

## Identification of reference microRNAs and suitability of archived hemopoietic samples for robust microRNA expression profiling

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### ABSTRACT

In many cancers, including neuroblastoma, microRNA (miRNA) expression profiling of peripheral blood (PB) and bone marrow (BM) may increase understanding of the metastatic process and lead to the identification of clinically informative biomarkers. The quality of miRNAs in PB and BM samples archived in PAXgene™ blood RNA tubes from large-scale clinical studies and the identity of reference miRNAs for standard reporting of data are to date unknown. In this study, we evaluated the reliability of expression profiling of 377 miRNAs using quantitative polymerase chain reaction (qPCR) in PB and BM samples ( $n = 90$ ) stored at  $-80\text{ }^{\circ}\text{C}$  for up to 5 years in PAXgene™ blood RNA tubes. There was no correlation with storage time and variation of expression for any single miRNA ( $r < 0.50$ ). The profile of miRNAs isolated as small RNAs or co-isolated with small/large RNAs was highly correlated ( $r = 0.96$ ). The mean expression of all miRNAs and the geNorm program identified miR-26a, miR-28-5p, and miR-24 as the most stable reference miRNAs. This study describes detailed methodologies for reliable miRNA isolation and profiling of PB and BM, including reference miRNAs for qPCR normalization, and demonstrates the suitability of clinical samples archived at  $-80\text{ }^{\circ}\text{C}$  into PAXgene™ blood RNA tubes for miRNA expression studies.

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In many solid cancers, such as neuroblastoma (NB)<sup>1</sup>, dissemination of tumor cells through the hemopoietic system is an indicator of poor outcome [1]. Therefore, body fluids such as peripheral blood (PB) and bone marrow (BM) are valuable sources of surrogate markers for prognostication and monitoring of disease status. This is exemplified by the predictive power of specific mRNAs detected in BM and PB from children with high-risk NB [2–5]. MicroRNAs (miRNAs) are distinct from many other biomarkers because of their pathogenic role in controlling gene regulatory networks in cancer development and progression [6–10]. Thus, miRNA expression studies in PB and BM may identify deregulated biological pathways that contribute to the survival and dissemination of tumor cells leading to metastasis and disease recurrence. Standardization of sample collection, analysis, and reporting is a prerequisite to ensure the reliability of gene expression data in large prospective clinical studies. Although the PAXgene™ blood RNA tubes and extraction kits are increasingly recommended for stabilization and isolation of messenger RNA (mRNA) species from PB and BM [11–19], their suitability for robust long-term stabilization of miRNAs remains to be estab-

lished [20–22]. In this study, we evaluate the options for isolating miRNAs from PAXgene™ blood RNA tubes and describe, for the first time, the consistency of miRNA expression profiles obtained in PB and BM samples stored for prospective clinical studies at  $-80\text{ }^{\circ}\text{C}$  for up to 5 years. We compared the expression of 380 miRNAs/small RNAs using TaqMan™ low-density polymerase chain reaction (PCR) arrays (TLDA) and identified suitable reference miRNAs to improve the accuracy of future miRNA expression studies in PB and BM samples.

### Materials and methods

#### Hemopoietic samples

BM aspirates (0.5 ml) and PB (2 ml) from children with high-risk NB ( $n = 40$ ), and BM ( $n = 9$ ) and PB ( $n = 9$ ) from healthy controls were collected in PAXgene™ blood RNA tubes and stored at  $-80\text{ }^{\circ}\text{C}$  for 48 h up to 70 months. To compare miRNA profiles obtained using small RNAs isolated separately or co-isolated with large RNAs, three PB samples from a healthy volunteer were collected in PAXgene™ blood RNA tubes. An additional four samples from another healthy volunteer were processed after 2 days, 1 month, 3 months, and 6 months at  $-80\text{ }^{\circ}\text{C}$ . Written informed consent was obtained from all participants and parents/legal guardians of the children involved in this study for the collection

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<sup>1</sup> Abbreviations used: NB, neuroblastoma; PB, peripheral blood; BM, bone marrow; miRNA, microRNA; mRNA, messenger RNA; PCR, polymerase chain reaction; TLDA, TaqMan™ low-density PCR array; RT, reverse transcription; qPCR, real-time quantitative PCR; C<sub>q</sub>, quantification cycle; PBMC, peripheral blood mononuclear cell.

and use of samples for research purposes. The study was approved by the CCLG (Children's Cancer and Leukemia Group) Biological Studies Group (MREC/98/4/023), the Leeds Teaching Hospital Trust Local Research Ethics Committee, and the Gaslini Institute Ethical Committee.

### Isolation of small RNAs

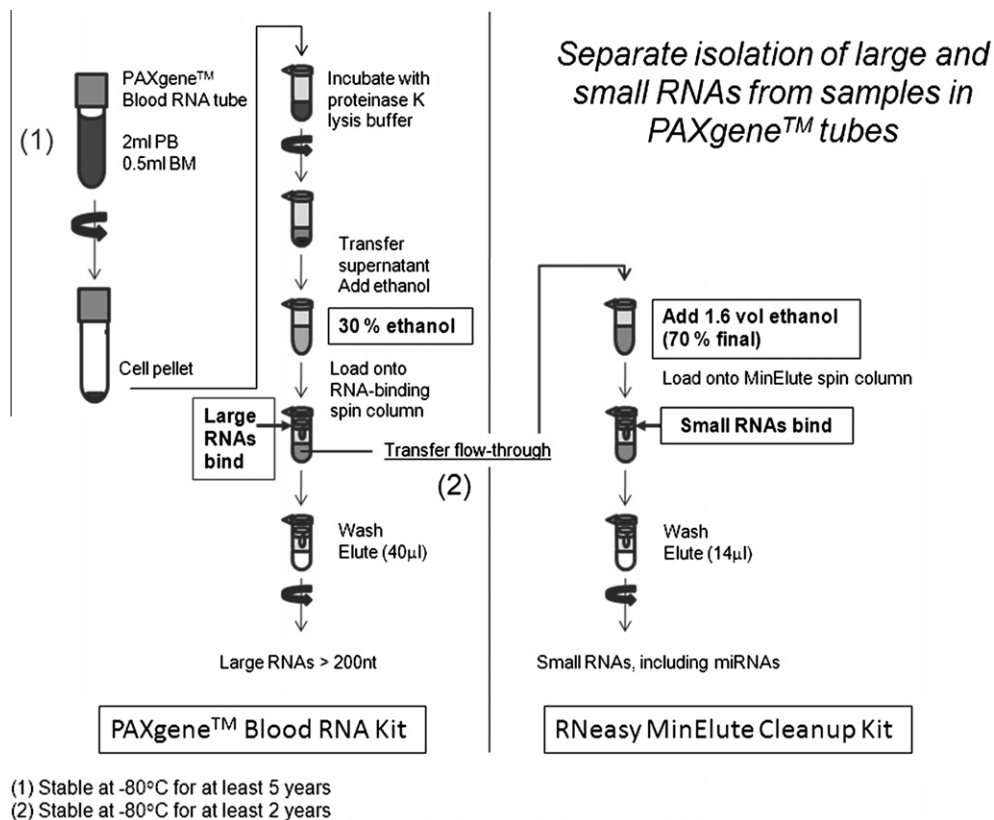
The miRNA profile of small RNAs isolated alone or co-isolated with large RNAs was investigated in PB samples from a healthy volunteer in three independent experiments. PB (2 ml) was collected into PAXgene™ blood RNA tubes and centrifuged at 3500g, and the resulting pellet was resuspended in 5 ml of RNase-free water. This was aliquoted into  $2 \times 2.5$  ml; one of these samples was processed according to protocol A for co-isolation of large and small RNAs (PAXgene™ Blood miRNA Kit, Qiagen, cat. no. 763134), and the second one was processed according to protocol B using the RNeasy MinElute Cleanup Kit (Qiagen, cat. no. 74204) for isolation of small RNAs from the flow-through that is usually discarded when isolating the large RNA fraction using the PAXgene™ Blood RNA Extraction Kit (Qiagen, cat. no. 762165) (Fig. 1). The concentration of recovered small RNAs was evaluated using the Nanodrop ND-1000 (<http://www.labtech.co.uk>). Because the optical density of small RNAs does not directly quantify the concentration of miRNAs, reflecting a large proportion of small ribosomal and transfer RNAs, the concentration of small RNAs for reverse transcription was standardized as that isolated from 400 ng of the large RNA fraction.

### Reverse transcription

The total RNA fraction (400 ng; protocol A) and the miRNA-enriched small RNA fraction (miRNA from the equivalent of 400 ng of large RNA; protocol B) were reverse transcribed using the miRNA reverse transcription (RT) kit and the stem-loop Megaplex RT primers pool A (Applied Biosystems) according to manufacturer's instructions. The volume ( $\mu\text{l}$ ) of the small RNA fraction for Megaplex RT was  $EQ/(CONC * DF)$ , where  $EQ$  is the equivalent amount of large RNAs (ng),  $CONC$  is the concentration of large RNAs (ng/ $\mu\text{l}$ ), and  $DF$  is the dilution factor (eluate volume large RNA/volume of small RNAs, e.g.,  $38 \mu\text{l}/12 \mu\text{l} = 3.17$ ).

### miRNA expression profiling

The expression profile of 380 miRNAs/small RNAs was measured by real-time quantitative PCR (qPCR) using the Applied Biosystems TLDA miRNA array (version 2.0), a 384-well microfluidic card containing 377 preloaded and optimized individual human TaqMan™ miRNA assay targets and 3 small RNA controls (RNU44, RNU48, and mammalian U6 in four replicates). The Megaplex RT products were mixed with TaqMan™ Universal PCR Master Mix-No AmpErase without preamplification, loaded onto the microfluidic card, and amplified on the Applied Biosystems 7900HT qPCR machine. Compliance with the MIQE (minimum information for publication of quantitative real-time PCR experiments) guidelines [23] is listed in Table S1 of Supplementary material. The quantification cycle ( $C_q$ ) for each miRNA was used in subsequent analysis [24];  $C_q$  is calculated using a threshold of 0.2 and a baseline that is automatically se-



**Fig. 1.** Workflow for isolation of miRNA-enriched fraction from samples in PAXgene™ blood RNA tubes (protocol B) adapted from manufacturer's manual. The small RNA fraction is retrieved using the RNeasy MinElute Cleanup column rather than the RNeasy column previously tested [20]. Stability of samples and of the flow-through containing small RNAs at  $-80^\circ\text{C}$  has been validated in this study. Co-isolation of large and small RNAs (protocol A) is performed according to the manufacturer's manual of the PAXgene™ Blood miRNA Kit.

lected by TaqMan™ SDS (sequence detection systems) software (version 2.3). The number of miRNAs detected (with  $C_q$  equal to or below 35; detection of a single molecule) and  $C_q$  values are reported as the means  $\pm$  standard deviations. To standardize reporting, the expression of each miRNA was deducted from the  $C_q$  value using the delta- $C_q$  method [25], and the mean  $C_q$  of all expressed miRNAs was used as a normalization factor [26].

#### Modeling to identify robust reference miRNAs

miRNAs that resembled the mean expression  $C_q$  value were identified by calculating the standard deviation of the difference between the  $C_q$  value of each miRNA and the mean  $C_q$  of all expressed miRNAs/small RNAs per sample [26]. Candidate reference miRNAs that most closely resembled the mean (lowest standard deviation) were ranked according to expression stability using the geNorm program [27]. The geNorm algorithm is based on the principle that the expression ratio of two suitable reference genes should be identical in all samples regardless of the experimental conditions or cell type. For every candidate reference gene, the pairwise variation ( $V$ ) with all other candidate genes is determined as the standard deviation of the  $\log_2$ -transformed expression ratios. The gene stability measure ( $M$ ) for a reference gene is the average  $V$  value for that gene against all other tested reference genes. Lower  $M$  values indicate increased stability of reference miRNAs across samples. The number of reference miRNAs was considered as optimal when the  $V$  value was below 0.15 [27].

#### Statistical analyses

Statistical analyses were performed using GraphPad Prism software (version 5.04). Correlation coefficients were determined using the nonparametric Spearman's correlation  $r$ . To allow for a correlation calculation of storage time with variation of  $C_q$  values of all miRNAs detected in at least one sample,  $C_q$  values above 35 were rescored to 35. Differences between groups were assessed by the nonparametric Mann–Whitney  $U$  test unless otherwise stated. A  $P$  value less than 0.05 was considered as statistically significant.

## Results

#### miRNA expression profiles in total RNA and miRNA-enriched samples

miRNA expression profiles of total RNA (protocol A) and miRNA-enriched (protocol B) (Fig. 1) samples isolated from the same PB were compared. The median total RNA yields were 3.6  $\mu\text{g}$  (range = 3.3–3.9) and 4.4  $\mu\text{g}$  (range = 4.3–4.6) when using protocols A and B, respectively. Following TLDA analyses, the number of detectable miRNAs was  $156.0 \pm 0.6$  with a mean expression  $C_q$  value of  $28.5 \pm 0.1$  when using the separate small RNA fraction (protocol B) and  $143.0 \pm 6.0$  with a mean expression  $C_q$  value of  $28.7 \pm 0.2$  when using the combined large and small RNA fraction (protocol A) (see Table S2 in Supplementary material). These were not significantly different from each other (Wilcoxon  $t$  test,  $P = 0.25$ ). The  $C_q$  values and normalized expression levels of miRNAs detected in both fractions were also highly correlated ( $r = 0.96$ ,  $P < 0.0001$ ) (Fig. 2). In subsequent experiments, miRNAs were isolated using protocol B to maximize the use of precious clinical material.

#### Yield of small RNAs from PB and BM samples

The median small RNA yield of 1.5  $\mu\text{g}$  (range = 0.4–5.0,  $n = 40$ ) from PB samples from children with NB was not significantly different ( $P = 0.061$ ) from the median yield of 1.09  $\mu\text{g}$  (range = 0.17–2.23,

$n = 9$ ) from PB samples from healthy controls. However, the median small RNA yield from BM samples from children with NB of 2.98  $\mu\text{g}$  (range = 0.7–22.4,  $n = 40$ ) was significantly different ( $P = 0.002$ ) from the median yield from BM samples from healthy controls (0.96  $\mu\text{g}$ , range = 0.6–4.3,  $n = 9$ ). There was a moderate correlation between the yield of large and small RNAs recovered from BM and PB samples ( $r = 0.57$ ,  $P < 0.001$  and  $r = 0.70$ ,  $P < 0.001$ , respectively). The yield of small RNAs did not correlate with storage time of sample at  $-80^\circ\text{C}$  in PAXgene™ blood RNA tubes (up to 70 months,  $r = 0.33$ ,  $P = 0.02$ ) or of the flow-through fraction containing small RNAs (up to 24 months,  $r = 0.006$ ,  $P = 0.97$ ).

#### Measurement of miRNAs in small RNA fraction from PB and BM samples by qPCR

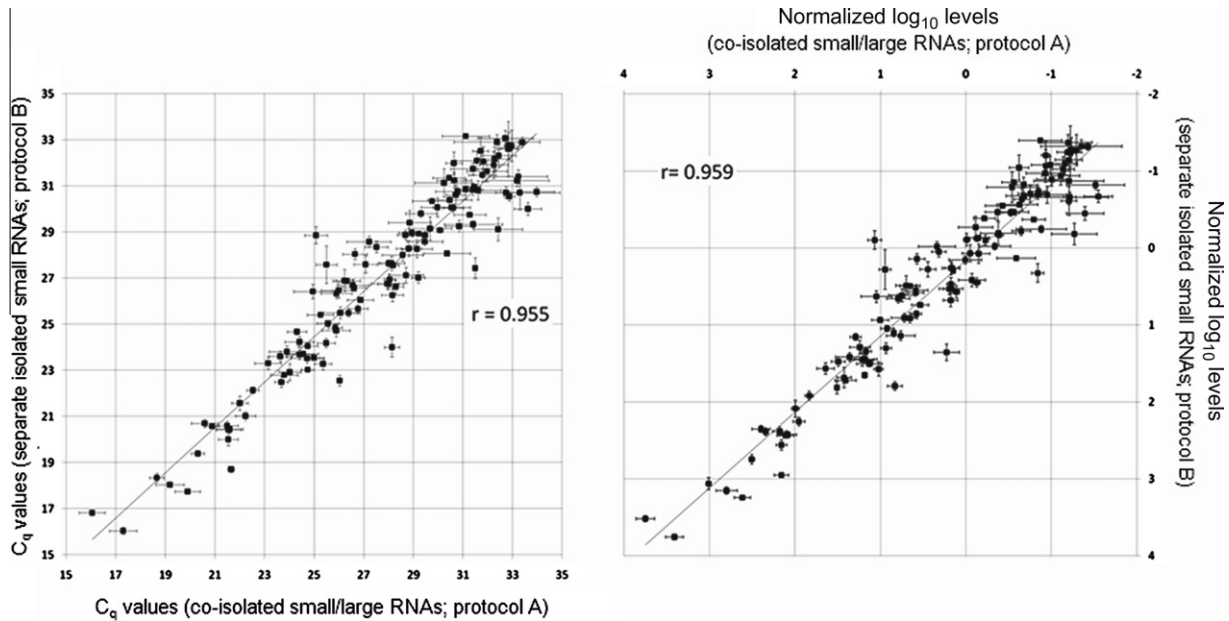
To accurately assess the quality of the miRNAs isolated within the small RNA fraction, we used TLDAs to measure expression of 377 miRNAs; any fluctuations in the quality of miRNAs would be reflected in the mean expression value of all miRNAs considered [26]. In this study, the mean expression  $C_q$  value of all detectable miRNAs did not differ significantly ( $P = 0.17$ ) between PB and BM samples ( $28.66 \pm 0.37$  and  $28.82 \pm 0.46$ , respectively). The number of miRNAs detected in PB samples from healthy volunteers was  $181 \pm 17$  (range = 145–202). Of these, 131 miRNAs were detected in all ( $n = 9$ ), and 163 were detected in 80% ( $n = 7/9$ ) of individual observations. This level of detection is higher than that previously reported in miRNAs isolated from white blood cells and plasma microvesicles using Trizol RNA extraction (108 miRNAs detected in 80% of individual observations,  $n = 51$ ) [28]. In PB samples from children with NB, the number of miRNAs detected was  $176 \pm 20$  (range = 134–202). The numbers of miRNAs detected in BM from healthy volunteers and BM from children with NB were  $188 \pm 14$  (range = 168–211) and  $206 \pm 22$  (range = 160–254), respectively. Together, these results demonstrate that the quality of miRNAs within the small RNA fraction isolated from 400 ng of the large RNA fraction is suitable for qPCR profiling and can be used to ensure consistency in the amount of material in the Megaplex RT reaction.

#### Profiling miRNAs using TLDAs is highly reproducible

To assess the interassay variability of miRNA TLDAs, we performed five independent TLDAs using the same Megaplex RT product prepared from the small RNA fraction of a PB sample from a healthy volunteer. The number of miRNAs detected in each of five replicate TLDAs (with  $C_q < 35$ ) ranged from 136 to 148, including 128 that were detected in all (see Table S3 in Supplementary material). Those miRNAs not detected in all replicates had  $C_q$  values above 33, consistent with low borderline expression. The  $C_q$  values of miRNAs detected in all replicates were highly correlated ( $r > 0.96$ ,  $P < 0.001$ ), with an interassay coefficient of variation of  $1.58 \pm 0.87\%$ . This variation is equivalent to a difference of less than 2 in  $C_q$  value, indicating that repeated measurements of the same sample could vary by up to 4-fold. This demonstrates that changes in miRNA expression can be detected with 95% confidence when of 4-fold or greater difference [29].

#### Level and profile of miRNAs are stabilized in PAXgene™ blood RNA tubes

There was no deterioration of miRNA following 6 months of storage at  $-80^\circ\text{C}$  in PAXgene™ blood RNA tubes (Fig. 3A). The same 177 miRNAs were detected in blood samples from a healthy volunteer processed after 2 days, 1 month, 3 months, and 6 months. Expression at 6 months compared with 2 days showed a coefficient of variation of  $C_q$  values of 1.63%; this coefficient of



**Fig. 2.** miRNA expression profiles of blood samples using the small RNA fraction isolated separately or co-isolated with large RNAs as template for TLDA. Scatterplots indicate high correlation of raw  $C_q$  values and normalized  $\log_{10}$  levels of miRNAs detected in both isolated fractions ( $r = 0.96$ ). Error bars indicate standard deviations across triplicate samples.

variation of  $C_q$  is similar to that observed for interassay variation (1.58%) (Fig. 3A). The normalized levels of each individual miRNA detected in samples stored for up to 6 months were not significantly different from those in samples stored for 2 days ( $P = 0.61$ , fold change =  $1.2 \pm 0.84$ ).

In comparing miRNA profiles of BM and PB samples stored in PAXgene™ blood RNA tubes at  $-80^\circ\text{C}$  for up to 70 months, there was no correlation between storage time and variation of the mean  $C_q$  value of all miRNAs detected on each qPCR array or of the  $C_q$  values of the three endogenous controls: the small RNAs U6, RNU44, and RNU48 ( $r < 0.28$ ) (Fig. 3B). Similarly, the variation in mean  $C_q$  value and the values of the endogenous small RNAs did not correlate with time of storage of the flow-through containing small RNA fraction ( $r < 0.21$ ) (Fig. 3C). Most important, there was no correlation between storage time and variation of  $C_q$  values of any of 270 miRNAs detected in at least 1 of 49 PB samples (median  $r = -0.04$ , range =  $-0.49$ – $0.47$ ) (see Table S4 in Supplementary material). Similarly, there was no correlation between storage time and variation of  $C_q$  values for any of 310 miRNAs detected in at least 1 of 49 BM samples (median  $r = -0.07$ , range =  $-0.44$ – $0.38$ ) (see Table S5 in Supplementary material). Together, these data indicate no deterioration of miRNAs during 5 years of storage in PAXgene™ blood RNA tubes at  $-80^\circ\text{C}$  or of small RNAs in the enriched flow-through fraction stored at  $-80^\circ\text{C}$  for at least 2 years prior to small RNA recovery on the RNeasy MinElute column.

#### Reference miRNA controls in hemopoietic compartments

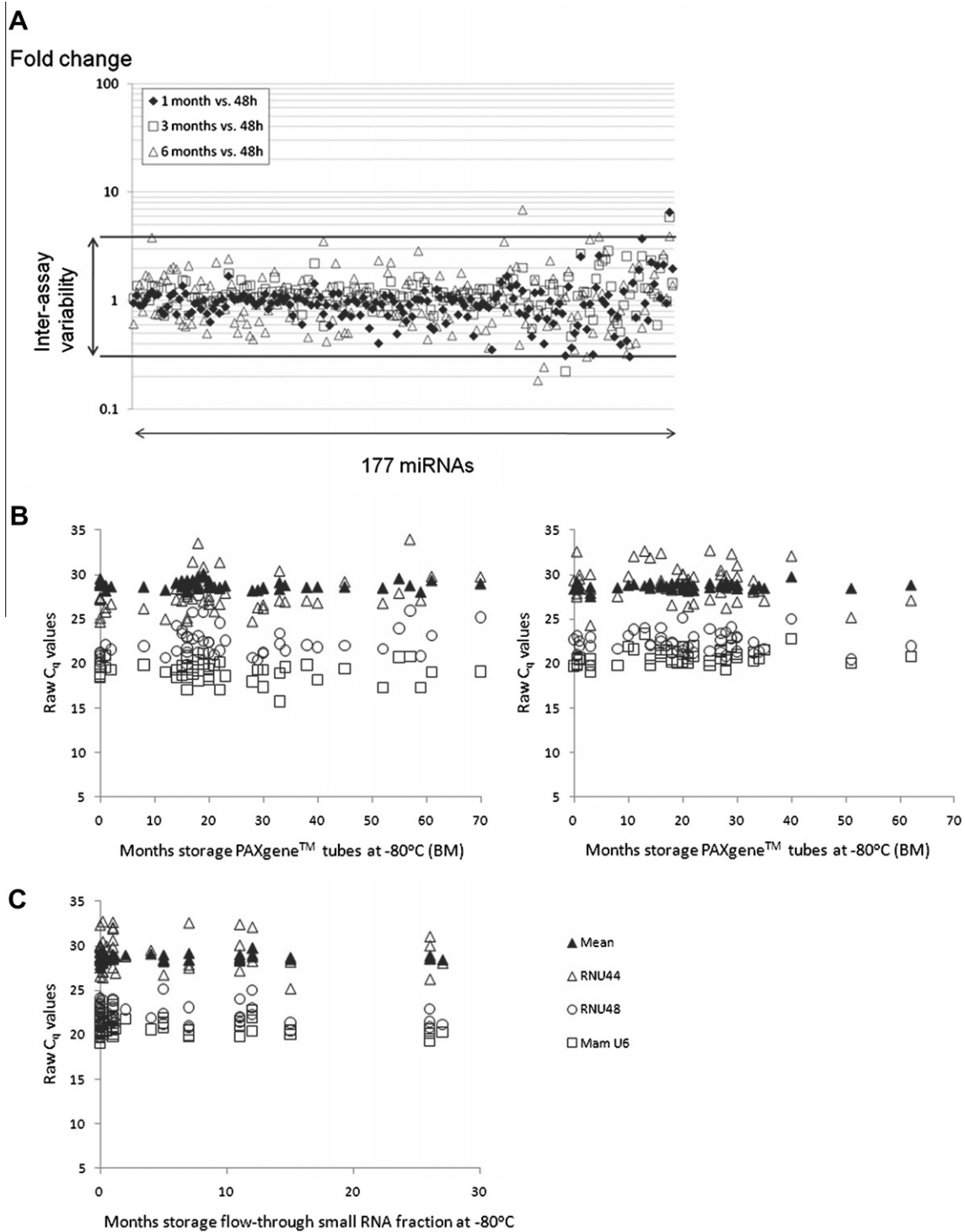
For studies that focus on a limited number of miRNAs, normalization using the mean expression of a panel of miRNAs is not reliable. Therefore, we sought to identify a suitable reference miRNA panel for standardization of miRNA expression in PB and BM. The expression stability of miRNAs that most closely resembled the mean expression value in each of the BM and PB datasets, with that of the small endogenous RNAs (RNU44, RNU48, and mammalian U6) and of two previously described reference miRNAs for BM (mir-30c and mir-140) [26], was investigated. The most stable and optimal number of these reference miRNAs was determined

using the geNorm stability ( $M$ ) and pairwise variation ( $V$ ) values. We identified several miRNAs with high expression stability in both the BM and PB datasets (Fig. 4) and with  $M$  values below the cutoff of 0.7 previously shown to eliminate most variable and outlying genes in microarray analyses [27]. The three most stable candidates across both BM and PB samples were miR-26a, miR-28-5p, and miR-24 ( $M = 0.57$ ,  $V3/4 = 0.15$ ). The geometric  $C_q$  mean value of miR-24, miR28-5p, and miR-26a obtained in BM samples from healthy donors did not differ significantly from the one in BM from children with NB ( $24.38 \pm 0.6$  and  $24.92 \pm 0.9$ , respectively,  $P = 0.10$ ), nor did it differ significantly from the one in PB from healthy donors and children with NB ( $25.30 \pm 0.9$  and  $25.60 \pm 0.9$ , respectively,  $P = 0.70$ ).

#### Discussion

Using the largest number of samples and miRNAs tested to date, this study has demonstrated that miRNA species are stable in hemopoietic samples stored in PAXgene™ blood RNA tubes at  $-80^\circ\text{C}$  for up to 5 years. The large and small RNAs co-isolated from PAXgene™ blood RNA tubes have previously been shown to be of comparable quality to RNAs isolated using Trizol-based extraction methods [22]. In this study, we demonstrated that the RNeasy MinElute Kit, when used with the PAXgene™ Blood RNA Kit, allows the purification of a separate miRNA-enriched fraction of similar quality to the total RNA fraction for miRNA qPCR profiling. The quantification of 377 miRNAs was unchanged in the small RNAs isolated separately or co-isolated with large RNAs. For studies where standard procedures have been introduced for analysis of mRNA expression in samples stored in PAXgene™ blood RNA tubes, and where large RNAs are isolated separately [14,30], we provide evidence that an miRNA-enriched fraction can be isolated and stored for up to 2 years at  $-80^\circ\text{C}$  prior to analysis. Another advantage of the specific isolation of small RNAs is that they can be recovered in a small elution volume, resulting in a 3-fold increase in concentration compared with that co-isolated with large RNAs. Increased concentration of small RNA preparations may facilitate the standardization of material for Megaplex RT-PCR.

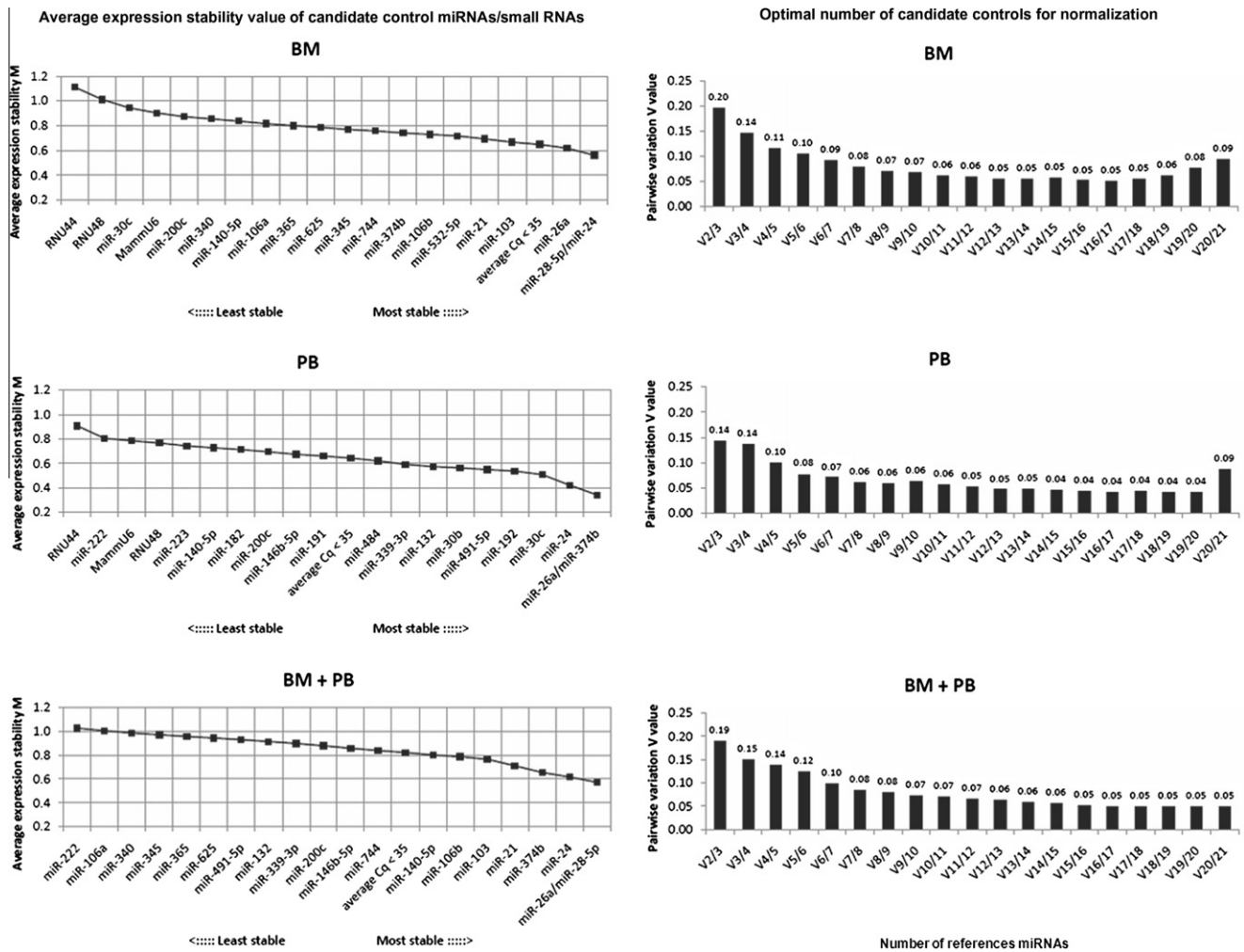




**Fig. 3.** miRNA stability up to 5 years at  $-80^{\circ}\text{C}$ . (A) Fold change in expression of each of 177 miRNAs detected (x axis) at 1, 3, and 6 months compared with 48 h storage at  $-80^{\circ}\text{C}$ . (B, C) Mean  $C_q$  values and  $C_q$  values of small RNAs RNU44, RNU48, and mammalian U6 measured in BM and PB samples in PAXgene™ blood RNA tubes (B) or in flow-through containing miRNAs from PB samples (C) stored for different times.

Interestingly, the number of miRNAs detected in PB from healthy volunteers using the separate small RNA isolation procedure was higher than that previously described using Trizol RNA extraction of combined peripheral blood mononuclear cells (PBMCs) and plasma microvesicles [28]. Previous studies have suggested that miRNA profiling of blood using PBMCs only might be misleading for diagnostic and prognostic assays due to inappropriate miRNA stabilization during PBMC isolation and loss of poten-

tially clinically relevant miRNAs from granulocytes and microvesicles [22]. The results from our study demonstrate that the PAXgene™ blood RNA tubes stabilize miRNAs from whole PB and BM and, therefore, may allow the detection of more clinically useful miRNA biomarkers. We found a significant increase in the level of enriched miRNAs in BM from children with NB compared with that in BM from healthy individuals, consistent with other studies reporting the detection of elevated levels of nucleic acid



**Fig. 4.** geNorm analysis of candidate reference miRNAs for qRT-PCR normalization across BM and PB samples. Shown is a ranking of candidate reference miRNAs that resemble the mean expression value and of other described endogenous controls: miR-30c, miR-140, and small RNAs according to average expression stability  $M$  value. The number of reference miRNAs was considered as optimal when the pairwise  $V$  value was less than 0.15.

in blood of cancer patients compared with healthy controls [31–33].

Together with the minimum manual handling steps, high reproducibility, and availability of individual validated primer–probe sets for each of 377 functionally annotated miRNAs, TLDA are highly suitable for standard profiling of a large number of retrospective clinical samples. In addition, TLDA eliminate the need for postarray analysis by qPCR that is required to confirm results when using hybridization-based microarray platforms. TLDA are also more sensitive, detecting a larger dynamic expression range than that of microarray platforms (7 vs. 3–4 logs) [34–36].

In accordance with a previous study, we found that the mean miRNA expression value was more reliable for normalization than endogenous small RNA controls [26]. Furthermore, we found that the geometric mean of miR-26a, miR-28-5p, and miR-24 was as reliable for accurate normalization of miRNA profiles in PB and BM.

This study demonstrates that miRNA expression profiling of PB and BM collected prospectively in a clinical setting is feasible and reliable when samples are collected into PAXgene™ blood RNA tubes and stored at  $-80^{\circ}\text{C}$  and data are reported against three miRNAs (miR-26a, miR-28-5p, and miR-24). Because improved outcome for cancer patients is anticipated by adjusting therapy according to prognostic and predictive biomarkers, we are currently investigating the hypothesis that miRNAs may constitute a new class of biomarkers in PB and BM for prognostication and

monitoring of disease status and response in children with NB, providing insight into the biology of the tumor cell and its microenvironment within those compartments.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.ab.2011.10.022](https://doi.org/10.1016/j.ab.2011.10.022).

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