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A phenol-free method for isolation of microRNA from biological fluids

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ABSTRACT

MicroRNAs (miRNAs) found in biological fluids such as blood and urine have been identified as promising biomarkers for many human disorders, including cancer, cardiopathies, and neurodegenerative diseases. However, circulating miRNAs are either encapsulated into vesicles or found in complexes with proteins and lipoproteins and, thus, require a special approach to their isolation. Acid phenol–chloroform extraction can solve this problem, but it is a labor-intensive procedure that relies heavily on the use of hazardous chemicals. Here we describe a fast and simple phenol-free protocol for miRNA isolation from biofluids. MiRNA is extracted from complexes with biopolymers by a high concentration of guanidine isothiocyanate combined with water/organic composition of solvents. Purification is finished using silica-based spin columns. Comparison of miRNA isolation from blood plasma and urine using the single-phase method and acid phenol–chloroform extraction by means of radioisotope spike-ins and quantitative real-time reverse transcription–polymerase chain reaction (qRT–PCR) showed similar performance of the two methods.

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MicroRNAs (miRNAs)[1] are a class of short noncoding RNAs that post-transcriptionally regulate gene expression. MiRNAs have been found in biological fluids such as blood and urine and have been identified as promising biomarkers for a wide range of human disorders, including cancer [1]. However, circulating miRNAs are either encapsulated into vesicles or found in complexes with proteins and lipoproteins [2–4]. That, along with high RNase activity in many biological fluids, calls for a special approach to their isolation.

For three decades, acid phenol–chloroform extraction has been considered to be a "gold standard" of RNA isolation. A great number of improvements and modifications to the protocol tailoring it to specific applications have been proposed, but the core principle has remained mostly unchanged [5]. It has proved to be efficient but also labor-intensive and heavily reliant on the use of hazardous chemicals such as phenol, which makes it inconvenient for common use. Surprisingly, during recent decades only a few other approaches to RNA isolation from biofluids have been reported, and even fewer have been introduced into laboratory practice (e.g., miRCURY, Exiqon). Thus, a more efficient way of miRNA isolation that makes the procedure less time-costly, less cumbersome, and more easily adaptable to automated systems and clinical needs is demanded by researchers and clinicians alike.

Here we describe a fast and simple protocol for miRNA isolation from biofluids that does not rely on phenol–chloroform extraction and does not require phase separation. A distinct feature of the protocol is destruction of RNA-containing complexes in a single-phase water/organic solvent solution. The release of miRNA from complexes and binding to carrier (silica) is facilitated by the use of chaotropic salt (guanidine isothiocyanate) and a combination of common organic solvents (chloroform and ethanol) (Russian patent application no. 2014137763, priority date 17 September 2014). The current protocol can be used to reliably isolate miRNA and similar small nucleic acids from plasma, serum, urine, and other biofluids.

Materials and methods

Samples of biological fluids

Blood samples of 50 healthy individuals were obtained from the Center of New Medical Technologies of the Institute of Chemical Biology and Fundamental Medicine SB RAS (ICBFM SB RAS, Novosibirsk, Russia) and the Novosibirsk Research Institute of

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[1] *Abbreviations used:* miRNA, microRNA; ICBFM SB RAS, Institute of Chemical Biology and Fundamental Medicine SB RAS; EDTA, ethylenediaminetetraacetic acid; RT, reverse transcription; MMLV, Moloney murine leukemia virus; dNTP, deoxynucleoside triphosphate; qPCR, quantitative real-time polymerase chain reaction; *Cq*, quantification cycle.

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Circulation Pathology of E.N. Meshalkin (Novosibirsk, Russia) after approval of the study by the ethics committees of both organizations. Written informed consent was provided by all participants. Venous blood was collected in ethylenediaminetetraacetic acid (EDTA) spray-coated vacutainers (BD, cat. no. 368589) and fractionated into plasma and blood cells within 4 h of blood sampling. Blood was centrifuged at 290*g* for 20 min, transferred into a new tube, and centrifuged a second time at 1200*g* for 20 min. The supernatant was stored frozen in aliquots at −20 °C.

Urine samples of 10 healthy individuals were obtained from the Center of New Medical Technologies of ICBFM SB RAS after approval of the study by the ethics committee. Written informed consent was provided by all participants. Urine was collected from participants and immediately clarified by two serial centrifugations at 400*g* and 17,000*g*, both at 20 °C for 20 min. The supernatant from the final centrifugation was stored frozen in aliquots at −20 °C.

Single-phase protocol for miRNA isolation from blood plasma

Frozen plasma samples were thawed and centrifuged at 3000*g* for 5 min to get rid of the cryoprecipitate. The supernatant was then used for isolation of RNA.

A sample of 150 μl plasma is mixed with 4.5 μl of 2-mercaptoethanol and 300 μl of denaturing buffer (6 M guanidine isothiocyanate and 15 mM Tris–acetate, pH 6.5) in a 2-ml Eppendorf propylene tube. The mixture is vortexed for 5 s and incubated for 5 min at room temperature. Then 1.35 ml of a 2:1 ethanol/chloroform solution is added (2 volumes of ethanol and an equal volume of chloroform), mixed thoroughly, and incubated for 5 min at room temperature. Before application to the silica column, the sample is centrifuged at a benchtop centrifuge at maximum speed (~10,000*g*) for 5 min. An opaque translucent pellet may appear in some samples, depending on the type and qualities of the sample. The supernatant is applied to a silica spin column (e.g., BioSilica, Novosibirsk, Russia) at vacuum manifold, or alternatively at a benchtop centrifuge, no more than 500 μl at a time. The flow-through is discarded.

The column is washed twice with 300 μl of washing buffer (1 M guanidine isothiocyanate, 2.5 mM Tris–acetate, 50% ethanol, 25% chloroform, and 1% 2-mercaptoethanol). After the second wash, the column is dried by centrifugation at 10,000*g* for 1 min. The flow-through is discarded.

The column is washed twice with 300 μl of washing buffer (10 mM Tris–HCl [pH 7.5], 0.1 M NaCl, and 75% ethanol). After the second wash, the column is dried by centrifugation at 10,000*g* for 1 min. The flow-through is discarded.

To elute the RNA from the column, 120 μl of BioSilica RNA elution solution or preheated 1 mM EDTA (pH 9.5) is applied to the center of the filter, centrifuged at 400*g* for 1 min, and then dried at 10,000*g* for 1 min. The column is discarded; the flow-through contains purified miRNA.

Single-phase protocol for miRNA isolation from urine

A sample of 400 μl clarified urine is mixed with 4.5 μl of 2-mercaptoethanol and 800 μl of denaturing buffer (6.75 M guanidine isothiocyanate and 15 mM Tris–acetate, pH 6.5) in a screw-cap tube (Sarstedt, Nuembrecht, Germany). The mixture is vortexed for 5 s, diluted with 1.2 ml of RNase-free water, mixed thoroughly with 7.2 ml of 2:1 ethanol/chloroform solution, and incubated for 1 to 5 min at room temperature.

Then the solution is transferred into a silica spin column, and RNA isolation is performed similarly to a previously described procedure for isolation from blood plasma.

Acid phenol–chloroform RNA isolation

RNA was isolated from 300 μl of blood plasma or 600 μl of clarified urine. Acid phenol–chloroform extraction was performed as described previously [5]. In the case of urine, pilot experiments demonstrated the necessity to reduce the final concentration of guanidine isothiocyanate before the addition of phenol to 1 M in order to increase the RNA yields (data not shown). After phase separation, the water phase was collected, mixed with an equal volume of ethanol, and purified using silica columns (BioSilica). Briefly, the sample is applied to a silica spin column at vacuum manifold, or alternatively at a benchtop centrifuge, no more than 500 μl at a time. The flow-through is discarded. The column is washed twice with 300 μl of washing buffer (1 M guanidine isothiocyanate, 2.5 mM Tris–acetate, 50% ethanol, 25% chloroform, and 1% 2-mercaptoethanol). After the second wash, the column is dried by centrifugation at 10,000*g* for 1 min. The flow-through is discarded. The column is washed twice with 300 μl of washing buffer (10 mM Tris–HCl [pH 7.5], 0.1 M NaCl, and 75% ethanol). After the second wash, the column is dried by centrifugation at 10,000*g* for 1 min. The flow-through is discarded. RNA is eluted from the column with 120 μl of BioSilica RNA elution solution or preheated 1 mM EDTA (pH 9.5) as described above.

RNA precipitation

To precipitate miRNA, 2 μl of glycogen (20 mg/ml) and 12 μl of 3 M sodium acetate (pH 7.5) were added to 120 μl of RNA eluate and mixed by vortexing. Then 132 μl of isopropanol was added and mixed by inversion and then by vortexing. The mixture was incubated for 30 min at −20 °C and then centrifuged at 12,000*g* for 10 min at 4 °C. The supernatant was carefully discarded.

The pellet was washed by 75% ethanol at 7500*g* for 5 min at 4 °C. The supernatant was carefully discarded.

Then the pellet was washed by 96% ethanol at 7500*g* for 5 min at 4 °C. The supernatant was carefully discarded, and the pellet was air-dried and dissolved in 15 μl of RNase-free water.

Isotope labeling, detection, and use of ^{32}P-labeled miRNA

Synthetic miR-21 oligoribonucleotide was 5′-labeled using [γ-^{32}P]ATP and T4 polynucleotide kinase (Fermentas, Vilnius, Lithuania) according to the manufacturer's specifications, separated by 16% polyacrylamide gel electrophoresis with 8 M urea, and isolated from gel using electroelution.

Samples containing radiolabeled oligoribonucleotide were measured against equal volumes of control solutions containing the same input amounts of oligoribonucleotide in water or RNA elution solution using a scintillation beta-counter.

Radiolabeled miR-21 was spiked into plasma and urine samples up to 150 nM concentration after the addition of 2-mercaptoethanol, and denaturing buffer and isolation procedures were executed as described above.

Reverse transcription and qRT–PCR

Reverse transcription (RT) on miRNA templates was performed as described by Chen and coworkers [6]. Stem–loop RT primers are listed in the online supplementary material. Each RT reaction was performed in a total volume of 10 μl and contained 3 μl of RNA, 50 nM of each miRNA-specific primer, 1 U of RiboLock RNase inhibitor (Fermentas), 100 U of Moloney murine leukemia virus (MMLV) reverse transcriptase (Fermentas), 2 μl of 5× MMLV reaction buffer (250 mM Tris–HCl [pH 8.3 at 25 °C], 250 mM KCl, 20 mM $MgCl_2$, and 50 mM DTT), and 250 μM of each deoxynucleoside triphosphate (dNTP). The reaction conditions were as

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