Examination and comparison of the RNA extraction methods using mouse serum

KEISUKE YAMAMOTO[1] and MITSURU CHIBA[1,2]

[1]Department of Bioscience and Laboratory Medicine, Graduate School of Health Sciences;
[2]Research Center for Biomedical Sciences, Hirosaki University, Hirosaki, Aomori 036-8564, Japan

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Abstract. Serum microRNAs (miRNAs) are considered useful as non-invasive biomarkers for different diseases. However, the optimal method for extracting RNAs from serum is currently unknown. In the present study, several RNA extraction kits were used to examine the optimal kit. RNAs were extracted from the serum of 8-week-old C57BL/6NJcl male mice following the protocol of each RNA extraction kit. The yield of the extracted RNA samples was calculated, and an Agilent Bioanalyzer was used to assess the electrophoretic patterns. An Agilent mouse miRNA microarray was utilized to confirm the expression patterns of the extracted RNA samples. The results revealed significant differences in RNA yields from the miRNeasy Serum/Plasma Advanced kit and mirVana™ PARIS™ RNA and Native Protein Purification Kit compared with almost all other samples. Further, two peaks were determined in the miRNeasy Serum/Plasma Advanced kit using a small RNAs kit of Agilent Bioanalyzer, including one at 20-40 nucleotides (nt) and another at ~40-100 nt, whereas the other reagents had a single peak. This revealed that the extracted RNAs may differ in composition based on the RNA extraction method. Some types of miRNAs were only detected with certain RNA extraction reagents. This suggested that different RNA extraction reagents may cause differences in the types of miRNAs detected. On the other hand, the miRNAs commonly expressed by the three RNA extraction reagents are highly correlated in expression levels.

Introduction

MicroRNAs (miRNAs) are small RNAs of ~21-24 nucleotides (nt) that generally regulate protein expression in cells and influence cellular processes (1,2). Particularly, miR-17 has been involved in adenosine deaminase expression that acts on RNA in melanoma stem cells and regulates the editing of dedicator of cytokinesis mRNA (3). MiRNAs are intracellularly expressed molecules, and they regulate gene expression in cells and are released from cells through various mechanisms (4,5). The well-known release mechanism is the release of extracellular vesicles (EVs), such as exosomes that contain miRNAs inside (6,7). MiRNAs are relatively stable and somewhat resistant to ribonuclease inside exosomes (8).

There are two major RNA extraction methods (9). One is Guanidinium Thiocyanate (GuSCN)-Phenol-Chloroform Extraction. In this method, GuSCN is added and homogenized, and then an acidic solution consisting mainly of sodium acetate, phenol and chloroform is added and centrifuged to separate the RNA in the aqueous layer and DNA in the organic layer. By adding isopropanol to the aqueous layer where the RNA is eluted, the RNA is precipitated and can be recovered. Another method is the silica matrix method. In this method, DNA and RNA are bound to silica-based filters or beads because of their high affinity for silica. In the case of RNA extraction, DNA and RNA are bound to silica filters or beads and washed against a sample of ethanol containing DNase. The advantages of the GuSCN-Phenol-Chloroform Extraction are that RNA can be extracted from basically any sample, and the RNA concentration can be adjusted to some extent by adjusting the amount of water. The disadvantage is that the RNA recovery rate is lower than that of the silica membrane filter base, and depends to some extent on the skill of the technician who performs the extraction. It is also environmentally unfriendly because of the use of phenol. The advantages of the silica filter base are that it does not use phenol, the extraction method is simple, and the recovery rate and purity are favorable. It is also beneficial for the environment since most of them do not use phenol. The disadvantages are that the extractable sample may vary depending on the extraction kit, and the amount of water used to elute the RNA is fixed, therefore it is not possible to adjust the concentration by oneself. Therefore, it is necessary to consider the advantages and disadvantages and use the RNA extraction method according to the purpose.

Previous studies have revealed the presence of small RNAs in blood and various body fluids (10). Serum miRNAs has been a potential biomarker in the diagnosis and prognosis of various diseases, including cancer (11-13), cardiovascular diseases (14)

Correspondence to: Dr Mitsuru Chiba, Department of Bioscience and Laboratory Medicine, Graduate School of Health Sciences, Hirosaki University, 66-1 Hon-cho, Hirosaki, Aomori 036-8564, Japan
E-mail: mchiba32@hirosaki-u.ac.jp

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and neurodegenerative diseases (15). Researchers frequently use different protocols and kits to perform serum RNA extractions for miRNA analysis (16). However, each extraction method has different principles, which may influence the yield and composition of the extracted serum RNA. Usually, having an RNA extraction kit that can recover the most RNAs from the serum is essential for comprehensive RNA analysis, such as microarrays, because it is easier to conduct analysis with higher concentrations of RNA. Therefore, the present study compared the RNA yield and composition of mice serum using five different RNA extraction kits.

Materials and methods

Mice and blood collection. A total of 24 male C57BL/6NJcl mice (8 weeks-old; body weight, 24.0±0.9 g) were purchased from CLEA Japan. All mice were provided a solid diet CE-2 (CLEA Japan) and water *ad libitum* and were housed in a conventional animal room with 12/12-h light/dark cycle. Mice were housed up to five mice per cage, and bedding, feed and water were changed weekly. Mice were observed 2-3 times per day for monitoring, and health or behavior abnormalities were not observed during the rearing period. Blood samples from all mice were collected by cardiac blood sampling under anesthesia with the inhalation anesthetic solution isoflurane (Pfizer) at the end of the 8-week time points. Small animal anesthesia machines (Muromachi Kikai) were used to anesthetize the mice. Isoflurane vaporized to a concentration of 4-5% was inhaled into the mice and maintained at 2-3% throughout the experiment, and blood was drawn from the mice's hearts. After anesthesia, ~0.5-1.0 ml of blood was received from the heart, and the mice were promptly cervically dislocated to minimize distress as a humane endpoint. The start of anesthesia to the end of blood collection took <10 min per animal. Death was confirmed by respiratory and cardiac arrest. All mice were euthanized immediately after the experiment. Serum samples that were separated using BD MicroTainer® SST (Nippon Becton Dickinson) blood collection tubes were used. Anticoagulants were not used. Serum was used because RNAs extracted from plasma contains platelet-derived RNAs (17). The collected blood was centrifuged at 6,000 x g for 3 min at room temperature to separate the serum. Serum was collected from 24 mice. The sera collected were not pooled and RNA was extracted from each individual. Serum at 100 μl was dispensed from one mouse in each group, which was considered as one sample. Four mice were used for each RNA extraction method. The present study was approved (approval no. AE01-2023-097-1) by the Hirosaki University Ethics Committee for Animal Experiments (Hirosaki, Japan), and was conducted under the Hirosaki University Guidelines for Animal Experiments.

RNA extraction. RNAs were extracted using serum of 100 μl from 8-week-old C57BL/6NJcl male mice and four reagents, including miRNeasy Serum/Plasma Advanced kit (cat. no. 217204), miRNeasy mini kit (cat. no. 217004; both from Qiagen KK), TRIzol-LS (cat. no. 10296028), and mirVana™ PARIS™ RNA and Native Protein Purification Kit (cat. no. AM1556; both from Thermo Fisher Scientific, Inc.), following the manufacturer's protocol. Additionally, an exoRNeasy midi kit (cat. no. 77144; Qiagen KK) in two ways was used to extract RNAs to determine the presence of RNAs in serum EVs. One method was performed following the manufacturer's protocol to extract EV RNAs from the serum. The other was utilized to extract RNAs from the aqueous layer from serum of 200 μl to which QIAzol Lysis Reagent (cat. no. 79306; Qiagen KK) of 700 μl was added and separated into two layers to determine the amount of total RNAs in the serum. The concentration of extracted RNAs was measured by Qubit™ microRNA Assay Kits (cat. no. Q32880.) and Qubit 4 Fluorometer (cat. no. Q33238; both from Thermo Fisher Scientific, Inc.) following the manufacturer's protocol. The RNA 6000 Pico 2100 bioanalyzer system (cat. no. 5067-1513) and bioanalyzer small RNA chip (cat. no. 5067-1548; both from Agilent Technologies, Inc.) were used to assess the size of extracted RNAs from 8-week-old C57BL/6NJcl mice.

miRNA microarray. RNAs of 1.8 ng extracted by the aforementioned extraction method were used for miRNA microarray analysis to examine serum miRNA expressions, following the manufacturer's instructions and as previously reported (18). The microRNA Spike In Kit (cat. no. 5190-1934; Agilent Technologies, Inc.) was used to perform quality checks of the microarray experiments. The RNA samples, Cyanine-3-labeled fluorescently, were hybridized to SurePrint G3 Mouse 8x60-K miRNA microarray slides (cat. no. G4872A; Agilent Technologies, Inc.) at 55˚C for 20 h. A SureScan Microarray Scanner (cat. no. G4900DA; Agilent Technologies, Inc.) was utilized to detect fluorescence signals using Agilent Feature Extraction 12.0 (Agilent Technologies, Inc.). As a method of evaluating Spike-In, Agilent Feature Extraction 12.0 was used to verify that the calculated values of LabelingSpike-InSignal and HybSpike-InSignal are each >2.5. This indicates that the microarray experiments are favorable. From all raw data obtained, excluding control probes, those with signal values 3-fold higher than the error value were selected by 'gls Gene Detected'. The selected raw data were normalized with quantile normalization and displayed logarithmically. These data were registered with the Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE246437).

Statistical analysis. The mann-whitney U-test and multiple-test Steel-Swass method was used to statistically analyze RNA-yield data. Spearman's rank correlation coefficient was utilized to assess the correlation coefficient of the microarrays. All tests were statistically processed with a sample size of four. The Statcel 4 software (OMS publication, https://oms-publ.main.jp/main/4steps4-hyo1/), was used for statistical analysis. Correlation coefficients were calculated and plotted using R (version 4.2.3).

Results

Comparison of the RNA yields from mouse serum. The RNA yields in mouse serum were compared for each of the RNA extraction reagents. Qubit™ microRNA Assay Kit was utilized to measure the RNA concentration, and the yield was calculated based on the amount of RNase-free water and RNA concentration. The yields of RNAs in the five RNA extraction reagents were compared. The

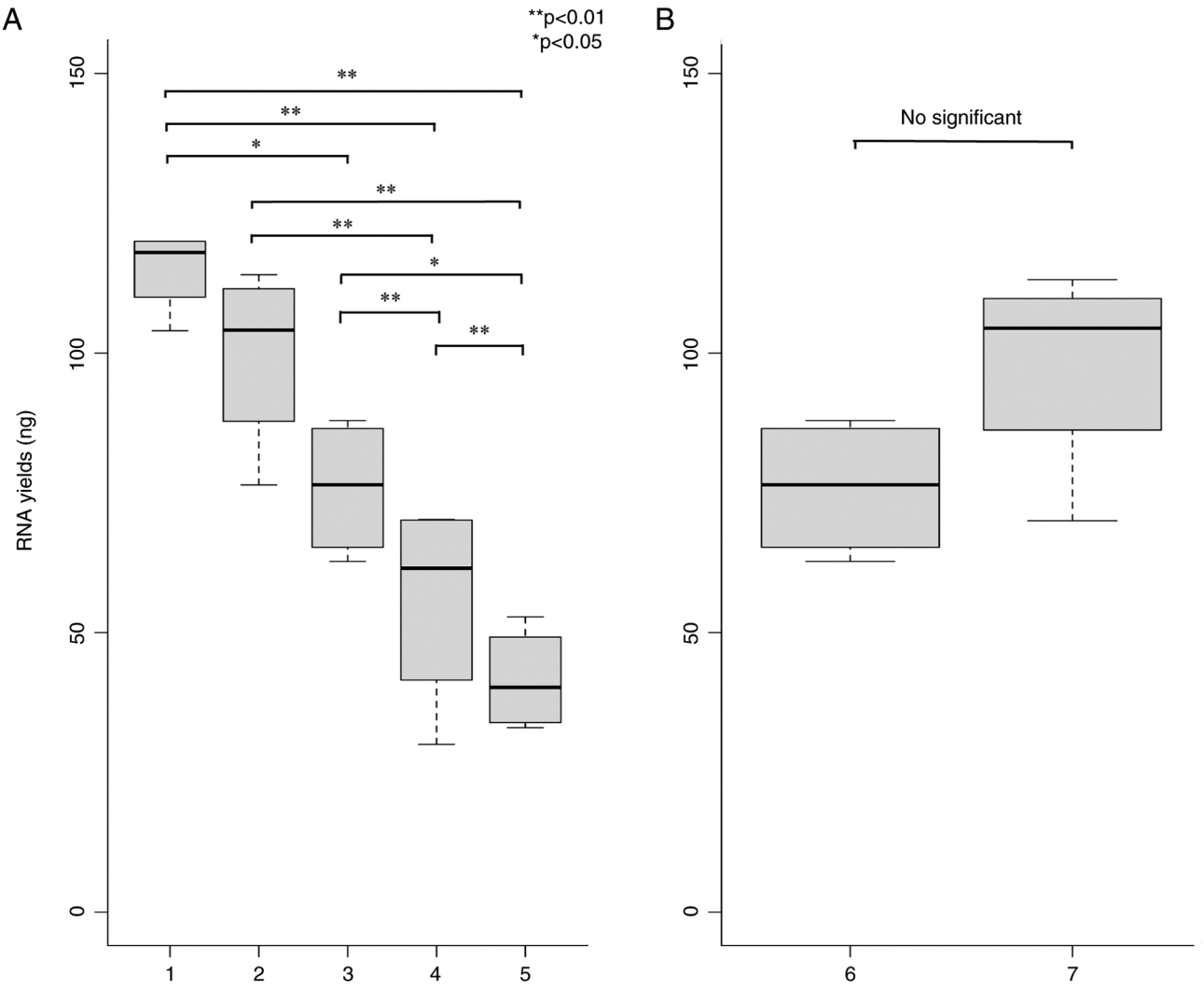

Figure 1. Comparison of RNA yield in serum by RNA extraction method. The RNA yield was calculated by each RNA extraction method. (A) RNA yields per 1 ml of serum for five different RNA extraction kits, including 1) mirVana™ PARIS™ RNA and Native Protein Purification Kit; 2) miRNeasy Serum/Plasma Advanced kit; 3) exoRNeasy midi kit total RNA extraction protocol; 4) miRNeasy mini kit; and 5) Trizol-LS. The Steel-Dwass method was used to identify statistical differences. *P<0.05 and **P<0.01. (B) Comparison of exoRNeasy midi kit following the manufacturer's protocol and exoRNeasy midi kit total RNA extraction protocol. 6) exoRNeasy midi kit total RNA extraction protocol; 7) exoRNeasy midi kit (manufacturer's protocol). The Mann-Whitney U-test was used to determine statistical differences. Significance levels were set at a risk ratio of P<0.05. Serum was collected from 24 mice. Four mice were used for each RNA extraction method. The same data were used for 3) and 6).

results demonstrated significantly different yields from the miRNeasy Serum/Plasma Advanced kit and the mirVana™ PARIS™ RNA and Native Protein Purification Kit (from the miRNeasy mini kit and TRIzol-LS (Fig. 1A). Thus, the miRNeasy Serum/Plasma Advanced kit or mirVana™ PARIS™ RNA and Native Protein Purification Kit should be used for the most efficient RNA extraction from mouse serum. Conversely, the exoRNeasy midi kit was used for two different extraction methods to assess the proportion of serum RNAs contained within EVs. The results revealed no statistically significant difference in yield between the two RNA extraction methods (Fig. 1B). This indicated the presence of most serum RNAs in the EVs in serum.

Several small RNAs are present in serum RNA. The RNA 6000 Pico 2100 bioanalyzer system and the bioanalyzer small RNA chip in the three RNA extraction reagents with the hightest RNA yields aforementioned were used to confirm RNA quality and size in serum. The results of the three RNA extraction reagents demonstrated that the RNA 6000 Pico bioanalyzer system confirmed small RNAs of <200 nt (Fig. 2A-C). The bioanalyzer small RNA chip detected 20-40 nt small RNAs (Fig. 2D-F). Further, serum RNAs extracted by miRNeasy Serum/Plasma Advanced kit exhibited another peak of ~40-100 nt using a bioanalyzer small RNA chip (Fig. 2D). This result indicated that most of the RNAs in the serum are small RNAs. Further, small RNAs from 40-100 nt was also efficiently extracted from serum using the miRNeasy Serum/Plasma Advanced kit. This revealed that the extracted RNAs may differ in composition based on the RNA extraction method.

Correlation between miRNA expression in mouse serum and RNA extraction reagents. The three RNA extraction reagents with the highest RNA yields were used to perform miRNA microarrays to determine the differences in serum miRNA expression obtained with each RNA extraction method and

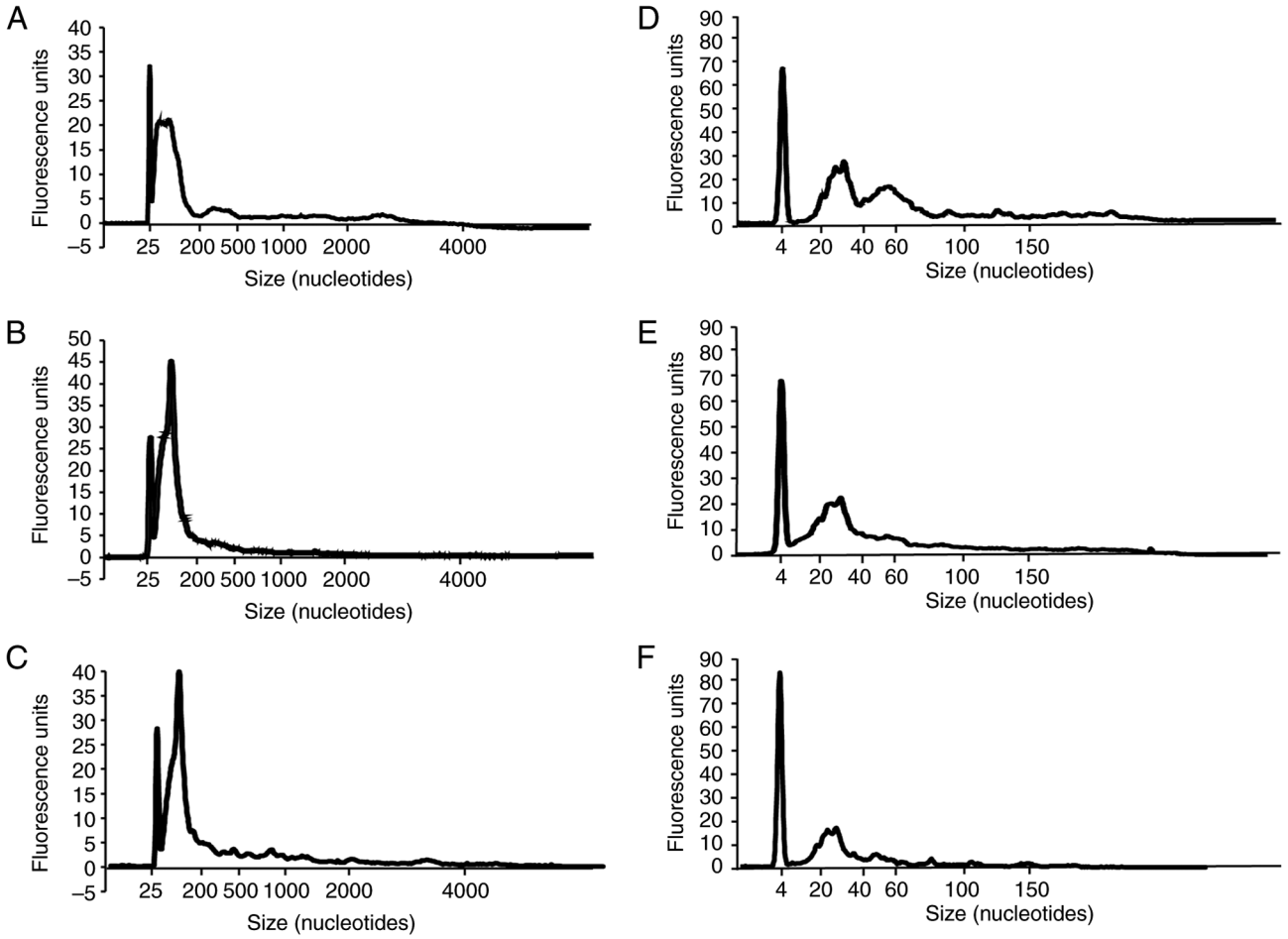

Figure 2. Comparison of size of serum RNA by RNA extraction method. An Agilent Bioanalyzer was utilized to electrophorese serum RNAs. The vertical axis indicates fluorescence intensity and the horizontal axis represents RNA size. (A) miRNeasy Serum/Plasma Advanced kit for the RNA 6000 Pico 2100 bioanalyzer system. (B) exoRNeasy midi kit total RNA extraction protocol for the RNA 6000 Pico 2100 bioanalyzer system. (C) mirVana™ PARIS™ RNA and Native Protein Purification Kit for the RNA 6000 Pico 2100 bioanalyzer system. (D) miRNeasy Serum/Plasma Advanced kit for the bioanalyzer small RNA chip. (E) exoRNeasy midi kit total RNA extraction protocol for the bioanalyzer small RNA chip. (F) mirVana™ PARIS™ RNA and Native Protein Purification Kit for the bioanalyzer small RNA chip. The RNA used in this experiment was the same sample extracted in Fig. 1.

examine the correlations. It was confirmed that there were no problems with the microarray experiment by quality check. These three RNA extraction reagents commonly expressed 84 types of miRNAs (Fig. 3A). However, some types of miRNAs were only detected with certain RNA extraction reagents. This suggested that different RNA extraction reagents may cause differences in the types of miRNAs detected. Furthermore, the correlation between the expression levels of commonly expressed miRNAs in all combinations of RNA extraction reagents was examined. There was a high correlation between the expression of common miRNAs detected by each RNA extraction reagent (Fig. 3B-D). These results indicated that the miRNAs commonly expressed by the three RNA extraction reagents are highly correlated in expression levels.

Discussion

The present study used various RNA extraction reagents to compare RNA yields, size and small RNAs components from mouse serum. Serum, not plasma, was used as the specimen. Plasma generally contains more platelets, and differences in the number of platelets in individuals may affect the amount and type of miRNAs in the plasma. It has been reported that platelets also contain high amounts of miRNAs (19). Therefore, serum was used to exclude the effect of platelet count on the amount and type of miRNAs.

RNA extraction methods using miRNeasy Serum/Plasma Advanced kit and mirVana™ PARIS™ RNA and Native Protein Purification Kit, following the manufacturer's protocol, demonstrated the highest RNA yields from 100 μl of mouse serum (Fig. 1A). Conversely, the yields of RNAs collected from the RNA extraction methods using the miRNeasy mini kit and TRIzol-LS were significantly lower than those of the aforementioned two methods (Fig. 1A). These results revealed that serum RNA extraction methods using the miRNeasy Serum/Plasma Advanced kit and mirVana™ PARIS™ RNA and Native Protein Purification Kit (efficiently collected serum RNAs in terms of RNA yields. However, the RNAs must be concentrated by ethanol precipitation or other methods to obtain high RNA concentrations, since the mirVana™ PARIS™ RNA and Native Protein Purification Kit elutes RNAs with 100 μl of RNase-free water.

Wright *et al* (20) revealed that miRNeasy Serum/Plasma Advanced kit is the best extraction kit for blood miRNAs

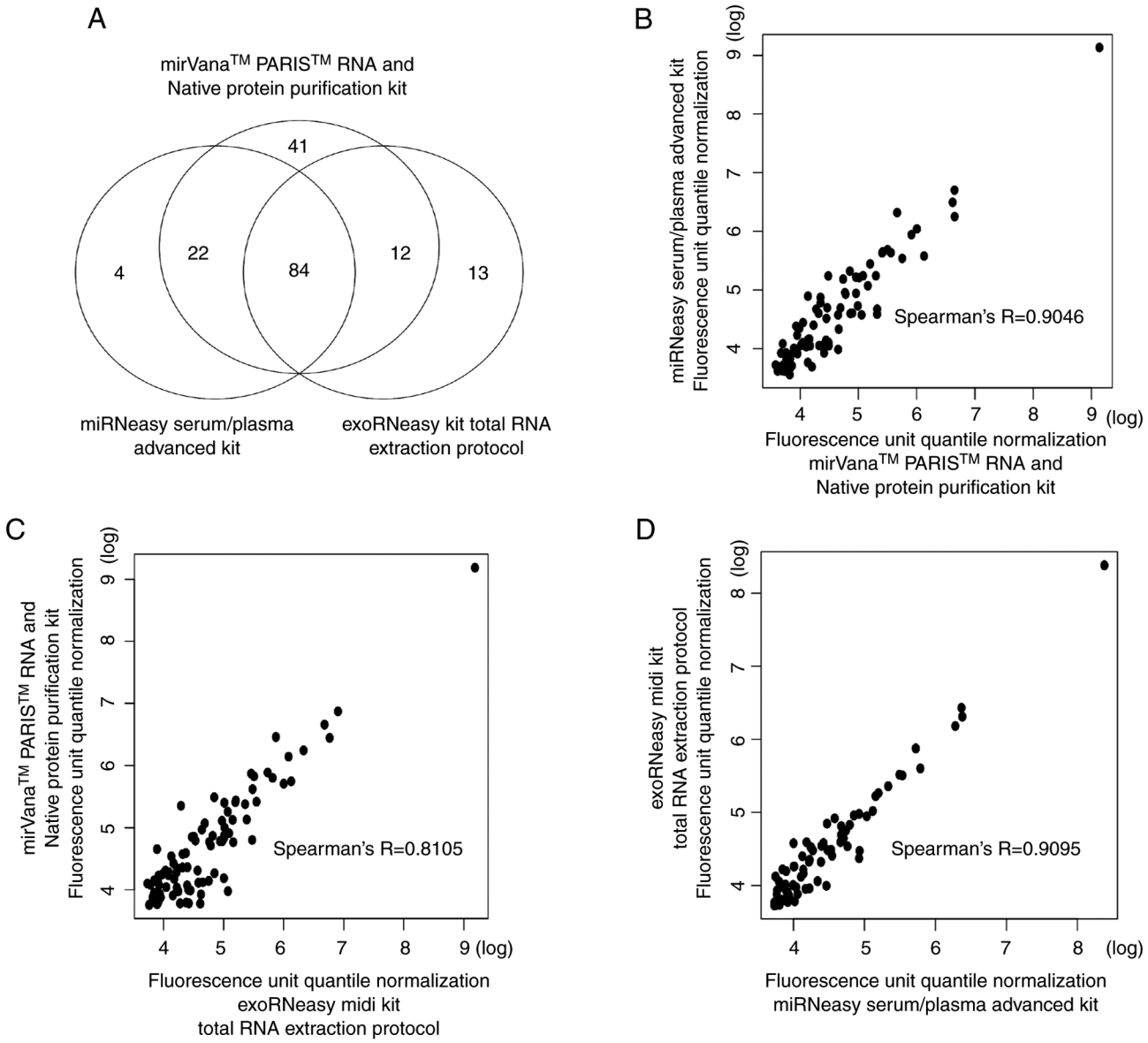

Figure 3. MiRNA expression detected by microarray. Venn diagram and correlation coefficients of miRNAs detected by Agilent miRNA microarray. (A) Venn diagram illustrating miRNAs confirmed to be expressed by the three RNA extraction reagents. (B) The y-axis demonstrates the miRNeasy Serum/Plasma Advanced kit and the x-axis indicates mirVana™ PARIS™ RNA and Native Protein Purification kit. (C) The y-axis exhibits mirVana™ PARIS™ RNA and Native Protein Purification kit and the x-axis represents the exoRNeasy midi kit total RNA extraction protocol. (D) The y-axis demonstrates the exoRNeasy midi kit total RNA extraction protocol and the x-axis indicates the miRNeasy Serum/Plasma Advanced kit. The RNA used in this experiment was the same sample extracted in Fig. 1.

using sheep serum as sample. This was consistent with the current results in terms of RNA yields and ease of use. Silica-based or magnetic beads-based was recommended for RNA extraction of hepatitis C virus in serum, as used in a recent study (21). The miRNeasy Serum/Plasma Advanced kit and mirVana™ PARIS™ RNA and Native Protein Purification Kit are silica-based kits, and TRIzol-LS is a guanidinium phenol-based kit. Silica-based RNA extraction was considered to be improved for RNA extraction using samples with low RNA content, such as serum.

There have been several studies on RNA yield from serum; Tang *et al* (22) in their study on RNA extraction from extracellular vesicle-derived RNA in human serum identified that the exoRNeasy kit had a higher RNA yield than Trizol-LS. Trakunram *et al* (23) also reported that RNA extraction of human serum demonstrated improved RNA yield and purity with the miRNeasy mini kit compared with Trizol-LS. These results are consistent with the present study, with improved RNA yield with the silica filter base than with the phenol base. The present study also used mouse serum, but similar results were confirmed with human serum.

RNAs in serum is generally contained inside EVs, such as exosomes (24-27), but the extent to which RNAs in serum is present in EVs remains unclear. Therefore, the RNA yields of the used methods were compared to extract EV RNAs and total RNAs in serum using the exoRNeasy midi kit. This result revealed no significant difference between the amount of serum EV RNAs and that of serum total RNAs, indicating that most of the serum RNA may be RNAs in EVs (Fig. 1B).

Two types of Agilent Bioanalyzer chips were used for RNA electrophoresis to determine the size of serum RNAs. The results revealed the peaks of small RNAs of <200 nt in RNA 6000 Pico 2100 bioanalyzer system, and the peaks of small RNAs of 20-40 nt in the bioanalyzer small RNA chip (Fig. 2). Interestingly, RNAs extracted from the miRNeasy Serum/Plasma Advanced kit demonstrated a reproducible bimodal pattern with peaks of ~40-100 nt (Fig. 2D). The peaks of small RNAs at 20-40 nt are mainly miRNAs, whereas the peaks at 40-100 nt are small RNAs that do not match the size of miRNAs. In the present study, sufficiently heat-treated serum RNAs were extracted, and the peak was not caused by miRNA duplication. RNAs that match this size may consist of precursor miRNAs (28), transfer RNAs (29,30) and small nucleolar RNAs (snoRNAs) (31,32). Fitz *et al* (33) demonstrated that snoRNAs are encapsulated within EVs and exist extracellularly, and that snoRNAs in the EVs in serum can be a diagnostic biomarker for Alzheimer's disease (33). The miRNeasy Serum/Plasma Advanced kit that efficiently collected 40-100 nt of small RNAs was unclear, but serum RNAs by miRNeasy Serum/Plasma Advanced kit may be suitable for extracting small RNAs other than miRNAs. The novelty of the present study was that it is the first, to the best of the authors' knowledge, to evaluate the miRNeasy Serum/Plasma Advanced kit for RNA extraction from serum. In addition, a bimodal pattern was observed in the miRNeasy Serum/Plasma Advanced Kit. This is also a novel result, as it had not been previously reported.

Further, 84 miRNAs were expressed in common with the three types that demonstrated the highest amount of RNA extraction, but some miRNAs were not confirmed to be expressed in common with the three types (Fig. 3A). No issues were concluded in using RNA extraction kits throughout the experiment. It has been recently reported that miRNA expression levels in blood are lower than cell/tissue expression levels (34). In the present study, RNA was extracted from 100 μl of serum, and it is expected that the number of miRNAs commonly detected in the three protocols will increase as the amount of specimen used increases. There are two possible reasons why the types of miRNAs do not completely match in all three extraction kits: One reason is that there are individual differences in the samples. The other may be a bias in the type of small RNAs extracted due to the characteristics of the RNA extraction method as demonstrated in Fig. 2.

A high correlation exists in miRNA expression levels among the RNA extraction kits examined, and any RNA extraction kit may be used when examining miRNA expressions with microarrays (Fig. 3B-D). However, the extraction efficiency of small RNAs other than miRNAs may differ based on the RNA extraction method. Next-generation sequencing or other methods are reqired to clarify the components of these 40-100 nt small RNAs in the future. In addition, the sample size in the present study was small and will need to be reexamined with a sufficient sample size in the future.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request The datasets generated and/or analyzed during the current study are available in the Gene Expression Omnibus repository (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE246437).

Authors' contributions

KY and MC were major contributors to performing the experiments and writing the manuscript. All authors read and approved the final manuscript. KY and MC confirm the authenticity of all the raw data.

Ethics approval and consent to participate

All experiments were performed in accordance with The Guideline for Animal Experimentation of Hirosaki University. The present study was approved (approval number: AE01-2023-097-1) by the Animal Research Committee of Hirosaki University (Hirosaki, Japan).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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