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Multicenter Evaluation of Circulating Plasma MicroRNA Extraction Technologies for the Development of Clinically Feasible Reverse Transcription Quantitative PCR and Next-Generation Sequencing Analytical Work Flows

Vera Kloten,[1†] Martin H.D. Neumann,[2†] Francesca Di Pasquale,[2] Markus Sprenger-Haussels,[2]
Jonathan M. Shaffer,[3] Martin Schlumpberger,[2] Andrei Herdean,[4] Fay Betsou,[6] Wim Ammerlaan,[6]
Taija af Hällström,[7,8,9] Elina Serkkola,[9] Tarja Forsman,[9] Evi Lianidou,[10] Robert Sjöback,[4] Mikael Kubista,[4,5]
Sebastian Bender,[11] Rita Lampignano,[1] Thomas Krahn,[1] and Thomas Schlange[1*]
for the CANCER-ID consortium

BACKGROUND: In human body fluids, microRNA (miRNA) can be found as circulating cell-free miRNA (cfmiRNA), as well as secreted into extracellular vesicles (EVmiRNA). miRNAs are being intensively evaluated as minimally invasive liquid biopsy biomarkers in patients with cancer. The growing interest in developing clinical assays for circulating miRNA necessitates careful consideration of confounding effects of preanalytical and analytical parameters.

METHODS: By using reverse transcription quantitative real-time PCR and next-generation sequencing (NGS), we compared extraction efficiencies of 5 different protocols for cfmiRNA and 2 protocols for EVmiRNA isolation in a multicentric manner. The efficiency of the different extraction methods was evaluated by measuring exogenously spiked cel-miR-39 and 6 targeted miRNAs in plasma from 20 healthy individuals.

RESULTS: There were significant differences between the tested methods. Although column-based extraction methods were highly effective for the isolation of endogenous miRNA, phenol extraction combined with column-based miRNA purification and ultracentrifugation resulted in lower quality and quantity of isolated miRNA. Among all extraction methods, the ubiquitously expressed miR-16 was represented with high abundance when compared with other targeted miRNAs. In addition, the use of miR-16 as an endogenous control for normalization of quantification cycle values resulted in a decreased variability of column-based cfmiRNA extraction methods. Cluster analysis of normalized NGS counts clearly indicated a method-dependent bias.

CONCLUSIONS: The choice of plasma miRNA extraction methods affects the selection of potential miRNA marker candidates and mechanistic interpretation of results, which should be done with caution, particularly across studies using different protocols.

In the past decade, molecular analysis of circulating nucleic acids in body fluids started to have a growing impact on the clinical treatment of patients with cancer. Liquid biopsy is a rapidly expanding field in translational cancer research and shows the potential to complement diagnostic and therapeutic care of patients with cancer.

Circulating biomarkers, including cancer-derived microRNA (miRNA),[12] have emerged as a new class of promising minimally invasive clinical biomarkers for liquid molecular profiling of patients with cancer *(1–4)*. miRNAs are short (about 22 nucleotides in length) non-

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[1] Bayer AG, Pharmaceutical Division, Biomarker Research, Wuppertal, Germany; [2] QIAGEN GmbH, Hilden, Germany; [3] QIAGEN, Frederick, MD; [4] TATAA Biocenter AB, Gothenburg, Sweden; [5]Institute of Biotechnology CAS, v. v. i., Vestec, Czech Republic; [6] Integrated BioBank of Luxembourg, Dudelange, Luxembourg; [7] AstraZeneca, Espoo, Finland; [8] Institute for Molecular Medicine Finland, University of Helsinki, Helsinki, Finland; [9] Orion Pharma, Orion Corporation, Espoo, Finland; [10] University of Athens, Athens, Greece; [11] Bayer AG, Pharmaceutical Division, Translational Assay Technology, Berlin, Germany.

[*] Address correspondence to this author at: Bayer AG, Pharmaceuticals Division, 42096 Wuppertal, Germany. E-mail thomas.schlange@bayer.com.

[†] V. Kloten and M.H.D. Neumann contributed equally to the work.

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[12] Nonstandard abbreviations: miRNA, microRNA; cfmiRNA, cell-free miRNA; EV, extracellular vesicle; EVmiRNA, extracellular vesicle derived miRNA; RT-qPCR, reverse transcription quantitative real-time PCR; NGS, next-generation sequencing; mirV, mirVana; miRCB, miRCURY Biofluids; PSRPM, Plasma/Serum RNA Purification Mini; miRSP, miRNeasy Serum/Plasma; miRA, miRNeasy Advanced Serum/Plasma; exoR, exoRNeasy Serum/Plasma; exoU, exosome ultracentrifugation; PPC, positive PCR control; miRTC, miRNA reverse transcription control; C_q, quantification cycle; PCA, principal component analysis; UMI, unique molecular index.

coding RNAs that regulate protein-coding gene expression by binding to a specific site in the 3′ untranslated regions of mRNA targets and, thus, promote their degradation and/or translational inhibition, thereby potentially contributing to cancer initiation and progression. The use of miRNA in body fluids as biomarkers may be associated with a number of advantages: (*a*) cell-free miRNA (cfmiRNA) is well-preserved in body fluids owing to an association with Argonaute 2 protein *(5, 6)* or high-density proteins *(7)*; (*b*) extracellular vesicles (EVs), primarily exosomes, have been identified as important carriers for miRNA, keeping RNAs protected from intercellular nucleases *(8, 9)*; and (*c*) circulating miRNA is highly stable over multiple freeze–thaw cycles, long-term storage, or treatment with RNase *(10)*. Therefore, a number of extraction methods for miRNA have been developed and commercialized in the past decade. Associated with this is the need for appropriate controls of preanalytic and analytic variables when considering circulating miRNA biomarkers in the clinical setting *(11–13)*. Analytical challenges such as low concentration, suboptimal RNA integrity, and high interindividual variability of miRNA expression must be controlled to establish clinically deployable miRNA biomarker assays. In addition, methods for the isolation of EVs differ in subpopulations, size, concentration, purity, and functionality of the extracted EVs *(14)*. Therefore, selecting appropriate extraction methods is a critical step in all areas of miRNA liquid biopsy research. Finally, standardization and benchmarking of downstream read-out technologies are of key importance to generate comparable data among different analytical laboratories or among different clinical studies.

Within the Innovative Medicines Initiative project CANCER-ID, standardization of preanalytical and analytical work flows for blood-based biomarkers is a key objective. Here, we report the results on the implementation of different miRNA extraction technologies in a multicentric ring study to establish standardized analytical work flows for plasma-derived miRNA analysis. Five different miRNA and 2 EV miRNA (EVmiRNA) isolation protocols were systematically compared in the study by using reverse transcription quantitative real-time PCR (RT-qPCR) and next-generation sequencing (NGS). The candidate miRNAs miR-16, miR-21 [reviewed by Bica-Pop et al. *(15)*], let-7a, and miR-191 were chosen not only because of their ubiquitous expression in a wide range of body fluids *(16, 17)* and their potential role as cancer biomarkers, but also because of ongoing discussion about their suitability for normalization *(14)*. In addition, the EV-associated miRNA candidates miR-122 (predominantly found outside of EVs) and the EV-associated miR-150 (highly enriched in EVs) were included.

Materials and Methods

STUDY DESIGN

For this study, 10 mL of K_2EDTA whole blood from 20 healthy donors (10 female, 62.6 ± 6.9 years of age; 10 male, 60.3 ± 5.6 years of age) was collected at the Zitha Clinic in Luxembourg under the Informed Consent CNER 201005/02 (approved by the local Luxembourg ethics committee) and processed to plasma at Integrated BioBank of Luxembourg. Systematic comparison of different extraction protocols for cfmiRNA and EVmiRNA from plasma was designed as a multicentric ring study. Six commercially available extraction kits and ultracentrifugation were performed by six CANCER-ID participating sites. The extracted miRNA was centrally analyzed using miScript qPCR and miRNA QIAseq (QIAGEN) (see Fig. 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol65/issue9).

PLASMA PROCESSING

Blood samples (10 mL) from all blood donors were obtained by venipuncture. Blood was collected in K_2EDTA vacutainers (BD). Blood tubes remained at ambient temperature until plasma was generated within 4 h after blood draw. Plasma samples were centrifuged at 1900*g* for 10 min at 4 °C. Supernatant was carefully transferred into 15-mL high-performance centrifuge tubes (VWR) and centrifuged a second time at 16000*g* for 10 min at 4 °C to remove cellular debris. From each tube, 4 mL of plasma was aliquoted in 2 portions of 2 mL, which were shipped to Bayer AG on dry ice; one freeze–thaw cycle was done while preparing the aliquots. Bayer AG prepared aliquots of the plasma samples depending on the extraction methods (Table 1).

EXOGENOUS CONTROL

Synthetic cel-miR-39 (*Caenorhabditis elegans*; 5′-UCACCGGGUGUAAAUCAGCUUG-3′) (QIAGEN) was added to each extraction. Ten picomoles of lyophilized synthetic cel-miR-39 was dissolved in 550 μL of nuclease-free water resulting in a 1+E10 copies/μL stock solution. Every participating site added 52.5 μL into the method-specific lysis buffer (volume according to vendor specifications). Based on this, the total amount spiked into the cDNA synthesis was 4.5 fmol for each extraction method. Adding 200 μL of RNase-free water to the reverse transcription reaction resulted in a cel-miR-39 concentration of 2.8+E07 copies/μL in each qPCR reaction. For ultracentrifugation, the spike-in was added at the EV lysis step and, therefore, does not reflect efficiency of the EV isolation step.

ISOLATION OF cfmiRNA

cfmiRNA was isolated from 200 μL of plasma using five commercially available extraction technologies (Table 1).

Table 1. RNA extraction kits utilized in this study and participation site.

Extraction site	Manufacturer	RNA extraction kit/protocol	Abbreviation	Principle of extraction
University of Athens	Thermo Fisher Scientific	mirVana	mirV	Phenol + spin column
IBBL	QIAGEN	miRNeasy S/P	miRSP	Phenol + spin column
QIAGEN	QIAGEN	miRNeasy Advanced S/P	miRA	Protein precipitation + spin column
TATAA	Norgen	P/S RNA Purification Mini	PSRPM	Proteinase K + spin column
Bayer AG	Exiqon	miRCURY Biofluids	miRCB	Protein precipitation + spin column
QIAGEN	QIAGEN	exoRNeasy S/P	exoR	EV: membrane affinity EVmiRNA: phenol + spin column
EV Core, University of Helsinki	QIAGEN	Exosome Ultracentrifugation + miRNeasy kit	exoU	EV: sedimentation EVmiRNA: phenol + spin column

Isolation was performed following manufacturers' recommendations. Total RNA, including cfmiRNA, was eluted in 100 μL of mirVana (mirV), 50 μL of miRCURY Biofluids (miRCB), 15 μL of Plasma/Serum RNA Purification Mini (PSRPM), and 14 μL of [miRNeasy Serum/Plasma (miRSP), miRNeasy Advanced Serum/Plasma (miRA)] nuclease-free water.

ISOLATION OF EVmiRNA

EVmiRNA was isolated from 1 mL of plasma using the exoRNeasy Serum/Plasma Midi [exoRNeasy Serum/Plasma (exoR)] kit (QIAGEN), which was performed according to the manufacturer's recommendations, and eluted in 14 μL of nuclease-free water. EV isolation using ultracentrifugation followed by membrane affinity miRNA extraction (Table 1) was performed as detailed below.

ULTRACENTRIFUGATION STEP

EDTA plasma samples (1 mL each) were thawed at 37 °C in a water bath and placed on ice. Samples were diluted 1:2 with cold sterile-filtered PBS (0.2 μm, Whatman TM, GE Healthcare Life Sciences). After gentle mixing by inversion, diluted plasma samples were filtered with 0.8-μm Millex AA syringe filters (Merck Millipore). EVs were then pelleted by ultracentrifugation at 100000*g* (K-factor, 123.0) for 2 h using a fixed-angle rotor TLA-55 (Beckman-Coulter) at 4 °C. The supernatant was carefully aspirated by pipetting, and each pellet was resuspended in 50 μL of ice-cold filtered PBS. EVs were frozen at −80 °C until use.

RNA EXTRACTION STEP

Total RNA was extracted from frozen EV concentrates with the miRNeasy Micro kit (QIAGEN) according to the manufacturer's protocol "Purification of Total RNA, Including miRNA, from Animal Cells." A DNase I treatment was not performed. Total RNA, including EVmiRNA, was eluted in 14 μL of RNase-free water and stored at −80 °C until use.

REVERSE TRANSCRIPTION

cDNA was generated from extracted cfmiRNA and EVmiRNA samples using the miScript II RT kit (QIAGEN), according to the manufacturer's instructions. Ten percent of each eluate {i.e., 10 μL [mirV], 5 μL [miRCB], 1.5 μL [PSRPM], and 1.4 μL [miRSP, miRA, exoR, exosome ultracentrifugation (exoU)]} was used for the cDNA synthesis in a 20-μL reaction using the miScript HiSpec buffer (QIAGEN). The reactions were incubated for 60 min at 37 °C and stopped at 95 °C for 5 min. Finally, cDNA was diluted in 200 μL of nuclease-free water.

QUANTITATIVE REAL-TIME PCR

Real-time PCR was carried out using the miScript SYBR Green PCR kit (QIAGEN) on a CFX-96 real-time PCR detection system (Bio-Rad Laboratories) according to the manufacturer's instructions. Twenty-five microliters of PCR reaction mix included 2.5 μL of prediluted cDNA, 12.5 μL of QuantiTect SYBR Green PCR Master Mix, 2.5 μL of miScript Universal Primer, and 7.5 μL of nuclease-free water. The reaction mixture was added to a custom miScript Primer Assay plate containing the lyophilized miRNA-specific primer assays for one 25-μL real-time PCR reaction/well. Besides selected miRNA-specific primer assays (let-7a-5p, miR-150–5p, miR-16–5p, miR-122–5p, miR-21–5p, miR-191–5p), several control

Table 2. Mean C_q values SD of 6 human miRNAs measured by RT-qPCR in 20 healthy individuals.

			Normalized to					
			cel-miR-39			miR-16		
	Mean raw C_q	SD raw C_q	C_q	SD C_q	%[a]	C_q	SD C_q	%[a]
let-7a	27.76	1.67	27.73	1.59	4.79	27.68	1.67	0.00
miR-16	24.15	1.48	24.12	1.50	−1.35	–	–	–
miR-21	26.98	1.74	26.95	1.17	32.76	26.91	1.80	−3.45
miR-122	30.23	2.43	30.20	2.39	1.65	30.16	2.49	−2.47
miR-150	27.49	1.73	27.46	1.67	3.47	27.42	1.81	−4.62
miR-191	29.45	1.81	29.42	1.95	−7.73	29.37	1.79	1.10

[a] Percentages indicate the proportion of normalized SD of C_q values relative to raw SD of C_q values.

primer assays were included: cel-miR-39–3p, positive PCR controls (PPCs) in duplicates, and miRNA reverse transcription control (miRTC) in triplicates. Samples were incubated at 95 °C for 15 min, followed by 40 amplification cycles of 94 °C for 15 s, 55 °C for 30 s, and 70 °C for 30 s. The ramp rate of the instrument was set at 1 °C/s. A melt curve analysis was performed following PCR cycling. The quantification cycle (C_q) value was defined using the regression mode.

NGS

NGS libraries were prepared from 5-μL aliquots using the QIAseq miRNA Library kit (QIAGEN). A detailed description of the libraries and NGS procedure is provided in Materials and Methods of the online Data Supplement.

RT-qPCR AND NGS DATA NORMALIZATION

Raw C_q values of targeted miRNA were normalized to the endogenous miR-16 *(16)* and to the spiked-in cel-miR-39 *(12)*:

$$\text{Target}_{\text{normalized}} = \text{Target}_{\text{raw}} - (\text{Control}_{\text{raw}} - \text{Control}_{\text{median run}}) \qquad (1)$$

where *Target* is the miRNA to be normalized, *Control* is miR-16 or cel-miR-39, *raw* is the sample C_q value, and *median run* is the median C_q value of 20 raw results produced by each technology. To this end, the standard variation was calculated from raw and normalized C_q values (Tables 2 and 3).

NGS data were preprocessed and analyzed using the MultiD GenEx software 7.0.1.473. Data normalization consisted of the following steps: (*a*) miRNA without any

Table 3. Mean C_q values and SD of different RNA extraction protocols.

			Normalized to					
			cel-miR-39			miR-16		
Code	Mean raw C_q[a]	SD raw C_q	C_q	SD C_q	%[b]	C_q	SD C_q	%[b]
mirV	28.00	2.14	27.65	2.20	−2.80	28.23	2.10	4.55
miRCB	26.39	2.13	26.67	2.26	−6.10	27.12	1.70	24.78
miRSP	29.14	2.69	29.21	2.58	4.09	29.92	1.87	27.52
miRA	26.61	2.47	26.24	2.43	1.62	27.37	1.65	32.10
PSRPM	27.84	2.24	27.88	2.22	0.89	28.58	1.34	39.64
exoR	26.80	3.18	26.9	3.11	2.20	27.38	3.11	0.00
exoU	28.98	2.25	28.98	2.24	0.44	29.55	2.15	4.02

[a] Mean C_q for all the miRNA tests by method.

[b] Percentages indicate the proportion of normalized SD of C_q values relative to raw SD of C_q values.

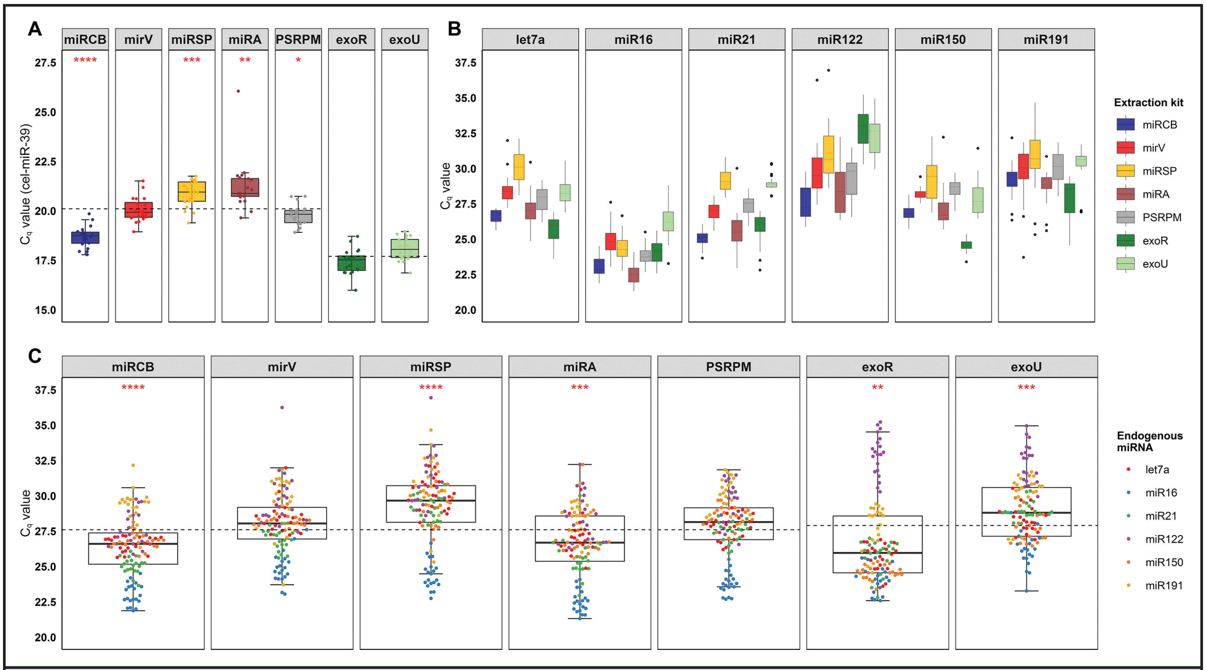

Fig. 1. Column-based RNA extraction technologies reveal high recovery of exogenous and endogenous miRNAs.

(A), Box plot analysis showing recovery of synthetic spiked-in cel-miR-39 among commercially available miRNA extraction technologies. Each dot represents a single plasma sample. (B), Box plot analysis showing 6 different targeted miRNAs to compare the extraction efficiency of 5 total RNA and 2 EVmiRNA extraction methods: miRCB, dark blue; mirV, red; miRSP, yellow; miRA, brown; PSRPM, gray; exoR, dark green; and ultracentrifugation followed by the exoU, light green. (C), Bee swarm plot analysis showing each extraction method among targeted miRNAs. The resulting miRNA quantity is reported as raw C_q value. The horizontal line in each box represents the mean; the error bars indicate the range; statistical analysis in (A) and (C) was performed using Student *t*-test with the base mean (dotted line) to compare all groups where *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$.

counts in all samples were removed; (*b*) all remaining values were converted to log2; (*c*) missing values were replaced with −1; and (*d*) data were normalized to total number of counts.

STATISTICAL ANALYSIS

The R (version 3.5.2) and R Studio software (R Studio) were used for statistical analysis and data visualization. Statistical comparisons were performed using Student *t*-test. Two-sided tests with $P < 0.05$ were considered statistically significant. Correlations were calculated using Pearson rank. Principal component analysis (PCA) of normalized NGS data was done using the *t*-test method with autoscaling of the column-wise scaling in the PCA step. Visualization of results in box plots, histogram, and PCA plot and heatmap was carried out using the package ggplot2.

Results

miRCB AND miRA EXTRACTED THE HIGHEST AMOUNTS OF ENDOGENOUS CANDIDATE miRNAs

First, total RNA, including cfmiRNA and EVmiRNA, was characterized by a customized miScript PCR panel including six different miRNA targets and distinct control targets. The inhibition control showed with a mean C_q^{PPC} value of 18.78 high-quality RNA across all extraction kits. In addition, C_q values of the miRTCs were examined using the values for the PPCs by calculating $\Delta C_q = \text{mean}C_q^{miRTC} - \text{mean}C_q^{PPC}$ revealing a mean value of 3.49 across all extraction kits, which indicated no inhibition of the reverse transcription reaction. Further, cel-miR-39 levels were evaluated (Fig. 1A). Recovery of cel-miR-39 in each case was estimated with respect to C_q values, thus translating lower C_q values into higher recovery of cel-miR-39.

In more detail, the miRSP (mean C_q, 20.87 ± 0.67) and the miRA (mean C_q, 21.24 ± 1.28) revealed significantly [$P < 0.001$ (miRSP), $P = 0.00133$ (miRA)] higher mean C_q values compared with the base mean (C_q, 20.13) among five cfmiRNA extraction methods. The mirV (mean C_q, 20.05 ± 0.62) and the PSRPM (mean C_q, 19.78 ± 0.52) were slightly lower than the cfmiRNA base mean, whereas the miRCB (mean C_q, 18.68 ± 0.53) showed a significantly ($P < 0.0001$) lower mean C_q value. Moreover, with the exoR (mean C_q, 17.43 ± 0.63)

Fig. 2. Multicentric evaluation of RNA extraction technologies shows variability between extraction sites and kits. Box plot analysis showing six different targeted miRNAs to compare the extraction efficiencies between different extraction sites and kits. The horizontal line in each box represents the mean; the error bars indicate the range. IBBL, Integrated BioBank of Luxembourg.

slightly lower and with the exoU (mean C_q, 18.05 ± 0.56) slightly higher C_q values compared with the base mean (C_q, 17.74) of EVmiRNA extraction protocols were observed.

Next, C_q values of targeted miRNA were evaluated. Each extracted miRNA was detected in varying amounts dependent on the extraction technology and site (Fig. 1B and Table 2). Importantly, differences up to 3 C_q values were shown between different extraction sites (Figs. 1B and 2). In addition, miRNA amounts showed a high interindividual variability, which was also influenced by the isolation method. miR-16 exhibited the highest expression (mean C_q, 24.15) and marginal variability (SD, 1.48) among all targeted miRNAs and extraction technologies. In addition, miR-21 was found highly expressed (mean C_q, 26.98 ± 1.74).

Subsequently, amounts of all miRNA targets were evaluated with respect to the extraction protocol (Fig. 1C and Table 3). The miRCB displayed the lowest overall mean C_q value of 26.4 for the six selected miRNA targets. With a C_q value of 26.6, the mean amount of endogenous miRNAs extracted with the miRA was similar. In addition, both kits showed a significantly ($P < 0.001$) lower mean C_q value compared with the base mean (C_q, 27.59) among five cfmiRNA extraction methods. In contrast, the mirV and PSRPM resulted in a mean C_q value of 28.0 and 27.8, respectively, while the miRSP showed the highest mean C_q value (29.1), which was significantly higher ($P < 0.0001$) compared with the base mean.

For EV-derived miRNA fractionations, exoU showed a significantly ($P < 0.001$) higher mean C_q value (29.0), whereas the exoR kit revealed a significantly ($P = 0.00196$) lower mean C_q value (26.89) compared with the base mean (C_q, 27.89) of EVmiRNA extraction protocols (Fig. 1C).

NGS ANALYSIS CONFIRMED HIGH EFFICIENCY AND ANALYTICAL PERFORMANCE OF THE miRCB AND miRA

Total RNA extraction methods were compared by applying the QIASeq miRNA library kit. The mapping distribution of different small RNAs is shown in Fig. 3 of the online Data Supplement. Box plot analysis (Fig. 3A) shows the total mapped miRNA read number in 20 healthy donors for each of the 7 RNA extraction protocols. Sequencing of miRNA resulted in a base mean of 2.89E+06 and 1.55E+06 reads for miRNA and EVmiRNA extraction protocols, respectively. The miRCB showed significantly ($P < 0.001$) higher reads (mean total reads, 3.73E+06) compared with the other miRNA protocols. Comparing EVmiRNA extraction protocols, the exoR revealed significantly ($P = 0.0036$; mean total reads, 2.35E+06) higher and the exoU significantly ($P < 0.0001$; mean total reads, 7.65+E05) lower read numbers. Supporting the RT-qPCR results, miR-16 displayed the highest unique molecular index (UMI) counts across all extraction technologies (Fig. 3B). miRNAs were distributed with heterogeneous mean counts among 20 healthy individuals depending on the extraction technology. In addition, a strong read repetition revealed that miRCB

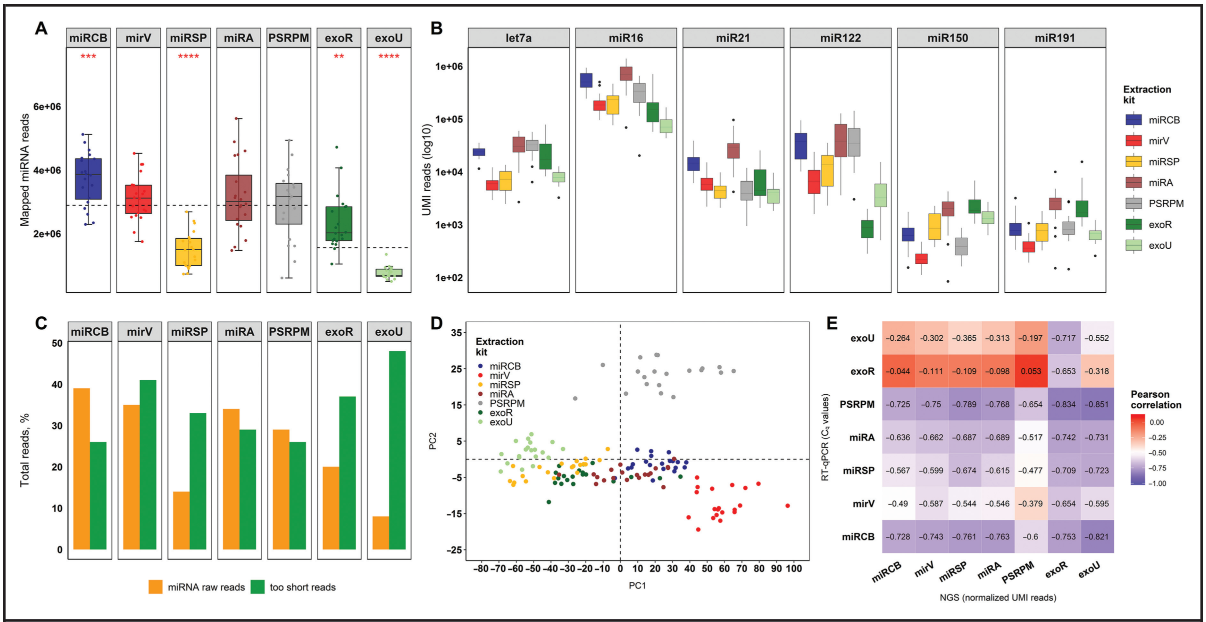

Fig. 3. Small RNA sequencing confirms high performance of miRCB and miRA isolation kits.

(A), Box plot analysis showing total reads among commercially available miRNA extraction technologies. Each dot represents one plasma sample. (B), Box plot analysis showing total counts of each endogenous miRNA among all technologies. The horizontal line in each box represents the mean; the error bars indicate the range; statistical analysis in (A) was performed using Student *t*-test with the base mean (dotted line) to compare all groups where $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$. (C), Bar plot showing the percentage of cumulative mapping of miRNA raw reads and "too short" (<16 bp insert sequences) reads. (D), PCA of miRNA data generated by NGS distinguishes four groups: (*a*) exoU, (*b*) PSRPM, (*c*) mirV, and (*d*) exoR, miRCB, miRA, and miRSP. The contribution of PC1 and PC2 to the overall variance in the data set is 61.9% and 4.53%, respectively. (E), Correlation matrix of extraction methods used in this study. Results of qPCR and NGS were compared. Correlation coefficients are color-coded in red ($r < -0.5$), white ($r = -0.5$) and blue ($r > -0.5$).

and miRA showed most cumulative miRNA molecules (Fig. 3C). This was not a consequence of more allocated total reads: While having the highest (miRCB, 39%; miRA, 34%) miRNA molecule counts, either similar or fewer total reads allocated during sequencing compared with the other protocols were obtained (Fig. 3A). Surprisingly, both exoR and exoU produced 37% and 48%, respectively, cumulative "too short" reads and fewer mapped miRNA molecules (Fig. 3C).

Moreover, we compared normalized total read counts produced by each extraction protocol. Global clustering by PCA clearly indicated distinct groups (bias) introduced by the miRNA purification methods (Fig. 3D). Four groups can be visually distinguished: (*a*) exoU, (*b*) PSRPM, (*c*) mirV, and (*d*) exoR, miRCB, miRA, and miRSP. Ultimately, we compared the C_q values and normalized UMI reads among all endogenous miRNAs. The correlation heatmap shown in Fig. 3E demonstrated an inverse correlation ($r > -0.5$) between RT-qPCR and NGS results for all extraction protocols. The strongest correlation was shown for the miRCB ($r = -0.728$). Interestingly, the cluster illustrated that EVmiRNA-determined C_q values only slightly correlated with UMI reads for cell-free RNA. Conversely, EVmiRNA-based UMI reads were strongly correlated with C_q values obtained for cell-free miRNA.

NORMALIZATION WITH ENDOGENOUS miR-16 DECREASED VARIABILITY OF COLUMN-BASED KITS

Mean raw C_q values of each targeted miRNA and extraction technology were normalized to cel-miR-39 or miR-16 (Tables 2 and 3). With the exception of miR-21 (32.8% decreased SD compared with raw C_q value SD), normalization to cel-miR-39 had no influence on variability of mean raw C_q values of each candidate miRNA among all extraction kits. In addition, normalization to miR-16 increased variability of candidate miRNAs (Table 2).

Interestingly, using miR-16 to normalize mean raw C_q values of each extraction kit among all candidate miRNAs (Table 3), we observed a decreased C_q value variability of column-based kits (miRCB, miRSP, miRA, and PSRPM) of 24.78%, 27.53%, 32.10%, and 39.64%, respectively. Normalization to cel-miR-39 left the variability largely unchanged or even increased it.

Discussion

Notwithstanding the growing number of published circulating miRNA studies in patients with cancer *(18–22)*, there is still no consensus on procedures and standardized protocols to use downstream analytical technologies, not even with respect to preanalytical sample handling in the clinical setting, which represents the first step in analytical work flows. In this study, we performed extraction using seven protocols at six different participating sites.

Several research groups have investigated circulating miRNA extraction by comparing different protocols *(12, 23–30)*. In our analysis, isolation of cfmiRNA based on the miRCB and the miRA resulted in low C_q values and high relative numbers of mapped miRNA reads over "too short" reads. In addition, NGS reads showed a tight cluster, as both column-based kits have a similar work flow to precipitate proteins and purify miRNA from the supernatant. It is important to note that after the acquisition of Exiqon by QIAGEN, the miRCB is no longer available. In contrast, the mirV and the PSRPM produced higher C_q values and a lower percentage of miRNA raw reads compared with too short reads. Using the miRSP, practical issues with phase separation in about 50% of samples occurred for unknown reasons. By doubling the QIAzol and chloroform this could be solved; however, only half of the doubled upper phase (600 μL) could be loaded in the QIAcube, resulting in a 1-C_q value increase and a lower number of miRNA raw reads. Taking this technical issue into account, the results of this study were comparable with another study *(23)*, demonstrating that RNA extraction with miRSP led to a 2- to 3-fold increase in RNA yield compared with the mirV kit. In addition, several studies *(24, 26, 27)* showed a high recovery of cfmiRNA using the miRSP. In our hands, the miRCB outperformed other column-based extraction technologies, which strengthens the results of other studies *(25, 30)*. In contrast, others *(12, 28)* found that the mirV and the miRNeasy produced the highest yield of recovery for spiked-in control miRNA, with the mirV obtaining a better performance than the miRNeasy. Interestingly, the exoR and exoU were prone to capture too short sequences. exoU revealed almost 50% of too short miRNA reads, in line with a previous study *(14)*. NGS data obtained by different extraction protocols and participation sites might be biased for a unique subpopulation of miRNA. Highly abundant miRNA are less affected by library preparation-induced biases, although this might be more problematic for low-abundance transcripts *(14)*. One limitation of the study is that we did not test each protocol at each site. However, routinely used standard protocols for the extraction of miRNA were used; in addition, experienced personnel extracted the miRNA. Nevertheless, there is still a chance that the performance of the protocol is influenced by the operator rather than by its technical specifications.

Normalization of raw C_q values using the exogenous cel-miR-39 left total variability unchanged. However, one must be aware that heterogeneous cel-miR-39 recovery between methods, which are probably introduced by spiked-in variations (e.g., different pipette sets and operators), could affect normalization. On the other hand, normalization to endogenous miR-16, commonly reported as an miRNA housekeeper *(16, 17, 31)*, decreased the variability of miRCB, miRSP, miRA, and PSRPM. Interestingly, the SDs of the mirV kit and both exoU and exoR kits remained similar. These results are in contrast to another study *(12)*, illustrating an increase in variance when using miRNA-16 as a normalizing factor. Global clustering of normalized UMI reads of endogenous miRNAs revealed a prominent cluster of miRCB, miRSP, miRA, and exoR. It becomes clear that every extraction method introduces a method-dependent bias. In this context, it might be difficult to compare the extraction kits, as there is no independent standard and every method extracts a unique subpopulation of miRNA. In addition, concerning the variability between extraction sites, the difference between biological groups (e.g., cancer patients vs healthy) should be >2 C_q values to overcome technical background noise. This underlines the fact that no normalization approach will eliminate all sources of variation (e.g., blood storage). However, without standardization, clinical application of miRNA will remain uncertain.

In conclusion, we report here that enriching cfmiRNA by the miRCB and miRA allows high recovery of endogenous miRNA. Working toward standardization, all technology providers should implement the possibility of analyzing identical sets of artificial miRNAs (e.g., cel-miR-39, ath-miRs) including variants and modifications; all biobanks should pay attention to developments in the preanalytical field to allow collection of samples most suitable for miRNA analysis; and all laboratories should participate in external quality assessment "processing" and analytical schemes to assess the performance of their miRNA extraction method(s) and their relative or absolute quantification assays and/or implement appropriate reference material. Our study underpins the necessity of performing standardized benchmarking studies to implement best practices of extraction and analysis platform performance for miRNA analysis in liquid biopsy.

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