

SUMMARY POINTS

- A controlled, internal isolation protocol based on a modified RecoverAll™ Total Nucleic Acid Isolation Kit procedure was implemented;
- This custom procedure allows extraction of high yields of total RNA including microRNA (miRNA) fraction from various archived fixed tissues;
- Reproducible and robust profiling of miRNA expression in FFPE tissues was enabled through optimization of microarray processing, data normalization, and analysis;
- High correlation values were observed between miRNA expression profiles generated from FFPE blocks up to 12 years old;
- Capability to interrogate miRNA expression in archived clinical samples should facilitate retrospective molecular studies for biomarker discovery and validation.

INTRODUCTION

Formalin-fixed, paraffin-embedded (FFPE) tissues constitute the most complete and widely available archived collection of specimens suitable for retrospective analysis. These archived tissues hold a wealth of information about disease progression, therapeutic response or toxicity, and offer a possibility for discovery of nucleic acid biomarkers with potential diagnostic and therapeutic value. However, the recovery of quality RNA from FFPE tissues can represent a challenge. The commonly used fixation process causes cross-linking between nucleic acids and proteins, irreversible and random fragmentation of RNA, and covalent modification of RNA. Variations within the fixation protocol among different clinical laboratories can further complicate the nucleic acid isolation and subsequent analysis of data derived from specimens of various sources.

microRNAs (miRNAs) are small RNA molecules (19-23 nucleotides long) that are well conserved both in plants and animals and regulate diverse cellular processes. Over the past few years, the involvement of miRNAs in human diseases, including several cancer types, has become increasingly clear. Due to their small size, miRNAs are less susceptible to damage during the fixation procedure and can later be more easily isolated from FFPE specimens than mRNAs. Small size and stability in clinical samples could make these small RNA species ideal candidates for retrospective molecular studies focused on the development of new therapeutics and diagnostics. In this pilot study, we evaluate the suitability of RNA isolated from lung tumor FFPE tissue specimen for miRNA expression profiling using qRT-PCR and microarray platforms.

MATERIALS AND METHODS

Fifteen pairs of lung tumor and normal adjacent FFPE tissues, varying between 1 to 12 years in age, were purchased from Phylogeny Inc. (Columbus, OH). Total RNA was isolated using RecoverAll™ Total Nucleic Acid Isolation Kit (Ambion, Austin, TX) according to the original manufacturer's protocol with custom modifications to enable high tissue throughput and a controlled isolation process. The recovery of miRNAs was assessed by qRT-PCR utilizing TaqMan® Assays (ABI, Foster City, CA) for a panel of 3 miRNA and 2 mRNA species, demonstrated to be stably expressed across different tissues. Total RNA from each sample was fractionated by polyacrylamide gel electrophoresis (PAGE) to enrich for small RNA species and subsequently end-labeled with biotin according to the modification of the *mirVana*™ miRNA labeling Kit protocol (Ambion, Austin, TX). Biotinylated miRNA fraction was hybridized to the DiscovArray™ expression miRNA profiling platform (Asuragen, Austin, TX) according to the standard Affymetrix procedure. The DiscovArray platform is a custom-manufactured Affymetrix array that contains probes for human miRNAs selected from multiple sources, including the Sanger miRBase database and published reports. Array data was normalized with a variance stabilization method (VSN).

RESULTS

Table 1. Yield and quality of total RNA isolated from 15 matched pairs of FFPE Lung Tumor and NAT tissue using RecoverAll Total Nucleic Acid Isolation Kit for FFPE Tissues (Ambion). SqCC- squamous cell carcinoma, AdCA- adenocarcinoma, LCC- large cell carcinoma, AdSqCC- adenosquamous cell carcinoma.

Embedding Year	Donor ID	Tissue	Diagnosis	Tumor Subtype	A260/280	Yield per 20 th m slice (µg)
1996	D-1	Lung	NSCLC	AdCA	1.97	4.29
1996		Lung	NAT		1.77	2.33
1996	D-2	Lung	NSCLC	LCC	1.96	5.04
1996		Lung	NAT		1.83	1.45
1996	D-3	Lung	NSCLC	AdSqCC	2.00	3.23
1996		Lung	NAT		1.83	1.96
1996	D-4	Lung	NSCLC	AdSqCC	2.02	4.35
1996		Lung	NAT		1.94	1.18
1996	D-5	Lung	NSCLC	AdSqCC	1.98	1.31
1996		Lung	NAT		1.93	0.88
2003	D-6	Lung	NSCLC	LCC	1.88	5.89
2003		Lung	NAT		1.90	1.47
2003	D-7	Lung	NSCLC	AdCA	1.88	2.67
2003		Lung	NAT		1.85	2.15
2003	D-8	Lung	NSCLC	LCC	1.60	0.89
2003		Lung	NAT		1.55	1.11
2003	D-9	Lung	NSCLC	AdCA	1.91	1.37
2003		Lung	NAT		1.78	0.36
2003	D-10	Lung	NSCLC	SqCC	1.84	1.05
2003		Lung	NAT		1.55	0.76
2006	D-11	Lung	NSCLC	SqCC	1.75	1.54
2006		Lung	NAT		1.54	0.75
2006	D-12	Lung	NSCLC	AdCA	1.60	0.87
2006		Lung	NAT		1.59	0.64
2006	D-13	Lung	NSCLC	LCC	1.74	1.63
2006		Lung	NAT		1.63	0.41
2006	D-14	Lung	NSCLC	AdCA	1.61	0.84
2006		Lung	NAT		1.54	0.63
2006	D-15	Lung	NSCLC	AdCA	1.52	1.14
2006		Lung	NAT		1.74	1.23

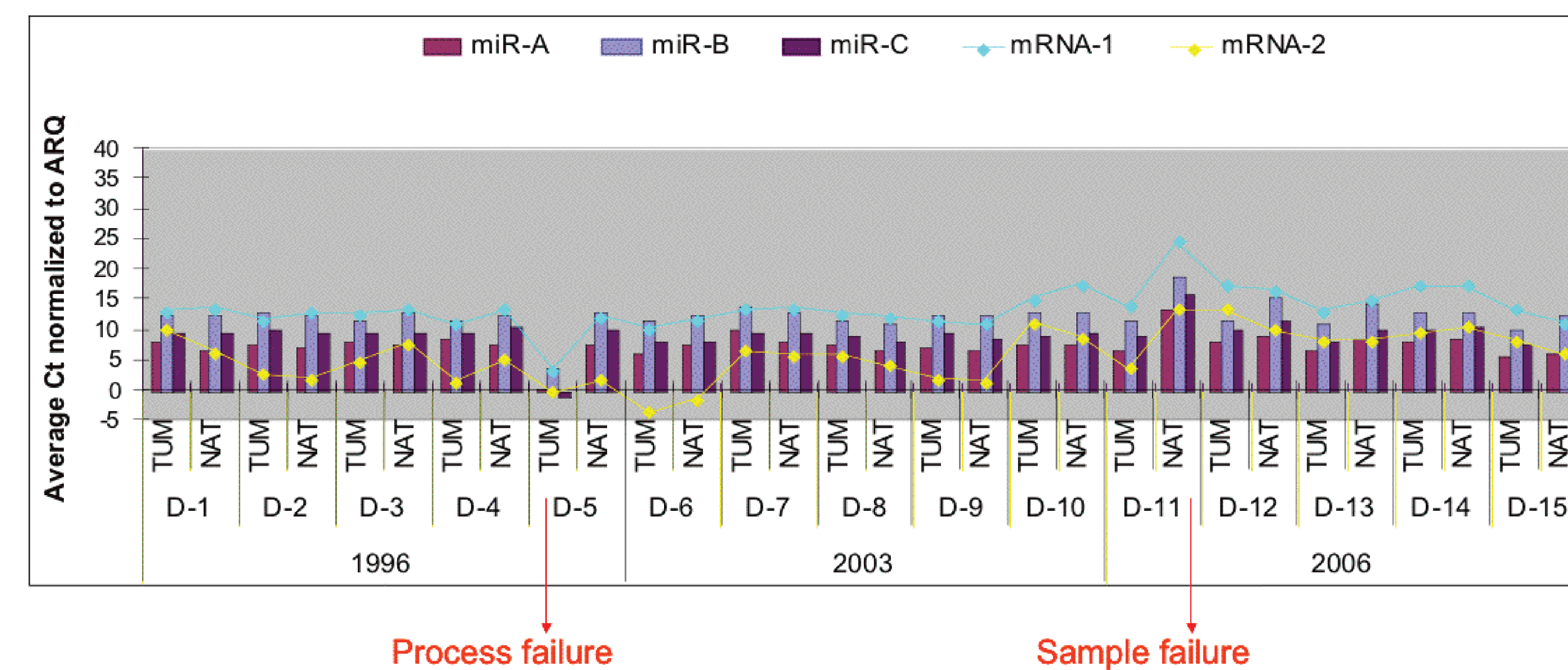


Figure 1. qRT-PCR amplification of a 3-miRNA and a 2-mRNA “normalizer” panel from 15 matched pairs of FFPE Lung Tumor and NAT. Average Ct values for each target were normalized to an internal isolation process control (Armored RNA® Quant™). Sample “D-4 TUM” failed the isolation process as determined by the low amplification of the process control. Sample “D-11 NAT” was isolated successfully, however shows low RNA content, as determined by poor amplification of miRNA and mRNA targets.

Figure 2. Scatter plots (A) and correlation ratios (B) between mean miRNA expression for FFPE lung NAT and lung tumor samples according to block age. Each group contained 5 samples.

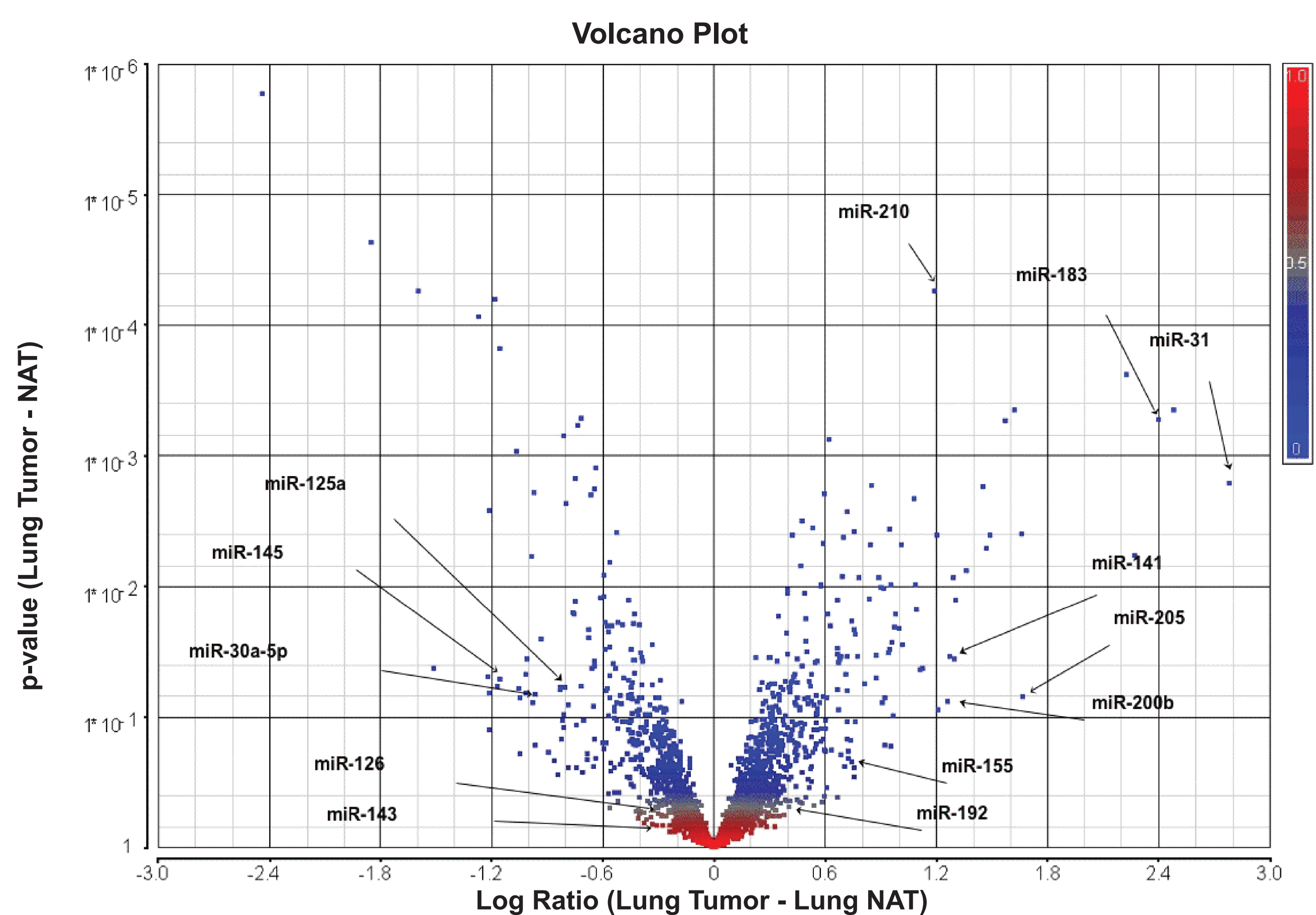
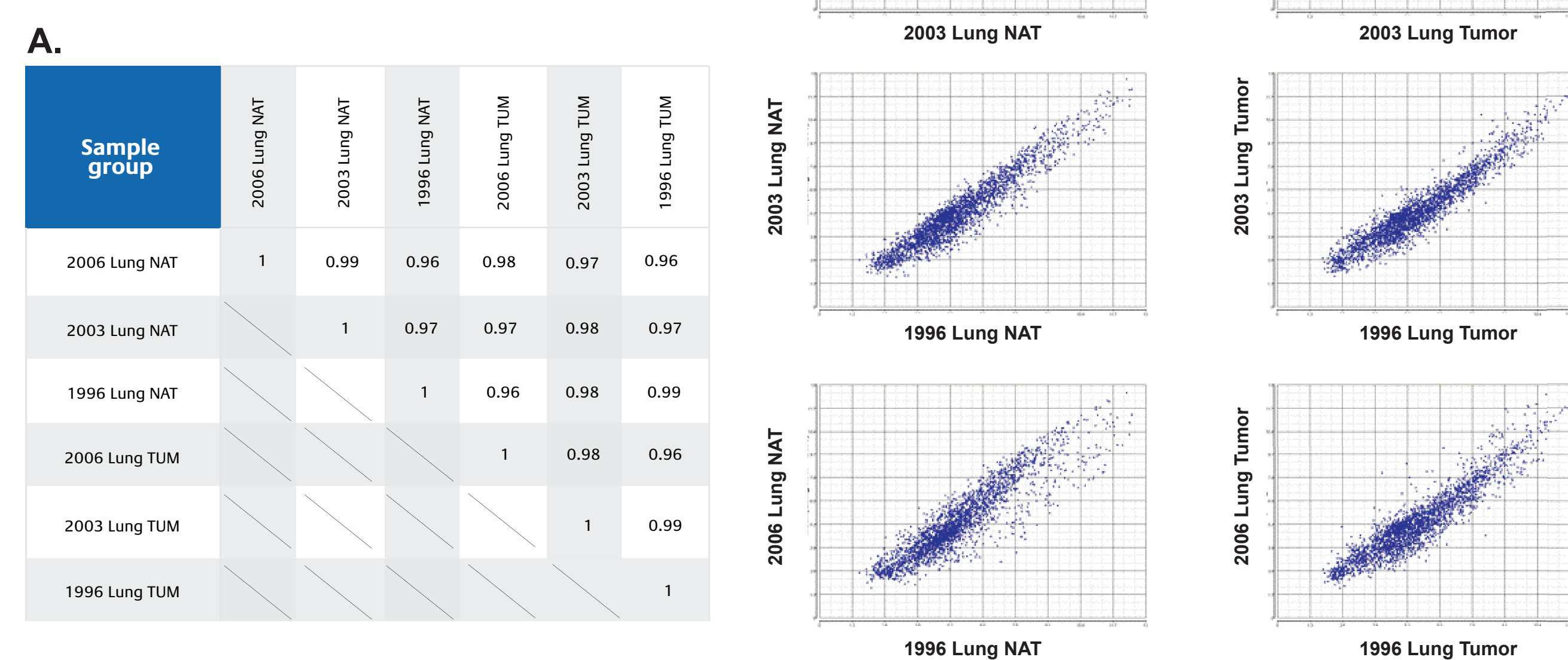


Figure 3A. Concordance between miRNAs shown to be differentially expressed in 15 pair Lung Tumor/NAT FFPE sample set and miRNAs reported in the literature. Mean difference in miRNA expression between 15 lung NAT and 15 lung tumor samples (matched). Blue color depicts low p-value, red- high p-value for a given miRNA.

miR Name	Expression change in cancer	Reference
has-mir-21	up	Yanaihara <i>et al.</i> (2006)
has-mir-210	up	Yanaihara <i>et al.</i> (2006)
has-mir-155	up	Yanaihara <i>et al.</i> (2006)
has-mir-143	down	Yanaihara <i>et al.</i> (2006)
has-mir-205	up	Yanaihara <i>et al.</i> (2006)
has-mir-126	down	Yanaihara <i>et al.</i> (2006)
has-mir-30a-5p	down	Yanaihara <i>et al.</i> (2006)
has-mir-145	down	Yanaihara <i>et al.</i> (2006)
has-mir-192	up	Yanaihara <i>et al.</i> (2006)
has-mir-203	up	Yanaihara <i>et al.</i> (2006)
has-mir-125a	down	Yanaihara <i>et al.</i> (2006)
has-mir-31*	up	Bandres <i>et al.</i> (2006)
has-mir-183*	up	Bandres <i>et al.</i> (2006)
has-mir-200b**	up	Meng <i>et al.</i> (2006)

* - in colorectal cancer
** - in human cholangiocarcinoma cell lines

Figure 3B. Concordance between miRNAs shown to be differentially expressed in the 15 pair lung tumor/NAT FFPE sample set and miRNAs reported in the literature. Examples of miRNAs confirmed in our experiment and reported in the literature to be differentially expressed in lung cancer and a subset of other cancers.

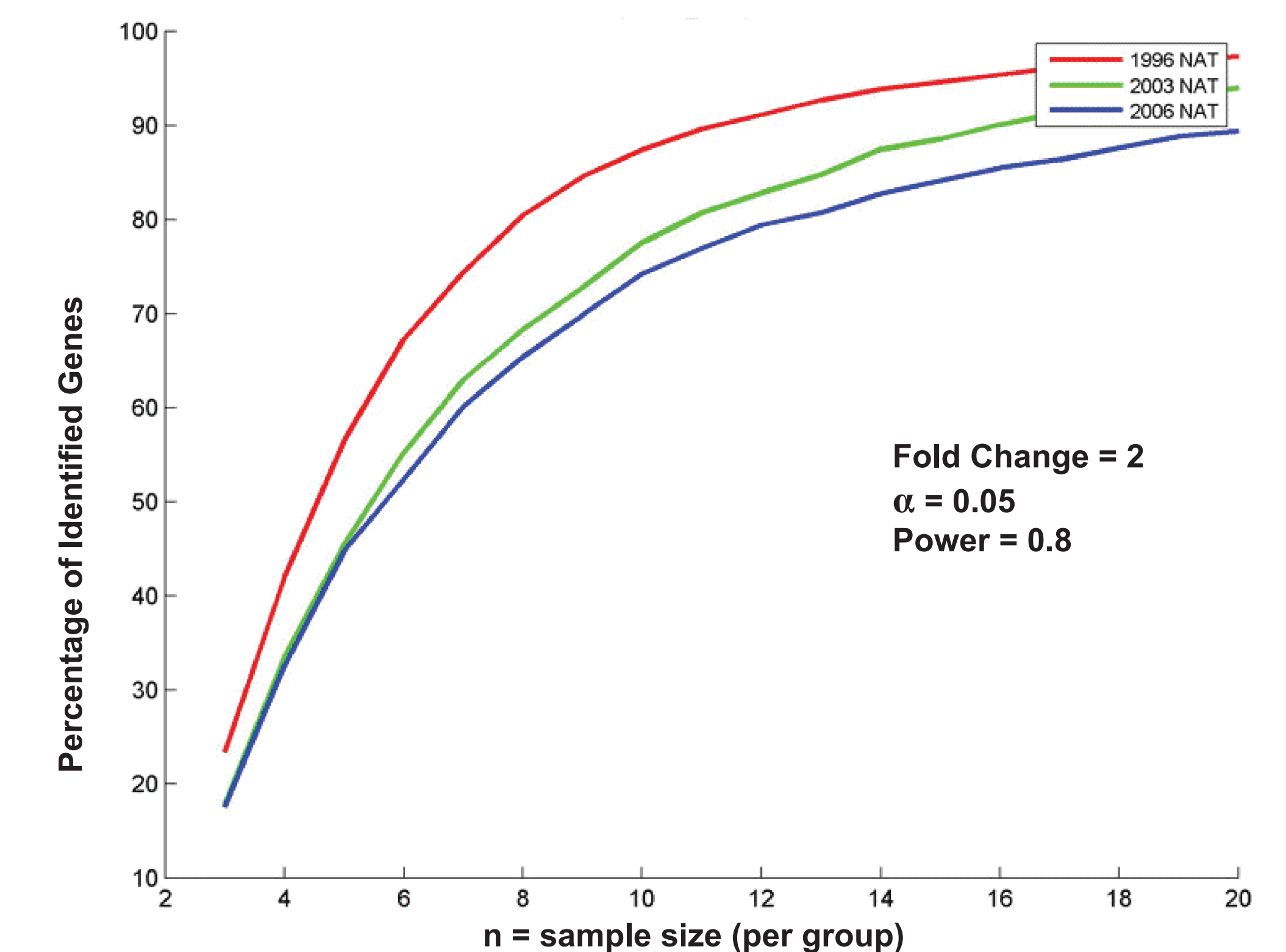


Figure 4. Statistical power analysis performed on 15 matched pairs of FFPE lung tumor and NAT in order to determine sample size required to detect miRNAs with a two-fold change in expression. According to this analysis, studies performed on sample sets of at least 8 samples, depending on age, may permit detection of at least 80% of the miRNAs with a 2-fold difference in expression.

CONCLUSION

Here we demonstrate that Asuragen's internal isolation protocol for FFPE tissues enables efficient isolation of total RNA including miRNA fraction. The generated miRNA expression data shows high correlation ratio ($R^2 > 0.9$) between different block ages within lung NAT and lung tumor tissue groups, indicating that classification of these tissue types can be achieved using archived specimen up to 12 years in age. Furthermore, the observed miRNA expression changes between lung NAT and lung tumor tissues reveal a set of miRNAs that is very concordant with miRNAs reported in the literature to be differentially expressed in lung cancer. Statistical power analysis performed on the FFPE lung sample set herein suggests that, depending on block, age, 8-13 samples per group would be required to detect 80% of the miRNAs with a two-fold change in expression.