MicroRNA Isolation and Analysis from Human Whole Blood

Jon Kemppainen, Stela Filipovic-Sadic, Joseph Ehardt, Timothy S. Davison, and Gary J. Latham. Asuragen, Inc. 2150 Woodward St., Austin, Texas 78744.

ABSTRACT

MicroRNAs (miRNAs) represent an exciting new class of small, regulatory biomolecules. Data to date suggests that as many as half of all genes may be regulated by miRNAs. Over the past few years, scientists have demonstrated that miRNA play important roles in processes as diverse as early development, cell proliferation and differentiation, apoptosis and fat metabolism, and oncogenesis. Indeed, tumor-associated, differentially expressed miRNAs, termed "oncomirs," have been found in more than a half dozen different cancers and the number of known oncomirs is growing rapidly.

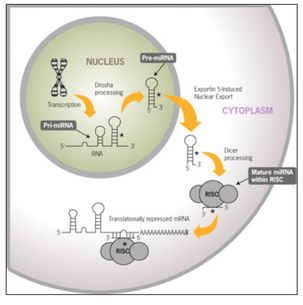
Blood is a readily accessible tissue that carries biological material from all reaches of the body, and is the preferred clinical sample for many diagnostic tests. Blood-borne cells such as lymphocytes, monocytes, neutrophils, eosinophils, and basophils contain molecular clues of infection, inflammation, and other hallmarks of disease. We hypothesize that miRNA biomarkers exist in blood, and further that these biomarkers will drive the development of future molecular diagnostics assays. However, technologies for isolating and analyzing the global miRNA profile in human whole blood are largely lacking. For example, the most widely used protocol for whole blood RNA stabilization and isolation, the PAXgene™ Blood RNA System, does not provide for the recovery of small RNA. As a result, we developed a novel methodology using the PAXgene stabilized blood that enables the isolation of mature, ~22-mer miRNAs. Using microarrays, we then compared the expression levels of hundreds of miRNAs in blood with the miRNA profile from nearly two dozen other normal human tissues. Statistical analysis enabled the identification of miRNAs that were relatively differentially expressed in whole blood with respect to the expression profiles from the reference tissues. The implications of these findings are discussed.

INTRODUCTION

MiRNAs are known to play key roles in a number of fundamental biological pathways. The discovery that miRNAs are differentially expressed in diseased tissues has created new opportunities for the diagnosis and treatment of a broad set of potential maladies, including cancer. The ideal clinical sample for any diagnostic test, however, should pose minimal inconvenience to the patient, be readily obtainable and archivable using simple, widely accepted methods, and provide appropriately sensitive, specific and robust information to enable clinical utility. For these reasons, blood has become the sample of choice for many diagnostic assays. We suggest that miRNA in blood may offer a foundation for the detection and/or stratification of many possible diseases.

The most commonly used procedure for the collection and stabilization of human whole blood is the PAXgene Blood RNA System. This product consists of a vacutainer tube that is prefilled with ~6.5 ml of a cationic detergent known as Catrimox-14. Whole blood is collected by venipuncture into the PAXgene tube, and the detergent ruptures cells, strips proteins from nucleic acids, and sequesters RNA into micelles that reportedly protect it from the high levels of nuclease activity in blood. RNA is isolated following a centrifugation and wash step that deposits a RNA precipitate that is further purified on a glass filter column. However, conventional glass filter column RNA isolation procedures do not efficiently recover RNA less than ~100 nucleotides, and, indeed, PreAnalytix, the company that sells the PAXgene product, does not support the recovery of small RNA such as miRNA. As a result, we developed an alternative protocol that accepts PAXgene-stabilized blood RNA and provides high yields of the miRNA fraction from total RNA. In the data to follow, we compare the performance of this alternative procedure, termed the Asuragen Blood miRNA Isolation Protocol, with the PAXgene procedure with respect to both miRNA and mRNA recovery, and functionality in gene expression assays.

Fig. 1. microRNA Biogenesis and Processing



EXPERIMENTAL METHODS

Whole blood was procured from 3 healthy human donors in PAXgene vacutainer tubes following Asuragen's approved blood collection protocol. Samples were frozen at -80C, and then thawed for 16 hr at ambient temperature. Samples from each donor were then divided into two groups of 3 tubes each and then processed by either the PAXgene protocol (according to the manufacturer's instructions) or the Asuragen protocol. Purified RNA from each was then assessed by two quality measures, namely the A260/280 ratio and RNA profile analysis by the Agilent 2100 bioanalyzer. RNA yields were determined spectrophotometrically using the Nanodrop 6000.

To quantify the mRNA and miRNA fractions, a series of RNA targets were interrogated by quantitative RT-PCR (qRT-PCR). The relative abundance of 6 different mRNAs was assessed using the TaqMan Gene Expression Assay (ABI), whereas miRNA abundance was determined using a proprietary miRNA qRT-PCR assay developed at Asuragen. All RNA data were normalized to 18S rRNA. Since the Asuragen miRNA isolation protocol yielded ~50-fold more miRNA than the PAXgene method, only this RNA was examined further by miRNA microarrays.

Samples for microRNA profiling studies were processed by Asuragen according to the company's standard operating procedures. The microRNA enriched fraction was obtained by passing 7 µg of total RNA through a flashPAGE™ Fractionator apparatus (Ambion, Inc., Austin, TX) and cleaned and concentrated using the flashPAGE Reaction Clean-Up Kit (Ambion, Inc., Austin, TX). The 3' ends of the RNA molecules were tailed and labeled using the mirVana™ miRNA Labeling Kit (Ambion, Inc., Austin, TX) according to the manufacturer's instructions. Amine-modified nucleotides were incorporated during the poly (A) polymerase mediated tailing reaction, and Cy3 succinimide esters (Amersham Biosciences (GE Healthcare), Piscataway, NJ) were conjugated to the amine moieties on the microRNAs. Hybridization to the mirVana miRNA Bioarrays V2 (Ambion, Inc., Austin, TX) was performed using the mirVana miRNA Bioarray Essentials Kit (Ambion, Inc., Austin, TX). Array fluorescence on the arrays was scanned using a GenePix 4200AL scanner (Molecular Devices, Union City, CA). The fluorescent signal associated with the probes and local background was extracted using GenePix Pro (version 6.0, Molecular Devices, Union City, CA).

Thresholding and signal scaling were generated using algorithms selected by Asuragen, as implemented as part of the microRNA Standard Service Premium Analysis (miSSP package). The background adjusted fluorescent values generated by GenePix Pro were normalized for each microRNA using a variation stabilization transformation method described by Huber et al., 2002.

RESULTS

As shown in Fig. 2 and Table 1, both the PAXgene and the Asuragen RNA purification protocols yielded RNA with clearly intact rRNA species and similar 28S/18S and RNA integrity numbers (RIN). Based upon the qRT-PCR results, the relative abundance of 6 distinct mRNA targets was comparable for both RNA isolation protocols (Fig. 3). However, the average yield of the miRNA fraction was 5.5 Ct's, or ~50-fold greater for the Asuragen protocol (Fig. 4). This level of miRNA enrichment was consistent across all 8 miRNAs tested. In addition, two other small RNAs, U6 and 5S, were 10-fold and 26-fold enriched, respectively, by the Asuragen procedure.

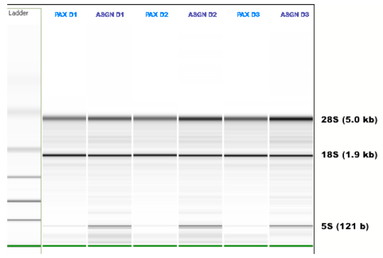


Fig. 2A. The Asuragen microRNA Isolation Protocol from PAXgene–stabilized Whole Blood Enables the Recovery of Small RNA

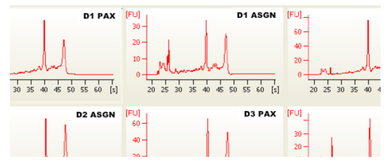


Fig. 2B. Bioanalyzer Profiles of Total RNA Isolated by the Asuragen (ASGN) and PAXgene (PAX) Protocols

		yield (ug/ml blood)	260/280	28S/18S	RIN
Donor 1	PAX	3.67	2.12	0.90	7.6
	ASGN	1.17	2.22	1.10	7.4
Donor 2	PAX	2.78	2.12	1.00	7.9
	ASGN	0.85	2.23	1.40	7.5
Donor 3	PAX	2.01	2.15	1.10	7.8
	ASGN	2.65	2.19	1.50	7.7

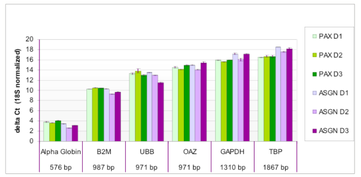
	average yield (ug/ml blood)	yield/ml ratio (PAX/ASGN)
PAX	2.82	1.81
ASGN	1.56	

Table 1. Comparison of Total RNA Yield and Intactness Metrics for Both PAXgene and the Asuragen RNA Isolation Protocols.

To further demonstrate the functionality of the recovered miRNA, we next analyzed the RNA purified using the Asuragen protocol on mirVana miRNA Bioarrays V2 (Fig. 5). This microarray presents 662 probe sets, including probes against 480 distinct human miRNAs. The array results for all 3 donors' RNA surpassed all internal QC metrics (process and specificity controls, negative controls, median background, etc.). As shown in Fig. 6, the signal intensities encompassed a large dynamic range, with many miRNAs undetected. The number absent calls was not unexpected given the extensive non-human miRNA content, and the fact that no tissue presents all known human miRNAs by microarray. Signal concordance among different donors was high (Fig. 7) with R2 values of at least 0.97. This correlation was not positively skewed by the large number of relatively low signals; indeed, if only signals significantly above background (>6) were considered, the R2 value remained >0.97.

To reveal differences between blood and other human tissues, we next compared this array dataset with a reference dataset of 21 normal human tissues. This analysis revealed a number of miRNAs that were extremely overrepresented in blood compared to other tissues (Table 2). Further study of these miRNAs may lend insights into the fundamental biology of blood-borne cells.

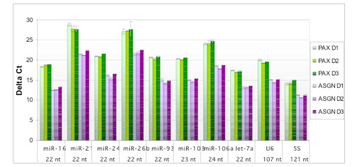
Fig. 3. PAXgene and Asuragen RNA Isolation Protocols Provide Comparable Representation of Select Transcripts after qRT-PCR



Delta Ct (18S normalized)	PAX D1	PAX D2	PAX D3	ASGN D1	ASGN D2	ASGN D3	Average Difference (Ct)
Alpha Globin	3.82	3.59	4.07	3.42	2.59	3.05	0.8
B2M	10.21	10.51	10.41	10.29	9.30	9.69	0.6
UBB	13.32	13.83	12.93	13.52	12.97	11.44	0.7
OAZ	14.52	14.11	14.91	14.95	14.07	15.46	-0.3
GAPDH	15.93	15.62	16.00	17.17	16.04	17.12	-0.9
TBP	16.51	16.72	16.66	18.52	17.58	18.18	-1.5

Isolated RNA samples were diluted in nuclease-free water and each target assayed by qRT-PCR with an input of 1 ng/reaction using the 7900HT Fast Real-Time PCR System (ABI). Real-time data was analyzed using the SDS 2.3 software.

Fig. 4. The Asuragen microRNA Isolation Protocol Recovers ~50-fold More microRNA than the PAXgene Protocol



Delta Ct (18S normalized)	PAX D1	PAX D2	PAX D3	ASGN D1	ASGN D2	ASGN D3	Ave Difference
miR-16	18.25	18.74	18.90	12.50	12.64	13.30	5.8
miR-21	28.80	27.73	27.63	21.42	21.19	22.39	6.4
miR-24	20.96	20.74	21.48	16.18	15.30	16.49	5.1
miR-26b	27.12	27.25	27.69	21.48	21.79	22.44	5.5
miR-93	20.65	20.20	20.74	15.04	14.22	14.81	5.8
miR-103	20.29	20.04	20.58	15.00	14.58	15.33	5.3
miR-106a	24.07	24.16	24.65	18.53	17.82	18.65	6.0
let-7a	17.46	16.93	17.18	13.13	13.10	13.48	4.0
U6	20.02	19.24	19.50	15.06	14.43	15.12	4.7
5S	14.11	14.26	14.90	11.26	10.65	11.19	3.4

Each mRNA and 18S RNA was assayed using the TaqMan® Gene Expression Assay (ABI) according to the supplier's instructions. The inputs of total RNA for each mRNA target were as follows: 1 ng: GAPDH (glyceraldehyde-3-phosphate dehydrogenase), UBB (ubiquitin B), OAZ (ornithine decarboxylase antizyme 1); 5 ng: B2M (beta-2-microglobulin), alpha globin (alpha 1 hemoglobin); and 20 ng: TBP (TATA binding proteinsoftware.

Fig. 5. Sample to microRNA Microarray Workflow

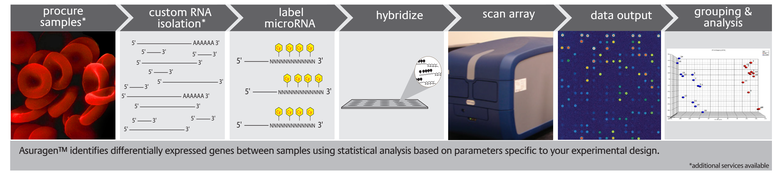


Fig. 6. Bicluster Analysis of Bioarray Detection of microRNA from Human Whole Blood

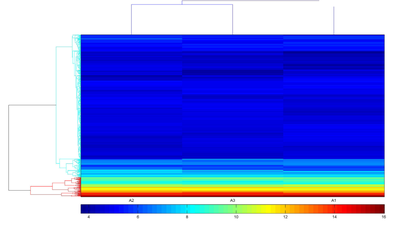


Fig. 7. The Global microRNA Profile from Human Whole Blood is Highly Concordant from Donor to Donor

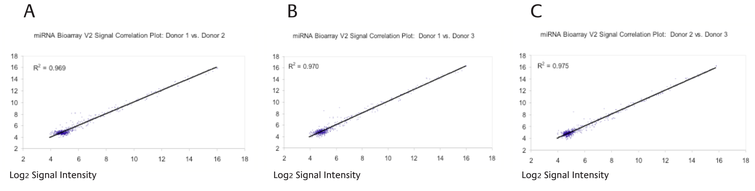


Table 2. A Comparison of the microRNA Microarray Profile from Human Whole Blood with a 21 Human Tissue microRNA Reference Dataset Reveals Many microRNAs that are Highly Enriched in Blood

miRNA	Mean Signal Log2, (Blood)	Mean Signal Log2, (Ref)	p-value	△ Expression (Blood - Ref)
ambi miR 7036	14.12	3.58	3.4E-20	10.54
mmu miR 383	13.58	4.67	1.5E-11	8.91
hsa miR 25	12.14	3.27	3.4E-21	8.86
ambi miR 7070	12.36	3.64	1.4E-12	8.72
hsa miR 126 AS	12.56	4.55	1.9E-06	8.01
hsa miR 200b	11.65	3.99	7.4E-12	7.66
ambi miR 7074	11.09	3.50	3.3E-17	7.59
mmu miR 330	9.67	3.27	2.1E-18	6.39
hsa miR 448	10.47	4.11	1.1E-09	6.36
ambi miR 7083	9.63	3.27	1.8E-18	6.36
hsa miR 10a	9.27	3.32	4.1E-15	5.95
hsa miR 326	9.29	3.47	2.3E-14	5.82
hsa miR 518c AS	8.83	3.28	1.9E-17	5.55
ambi miR 7062	8.72	3.27	3.5E-17	5.45
hsa miR 518c	9.36	3.93	1E-08	5.44
hsa miR 373	13.04	7.63	2.4E-07	5.42
hsa miR 181a	14.10	8.81	0.00125	5.29
hsa miR 375	11.03	5.92	3.3E-05	5.12
hsa miR 24	8.38	3.38	7.6E-15	5.00

CONCLUSIONS

- An RNA isolation procedure from PAXgene-stabilized whole blood is described that recovers highly intact total RNA and comparable representation of mRNA to the standard PAXgene purification protocol, but purifies >10-fold more miRNA and small RNA content.
- The RNA isolated using this protocol is compatible with both qRT-PCR and microarray expression profiling.
- The donor-to-donor miRNA profiles from the whole blood of healthy volunteers are highly concordant.
- A number of miRNAs were identified that are highly enriched in blood compared to a reference tissue microarray dataset of 21 normal tissues.