Analytical Biochemistry 421 (2012) 566–572

Contents lists available at SciVerse ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

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

Virginie F. Viprey [a,*], Maria V. Corrias [b], Susan A. Burchill [a]

[a] Children's Cancer Research Group, Leeds Institute of Molecular Medicine, Section of Experimental Oncology, Leeds LS9 7TF, UK
[b] Laboratory of Oncology, Gaslini Institute, 16147 Genoa, Italy

ARTICLE INFO

Article history:
Received 2 September 2011
Accepted 12 October 2011
Available online 18 October 2011

Keywords:
MicroRNAs
RT-qPCR
Blood
Bone marrow
Reference microRNAs
PAXgene™ blood RNA tube
Long-term storage
Neuroblastoma

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 °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 °C into PAXgene™ blood RNA tubes for miRNA expression studies.

In many solid cancers, such as neuroblastoma (NB)[1], 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 established [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 °C for up to 5 years. We compared the expression of 380 miRNAs/small RNAs using TaqMan™ low-density polymerase chain reaction (PCR) arrays (TLDAs) 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 °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 °C. Written informed consent was obtained from all participants and parents/legal guardians of the children involved in this study for the collection

* Corresponding author. Fax: +44 113 242 9886.
E-mail address: v.f.viprey@leeds.ac.uk (V.F. Viprey).

[1] *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_q, quantification cycle; PBMC, peripheral blood mononuclear cell.

0003-2697/$ - see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.ab.2011.10.022

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 3500*g*, and the resulting pellet was resuspended in 5 ml of RNase-free water. This was aliquoted into 2 × 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 (μl) 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/μl), and DF is the dilution factor (eluate volume large RNA/volume of small RNAs, e.g., 38 μl/12 μl = 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-

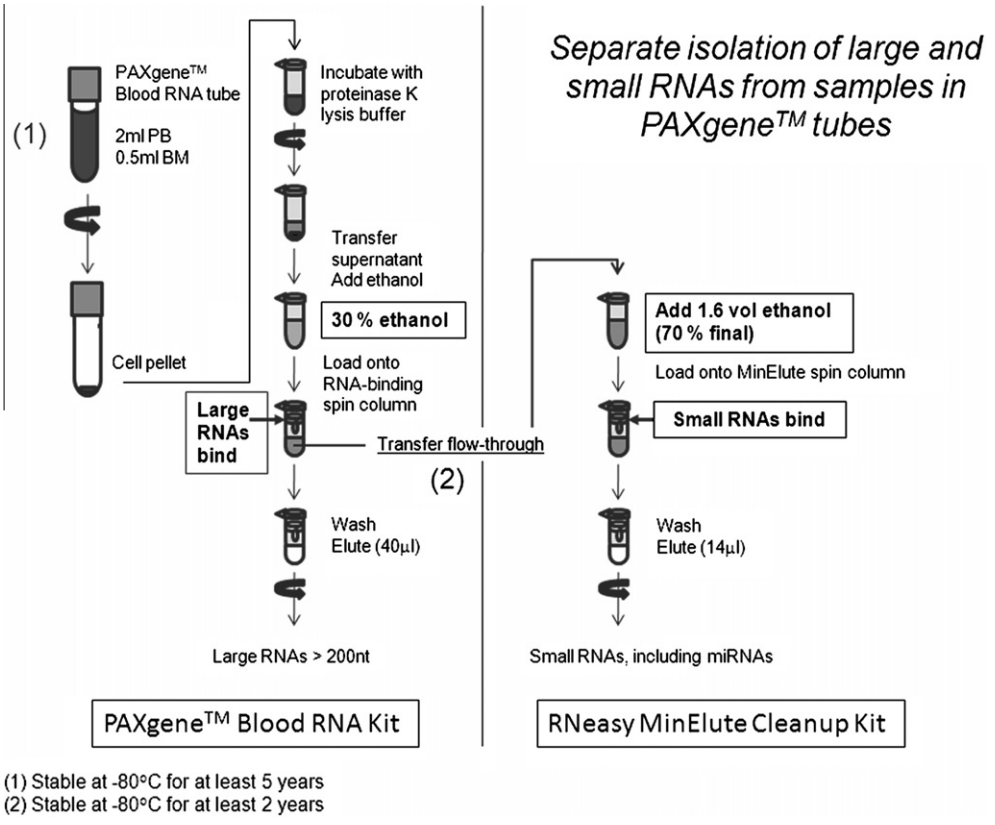

Fig.1. Workflow for isolation of miRNA-enriched fraction from samples in PAXgene™ blood RNA tubes (protocol B) adapted from manufacturer's manual. The small RNA fraction is retrieved using the RNeasy MinElute Cleanup column rather than the RNeasy column previously tested [20]. Stability of samples and of the flow-through containing small RNAs at −80 °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.