Clinical Chemistry 57:6
833–840 (2011)

Analysis of Circulating MicroRNA: Preanalytical and Analytical Challenges

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BACKGROUND: There is great interest in circulating microRNAs (miRNAs) as disease biomarkers. Translating promising miRNAs into validated clinical tests requires the characterization of many preanalytical and analytical parameters.

METHODS: miRNAs were extracted from serum and plasma samples of healthy volunteers, and miRNAs known to be present in serum and plasma (miR-15b, miR-16, miR-24, and miR-122) were amplified by reverse-transcription quantitative PCR. Stability and the effects of hemolysis were determined. Assay variation and its components, including the effect of adding control miRNA, were assessed by nested ANOVA.

RESULTS: miRNA concentrations were higher in plasma than in serum. Processing of plasma to remove subcellular/cellular components reduced miRNA concentrations to those of serum. The miRNAs analyzed were stable refrigerated or frozen for up to 72 h and were stable at room temperature for 24 h. Hemolysis increased the apparent concentration of 3 of the miRNAs. The total variability of replicate miRNA concentrations was <2.0-fold, with most of the variability attributable to the extraction process and interassay imprecision. Normalizing results to those of spiked exogenous control miRNAs did not improve this variability.

CONCLUSIONS: Detailed validation of the preanalytical steps affecting miRNA detection and quantification is critical when considering the use of individual miRNAs as clinical biomarkers. Unless these causes of imprecision are considered and mitigated, only miRNAs that are extremely up- or downregulated will be suitable as clinical biomarkers.

MicroRNAs (miRNAs)[3] are short noncoding RNA sequences that regulate gene expression by inducing mRNA degradation or inhibiting mRNA translation into protein *(1)*. Dysregulation of miRNAs has been associated with many human diseases *(2)*. The detection of miRNAs in plasma and serum samples *(3, 4)* has raised the possibility that they might serve as noninvasive biomarkers for the diagnosis of a wide range of disorders—from acute myocardial infarction, congestive heart failure, and drug-induced liver injury *(5–7)* to various malignancies *(3, 4, 8–10)*. miRNAs seem to be resistant to rapid degradation by ribonucleases (RNases), an issue that has confounded the use of circulating mRNAs for clinical diagnosis *(3, 4, 11–13)*.

The route from proof of principle to the creation of reliable and reproducible miRNA clinical tests is a long one. Many of the preanalytical variables that might affect the clinical utility of miRNAs have yet to be studied in any detail. For example, there are few data on short-term stability. The effects of common assay interferences, such as hemolysis, remain to be explored. Similarly, the systematic differences between sample types remain largely unknown. Some investigators hypothesize that miRNAs are present in the circulation within exosomes and microvesicles, which are preferentially shed from diseased cells *(14–16)*. In plasma samples, this disease-specific signature might be overwhelmed by miRNAs contained in platelets.

To date, only a few studies have assessed miRNAs with respect to assay analytical parameters *(9, 17–19)*. The expected magnitude of assay imprecision is not known, nor are the relative contributions to assay imprecision from components including intra- and interassay imprecision, miRNA extraction, reverse transcription (RT), real-time PCR, and normalization (or not) to spiked or internal-control miRNAs. Therefore, we studied the effects of sample type, miRNA stability, intra- and interassay imprecision and its components,

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Received September 30, 2010; accepted March 24, 2011.
Previously published online at DOI: 10.1373/clinchem.2010.157198

[3] Nonstandard abbreviations: miRNA, microRNA; RT, reverse transcription; RNase, ribonuclease; cel, *Caenorhabditis elegans* (miRNA); RT-qPCR, RT quantitative PCR; Cq, quantification cycle.

and hemolysis on the reproducibility and reliability of quantification of circulating miRNAs.

Materials and Methods

STUDY PARTICIPANTS AND SAMPLE PROCESSING

The Mayo Clinic Institutional Review Board approved this study. Blood from healthy nonfasting male and female volunteers was drawn in the morning into serum separator or EDTA-containing tubes (BD Biosciences). The collection tubes were processed according to our routine preanalytical protocol for clinical serum and plasma samples. Tubes were rotated end-over-end at room temperature for 30 min and centrifuged at 795*g* (Beckman JB-6; Beckman Coulter) at 4 °C for 20 min. The serum or plasma sample was aliquoted into 1.5-mL RNase-free Eppendorf tubes (Ambion).

Where indicated below, we removed cellular contaminants by subjecting serum or plasma samples to additional centrifugation at 15 000*g* (Eppendorf 5417R) for 10 min, which produced a visible pellet in the plasma samples. The pellet was subjected to staining and manual cell differentiation. The supernatant was isolated and centrifuged again, at 355 000*g* (Beckman TLA 100.2; Beckman Coulter) for 1 h at 4 °C, to remove exosomes, microvesicles, and other smaller subcellular components.

miRNA EXTRACTION

Unless stated otherwise, serum and plasma samples were immediately extracted after rotation and centrifugation of the blood sample. The *mir*Vana™ PARIS™ Kit (Ambion) was found to be the best-performing column-based kit for isolating RNAs (see the supplemental file in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol57/issue6) and was therefore used for the remainder of the study. Total RNA, including miRNA, was extracted with 2 minor modifications to the liquid-sample protocol, as follows. First, 400 μL serum or plasma was lysed with an equal volume of 2× Denaturing Solution. Second, a mixture of 50 pmol/L each of 3 synthesized *Caenorhabditis elegans* (cel) miRNAs (cel-miR-39, 5′-UCACCGGGUGUAAAUCAGCUUG-3′; cel-miR-54, 5′-UACCCGUAAUCUUCAUAAUCCGAG; and cel-miR-238, 5′-UUUGUACUCCGAUGCCAUUCAGA-3′) (Integrated DNA Technologies) was added as internal calibrators to monitor extraction efficiency, as has previously been described *(3)*. The remainder of the extraction was performed according to the manufacturer's instructions. miRNA was eluted with 100 μL of Elution Solution. Eluted RNA was stored at −20 °C until further analysis.

REVERSE TRANSCRIPTION

In initial experiments, we observed a poor correlation between the RNA concentration as measured with a NanoDrop spectrophotometer (Thermo Scientific) and the ability to amplify miRNA. To overcome this problem, we kept both the volume of serum that was extracted and the volume of extracted eluent used for RT quantitative PCR (RT-qPCR) consistent throughout this study. The TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems) and miRNA-specific stem–loop primers (Applied Biosystems) were used for miRNA RT. Each reaction consisted of 5 μL eluted RNA and 10 μL master mix, prepared in our laboratory by using Applied Biosystems components [4.16 μL nuclease-free H_2O, 3 μL 5× TaqMan microRNA RT primer, 1.5 μL 10× RT buffer, 0.19 μL of 20 U/μL RNase inhibitor, 0.15 μL of 100 mmol/L deoxynucleoside triphosphates, and 1 μL of 50 U/μL MultiScribe reverse transcriptase]. RT was carried out in an MJ Research Thermal Cycler (Bio-Rad Laboratories) at 16 °C for 30 min, 42 °C for 30 min, and 85 °C for 5 min. A no-RT negative control was included in each experiment to ensure that PCR products were not due to contamination by genomic DNA. The negative control produced no detectable signal in any of the experiments.

qPCR ANALYSIS

miRNAs were quantified by 6-carboxyfluorescein–based qPCR with TaqMan Fast Universal PCR Master Mix (Applied Biosystems) and individual miRNA primers and hydrolysis probes (Applied Biosystems). We combined 2 μL RT product with 7 μL nuclease-free H_2O, 10 μL TaqMan Fast Universal PCR Master Mix (2×), and 1 μL TaqMan MicroRNA Assay (20×) primer. qPCR was performed on the StepOnePlus Real Time PCR instrument (Applied Biosystems) as follows: 95 °C for 20 s followed by 40 cycles of 95 °C for 1 s and 60 °C for 20 s. The quantification cycle (Cq) was determined with instrument default threshold settings (10 SDs above the mean fluorescence of the baseline cycle). Cq and miRNA copy number are related by a $\log_2$ factor; a change in the Cq of 1 represents a 2-fold change in miRNA copy number.

SPECIMEN COMPARISON

Paired serum and plasma samples were obtained from 10 healthy individuals. RNA was extracted from the samples with and without the 1 or 2 additional high-speed centrifugation steps described above, and miRNA concentrations were compared after RT-qPCR (see above).

miRNA STABILITY

Blood samples were collected from 5 healthy individuals into plain tubes. We extracted a serum aliquot from each individual immediately after centrifugation of the

serum; this aliquot served as the 0-h control. The remainder of the serum was stored at room temperature, 4 °C, or −20 °C for 24, 48, or 72 h. These times were chosen to represent typical short-term transport and storage conditions encountered in the clinical laboratory.

ASSESSMENT OF THE EFFECTS OF HEMOLYSIS

Blood samples were collected from 8 healthy individuals into plain tubes. Serum and erythrocytes were separated by centrifugation as described above and frozen at −20 °C. Serum samples contained minimal hemoglobin [<10 mg/dL (<0.1 g/L) as measured on a Roche Cobas 6000 instrument (Roche)]. A hemolysate prepared from the erythrocyte fraction of each individual was added back to the serum of the same individual to obtain hemoglobin concentrations of 25–1000 mg/dL (0.250–10.0 g/L). The samples were then subjected to RNA extraction, RT, and qPCR as described above.

PRECISION

We designed and performed nested precision experiments for analysis by nested ANOVA as described by Tichopad et al. *(20)*. In brief, serum was obtained from the same 3 individuals over 4 days. The serum sample obtained from each individual was simultaneously extracted in triplicate. The resulting 9 extractions were run in triplicate on 1 plate for RT; the 27 RT products were then run in triplicate on 1 plate for qPCR. This nested design performed over 4 days yielded 324 Cq values for analysis (3 participants × 3 extractions × 3 RTs × 3 qPCRs × 4 days).

INTERINDIVIDUAL VARIABILITY

Interindividual variability was estimated by obtaining serum samples from 26 healthy male and female volunteers over 4 days. miR-15b, miR-16, and miR-24 were quantified from these samples as described above.

STATISTICAL ANALYSIS AND RT-qPCR NORMALIZATION

The Wilcoxon signed rank test was used to compare miRNA concentrations in plasma and serum samples and to compare the effects of various preanalytical centrifugation procedures. To analyze miRNA stability, we used repeated-measures ANOVA to compare miRNA concentrations at various time points. A P value <0.05 was considered statistically significant.

Variance components were estimated with a fully nested random-effects model and the method of moments *(21)*. The effect of blood draw (experiment) was nested within patient; extraction was nested within blood draw; RT was nested within extraction; and qPCR (residual effect) was nested within RT. All analyses were performed with SAS version 9 (SAS Institute).

Where indicated, miRNA-quantification results were normalized to an internal control [the ubiquitously produced microRNA miR-16 *(12, 19, 22)*] or to a spiked control [cel-miR-39; the mean of cel-miR-39 and cel-miR-54; or the mean of cel-miR-39, cel-miR-54, and cel-miR-238 *(3)*]:

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

where "Target" is the miRNA to be normalized; "Control" is miR-16, cel-miR-39, the mean Cq of cel-miR-39 and cel-miR-54, or the mean Cq of cel-miR-39, cel-miR-54, and cel-miR-238; "raw" is the instrument Cq result for that sample; and "median run" is the median Cq of all 81 raw results for the control miRNA for a nested precision run. The total variance was then calculated from these normalized Cq results.

Results

QUANTIFICATION OF miRNA IN PLASMA AND SERUM

After routine sample processing, concentrations of miR-15b, miR-16, and miR-24 were significantly higher in plasma than in serum: (*a*) median miR-15b Cq, 23.9 in plasma vs 29.3 in serum ($P = 0.002$); (*b*) median miR-16 Cq, 20.4 in plasma vs 23.6 in serum ($P = 0.02$); (*c*) median miR-24 Cq, 20.4 in plasma vs 25.7 in serum ($P = 0.002$) (Fig. 1). A synthetic *C. elegans* miRNA (cel-miR-54) added to serum and plasma samples before extraction, showed similar concentrations in serum and plasma.

The plasma and serum samples underwent additional centrifugation steps to determine the cause of the difference between sample types. miR-15b and miR-24 concentrations were significantly reduced in plasma after the 15 000*g* spin by a mean Cq of 7.9: median miR-15b Cq, 31.6 ($P = 0.004$); median miR-24 Cq, 27.1 ($P = 0.002$). miR-16 concentrations were reduced by approximately 2 Cq: median miR-16 Cq, 22.2 ($P = 0.02$) (Fig. 1). The 15 000*g* centrifugation step had much less of an effect on serum samples, reducing miRNA concentrations by approximately 1 Cq: median miR-15b Cq, 30.6 ($P = 0.02$); median miR-16 Cq, 23.6 ($P = 0.92$); median miR-24 Cq, 26.8 ($P = 0.002$). An additional centrifugation step at 355 000*g* had a minor effect on miR-15b in both plasma and serum samples, compared with the concentration in the 15 000*g* samples. This centrifugation step had an insignificant effect on miR-16 and miR-24: median miR-15b Cq, 32.6 in plasma ($P = 0.008$) and 31.6 in serum ($P = 0.002$); median miR-16 Cq, 22.2 in plasma ($P = 0.75$) and 23.1 in serum ($P = 0.46$); median miR-24 Cq, 29.3 in plasma ($P = 0.08$) and 27.4 in serum ($P = 0.06$). This finding indicates that the higher concentrations of

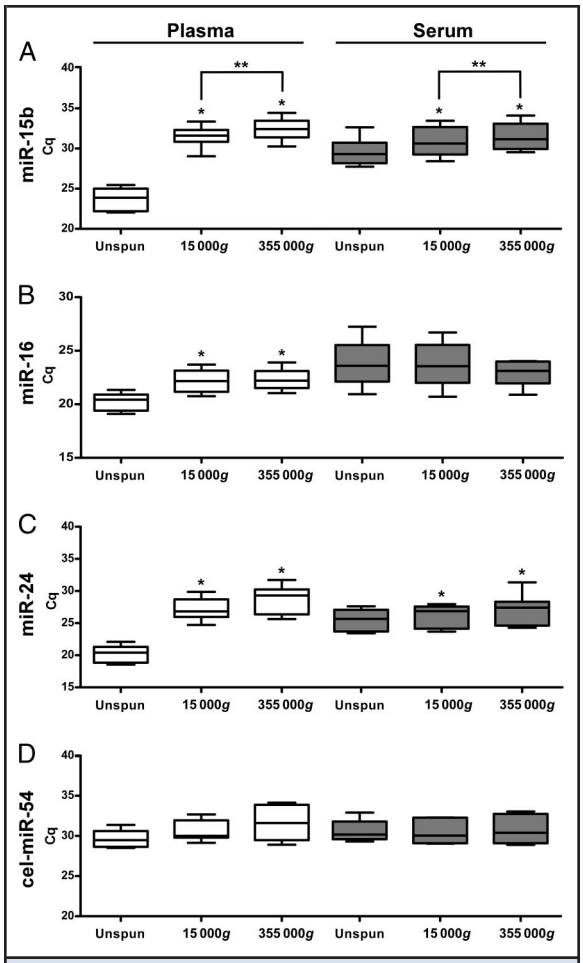

Fig. 1. miRNA concentrations in serum and plasma after centrifugation.

Paired plasma (left 3 columns) and serum (right 3 columns) samples were obtained from 10 healthy individuals. We spiked 400 μL of the uncentrifuged (Unspun) and 2 supernatant aliquots with *C. elegans* cel-miR-54, extracted the RNA, and analyzed the samples for miR-15b (A), miR-16 (B), miR-24 (C), and cel-miR-54 (D). The resulting miRNA concentrations are reported as raw Cq values. The boxes represent the 25th and 75th percentiles; the horizontal line in each box represents the mean; the error bars indicate the range. Significant differences ($P < 0.05$, Wilcoxon signed rank test) from the unspun control (*) and significant differences between the 15 000*g* and 355 000*g* centrifugation steps (**) are indicated.

these miRNAs in plasma compared to serum are mainly due to the presence of cellular contaminants, which microscopical examination of the pellet revealed to be platelets (data not shown). The additional 355 000*g* ultracentrifugation produced only a minor decrease in miRNA concentrations, indicating that exosomes and other small vesicles contribute minimally to the concentration of these miRNAs in the circulation of healthy volunteers. To minimize the variation introduced by variable amounts of platelet contamination, we used serum samples for the remainder of the study.

miRNA STABILITY

Endogenous miRNA concentrations showed a mean increase in Cq of 0.48, 1.12, and 1.07 after 24, 48, and 72 h, respectively, at ambient temperature (Fig. 2). Storage at 4 °C for 72 h yielded a mean increase of 0.67 Cq, whereas storage at −20 °C for 72 h yielded a mean increase of 1.02 Cq. These increases are statistically significant ($P < 0.05$, see Fig. 2 for details) and suggest miRNA degradation; however, because the samples were extracted on different days, it is difficult to determine how much of the Cq increases can be attributed to degradation vs interassay imprecision (see below).

EFFECTS OF HEMOLYSIS

In addition to platelet contamination, miRNA quantification might be affected by the presence of erythrocyte contamination and hemolysis, because many miRNAs are present in erythrocytes *(23, 24)*. The concentrations of both miR-15b and miR-16 increased substantially with hemolysis, from ΔCq values of 0.91 and 2.13, respectively, at 25 mg/dL (0.25 g/L) hemoglobin to ΔCq values of 7.78 and 6.98 at 1200 mg/dL (12.0 g/L) hemoglobin (Fig. 3). miR-24 concentrations were less affected, because a change of >2.0 Cq was not observed until the hemoglobin concentration had reached 600 mg/dL (6.0 g/L). A third endogenous miRNA, miR-122, was not significantly affected by hemoglobin concentrations up to 1200 mg/dL (12.0 g/L). Spiked cel-miR-39 was also not affected by hemolysis, indicating that the changes observed for miR-15b, miR-16, and miR-24 are likely due to the release of these miRNAs from erythrocytes and not a direct effect of hemoglobin or other erythrocyte components on the miRNA extraction, RT, or amplification process.

ASSAY AND INDIVIDUAL VARIANCE AND THE EFFECT OF NORMALIZATION

Intra- and interassay variances for 3 endogenous miRNAs and a spiked *C. elegans* miRNA control are summarized in Table 1. The total variance (σ^2) ranged from 0.521 Cq for miR-24 to 1.872 Cq for miR-15b, corresponding to a 1.43-fold to 3.66-fold variation in the recovery of miRNA copies. Interassay variance contributed to most of this total variance (66%–88% of the total assay variance).

The intraassay variance was ≤0.3 Cq for all of the miRNAs analyzed. The RNA-extraction step showed the least reproducibility and introduced most of the

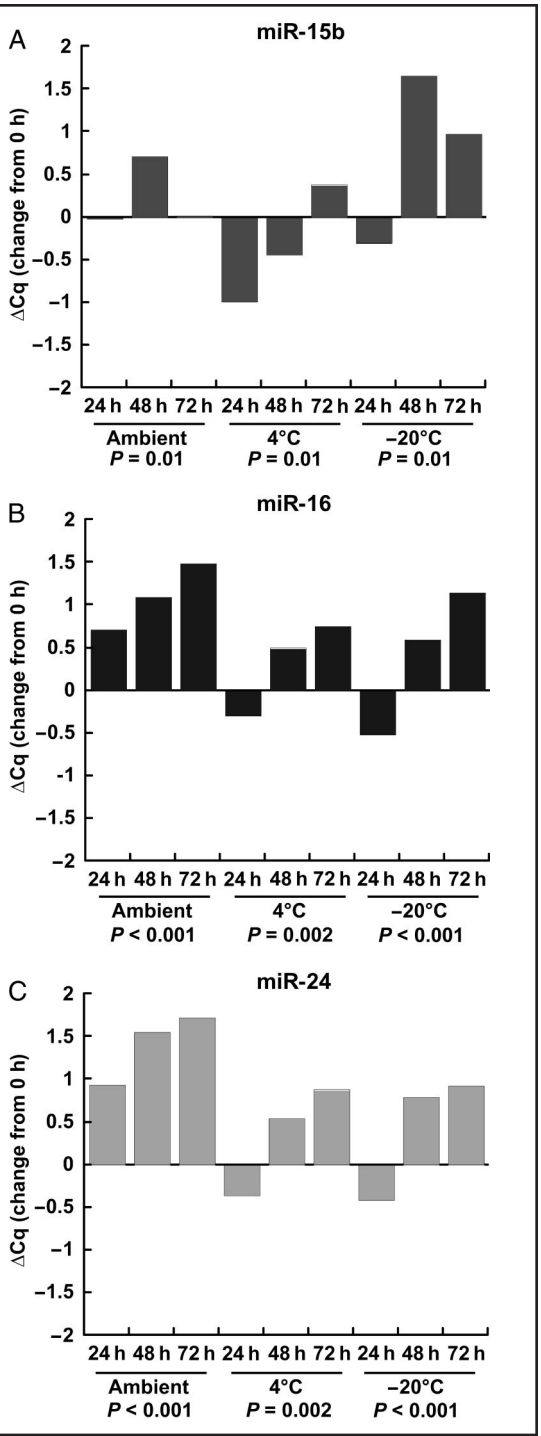

Fig. 2. miRNA stability for miR-15b (A), miR-16 (B), and miR-24 (C) results.

Results are presented as the difference in raw Cq values from the 0-h control. Positive differences represent decreases in miRNA concentration, and negative differences represent increases in miRNA concentration. *P* values were determined by using repeated measures ANOVA.

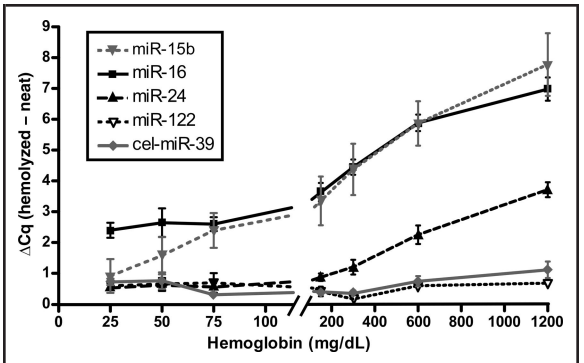

Fig. 3. Effect of hemolysis on miRNA concentrations.

Results are reported as the difference in raw Cq values between the hemolyzed sample and unhemolyzed (Neat) sample. The *x* axis is broken between 100 and 200 mg/dL (1.0 and 2.0 g/L) to highlight the effect of low hemoglobin concentrations. Positive results represent increases in miRNA concentration. Error bars represent the SD.

intraassay imprecision (77%–92% of intraassay imprecision). The percentage of the variance introduced by the RNA-extraction step was consistent between the tested miRNAs, although they had different total assay imprecision values. The RT and qPCR steps were the most reproducible and contributed minimally to the overall assay imprecision (<1%–6% and 6%–17%, respectively, of the intraassay imprecision).

The interindividual variability for these miRNAs was examined by quantifying miR-15b, miR-16, and miR-24 in 26 healthy individuals. The total variance of miR-15b was 0.58, lower than that observed for the 3 individuals in the nested experiment described above (Table 2). The total variance for miR-16 and miR-24, however, was 1.54 Cq and 1.24 Cq, respectively, higher than that observed in the nested experiment, suggesting that these miRNAs have higher interindividual variability than miR-15b.

Nested precision results were normalized with miRNA controls described in previous studies to determine if normalization reduces imprecision. miRNA results from a fifth representative day of the nested precision experiments were normalized to miR-16, cel-miR-39, the mean of cel-miR-39 and cel-miR-54, or the mean of cel-miR-39, cel-miR-54, and cel-miR-238, as described in Materials and Methods. Total variances from the raw and normalized results are listed in Table 3. Normalizing to miR-16 increased the total imprecision in miRNA results, whereas normalization to the *C. elegans* miRNA controls left the total imprecision largely unchanged. Normalizing to the mean of 2 or 3 exog-

Table 1. Nested precision experiments.[a]

	miR-15b	miR-16	miR-24	cel-miR-39
Mean Cq	27.44	22.51	27.25	27.09
Variance, Cq				
Intraassay	0.224	0.129	0.131	0.300
RNA extraction	0.172 (77%)	0.110 (85%)	0.120 (92%)	0.273 (91%)
RT	0.014 (6%)	0.004 (3%)	0.001 (<1%)	0.008 (3%)
qPCR	0.038 (17%)	0.015 (12%)	0.010 (8%)	0.019 (6%)
Interassay	1.648	0.401	0.390	0.576
Total	1.872	0.530	0.521	0.876

[a] Results obtained from serum samples of 3 individuals where miRNA was extracted over 4 days. Variance is reported as raw Cq. Percentages in parentheses indicate the proportion of total intraassay variance.

enous miRNAs did not improve total imprecision, compared with normalizing to cel-miR-39 alone.

Discussion

This study highlights several of the preanalytical and analytical variables affecting the quantification of miRNA in serum and plasma samples. To our knowledge, this report is the first publication to provide such detailed information. We demonstrated that the choice of specimen, the presence of cellular contaminants such as platelets or erythrocytes, and the imprecision introduced from the day-to-day setup of the extraction and amplification procedures are critical to investigating the utility of miRNAs as circulating biomarkers.

We confirmed previous observations of the stability of miRNAs in serum samples *(3)*. Refrigerating or freezing samples is recommended to reduce miRNA degradation. We also found that the choice of sample type can have a profound effect on miRNA concentrations. Circulating endogenous miRNAs miR-15b, miR-16, and miR-24 were present at higher concentrations in plasma samples than in serum samples. This result contrasts with previous findings that reported no difference between plasma and serum in the concentrations of these miRNAs *(3)*. The differences could be due to differences in preanalytical variables, such as blood tube type or phlebotomy protocols (e.g., hemolysis would minimize sample type differences), or to differences in sample-processing protocols (routine double centrifugation or filtration would minimize discrepancies). In the healthy volunteer cohort, little miRNA seemed to be in the exosome/microvesicle fraction. These amounts could well be different in various diseases states, however, and other studies have demonstrated varying yields of different circulating miRNAs after plasma filtration *(12)*. Although these findings highlight the need for further detailed studies, it is clear that serum and plasma cannot be automatically assumed to be interchangeable with regard to miRNA concentrations. An analysis of extraction variation should be performed for each sample type. Studies of circulating miRNAs should detail the centrifugation protocol used to isolate serum or plasma to ensure that subsequent studies can accurately compare and reproduce the results.

Table 2. Total variance of miR-15b, miR-16, and miR-24 for samples from 26 individuals.[a]

	Mean Cq	Total variance, Cq
miR-15b	27.56	0.58
miR-16	23.57	1.54
miR-24	27.07	1.24

[a] Results obtained from serum samples of 26 individuals extracted and run over 4 days.

Another important finding is that sample hemolysis can produce artificially high miRNA concentrations. miR-15b and miR-16 concentrations were highly susceptible to hemolysis, causing an increase in Cq of 1–2 (i.e., a 2- to 4-fold increase in copy number) with as little as 25 mg/dL (0.25 g/L) hemoglobin, whereas similar increases in miR-24 were not observed until 600 mg/dL (6.0 g/L) hemoglobin. In contrast, miR-122 concentrations were not significantly affected at the hemoglobin concentrations tested. miR-15b, miR-16, and miR-24 have previously been reported to be present in isolated erythrocytes; however, miR-122 was not detected in these studies *(23–25)*. Similar to our findings of miRNAs in residual platelets, miRNAs that are present at higher concentrations in erythrocytes could be a confounding factor in hemolyzed samples.

Table 3. Effect of normalization on assay imprecision.[a]

	Variance, Cq	Variance after normalization			
		miR-16, Cq	cel-miR-39, Cq	cel-miR-39 + cel-miR-54, Cq	cel-miR-39 + cel-miR-54 + cel-miR-238, Cq
miR-15b	0.11	0.25	0.13	0.15	0.15
miR-16	0.13	—	0.12	0.10	0.09
miR-24	0.11	0.23	0.13	0.15	0.14

[a] Results obtained from an additional nested precision experiment as described for Table 1 and reported as total intraassay variance (Cq).

Assay imprecision had a significant effect on the reproducibility of miRNA measurements. Intraassay imprecision was modest, with variances of <1 Cq. Nevertheless, that translates into a 2-fold difference in miRNA copy number. Most of this variation was due to the RNA-extraction process. Because miRNAs are very short sequences and are present at low concentrations in blood, performing consistent column extractions may be challenging. Alternative protocols, such as liquid–liquid extraction, might produce a higher miRNA yield and lower imprecision; however, such methods may be more cumbersome to implement in the clinical laboratory. Our preliminary studies indicate that further modifications of the column-based miRNA-extraction protocol or the use of liquid–liquid extraction protocols can decrease the observed assay imprecision. For example, a longer incubation of serum with denaturation buffer before adding the *C. elegans* miRNA mixture decreased the total assay variance for the spiked miRNA (see Table 2 in the online Data Supplement). This finding suggests that RNases are not completely denatured after the recommended denaturation time, likely increasing assay imprecision for these exogenous miRNAs. In addition, a standard liquid–liquid extraction protocol using TRIzol LS (Invitrogen) increased the yields of all miRNAs and decreased the total intraassay variance, compared with the *mir*Vana column-based protocol (see Table 3 in the online Data Supplement). Together, these observations highlight the need for overall improvements in the procedures and technologies used to extract miRNA from biological fluids.

In this study, interassay imprecision contributed most of the total assay variance. miRNA results from assays run on different days could be misconstrued as physiological differences instead of interassay variance. Assays should therefore include well-characterized control samples in every run to account for the interassay variance. The reproducibility studies also emphasize the importance of choosing internal and external controls for miRNA analysis. The spiked exogenous *C. elegans* miRNAs cel-miR-39, cel-miR-54, and cel-miR-238, which previously have been reported as assay-normalization controls *(3)*, did not significantly improve assay imprecision. Endogenous miR-16, an internal control that has been used to normalize the results of miRNAs of interest in many studies *(9, 19, 26–30)*, fared even worse when it was used as a normalizing factor. This result may be because miR-16 was shown to be particularly susceptible to hemolysis. Unless samples are strictly monitored for hemolysis, this factor could contribute to high variation in miR-16 results. Therefore, this miRNA may not be an ideal choice as an internal control.

In conclusion, this study aimed to investigate some of the preanalytical and analytical variables that could influence the accurate analysis of circulating miRNAs in clinical practice. Although this study focused on a small number of miRNAs, the results highlight our notion that all of the parameters we have analyzed should be examined for each candidate miRNA to determine the effects of sample type, assay choice, individual variation, stability, and the effect of hemolysis. Candidate miRNAs can then be selected on the grounds of displaying a difference between control and disease groups that greatly exceeds the range of observed assay variances. Unless these steps are taken, even the most promising miRNA biomarkers may fail in clinical practice.

Author Contributions: *All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.*

Authors' Disclosures or Potential Conflicts of Interest: *No authors declared any potential conflicts of interest.*

Role of Sponsor: The funding organizations played no role in the design of study, choice of enrolled patients, review and interpretation of data, or preparation or approval of manuscript.

Acknowledgments: We gratefully thank Scott Kaufmann's laboratory for the use of the ultracentrifuge equipment.

References

1. Bushati N, Cohen SM. MicroRNA functions. Annu Rev Cell Dev Biol 2007;23:175–205.
2. Lu J, Getz G, Miska EA, Alvarez-Saavedra E, Lamb J, Peck D, et al. MicroRNA expression profiles classify human cancers. Nature 2005;435:834–8.
3. Mitchell PS, Parkin RK, Kroh EM, Fritz BR, Wyman SK, Pogosova-Agadjanyan EL, et al. Circulating microRNAs as stable blood-based markers for cancer detection. Proc Natl Acad Sci U S A 2008; 105:10513–8.
4. Chen X, Ba Y, Ma L, Cai X, Yin Y, Wang K, et al. Characterization of microRNAs in serum: a novel class of biomarkers for diagnosis of cancer and other diseases. Cell Res 2008;18:997–1006.
5. Wang K, Zhang S, Marzolf B, Troisch P, Brightman A, Hu Z, et al. Circulating microRNAs, potential biomarkers for drug-induced liver injury. Proc Natl Acad Sci U S A 2009;106:4402–7.
6. Wang GK, Zhu JQ, Zhang JT, Li Q, Li Y, He J, et al. Circulating microRNA: a novel potential biomarker for early diagnosis of acute myocardial infarction in humans. Eur Heart J 2010;31:659–66.
7. Tijsen AJ, Creemers EE, Moerland PD, de Windt LJ, van der Wal AC, Kok WE, Pinto YM. miR423–5p as a circulating biomarker for heart failure. Circ Res 2010;106:1035–9.
8. Ng EK, Chong WW, Jin H, Lam EK, Shin VY, Yu J, et al. Differential expression of microRNAs in plasma of patients with colorectal cancer: a potential marker for colorectal cancer screening. Gut 2009;58:1375–81.
9. Huang Z, Huang D, Ni S, Peng Z, Sheng W, Du X. Plasma microRNAs are promising novel biomarkers for early detection of colorectal cancer. Int J Cancer 2010;127:118–26.
10. Taylor DD, Gercel-Taylor C. MicroRNA signatures of tumor-derived exosomes as diagnostic biomarkers of ovarian cancer. Gynecol Oncol 2008; 110:13–21.
11. Gilad S, Meiri E, Yogev Y, Benjamin S, Lebanony D, Yerushalmi N, et al. Serum microRNAs are promising novel biomarkers. PLoS One 2008;3: e3148.
12. Chim SS, Shing TK, Hung EC, Leung TY, Lau TK, Chiu RW, Lo YM. Detection and characterization of placental microRNAs in maternal plasma. Clin Chem 2008;54:482–90.
13. Jung M, Schaefer A, Steiner I, Kempkensteffen C, Stephan C, Erbersdobler A, Jung K. Robust microRNA stability in degraded RNA preparations from human tissue and cell samples. Clin Chem 2010;56:998–1006.
14. Skog J, Wurdinger T, van Rijn S, Meijer DH, Gainche L, Sena-Esteves M, et al. Glioblastoma microvesicles transport RNA and proteins that promote tumour growth and provide diagnostic biomarkers. Nat Cell Biol 2008;10:1470–6.
15. Valadi H, Ekstrom K, Bossios A, Sjostrand M, Lee JJ, Lotvall JO. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. Nat Cell Biol 2007;9:654–9.
16. Hunter MP, Ismail N, Zhang X, Aguda BD, Lee EJ, Yu L, et al. Detection of microRNA expression in human peripheral blood microvesicles. PLoS One 2008;3:e3694.
17. Mestdagh P, Van Vlierberghe P, De Weer A, Muth D, Westermann F, Speleman F, Vandesompele J. A novel and universal method for microRNA RT-qPCR data normalization. Genome Biol 2009; 10:R64.
18. Becker C, Hammerle-Fickinger A, Riedmaier I, Pfaffl MW. mRNA and microRNA quality control for RT-qPCR analysis. Methods 2010;50: 237–43.
19. Davoren PA, McNeill RE, Lowery AJ, Kerin MJ, Miller N. Identification of suitable endogenous control genes for microRNA gene expression analysis in human breast cancer. BMC Mol Biol 2008;9:76.
20. Tichopad A, Kitchen R, Riedmaier I, Becker C, Stahlberg A, Kubista M. Design and optimization of reverse-transcription quantitative PCR experiments. Clin Chem 2009;55:1816–23.
21. Searle SR, Casella G, McCulloch CE. Variance components. New York: Wiley; 1992. xxiii, 501 p.
22. Lagos-Quintana M, Rauhut R, Yalcin A, Meyer J, Lendeckel W, Tuschl T. Identification of tissue-specific microRNAs from mouse. Curr Biol 2002; 12:735–9.
23. Kannan M, Atreya C. Differential profiling of human red blood cells during storage for 52 selected microRNAs. Transfusion 2010;50:1581–8.
24. Chen SY, Wang Y, Telen MJ, Chi JT. The genomic analysis of erythrocyte microRNA expression in sickle cell diseases. PLoS One 2008;3:e2360.
25. Vaz C, Ahmad HM, Sharma P, Gupta R, Kumar L, Kulshreshtha R, Bhattacharya A. Analysis of microRNA transcriptome by deep sequencing of small RNA libraries of peripheral blood. BMC Genomics 2010;11:288.
26. Miyachi M, Tsuchiya K, Yoshida H, Yagyu S, Kikuchi K, Misawa A, et al. Circulating muscle-specific microRNA, miR-206, as a potential diagnostic marker for rhabdomyosarcoma. Biochem Biophys Res Commun 2010;400:89–93.
27. Mattie MD, Benz CC, Bowers J, Sensinger K, Wong L, Scott GK, et al. Optimized high-throughput microRNA expression profiling provides novel biomarker assessment of clinical prostate and breast cancer biopsies. Mol Cancer 2006;5:24.
28. Roy S, Khanna S, Hussain SR, Biswas S, Azad A, Rink C, et al. MicroRNA expression in response to murine myocardial infarction: miR-21 regulates fibroblast metalloprotease-2 via phosphatase and tensin homologue. Cardiovasc Res 2009;82:21–9.
29. Chiang CW, Huang Y, Leong KW, Chen LC, Chen HC, Chen SJ, Chou CK. PKCalpha mediated induction of miR-101 in human hepatoma HepG2 cells. J Biomed Sci 2010;17:35.
30. Greco S, De Simone M, Colussi C, Zaccagnini G, Fasanaro P, Pescatori M, et al. Common micro-RNA signature in skeletal muscle damage and regeneration induced by Duchenne muscular dystrophy and acute ischemia. FASEB J 2009;23: 3335–46.