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Mini Review

MicroRNA isolation and stability in stored RNA samples

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ABSTRACT

MicroRNAs (miRNAs) are small RNA molecules, which act as post-transcriptional regulators of a gene expression, with important functions within the cell physiology. Whilst many authors have focused on the study of miRNA expression in physiological and pathological processes, various technical variables related to miRNA isolation have simultaneously emerged and the stability of the stored miRNA samples has been questioned. A robust method for RNA isolation is essential for reproducible results and miRNAs instability in the stored samples would make for an alarming situation for most expression studies. Here these issues are discussed and we investigate the stability of miRNAs isolated from clinical samples of B lymphocytes (chronic lymphocytic leukemia) by the most commonly utilized method based on a Trizol/TRI-Reagent solution (RNAs stored at −80 °C). To assess the stability of miRNAs, a Real Time-PCR analysis was performed for a panel of 29 miRNAs from a freshly isolated RNA sample and after 14 days storage at −80 °C. Furthermore, a Real Time-PCR analysis was repeatedly performed for a stored RNA sample over a period of ~10 months. We observed high stability of isolated miRNAs and respective cDNAs. The reproducibility and efficiency of the Trizol/TRI-Reagent isolation method was also tested and compared to the mirVana Isolation kit (Ambion) and RNeasy kit (Qiagen). In conclusion, Trizol/TRI-Reagent based isolation is a robust reproducible method, and obtained miRNA samples do not show any tendency to degradation when properly stored and handled.

Introduction

MicroRNAs (miRNAs) are short 20–22 nucleotide RNA molecules that function as post-transcriptional regulators of the gene expression. MicroRNA mediated gene silencing pathways have essential roles in development, cell differentiation, cell proliferation, cell death, chromosome structure and virus resistance. These highly conserved RNAs constitute about 3–5% of predicted genes in human/animal genomes, and 20–30% of protein-coding genes are probably regulated by miRNAs. Many evolutionary conserved miRNAs were discovered and public miRBase database contains in total more than 10800 miR sequences annotated for 115 species (version 14—September 2009; www.mirbase.org). MicroRNA genes are non-randomly distributed in the genomes and one half of approximately 700 known human miRNAs are found in clusters and transcribed as polycistronic primary transcripts [1,2].

The transcription of a microRNA is followed with processing by Drosha and Dicer enzymes and finally the miRNA is associated with the RNA-induced silencing complex (RISC) [2–4]. This complex binds target mRNAs through partially complementary sequences and reduces their translation and/or stability [5]. MicroRNA navigated post-transcriptional processes add more complexity to the conceptions of the proteome orchestration. Regulation mediated by microRNAs has a large impact on the gene expression because, according to the published data and the computational predictions, a single miRNA can target dozens of genes. Many recent studies have shown that miRNAs have specific expression patterns in each cell type and tissue. Moreover, miRNAs are aberrantly expressed in practically all solid tumors and hematological malignancies, suggesting that they could function as oncogenes or tumor suppressors [6–8]. Numerous authors have reported that each cancer tissue has a specific microRNA signature and this can be effectively used as a novel cancer classification tool [9].

MicroRNA expression studies also require the introduction of novel techniques like microarray platforms and Real Time-PCR based approaches. Simultaneously, a need for robust miRNA isolation techniques have emerged and the stability of stored miRNA samples isolated by these methods has been questioned. Bravo et al. [10] reported that the preparation methods commonly used for miRNA isolation yield highly unstable miRNAs. Here we investigate the stability of miRNAs isolated from the clinical samples of B lymphocytes (from chronic lymphocytic leukemia patients) and demonstrate that it is possible to obtain highly reliable results with a Trizol/TRI-Reagent based isolation procedure.

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MicroRNA isolation

The quality of the miRNA expression profiles largely depends on the input RNA material and a robust RNA isolation method is essential for reproducible results. Whilst the miRNA expression profiling platforms have received significant interest in their optimalization [11–17], less attention in general has been focused on miRNA isolation and the storage of the samples [10,18,19]. Isolation of the RNA sample could be of great influence for the general experimental quality irrespectively of the technical intricacies of a particular expression profiling platform. This has been previously shown for the classical mRNA expression studies [20–23] and a few recent studies have demonstrated the apparent influence of different RNA isolation methods for miRNA expression profiling [18,24]. Present publications do not themselves recommend a particular RNA isolation method as superior to another. However, the application of the Trizol/TRI-Reagent followed by alcohol precipitation is the most widespread method for the isolation of total RNAs including miRNAs, which enables researchers to analyze all RNAs from one sample. The classical spin column based kits used for "total RNA isolation" are not suitable for miRNA expression profiling because they do not effectively recover RNAs smaller than 200 nt (illustrated for Qiagen RNeasy Mini kit in Fig. 1A and B). There is also a variety of commercially available methods for the isolation of samples enriched in small RNAs (supplied by Ambion, Stratagene, Roche, Sigma–Aldrich, Invitrogen; etc.) and simply due to historical reasons the mirVana miRNA Isolation kit (Ambion) is the most utilized one. The isolation of enriched small RNAs instead of total RNA can be of particular benefit mainly for microarray studies because of more intensive "expression signals". Fig. 1(C) illustrates how these different isolation methods influence the recovery and expression analyses of small RNAs. Interestingly, small nucleolar RNAs RNU38B (69 nt) and U6B (105 nt) used frequently as normalization genes were not enriched/depleted with the same efficacy by these methods compared to miRNAs.

MicroRNA stability in stored RNA samples

The expression profiling results can be potentially influenced not only by the method used to isolate the miRNAs, but also by the RNA storage conditions and handling. Bravo et al. [10] reported that miRNAs isolated by the previously discussed TRI-Reagent method or mirVana Isolation kit and stored at −80 °C are highly unstable. They described the potential degradation of the stored miRNAs and respective cDNA in few days after isolation. This would be an alarming situation for most expression based studies and time laborious microarray experiments. Therefore we focused on detailed analyses of the robustness of Trizol/TRI-Reagent based isolation and the stability of miRNAs in obtained samples stored at −80 °C.

The Trizol (Invitrogen) or TRI-Reagent (Sigma–Aldrich; MRC) preparation are well suitable for purifying quality RNA samples and the usage of these reagents ensures that the samples will be practically free of genomic DNA [25]. Unfortunately, further enzymatic reactions can be inhibited by Trizol/TRI-Reagent contamination, but it is infrequent according to our knowledge. MicroRNAs extraction should be performed following the manufacture's protocol or with a modified overnight −20 °C precipitation step for samples with expected lower RNA yield; this step can also be replaced by ethanol precipitation instead of the isopropyl alcohol [14]. It is recommended to store the obtained RNAs at high concentrations (>100 ng/μL) until further use. We assessed the concentration of isolated RNA by Nanodrop-1000 and the RNA quality was controlled by capillary electrophoresis (Agilent Bioanalyzer RNA 6000 Nano Assay) prior to any expression profiling. The Agilent

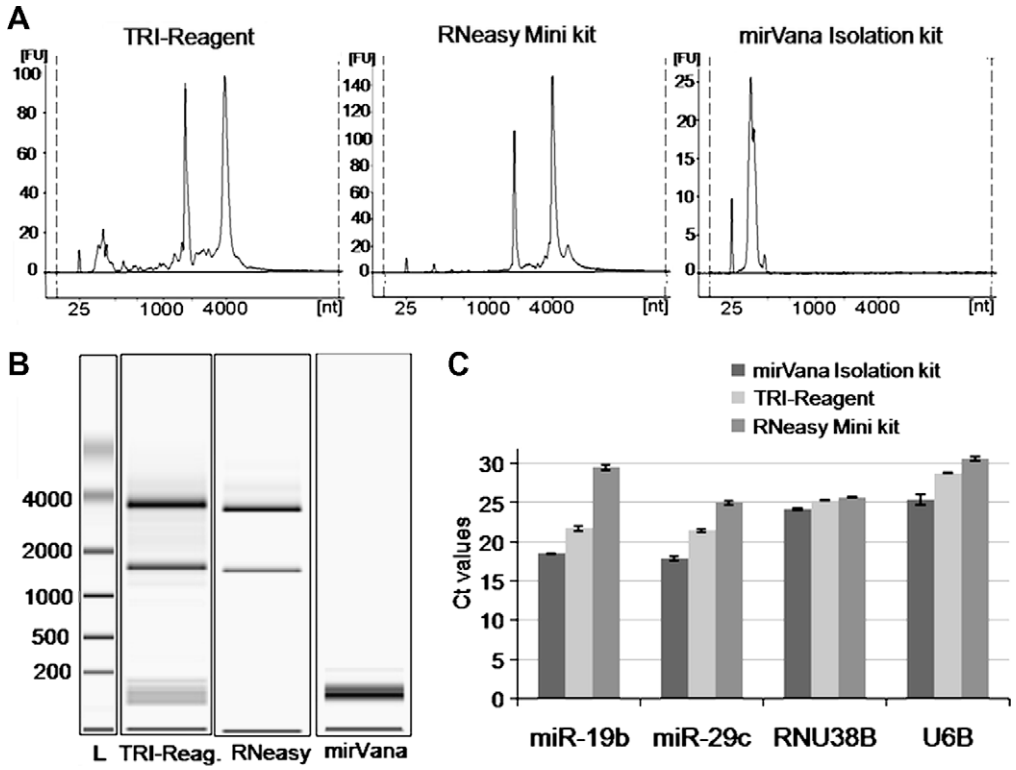

Fig. 1. (A,B) Capillary electrophoresis (Bioanalyzer RNA 6000 Nano Assay, Agilent) of RNAs isolated by TRI-Reagent (total RNA containing small RNAs), mirVana Isolation kit-Ambion (enriched in small RNAs), and by RNeasy Mini kit columns-Qiagen (total RNA depleted of small RNAs). (C) Illustrates how these different isolation methods influence the expression analyses of small RNAs (TaqMan miRNA Assays, ABI). Indicated Ct values are inversely proportional to the expression of RNAs. Interestingly, small nucleolar RNAs RNU38B (69 nt) and U6B (105 nt) used frequently as normalization genes were not enriched/depleted with the same efficancy by these methods compared to miRNAs. Error bars represent standard deviation of three performed RT-qPCR replicates. L stands for ladder.

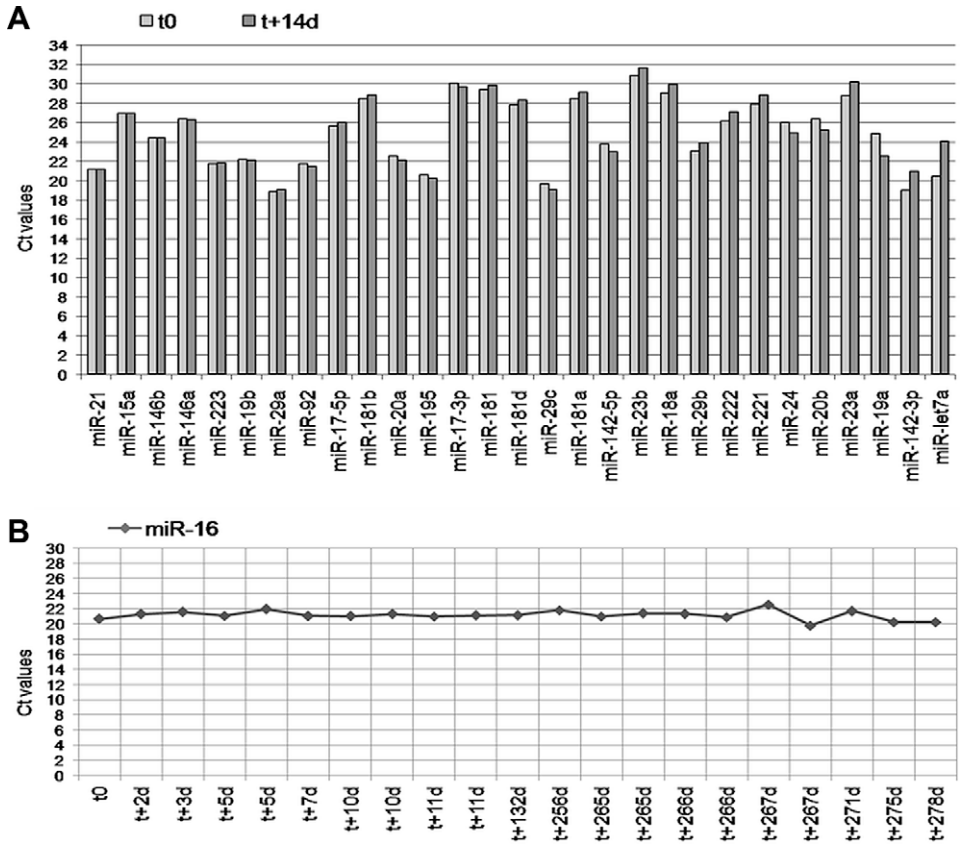

Fig. 2. (A) Real Time-PCR analyses (TaqMan miRNA Assays, ABI) for 29 miRNAs from freshly isolated RNA and after 14 days storage at −80 °C. Isolated miRNAs stored under these conditions did not show any tendency to degradation (correlation coefficient $R^2 = 0.92$). (B) Real Time-PCR (TaqMan miRNA Assays, ABI) analysis of RNA sample isolated by TRI-Reagent and stored at −80 °C over a period of ~10 months. Time points in days after isolation ($t + 0$) are indicated. The technical variability of the method can be illustrated by performing reverse transcription and RT-qPCR analysis more times in the same day (i.e. $t + 5$, $t + 10$, $t + 11$, $t + 265$, $t + 267$). Total RNA for both experiments was isolated by TRI-Reagent (MRC) according to the manufacturer's protocol and RNA was kept diluted to a concentration of 100 ng/μL at −80 °C in RNase free water.

Bioanalyzer "RNA Integrity Number" provides a score (range 0–10) measured as degradation/integrity of ribosomal peaks. Small RNAs are visible as an apparent band on the capillary gel (the RIN reached >9.0 for the used total RNA samples, Fig. 1B).

Maintenance of an RNase free environment can be vital to preventing RNA degradation and generation of reproducible results. Commercially available RNase free products, reagent bottles, tubes, vials etc. were used and other materials and gloves were cleaned with solutions designed to inactivate RNases (e.g. RNaseZAP, Ambion; RNase Away, Sigma–Aldrich).

To assess the stability of the miRNAs in obtained RNA samples a Real Time-PCR analysis was performed for a panel of 29 miRNAs

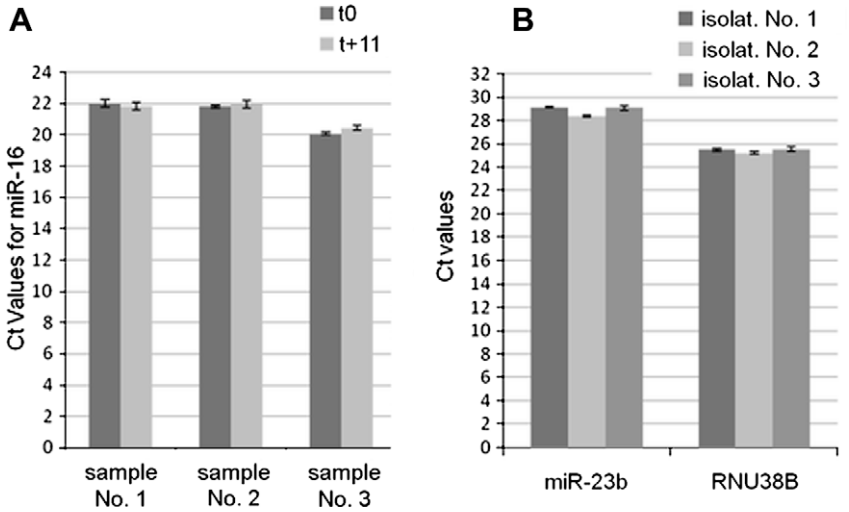

Fig. 3. (A) The stability of cDNAs derived from three distinct samples of total RNAs. The Real Time-PCR was performed immediately following cDNA preparation and from the same cDNA stored 11 days at −20 °C. Reverse transcription and Real Time-PCR quantification was performed according to the manufacturer's protocol (TaqMan miRNA Assays, ABI. (B) The TRI-Reagent RNA isolation giving identical results for miRNA quantification when repeated three times for the same sample. RNU38B is a member of small nucleolar RNAs, which are commonly used for miRNA data normalization. Error bars represent standard deviation of three performed RT-qPCR replicates.

from freshly isolated RNA and after 14 days storage at −80 °C (Fig. 2A). Moreover, a Real Time-PCR analysis was repeatedly performed for a stored RNA sample over a period of ~10 months. Surprisingly, the expression of the tested microRNAs was stable during all this long period of time (Fig. 2B). Isolated miRNAs stored under these conditions did not show any tendency to degradation (correlation coefficient $R^2 = 0.92$). The small observed differences were caused by technical variability of the Real Time-PCR itself (±1 Ct value), which can also be illustrated by performing reverse transcription and RT-qPCR for a RNA sample more times in the same day (Fig. 2B). Moreover, respective cDNAs ($n = 3$) were stable after 11 days storage at −20 °C in RNase free water (Fig. 3A). Thus RNase free conditions and proper handling can preserve the potential degradation of miRNAs and cDNAs. Finally, the Trizol/TRI-Reagent isolation is indeed a robust method, which gives consistent results when isolation and expression analysis was repeated more times for the same sample (Fig. 3B).

In conclusion, miRNAs obtained by the use of Trizol/TRI-Reagent solution are highly stable and this method remains a "gold standard" for miRNA isolation. In our hands, the storage of RNAs at −80 °C for long time periods is associated with reproducible results and also the cDNAs stored at −20 °C are suitable for expression studies. We agree with others that working with miRNA samples in RNase free conditions demands essential proper handling for a valid comparison of samples.

Acknowledgments

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References

[1] M. Lagos-Quintana, R. Rauhut, W. Lendeckel, T. Tuschl, Identification of novel genes coding for small expressed RNAs, Science 294 (2001) 853–858.
[2] Y. Lee, K. Jeon, J.T. Lee, S. Kim, V.N. Kim, MicroRNA maturation: stepwise processing and subcellular localization, EMBO J. 21 (2002) 4663–4670.
[3] R. Yi, Y. Qin, I.G. Macara, B.R. Cullen, Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs, Genes Dev. 17 (2003) 3011–3016.
[4] E. Lund, S. Guttinger, A. Calado, J.E. Dahlberg, U. Kutay, Nuclear export of microRNA precursors, Science 303 (2004) 95–98.
[5] W. Filipowicz, S.N. Bhattacharyya, N. Sonenberg, Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? Nat. Rev. Genet. 9 (2008) 102–114.
[6] Esquela-Kerscher, F.J. Slack, Oncomirs—microRNAs with a role in cancer, Nat. Rev. Cancer 6 (2006) 259–269.
[7] R. Visone, C.M. Croce, MiRNAs and cancer, Am. J. Pathol. 174 (2009) 1131–1138.
[8] M. Mraz, S. Pospisilova, K. Malinova, I. Slapak, J. Mayer, MicroRNAs in chronic lymphocytic leukemia pathogenesis and disease subtypes, Leuk. Lymphoma 50 (2009) 506–509.
[9] J. Lu, G. Getz, E.A. Miska, E. Alvarez-Saavedra, J. Lamb, D. Peck, A. Sweet-Cordero, B.L. Ebert, R.H. Mak, A.A. Ferrando, J.R. Downing, T. Jacks, H.R. Horvitz, T.R. Golub, MicroRNA expression profiles classify human cancers, Nature 435 (2005) 834–838.
[10] V. Bravo, S. Rosero, C. Ricordi, R.L. Pastori, Instability of miRNA and cDNAs derivatives in RNA preparations, Biochem. Biophys. Res. Commun. 353 (2007) 1052–1055.
[11] J.Q. Yin, R.C. Zhao, Identifying expression of new small RNAs by microarrays, Methods 43 (2007) 123–130.
[12] J.Q. Yin, R.C. Zhao, K.V. Morris, Profiling microRNA expression with microarrays, Trends Biotechnol. 26 (2008) 70–76.
[13] J. Shingara, K. Keiger, J. Shelton, W. Laosinchai-Wolf, P. Powers, R. Conrad, D. Brown, E. Labourier, An optimized isolation and labeling platform for accurate microRNA expression profiling, RNA 11 (2005) 1461–1470.
[14] M. Castoldi, V. Benes, M.W. Hentze, M.U. Muckenthaler, MiChip: a microarray platform for expression profiling of microRNAs based on locked nucleic acid (LNA) oligonucleotide capture probes, Methods 43 (2007) 146–152.
[15] M. Castoldi, S. Schmidt, V. Benes, M.W. Hentze, M.U. Muckenthaler, MiChip: an array-based method for microRNA expression profiling using locked nucleic acid capture probes, Nat. Protoc. 3 (2008) 321–329.
[16] C.G. Liu, R. Spizzo, G.A. Calin, C.M. Croce, Expression profiling of microRNA using oligo DNA arrays, Methods 44 (2008) 22–30.
[17] T.D. Schmittgen, E.J. Lee, J. Jiang, A. Sarkar, L. Yang, T.S. Elton, C. Chen, Real-time PCR quantification of precursor and mature microRNA, Methods 44 (2008) 31–38.
[18] W.X. Wang, B.R. Wilfred, D.A. Baldwin, R.B. Isett, N. Ren, A. Stromberg, P.T. Nelson, Focus on RNA isolation: obtaining RNA for microRNA (miRNA) expression profiling analyses of neural tissue, Biochim. Biophys. Acta 1779 (2008) 749–757.
[19] M. Doleshal, A.A. Magotra, B. Choudhury, B.D. Cannon, E. Labourier, A.E. Szafranska, Evaluation and validation of total RNA extraction methods for microRNA expression analyses in formalin-fixed, paraffin-embedded tissues, J. Mol. Diagn. 10 (2008) 203–211.
[20] J. Botling, K. Edlund, U. Segersten, S. Tahmasebpoor, M. Engström, M. Sundström, P.U. Malmström, P. Micke, Impact of thawing on RNA integrity and gene expression analysis in fresh frozen tissue, Diagn. Mol. Pathol. 18 (2009) 44–52.
[21] F.J. Staal, G. Cario, G. Cazzaniga, T. Haferlach, M. Heuser, W.K. Hofmann, K. Mills, M. Schrappe, M. Stanulla, L.U. Wingen, J.J. van Dongen, B. Schlegelberger, Consensus guidelines for microarray gene expression analyses in leukemia from three European leukemia networks, Leukemia 20 (2006) 1385–1392.
[22] S. Leonard, J. Logel, D. Luthman, M. Casanova, D. Kirch, R. Freedman, Biological stability of mRNA isolated from human postmortem brain collections, Biol. Psychiatry 33 (1993) 456–466.
[23] V.M. Van Deerlin, L.H. Gill, P.T. Nelson, Optimizing gene expression analysis in archival brain tissue, Neurochem. Res. 27 (2002) 993–1003.
[24] P.T. Nelson, W.X. Wang, B.R. Wilfred, G. Tang, Technical variables in high-throughput miRNA expression profiling: much work remains to be done, Biochim. Biophys. Acta 1779 (2008) 758–765.
[25] P. Chomczynski, N. Sacchi, Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction, Anal. Biochem. 162 (1987) 156–159.