# Bead Array–Based microRNA Expression Profiling of Peripheral Blood and the Impact of Different RNA Isolation Approaches

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**Blood-based mRNA expression profiling has already become an important issue in clinical applications. More recently, the characterization of the small RNA transcriptome offers additional avenues for diagnostic approaches. However, when applying miRNA expression profiling in routine clinical settings, the method of RNA preservation and the manner of RNA extraction as well as the reliability of the miRNA profiling procedure have to be carefully considered. Here we evaluate a recently introduced bead array–based technology as a robust method for the generation of blood-based human miRNA expression profiles. Importantly the comparison of different RNA extraction strategies resulted in dissimilar profiles depending on the RNA extraction method as well as on the underlying source. Expression profiles obtained from peripheral mononuclear cells (PBMCs) substantially differed from those of whole blood samples, whereby both sources per se yielded reproducible and reliable results. Expression profiles were also distinct when using either fresh or frozen PBMCs. Moreover RNA size fractioning resulted in discriminative miRNA expression profiles compared with total RNA based profiles. This study outlines important steps toward the establishment of a robust strategy for blood-based miRNA profiling and provides a reliable strategy for its implementation in routine handling for diagnostic purposes.** *(J Mol Diagn 2010, 12:000–000; DOI: 10.2353/jmoldx.2010.090116)*

Gene expression profiling has already been widely accepted as a powerful tool to investigate the transcriptome of a given source to describe disease-specific signatures as well as to identify pathogenetic relevant genes on its deregulated transcription. In peripheral blood, gene expression profiling is used to characterize specific signatures for differential diagnosis of hematological neoplasias such as leukemia and also to associate specific blood-based signatures to the occurrence of a variety of other diseases such as infectious disease, cardiovascular disease, autoimmune disease, and cancer.[1] In general, peripheral blood represents an archive of all ongoing processes in an organism and is easily accessible in clinical settings. Therefore, its use for diagnostic and prognostic applications is widely established. However, the analysis of the blood-derived RNA transcriptome requires serial mandatory preconditions. Most important, the RNA should be stabilized immediately after blood taken because even short-time storage of blood cells either at room temperature or under freezing conditions introduces dramatic changes at least to the mRNA composition.[2]

More recently, the discovery of noncoding small RNA transcripts including microRNA (miRNA) has opened new promising avenues for diagnostic implementations of transcriptomics. miRNAs represent a family of functional RNAs of 19 to 23 nucleotides (nt) cleaved from 60- to 110-nt hairpin precursor microRNA (pre miRNA) by Dicer, which is a RNase III enzyme.[3] Pre miRNAs in turn are processed from the primary transcript (pri RNA) by the RNase III Drosha.[4] The regulatory role of miRNAs is conducted by translational repression or degradation of specific target mRNAs.[5,6] MiRNAs exert important roles in developmental

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processes, cell proliferation, hematopoietic differentiation, regulation of lymphoid subset lineage development, oncogenic transformation, and apoptosis.[7] Many reports have already described altered expression of miRNAs in cancer samples compared with normal tissues including breast cancer,[8] sarcomas,[9] leukemias,[10,11] lymphomas,[12] prostate cancer,[13] and autoimmune diseases.[14] These data indicate that analyzing miRNA expression can be used to define tumor subtypes, to identify new clinical and prognostic markers, and to classify human cancer entities. One important result of these efforts is that expression profiling derived from miRNAs can be used for cancer classification with more accuracy than mRNA expression profiles.[15] Interestingly, miRNAs were reported to be actively secreted by tumor cells through the formation of microvesicles.[16,17] Such microvesicles can be traced back in peripheral blood,[17] leading to the assumption that peripheral blood might be a perfect source to monitor tumor-associated miRNA expression signatures for early diagnosis and prediction of therapeutic outcome. Subsequently, most recently blood-based disease specific miRNA signatures were identified in lung cancer patients,[18] patients suffering from multiple sclerosis,[19] as well as in young stroke patients.[20]

Until now, several methods have been developed to allow miRNA identification and quantification including cloning approaches,[3,21] Northern blots,[22,23] real-time PCR,[24] bead-based flow cytometric approaches,[15,25] and customized microarray-based methods.[26–28] Technical issues regarding high-throughput miRNA expression profiling are discussed by various authors.[29,30] With regard to peripheral blood, most of these studies were based on RNA extracted from either whole blood or extracted peripheral blood mononuclear cells (PBMCs).

More recently, a bead array–based assay was introduced comprising 735 human miRNAs allowing high-throughput expression profiling in a large number of samples.[31] In the present study we evaluated this newly introduced array platform for miRNA expression profiling of peripheral blood. We assessed miRNA expression in whole blood as well as in separated PBMCs from healthy individuals. To evaluate the performance of the microarray we studied several aspects of technical reproducibility of the array results, the effect of different normalization approaches, and the influence of total RNA input amount on miRNA expression pattern results. Moreover, we addressed the question whether the enrichment of small RNA fraction in comparison with the use of total RNA improves results of miRNA expression profiling by, e.g., reducing nonspecific hybridization of longer miRNA precursors or interference of target mRNA molecules within the assay. The influence of cryopreservation of isolated PBMCs on miRNA expression profiles was investigated by comparing cryopreserved PBMCs to their directly lysed matched counterparts. Furthermore, we compared whole blood–derived PAXgene-based RNA to a TRIZOL-based approach using PBMCs. Microarray expression values were exemplarily validated by qPCR of selected miRNA sequences. Our comprehensive miRNA screen is intended to serve as a reference for future studies assessing peripheral blood in the context of diagnosis and monitoring of certain diseases in clinical studies.

## Materials and Methods

### Subject Information and Blood Sample Collection

Blood samples from 29 apparently healthy blood donors were collected in two cell preparation tubes with sodium citrate (CPT, Becton Dickinson, Heidelberg, Germany) after written informed consent had been obtained and after approval by the institutional review board. PBMCs were prepared following the manufacturer's protocol.

To evaluate the influence of freezing cells in FCS with 10% (v/v) DMSO and storage in liquid nitrogen on miRNA stability, one portion of the freshly isolated PBMCs was lysed in TRIZOL® reagent (1 ml/1 × 10$^7$ PBMCs, Invitrogen, Karlsruhe, Germany) and stored at −80°C until further processing. The other portion of isolated PBMCs was resuspended in FCS with 10% (v/v) DMSO (1 ml/1 × 10$^7$ PBMCs) and frozen at −80°C. The next day frozen PBMCs were cryopreserved in liquid nitrogen for several days to weeks until further processing.

To analyze the difference between total RNA and less abundant low-molecular-weight (LMW) RNA, we collected blood samples from additional six healthy blood doners.

### RNA Isolation

Total RNA from PBMCs stored in TRIZOL® was isolated according to the manufacturer's protocol. For RNA isolation from PBMCs stored in liquid nitrogen, cells were removed from liquid nitrogen and transferred to a 37°C water bath until thawing. The thawed cell suspension was quickly transferred to 40 ml chilled RPMI medium and centrifuged at room temperature at 400 × *g* for 10 minutes. Supernatant was removed, and PBMCs were washed once with 50 ml of room temperature RPMI and centrifuged at room temperature at 400 × *g* for 10 minutes. Supernatant was completely removed, and cells were subsequently lysed in TRIZOL® reagent. RNA isolation was then performed according to the manufacturer's protocol.

LMW RNA molecules were enriched using Invitrogen's PureLink miRNA Isolation Kit (Invitrogen, Karlsruhe, Germany) according to manufacture's protocol. LMW RNAs (50 to 350 ng) were enriched from 1 to 2 $\mu$g of total RNAs, and 10 to 70 ng of the enriched RNAs were used for sample labeling and array hybridization.

For the comparison of isolation techniques blood samples from six apparently healthy blood donors were collected either in CPT or in PAXgene Blood RNA Tubes (PreAnalytiX, Hombrechtikon, Switzerland). PAXgene Blood RNA Tubes were stored at −20°C until further processing. PBMCs were prepared from CPT following the manufacturer's protocol and stored at −80°C. Total RNA from PBMCs was isolated using TRIZOL® reagent (Invitrogen) according to the manufacturer's protocol. RNA from PAXgene Blood RNA Tubes was isolated using the PAXgene Blood miRNA $\beta$ Version extraction kit.

## Analysis of RNA Samples

Total RNA was quantified by UV-spectroscopy at 260 nm. The quality of the isolated RNA samples was determined by measuring the A260/A280 ratio, and the integrity of the ribosomal 28s and 18s bands was determined by agarose-gel electrophoresis.

## MiRNA-Microarray Procedure

MicroRNA expression profiling was performed using the MicroRNA Profiling β-Test Assay Kit for Sentrix Array Matrixes (Illumina, CA). This system is a modification of the high throughput gene expression profiling assay DASL® (cDNA-mediated annealing, selection, extension, and ligation),[32] which provides a novel highly multiplexed assay and 96-sample Sentrix Array Matrix (SAM) readout.

The human miRNA panel used contained 735 miRNA-specific oligos detecting 470 well-annotated human miRNA sequences (miRBase: *http://microrna.sanger.ac.uk/*, version 9.1, date of accession 02/2007) and 265 potential miRNAs that were identified recently.[33,34]

The miRNA microarray assays were generally performed with 500 ng total RNA if not otherwise stated. All steps were performed according to the manufacturer's protocol. In brief, in a first step input RNA is polyadenylated and converted to cDNA using a biotinylated oligo-dT primer with a universal PCR sequence at its 5′ end. In a second step the biotinylated cDNA is annealed to the 735 miRNA-specific oligos. The mixture is bound to streptavidin-conjugated paramagnetic particles to select cDNA/oligo complexes. After washing out mis-hybridized and non-hybridized oligos, a polymerase is added to extent the miRNA-specific oligos. Oligos are only extended if their 3′ bases are complementary to the cognate sequence in the cDNA template.

A PCR-based universal amplification of the extended miRNA specific primers is then performed, creating fluorescently labeled products. The labeled PCR products correspond to specific original miRNAs in the RNA sample. After PCR amplification the single-stranded PCR products are prepared for hybridization to the SAM. PCR products were hybridized for 16 hours to the beads on the arrays containing complementary address sequences. After hybridization signal intensities at each address location were measured using Illumina BeadArray Reader 500× (Illumina, CA). The intensities of the signals correspond to the quantity of the respective miRNA in the original sample.

## Statistical and Bioinformatics Analysis

Raw data extraction of miRNA microarrays was performed with Illumina Beadstudio 3.1.1.0 software using the Beadstudio Gene Expression Analysis Module 3.1.8. All further analysis was performed in R statistical software (version 2.8.0) using Bioconductor packages.[35] Prior analysis data quality assessment was performed, and samples with lower overall intensity distributions and decreased number of miRNA transcripts detected as present were excluded from further analysis. For further analysis we used quantile normalization implemented in the Bioconductor affy package.

Variable miRNAs were defined by a coefficient of variation (SD/mean) between 0.5 and 10. Determination of present calls was based on the detection $P$ value assessed by Beadstudio software; a miRNA transcript was called present if the detection $P$ value was <0.05. Otherwise the miRNA transcript was called absent. Differentially expressed miRNAs were selected using a fold-change/$P$ value filter with the following criteria: Only $P$ values smaller than 0.05 and an expression change higher than twofold and a difference between mean intensity signals greater 100 were considered statistically significant for further analysis. The Benjamini–Hochberg method was used to adjust the raw $P$ values to control the false discovery rate. The fold-change was calculated by dividing the mean intensity of the miRNAs in one group by that in the other group. If this number was less than one, the negative reciprocal was used.

Hierarchical cluster analysis was performed using the hcluster method in R. Before clustering, the data were log2 transformed. Distances of the samples were calculated using Pearson correlation, and clusters were formed by taking the average of each cluster. Principle component analysis was performed using the pcurve package in R. When visualizing principle component analysis results, the first three principal components (coordinates) were z-transformed (mean, 0, SD = 1) and subsequently plotted in three dimensions.

## Validation of miRNA Expression Results

Quantitative (q) PCR analysis of a selected number of miRNA targets was performed on the six aforementioned blood samples from healthy donors. Three RNA isolation approaches were compared: total RNA, enriched small RNA, and PAXgene-isolated RNA. Twelve miRNAs were selected (hsa-miR-100, 125a, 125b, 135a, 146a, 150, 17-3p, 221, 26a, 31, 93, and 328), and data were produced as described previously[31] using those RNA samples that were also analyzed in array based miRNA profiling. Absolute association of normalized miRNA array expression intensities (log10) versus the negative cycle treshhold (Ct) value was explored via Spearman's correlation coefficient.

## Results

### High Technical Reproducibility of the miRNA Assay in Peripheral Blood

Before analyzing miRNA expression profiles in peripheral blood we addressed technical aspects of the newly introduced microarray technology. Therefore, the technical reproducibility of miRNA profiles was evaluated within and between different SAM devices, which allow the assessment of 735 miRNA profiles of 96 samples in parallel. First, RNA samples derived from PBMCs of 11 different healthy donors (biological replicates) were analyzed in triplicates on one SAM (intra-SAM reproducibility), and in the

next step the same 11 RNA samples used for the intra-SAM evaluation were analyzed on two additional SAMs (inter-SAM reproducibility). Before analyzing the data, quality assessment was performed according to the following criteria: lower overall intensity distributions and decreased number of miRNA transcripts detected as present with both expression means and present calls below the 10% quantile. In general, good intra-SAM as well as inter-SAM replication was achieved (Figure 1A). Of the 55 technical replicates, only six replicates were identified as hybridization outliers. As demonstrated in Supplemental Figure S1A at *http://jmd.amjpathol.org*, technical outliers exhibit both decreased median signal intensity and lower amount of present calls in box graph plots. Furthermore, the reduced correlation of the outliers results in widespread scatter plots, as shown for replicate three of sample one in comparison with replicates one and two (see Supplemental Figure S1B at *http://jmd.amjpathol.org*). These five samples were removed from further analysis. Different normalization procedures were compared between replicate samples including quantile, variance stabilization normalization (VSN), invariant, and loess normalization (see *Materials and Methods*). Similar to results published by Rao et al,[36] in our miRNA dataset the highest overall correlation between samples and the lowest variance was observed after quantile normalization (Supplemental Figure S2 at *http://jmd.amjpathol.org*). Hence, quantile normalization was used in all following analyses.

The intra- and inter-SAM reproducibility of the replicates was estimated by calculating the Pearson correlations ($r^2$) for all pair-wise combinations of individual miRNA profiles within a given sample. The overall mean correlation coefficient in intrareproducibility was 0.9933 ± 0.0066. For inter-SAM analysis the overall mean correlation coefficient was 0.9880 ± 0.0069. Both calculated $r^2$-values are displayed in Figure 1A. Taken together, intra- and inter-SAM reproducibility of the miRNA microarray assay was very high. To assess whether the variability in miRNA expression among the 29 healthy subjects is associated with specific interindividual parameters, we correlated the expression values of all miRNAs with age and gender (see Supplemental Table S1 at *http://jmd.amjpathol.org*). Only one miRNA (hsa-miR-126*) had a significant moderate correlation with age ($r^2 = 0.69$), and none was significantly correlated with gender.

To test the lowest amount of total RNA derived from PBMCs, which still yields reliable results in the miRNA assay, a titration experiment was performed using a broad range of input amounts of total RNA (2, 12.5, 25, 50, 100, 200, 500 ng) from three healthy individuals, which were tested in triplicates. Analysis of variance was performed to assess the reproducibility of miRNA microarray data in the titration experiments by comparing all RNA input amounts below 500 ng against the 500 ng RNA reference, and the correlation within each biological replicate were calculated. As demonstrated in Figure 1B, correlations remained relatively constant when using 100 to 500 ng total RNA (mean $r^2 = 0.97$) indicating that the input of 100 ng total RNA still leads to reliable results. Furthermore, data were still reproducible for replicates with 2 ng total RNA ($r^2 > 0.9$). When comparing all input amounts of 2, 12.5, 25, 50, 100, and 200 ng to the reference of 500 ng, there was a statistically significant difference (*t* test *P* value <0.05) for the samples with 50 ng or less input amount indicating that less than 100 ng total RNA is not sufficient to produce reliable results in peripheral blood derived samples.
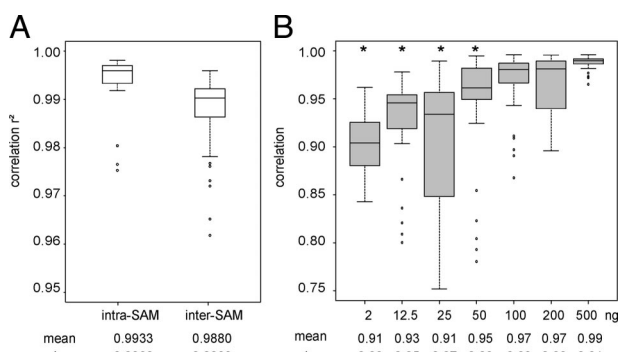


**Figure 1.** Analysis of peripheral blood samples with miRNA microarrays. **A:** Data reproducibility between and within Sentrix-Array Matrix (SAM) devices. The intra- and inter-SAM data reproducibility was estimated by calculating the Pearson correlation coefficients ($r^2$ -values) for all pair-wise combinations of technical triplicates derived from 11 different blood donors (biological replicates) analyzed on the same and on different SAM. Prior analysis technical outliers were identified exhibiting lower overall intensity distributions and decreased number of miRNA transcripts detected as present. Intra- and inter-SAM correlations are calculated using quantile normalized data and are displayed in boxplots. The boundary of the box closest to zero indicates the 25th percentile, the line within the box marks the median, and the boundary of the box farthest from zero indicates the 75th percentile. Whiskers above and below the box indicate the 90th and 10th percentiles. Outliers are plotted as dots. Mean value and SD are displayed below. **B:** Analysis of total RNA input on overall performance of miRNA microarrays. Correlation coefficients were estimated between groups of total RNA input amount <500 ng versus the 500 ng group in the quantile normalized data set. The boundary of the box closest to zero indicates the 25th percentile, the line within the box marks the median, and the boundary of the box farthest from zero indicates the 75th percentile. Whiskers above and below the box indicate the 90th and 10th percentiles. Outliers are plotted as dots. The significance of differences between the groups was tested with unpaired *t* test. * indicates a significant difference in comparison with the 500 ng group with a *P* value <0.01. Mean value and SD are displayed below.

### miRNA Assay Specificity of Blood-Derived Total versus Enriched LMW RNA miRNA Expression Profiles

Next, we were interested in whether the presence of other RNA molecules such as mRNA including miRNA precursor RNA or ribosomal RNA had any influence on the blood expression profiling of miRNAs. To address this question, we compared PBMCs derived miRNA profiles of total RNA to enriched LMW RNA profiles from six healthy individuals. Therefore, fractions of total RNA as well as of less abundant LMW-enriched RNA below 200 bp were extracted. In contrast to the total RNA fraction, pri-miRNA sequences as well as mRNAs and ribosomal RNAs should be depleted in the LMW RNA fraction. The mean overall correlation coefficient between samples for total RNA was 0.9907 ± 0.0043 and for enriched LMW-RNA 0.9786 ± 0.0113, indicating a high similarity between the samples and showing that LMW RNA enrichment procedure introduces only little variation (data not shown).
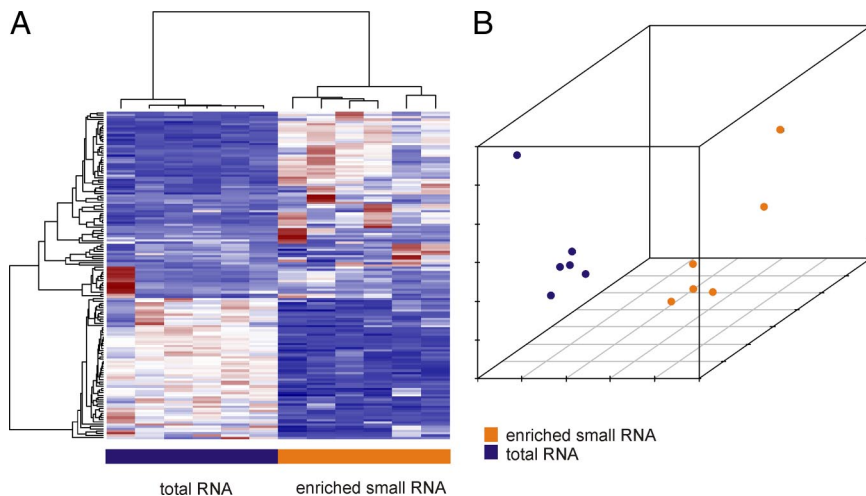
**Figure 2.** Difference between total RNA and enriched small RNA in miRNA expression profile. **A:** Unsupervised hierarchical cluster analysis of total and enriched small RNA. Hierarchical clustering was performed on quantile normalized data (suffix A, orange bar = enriched small RNA; suffix B, blue bar = total RNA) using the most variable miRNAs in the dataset (SD/mean 0.5 to 10). **B:** Principal component analysis of total and enriched small RNA. Principal component analysis of either total RNA (blue) or enriched small RNA (orange) was performed on quantile normalized data using the most variable miRNAs in the dataset (SD/mean 0.5 to 10).

Next the miRNA expression profiles of total and enriched LMW-RNA were compared. By statistically testing for differentially expressed miRNAs, 134 miRNAs of 735 (18%) were identified to be significantly changed in the process of LMW RNA enrichment (fold-change > ±2, total difference in mean signal intensity between both groups >100 and Benjamini–Hochberg adjusted *P* value <0.05). In total 60 transcripts exhibited diminished signal intensities in enriched LMW-RNA and 74 transcripts had increased signal intensities. Thereof, 24 transcripts were predicted miRNA sequences and 110 were known miRNAs. Supplemental Table S2 at *http://jmd.amjpathol.org* summarizes these transcripts. Unsupervised classification analysis, hierarchical clustering, and principal component analysis based on the most variable transcripts in this dataset demonstrate a clear separation of the expression profile between total and enriched small RNA as shown in Figure 2, A and B.

Taken together, there is a significant difference in the expression profiles between total and enriched LMW RNA, indicating that the miRNA assay is sensitive to size fractionation.

## Effect of Cryopreservation of PBMCs on miRNA Expression Profiles in Blood Samples of Healthy Individuals

Based on our experience with gene expression data, we have clear evidence that sample storage and preparation techniques influence gene expression results.[2] Because many tissues and cells from clinical samples are often stored for long time periods in liquid nitrogen (i.e., to allow retrospective gene or miRNA expression analysis), it is important to know whether expression profiles remain stable or undergo *in vitro* changes. We therefore assessed whether the storage of viable cells in liquid nitrogen has any impact on the stability of miRNA transcripts. For this purpose, miRNA expression profiles derived from total RNA obtained from fresh or frozen PBMCs (see *Materials and Methods*) of 29 healthy individuals were compared.

Although the mean overall Pearson correlation coefficient for the sample-wise comparison between fresh and frozen PBMCs was 0.9291 ± 0.0292, the hierarchical cluster analysis clearly demonstrated an incisive effect
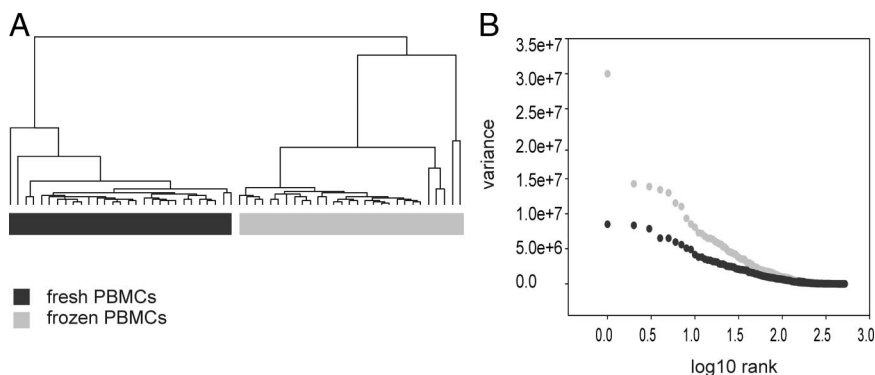


**Figure 3.** Analysis of effect on sample handling on miRNA expression profiles. **A:** Unsupervised hierarchical cluster analysis on freshly isolated or cryopreserved PBMC samples. RNA was prepared either directly after lysis in Trizol (suffix A, dark gray bar = fresh PBMCs) or stored in liquid nitrogen for several days up to several weeks (suffix B, light gray bar = frozen PBMCs) before RNA preparation. Hierarchical clustering was performed on quantile normalized data using the most variable miRNAs in the dataset (SD/mean 0.5 to 10). For hierarchical clustering only the dendrogram is shown. **B:** Overall variance in fresh and frozen PBMC samples. Overall variance in fresh PBMC (dark gray) and frozen PBMC (light gray) samples was ranked and plotted. Variance was calculated in quantile normalized data for each miRNA transcript that was called present (detection *P* value <0.05) in at least one-third of the 29 samples.

on miRNA expression profiles, resulting in a clear separation of fresh PBMCs from frozen specimens (Figure 3A). None of the related sample pairs clustered together or were grouped together, indicating that fresh and frozen cells cannot be compared directly.

Comparison analysis revealed 38 miRNA transcripts (=5.17%) that significantly differ (fold-change > ±2, total difference in mean signal intensity between both groups >100 and Benjamini–Hochberg adjusted *P* value <0.05) between both groups with 21 transcripts exhibiting diminished signal intensities in frozen PBMCs samples and 17 transcripts with increased signal intensities including, e.g., miRNAs 132 and let-7b, which were described as clinically relevant earlier.[5,9,41] These transcripts are summarized in Supplemental Table S3 at *http://jmd.amjpathol.org.* Evaluating the variability of miRNA expression data in both groups revealed an overall higher variability in the freshly prepared PBMC group (Figure 3B), indicating that the freezing procedure might affect miRNA transcripts responsible for interindividual variation in healthy subjects.

## Comparison of miRNA Expression Profiles Derived from PBMCs and From Whole Blood Samples

To determine the effect of different blood cell populations and their influence on miRNA expression profiling, we compared RNA derived from PBMCs to RNA from whole blood samples. Therefore, blood samples from six donors were collected into either CPT tubes followed by PBMC separation and total RNA isolation using TRIZOL or were collected into PAXgene Blood RNA Tubes followed by direct RNA extraction using the newly introduced PreAnalytiX's PAXgene Blood miRNA *β* Version kit.

There was no relevant difference in the amount and quality of the obtained RNA. When assessing miRNA microarray quality both RNA isolation techniques revealed comparable results. This is demonstrated by low variances in the signal intensity distributions and overall percent present calls (Figure 4A). No statistically significant difference in mean expression intensity or detection rate was seen. The same is true for intragroup correlation being 0.9639 ± 0.02 for the whole blood derived RNA and 0.9796 ± 0.02 for RNA from PBMCs. When comparing variance derived from PBMCs and whole blood samples we observed increased variance in the whole blood samples (*t* test *P* value <0.05) probably attributable to the increased heterogeneity of cell types in whole blood compared with the PBMC fraction. The intragroup variance is displayed in Figure 4B. The clear differences between whole blood versus PBMC-derived profiles led to a total of 158 miRNAs (21.55%) differentially expressed miRNAs (*P* value <0.05, fold-change > ±2) and a mean $r^2$ between sources of 0.7889 ± 0.180. Of these 158 miRNAs, 92 are up- and 66 are down-regulated in whole blood–derived RNA samples. Among these less abundant miRNAs are two members of the let-7 family (let-7f and let-7g) and mir-21, which have been shown to be involved in different disease areas.[37–39] A summary of the
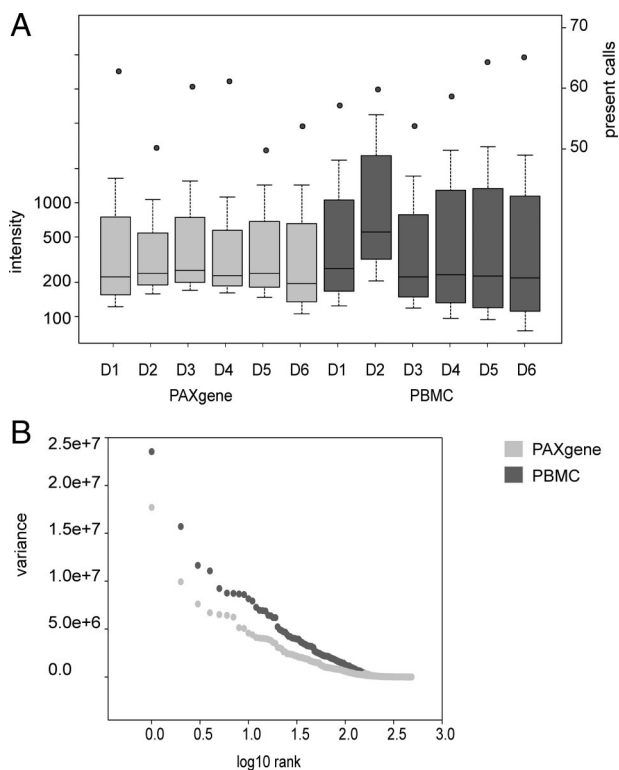


**Figure 4.** Comparison of two different RNA extraction methods. **A:** Comparison analysis of whole blood and PBMC samples. The distribution of signal intensities is presented in a series of 12 box and whiskers plots for two RNA extraction methods of the same six healthy samples. The boundary of the box closest to zero indicates the 25th percentile, the line within the box marks the median, and the boundary of the box farthest from zero indicates the 75th percentile. Whiskers above and below the box indicate the 90th and 10th percentiles. The percentage of probes detected as present with a *P* value <0.05 on the array are noted as the dot plot with the secondary axis. PAXgene samples are displayed in light gray, and the corresponding PBMC samples are displayed in dark gray. **B:** Variance in PAXgene and PBMC samples. Overall variance in PAXgene (light gray) and PBMC (dark gray) samples was ranked and plotted. Variance was calculated in quantile normalized data for each miRNA transcript that was called present (detection *P* value <0.05) in at least one-third of the 12 samples. Statistical difference between corresponding whole blood and PBMC variance was calculated using paired *t* test and resulted in a *P* value <0.05.

158 differentially expressed miRNAs is provided in Supplemental Table S4 at *http://jmd.amjpathol.org*.

## qPCR Validation of miRNA Array Data

To verify the accuracy of this bead-based technology and to validate miRNA expression, qPCR data were explored for 12 miRNAs for blood samples derived from the six aforementioned healthy donors. Three RNA isolation approaches were chosen: total RNA, enriched small RNA, and PAXgene RNA. In all three comparisons absolute normalized expression values from the miRNA array were highly correlated to qPCR Ct values as demonstrated in Figure 5, A–C with Spearman's correlation $r^2$ values of 0.9597 for total RNA samples, 0.8346 for enriched small RNA samples, and 0.8873 for PAXgene RNA samples.

**A** total RNA
correlation r² = 0.9597

**B** enriched small RNA
correlation r² = 0.8346
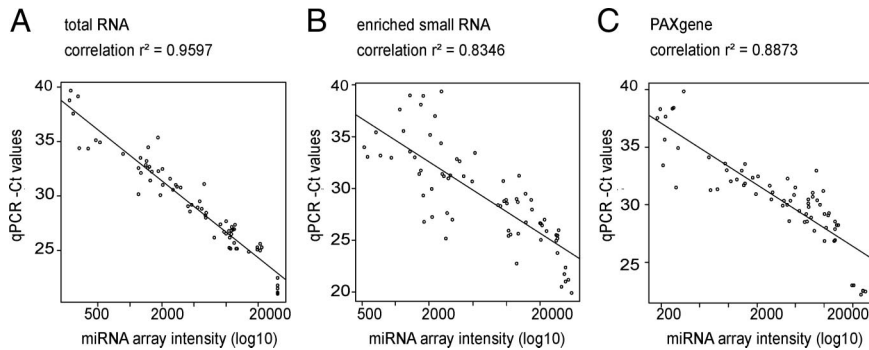
**C** PAXgene
correlation r² = 0.8873



**Figure 5.** Correlation of miRNA array and qPCR expression. Correlation between array and qPCR results are displayed in a scatterplot illustrating the association of normalized miRNA array expression (log10) versus the Ct value for six aforementioned total RNA (**A**), enriched small RNA (**B**), and PAXgene samples (**C**) together with a linear regression fit line. Spearman's correlation coefficients were calculated as well for each RNA isolation technique.

## Discussion

We have studied the performance of a miRNA expression profiling microarray platform when assessing samples derived from peripheral blood in healthy subjects. More importantly, we addressed reproducibility and sample handling issues as well as the influence of different blood-based RNA extraction methods.

To study the reproducibility of data generated on this new miRNA array platform we evaluated several technical replicates derived from peripheral blood samples of healthy subjects. Before data analysis, we checked the quality of every miRNA array and removed samples with lower technical quality from further processing. Next we compared different normalization methods and finally used quantile normalization, because this method showed the highest overall correlation between samples and the lowest variance among replicated samples. These results are in line with the results derived from the study of Rao et al.[36] A high reproducibility of miRNA expression data was estimated by calculating correlation coefficients of technical replicates on the same as well as on different SAM devices. All technical replicates analyzed revealed correlation coefficients >0.98 indicating high reproducibility and reliability of this miRNA microarray assay. Moreover, variability within the miRNA expression profiles was not correlated with gender or (except one miRNA) with age. We thus conclude that the variability within the gene expression profiles is not predominantly influenced by these conditions. A high sensitivity of the miRNA assay method was shown for tissue samples and cell lines by Chen et al,[31] and we extend these findings demonstrating that highly reproducible miRNA expression profiles are generated with 100 to 200 ng total RNA input from PBMCs and stabilized whole blood.

In contrast to mRNA profiling technologies, miRNA profiling must take into account the difference between mature miRNAs and their precursors. The interference of signals from pri-miRNAs and other larger RNAs exhibiting miRNA target sequences has therefore to be avoided.[40,41] Chen et al[31] showed that the method evaluated in this study generates similar expression profiles with total RNA and enriched LMW-RNA having a correlation greater than 0.97 using a prostate adenocarcinoma cell line (PC-3). The data of our study clearly indicate that assay results are different if using total RNA or enriched LMW-RNA derived from peripheral blood. However, reproducibility

and correlation were very high if results were compared between assays using the same preprocessing procedures ($r^2 > 0.99$), showing that the enrichment procedure itself did not add variation to the measurement. Thus, enrichment of LMW-RNA can, on one hand, lower cross-hybridization through inactive precursors of the miRNAs present in total RNA or through additional mRNA interference. On the other hand, this procedure might also lead to a biased composition of mature miRNAs lacking, for example, less abundant active miRNA sequences. Both phenomena could account for the variations we observed when using RNA enrichment. However, because total RNA as well as enriched LMW-RNA thereof give reliable and reproducible results, both methods should be valid for diagnostic purposes when taking into account its specific limitations.

Cryopreservation of clinical specimens, including PBMCs, is a commonly used technique in many clinical trials. In particular for multicenter studies, the performance of batch testing of harvested frozen samples in a central diagnostic unit is often preferred on the independent analysis of fresh samples at different study sites. However, cryopreservation is associated with adverse effects on subsequent functional studies in comparison with those performed with freshly isolated cells (e.g., it has been described recently that cryopreservation of PBMCs results in loss of T cell responses following long-term cryopreservation[42]). Cryopreservation also seems to have an influence on dendritic cells (DCs) monitored by affecting the proportion of myeloid DCs and plasmacytoid DCs and expression levels of DC-associated genes.[43] Furthermore, it can affect cell viability.[44,45] Both the freezing process as such as well as the addition of DMSO enabling the asservation of viable cells could account for these alterations, because DMSO is already known to induce changes to the transcriptome under certain conditions.[46] Here we estimated the influence of cryopreservation of isolated PBMCs on miRNA expression profiles by comparing cryopreserved PBMCs with their matched counterparts directly lysed after isolation. We clearly demonstrated that the miRNA profiles of cryopreserved PBMC samples were not comparable with freshly isolated PBMCs, similar to findings obtained in mRNA expression studies.[2,47] One interesting finding is the increase in signal intensity of miR-132 and let-7b in samples prepared from frozen PBMC samples in com-

parison with freshly isolated PBMCs. MiR-132 was recently identified as up-regulated in malignant B-cells derived from B-CLL patients in comparison with B-cells derived from healthy individuals,[11] and members of the let-7 family are known to regulate transcripts of the proto-oncogene RAS, which are down-regulated in lung cancer specimen.[48] The fact that the expression of these apparently informative miRNAs in diseased tissues seems to be affected by simple sample preparation issues underlines the necessity of uniformly prepared samples for miRNA analysis. A careful and uniform selection of sample material therefore needs to be assured within a study, and attention needs to be paid to those transcripts that are affected by sample handling procedures.

In a clinical setting peripheral blood is the most widely used tissue for disease monitoring. If miRNA expression profiling will become a routine tool for diagnostic and prognostic clinical studies, it is crucial to understand the differences by using either whole blood samples or isolated PBMCs. For gene expression studies of whole blood samples, RNA derived from PAXgene Blood RNA tubes is clinically the most suitable methodology,[2,49–51] because of immediate stabilization of original expression profiles in blood cells. Moreover according to the manufacturers' protocol blood-filled PAXgene tubes can be stored for up to 50 month at $-80°C$ without alteration of the RNA composition. We compared miRNA expression profiles derived from PBMCs using TRIZOL isolation and from whole blood using a PAXgene Blood miRNA Kit $\beta$ Version extraction kit. Most importantly, we assessed the newly introduced PAXgene isolation method as a robust technique for total RNA including miRNA extraction of blood samples whose RNA quality is comparable with RNA obtained from PBMCs using TRIZOL. Analysis of amount of present miRNAs, overall signal intensity, and intragroup correlation gave similar results. Akin to mRNA expression profiling, whole blood–derived miRNA profiles exhibit higher variance and results in miRNAs significantly differentially expressed in comparison with the PBMC-derived profile. Among the miRNAs that are more abundant in PBMCs compared with whole blood, the altered expression of two members of the let-7 family (let-7f and let-7g) and miR-21[37,38] demonstrated again that sample handling is crucial when analyzing expression profiles of potentially clinically relevant miRNA genes. As a higher amount of the differentially expressed miRNAs is up-regulated in PBMCs, those miRNAs could have emerged because of different regulation processes during the PBMC isolation procedure. Alternatively, the additional presence of miRNA transcripts derived from red blood cells,[2] granulocytes, as well as from serum in whole blood RNA samples might lead to different expression profiles when comparing to PBMCs. These miRNAs as well as transcripts that are secreted via microvesicles[13] are of course lost if only analyzing isolated PBMCs. Therefore, using PBMCs might lead to misleading results in diagnostic or prognostic assays.

Finally the expression of 12 miRNAs was determined by qPCR as another established expression technology. Good correlation coefficients between miRNA array expression levels and qPCR values were assessed for total RNA and enriched small RNA as previously reported.[31,52] PAXgene samples performed similarly well. We therefore conclude that the bead array platform revealed reliable results compared with the hitherto used qPCR method as gold standard for miRNA profiling.

Taken together, we present clear evidence that highly reproducible and reliable miRNA profiles of primary human peripheral mononuclear cells as well as of whole blood samples are obtained by the miRNA bead array technology. We clearly demonstrate that sample handling and the choice of either PBMCs or whole blood is a rather critical issue when assessing miRNA profiles. Furthermore, we evaluated that the assay monitors different expression profiles when RNA size fractionation is performed. This comprehensive dataset of miRNA profiles derived from peripheral blood of healthy individuals might serve as a valuable resource for future steps toward the establishment of a comprehensive global miRNA profile of human peripheral blood for diagnostic as well as prognostic purposes.

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## References

1. Staratschek-Jox A, Classen S, Gaarz A, Debey-Pascher S, Schultze JL: Blood-based transcriptomics: leukemias and beyond. Expert Rev Mol Diagn 2009, 9:271–280
2. Debey S, Schoenbeck U, Hellmich M, Gathof BS, Pillai R, Zander T, Schultze JL: Comparison of different isolation techniques prior gene expression profiling of blood derived cells: impact on physiological responses, on overall expression and the role of different cell types. Pharmacogenomics J 2004, 4:193–207
3. Lagos-Quintana M, Rauhut R, Lendeckel W, Tuschl T: Identification of novel genes coding for small expressed RNAs. Science 2001, 294:853–858
4. Jinek M, Doudna JA: A three-dimensional view of the molecular machinery of RNA interference. Nature 2009, 457:405–412
5. Bartel DP: MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 2004, 116:281–297
6. Valencia-Sanchez MA, Liu J, Hannon GJ, Parker R: Control of translation and mRNA degradation by miRNAs and siRNAs. Genes Dev 2006, 20:515–524
7. Ambros V: The functions of animal microRNAs. Nature 2004, 431:350–355
8. Iorio MV, Ferracin M, Liu CG, Veronese A, Spizzo R, Sabbioni S, Magri E, Pedriali M, Fabbri M, Campiglio M, Menard S, Palazzo JP, Rosenberg A, Musiani P, Volinia S, Nenci I, Calin GA, Querzoli P, Negrini M, Croce CM: MicroRNA gene expression deregulation in human breast cancer. Cancer Res 2005, 65:7065–7070
9. Subramanian S, Lui WO, Lee CH, Espinosa I, Nielsen TO, Heinrich MC, Corless CL, Fire AZ, van de Rijn M: MicroRNA expression signature of human sarcomas. Oncogene 2008, 27:2015–2026
10. Calin GA, Ferracin M, Cimmino A, Di Leva G, Shimizu M, Wojcik SE, Iorio MV, Visone R, Sever NI, Fabbri M, Iuliano R, Palumbo T, Pichiorri F, Roldo C, Garzon R, Sevignani C, Rassenti L, Alder H, Volinia S, Liu CG, Kipps TJ, Negrini M, Croce CM: A MicroRNA signature associated with prognosis and progression in chronic lymphocytic leukemia. N Engl J Med 2005, 353:1793–1801

11. Calin GA, Liu CG, Sevignani C, Ferracin M, Felli N, Dumitru CD, Shimizu M, Cimmino A, Zupo S, Dono M, Dell'Aquila ML, Alder H, Rassenti L, Kipps TJ, Bullrich F, Negrini M, Croce CM: MicroRNA profiling reveals distinct signatures in B cell chronic lymphocytic leukemias. Proc Natl Acad Sci USA 2004, 101:11755–11760

12. Lawrie CH, Soneji S, Marafioti T, Cooper CD, Palazzo S, Paterson JC, Cattan H, Enver T, Mager R, Boultwood J, Wainscoat JS, Hatton CS: MicroRNA expression distinguishes between germinal center B cell-like and activated B cell-like subtypes of diffuse large B cell lymphoma. Int J Cancer 2007, 121:1156–1161

13. Mattie MD, Benz CC, Bowers J, Sensinger K, Wong L, Scott GK, Fedele V, Ginzinger D, Getts R, Haqq C: Optimized high-throughput microRNA expression profiling provides novel biomarker assessment of clinical prostate and breast cancer biopsies. Mol Cancer 2006, 5:24

14. Dai Y, Huang YS, Tang M, Lv TY, Hu CX, Tan YH, Xu ZM, Yin YB: Microarray analysis of microRNA expression in peripheral blood cells of systemic lupus erythematosus patients. Lupus 2007, 16:939–946

15. Lu J, Getz G, Miska EA, Alvarez-Saavedra E, Lamb J, Peck D, Sweet-Cordero A, Ebert BL, Mak RH, Ferrando AA, Downing JR, Jacks T, Horvitz HR, Golub TR: MicroRNA expression profiles classify human cancers. Nature 2005, 435:834–838

16. Valadi H, Ekstrom K, Bossios A, Sjostrand M, Lee JJ, Lotvall JO: Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. Nat Cell Biol 2007, 9:654–659

17. Skog J, Wurdinger T, van Rijn S, Meijer DH, Gainche L, Sena-Esteves M, Curry WT Jr, Carter BS, Krichevsky AM, Breakefield XO: Glioblastoma microvesicles transport RNA and proteins that promote tumour growth and provide diagnostic biomarkers. Nat Cell Biol 2008, 10:1470–1476

18. Keller A, Leidinger P, Borries A, Wendschlag A, Wucherpfennig F, Scheffler M, Huwer H, Lenhof HP, Meese E: miRNAs in lung cancer - studying complex fingerprints in patient's blood cells by microarray experiments. BMC Cancer 2009, 9:353

19. Keller A, Leidinger P, Lange J, Borries A, Schroers H, Scheffler M, Lenhof HP, Ruprecht K, Meese E: Multiple sclerosis: microRNA expression profiles accurately differentiate patients with relapsing-remitting disease from healthy controls. PLoS ONE 2009, 4:e7440

20. Tan KS, Armugam A, Sepramaniam S, Lim KY, Setyowati KD, Wang CW, Jeyaseelan K: Expression profile of MicroRNAs in young stroke patients. PLoS ONE 2009, 4:e7689

21. Lau NC, Lim LP, Weinstein EG, Bartel DP: An abundant class of tiny RNAs with probable regulatory roles in Caenorhabditis elegans. Science 2001, 294:858–862

22. Cummins JM, Velculescu VW: Implications of micro-RNA profiling for cancer diagnosis. Oncogene 2006, 25:6220–6227

23. Sempere LF, Freemantle S, Pitha-Rowe I, Moss E, Dmitrovsky E, Ambros V: Expression profiling of mammalian microRNAs uncovers a subset of brain-expressed microRNAs with possible roles in murine and human neuronal differentiation. Genome Biol 2004, 5:R13

24. Chen C, Ridzon DA, Broomer AJ, Zhou Z, Lee DH, Nguyen JT, Barbisin M, Xu NL, Mahuvakar VR, Andersen MR, Lao KQ, Livak KJ, Guegler KJ: Real-time quantification of microRNAs by stem-loop RT-PCR. Nucleic Acids Res 2005, 33:e179

25. Jiang J, Lee EJ, Gusev Y, Schmittgen TD: Real-time expression profiling of microRNA precursors in human cancer cell lines. Nucleic Acids Res 2005, 33:5394–5403

26. Beuvink I, Kolb FA, Budach W, Garnier A, Lange J, Natt F, Dengler U, Hall J, Filipowicz W, Weiler J: A novel microarray approach reveals new tissue-specific signatures of known and predicted mammalian microRNAs. Nucleic Acids Res 2007, 35:e52

27. Castoldi M, Schmidt S, Benes V, Noerholm M, Kulozik AE, Hentze MW, Muckenthaler MU: A sensitive array for microRNA expression profiling (miChip) based on locked nucleic acids (LNA). RNA 2006, 12:913–920

28. Wang H, Ach RA, Curry B: Direct and sensitive miRNA profiling from low-input total RNA. RNA 2007, 13:151–159

29. Nelson PT, Wang WX, Wilfred BR, Tang G: Technical variables in high-throughput miRNA expression profiling: much work remains to be done. Biochim Biophys Acta 2008, 1779:758–765

30. Wang WX, Wilfred BR, Baldwin DA, Isett RB, Ren N, Stromberg A, Nelson PT: Focus on RNA isolation: obtaining RNA for microRNA (miRNA) expression profiling analyses of neural tissue. Biochim Biophys Acta 2008, 1779:749–757

31. Chen J, Lozach J, Garcia EW, Barnes B, Luo S, Mikoulitch I, Zhou L, Schroth G, Fan JB: Highly sensitive and specific microRNA expression profiling using BeadArray technology. Nucleic Acids Res 2008, 36:e87

32. Fan JB, Yeakley JM, Bibikova M, Chudin E, Wickham E, Chen J, Doucet D, Rigault P, Zhang B, Shen R, McBride C, Li HR, Fu XD, Oliphant A, Barker DL, Chee MS: A versatile assay for high-throughput gene expression profiling on universal array matrices. Genome Res 2004, 14:878–885

33. Berezikov E, Thuemmler F, van Laake LW, Kondova I, Bontrop R, Cuppen E, Plasterk RH: Diversity of microRNAs in human and chimpanzee brain. Nat Genet 2006, 38:1375–1377

34. Berezikov E, van Tetering G, Verheul M, van de Belt J, van Laake L, Vos J, Verloop R, van de Wetering M, Guryev V, Takada S, van Zonneveld AJ, Mano H, Plasterk R, Cuppen E: Many novel mammalian microRNA candidates identified by extensive cloning and RAKE analysis. Genome Res 2006, 16:1289–1298

35. Gentleman RC, Carey VJ, Bates DM, Bolstad B, Dettling M, Dudoit S, Ellis B, Gautier L, Ge Y, Gentry J, Hornik K, Hothorn T, Huber W,Iacus S, Irizarry R, Leisch F, Li C, Maechler M, Rossini AJ, Sawitzki G, Smith C, Smyth G, Tierney L, Yang JY, Zhang J: Bioconductor: open software development for computational biology and bioinformatics. Genome Biol 2004, 5:R80

36. Rao Y, Lee Y, Jarjoura D, Ruppert AS, Liu CG, Hsu JC, Hagan JP: A comparison of normalization techniques for microRNA microarray data. Stat Appl Genet Mol Biol 2008, 7:Article22

37. Gabriely G, Wurdinger T, Kesari S, Esau CC, Burchard J, Linsley PS, Krichevsky AM: MicroRNA 21 promotes glioma invasion by targeting matrix metalloproteinase regulators. Mol Cell Biol 2008, 28: 5369–5380

38. Yan LX, Huang XF, Shao Q, Huang MY, Deng L, Wu QL, Zeng YX, Shao JY: MicroRNA miR-21 overexpression in human breast cancer is associated with advanced clinical stage, lymph node metastasis and patient poor prognosis. RNA 2008, 14:2348–2360

39. Yanaihara N, Caplen N, Bowman, Seike M, Kumamoto K, Yi M, Stephens RM, Okamoto A, Yokota J, Tanaka T, Calin GA, Liu CG, Croce CM, Harris CC: Unique microRNA molecular profiles in lung cancer diagnosis and prognosis. Cancer Cell 2006, 9:189–198

40. Shingara J, Keiger K, Shelton J, Laosinchai-Wolf W, Powers P, Conrad R, Brown D: E. Labourier, an optimized isolation and labeling platform for accurate microRNA expression profiling. RNA 2005, 11:1461–1470

41. Davison TS, Johnson CD, Andruss BF: Analyzing micro-RNA expression using microarrays. Methods Enzymol 2006, 411:14–34

42. Owen RE, Sinclair E, Emu B, Heitman JW, Hirschkorn DF, Epling CL, Tan QX, Custer B, Harris JM, Jacobson MA, McCune JM, Martin JN, Hecht FM, Deeks SG, Norris PJ Loss of T cell responses following long-term cryopreservation. J Immunol Methods 2007, 326:93–115

43. Gerrits JH, Athanassopoulos P, Vaessen LM, Klepper M, Weimar W, van Besouw NM: Peripheral blood manipulation significantly affects the result of dendritic cell monitoring. Transpl Immunol 2007, 17:169–177

44. Weinberg A, Zhang L, Brown D, Erice A, Polsky B, Hirsch MS, Owens, Lamb K: Viability and functional activity of cryopreserved mononuclear cells. Clin Diagn Lab Immunol 2000, 7:714–716

45. Disis ML, dela Rosa C, Goodell V, Kuan LY, Chang JC, Kuus-Reichel K, Clay TM, Kim Lyerly H, Bhatia S, Ghanekar SA, Maino VC, Maecker HT: Maximizing the retention of antigen specific lymphocyte function after cryopreservation. J Immunol Methods 2006, 308:13–18

46. Peng CF, Wei Y, Levsky JM, McDonald TV, Childs G, Kitsis RN: Microarray analysis of global changes in gene expression during cardiac myocyte differentiation. Physiol Genomics 2002, 9:145–155

47. Feezor RJ, Baker HV, Mindrinos M, Hayden D, Tannahill CL, Brownstein BH, Fay A, MacMillan S, Laramie J, Xiao W, Moldawer LL, Cobb JP, Laudanski K, Miller-Graziano CL, Maier RV, Schoenfeld D, Davis RW, Tompkins RG: Whole blood and leukocyte RNA isolation for gene expression analyses. Physiol Genomics 2004, 19:247–254

48. Johnson SM, Grosshans H, Shingara J, Byrom M, Jarvis R, Cheng A, Labourier E, Reinert KL, Brown D, Slack FJ: RAS is regulated by the let-7 microRNA family. Cell 2005, 120:635–647

49. Vartanian K, Slottke R, Johnstone T, Casale A, Planck SR, Choi D, Smith JR, Rosenbaum JT, Harrington CA: Gene expression profiling

of whole blood: comparison of target preparation methods for accurate and reproducible microarray analysis. BMC Genomics 2009, 10:2

50. Kruhoffer M, Dyrskjot L, Voss T, Lindberg RL, Wyrich R, Thykjaer T, Orntoft TF: Isolation of microarray-grade total RNA, microRNA, and DNA from a single PAXgene blood RNA tube. J Mol Diagn 2007, 9:452–458

51. Tian Z, Palmer N, Schmid P, Yao H, Galdzicki M, Berger B, Wu E,

Kohane IS: A practical platform for blood biomarker study by using global gene expression profiling of peripheral whole blood. PLoS ONE 2009, 4:e5157

52. Cunningham JM, Oberg AL, Borralho PM, Kren BT, French AJ, Wang L, Bot BM, Morlan BW, Silverstein KA, Staggs R, Zeng Y, Lamblin AF, Hilker CA, Fan JB, Steer CJ, Thibodeau SN: Evaluation of a new high-dimensional miRNA profiling platform. BMC Med Genomics 2009, 2:57