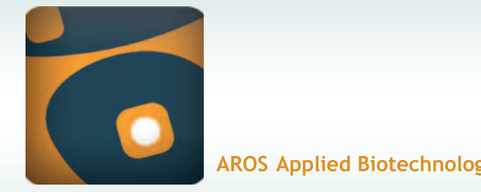
Isolation of MicroRNAs and Other Small RNA Species from PAXgene® Blood RNA Tubes

[1]Thorsten Voss, [2]Mogens Kruhøffer, [1]Maren Wengeler, [1]Franziska Heese & [3]Ralf Wyrich

[1]QIAGEN R&D Department, QIAGEN GmbH, Hilden, Germany; [2]AROS Applied Biotechnologies A/S, R&D Department, Aarhus, Denmark; [3]PreAnalytiX GmbH, Hombrechtikon, Switzerland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Introduction/Background

Pathological conditions, both systemic and tissue-specific, have been shown to be detectable in blood gene expression profiles. The RNA content and profile of unstabilized blood specimens, however, are altered post-phlebotomy due to RNA degradation and gene induction[1,2] leading to erroneous results in quantitative reverse transcriptase PCR (qRT-PCR) assays or microarray analysis. The need for stabilization of cellular RNA for accurate gene expression profiling in blood is widely accepted, and the PAXgene Blood RNA System for stabilization and isolation of cellular RNA is commonly used to address this problem. The PAXgene Blood RNA System was originally designed to isolate high molecular weight intracellular RNA species, such as messenger RNA (mRNA), rather than smaller RNAs such as micro RNA (miRNA). A modified protocol which allowed the separate isolation of small RNAs from PAXgene Blood RNA tubes was recently published.[3] We have now developed a chemistry and protocol that maximizes yields of small RNAs and other cellular RNA species from PAXgene Blood RNA Tubes in one eluate.

The PAXgene Blood miRNA Kit is intended for research use only. Not for use in diagnostic procedures.

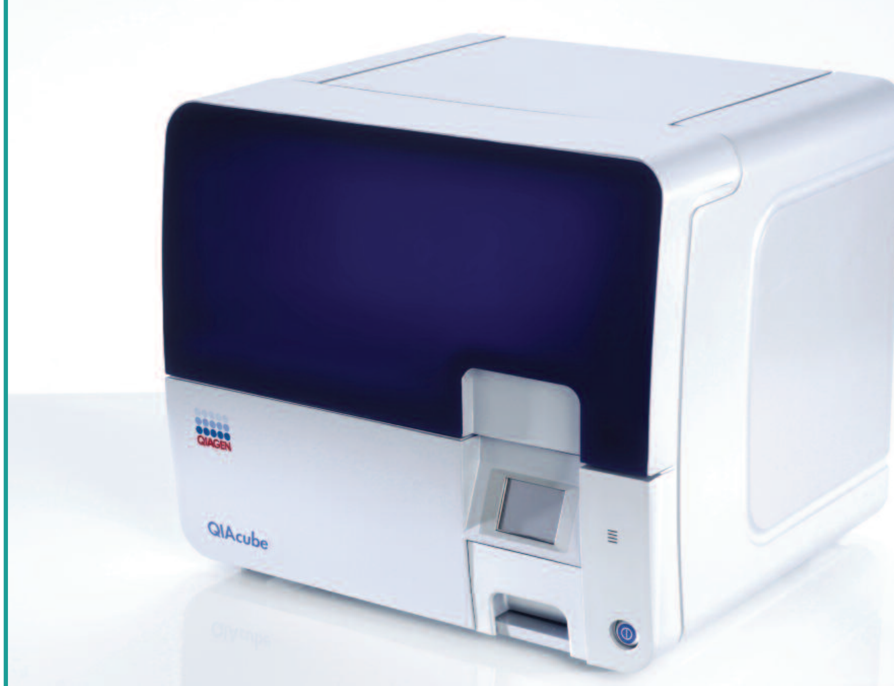

Figure 1. QIAcube® instrument, for automated PAXgene Blood miRNA purification.

Materials and Methods

Blood was collected in PAXgene Blood RNA Tubes (PreAnalytiX) from consented healthy adults, stored for 20-24 h at room temperature and frozen at -20°C. The tubes were thawed 2h before processing. Besides the new PAXgene Blood miRNA Kit (Fig. 2), we used the manual PAXgene Blood RNA Kit (both PreAnalytiX) and the TRI-X method[3] as references. The quantity and purity of the RNA samples were analyzed by spectrophotometric analysis. The integrity and enrichment of small RNAs were determined on an Agilent 2100 BioAnalyser® by using the Nano and small RNA LabChip® as well as with classical gel analysis. Purified RNA was analyzed for genomic DNA contamination and the presence of PCR inhibitors. Downstream methods of quantitative RT-PCR assays, SYBR green or probe based (QIAGEN or Life Technologies) assays, as well as low-density array formats (Life Technologies) were used to evaluate mRNAs and miRNAs. These assays were performed by using 7700 or 7900 PCR systems (Life Technologies). Data were analysed with the MeV ver. 4.4 software (http://www.tm4.org/).

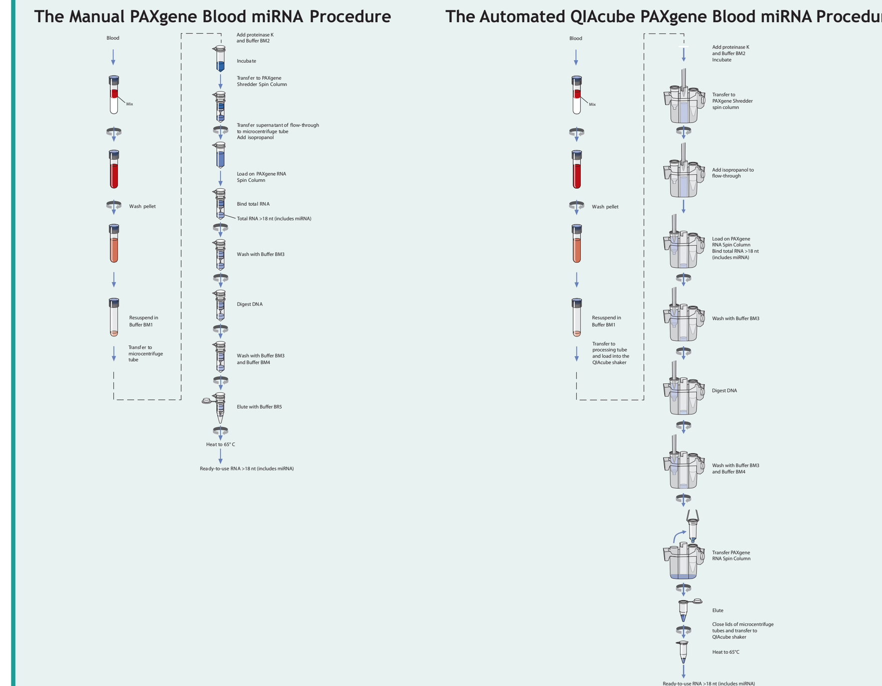

Figure 2. PAXgene Blood miRNA workflow, manual and automated.

Results - QC data

- Total RNA yields were ≥ 3µg for 99% of all samples with a WBC of 4.8-11 x 10^6/ml. Average yield was 6-8µg per tube.
- Very low amounts of gDNA (all below 1% of total nucleic acids) were present in the eluates
- No interference with qRT-PCRs when detecting different mRNA transcripts (Fig. 3).

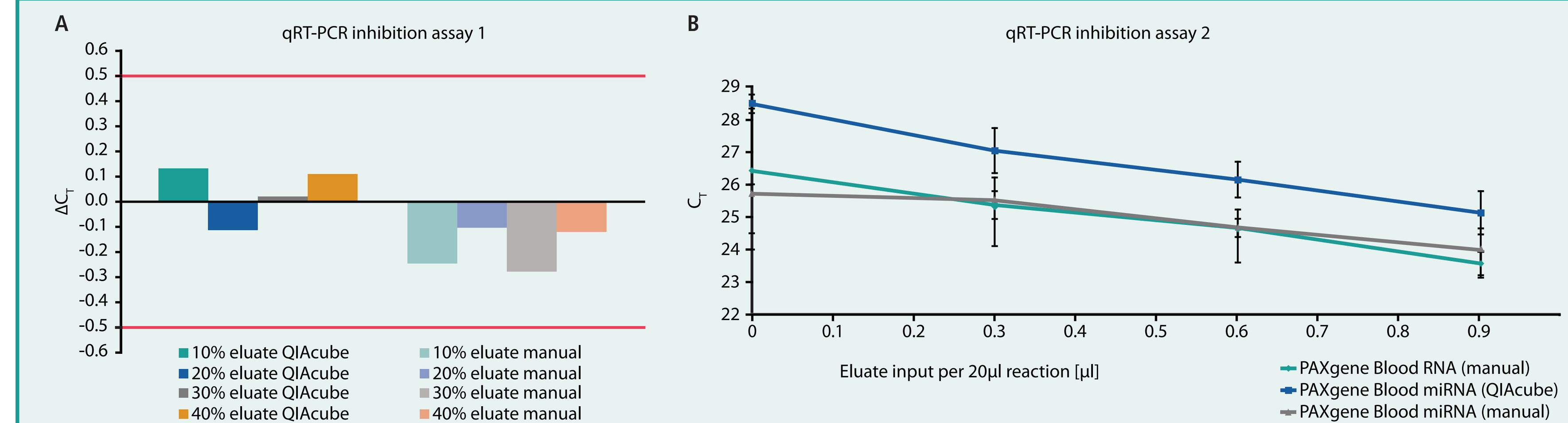

Figure 3. A) PAXgene Blood miRNA eluates from buffer runs were added to a quantitative, real-time RT-PCR assay (β-Actin) at the percentage indicated. A reaction is defined as inhibited if the ΔC_T compared to a unspiked control is ≥ 0.5. B) Increasing amounts of eluates from sample runs were added to another quantitative, real-time RT-PCR assay (IL1β). Between each data point a decrease of 1 C_T should be observed if the reaction is not inhibited.

Results - Enrichment of small RNAs

- Small RNAs were clearly enriched in samples processed with the PAXgene Blood miRNA Kit
- RIN values are comparable to those achieved with the PAXgene blood RNA Kit

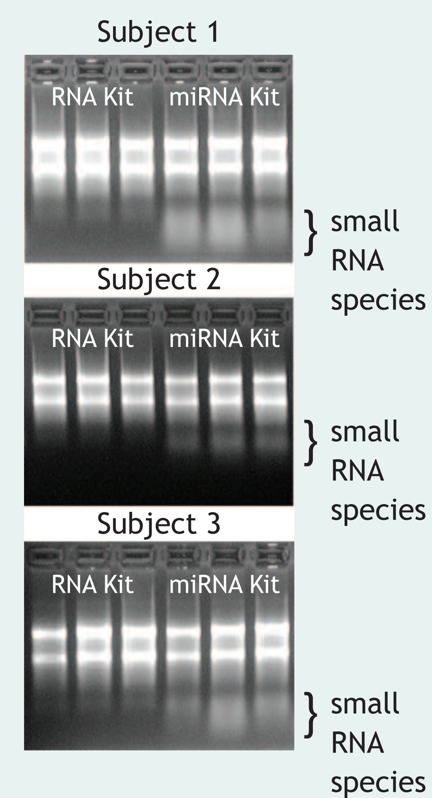

Figure 4. Analysis of eluates by agarose gel electrophoresis.

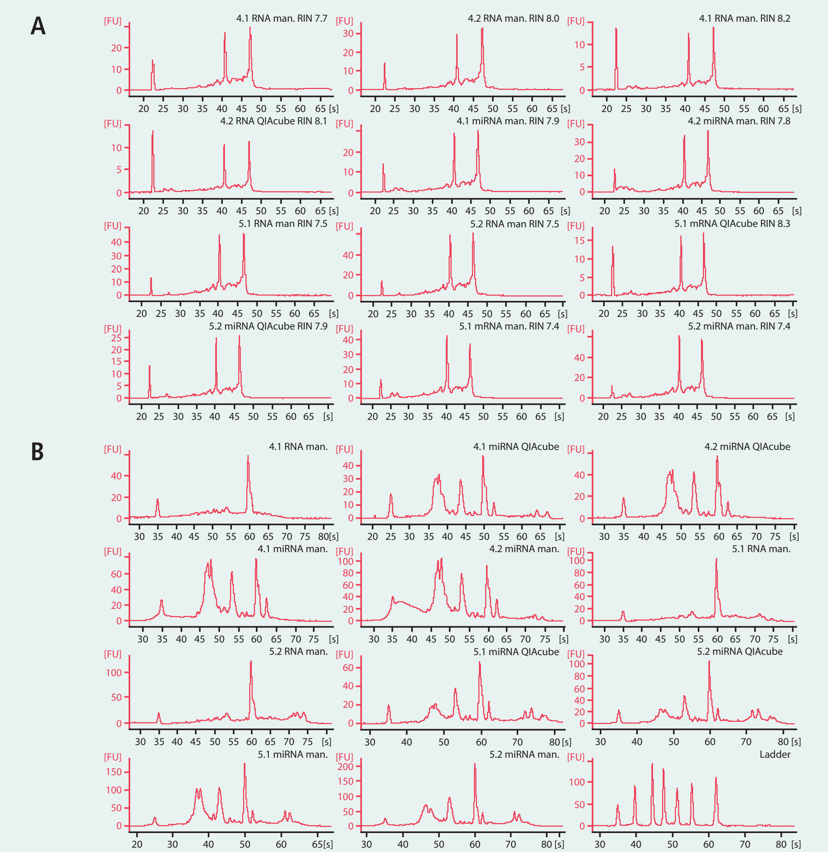

Figure 5. Eluate analysis with the Agilent Nano (A) and Small RNA LabChip (B). Y-axis represents fluorescence units (FU) and the X-axis runtime in seconds (s).

Results - SybrGreen based assays

- All four miRNAs were clearly enriched in samples processed with the PAXgene Blood miRNA Kit

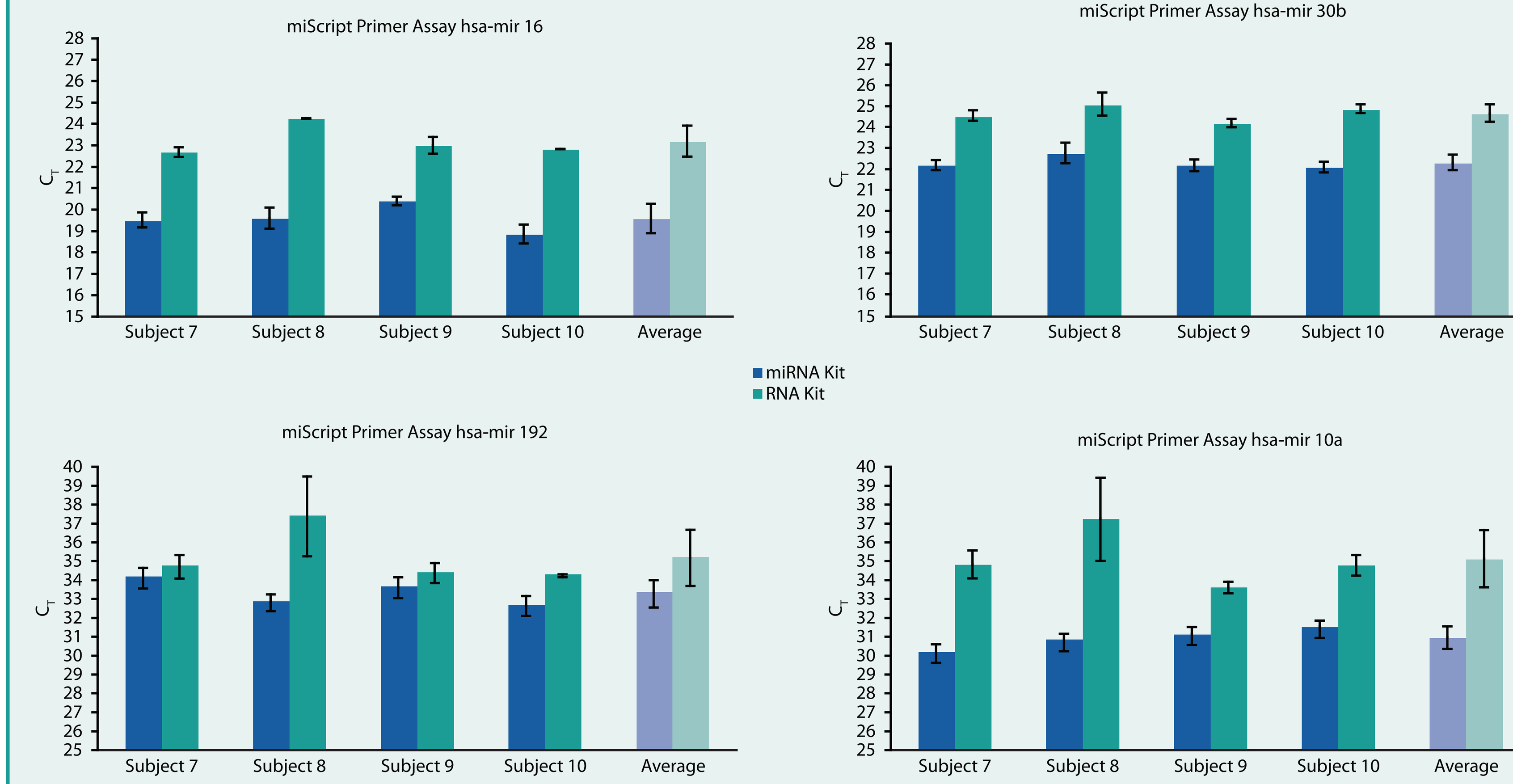

Figure 6. Results of four different miScript Primer Assays comparing the eluates generated with the PAXgene Blood miRNA or the PAXgene Blood RNA Kit.

Results - Probe based assays

- All four miRNAs were clearly enriched in samples processed with the PAXgene Blood miRNA Kit.

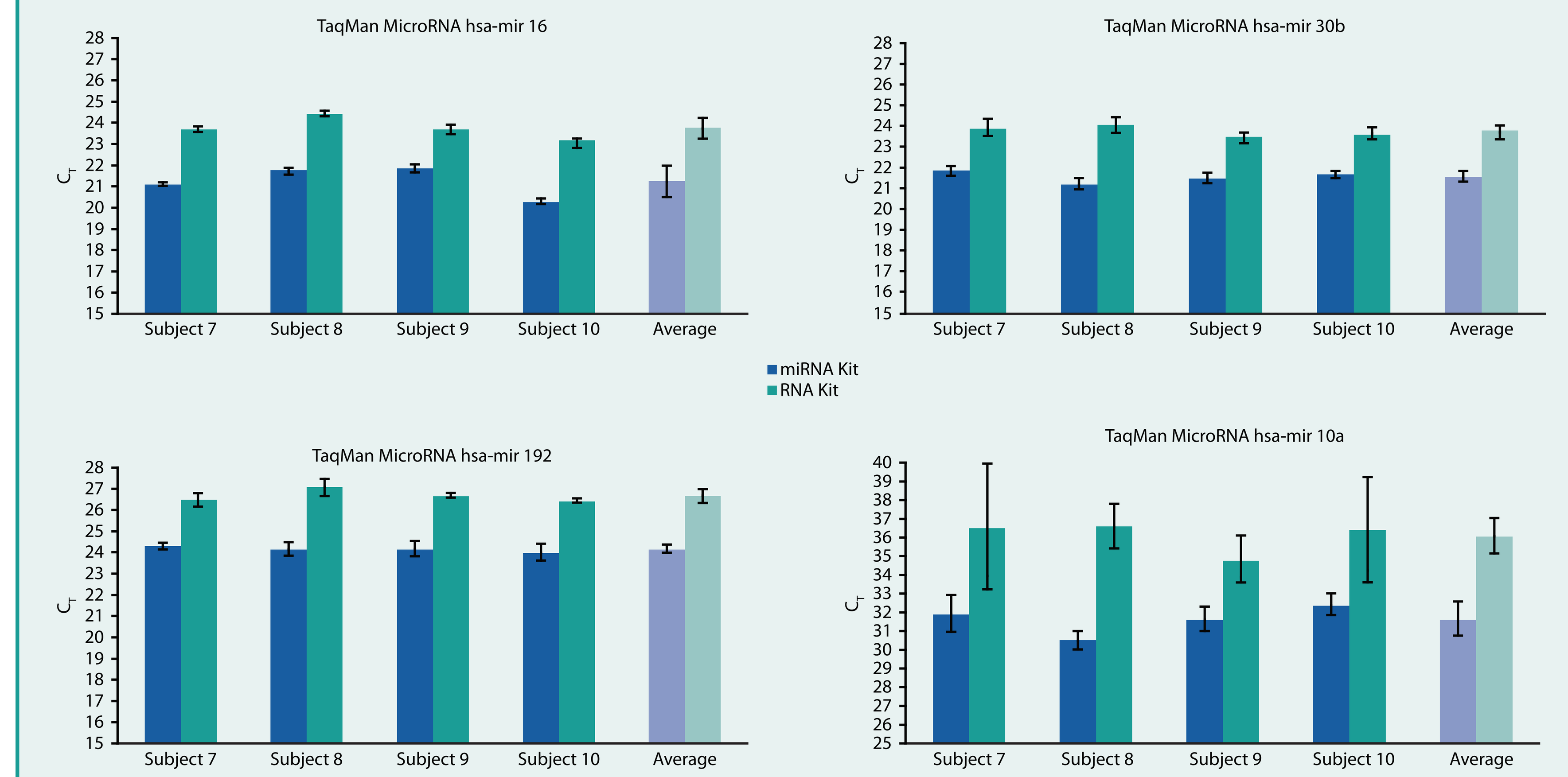

Figure 7. Results of four different TaqMan MicroRNA assays comparing the eluates generated with the PAXgene Blood miRNA or the PAXgene Blood RNA Kit.

Results - Low density arrays

- 728 different miRNAs placed on two low density array (LDA) cards (A and B) were analyzed in parallel with duplicates from two subjects
- With the PAXgene Blood miRNA kit significantly more target could be detected
- More than 90% of the targets detected with all three methods were enriched by the PAXgene Blood miRNA Kit

Table 1. Detected of miRNA on ABI® TaqMan® Human MicroRNA Array Set v2.0

RNA species	Method	Subject	Samples	Yield (µg)	260nm/280nm	RIN	LDA cards	miRNA detected (%)
Large	PAXgene Blood RNA	KK	n = 6	7.8	2.1	9.3	n=2	62.0
		JG	n = 6	5.4	2.1			
		LI	n = 6	9.1	2.1		n=2	64.5
Large & small	PAXgene Blood miRNA	KK	n = 6	8.5	2.1	8.8	n=2	74.3
		JG	n = 6	6.0	2.2			
		LI	n = 6	11.5	2.1		n=2	75.5
Small	TRI-X	KK	n = 6	nd	nd	nd	n=2	75.2
		JG	n = 6	nd	nd	nd		
		LI	n = 6	nd	nd	nd	n=2	75.0

Table 2. miRNA Enrichment Compared Between Targets Detected with All Three Methods

	Experiment 1				Experiment 2	
	Subject KK_1		Subject JG_1		Subject KK_2	Subject JG_2
Comparison	RNA Kit – miRNA Kit	RNA Kit – TRI-X	RNA Kit – miRNA Kit	RNA Kit – TRI-X	RNA Kit – miRNA Kit	RNA Kit – miRNA Kit
Mean ΔC_T	2,4	2,5	2,4	2,0	2,7	2,2
Enriched	92% (n=257)	81% (n=227)	91% (n=256)	78% (n=218)	96% (n=270)	89% (n=250)
Not-enriched	8% (n=23)	19% (n=53)	9% (n=24)	22% (n=62)	4% (n=10)	11% (n=30)

Results - Low density arrays

- Principal component analysis showed expected clustering within the three tested methods which contains three different compositions of cellular RNA (PAXgene blood miRNA Kit large and small RNA species, PAXgene Blood RNA Kit enriched large RNA species, and TRI-X enriched small RNA species).

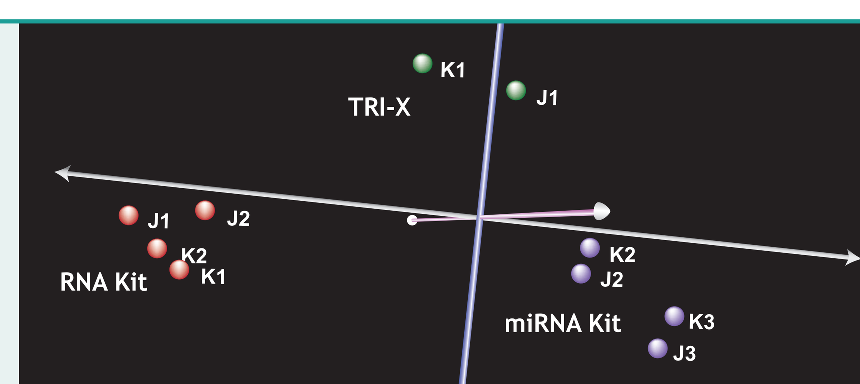

Figure 8. Principal component analysis from data generated with ABI TaqMan Human MicroRNA Array Set v2.0. Data were analysed with MeV ver. 4.4 software. Data of absent miRNA targets were excluded. Blue dots represent samples isolated with the PAXgene Blood miRNA Kit (miRNA Kit), red dots samples isolated with the PAXgene Blood RNA Kit (RNA Kit), and green dots samples isolated with the TRI-X method. K1 means sample KK1, K2 sample KK2, J1 sample LJ1 and J2 sample LJ2.

Summary

- Clear enrichment of small RNA species in eluates generated with the PAXgene Blood miRNA Kit could be shown by electrophoretical methods.
- Clear enrichment of miRNAs could be shown by using two different realtime RT-PCR systems in single tube as well as in low density array format.
- Purification using the PAXgene Blood miRNA Kit provided RNA within specification for purity (260nm/280nm ratio: 1.8 - 2.2) and DNA contamination (≤ 1% w/w).
- Manual and automated isolation procedures lead to comparable results.

Conclusion

- These results show that the dedicated isolation procedures for small RNAs based on the PAXgene Blood RNA Tube result in high quality enrichment of these RNA species which are ready for direct use in sensitive downstream research applications like real-time RT-PCR based on different formats like SybrGreen, TaqMan probes or LDA cards.

References

1. Rainen et al. *Clin Chem.* 2002;48:1883-90.
2. Müller et al. *Leukemia.* 2002;16:2395-99.
3. Kruhøffer et al. *J Mol Diag.* 2007;4:452-8.

Trademarks: PAXgene (PreAnalytiX); QIAGEN, QIAcube (QIAGEN Group); Bioanalyser (Agilent), LabChip (Caliper Life Sciences); ABI (Applied Biosystems LLC), TaqMan (Roche Diagnostics).

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