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Variability in microRNA recovery from plasma: Comparison of five commercial kits

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ABSTRACT

Numerous studies have indicated that microRNAs (miRNAs) are present and stable in multiple biological fluids, suggesting a great potential as biomarkers for molecular diagnostics and prognostics. Variations in the amount of starting material and isolation method to obtain miRNA may introduce bias and contribute to quantification errors. Given these concerns, we compared five commercially available kits for serum/plasma miRNA isolation to determine whether the plasma miRNA profile varies with the isolation method. We isolated miRNAs in blood plasma from colorectal cancer patients and healthy donors with five commercially available kits: Exiqon, Norgen, Macherey–Nagel, Qiagen, and Zymo Research. First, we assessed the robustness of the RNA isolation process and the quality of isolated miRNAs with the miRCURY microRNA QC PCR Panel (Exiqon), which contains six RNA spike-ins for quality control of RNA isolation (UniSp2, -4, and -5), complementary DNA (cDNA) synthesis (UniSp6 and cel-miR-39-3p), and polymerase chain reaction (PCR) amplification (UniSp3). This panel also includes circulating human miR-103, miR-191, miR-23a, and miR-451. Second, to evaluate the variability in miRNA profiling in relation to the extraction method, we analyzed plasma levels of candidate miRNA biomarkers for colorectal cancer (miR-18a, miR-21, and miR-29a). To determine PCR efficiencies per amplicon and per sample, we used LinRegPCR software. We found that all isolation methods were suitable for extracting miRNA from plasma samples and that all had similar C_q values in the three steps analyzed: RNA isolation, cDNA synthesis, and quantitative reverse transcription (qRT)–PCR. However, although the PCR replicates were excellent, the intersample variability of the spike-ins was unsatisfactorily high and all kits yielded suboptimal PCR efficiencies for some amplicons. Overall, our results underline the great difficulties involved in measuring miRNAs in plasma. The use of spike-ins is critical to control technical factors that affect final miRNA levels. We recommend that researchers investigating circulating miRNAs verify the PCR efficiency for each amplicon because quantification may be influenced by sample and PCR components.

MicroRNAs (miRNAs) comprise a family of highly conserved small noncoding RNAs (19–24 nt) that regulate post-transcriptional gene expression; miRNAs play a critical role in many biological processes, such as differentiation, proliferation, and apoptosis, and have been implicated in several diseases. The human genome contains more than 2500 mature miRNAs, and these regulate approximately 60% of all protein-coding genes [1,2]. The biogenesis of miRNAs is a complex multistep process that involves the RNase III enzymes Drosha and Dicer and ultimately results in the production of mature miRNAs of approximately 22 nt [3]. The mature miRNA strand is incorporated into an RNA-induced silencing complex (RISC), which directs the regulation of messenger RNA (mRNA), either by directly causing mRNA degradation or, more commonly, by binding to the target mRNA and

Abbreviations: miRNA, microRNA; mRNA, messenger RNA; qRT–PCR, quantitative reverse transcription–polymerase chain reaction; cDNA, complementary DNA; EDTA, ethylenediaminetetraacetic acid; CV, coefficient of variation; ANOVA, analysis of variance.

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preventing its translation; in very few cases, it increases the expression of the target mRNA by stabilizing it.

Numerous studies have demonstrated that miRNAs are readily detectable in body fluids, including plasma, serum, urine, saliva, and milk [4]. The first class of circulating miRNAs to be detected was placental miRNAs in maternal plasma during pregnancy [5]. Another study found higher serum levels of tumor-associated miRNAs in lymphoma patients than in healthy controls [6]. Although the origin and function of extracellular circulating miRNA are unknown, interest in miRNAs as noninvasive biomarkers in circulating blood is growing. Numerous studies have shown aberrant expression of circulating miRNAs in cancer [7–9], diabetes [7], acute myocardial infarction [10], and organ damage [11].

One key advantage of miRNAs as potential biomarkers is their stability. Cell-free miRNAs in body fluids are stable even after repeated freeze–thaw cycles and prolonged storage [12]. The surprising stability of cell-free miRNAs in body fluids has been attributed to encapsulation in membrane-bound vesicles, such as exosomes [13,14], and to their association with RNA-binding protein complexes, such as Argonaute2 [15] and lipoprotein complexes (high-density lipoproteins) [16].

Although circulating miRNAs are promising candidates for biomarkers, several challenges must be overcome before miRNAs can be used for diagnosis and monitoring. First, total circulating miRNAs are in the femtomolar range, and plasma, serum, and other biofluids contain only a few nanograms per microgram of RNA [17], so “normal” RNA quality control techniques such as capillary electrophoresis, fluorometry, and spectrometry are unsuitable. Second, not all miRNAs detected in biofluids are circulating extracellular miRNAs given that hemolysis and other processes can lead to sample contamination with cellular miRNAs and to quantification errors [18–20]. Third, biofluids may contain inhibitors of the reverse transcriptase and polymerase enzymes used in the quantitative reverse transcription–polymerase chain reaction (qRT–PCR), which may survive RNA purification. Finally, serum and plasma samples typically do not contain larger RNA species (e.g., 5S, U6, small nucleolar RNAs [snoRNAs]) that are sometimes used as internal controls in miRNA quantification studies. All of these points—low concentrations, sample contamination with leukocyte miRNAs, presence of inhibitors, and lack of non-miRNA species—are critical for choosing the right internal controls for normalization.

Another obstacle in the way of obtaining useful miRNA profiles from serum and plasma samples is that not all circulating miRNAs are associated with the development of a particular disease state. Recent investigations suggest that extracellular miRNAs are more than just by-products of cellular activity. Some might have signaling function during physiological and pathological processes [21]. The presence of these cellular RNA species that come from different body homeostatic processes but are unrelated to disease states may modify the miRNA profile of serum and plasma, resulting in a distorted and nonreproducible profile.

Given these concerns, an effective and robust isolation method that produces highly consistent results across different samples is critical for accurate quantification of circulating miRNAs. In this study, we aimed to determine whether the miRNA profile in plasma samples depends on the RNA isolation method used and to identify the kit that has the least technical variation in the quantification of miRNAs in plasma and serum.

To do this, we collected and aliquoted plasma samples from controls and colorectal cancer patients, extracted total RNA using five commercially available kits for biofluids, and analyzed the variability between samples and kits by adding synthetic spike-ins in key steps of the process: RNA isolation, complementary DNA (cDNA) synthesis, and qRT–PCR.

Materials and methods

Study design and ethics statement

The study was approved by the institutional medical ethical boards of our hospital (Corporació Sanitària Parc Taulí, Institut Universitari Parc Taulí–UAB). All donors provided written informed consent prior to participation.

This study comprised three parts: (i) isolation of RNA from plasma of six subjects using five kits and five RNA spike-ins, (ii) miRNA analysis using a QC PCR Panel predesigned for quality control and for the study of colorectal cancer miRNAs, and (iii) validation of a selection of spike-ins and miRNAs in a selection of kits in eight independent samples (Fig. 1).

Blood processing

Peripheral blood samples from eight individuals (four colorectal cancer patients and four healthy donors matched for age and sex) were collected in ethylenediaminetetraacetic acid (EDTA)-containing Vacutainer tubes and centrifuged at 3500*g* for 10 min at 4 °C. Plasma from each donor was carefully removed and stored at −70 °C in six 300-μl aliquots until analysis. To avoid possible differences due to blood collection and processing, samples were collected from patients and controls in the same place and period of time.

RNA isolation

Total RNA of the same six samples was extracted from plasma using the following five commercial kits designed for the isolation of RNA from serum or plasma: (E) miRCURY RNA Isolation Kits–Biofluids (ref. no. 300113, Exiqon, Vedbaek, Denmark), (N) Plasma/Serum Circulating and Exosomal RNA Purification Mini Kit (ref. no. 51040, Norgen, Thorold, Ontario, Canada), (MN) NucleoSpin miRNA Plasma (ref. no. 740981, Macherey–Nagel, Düren, Germany), (Q) miRNeasy Serum/Plasma Kit (ref. no. 4938635, Qiagen, Toronto, Ontario, Canada), and (ZR) Direct-zol RNA MiniPrep (ref. no. R2050, Zymo Research, Irvine, CA, USA). The latter two kits, Q and ZR, require a phenol/chloroform reagent prior to spin column RNA purification; we used QIAzol reagent (Qiagen).

In all cases, 1 μl of the spike-in mix containing synthetic UniSp2, UniSp4, and UniSp5 (RNA Spike-in Kit, Exiqon) was added to the sample immediately after the first RNA isolation buffer of each kit. The manufacturer dried the RNA spike-in mix before shipping, and we resuspended, aliquoted, and stored it at −20 °C to avoid freeze–thaw cycles. All samples were purified according to the manufacturer's instructions for each kit without recombinant DNAse digest, and the starting and elution volumes were adjusted as reported in Table 1. Finally, eluted RNAs were aliquoted and stored at −70 °C until analysis.

cDNA synthesis and real-time PCR

For cDNA synthesis, we used the miRCURY LNA Universal RT microRNA PCR Kit (Exiqon). Briefly, 2 μl of total RNA isolated from plasma and 1 μl of the mix containing two synthetic spike-ins (UniSp6 and cel-39-3p, RNA Spike-in Kit, Exiqon) were polyadenylated, and cDNA was synthesized using a poly(dT) primer with a 3′ degenerate anchor and a 5′ universal tag. The reaction was performed at 42 °C for 60 min and inactivated at 95 °C for 5 min in a thermocycler (Analytik Jena, Jena, Germany). We synthesized cDNA from each sample twice; one was used in the QC PCR Panel, and the other was used for miRNA biomarker profiling. cDNA underwent qRT–PCR immediately without freezing.