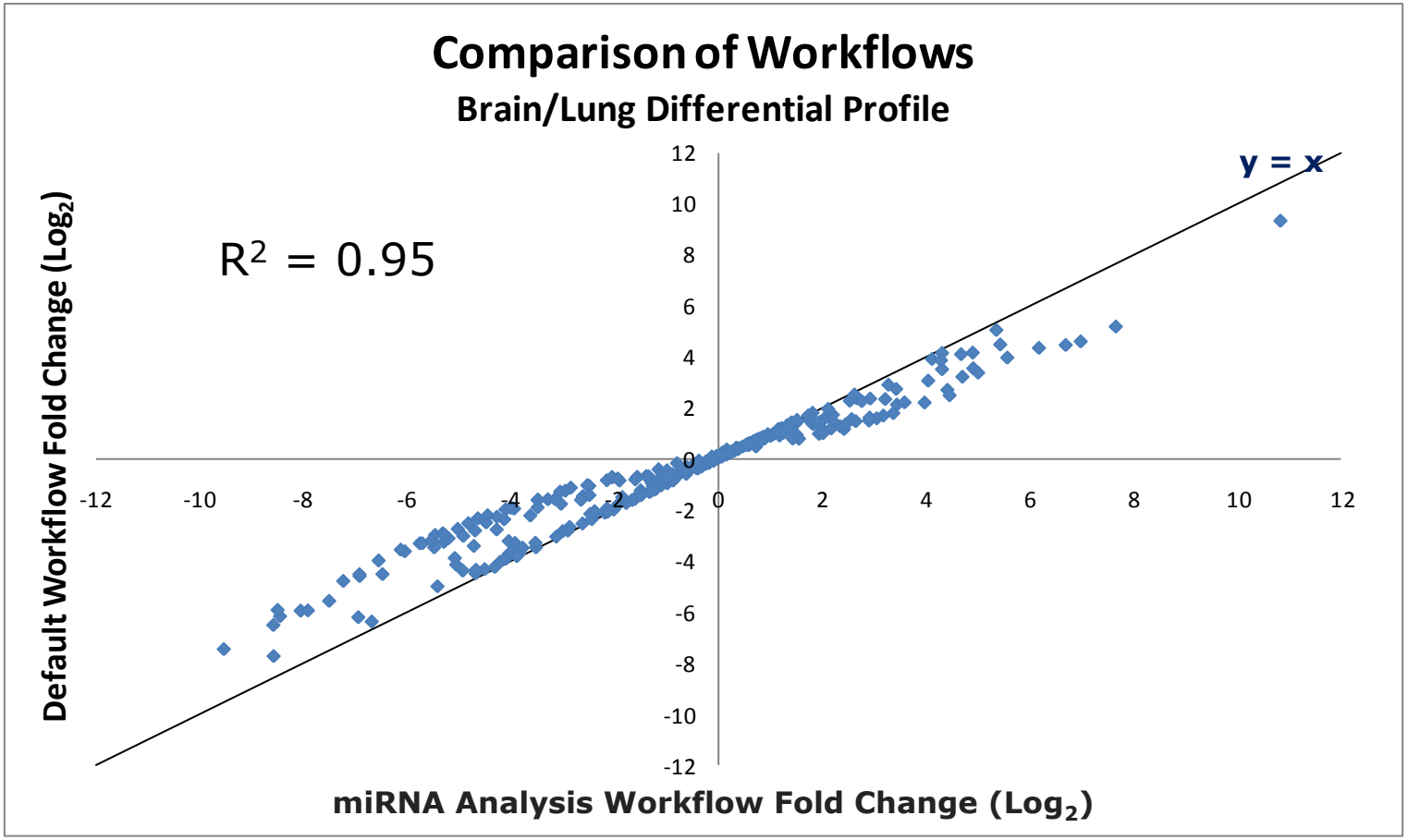
Comparison of MicroRNA QC Tool Workflows



If a microRNA is TRUE in both Lung and Brain, there isn't significant difference between the workflows (data tends to be along the y=x line). If a microRNA is TRUE in one tissue and FALSE in the other tissue, the Fold Change is higher in the miRNA Analysis workflow (data is further from the y=x line).

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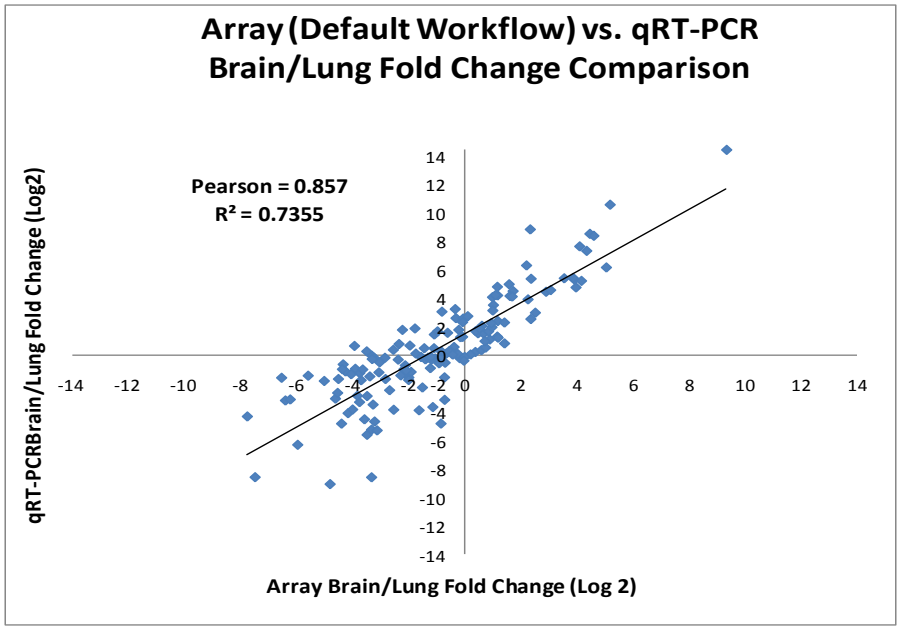
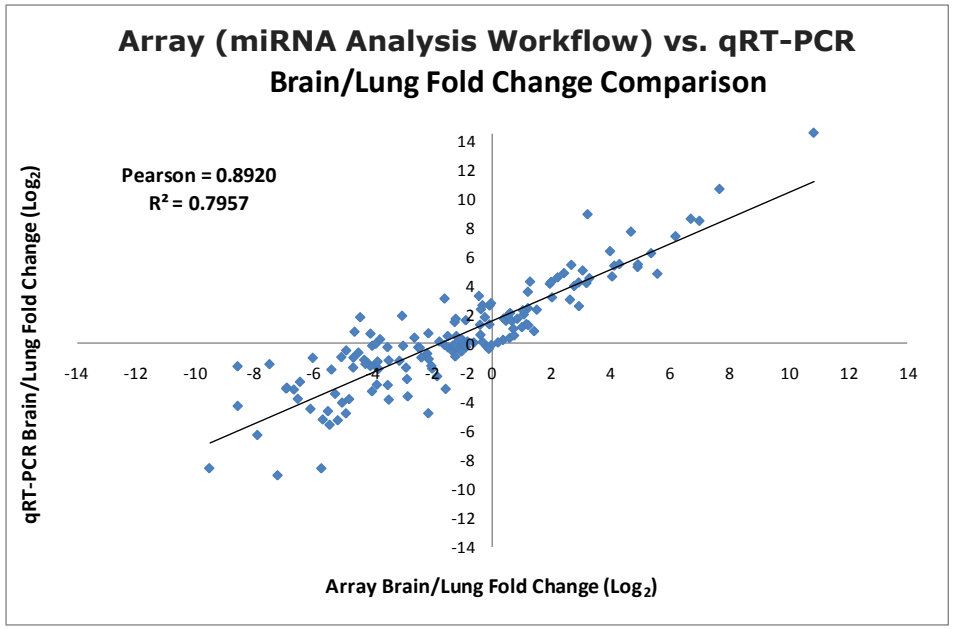
Comparison to qRT-PCR

qRT-PCR: Ct values were downloaded from Ambion TechNotes 14(2) – May 2007: miRNA Expression in FirstChoice® Human Brain Reference RNA (http://www.ambion.com/techlib/tn/142/5.html). To determine Fold Change, brain Ct values were subtracted from lung Ct values. To compare to array data, Brain/Lung Fold Changes were plotted for 149 miRs in common.





Array data generated with miRNA Analysis workflow better correlates to qRT-PCR data, than array data generated with Default workflow.

Summary and Reference

- miRNA QC Tool is easy-to-use
- A variety of workflows can be compared after CEL files are generated
- Depending on the experimental design, different workflows can be used
- For more information:

Irizarry RA, Hobbs B, Collin F, Beazer-Barclay YD, Antonellis KJ, Scherf U, Speed TP. Exploration, normalization, and summaries of high density oligonucleotide array probe level data. *Biostatistics* 2003 Apr.4 (2):249-64.

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Ordering Information

- **To order**

Part Number	Description	Quantity
901910	FlashTag™ Biotin HSR RNA Labeling Kit	10 reactions
901911	FlashTag™ Biotin HSR RNA Labeling Kit	30 reactions

- **Visit www.affymetrix.com for the latest information**

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