Establishment of miRNA profile from human whole blood in combination with other genetic data

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Introduction

Profiling of small regulatory RNA (miRNA) as well as messenger RNA (mRNA) expression changes in human tissue holds much promise for the development of genetic markers of disease. Genetic data generated during drug testing or retrospective clinical studies can often be used for identification of biomarkers. Pathological conditions in organs are often detectable in expression profiles from blood samples. Here we present research studies we have initiated to identify miRNA and mRNA signatures in blood.

As a first step, we aimed to minimize experimental noise by optimizing the procedures of blood sample collection, extraction of nucleic acids and expression analyses. For sample collection we chose the PAXgene™ Blood RNA system as it has the ability to "freeze" gene expression profiles at the time of blood collection (1, 2). In addition to total RNA extraction, for which the collection system was originally developed, we developed a procedure for the isolation of small RNA species. The procedure was developed for automatic extraction on a BioRobot® MDx as this system is suitable for large-scale experiments and is well documented in terms of reproducibility, sample tracking, and safety (3).

In order to explore the validity of this procedure, we analyzed a collection of several hundred blood samples. We present data that illustrate technological and biological efficiency of extraction of miRNA and total RNA from a single PAXgene RNA tube. The technological feasibility is illustrated through RNA yield and quality as well as testing it with quantitative PCR assays, high-content miRNA microarrays, and expression arrays. These profiles will help to define a golden healthy expression profile for comparison with disease profiles which are currently being established from comprehensive expression profiling studies.

1. Müller, M.C. et al. (2002) Leukemia **16**, 2395.

2. Rainen, L. et al. (2002) Clin. Chem. **48**, 1883.

3. Kruhøffer, M. et al. (2007) J. Molec. Diagn. **9**, 452.

Materials and methods

Tri-X is a combination of 3 isolation methods starting with 2.5 ml whole blood collected in PAXgene Blood RNA Tubes (PreAnalytiX). The workflow is described in Figure 2. Blood samples from healthy donors were collected and frozen at –80°C within 2 hours after blood withdrawal. The frozen samples were thawed for 16 hours at room temperature in batches of 48 before processing. The automated RNA isolation with the PAXgene Blood RNA MDx Kit (PreAnalytiX) was carried out on a BioRobot MDx (QIAGEN, Figure 1). The small RNA fraction containing the miRNA was pipetted onto a new RNA binding plate (RNeasy® 96 Kit, QIAGEN), and miRNA was procured by adjusting the binding conditions. The membrane was then washed according to the manufacturer's instruction, and finally the miRNAs were eluted. For genomic DNA isolation, the 1 ml aliquots were processed with the QIAamp® DNA Blood Mini Kit or the QIAamp DNA Blood BioRobot MDx Kit (QIAGEN) according to the manufacturer's instructions.

Affymetrix Genechip® Human Mapping 250K SNP arrays (Affymetrix) were used for DNA analysis. All procedures were performed according to Affymetrix standard protocols. Gene expression analyses were performed using the Affymetrix HG U133 plus 2.0 Genechip (Affymetrix). For labeling the MessageAmp™ II Biotin Enhanced labeling kit (Ambion) was used . All labeling reactions were performed either with or without SnX™ globin depletion reagent (AROS Applied Biotechnology). miRNA expression profiling was performed using LNA probes (miRCURY™ LNA Array Probeset V7.1, Exiqon) spotted in duplicate on CodeLink slides (GE Healthcare). miRNA equivalent to 2 µg of total RNA, isolated from the same PAXgene Blood RNA Tube, was used as starting material in the HY3™ enzymatic labeling reaction (miRCURY™ LNA Array, Hy3/Hy5™ labeling kit, Exiqon). We used TaqMan® miRNA assays (Applied Biosystems) for measuring the miRNA expression quantitatively.

Materials and methods — Instrumentation and workflow

Figure 1. BioRobot MDx workstation. Used for the PAXgene Blood RNA MDx Kit and the QIAamp DNA Blood BioRobot MDx Kit as part of the the TRI-X procedure.

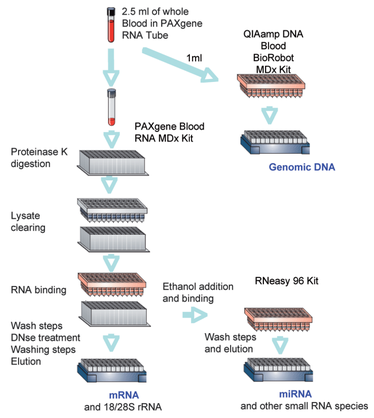

Figure 2. Tri-X procedure. Tri-X starts with 2.5 ml whole blood collected in a single PAXgene Blood RNA Tube. Genomic DNA is isolated from a 1 ml aliquot using the QIAamp DNA Blood BioRobot MDx protocol. The remaining sample is extracted using the PAXgene Blood RNA MDx procedure to isolate miRNAs and large rRNA fragments. Small RNA species, including miRNA, are isolated from the flow-through of the PAXgene Blood RNA MDx protocol binding step via a modified RNeasy 96 procedure.

Results — QC data

- Genomic DNA: sufficient yield for both 250K SNP microarrays from 97% of all samples
- Large RNAs: sufficient yield for gene expression microarray from 96% of all samples, mean RIN = 8.7 ± 0.47
- Small RNAs: clear enrichment, hsa-mR-30b quantitative RT-PCR assays used as quality control

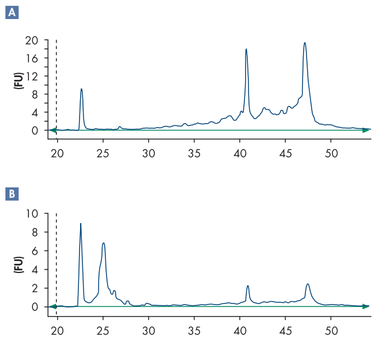

Figure 3. Electropherograms from Agilent bioanalyzer. The Y-axis represents fluorescence units (FU) and the X-axis the runtime (s). A Typical picture of a total RNA preparation with the PAXgene Blood RNA MDx protocol. The bands of the 18S and 28S rRNA fragments are clearly visible and the RIN score is 8.7. B Typical picture of a small RNA preparation isolated from the flow-through from the PAXgene Blood RNA MDx binding step.

gDNA*		Large RNAs†	N	
Yield (ng)	N	Yield (µg)	Eluate	Conc
<250	1	<1	0	6
250–500	3	1–5	67	110
500–1000	36	>5	76	28
1000–1500	54			
1500–2000	25			
>2000	25			

* Yield of genomic DNA isolated from 1 ml aliquots of blood from 144 donors.
† Corresponding yield of total RNA before (Eluate) and after ethanol concentration (Conc).

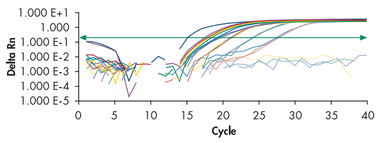

Figure 4. Amplification plot from the has-miR-30b quantitative RT-PCR assay used for quality control of the small RNA fraction eluates.

Results — Genotyping

- Call rates were essentially identical to those achieved with controls
- Gender was correctly determined
- Similar homo- and heterozygotic calls within each group
- IBS metric correlation heat map shows that the control genomic DNA and the individual donor genomic DNA was scored as identical within the groups and different between the groups

Table 2. Report data for 6 Affymetrix Human Mapping 250K SNP Genechips

	Called Gender	SNP Call	AA Call	AB Call	BB Call
Control-1	male	94.48%	38.35%	25.43%	36.22%
Control-2	male	92.84%	38.83%	24.16%	37.01%
Control-3	male	95.99%	38.22%	25.37%	36.41%
Donor1-1	male	93.54%	38.57%	24.52%	36.91%
Donor1-2	male	93.80%	38.77%	24.19%	37.04%
Donor1-3	male	92.13%	38.77%	24.69%	36.54%
Donor2-1	male	92.98%	38.61%	25.02%	36.37%
Donor2-2	male	92.45%	39.23%	23.08%	37.70%
Donor2-3	male	95.41%	38.12%	25.70%	36.18%
Donor3-1	female	94.15%	37.72%	26.16%	36.12%
Donor3-2	female	93.22%	38.38%	25.31%	36.31%
Donor3-3	female	95.17%	37.89%	26.27%	35.84%

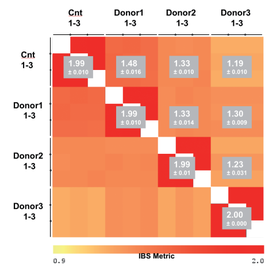

Figure 5. Sample mismatch analysis showing IBS scores of chip to chip comparisons of 250K SNP array. Heat map showing the result of a sample mismatch analysis comparing all SNP arrays against each other. The color of each square indicates the Identity By State (IBS) score between SNP samples ranging from 0.9 to 2.0. The gray boxes indicate the average IBS score and standard deviation between each group. **Cnt**: Control DNA.

Results — Gene expression

- RNA isolated by TRI-X showed good performance on GeneChip U133 plus 2.0 arrays
- Present call rate was comparable to published data
- Globin blocking with SnX reagent was as successful as PNA based method
- SnX method needs much less RNA input

Table 3. Performance of the expression analysis using GeneChip U133 plus 2.0

without SnX			
total ship fluorescence	present calls		GAPDH 3′-5′
scaling factor	N	%	ratio
3.7 (±0.89)	7.077 (±599)	34.4 (±2.7)	1.87 (±0.89)
with SnX			
total ship fluorescence	present calls		GAPDH 3′-5′
scaling factor	N	%	ratio
2.3 (±0.34)	9.731 (±347)	43.7 (±1.6)	1.42 (±0.31)

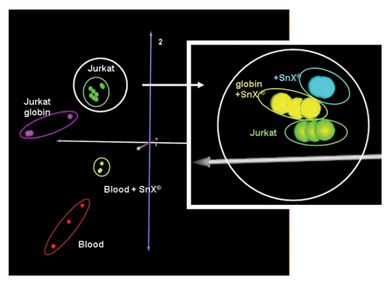

Figure 6. Principle component analysis. Validation of the SnX protocol using blood RNA as well as the GeneChip Globin-Reduction RNA Controls kit (Affymetrix) that provides both positive (Jurkat RNA + globin) and negative (Jurkat RNA) control RNA samples.

Results — MicroRNA expression

- Expression of 338 human miRNAs were examined in the TRI-X small RNA fraction from 4 healthy donors
- 135 miRNAs were detected as present and 27 miRNAs as marginal present
- Signals of additional 17 miRNAs lay near the detection limit of the array
- 140 miRNAs were designated as absent

Table 4. miRNA species detected in blood collected from four healthy donors in PAXgene Blood RNA Tubes

A

Probe name	Probe name	Probe name	Probe name	Probe name
hsa-miR-142-3p	hsa-miR-520d*	hsa-miR-7_MM1	hsa-miR-339	hsa-miR-498
hsa-miR-92	hsa-miR-186	hsa-miR-423	hsa-miR-519e*	hsa-miR-331
hsa-miR-144	hsa-miR-107	hsa-miR-29b	hsa-miR-487a	hsa-miR-335
hsa-miR-27b	hsa-miR-30c_MM2	hsa-miR-20a	hsa-let-7i	hsa-miR-10a
hsa-miR-202	hsa-miR-18a	hsa-miR-503	hsa-miR-199a	hsa-let-7a_MM2
hsa-miR-451	hsa-miR-191	hsa-miR-150	hsa-miR-18b	hsa-miR-519e
hsa-miR-30b	hsa-miR-346	hsa-miR-130a	hsa-miR-27a	hsa-miR-182
hsa-miR-195	hsa-miR-494	hsa-miR-106b	hsa-miR-155_MM1	hsa-let-7b
hsa-miR-452	hsa-miR-19a	hsa-miR-299-3p	hsa-miR-152	hsa-miR-22
hsa-miR-212	hsa-miR-26b	hsa-let-7e	hsa-miR-194	hsa-miR-518c*
hsa-miR-527-518a-2*	hsa-miR-198	hsa-miR-26a	hsa-miR-485-3p	hsa-miR-502
hsa-miR-15b	hsa-miR-20a	hsa-miR-326	hsa-let-7f	hsa-miR-373*
hsa-miR-30d	hsa-miR-193a	hsa-miR-185	hsa-miR-146b	hsa-miR-510
hsa-miR-486	hsa-miR-29c	hsa-miR-19b	hsa-miR-143	hsa-miR-193b
hsa-miR-484	hsa-let-7a	hsa-miR-189	hsa-miR-151_MM1	hsa-miR-130b
hsa-miR-223	hsa-miR-30e-5p	hsa-miR-368	hsa-miR-214	hsa-miR-492
hsa-miR-142-5p	hsa-miR-129	hsa-miR-23b	hsa-miR-365	hsa-miR-98
hsa-miR-25	hsa-miR-370	hsa-miR-93	hsa-miR-519d	hsa-miR-526c
hsa-miR-30c	hsa-miR-342	hsa-miR-188	hsa-miR-95	hsa-miR-512-5p
hsa-miR-17-5p	hsa-miR-23a	hsa-miR-29a	hsa-miR-525	hsa-miR-125b
hsa-miR-324-5p	hsa-miR-145	hsa-miR-101	hsa-miR-345_MM1	hsa-miR-203
hsa-miR-151	hsa-miR-20b	hsa-let-7c	hsa-miR-340	hsa-miR-17-3p_MM2
hsa-miR-30a-5p	hsa-miR-31	hsa-miR-146a	hsa-miR-101_MM1	hsa-miR-320
hsa-miR-453	hsa-miR-106b_MM2	hsa-miR-338	hsa-miR-513	hsa-miR-184
hsa-miR-106a	hsa-miR-21	hsa-let-7g	hsa-miR-200b	hsa-miR-219
hsa-miR-16	hsa-miR-127	hsa-miR-181a	hsa-miR-374	hsa-miR-143_MM2
hsa-miR-15a	hsa-miR-296	hsa-miR-17-3p	hsa-miR-24	hsa-miR-330_MM1

B

Probe name
hsa-miR-105
hsa-miR-100
hsa-miR-514
hsa-miR-455
hsa-miR-148b
hsa-miR-140
hsa-miR-133a-133b
hsa-miR-432*
hsa-miR-202*
hsa-miR-155
hsa-miR-32
hsa-miR-147
hsa-miR-30-3p
hsa-miR-382
hsa-miR-133b
hsa-miR-187
hsa-miR-325
hsa-miR-7
hsa-miR-205
hsa-miR-328
hsa-miR-148a
hsa-miR-125a
hsa-miR-375
hsa-miR-489
hsa-miR-483
hsa-miR-200c
hsa-miR-141

A miRNAs designated as present which means detected in 10–16 spots and
B miRNAs designated as marginal present, which means detected in 6–9 spots.

Results — Differentially expressed microRNAs

- Donor-dependant expression patterns were observed for 15 miRNAs
- The expression pattern of 4 additional miRNAs were classified as marginal differential
- All miRNAs described by Georgantas et al. and Landgraf et al. could be detected in this study

Table 5. Differentially expressed miRNAs detected in blood collected from healthy donors (D1–D4)

	Detected spots each person*			
Probe Name	D1	D2	D3	D4
hsa-miR-220	2	4	4	1
hsa-miR-367	4	1	2	1
hsa-miR-499	2	4	1	2
hsa-miR-363	1	3	4	1
hsa-let-7d	1	2	3	4
hsa-miR-302b	3	0	1	1
hsa-miR-206	4	0	1	3
hsa-miR-500	1	4	0	1
hsa-miR-299-5p	2	3	4	1
hsa-miR-302a*	3	0	1	1
hsa-miR-337	2	4	1	0
hsa-miR-1	3	0	1	1
hsa-miR-149	3	0	1	1
hsa-miR-190	0	1	0	3
hsa-miR-516-3P	0	0	0	3

Table 6. Comparison with findings in recent literature

	expressions described by	
miRNA name	Georgantas et al.§	Landgraf et al.¶
hsa-miR-142		hemapoetic
hsa-miR-144		hemapoetic
hsa-miR-202	PBSC & BM	
hsa-miR-30b	PBSC & BM	
hsa-miR-30d	PBSC & BM	
hsa-miR-223	PBSC & BM	
hsa-miR-25	PBSC & BM	
hsa-miR-29c	only in BM	
hsa-miR-23a	PBSC & BM	
hsa-miR-20a	PBSC & BM	
hsa-miR-150		hemapoetic
hsa-miR-23b	PBSC & BM	
hsa-miR-27a	only in BM	
hsa-miR-155		hemapoetic

§ Georgantas, R.W. et al. (2007) CD34+ hematopoietic stem-progenitor cell microRNA expression and function: A circuit diagram of differentiation control Proc. Natl. Acad. Sci. USA 104, 2750.

¶ Landgraf, P. et al. (2007) A mammalian microRNA expression atlas based on small RNA library sequencing. Cell 129, 1401.

Summary and conclusions

- Yield and quality of total RNA, microRNA, and DNA procured from a single PAXgene Blood RNA Tube with the TRI-X method is sufficient for downstream microarray analysis, and therefore it enables complete nucleic acid profiling from a single blood sample.
- From 338 tested human miRNA species, 162 could be detected as present and 140 as absent in blood.
- We found that at least 15 miRNAs were differential expressed within our group of donors.
- All 14 miRNAs that were described by other researcher to be expressed and/or have a specific expression pattern in hematopoietic cells were found in this study as well.
- In future studies, such combined nucleic acid profiles could help to define a golden healthy expression profile for comparison with disease profiles.

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The PAXgene Blood RNA MDx system, BioRobot MDx, QIAamp DNA Blood Mini Kit, and QIAamp DNA Blood BioRobot MDx Kit are intended for general laboratory use. The RNeasy 96 Kit is intended for research use. No claim or representation is intended to provide information for the diagnosis, prevention, or treatment of a disease. The combination of the PAXgene Blood RNA MDx System with the Affymetrix® GeneChip® probe arrays as described in the section "Materials and methods" is intended For Research Use Only. Not for Use in Diagnostic Procedures.

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