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Method and Technical Advance

Centrifugation: an important pre-analytic procedure that influences plasma microRNA quantification during blood processing

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Abstract

Circulating microRNAs are robustly present in plasma or serum and have become a research focus as biomarkers for tumor diagnosis and prognosis. Centrifugation is a necessary procedure for obtaining high-quality blood supernatant. Herein, we investigated one-step and two-step centrifugations, two centrifugal methods routinely used in microRNA study, to explore their effects on plasma microRNA quantification. The microRNAs obtained from one-step and two-step centrifugations were quantified by microarray and TaqMan-based real-time quantitative polymerase chain reaction (Q-PCR). Dynamic light scattering was performed to explore the difference underlying the two centrifugal methods. The results from the microarray containing 1,347 microRNAs showed that the signal detection rate was greatly decreased in the plasma sample prepared by two-step centrifugation. More importantly, the microRNAs missing in this plasma sample could be recovered and detected in the precipitate generated from the second centrifugation. Consistent with the results from microarray, a marked decrease of three representative microRNAs in two-step centrifugal plasma was validated by Q-PCR. According to the size distribution of all nanoparticles in plasma, there were fewer nanoparticles with size >1,000 nm in two-step centrifugal plasma. Our experiments directly demonstrated that different centrifugation methods produced distinct quantities of plasma microRNAs. Thus, exosomes or protein complexes containing microRNAs may be involved in large nanoparticle formation and may be precipitated after two-step centrifugation. Our results remind us that sample processing methods should be first considered in conducting research.

Key words Centrifugation, microRNA quantification, one-step centrifugation, two-step centrifugation

Ideal biomarkers for clinical application should be easily accessible. Therefore, biomarkers that can be sampled from body fluids such as plasma and serum are particularly desirable[1]. MicroRNAs are stable and abundant in plasma and serum and thus have become a research focus as biomarkers for tumor diagnosis and prognosis[2,3]. Indeed, microRNAs have been researched and validated in many types of tumors as valuable biomarkers[3-16]. Quantification of circulating microRNAs with sufficient sensitivity and precision is the prerequisite for developing effective diagnostic tools. However, it is affected by several procedures, including procedures for sample collection and processing (e.g., centrifugation), RNA extraction (e.g., extraction reagents), and quantitative polymerase chain reaction (Q-PCR) (e.g., internal reference selection for normalization)[17].

Plasma is the cell-free supernatant obtained by centrifugation of anticoagulated whole blood. When generating plasma during blood processing, there is a risk of contamination by cells from the cellular pellet when aspirating, which is important because cells have a high concentration of microRNAs[17]. By contrast, serum is the cell-free supernatant obtained from centrifugation of whole blood that clots due to the absence of anticoagulant. When generating serum, microRNA-containing vesicles could potentially be released as a

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result of cell lysis[9]. Therefore, thorough centrifugation is a necessary and important step in blood processing.

To the best of our knowledge, there are two primary centrifugation methods for plasma or serum preparation: one-step centrifugation, in which blood is only centrifuged at 820–2,800 ×g[5,6,15,16], and two-step centrifugation, in which blood is centrifuged at 12,000–16,000 ×g after centrifugation at 820–2,800 ×g[7,8,10,12-14]. Here, we investigated whether these two methods had distinct effects on plasma microRNA collection.

Materials and Methods

Blood sample collection

Two patients with nasopharyngeal carcinoma (NPC) were recruited. They all signed informed consent, and blood samples were collected by trained staff interviewers. This study was approved by the Human Ethics Committee, Sun Yat-sen University Cancer Center.

Plasma preparation and RNA isolation

For plasma preparation, peripheral blood (8 mL) was drawn into tubes coated with EDTA. Within 30 min, samples were centrifuged at 820 ×g for 10 min, generating the one-step centrifugation plasma sample (about 4 mL). Then, 1 mL aliquots of the plasma were transferred to 1.5 mL tubes and centrifuged at 16,000 ×*g* for another 10 min. After this step, white particles (probably cellular debris) were collected at the bottom of the tube and the supernatant, the two-step centrifugation plasma sample, was obtained and transferred to a fresh tube (about 2 mL). All components of the whole blood, including both the one-step and two-step centrifugation plasma samples and precipitates, were then stored at –80°C immediately.

For RNA isolation, total RNA was extracted from 400 μL plasma using the miRVana PARIS microRNA Isolation Kit according to the manufacturer's instructions (Ambion, Austin, TX). RNA concentration was quantified using a NanoDrop 1000 Spectrophotometer (NanoDrop Technologies, Waltham, MA).

Reverse transcription and Q-PCR

Two microRNAs (miR-BART5 and miR-BART16) originating from Epstein-Barr virus (EBV) and U6 non-coding small nuclear RNA (snRNA) were selected as representative RNAs. Using a TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, Calif), 40 ng total RNA from plasma was subjected to reverse transcription according to the manufacturer's protocol. Reverse transcription and following Q-PCR were performed using custom TaqMan microRNA assays (Applied Biosystems, Foster City, CA) specific to the corresponding mature sequence obtained from miRBase (www.miRBase.org). The reaction was run for 40 cycles (95°C, 10 min; 95°C, 15 s and 60°C, 1 min for 40 cycles). All cycle threshold (Ct) values were determined in real time with an ABI 7900HT system and analyzed with SDS Relative Quantification Software 2.2.3 (Applied Biosystems). All reactions were performed in triplicate.

Microarray analysis

MicroRNA microarrays from Agilent Technologies were used to profile microRNAs and to further identify the difference between one-step and two-step centrifugation plasma samples. Microarray analysis was performed after RNA quality control. The details of microarray hybridizations were described in a previous publication[12].

Dynamic light scattering measurement

The size and size distribution of nanoparticles in plasma were measured by dynamic light scattering (DLS) using a Malvern ZS90 equipped with a 532 nm laser beam and a scattering angle of 90°. The measurements were performed at 25°C without further dilution of the plasma samples. For each sample, size distribution measurement was performed for 15 cycles per run.

Results

The total signal detection rate of plasma microRNAs on microarray was decreased after two-step centrifugation

Microarray was used to get an overall view of microRNAs in the plasma prepared from one-step and two-step centrifugations. The plasma prepared from one-step and two-step centrifugations and the precipitate generated in the second centrifugation were all obtained from each patient (**Figure 1A**). Six Agilent microarrays were conducted and plasma microRNA profiles were compared for each sample. There were a total of 1,347 microRNAs on the microarray. For sample 1, 284 microRNAs (21.1%) were detected in plasma prepared from one-step centrifugation, whereas 206 microRNAs (15.3%) were detected in plasma prepared from two-step centrifugation (**Figure 1B**). Five microRNAs were only detected in plasma from two-step centrifugation and 83 only in plasma from one-step centrifugation, whereas 201 were detected in both plasma preparations. Interestingly, all 83 microRNAs not present in the plasma prepared from two-step centrifugation were present in the precipitate, suggesting they were pulled out of solution at high centrifugal speed (16,000 ×*g* for 10 min in this study). The same trends were observed for sample 2. Specifically, 250 microRNAs were detected in plasma from one-step centrifugation, and 195 microRNAs were detected in plasma from two-step centrifugation (**Figure 1B**). Two microRNAs were only detected in plasma from two-step

centrifugation and 57 only in plasma from one-step centrifugation, whereas 193 were detected in both plasma preparations. As with sample 1, 52 of the 57 microRNAs not present in the plasma from two-step centrifugation were detected in the precipitate. This difference demonstrates the direct influence of centrifugation on developing a microRNA-based diagnostic tool.

The content of representative microRNAs after two-step centrifugation was greatly decreased

EBV, a pro-oncogenic virus involved in a variety of human tumors, was the first virus found to encode microRNAs[18]. To date, 44 mature viral microRNAs targeting viral and cellular genes have been discovered[19]. Mature EBV-encoded microRNAs are secreted through exosomes, a vesicle ranging in size from 30 to 150 nm[20] and known to mediate short- and long-range cell-cell communication[20-22]. Considering the mechanism of entering blood stream, two microRNAs from EBV—miR-BART5 and miR-BART16—were selected and quantified by Q-PCR. The Ct values differed markedly in plasma preparations from the same sample. The Ct values for miR-BART5 in plasma prepared from one-step and two-step centrifugations were 33.5 and 40 in sample 1, and were 37.4 and 40 in sample 2 (**Figure 2A**). Similar results were obtained for miR-bart16 (**Figure 2B**). We also investigated U6 snRNA, the non-coding small nuclear RNA (snRNA) component of U6 small nuclear ribonucleoprotein (snRNP) that is commonly used as an internal reference. Consistent with the results from EBV microRNAs, plasma prepared from two-step centrifugation showed a higher Ct value Ct = 40 (**Figure 2C**). These results demonstrate that high-speed centrifugation decreased the total number and content of microRNAs in plasma.

The size distribution of nanoparticles differed in plasma prepared from one-step and two-step centrifugations

MicroRNAs encoded by EBV are secreted outside of cells, even into the blood stream, through exosomes[20]. In addition, human microRNAs are also carried by protein complexes, such as argonaute 2 complexes, apart from the exosome[23]. One hypothesis is that exosomes, nanoparticles 30–150 nm in size, and argonatute 2 complexes may form large nanoparticles in plasma. To test this hypothesis, we measured the size distribution of nanoparticles in one-step and two-step centrifugation plasma preparations. For sample 1, plasma prepared from one-step centrifugation contained many nanoparticles >1,000 nm in size. We expected these large nanoparticles to disappear after the second centrifugation, but some were still present (**Figure 3A**). For sample 2, similar results were obtained (**Figure 3B**). We also calculated the overall mean diameter of nanoparticles. In plasma prepared from one-step and two-step centrifugations, the mean diameters of nanoparticles were 62.9 nm and 169.9 nm, respectively, for sample 1 and 28.6 nm and 152.0 nm, respectively, for sample 2 (**Figure 3C**). These results provide evidence that these two centrifugations produced different particles collection.

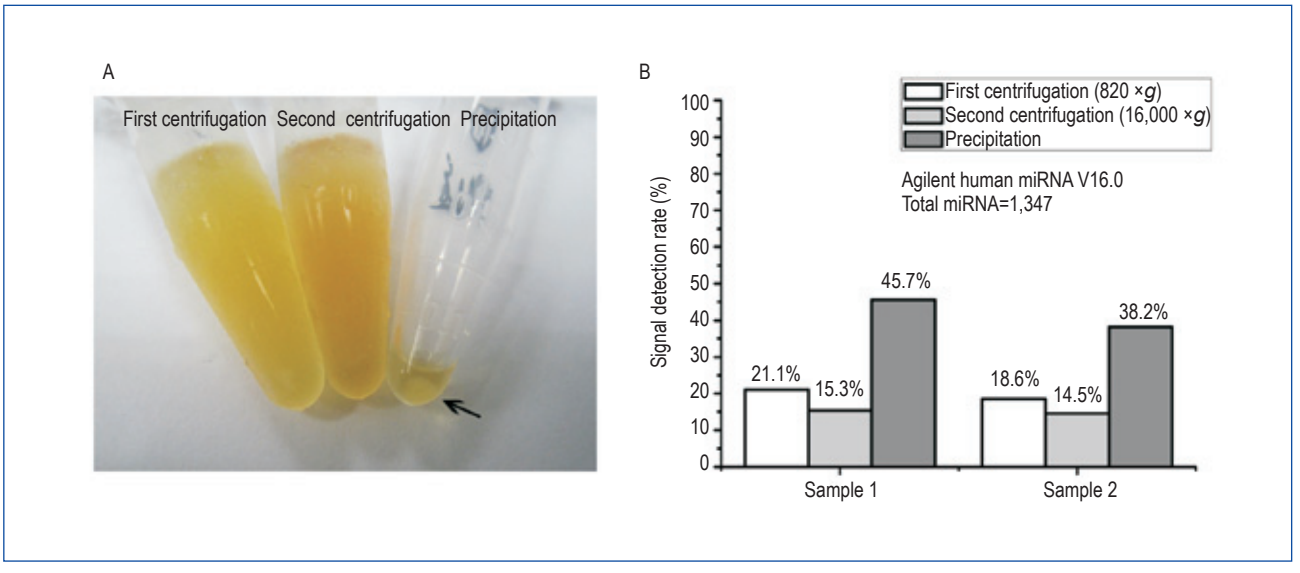

Figure 1. **Signal detection rate of microRNAs.** A, the one-step (left) and two-step (middle) centrifugation plasma preparations and the precipitate (right) generated after the second centrifugation were obtained from each patient. B, according to the positive signals on the microarray, the signal detection rate of microRNAs was calculated. There is a marked decrease in signal detection rate after the second centrifugation in both samples.

Discussion

The clinical effectiveness of microRNAs as biomarkers is likely affected by specimen collection and processing, RNA extraction, Q-PCR and other procedures or factors. In this study, we focused on centrifugation, a necessary and important pre-analytic procedure for obtaining high-quality blood supernatant. We report here that high-speed centrifugation decreased the total number and content of microRNAs in plasma.

For plasma preparation, peripheral blood was centrifuged at 820 ×g for 10 min and then 16,000 ×g for another 10 min. After the second centrifugation, there was a white precipitate at the bottom of the tube (**Figure 1A**). In other studies, this precipitate was reported as cellular debris[8,10,12,14]. We showed that RNA molecules could be extracted from this precipitate, producing a higher signal detection rate on the microarray (**Figure 1B**). Currently, there are two known mechanisms by which microRNAs enter the blood: by exosomes, typical nanoparticles that range in size from 30 to 150 nm[20], and by protein complexes, such as argonaute 2 complexes[23]. Our results strongly implied that exosomes or protein complexes carrying microRNAs were probably combined with cellular debris and then precipitated at high centrifugal speed. Theoretically, these large particles would disappear after second centrifugation. However, nanoparticles with size >1,000 nm re-formed after the second centrifugation (**Figures 3A** and **3B**). Therefore, apart from combining with cellular debris, the exosomes or protein complexes containing microRNAs may be polymerized and participate in formation of large nanoparticles, which also comprised the white precipitate after the second centrifugation.

Compared to microRNAs in tissues, the content of the same EBV microRNAs in plasma was greatly decreased. This was confirmed by the difference of Ct value between positive control group and the experiment group in our study (**Figures 2A** and **2B**). Wherein, the total RNA from NPC tissues was used as the positive groups while the total RNA from plasma was used as the experiment groups. Furthermore, the Ct value increased to 40 after the second centrifugation, reflecting the disappearance of these microRNAs in the plasma from two-step centrifugation (**Figures 2A** and **2B**). To confirm this, we investigated U6 snRNA, which is commonly used as an internal reference for tissue samples, and found results consistent

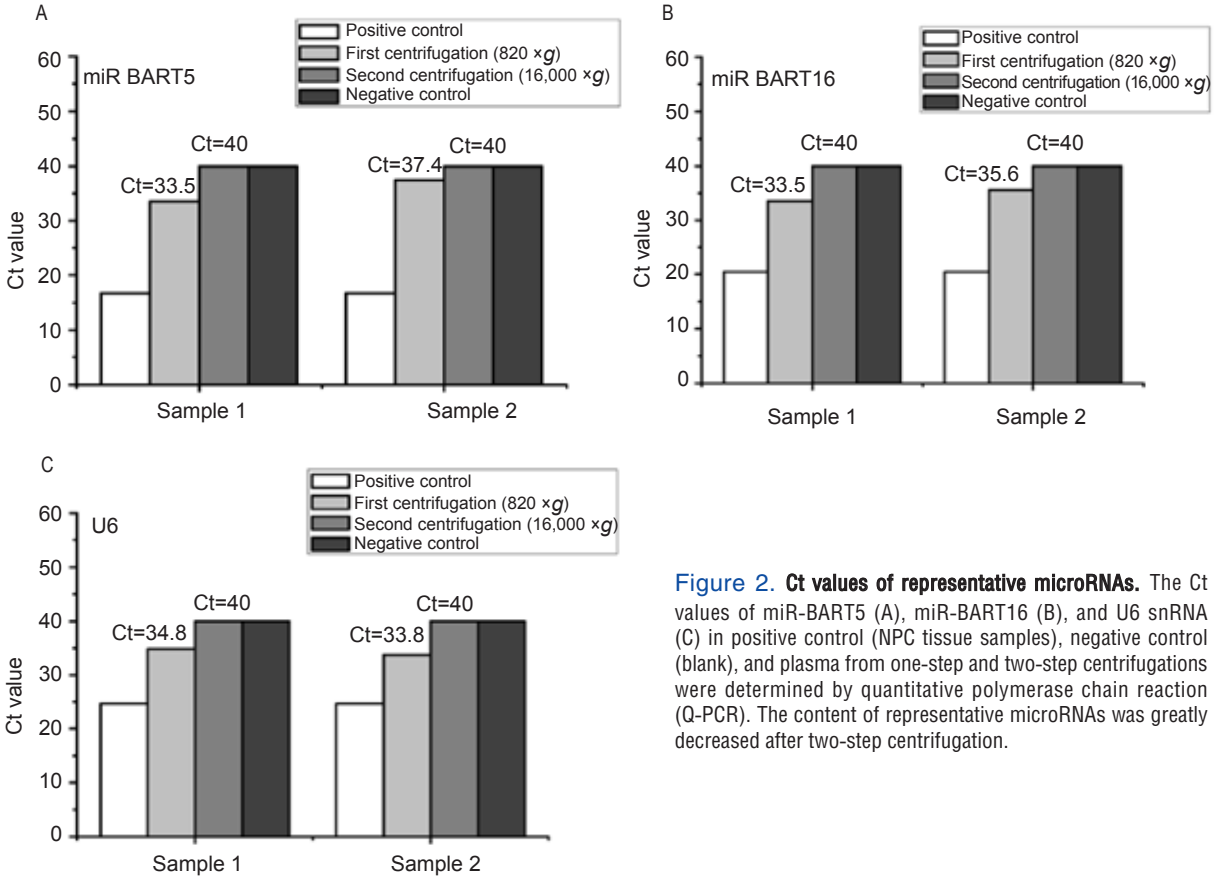

Figure 2. **Ct values of representative microRNAs.** The Ct values of miR-BART5 (A), miR-BART16 (B), and U6 snRNA (C) in positive control (NPC tissue samples), negative control (blank), and plasma from one-step and two-step centrifugations were determined by quantitative polymerase chain reaction (Q-PCR). The content of representative microRNAs was greatly decreased after two-step centrifugation.

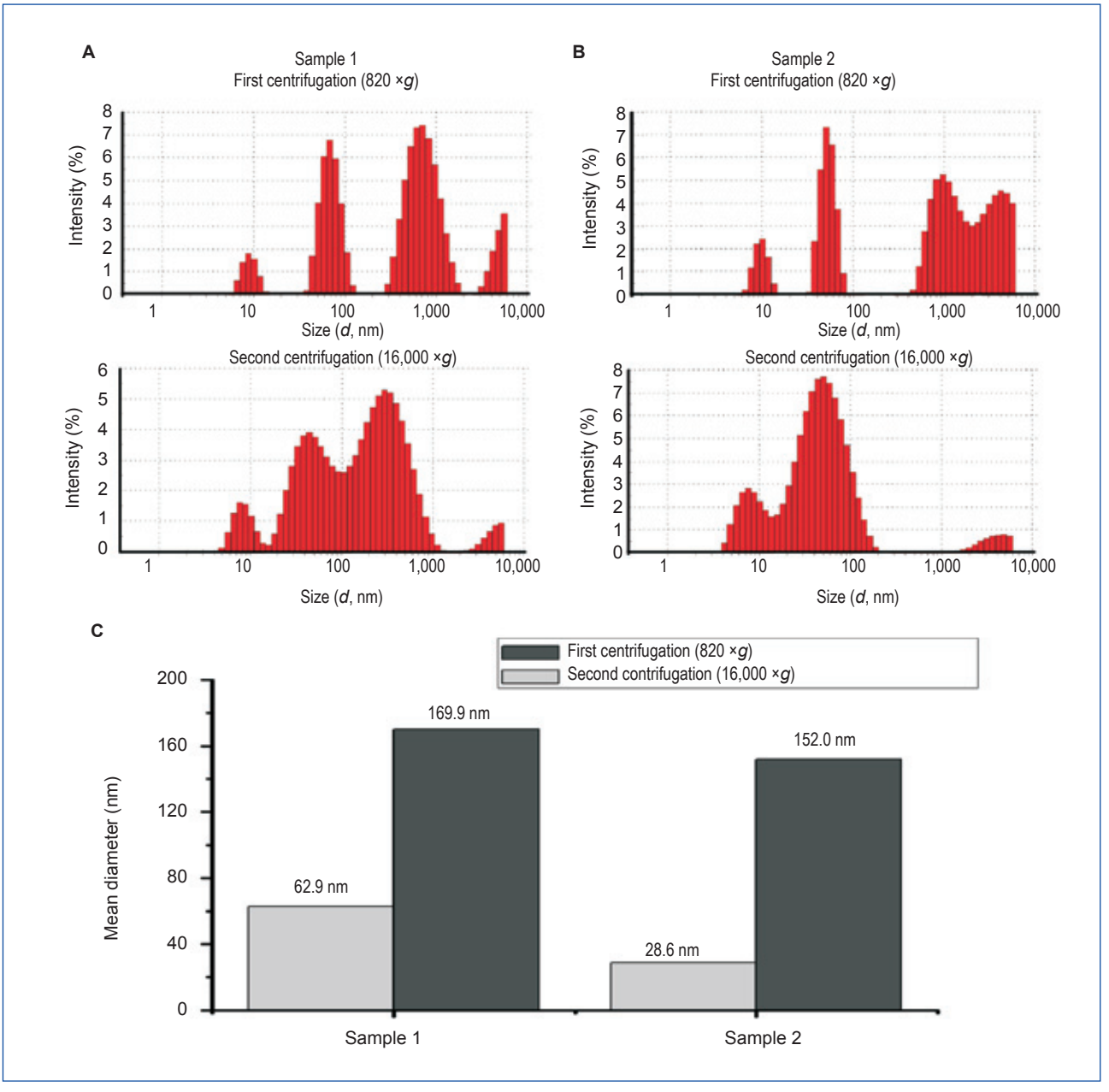

Figure 3. **Size distribution of nanoparticles in different plasma preparations.** A, size distribution of nanoparticles in plasma from one-step and two-step centrifugations in sample 1. B, size distribution of nanoparticles in plasma from one-step and two-step centrifugations in sample 2. C, the overall mean diameter of all nanoparticles in sample 1 and sample 2.

with those observed with EBV microRNAs (**Figure 2C**). These findings directly demonstrate the important influence of centrifugation on nucleic acid in plasma.

Although developing microRNAs as circulating biomarkers has become a hot topic, an effective normalization strategy has not yet been established[17]. Unlike U6 snRNA, an endogenous internal control in tissues, specific microRNAs have not been widely accepted as suitable internal controls in plasma or serum. To date, two strategies for normalization have been reported: one based on spiked-in microRNAs, such as cel-miR-39 and cel-miR-238[5,15,16], and the other based on endogenous microRNAs, such as miR-16 and miR-1228[10,12,13]. In the present study, we detected 201 microRNAs in sample 1 and 193 in sample 2 (**Figure 1**). A total of 188 microRNAs were detected on all four plasma microarrays (other two microarrays

for precipitate). Among them, some microRNAs showed stable signal intensity in plasma prepared from both one-step and two-step centrifugations. By comparing the signal intensity of microRNAs between different samples, housekeeping microRNAs that are suitable endogenous controls may be determined in the future.

There are usually several types of blood-based biomarkers for one human cancer[24]. In nasopharyngeal carcinoma, for example, these include proteins, such as EBV antibodies, and tumor-derived nucleic acids, such as EBV DNA. These biomarkers have been used for cancer screening and detection. Now, circulating microRNAs have emerged as potential biomarkers for cancer and are expected to play an important role in tumor diagnosis and prognosis. Our results show that the addition of one centrifugation changes microRNA output and detection. Therefore, the sample processing methods should always be considered in microRNA studies as well as in developing other types of blood-based biomarkers.

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