

Data Set

FlashTag™ Biotin

for

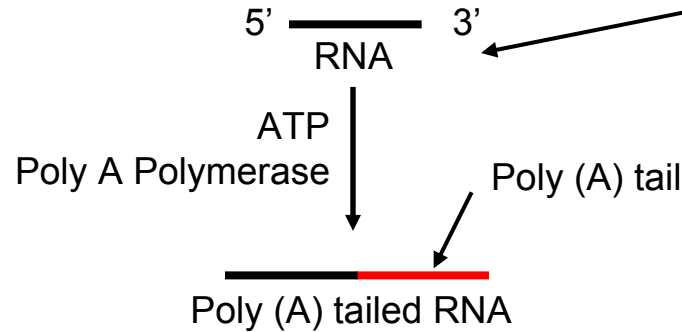
Affymetrix® GeneChip® miRNA Arrays

Genisphere®

SIGNAL + SAMPLE AMPLIFICATION PRODUCTS

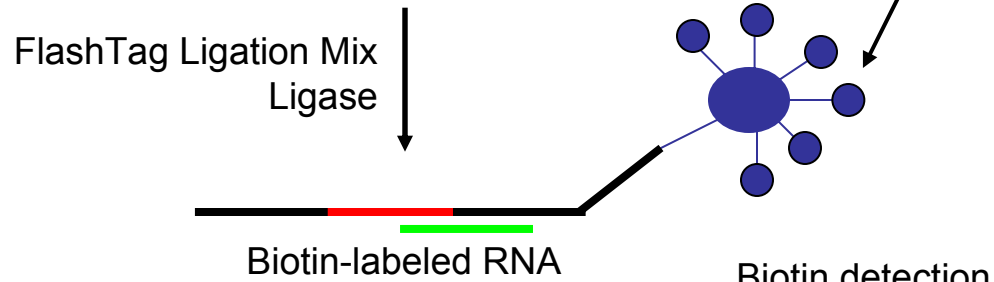
- FlashTag Biotin Procedure
 - 2 Steps; 45 minutes
 - No purification
- ELOSA QC Assay
 - Confirms FlashTag Biotin labeling of RNA samples
 - Proceed to Array Hybridization
- Controls
- Workflow

**1 Poly (A) Tailing
(15 minutes)**

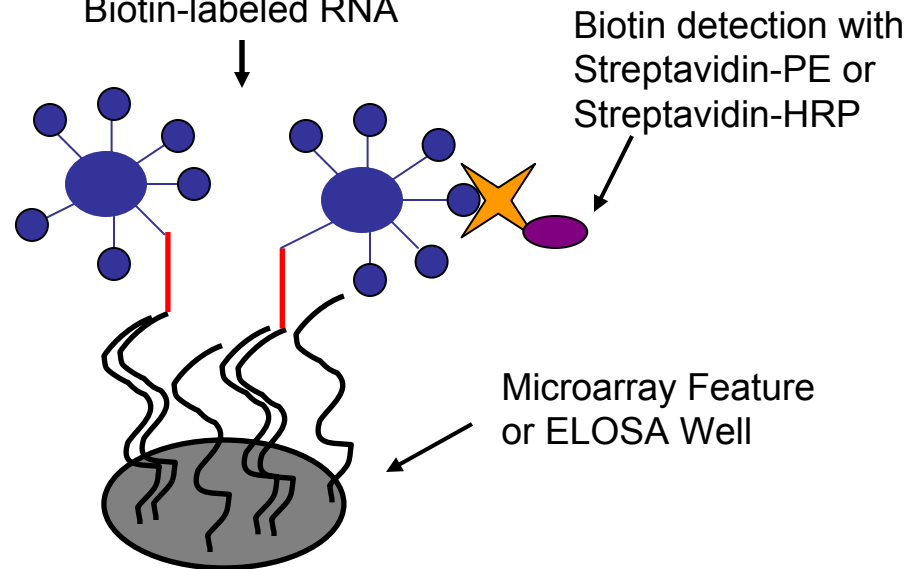


**Vial 8: RNA Spike
Control Oligos**

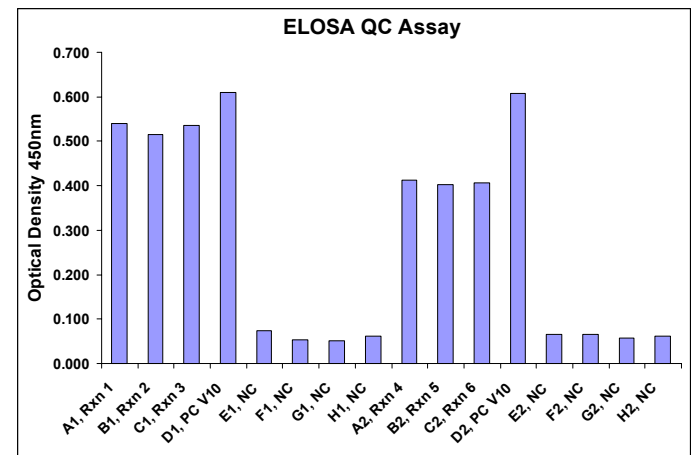
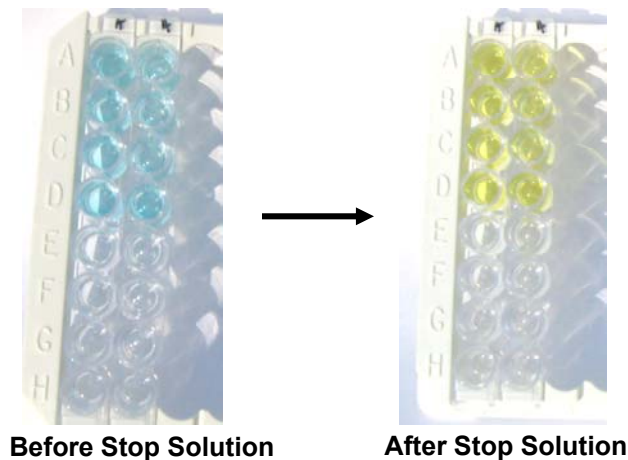
**2 Ligation
(30 minutes)**

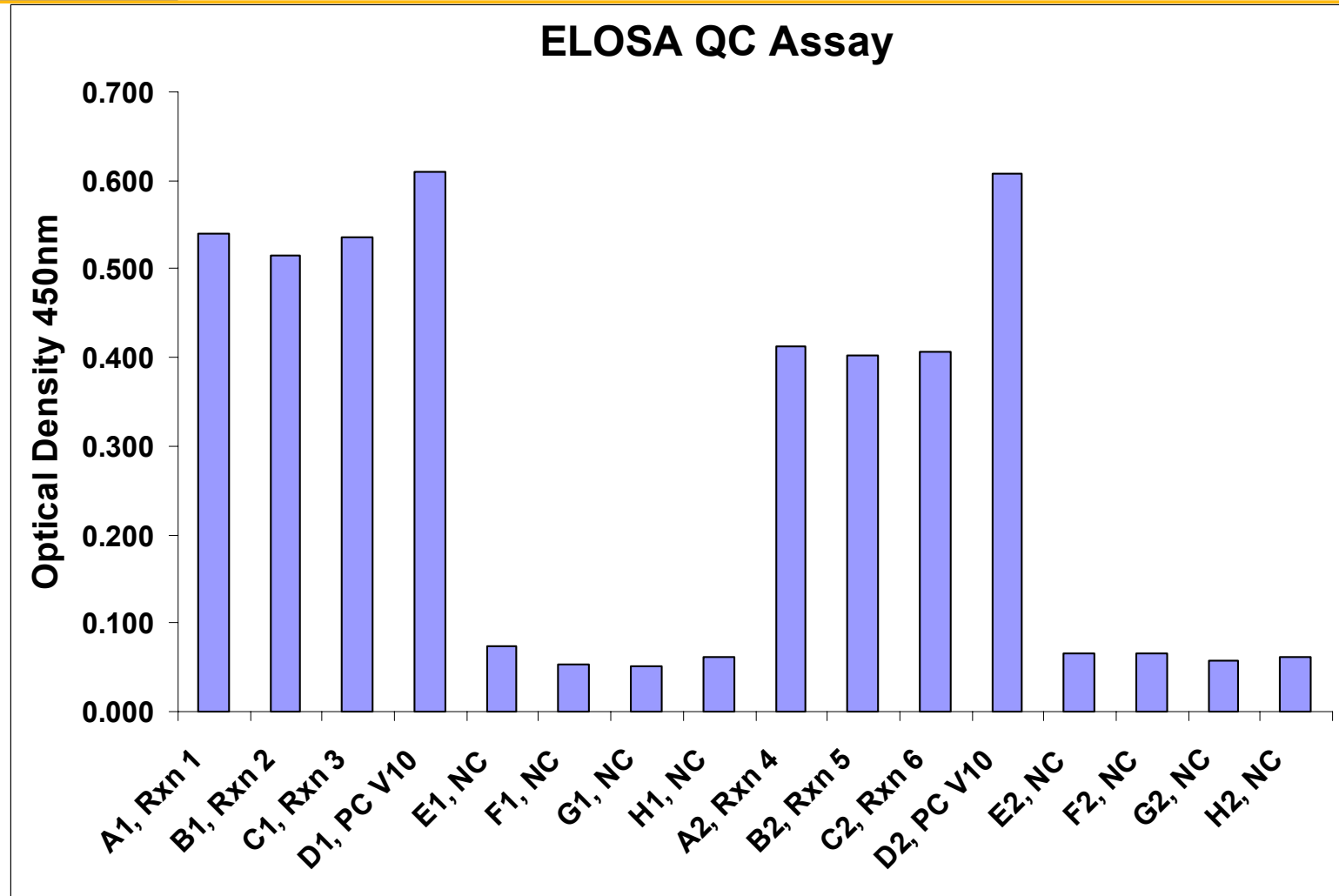


3 Analysis



- The ELOSA QC Assay confirms FlashTag Biotin labeling of RNA samples
 - RNA Spike Control Oligos (Vial 8) are added to RNA sample and biotinylated during the FlashTag process
 - Biotinylated RNA samples are hybridized to 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 itself
 - Results can be read visually: enzymatic color reactions indicate a positive result
 - Results can be quantitated and documented with a plate reader or instrument capable of reading absorbance at 450nm





- Samples: 6 reactions, NC: Negative Control, PC V10: Positive Control (Vial 10)
- Criteria: Readings of greater than 0.10 OD (450nm) over a negative control should be considered positive. Proceed with Array Hybridization.

FlashTag Biotin RNA Labeling Kit

- Vial 1 10X Reaction Buffer
 - Vial 2 25mM MnCl₂
 - 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 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)

***flash*tag RNA Labeling
and Hybridization****ELOSA QC Assay****Prior to
Assay**Sample Preparation
RNA Purification and
QuantificationCoat Wells with ELOSA
Spotting Oligos (Vial 9),
overnight incubation**Day 1**Poly (A) Tailing, 15 minutes
FlashTag Ligation, 30 minutes

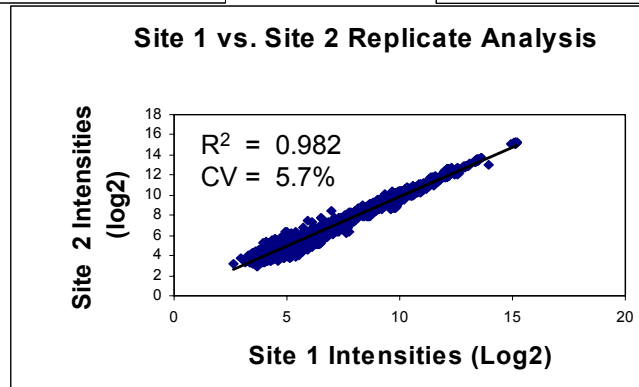
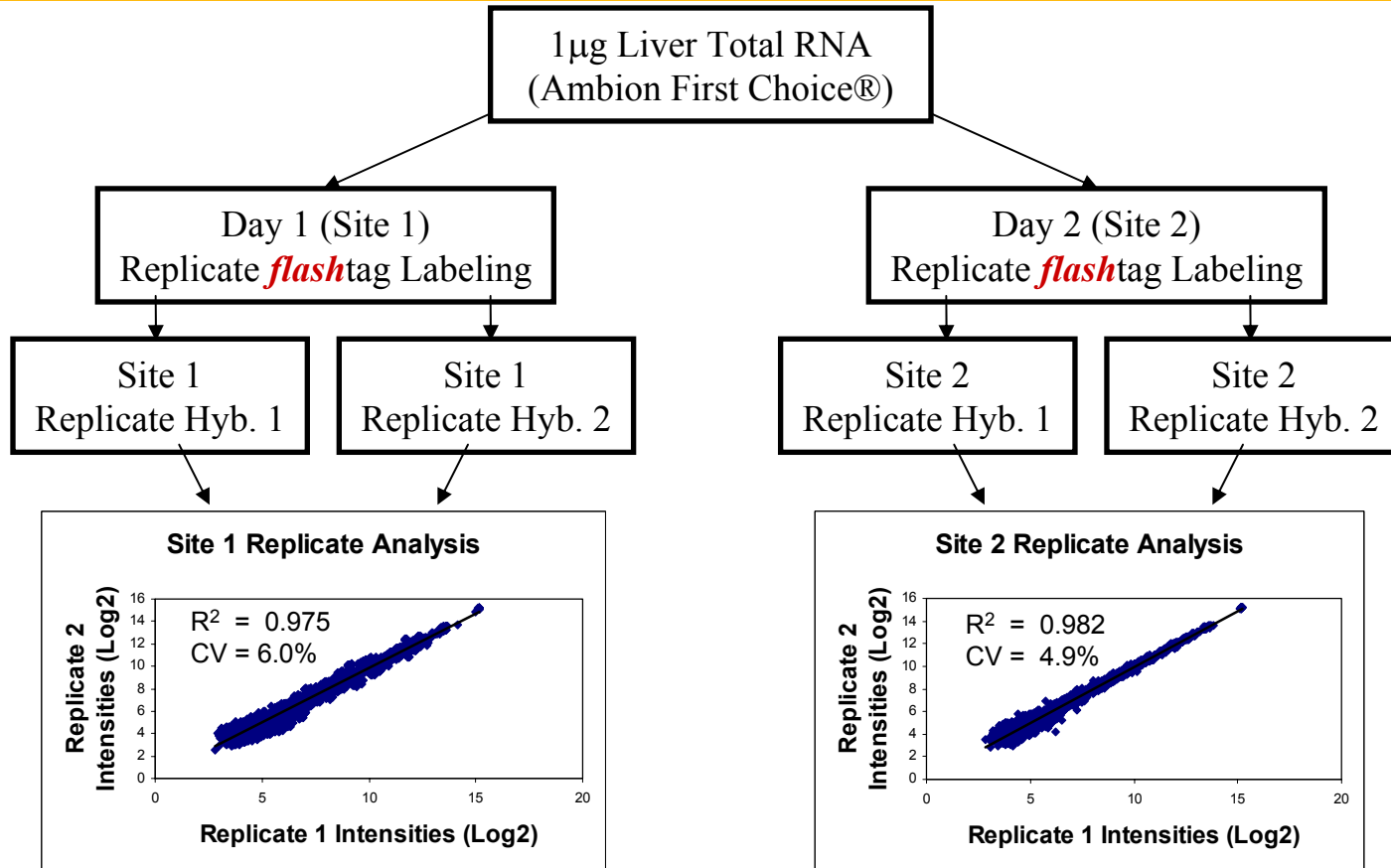
Washing and Blocking plate, 1 hour

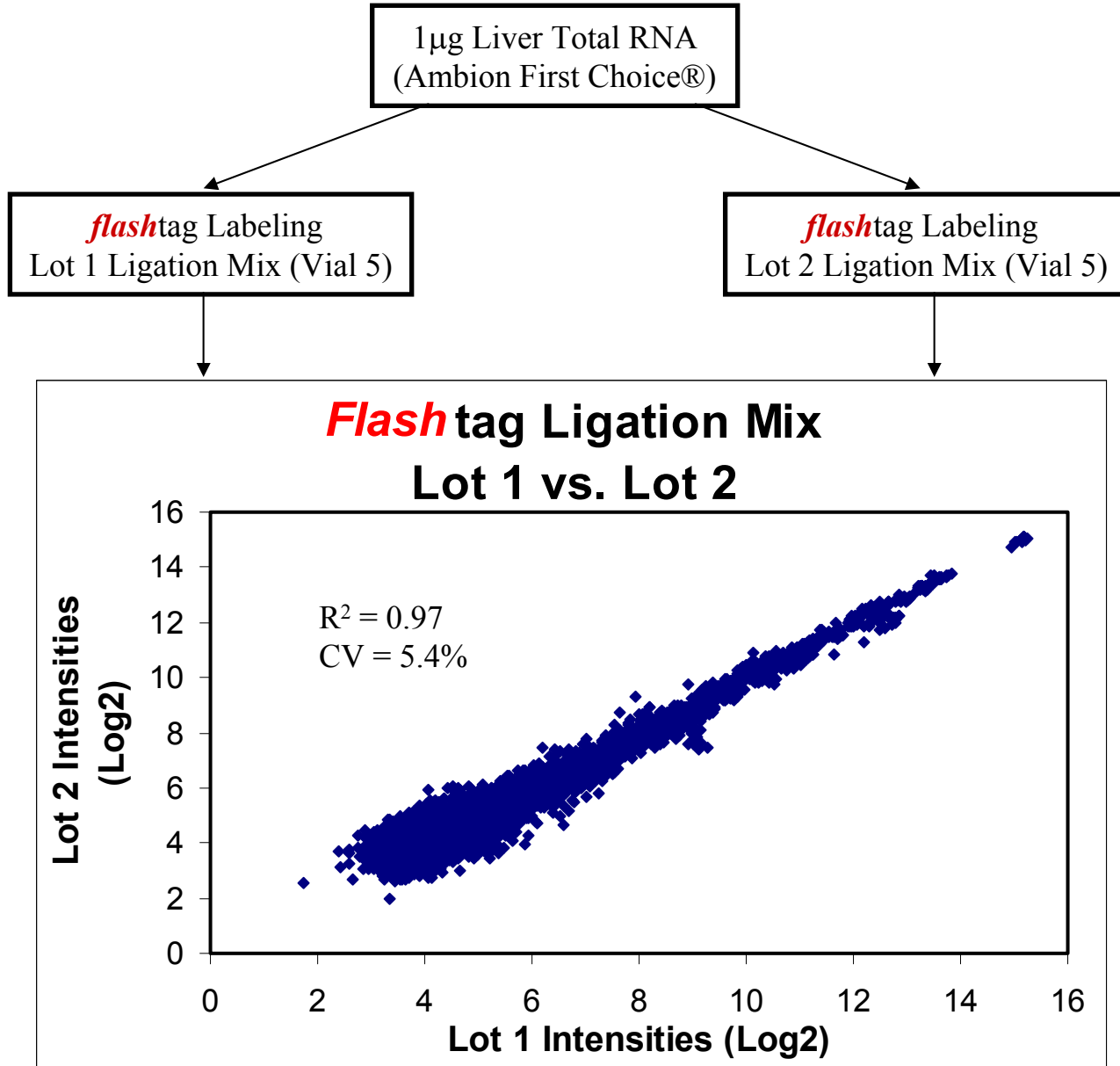
Sample Hybridization
SA-HRP Binding
Signal Development } 2 hoursmiRNA GeneChip
Hybridization, 16 hours

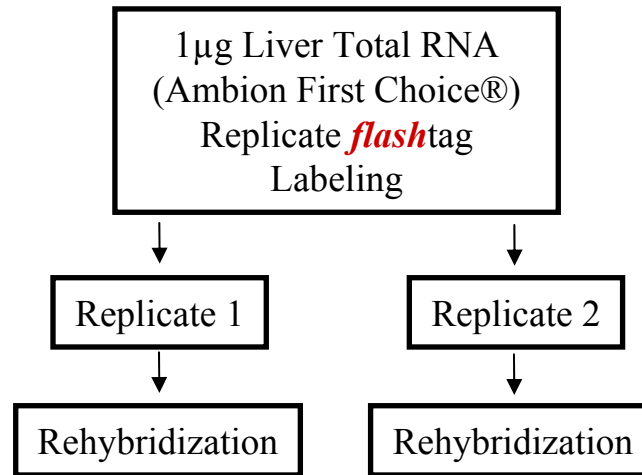
Successful QC

Day 2Array Washing, Staining,
and Scanning

- Reproducibility: $CVs \leq 10\%$
- Sensitivity: ≤ 10 attomoles
- Dynamic Range: $4 \log_{10}$
- Specificity: 1 nucleotide discrimination
- Differential Profiling
- Total RNA and Low Molecular Weight (LMW) RNA Labeling
 - 0.1-3 μ g Total RNA or the LMW RNA enriched from 0.1-3 μ g Total RNA







Summary of Rehyridization Experiment				
	Rep 1	Rep 1 Rehyb	Rep 2	Rep 2 Rehyb
Mean Intensity	357.1	275.3	377.1	278.8
Mean Background Intensity	69.0	45.2	70.9	45.8
Number of Probes	45930	45930	45930	45930
Number of Detected Probes	7627	7560	7572	7746
Number of Probe Sets	7788	7788	7788	7788
Number of Detected Probe Sets	1963	1840	1957	1896

Pearson Correlations				
	Rep 1	Rep 1 Rehyb	Rep 2	Rep 2 Rehyb
Rep 1	1.000	0.984	0.998	0.981
Rep 1 Rehyb	0.984	1.000	0.978	0.999
Rep 2	0.998	0.978	1.000	0.976
Rep 2 Rehyb	0.981	0.999	0.976	1.000

To determine sensitivity, 0.5-7400 amoles of 6 synthetic RNA spikes (22 bases), complementary to features present on the GeneChip miRNA Array, were titrated in a background of 1 μ g of human liver total RNA (Ambion First Choice®), **flash**tag labeled, and hybridized to Affymetrix GeneChip miRNA Arrays. For analysis, signal-background (s-b) intensities were determined for each spike miRNA input concentration and plotted (Figure 1). S-b intensities were also determined for the liver-specific miRNAs and the distribution plotted (Figure 2).

Figure 1

Titration of Synthetic miRNA

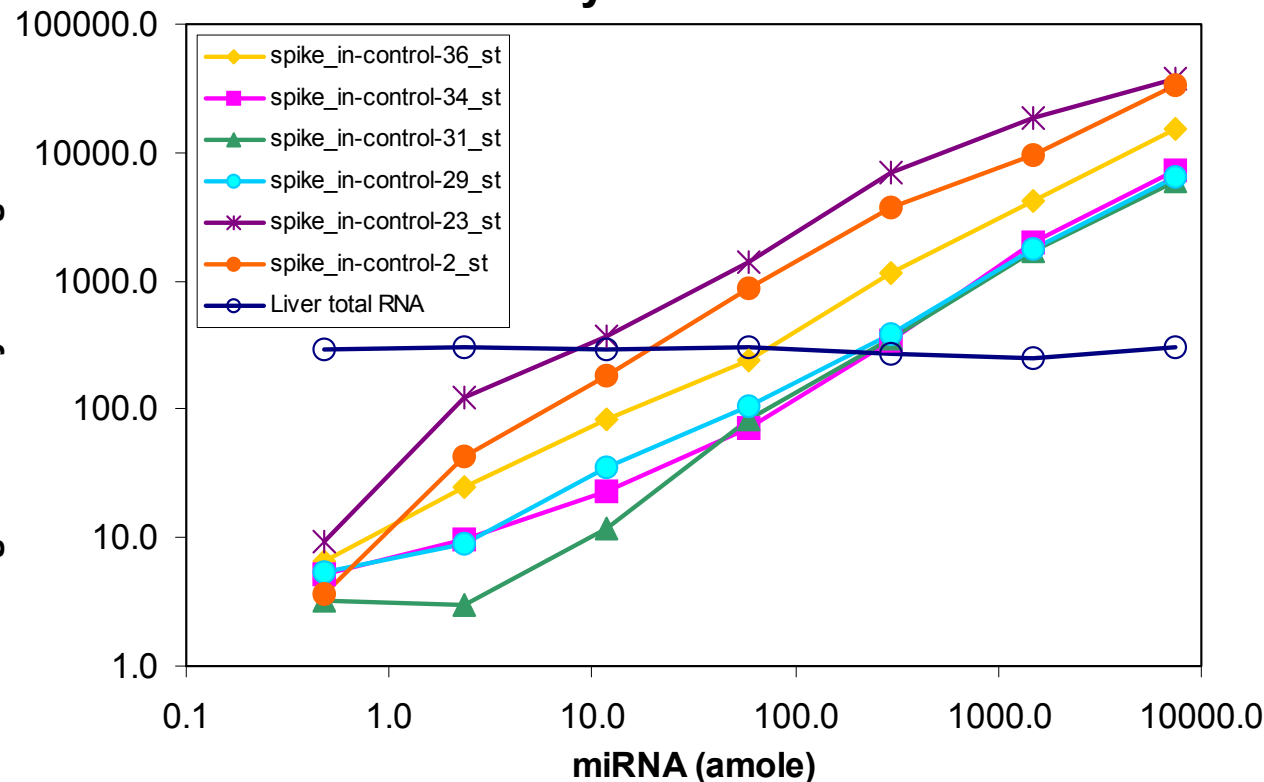
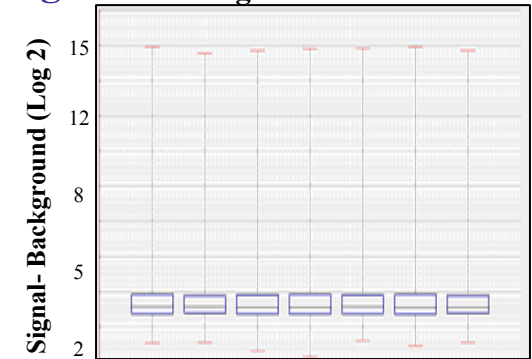


Figure 2

Signal Distribution



Mean intensity for all arrays: 390 +/- 40

Mean Background: 55 +/- 9

Probe sets detected: 2005 +/- 25

Assay Dynamic Range (signal - background)

Spike Titration: 4 Log(10)

1 μ g Tissue: 4 Log(10)

(s-b = ~5 - 32,000 units)

To determine specificity, synthetic hsa-let7a, 7b, 7c, and 7f were individually **flash**tag labeled in a background of 26 different synthetic miRNAs and hybridized to Affymetrix GeneChip miRNA Arrays. As a control, the same 26 synthetic miRNAs were hybridized to confirm no cross-reactivity with the let7 family arrayed probes (data not shown). For analysis, signals were normalized to the perfect match probe-target for each array.

		FlashTag + Affymetrix				miRNA	Sequence
		Labeled MicroRNA					
		Let 7a	Let 7b	Let 7c	Let 7f		
Array Feature	7a	100%	31%	17%	4%	let-7a	UGAGGUAGUAGGUUGUAUAGUU
	7b	4%	100%	4%	0%	let-7b	UGAGGUAGUAGGUUGUGUGGUU
	7c	8%	22%	100%	1%	let-7c	UGAGGUAGUAGGUUGUAUGGUU
	7d	8%	4%	2%	2%	let-7d	AGAGGUAGUAGGUUGCAUAGUU
	7e	1%	0%	0%	0%	let-7e	UGAGGUAGGAGGUUGUAUAGUU
	7f	4%	1%	1%	100%	let-7f	UGAGGUAGUAGAUUGUAUAGUU
	7g	0%	0%	0%	0%	let-7g	UGAGGUAGUAGUUUGUACAGUU
	7i	0%	0%	0%	0%	let-7i	UGAGGUAGUAGUUUGUGCUGUU

To further study specificity, two pools of perfect match (PM) and mis-matched (MM) hsa-let7-like miRNA oligos were designed and synthesized using Control Spike Sequences present on the Affymetrix GeneChip miRNA Array

Pool A: Control Spike 2, 23, 34 (PM) and 29, 31, 36 (MM) – 50pg each

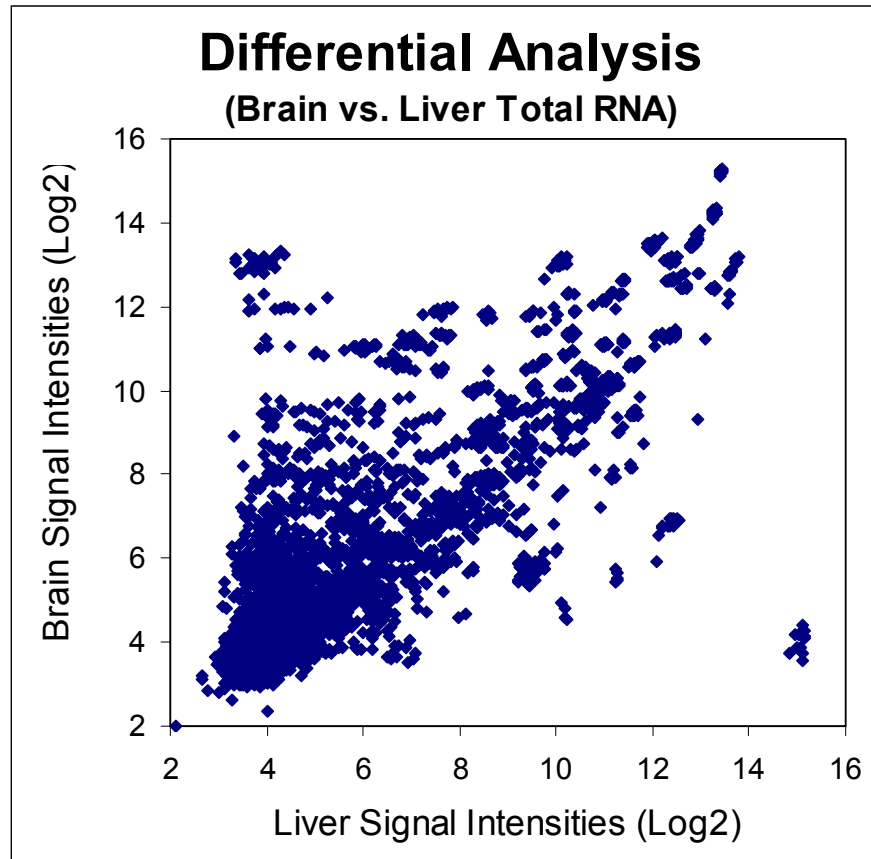
Pool B: Control Spike 29, 31, 36 (PM) and 2, 23, 34 (MM) – 50pg each

Pool A & B synthetic spikes were combined with 1 µg of human liver total RNA (Ambion First Choice®), **flash**tag labeled, and hybridized to Affymetrix GeneChip miRNA Arrays. For analysis, signals were normalized to the perfect match probe-target for each array.

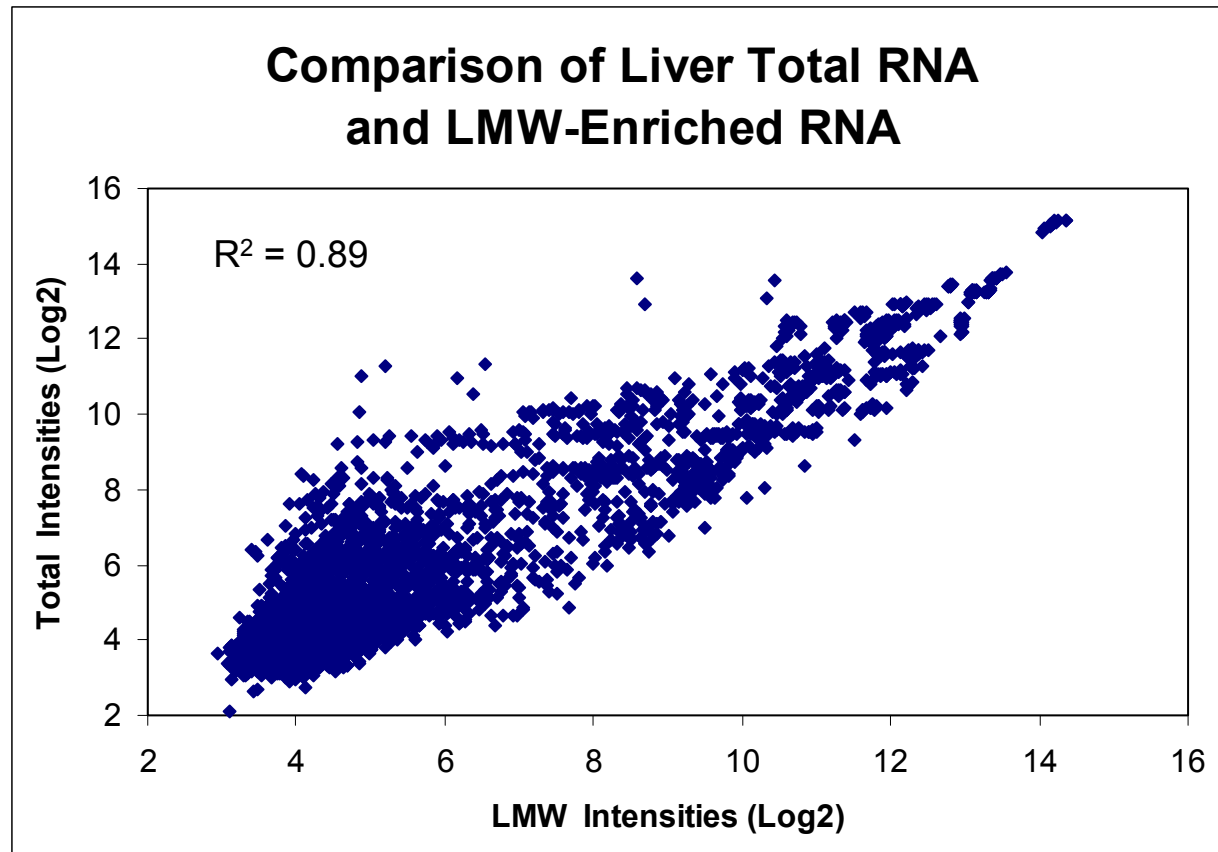
Spike	Perfect Match (PM) Sequence	Mis-Match (MM) Sequence	Cross-reactivity %
Control 2	RYYRYRRYRYYGRYRRYRRYRR	RYYRYRRYRYY A RYRRYRRYRR	10.4%
Control 23	YYYRYRRCRYRYRYYYRRYR	YYYRYRRC C RYRYRYYYRRYR	2.3%
Control 29	YRRYRRYRRYRRYRRYRGYR	YRRYRRYRRYRRYRRY A RYR	0.7%
Control 31	YRRYRRYRRYRRYRRYRRYR	YRRYRRYRRYRRYRRY U YYYR	0.1%
Control 34	URRYRYRRYRRYRRYRRYR	A RRRYRYRRYRRYRRY U RYRYR	1.5%
Control 36	RRRYRYRRYRRYRRYRRYR	RRRYRYRRYRRYRRY G YY G YR	8.7%

R = Purine ; Y = Pyrimidine

Duplicate reactions of 1 μ g each of human brain and human liver total RNA (Ambion First Choice®) were **flashtag** labeled and hybridized to Affymetrix GeneChip miRNA Arrays. For analysis, the duplicate background subtracted signals were normalized and averaged, and signal intensities were plotted for differential analysis.



1 μ g of Liver total RNA (Ambion First Choice®) was enriched for Low Molecular Weight (LMW) RNA using a YM100 microconcentrator (Millipore) and **flashtag** labeled. For comparison, 1 μ g of Liver total RNA was **flashtag** labeled. Both labeled RNAs were hybridized to Affymetrix GeneChip miRNA Arrays. For analysis, background subtracted signals were normalized and signal intensities were plotted.



Titrated amounts (2 μ g to 0.1 μ g) of Liver total RNA (Ambion First Choice®) were *flashtag* labeled in duplicate and hybridized to Affymetrix GeneChip miRNA Arrays. In Figure 1, the Project Description Table was used to calculate the number of detected probe sets and the percentage of detected probe sets divided by the total probe sets. In Figure 2, CEL files were individually exported and averaged, and R² correlations to 1 μ g were generated.

Figure 1

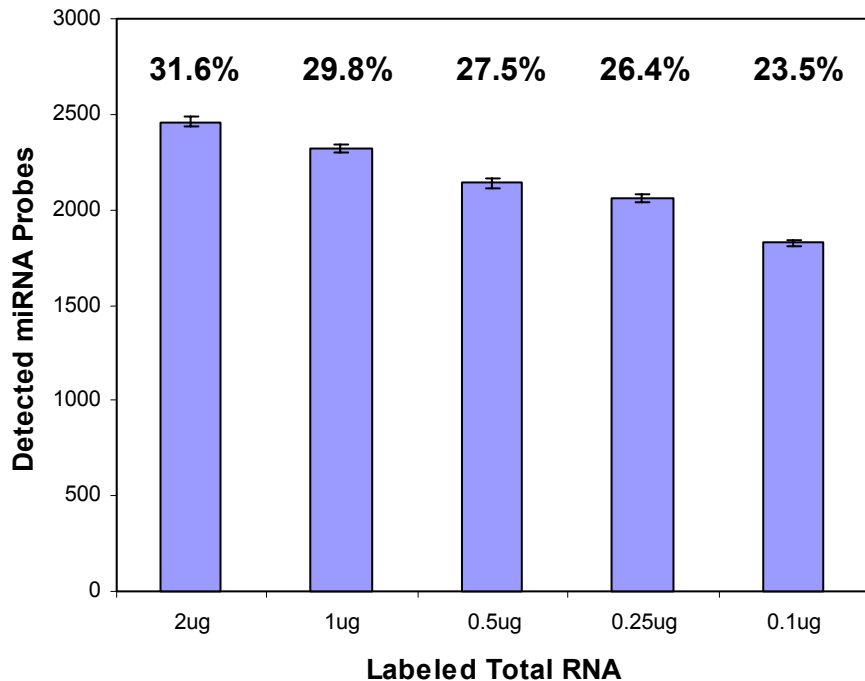
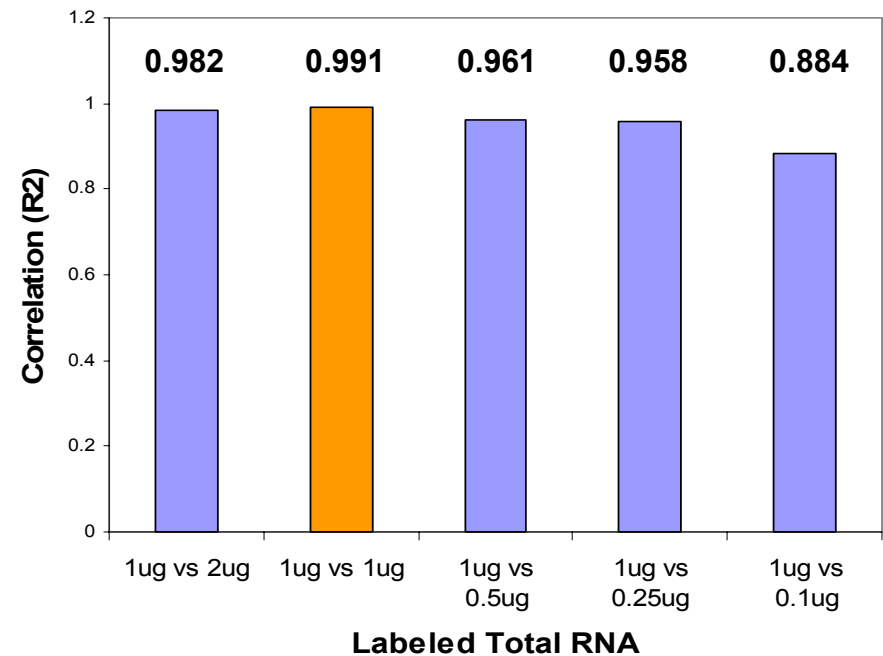


Figure 2



- FlashTag™ Biotin RNA Labeling Kit for Affymetrix® GeneChip® miRNA Arrays
 - FT10AFYB 10 Assay Kit
 - FT30AFYB 30 Assay Kit
- http://www.genisphere.com/array_detection_flashtag_biotin.html
 - Protocol
 - Frequently Asked Questions

- **Customer Service**
 - Email: info@genisphere.com
 - Phone: 1-877-888-3362 or 215-996-3002
 - FAX: 1-877-329-3362 or 215-996-3070
- **Technical Support**
 - Email: techsupport@genisphere.com
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