Short Communication

Validation of RNA Extraction Procedures Focused on Micro RNA Expression Analysis

(RNA preparation / miRNA / RIN)

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Abstract. The sampling procedure is a crucial step in every kind of experiment. This is also true in gene expression profiling experiments, where high quality and sufficient quantity of extracted RNA plays a significant role in the experimental outcome. We have compared five different RNA extraction protocols from peripheral blood/PBMCs with the aim to define the most suitable method for the miRNA expression profiling experiments. Convincing results in terms of highest quantity and quality were obtained by the TRIzol-chloroform extraction method. The total RNA obtained using this method contained the highest portion of good-quality miRNA molecules, which was also confirmed by gene-specific real-time PCR experiments.

Introduction

Small non-coding RNA molecules are molecules that are involved in the network of epigenetic regulation of gene expression. The group of small RNA molecules typically includes the tRNAs, rRNAs, snoRNAs, piRNAs, siRNAs and miRNAs. Epigenetic modifications including changes in miRNA expression profiles after viral infection or in association with some medications have been identified recently. The high significance of miRNAs has also been recognized in recent projects in the field of applied research. Studies on structural gene polymorphisms that render susceptibility to diseases are complemented by studies of the regulatory networks mediated by miRNAs. Moreover, the miRNAs can be a limiting factor in the disease onset. These molecules are tissue-specific, responsible for expression regulation at the post-transcriptional level, or for degradation or translational protein regression (Pratt and MacRae, 2009). Matured miRNAs are part of the active regulatory complex, called RNA-induced silencing complex (RISC). The mechanism of action of miRNAs in the regulation of gene expression is based on binding the miRNA to the regulatory region of mRNA, or it may also cause modification of histones or methylation of the promoter region of the genes (Hawkins and Morris, 2008). The ability of miRNAs to regulate mRNA expression promotes miRNAs to be significant prognostic and therapeutic targets for a variety of disorders, including cancer or autoimmune diseases.

The aim of this study was to find and confirm the most reliable method for RNA extraction in the context of preservation of the small RNA molecule fraction.

Material and Methods

We have compared five different methods widely used for RNA preparation from either cell suspensions or whole peripheral blood. Two methods for RNA preparation from a PAXgene tube (PAXgene Blood RNA Kit, PAXgene Blood miRNA Kit, both from Qiagen (Hilden, Germany)) were used, along with one column-based method for RNA extraction from fresh peripheral blood (RNeasy Mini Kit, Qiagen), one fully automated method based on magnetic bead separation of nucleic acids (MagNA Pure Compact RNA Isolation kit, Roche, Basel, Switzerland) and one TRIzol-based method (TRIzol®, Life Technologies, Carlsbad, CA) (Kruhoffer et al., 2007). We prepared the RNA from 28 biological samples of whole blood or suspension of PBMCs extracted from peripheral blood. Each RNA extraction method was used for at least three different samples. The RNA extraction procedure followed the manufacturer's instructions, when available (PAXgene Blood

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Abbreviations: miRNA – micro RNA, PBMCs – peripheral blood mononuclear cells, piRNA – Piwi-interacting RNA, siRNA – small interfering RNA, snoRNA – small nucleolar RNA, RISC – RNA-induced silencing complex.

Table 1. Detailed information on the quality, quantity and portion of miRNA present in RNA prepared by different extraction methods

RNA extraction method	Number of cells ($\times 10^6$)	RNA quantity total (ng)	RNA quantity / 10^6 cells (ng)	Absorbance 230/260	Absorbance 260/280	RNA Integrity Number (RIN)	miRNA (%)	miRNA concentration (pg/µl)
*QIAGEN – PAXgene Blood RNA Kit	18.5	5628	304.2	1.66	2.09	9	76%	12.6
*QIAGEN – PAXgene Blood miRNA Kit	18.5	9434	509.9	1.54	2	8.6	92%	40.6
** QIAGEN – Rneasy Mini Kit	11	8997	817.9	2.1	2.1	7.8	92%	10.5
*Roche – MagNA Pure Compact RNA Isolation Kit	1.48	805	543.9	1.59	2.31	8.6	65%	66.2
** TRIzol-chloroform extraction	11	12306	1118.7	2.01	2.14	9.1	95%	551.4

* whole peripheral blood used as a source of RNA
** PBMCs isolated from peripheral blood used as a source of RNA

RNA Kit, PAXgene Blood miRNA Kit, RNeasy Mini Kit, MagNA Pure Compact RNA Isolation kit). For the TRIzol-based RNA extraction, we used our home-made protocol (Chomczynski and Sacchi, 1987). The methods, total number of samples and concentration overview are listed in Table 1. All extracted RNA samples were analysed for the quality of total RNA, presence of small RNA and the portion of miRNAs. For the quality measurement and calculation of the portion of miRNA molecules, we used the Agilent RNA 6000 Nano Kit and Agilent Small RNA Kit (both from Agilent, Santa Clara, CA). The quality shown as RIN (RNA Integrity Number) and portion of miRNA (%) was calculated using the Agilent Bioanalyzer 2100 machine and corresponding software (Agilent 2100 Expert Software, Agilent). The presence of gene-specific miRNA molecules was analysed using gene-specific miRNA assays (Life Technologies).

Results and Discussion

The quality of extracted RNA was comparable across all the used methods (RIN = 8.6 (7.6–9.1), Table 1). However, we have found significant differences in the presence of small RNA molecules and in the portion of miRNA of the total RNA samples measured by Agilent Bioanalyzer 2100 (Agilent). The portion of miRNA (concentration and percentage) was calculated using the information obtained from Bioanalyzer 2100. This information included data about the concentration and amount of all small RNA molecules with length up to 60 bp. Nevertheless, no physical measurement method is available to date to evaluate the exact amount or quality of miRNAs. Therefore, all molecules with the length starting from 20 bp and ending with 60 bp present in our RNA samples were considered to be miRNA molecules. The highest amount of miRNAs was found in the samples prepared by the TRIzol-chloroform extraction method (95 %). On the other hand, the lowest portion of miRNAs in extracted RNA was obtained by the MagNA Pure Compact RNA Isolation Kit (65 %) and PAXgene Blood RNA Kit (76 %). The other two methods (PAXgene Blood miRNA Kit and RNeasy Mini Kit) seemingly guaranteed a constant amount of miRNA across all the corresponding samples (92 %). The difference in the amount of miRNAs prepared by the TRIzol-chloroform extraction method and the magnetic bead-based extraction method reached statistical significance with $P < 0.05$ (Fig. 1A). Similarly, the portion of miRNA molecules in RNA prepared by the TRIzol-chloroform extraction method was significantly higher than the portion of miRNAs in RNA prepared by the PAXgene Blood RNA Kit ($P < 0.01$) (Fig. 1A). No statistically significant differences were found when the other two extraction methods were compared with the TRIzol-chloroform method (Fig. 1A). However, the median portion of miRNAs present in RNA prepared with PAXgene Blood miRNA and RNeasy Mini Kit was significantly higher when compared with the other median values of the miRNA portion found in RNA samples prepared with the MagNA Pure Compact RNA Isolation Kit and PAXgene Blood RNA Kit (Fig. 1A).

To establish the specific amount of miRNA molecules in our extracted RNA samples, we also performed gene-specific miRNA detection of four miRNA molecules normally used as housekeeping control genes in miRNA expression profiling experiments (RNU6B, U47, RNU44, RNU48). For this purpose, gene-specific miRNA assays from Life Technologies were used. Using the absolute quantification approach, we calculated the total amount of input RNA used for cDNA reverse transcription. As a final value for miRNA amount shown in Fig. 1B, we used the median value of RNA amount calculated from all four genes. The calibration curve was prepared using the 10-fold dilution of standard total RNA template provided by Life Technologies. With this approach we found out that the highest amount of miRNA molecules was present in samples prepared by the TRIzol-chloroform extraction method; the lowest portion of miRNAs was found to be in samples prepared

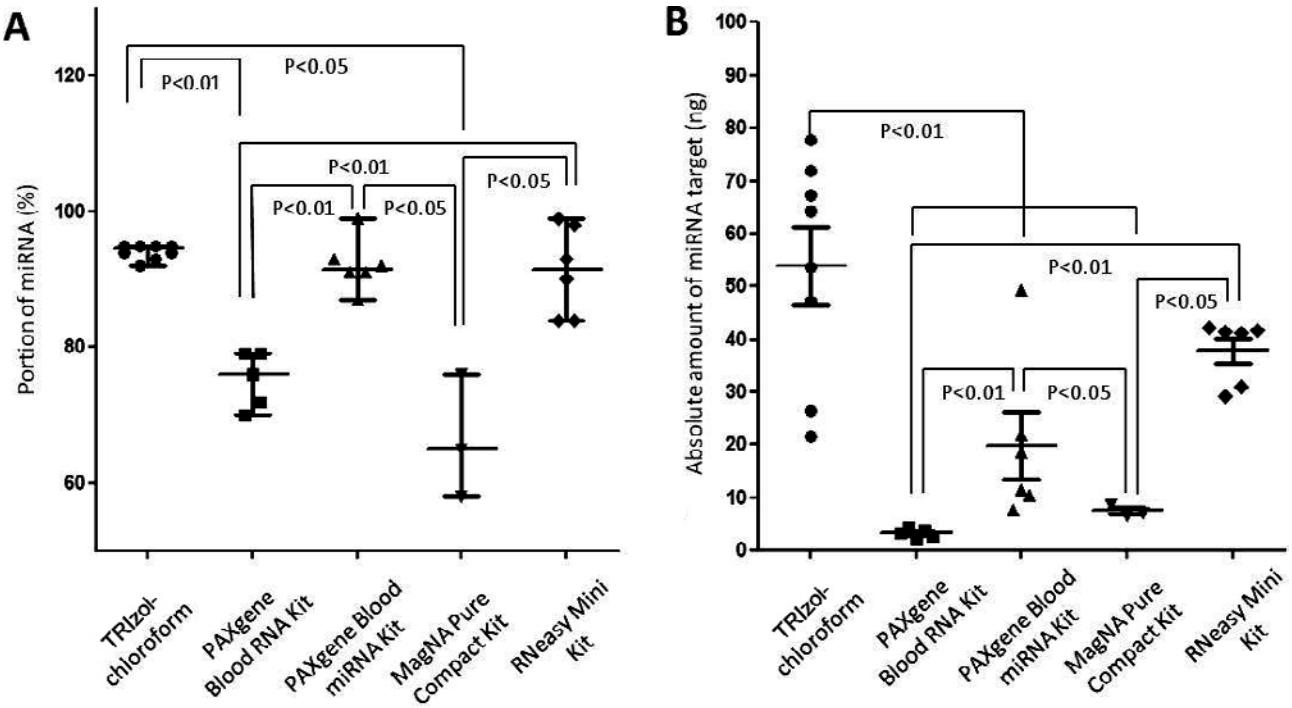

Fig. 1. Quantity of miRNA molecules evaluated using different methods. **(A)** Amount of miRNA molecules expressed as a portion out of all small RNA molecules (%) detected with Bioanalyzer 2100. A significantly higher portion of miRNA molecules is present in total RNA extracted using the TRIzol-chloroform method in comparison with PAXgene Blood RNA Kit ($P < 0.01$) and MagNA Pure Compact Kit ($P < 0.05$). A higher portion of miRNAs was also detected in RNA prepared with PAXgene Blood miRNA Kit and RNeasy Mini Kit ($P < 0.01$ when compared with PAXgene Blood RNA Kit; $P < 0.05$ when compared with MagNA Pure Compact Kit). **(B)** Gene-specific real-time PCR quantification results for selected housekeeping miRNA molecules. A significantly higher amount of miRNAs was found in RNA extracted using the TRIzol-chloroform method ($P < 0.01$ when compared with the PAXgene Blood RNA Kit, MagNA Pure Compact Kit and PAXgene Blood miRNA Kit method, respectively). A higher amount of miRNA molecules was also detected in RNA prepared with RNeasy Mini Kit and PAXgene Blood miRNA Kit when compared with the PAXgene Blood RNA Kit ($P < 0.01$) and MagNA Pure Compact Kit ($P < 0.05$).

by the PAXgene Blood RNA Kit method (Fig. 1B). The difference in the amount of miRNAs between TRIzol-chloroform extraction and PAXgene Blood RNA Kit, PAXgene Blood miRNA Kit or MagNA Pure Compact RNA Isolation Kit reached statistical significance ($P < 0.01$; Fig. 1B). The TRIzol-chloroform extraction method is widely used in expression profiling studies (Bilgin et al., 2009). For its generality it is used for RNA preparation from various biological samples such as animal tissues or plant tissues (Verhofstede et al., 1996; Fukabori et al., 2006; Ruettger et al., 2010; Ma and Yang 2011). The biggest pro of this extraction method is, as already mentioned, its generality. However, it is time-consuming and exacting on laboratory skills. A significant portion of miRNAs is expectable in RNA prepared with the PAXgene Blood miRNA Kit. However, the total amount of miRNA molecules was hardly comparable with the RNA prepared by the TRIzol-chloroform method. The use of blood stored in PAXgene tubes for RNA and miRNA detection was already discussed by Hammerle-Fickinger et al. (2010). Based on the technology used for RNA purification in RNeasy Mini Kit, we did not expect a very high portion of miRNAs. However, the amount of miRNA molecules was comparable to those found in RNA prepared with the PAXgene Blood miRNA Kit and even higher in the case of U47 and RNU44 (data not shown). Nevertheless, the gene-specific detection of miRNA molecules showed inconsistency in the amount of miRNAs in total RNA prepared using the RNeasy Mini Kit method. The amount of specific miRNAs was highly dependent on the size of the miRNA target. The technology itself should not allow extracting molecules smaller than 200 bp as stated by the manufacturer. However, our experiments show that the capacity of the binding membrane in the column is higher and it is able to capture molecules with the length starting at about 60 bp. The last two methods tested, the PAXgene Blood RNA Kit- and the MagNA Pure Compact RNA Isolation Kit-based approaches, do not seem to be suitable for miRNA preparation, as shown by the gene-specific miRNA detection approach as well as by the quality measurement using Bioanalyzer 2100.

Preparation of RNA is a crucial starting point in all expression analysis studies. The quality and specific template quantity of RNA can affect the amplification efficiency and also the final expression values. This is important in mRNA expression studies as well as in

miRNA expression profiling studies. The RNA extraction procedure, especially with the aim to investigate the miRNA expression profiles, must therefore be selected very precisely and with care. The TRIzol-chloroform extraction procedure presented here is the most suitable for large-scale experiments. The quality and quantity of purified total RNA and miRNAs was measured by Bioanalyzer 2100 and additionally by gene-specific real-time PCR experiments. We conclude that the yield and quality of total RNA as well as the portion of miRNAs prepared by the TRIzol-chloroform extraction procedure is sufficient for downstream expression profiling analysis.

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