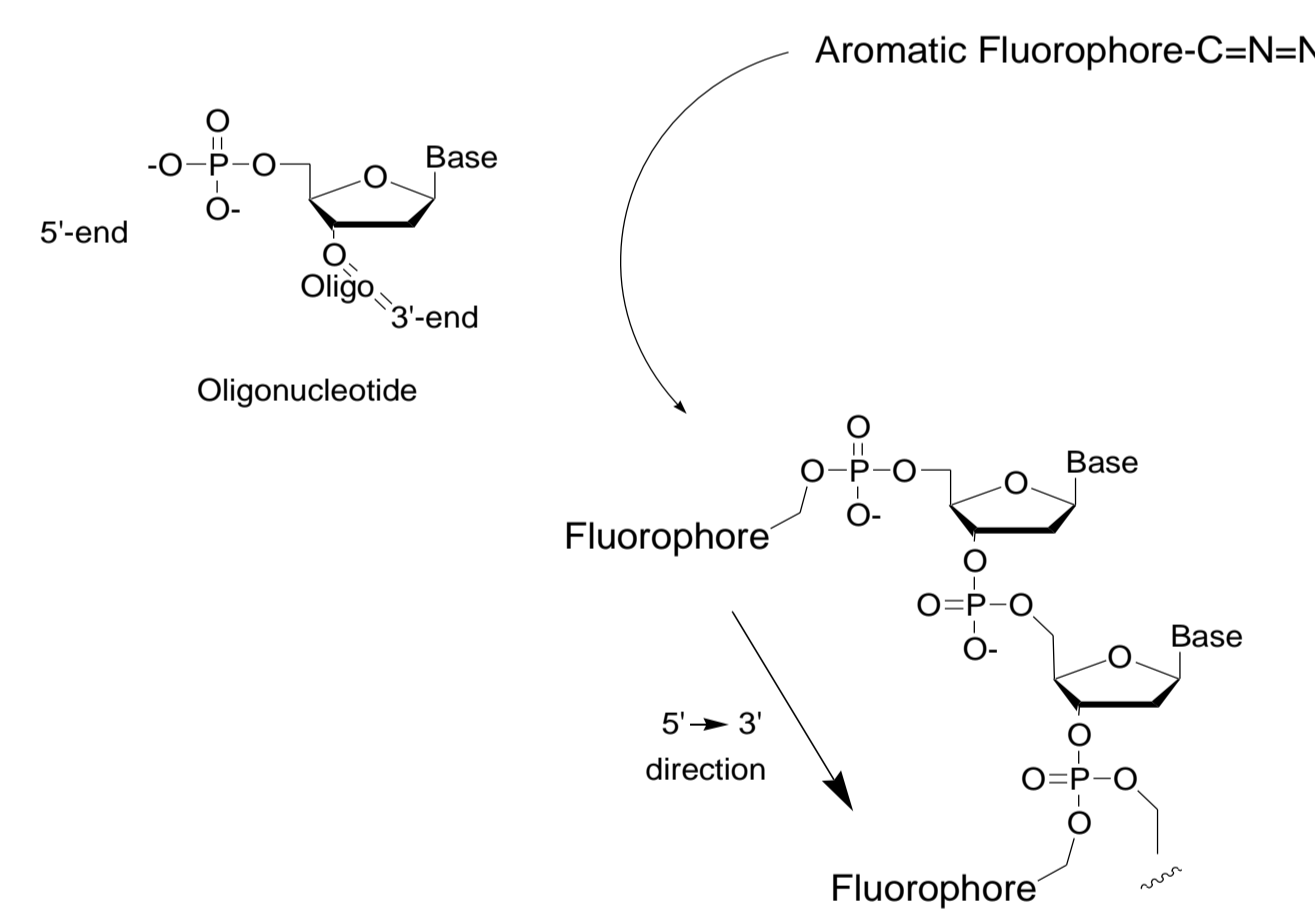


Introduction

Problems with current genomic microarray analysis techniques include hybridization perturbation caused by base labeling and enzyme-introduced sequence bias for labeling. To solve these problems, we have developed a series of new ultrasensitive labeling reagents for directly labeling DNA or RNA samples isolated from live cells or tissues or prepared by PCR. From their unique diazo reactive group as well as ³¹P-NMR studies, these labeling reagents have been found to directly modify terminal and backbone phosphate groups of DNA or RNA samples. We developed these new reagents for use in fluorescent (TAMRA, Cy3, Coumarin) as well as hapten (biotin) labeling protocols. The new labeling reagents have been shown to cause less hybridization perturbation.

The effects of the direct labeling method on hybridization efficiency as well as comparison with existing techniques were examined. Biotin labeling of miRNA isolated from NIH3T3 murine fibroblasts was hybridized to Affymetrix GeneChip miRNA (v1) arrays and compared to RNA ligase-based labeling which is the adopted labeling method used in most miRNA microarray protocols. We observed a 0.68 Pearson Correlation Coefficient between these two labeling methods, with the new direct labeling reagent detecting 24.4% more sequences. A 13.3% bias in labeling was found for sequences ending with a 3'-uracil for the enzymatic labeling method versus a 1.7% bias for our chemical labeling method. Certain hairpin sequences also exhibited biased labeling. Reports from multiple labs regarding enzyme-based bias in labeling are consistent with these observations. To confirm the differential labeling is due to the enzymatic sequence bias, we also compared biotin labeled miRNA with a chloroethylamino reactive biotin direct-labeling reagent which directly modifies the bases of nucleotides. We observed a 0.97 Pearson Correlation Coefficient with 17.8% higher mean intensity using our new direct labeling over the chloroethylamino direct labeling biotin system. In addition, all four miRNA probes miR-{467a,328,206,214}, which lack G,A,C and U respectively, were labeled and exhibited minimal sequence dependence compared with a 10% hybridization bias for the reactive chloroethylamino biotin labeling of miR-328 and 214 over miR-467a and 206. We also isolated miRNA from both etoposide-treated and non-treated NIH3T3 cells, and labeled them using our direct biotin labeling reagent and analyzed the miRNA patterns using the miRNA Affymetrix (v1) arrays. The differential expression miRNA patterns obtained upon etoposide treatment were consistent with the literature as well as confirmed by northern blot and qPCR. Uses of these methods to determine the pattern of gene expression upon therapeutic treatment as well as for pharmacokinetic analysis of DNA or RNA based drug therapies have been developed. This work is supported by NSF grant IIP-0923953.

Labeling through both terminal and internal phosphates



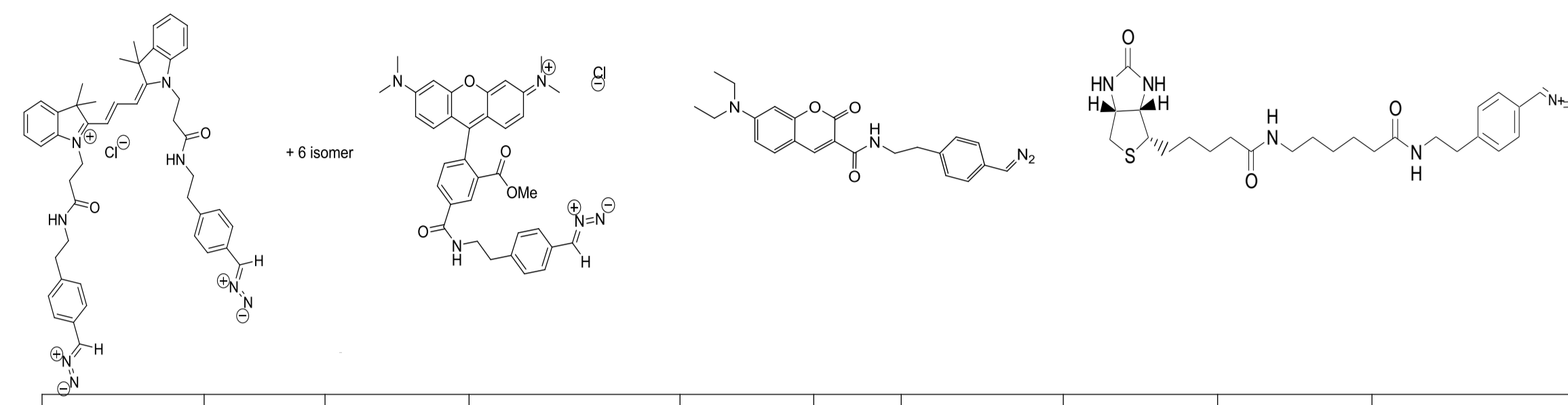
Labeling Systems

- The labeling compounds were prepared from standard fluorescent dyes and biotin-X using an aminoethylbenzaldehyde linking arm.
- Stable tri-isopropylsulfonfyl hydrazide or toluenesulfonylhydrazide intermediates were prepared from the aldehyde precursors.
- The hydrazide intermediates could be efficiently converted to the active diazo labeling agents using either strong basic conditions or with convenient polymer based reagents.
- The active diazo labeling reagents were stable for several weeks at -20°C
- Labeling of dsDNA, ssDNA, oligonucleotides or RNA was accomplished in aqueous solution using an excess of the active diazo dye or labeling reagents for 1 hour at 37°C.
- The labeled DNA or RNA could be purified by ethanol precipitation, silica gel spin column (Qiagen) or using gel electrophoresis.
- The labeled DNA or RNA was stable for several weeks when stored at -80°C in H₂O or appropriate buffer.

Evaluating the properties of labeled DNAs and RNAs

- Analysis of the labeled DNA or RNA by gel electrophoresis exhibited expected shifts due to charge changes upon labeling.
- Hybridization assays using two fluorophores and fluorescence resonance energy transfer (FRET) or by gel shifts indicated efficient hybridization.
- The degree of substitution was dependent upon fluorophore structure, varied from 12-120 dyes / 1000 bp.
- Dye incorporation into nucleic acids is proportional to dye/DNA(RNA) ratio. The higher the ratio, the higher the incorporation.
- Photostability of labeled DNA : measure of fluorescence over time indicated minimal photobleaching.
- Fluorescence In Situ Hybridization (FISH) analysis using Cy3 and TAMRA labeled satellite sequence probes toward centromere regions of chromosomes gave specific and bright signals.
- aRNA (amplified or anti-sense RNA) or miRNA (micro RNA) array analyses with biotin-X labeling gave expected labeling efficiency and excellent hybridization to the arrayed oligonucleotides.

Structures of diazo labeling reagents



	DNA oligomer	Oligo conc (ng/ul)	Oligomer Ext. Coeff (L/mole.cm)	maximum abs	OD	Dye Ext. Coeff	Dye conc. (moles/L)	Oligo conc (mole/L)	DOS (dyes/1000 base)
TAMRA M0776	36mer	289.6	329800	552nm	0.029	95000	3.0526E-07	2.6997E-05	11.3
Fluorescein M1544	36mer	266.7	329800	490nm	0.015	93000	1.6129E-07	2.4862E-05	6.5
Cy3 M1559	36mer	235.6	329800	546nm	0.319	130000	2.4538E-06	2.1963E-05	111.7
Cy5 M1560	36mer	171.5	329800	651nm	0.003	150000	0.00000002	1.5988E-05	1.3
Coumarin M1726	36mer	170	329800	423nm	0.071	57000	1.2456E-06	1.5848E-05	78.6

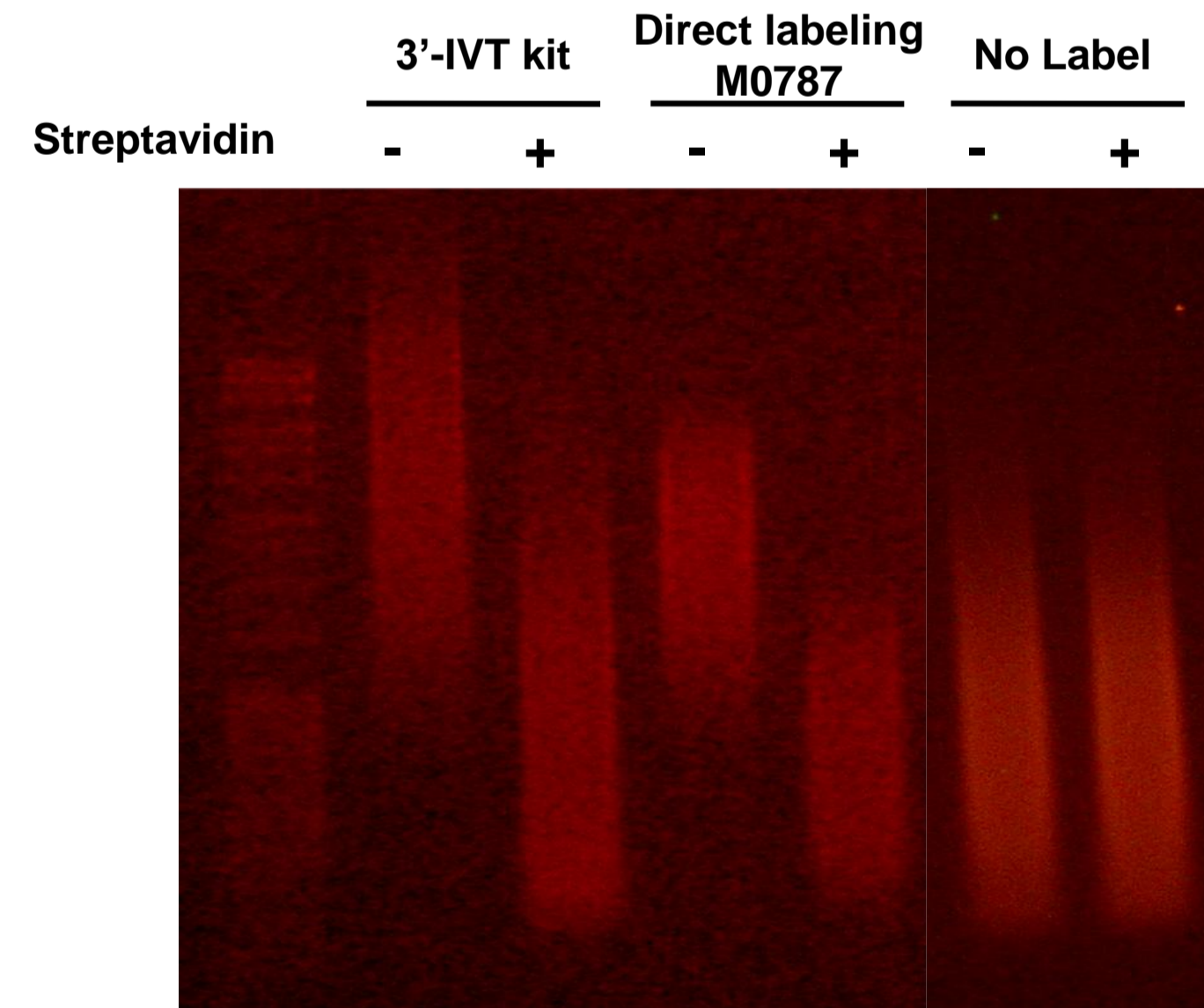
Figure 1: Structures of our direct labeling reagents and evaluation of their incorporation/ degree of substitution (DOS) using a 36-mer oligonucleotide.

³¹P-NMR confirmed the labeling of nucleotides on phosphate backbone



Figure 2: Analysis by ³¹P NMR revealed changes in phosphate peaks before (A) and after (B) labeling of an 18-mer oligonucleotide sample using the Coumarin (M1726) labeling dye.

Microarray analysis of aRNA labeled with different systems



Compare Affy labeled and Biotin-X labeled aRNA in microarray

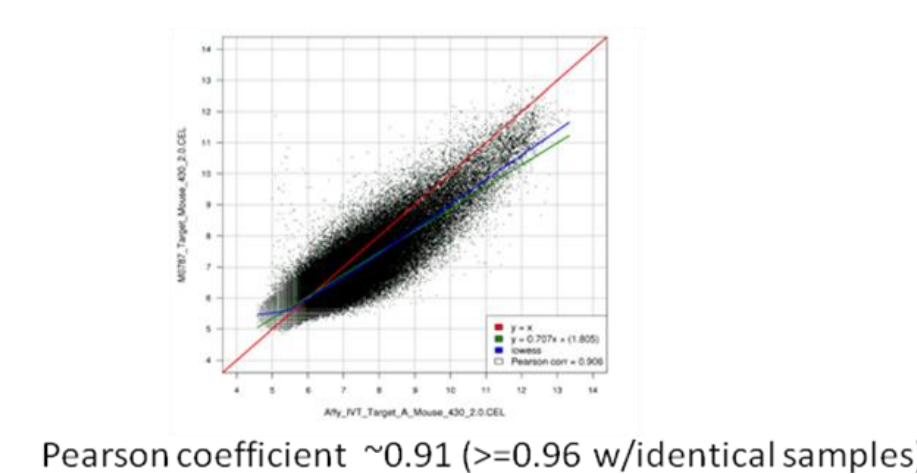
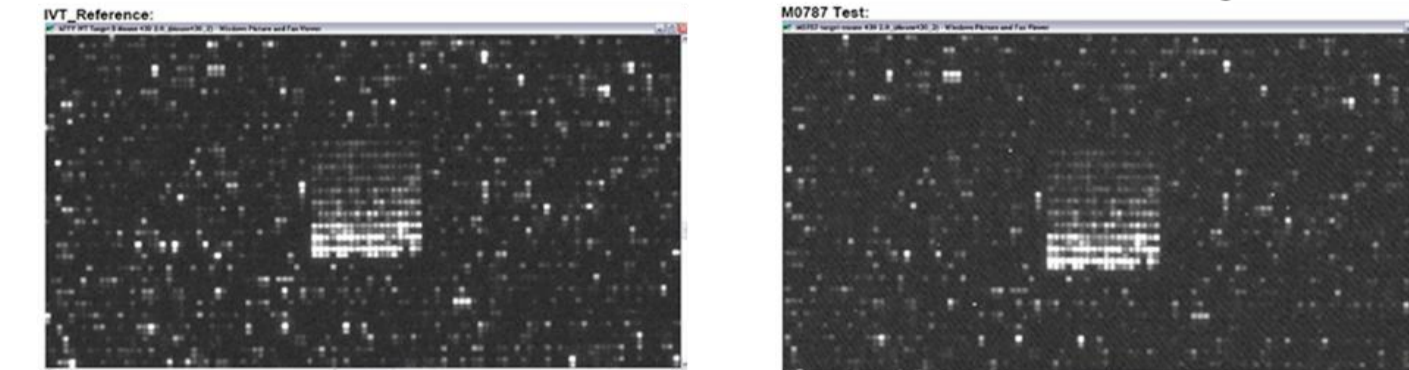


Figure 3: aRNA from mouse fibroblast (NIH3T3) cells labeled with our Biotin-X labeling reagent (M0787) generated equivalent signals as aRNA labeled with the Affymetrix 3'-IVT kit in microarray analysis.

Comparing different labeling in miRNA array analysis

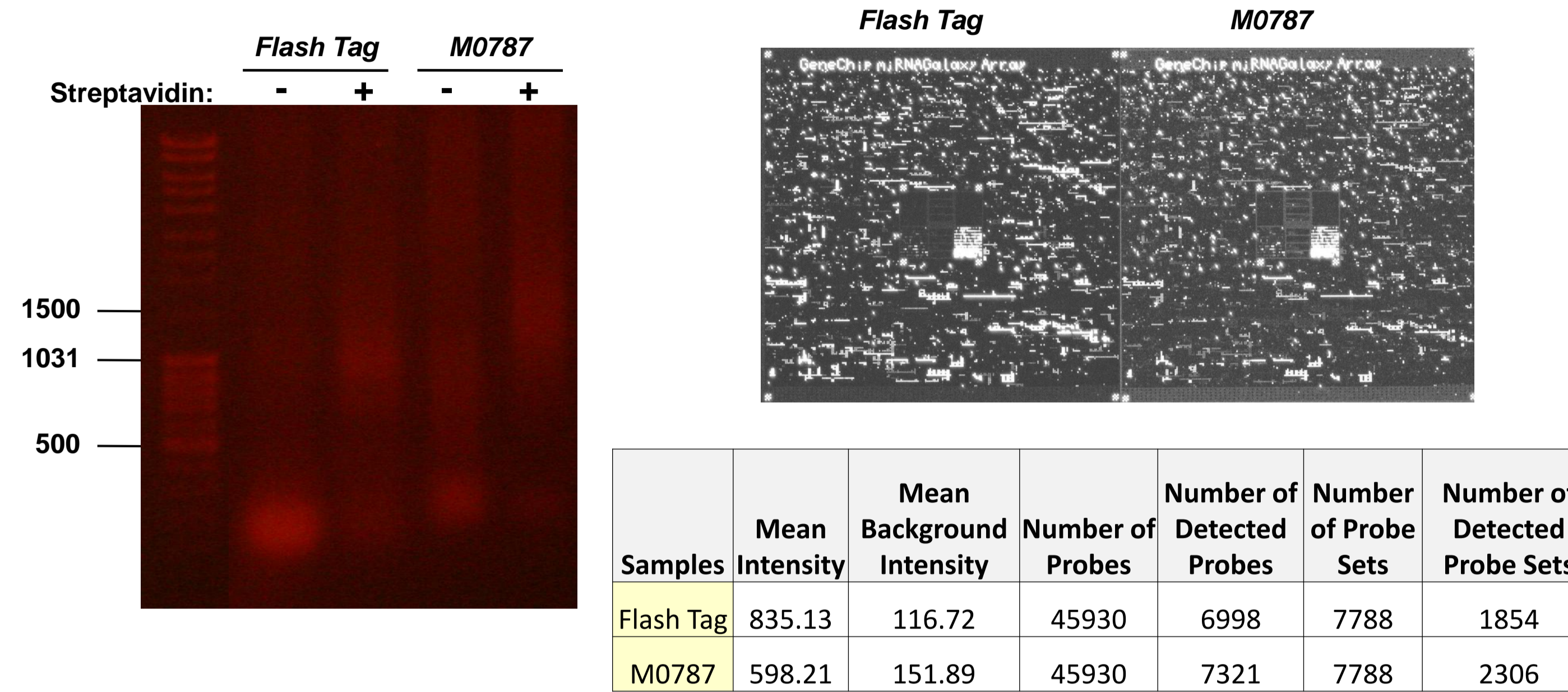
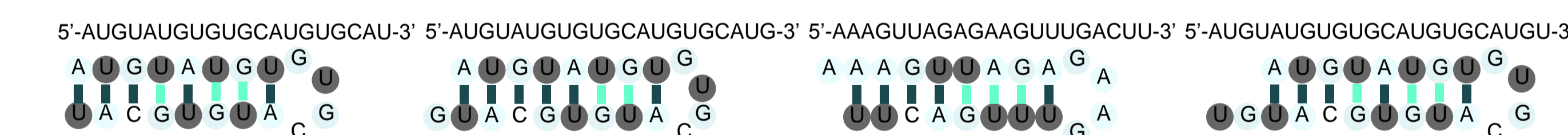
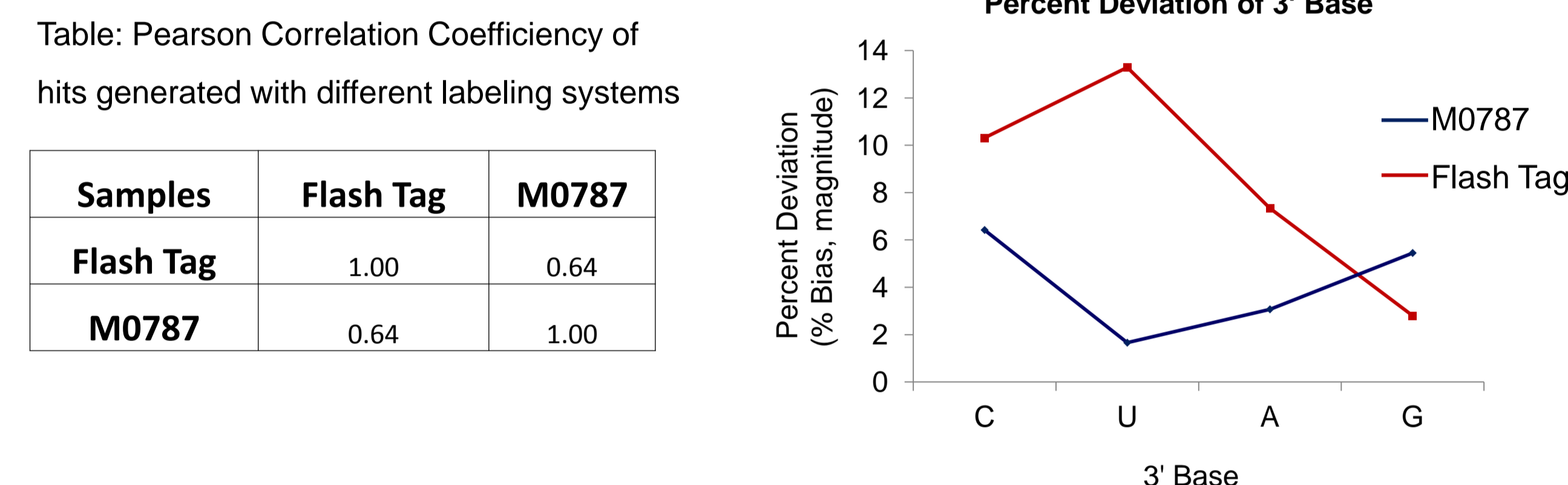


Figure 4: Total RNA including miRNA was labeled with either the direct biotin-X (M0787) labeling reagent or 3DNA Flash Tag Biotin labeling kit. Same amount of both labeled samples were applied for hybridization to miRNA array chips. The table revealed the direct labeling reagent (M0787) had more positive hits versus 3DNA Flash Tag Biotin labeling (7321 vs 6998).

Enzyme based labeling is prone to sequence bias in labeling



Flash Tag	False	False	False	False
M0787	True	True	True	False/True

Figure 5: A 64% correlation of hits were observed with our direct chemical vs enzyme-based (Flash Tag) labeling. Further statistical analysis revealed biased labeling at the 3' end with Flash Tag labeling system while our direct labeling method showed much reduced bias. Also miRNA hits with long hairpin structures (>=8 predicted by Nussinov-Jacobson Algorithm) were more prone to be identified in array analysis with our direct labeling method rather than the enzyme-based.

Unbiased labeling with direct chemical labeling method

Samples	Mean Intensity	Mean Background Intensity	Number of Probes	Number of Detected Probes	Number of Probe Sets	Number of Detected Probe Sets
Label IT	480.97	130.64	45930	7595	7788	2367
M0787	566.81	173.96	45930	8403	7788	2538

Table: Pearson Correlation Coefficiency of hits

Samples	M0787	Label IT
M0787	1.00	0.97
Label IT	0.97	1.00

Figure 6: Comparison of the miRNA hits from array analysis with samples labeled with our diazo labeling reagent vs Label IT from Mirus. 97% correlation was observed.

Differential expression of miRNA upon Etoposide treatment

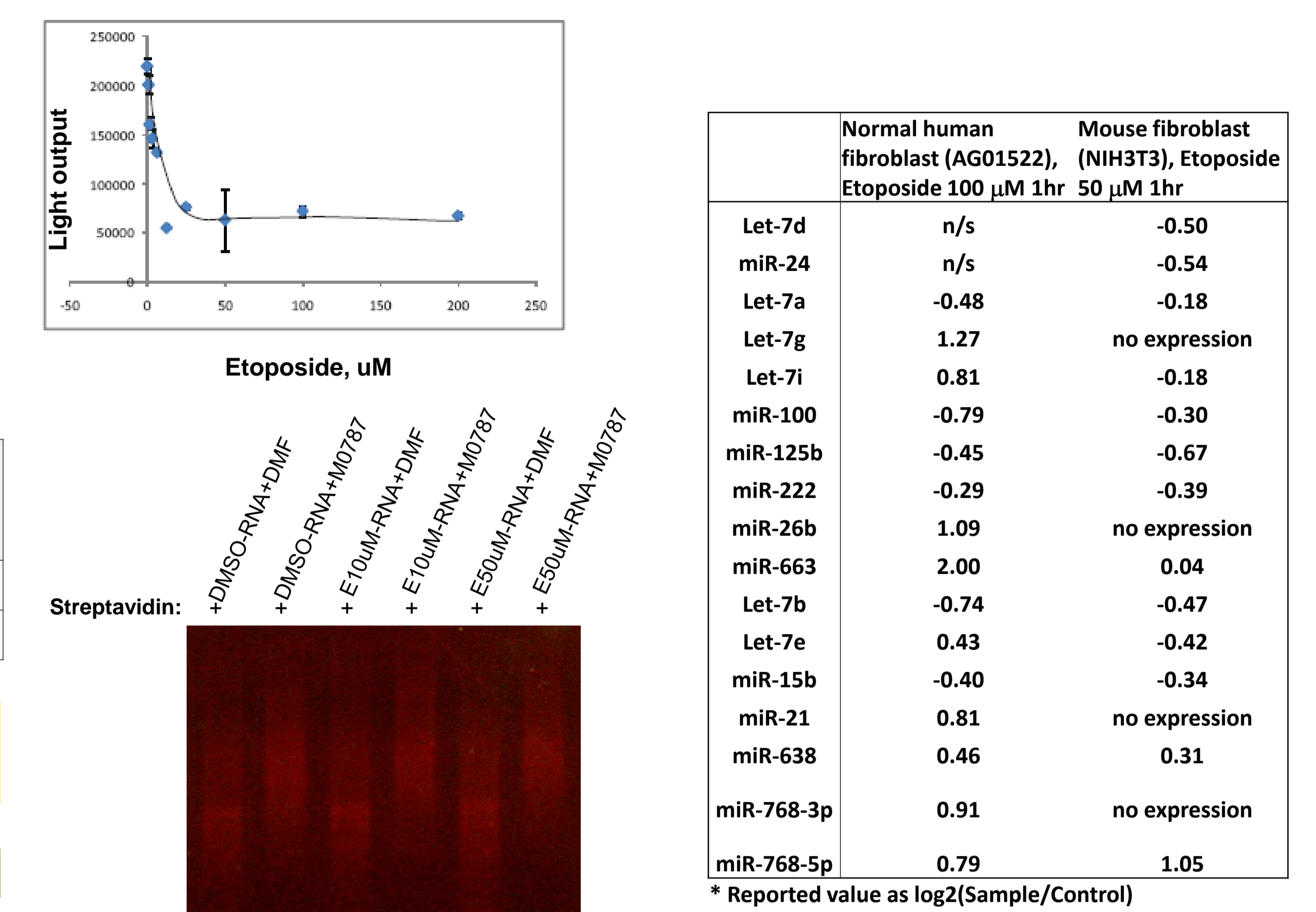


Figure 7: Mouse fibroblast (NIH3T3) cells were treated with Etoposide (10 or 50uM) and miRNAs isolated, labeled with our direct Biotin-X labeling reagent, and applied to miRNA array analysis. The change of miRNA expression was evaluated by comparing treated vs control samples.

Conclusions and Discussions

In order to produce an improved DNA and RNA labeling system, we developed a set of active fluorophore and biotin-X labeling compounds that can be used to directly label dsDNA, ssDNA mRNA, miRNA and oligonucleotide probes. Our *in vitro* testing indicates:

- Up to four color (multiplexed) labeling of DNA and RNA samples can be accomplished.
- The incorporation of dyes/labels into DNA and RNA samples can be controlled with optimized dye/nucleotide ratio.
- The labeling reagents are stable under standard microarray and gel based assay conditions, and exhibit improved photostability compared with currently available methods.
- Microarray analysis indicated that the labeling reagents perform as well or better than existing methods with reduced bias as to nucleotide sequence or hairpin structures.
- Analysis of miRNA expression levels by array analysis with the new direct labeling reagents revealed differential expression from cell/tissue analysis upon treatment with anti-neoplastic reagents.
- In addition to its application in array analysis, the labeling reagents are amenable to *in situ* hybridization and gel based assay systems.
- A new system for producing the active diazomethane labeling compounds *in situ*, using a polymer based activation step providing high stability, improved purity and long-shelf life was developed.
- The combined results of this work clearly indicated the potential of utilizing the direct labeling reagents for analysis of DNA and RNA expression levels from live cells and tissues *in vivo*, enabling the exciting commercial applications in numerous biotechnology areas.
- The new DNA/RNA labeling reagents are available under the trademark "OliGlo™". The reagents are available for both microarray, and *in situ* hybridization applications in which the protocols have been developed and optimized.

References

- Van Gelder R, von Zastrow ME, Dement WC, Barchas JD, and Eberwine JH., (1990) "Amplified RNA synthesized from limited quantities of heterogeneous cDNA." Proc. Natl. Acad. Sci. USA 87: 1663-1667.
- Vitale D, (2001) "Optimizing cRNA fragmentation for microarray experiments using the Agilent 2100 bioanalyzer." Agilent Technologies, Publication Number 5988-3119EN, pp.1-8.
- Whitaker JE, Haugland RP, Ryan D, Hewitt PC, Haugland RP (1992) "Fluorescent rhodol derivatives: versatile, photostable labels and tracers." Anal. Biochem. 207: 267-279.
- Nimura N, Kinoshita T, Yoshida T, Uetake A, Nakai C (1988) "1-Pyrenyldiazomethane as a fluorescent labeling reagent for liquid chromatographic determination of carboxylic acids." Anal. Chem. 60:2067-2070.
- Laayoun A, Kotera M, Sothier I, Trevisiol E, Bernal-Mendez E, Bourget C, Menou L, Lhomme J, Troesch A, (2003) "Aryldiazomethanes for Universal Labeling of Nucleic Acids and Analysis on DNA Chips." Bioconjugate Chem. 14: 1298-1306.
- Shiga M, He P, Sagara F, Ueno K, Sasamoto K (1993) "Synthesis of a Novel Biotin Derivative That Bears a Diazo Group as the Reactive Site." Anal. Sci. 9: 553-556.