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Quantification of Circulating miRNAs in Plasma

Effect of Preanalytical and Analytical Parameters on Their Isolation and Stability

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Circulating miRNAs are intensively evaluated as promising blood-based biomarkers. This growing interest in developing assays for circulating miRNAs necessitates careful consideration of the effects of preanalytical and analytical parameters on the isolation, stability, and quantification of circulating miRNAs. By using quantitative stem-loop RT-PCR, we compared the relative efficiencies of four miRNA isolation systems and different storage conditions. The effect of the data normalization procedure on the quantification of circulating miRNA levels in plasma from 30 healthy individuals and 30 patients with non–small cell lung carcinoma was estimated by measuring endogenous hsa-miR-21 and hsa-miR-16 and exogenous cel-miR-39 that was spiked in all samples at the same concentration. Silica column–based RNA extraction methods are more effective and reliable with respect to TRIzol LS. Endogenous circulating miRNA levels are unstable when plasma is stored at 4°C, and samples should be kept at −70°C, where the extracted miRNAs remain stable for up to 1 year. When normalization is based on combined endogenous and exogenous control miRNAs, differences in miRNA recovery and differences in cDNA synthesis between samples are compensated. Using this normalization procedure and hsa-miR-21 as a biomarker, we could clearly discriminate healthy individuals from patients with cancer. Experimental handling and the use of exogenous and endogenous controls for normalization are critical for the reliable quantification of circulating miRNA levels in plasma. *(J Mol Diagn 2013, 15: 827–834; http://dx.doi.org/10.1016/j.jmoldx.2013.07.005)*

miRNAs are a class of small, endogenous, noncoding, single-strand RNAs that can negatively regulate gene expression by binding to specific complementary sites at the 3′ untranslated region of target mRNAs, causing translational repression or transcript degradation. miRNAs are involved in many important biological processes, such as cell proliferation, differentiation, and apoptosis.[1] Because half of the miRNA genes in humans are located at fragile chromosomal regions that display deletions, amplifications, or translocations, aberrant expression of miRNAs occurs frequently, and thus miRNAs may act as oncogenes or tumor suppressor genes, depending on their target genes.[2,3] Deregulation of miRNA expression levels has been detected in many human tumor types and plays a critical role in cancer pathogenesis.[4,5] Despite the fact that there are

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<2000 human miRNAs, recent studies estimate that more than one-third of the cellular transcriptome is regulated by miRNAs.

Numerous recent studies have shown that miRNAs are rapidly released from tissues into the circulation in many pathologic conditions.[1] The presence of circulating miRNAs in peripheral blood in a highly stable manner that is protected from degradation conditions and factors such as boiling, extreme pH values, and endogenous RNase activity has been clearly demonstrated.[6,7] This relatively high stability of miRNAs in biofluids such as plasma, serum, urine, and saliva and the ability of miRNA expression profiles to accurately classify discrete tissue types and disease states have positioned miRNAs as promising noninvasive new biomarkers for a wide range of diagnostic applications. In blood, miRNAs can circulate withstanding degradation through their inclusion in microvesicles or exosomes that are secreted from cells or by binding to high-density lipoproteins[8] or to the argonaute 2 protein complex.[9] This, combined with the strong link between their deregulation and cancer development and progression, highlights the potential of these molecules as promising noninvasive biomarkers. Actually, to date, many research reports have demonstrated that circulating miRNA profiles can reflect physiologic and pathologic conditions.[6,7,10,11] In a variety of solid tumors and hematopoietic malignancies, cell-free miRNAs displayed diagnostic and prognostic value, enhancing the challenge of exploiting circulating miRNAs as molecular biomarkers for early tumor detection, diagnosis, and prognosis as well as monitoring of therapeutic response.[12,13]

This growing interest in developing circulating miRNAs as blood-based biomarkers necessitates very careful consideration of the effects of various preanalytical and analytical parameters on their measurements. Preanalytical parameters, such as sample handling and storage conditions before processing, play a significant role in the reliability and reproducibility of circulating miRNA quantification.[14–17] Besides, a technical hurdle to the study of miRNA expression is the ability to reliably and efficiently extract miRNAs from biological samples because of their small size and their attachment to lipids and proteins. Recently, several commercial extraction kits have become available that seek to optimize the extraction of small RNAs, either in conjunction with full-length total RNA or as a fraction enriched for small RNAs, and exogenous synthetic miRNAs have been proposed as external controls to normalize sample-to-sample variations in RNA isolation procedures.[7,18] In conclusion, it is important to establish standardized protocols for blood collection, sample storage conditions, inclusion of exogenous and endogenous miRNA controls for each clinical sample, and standardized calculations for normalization of the results to ensure the reproducible and accurate quantification of circulating miRNA levels so that miRNA analysis can be implemented in the clinical laboratory setting.[19,20]

The aim of this study was to evaluate the effects of preanalytical and analytical parameters on the isolation, stability, and quantification of circulating miRNAs in plasma. We compared the relative efficiencies of four different miRNA isolation systems and evaluated the effects of different storage conditions and storage periods on the quantification of plasma miRNA levels. Moreover, we evaluated the use of exogenous synthetic miRNAs as external controls for normalization of sample-to-sample variations in RNA isolation procedures and the use of endogenous miRNAs as normalizers for miRNA quantification.

Materials and Methods

Sample Collection

For the evaluation of four different protocols for the extraction of circulating miRNAs, whole blood samples were collected from healthy individuals in EDTA-containing tubes (BD Vacutainer; Becton Dickinson and Company, Franklin Lakes, NJ) and subjected to centrifugation at 2000 × *g* for 10 minutes at room temperature. The upper plasma layer was immediately pooled after centrifugation and was aliquoted into RNase-free tubes. These aliquots were used on the same day for the extraction of circulating miRNAs by four different extraction protocols. The same practice was followed for studying the stability of circulating miRNAs in plasma under different storage conditions. In this case, different aliquots of pooled plasma samples from healthy individuals were stored at different temperatures for different periods until the extraction of circulating miRNAs. One plasma aliquot was immediately extracted (baseline control, 0 hour), whereas the remaining aliquots were stored for 24 hours, 48 hours, 1 month, and 4 months at 4°C, −20°C, and −70°C until miRNA extraction. These periods were selected to represent short-term sample processing in a clinical laboratory and long-term sample storage for future processing. Moreover, we used plasma samples from 30 healthy individuals and 30 patients with non−small cell lung cancer (NSCLC) to evaluate the effect of different normalization procedures on the quantification of circulating miRNA levels in plasma.

Exogenous and Endogenous miRNA Controls

In all cases, a synthetic miRNA [*Caenorhabditis elegans* cel-miR-39 (5′-UCACCGGGUGUAAAUCAGCUUG-3′); miScript miRNA mimic (Qiagen Inc., Valencia, CA)] was added to all the plasma aliquots as an exogenous miRNA spiked-in control after addition of the denaturating solution to allow for normalization of sample-to-sample variation in the RNA isolation procedure. Moreover, mature hsa-miR-21 (5′-UAGCUUAUCAGACUGAUGUUGA-3′) and mature hsa-miR-16 (5′-UAGCAGCACGUAAAUAUUGGCG-3′) were used as endogenous miRNA controls.

Extraction of Circulating miRNAs from Plasma

Before starting the miRNA extraction procedure, pooled plasma samples were subjected to a second centrifugation at 12,000 × *g* for 15 minutes at 4°C to remove all cellular debris. Subsequently, 25 fmol of the exogenous miRNA spiked-in control (cel-miR-39) was added to all the plasma aliquots after addition of the denaturating solution to allow for normalization of sample-to-sample variation in the RNA isolation procedure. Thereafter, isolation of plasma miRNAs was performed according to the manufacturer's instructions for each of the examined extraction protocols: i) a standard liquid-liquid extraction protocol using TRIzol LS (Invitrogen, Life Sciences, Carlsbad, CA) that is used for the isolation of total RNA: 250 μL of plasma and 750 μL of denaturing solution, ii) miRNeasy mini kit (Qiagen GmbH, Hilden, Germany): 200 μL of plasma and 1 mL of QIAzol lysis reagent, iii) *mir*Vana PARIS kit (Ambion Inc., Life Sciences, Austin, TX): 200 μL of plasma and 200 μL of denaturing solution, and iv) miRNA purification kit (Norgen Biotek Corp., Thorold, ON, Canada): 1 mL of plasma and 1.5 mL of lysis solution.

Reverse Transcription

In all cases, cDNA was synthesized using the TaqMan miRNA reverse transcription kit and miRNA-specific stem-loop primers (both from Applied Biosystems, Life Sciences, Foster City, CA) in 15 μL of total volume reaction. Each reaction consisted of 5 μL of eluted plasma miRNAs and 10 μL of master mix [4.16 μL of nuclease-free H_2O, 3 μL of TaqMan miRNA reverse transcription−specific primer, 1.5 μL of reverse transcription buffer, 0.19 μL of 20 U/μL RNase inhibitor, 0.15 μL of 100 mmol/L dNTPs, and 1 μL of 50 U/μL MultiScribe Reverse Transcriptase (Applied Biosystems, Life Sciences)]. Reverse transcription reaction mixture was incubated at 16°C for 30 minutes, at 42°C for 30 minutes, and at 85°C for 5 minutes and then was held at 4°C. A no−reverse transcription negative control was included in each experiment to ensure that the PCR products were not due to contamination by genomic DNA. The negative control produced no detectable signal in any of the experiments.

Quantitative RT-PCR

Circulating miRNA levels were quantified by using TaqMan miRNA assays (Applied Biosystems, Life Sciences), according to the manufacturer's protocols. Quantitative RT-PCR was performed in a final volume of 10 μL containing 2 μL of cDNA template, 2 μL of nuclease-free water, 1 μL of 20× primer/probe mix from the TaqMan miRNA assay, and 5 μL of 2× TaqMan universal PCR master mix (Applied Biosystems, Life Sciences). All the reactions were run in triplicate using the LightCycler 2.0 real-time PCR instrument (Roche Applied Science, Indianapolis, IN). The reaction mixture was incubated at 95°C for 10 minutes, followed by 45 cycles of 95°C for 15 seconds and 60°C for 1 minute. Quantification cycle (Cq) values were calculated using LightCycler software version 4.05 (Roche Applied Science). Relative quantification was based on the ΔΔCq method as described by Livak and Schmittgen.[21]

Statistical Analysis

Statistical analysis was performed using the SPSS statistical package (version 21; SPSS Inc., Chicago, IL). Differences in mean Cq values for endogenous and exogenous miRNAs according to the extraction method used were estimated by the *U*-test. The same statistical test was also used for the evaluation of differences in mean Cq values of extracted miRNAs according to the different storage periods. Circulating miRNA stability concerning different storage times and different temperatures was calculated by the repeated-measures analysis of variance test. A $P < 0.05$ was considered statistically significant.

Results

We evaluated the efficacy and analytical performance of all these different protocols and experimental conditions by quantifying the levels of the spiked-in exogenous control miRNA cel-miR-39. Recovery of cel-miR-39 in each case was estimated with respect to the levels of an equivalent amount of cel-miR-39 copies that we added to the eluted RNA after the extraction step in each case (representing 100% recovery), when we evaluated the different extraction protocols. In parallel, in all the samples, endogenous hsa-miR-21 and hsa-miR-16 levels were also quantified by quantitative RT-PCR (RT-qPCR).

Optimization of the TRIzol LS Protocol for the Isolation of Circulating miRNAs from Plasma

We first evaluated the efficacy of TRIzol LS reagent, a widely applied method for the isolation of total RNA, for the extraction of circulating miRNAs. Therefore, we first tried to optimize the TRIzol LS protocol for isolating circulating miRNAs from plasma samples with respect to the percentage yield by examining the effects of the initial plasma volume, temperature, and incubation time of the RNA precipitation step. We used 200, 250, 300, and 500 μL of plasma and performed the precipitation step of RNA at room temperature for 10 minutes (according to the manufacturer's recommendations) or overnight at −20°C. We evaluated the efficacy and analytical performance of these different conditions by quantifying the levels of a spiked-in exogenous miRNA, cel-miR-39, that was added as an exogenous control. Recovery of cel-miR-39 in each case was estimated with respect to the levels of an equivalent amount of cel-miR-39 copies that we added to the eluted RNA after the extraction step in each case (representing 100% recovery). In parallel, in all these samples, the endogenous hsa-miR-21 was also quantified

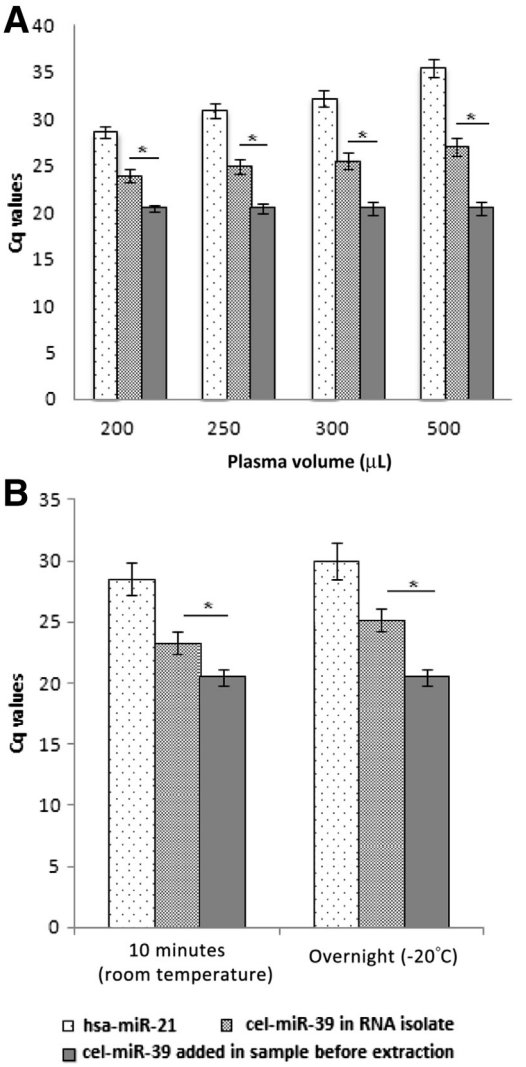

Figure 1 Isolation of plasma circulating miRNAs using TRIzol LS reagent. Raw Cq values for endogenous hsa-miR-21 after total RNA extraction, exogenous cel-miR-39 added in sample before total RNA extraction, and exogenous cel-miR-39 recovered after extraction are shown starting from different plasma volumes (**A**) and as the effect of temperature and time of the precipitation step (**B**). The data represent the means ± SD of three independent experiments. Statistical evaluation was based on the *U*-test. $*P < 0.05$.

by RT-qPCR. According to the present results, better recovery for cel-miR-39 (exogenous control) and hsa-miR-21 (endogenous miRNA) was achieved when starting from 200 μL of plasma and when the RNA precipitation step was performed at room temperature for 10 minutes (Figure 1).

Comparison of the Efficacy and Analytical Performance of Four Different Commercially Available Protocols for the Extraction of Circulating miRNAs

To compare the efficacy and analytical performance of the four different commercially available kits, we used the manufacturer-suggested plasma volume and instructions except for TRIzol LS reagent, where we used 200 μL of plasma, which we found optimal as previously shown. All the experiments were performed in triplicate for the whole analytical procedure. These different isolation approaches were compared with respect to the percentage yield for cel-miR-39 used as an exogenous control and the expression levels of hsa-miR-21 used as an endogenous miRNA, as described previously herein. As can be seen in Figure 2, by using TRIzol LS reagent, we got lower percentage yields from plasma samples for cel-miR-39 and hsa-miR-21 because the recovery of spiked-in cel-miR-39 by using TRIzol LS was significantly lower compared with the *mir*Vana PARIS kit ($P = 0.039$). Taking into account the amount of sample required in each case, we preferred to proceed further with the *mir*Vana PARIS kit.

Effect of Storage Conditions on the Stability of Circulating miRNAs in Plasma

We investigated the stability of circulating miRNAs in plasma at different storage temperatures and for different periods by measuring the levels of the exogenous control

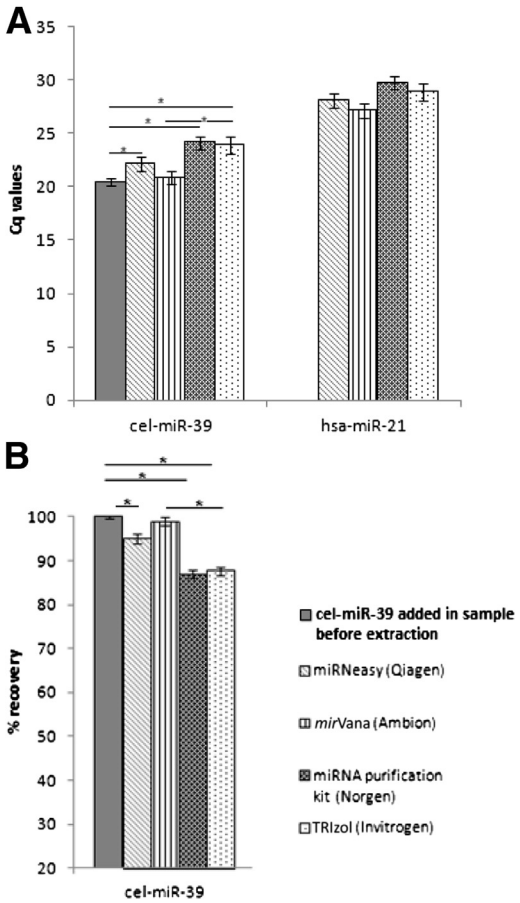

Figure 2 **A**: RT-qPCR raw Cq values for endogenous hsa-miR-21 after total RNA extraction, exogenous cel-miR-39 added in sample before total RNA extraction, and exogenous cel-miR-39 recovered after extraction from plasma samples using different commercially available extraction protocols. **B**: Recovery rates for the exogenous spiked-in control cel-miR-39. The data represent the means ± SD of three independent experiments. Statistical evaluation was based on the *U*-test. $*P < 0.05$.

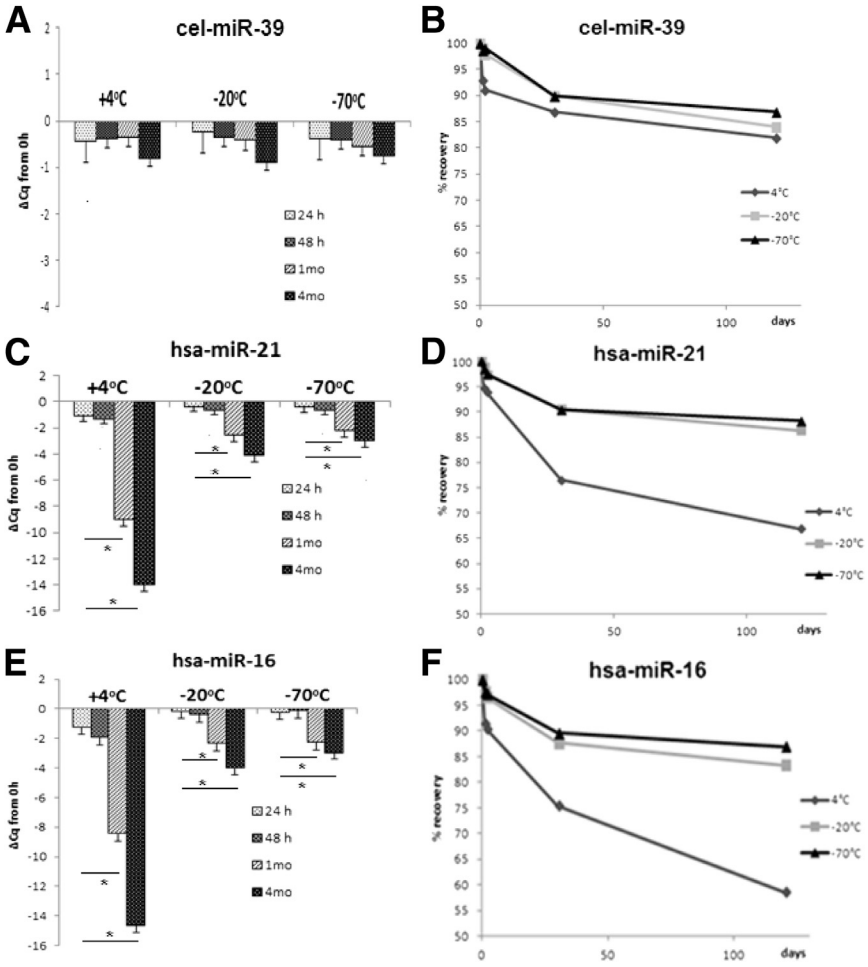

Figure 3 Stability study of circulating miRNAs in plasma at different storage time and temperature conditions. **A** and **B**: cel-miR-39. **C** and **D**: hsa-miR-21. **E** and **F**: hsa-miR-16. The results are presented as differences in raw Cq values from the 0-hour control (0h) (positive differences represent decreases and negative differences represent increases in miRNA concentrations) and as percentage recoveries from the 0h for each miRNA. The data represent the means ± SD of three independent experiments. Statistical evaluation was based on the *U*-test. $^*P < 0.05$.

(cel-miR-39) and two endogenous miRNAs (hsa-miR-21 and hsa-miR-16) using RT-qPCR. All the experiments were performed in triplicate for the whole analytical procedure. The same amount of exogenous control (cel-miR-39) was spiked-in after thawing in all the plasma samples to evaluate whether the detected differences in recovery rates were due to the storage conditions only and not to errors of the extraction procedure used. The results clearly indicate that endogenous circulating miRNA levels are unstable when plasma specimens are stored at 4°C after 24 hours, 48 hours, 1 month, and 4 months because there was an increase in Cq values in all cases (Figure 3). Storage of plasma samples at −20°C or −70°C for 4 months led to increases of approximately 4.0 and 3.0 in Cq values, respectively, indicating that for long-term maintenance of plasma samples, −20°C or −70°C temperature conditions are more appropriate ($P < 0.05$). In parallel, the levels of exogenous cel-miR-39 for each extraction on different days seem to be almost equal, thus eliminating the possibility that the increased Cq values are attributed to sample-to-sample variation during their extraction (Figure 3). Thus, these differences in Cq values for the endogenous miRNAs that are statistically significant intimate the degradation of miRNAs during long-term plasma storage.

Effect of Storage Conditions on the Stability of Extracted miRNAs

Additionally, we evaluated the stability of extracted miRNAs at −70°C for a storage period of up to 12 months by quantifying cel-miR-39 levels after 2, 6, 8, 9, and 12 months. These results indicate that extracted miRNAs are very stable when stored at −70°C for 1 year because slight differences in Cq values were not significant ($P > 0.05$) (Figure 4).

Effect of Different Normalization Procedures on the Quantification of Circulating miRNAs in Plasma

We first isolated circulating miRNAs from plasma samples from 30 healthy individuals and 30 patients with NSCLC by using the *mir*Vana PARIS kit. We evaluated the effect of different normalization procedures on the quantification of circulating miRNAs in these plasma samples by measuring endogenous hsa-miR-21 and hsa-miR-16 levels and levels of the exogenous control cel-miR-39 that was spiked in all the samples at the same concentration. The concentration of hsa-miR-21 in plasma was quantified by normalizing with respect to i) hsa-miR-16, used as an endogenous control of stable expression (Figure 5A); ii) exogenous control

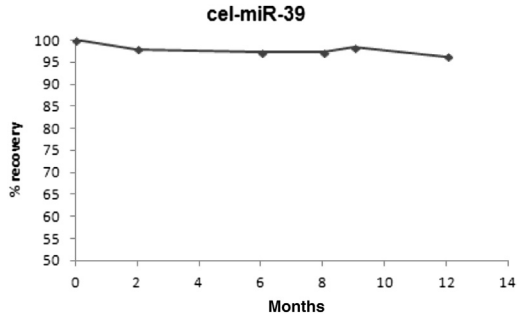

Figure 4 Percentage recovery for exogenous cel-miR-39 before and after storage of extracted miRNAs for 2, 6, 8, 9, and 12 months. The data represent the means of three independent measurements.

cel-miR-39 (Figure 5B); and iii) a combination of the two, where the expression levels of both hsa-miR-21 and the endogenous control hsa-miR-16 were normalized with respect to the exogenous control cel-miR-39, and then ΔCq was estimated (Figure 5C). These results clearly suggest that when normalization is based on a combination of an endogenous and an exogenous control miRNA, differences in the recovery of miRNAs from plasma and differences in cDNA synthesis between samples are compensated. By using this normalization procedure, we could clearly discriminate controls from patients with cancer when hsa-miR-21 was used as a biomarker.

Discussion

Circulating miRNAs are promising biomarkers for various diseases, especially cancer. However, their establishment in the clinical laboratory setting requires highly sensitive, reproducible, reliable, and robust assays enabling their accurate quantification in plasma and serum. To achieve this goal, a variety of preanalytical and analytical parameters that can influence their isolation and stability should be checked. Moreover, there is a great demand for robust and reliable approaches for the normalization of miRNA quantitative data.

Herein we evaluated the effect of preanalytical and analytical parameters on the isolation, stability, and quantification of circulating miRNAs in plasma. We compared the relative efficiencies of four different miRNA isolation systems and evaluated the effect of different storage conditions and storage times on the quantification of plasma miRNA levels and evaluated the use of exogenous synthetic miRNAs as external controls for data normalization of sample-to-sample variations in RNA isolation procedures and the use of endogenous miRNAs as normalizers for miRNA quantification.

Recently, a variety of studies have addressed some important steps for the establishment of robust strategies for blood-based miRNA profiling toward its implementation in routine handling for diagnostic purposes. It has already been reported that preanalytical issues, such as endogenous serum factors that are copurified with miRNAs and anticoagulant agents used during collection, can seriously influence the quantification of circulating miRNA levels[22] and that sample hemolysis alters miRNA content in plasma or serum[23]; however, the extraction method used and the underlying source have been found to result in dissimilar miRNA expression profiles.[24] Li and Kowdley [25] evaluated three commonly used commercial miRNA isolation kits for the best performance by comparing RNA quality and yield. In a very recent study, Callari et al[26] investigated whether circulating miRNAs can be reliably analyzed by microarrays from archival plasma samples. Moreover, the miRNA quantification strategy followed and especially the data normalization procedure have a significant effect on the reliability of miRNA quantification.[27,28]

The small size of miRNAs and their binding on lipids and proteins while in circulation in body fluids affects their reproducible recovery from plasma samples. These results on the analytical performance of three different column-based isolation protocols and that of TRIzol LS reagent indicated that column-based protocols are more effective and reliable and display a remarkable reproducibility concerning the isolation of cell-free miRNAs. By using TRIzol LS reagent based on the phenol:chloroform extraction, we could not achieve the same efficacy for endogenous and exogenous miRNAs despite the optimization steps that we performed before the comparison study. Among the commercially available kits that we evaluated, the *mir*Vana PARIS kit was more reproducible with a higher miRNA yield. These results were consistent with previous findings

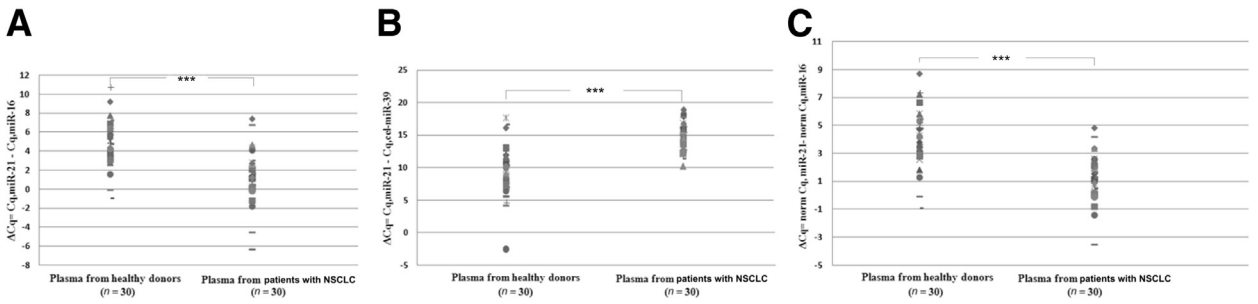

Figure 5 Effects of different normalization procedures on the quantification of circulating miRNA levels in the plasma of healthy individuals ($n = 30$) and patients with NSCLC ($n = 30$). The concentration of hsa-miR-21 in plasma was quantified by normalizing with respect to hsa-miR-16 (**A**), exogenous control cel-miR-39 (**B**), and a combination of both (**C**). $***P < 0.001$.

by McDonald et al,[14] who reported that the *mir*Vana PARIS kit was found to be the best-performing column-based kit for isolating RNAs among four miRNA extraction kits compared (two of them were the *mir*Vana PARIS kit from Ambion Inc. and the miRNeasy mini kit from Qiagen Inc., which were also used in the present study). However, the present results were in contrast with previous findings from the same group who also reported that TRIzol LS extraction matches or even surpasses the performance of the *mir*Vana PARIS kit.[14]

Sample storage conditions can seriously affect the accuracy and reliability of analytical results. We estimated the stability of circulating miRNAs in identical plasma samples under different temperature conditions for different periods. These results indicate that for the accurate quantification of cell-free miRNAs, the isolation process should be conducted within 48 hours of sample collection when keeping plasma samples at −20°C or −70°C, whereas storage at 4°C, even for 24 hours, leads to a significant reduction in circulating miRNA levels. When long-term storage of plasma samples is required, then −70°C rather −20°C should be used to avoid extensive miRNA degradation. Similar results have been presented by Grasedieck et al[29] with respect to the impact of serum storage conditions on miRNA stability.

We also evaluated the stability of miRNAs in the extraction buffer because this is very useful in the clinical laboratory setting, especially where novel findings suggest testing additional miRNAs in archived miRNA extraction samples. The results indicated that extracted miRNAs can remain stable for ≥1 year at −70°C. However, some studies report high instability and significant degradation of miRNAs 3 days after their isolation,[30] whereas other studies report high stability of isolated miRNAs over approximately 10 months.[31] One explanation could be the use of different elution buffers for miRNA storage in each case. The present results indicate that the isolated miRNA fraction from plasma samples using the *mir*Vana PARIS kit can be available for a long time at deep freeze.

Finally, we assessed the use of endogenous and exogenous controls for qPCR data normalization. This is a very critical issue to ensure the robustness of data owing to all the previously referred technical variations. In the field of circulating miRNAs, there is no generally accepted standard procedure for data normalization. One choice that is almost generally accepted now is the spiking of synthetic miRNAs into plasma samples before the extraction process and the measurement of their levels by RT-qPCR as normalizers.[7,28] Alternatively, endogenous miRNAs with low variability among the samples have also been used as normalizers by many research groups.[18,32] In the present study, we evaluated the effect of the data normalization procedure on the quantification of circulating miRNA levels in plasma from 30 healthy individuals and 30 patients with NSCLC by measuring levels of endogenous hsa-miR-21 and hsa-miR-16 and exogenous cel-miR-39 that was spiked in all samples at the same concentration. These data suggest that when normalization is based on a combination of an endogenous and an exogenous control miRNA, differences in miRNA recovery and differences in cDNA synthesis between samples are compensated. Using this normalization procedure and hsa-miR-21 as a biomarker, we could clearly discriminate healthy individuals from patients with cancer. It has been clearly shown that the expression of hsa-miR-21 can be differentiated between NSCLC and controls for fresh frozen tissue pairs[33,34] and plasma.[34–36] There is no gold standard to normalize Cq values and, thus, determine the actual quantitative level of these expression measurements and the magnitude of the difference between cancer and healthy plasma samples. This has led to many different approaches for miRNA normalization in plasma samples. We strongly believe that the proposed approach combines the advantages of an endogenous control, reassuring us of the sample quality, and an exogenous control, helping correct for differences in recovery rates between different samples.

These results are in agreement with those of Blondal et al,[37] who also reported a similar normalization approach for high-quality data from miRNA expression profiling studies.

Conclusion

Consistent with previous studies, our results confirm that preanalytical and analytical variables can seriously affect the reliability and reproducibility of circulating miRNA quantification. In particular, the use of exogenous and endogenous controls for normalization is critical for the reliable quantification of circulating miRNA levels in plasma. These factors should be taken into account to translate analysis of circulating miRNAs in body fluids into validated clinical tests in a routine clinical setting.

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