

# Plasma Components Affect Accuracy of Circulating Cancer-Related MicroRNA Quantitation

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**Circulating microRNAs (miRNAs) have emerged as candidate biomarkers of various diseases and conditions including malignancy and pregnancy. This approach requires sensitive and accurate quantitation of miRNA concentrations in body fluids. Herein we report that enzyme-based miRNA quantitation, which is currently the mainstream approach for identifying differences in miRNA abundance among samples, is skewed by endogenous serum factors that co-purify with miRNAs and anticoagulant agents used during collection. Of importance, different miRNAs were affected to varying extent among patient samples. By developing measures to overcome these interfering activities, we increased the accuracy, and improved the sensitivity of miRNA detection up to 30-fold. Overall, the present study outlines key factors that prevent accurate miRNA quantitation in body fluids and provides approaches that enable faithful quantitation of miRNA abundance in body fluids. (*J Mol Diagn* 2012, 14:71–80; DOI: 10.1016/j.jmoldx.2011.09.002)**

MicroRNAs (miRNAs) are small non-coding RNA sequences of approximately 19 to 22 nucleotides that modulate the activity of specific mRNA targets in development, differentiation, or disease, typically by compromising mRNA stability or interfering with translation (reviewed by Almeida et al,<sup>1</sup> Corcoran et al,<sup>2</sup> Brase et al,<sup>3</sup> Zen and Zhang,<sup>4</sup> and Kosaka et al<sup>5</sup>). Recently, miRNAs circulating in body fluids, and in blood serum in particular, have emerged as promising markers of disease and other processes (re-

viewed in references<sup>1–5</sup>). This application raises the need to accurately identify and quantitate miRNAs in samples collected from patients.

The mainstream approach to identification and quantification of miRNAs uses quantitative RT-PCR (RT-qPCR). With RT-qPCR, changes in plasma and serum miRNA profiles have been reported to reflect various physiologic and pathologic conditions including diagnostic and prognostic values for colorectal, breast, gastric, lung, oral, ovarian, pancreatic, and prostate cancers and for leukemia and lymphoma<sup>1–5</sup> and other diseases and conditions.<sup>6–9</sup> The increasing use of cell-free circulating miRNA for diagnostic and prognostic purposes, as for any such marker, requires assurance that the measured concentration represents the actual amount in the samples. Such assurance is often lacking.<sup>10</sup> The problem is exacerbated by the common assumption that a protocol developed for one study is applicable to other studies.<sup>11</sup> Overall, few methods<sup>12</sup> and improvements<sup>13</sup> have been offered,<sup>14</sup> and commonly used approaches lack required accuracy.<sup>15</sup>

The objective of the present study was to standardize and optimize miRNA detection for biomarker studies. We quantified two miRNAs that are implicated in distinct processes. One was miR-16, which acts as a tumor suppressor, is UV-inducible, is p53-regulated, and is deregulated or lost in some cancers.<sup>16</sup> MiR-16 has also been used to normalize quantitation of circulating miRNAs in breast

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cancer studies.<sup>17–19</sup> The other miRNA, miR-223, has been implicated in pregnancy, other conditions, and malignant disease.<sup>6,20,21</sup> Devising reliable approaches for accurate quantitation of circulating miRNAs is important for assessment of their potential as biomarkers.

## Materials and Methods

### Collection of Blood Samples

Fresh blood samples (5 mL) were either collected from healthy adults, received from the Susan G. Komen Foundation for the Cure Tissue Bank at the IU Simon Cancer Center (Indiana University, Indianapolis, IN), or supplied by Jeffrey Martin, MD of the AIDS Cancer and Specimen Resource (University of California, San Francisco, CA). Blood was collected in Vacutainer tubes (BD Diagnostics, La Jolla, CA) containing 10 mL heparin (sodium heparin, 143 USP units), 4.0 mL EDTA (EDTA, 7.2 mg), 4.5 mL sodium citrate (sodium citrate, 0.105 mmol/L), or 2 mL sodium fluoride and potassium oxalate (NaF/KOx, 5 mg/4 mg). Serum (7.5 mL) was collected in BD SST Vacutainer tubes (BD Diagnostics). Plasma was separated from red blood cells promptly to prevent loss of components<sup>22</sup> or hemolysis.<sup>23</sup> Blood was allowed to coagulate for 15 minutes at room temperature before prompt centrifugation. The study was approved by the Rosalind Franklin University of Medicine and Science institutional review board under protocols 004 and 005 PATH, and all donors provided written consent.

### Processing of Blood Samples

Fresh plasma and serum were obtained by centrifugation of blood samples at  $200 \times g$  for 15 minutes at 4°C. Supernatants were removed and collected in 15-mL polypropylene tubes. The plasma was centrifuged twice at  $800 \times g$  for 15 minutes at 4°C to obtain cell-free plasma. After the second centrifugation, supernatants were collected and passed through 0.45- $\mu\text{m}$  pore-size filters (Pall Corp., Port Washington, NY). Plasma and serum were divided into 200-, 50- and 10- $\mu\text{L}$  samples, and total volumes were adjusted to 200  $\mu\text{L}$  using water. A synthetic RNA (SYNTH, formerly INT,<sup>18</sup> 250 fmol/ $\mu\text{L}$ ) was added, and samples were analyzed immediately or were flash-frozen.

### Heparinase Treatment of Samples

Digestion of plasma samples using heparinase I (H2519; Sigma-Aldrich Corp., St. Louis, MO) was performed according to the manufacturer's protocol, as follows. Heparinase I (55 units) was dissolved at 1 mg/mL in 20 mmol/L Tris-HCl (pH 7.5), 50 mmol/L NaCl, 4 mmol/L  $\text{CaCl}_2$ , and 0.01% bovine serum albumin, and was added to a final concentration of 143 USP units per 10 mL of sample, which is expected to nearly completely remove heparin.<sup>24</sup> All digestions were performed for 1 hour at room temperature, and heparinase was removed using phenol-chloroform extractions.

### Post-Collection Treatment of Samples Using NaF/KOx

NaF (S-6776; Sigma-Aldrich Corp.) and KOx (P5311; Aqua Solutions, Deer Park, TX) were used for the experiments. Five milliliters blood was collected in NaF/KOx Vacutainer tubes containing 5 mg NaF and 4 mg KOx, effecting a final concentration of 1  $\mu\text{g}/\mu\text{L}$  NaF and 0.8  $\mu\text{g}/\mu\text{L}$  KOx (1X). Therefore, 50- $\mu\text{L}$  serum and EDTA samples were supplemented with NaF/KOx at 8X (8  $\mu\text{g}/\mu\text{L}$  NaF/6.4  $\mu\text{g}/\mu\text{L}$  KOx), 4X (4  $\mu\text{g}/\mu\text{L}$  NaF/3.2  $\mu\text{g}/\mu\text{L}$  KOx), 2X (2  $\mu\text{g}/\mu\text{L}$  NaF/1.6  $\mu\text{g}/\mu\text{L}$  KOx), 1X (1  $\mu\text{g}/\mu\text{L}$  NaF/0.8  $\mu\text{g}/\mu\text{L}$  KOx), and 0.5X (0.5  $\mu\text{g}/\mu\text{L}$  NaF/0.4  $\mu\text{g}/\mu\text{L}$  KOx), as indicated.

### Determination of Exosomal miRNA Stability in Blood Plasma and Whole Blood

Blood was collected in EDTA Vacutainer tubes. Half was processed to produce cell-free plasma, and the other half was left intact. To test the stability in blood of miRNAs released from cells in culture, aliquots of the plasma and serum were supplemented with exosomal miRNA of BC3 cells and incubated for the indicated time at 10°C in a continuously revolving tube rotator.

### RNA Isolation

SDS was added to 200- $\mu\text{L}$  samples of plasma or serum for a final concentration of 0.5% where indicated. This preparation was extracted using 500  $\mu\text{L}$  or 1 mL TRIzol LS reagent (Invitrogen Corp., Carlsbad, CA) and incubated for 10 minutes at room temperature followed by 100 or 200  $\mu\text{L}$  chloroform. The mixture was centrifuged at  $12,000 \times g$  for 16 minutes, and the aqueous layer was transferred to a new tube. Where indicated, this preparation was extracted one to three times using acidic phenol/chloroform (0966; Amresco Corp., Solon, OH). The resulting aqueous phase was transferred to a new tube and applied to a PureLink miRNA isolation kit (Invitrogen Corp.) as indicated, and was processed according to the manufacturer's recommendations. RNA was eluted with 50  $\mu\text{L}$  RNase-free water and stored at  $-70^\circ\text{C}$  or used immediately.

### Cell Culture

Exosomes and other particulates were collected from cells in culture as described.<sup>18</sup> In brief, after 5 days of culturing, the medium was collected, centrifuged at  $300 \times g$  for 15 minutes, and filtered through a 0.45- $\mu\text{m}$  filter to remove cell debris. The supernatant was centrifuged at  $70,000 \times g$  to collect particulates including exosomes, and was resuspended with 100  $\mu\text{L}$  PBS. BeWo cells were purchased from American Type Culture Collection (CCL-98; Manassas, VA) and cultured in F-12K (Mediatech, Inc., Manassas, VA) or RPMI 1640 (HyClone Laboratories, Inc., Logan, UT) with 10% or 20% fetal bovine serum. To remove bovine particulates, including exosomes, fetal bovine serum was ultracentrifuged at

70,000 × *g* for 2 hours, and the collected supernatant was added to culture medium. BC3 cells were cultured as described.<sup>25</sup>

### Reverse Transcription

For studies using fresh plasma, serum, and samples from the Susan G. Komen Foundation for the Cure Tissue Bank, 10 μL of the 50 μL extracted RNA was used as input into a reverse transcriptase reaction (Superscript III; Invitrogen Corp.) with miRNA-specific stem-loop primers in the Duelli laboratory as described.<sup>18,26</sup> The thermal cycles used to amplify the samples were 65°C for 5 minutes, 50°C for 60 minutes, and 70°C for 15 minutes. Samples from AIDS Cancer and Resource and samples including miRNAs released from BC3 cells were analyzed using primers obtained from Applied Biosystems, Inc. (Foster City, CA), also using miRNA-specific stem-loop primers in the Cullen laboratory as described.<sup>26,27</sup>

### MicroRNA Quantitation Using Taq-Based PCR

qPCR reactions were performed as described using SYBR Green or TaqMan (Applied Biosystems, Inc.), as noted.<sup>18</sup> Four percent of the cDNA produced in the reverse transcriptase reaction was amplified in MicroAmp Optical 96-well reaction plates in triplicate 20-μL reactions using a thermocycler (7900HT; Applied Biosystems, Inc.) using the cycles 95°C for 10 minutes, 40 cycles at 95°C for 15 seconds, 60°C for 1 minute, and hold at 4°C. Raw data were analyzed using SDS Relative Quantitation Software version 2.2.3 (Applied Biosystems, Inc.), in general using the automatic cycle threshold (Ct) setting for assigning baseline and threshold for Ct determination. MiRNA abundance was measured using computing amoles based on comparing Ct values of samples with dilutions of a synthetic DNA corresponding to the cDNA produced at reverse transcription for each miRNA measured to make a standard curve. The amplification efficiency, a measure of the number of template duplications per PCR amplification cycle, was calculated using the equation  $(T2/T1)^{1/(CT2_{ave}-CT1_{ave})} - 1$ .<sup>28</sup>

### PCR Using Other Polymerases

GoTaq Green (Promega Corp., Madison, WI) PCR was performed in 25-μL volumes according to the manufacturer's instructions. The initial denaturation step was 5 minutes at 95°C, followed by 40 cycles of 15 seconds at 95°C, 30 seconds at 50°C, 30 seconds at 72°C, and a final extension of 5 minutes at 73°C.

Hemo KlenTaq<sup>29</sup> (New England BioLabs, Inc., Ipswich, MA) PCR<sup>18</sup> was performed in 25-μL volumes according to the manufacturer's instructions, including attempts to reduce nonspecific priming by assembling PCR reactions on ice and transfer of reactions to the thermocycler preheated to 95°C. PCR using cocktails containing GoTaq DNA polymerase and Hemo KlenTaq polymerase were performed using GoTaq qPCR Mastermix (Promega Corp.) for SYBR Green

quantitation or TaqMan Universal PCR Mastermix (Applied Biosystems, Inc.).

Phire and Phusion enzymes were used according to supplier specifications (New England Biolabs, Inc.) for end-point PCR or were used with GoTaq qPCR Mastermix (Promega Corp.) for SYBR Green or with TaqMan Universal PCR Mastermix (Applied Biosystems, Inc.) for quantitation.

### Polyacrylamide Gel Electrophoresis

Native polyacrylamide gel electrophoresis (PAGE) of PCR products was performed as described.<sup>18</sup> A 10-pb ladder (marker; Invitrogen Corp.) was used for sizing PCR products in all experiments. Typically, 8 μL of each PCR sample was analyzed using PAGE.

### Statistical Analysis

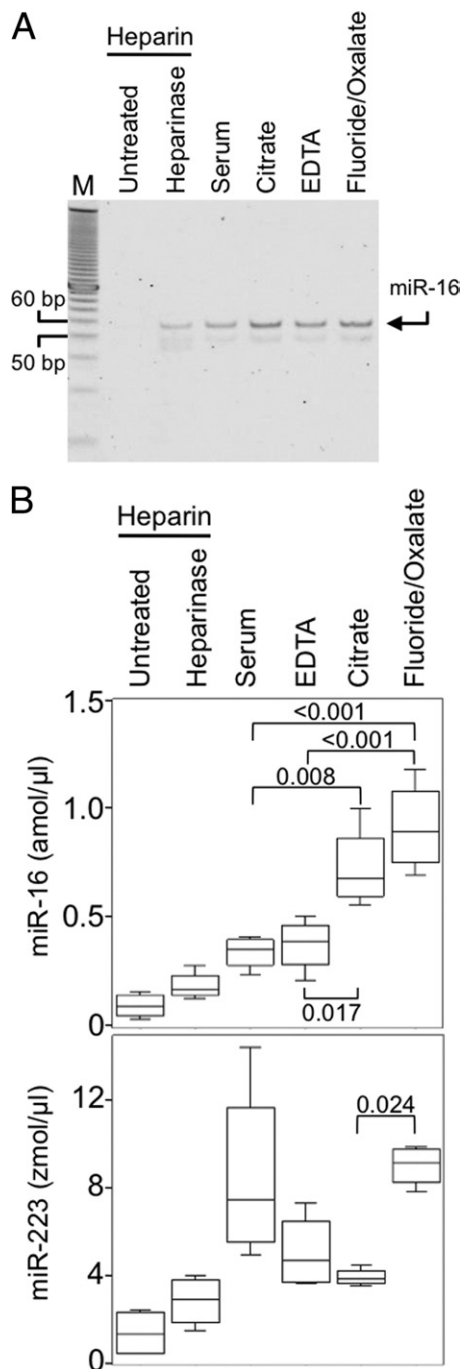
Statistical analysis was performed using one-way analysis of variance followed by Tukey's honestly significant difference test where indicated. Column graphed data are given as mean ± 1 SD. Data points were compared using the unpaired one-tailed Student's *t*-test unless otherwise indicated, and *P* values are given in the text, figures, or legends as are the number of independent experiments.

## Results

### NaF and KOx Improve miRNA Quantitation

Various anticoagulants and blood stabilizers are used for collection of plasma but not serum. Therefore, we tested whether the choice of collection method affected miRNA quantitation. Blood was collected in Vacutainer tubes containing either an anticoagulant (EDTA, heparin, sodium citrate, or NaF/KOx) or no anticoagulant. Blood was collected into the Vacutainer tubes in immediate succession using a single venipuncture per person (see *Materials and Methods*) to ensure nearly identical blood composition at the time of blood collection. Blood was drawn from four individuals of different ethnic origin, sex, and age on three separate dates.

miRNA abundance was measured using RT-PCR (Figure 1A) and qPCR of reverse-transcribed miRNAs using SYBR Green (Figure 1B). Reproducibility of miRNA quantitation depended on the blood collection method, with best results obtained by collecting into tubes containing NaF/KOx (Figure 1B). Although in blood plasma, miR-16 is about 500-fold more abundant than miR-223, the recovery and accurate detection of both miRNAs depended on the plasma collection method. Therefore, differences in detection resulting from the choice of blood collection method likely applies to other circulating miRNAs regardless of abundance. Furthermore, quantitation of miR-223 in serum yielded more variable results than most other collection methods, which suggests that at least for some miRNAs, collection of blood as plasma and the choice of anticoagulant can improve detection.



**Figure 1.** Fluoride and oxalate increase quantitation efficiency of circulating miRNAs. **A:** MiR-16 detection is affected by the blood collection method. Plasma and serum samples were collected from four individuals on three occasions. MiR-16 was amplified using RT-PCR, and was separated using PAGE. **Arrow,** miR-16 as determined by sizing and sequencing. **B:** Collection of blood in the presence of EDTA, citrate, or NaF/KOx increases miRNA detection. MiR-16 and miR-223 were quantified using SYBR Green RT-qPCR approaches. Data were analyzed using one-way analysis of variance followed by Tukey's honestly significant difference test. Middle bar represents the median; top and bottom of the box represent the 75th and 25th percentiles, respectively. Tukey's fences indicate the range of the values. *P* values are labeled above or below brackets. Chemicals listed indicate the coagulant and/or blood stabilization agents present in the particular Vacutainer tube used for the blood collection (see *Materials and Methods*). Heparinase indicates addition of heparinase 1 to the sample (see *Materials and Methods*). **Lower panel:** MiR-223 detection is robust in NaF/KOx. Detection of miR-16 or miR-223 was less in heparinized plasma than in serum. Heparinase treatment did not always improve detection of miR-16 (*P* = 0.082) or miR-223 (*P* = 0.125) in heparinized plasma. DNA length of the bands in the marker is indicated by 50 and 60 bp.

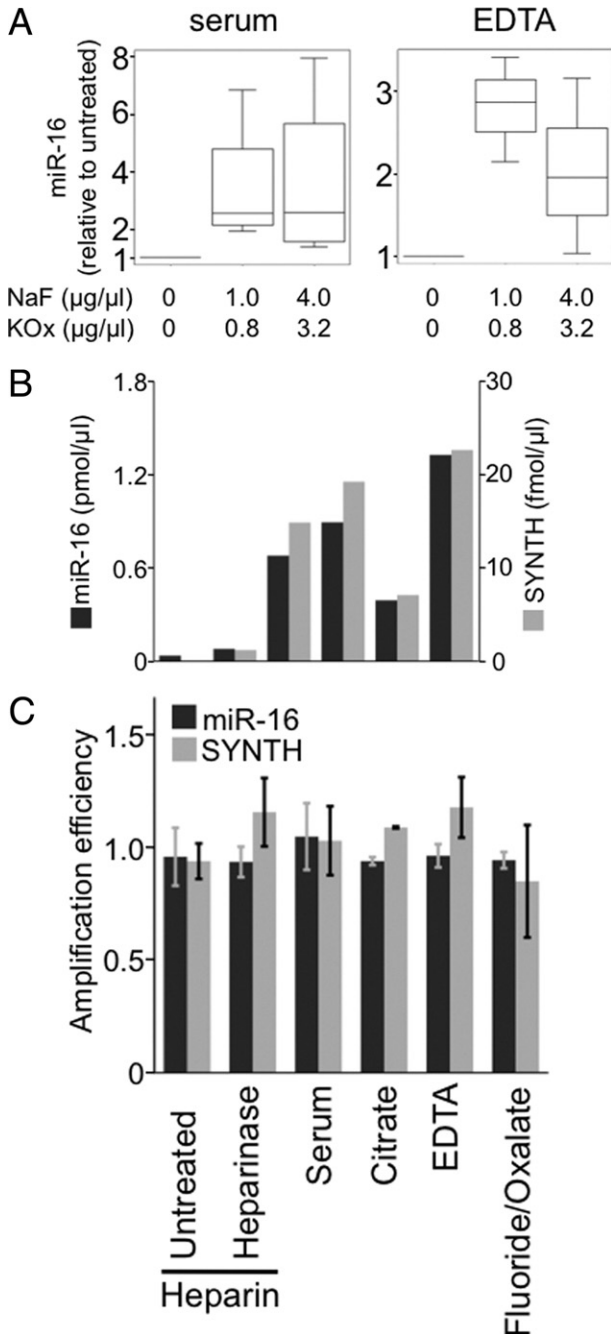
### NaF and KOx Improve the Sensitivity of miRNA Detection after Blood Collection

Maintenance of RNA stability during and after blood collection is an important factor for accurate miRNA quantitation and may depend on the anticoagulant used to collect the sample. For example, NaF/KOx is thought to enable greater sensitivity in the quantitation of other molecules in the blood including glucose,<sup>22</sup> alcohol, and opioids<sup>30</sup> by preventing their degradation at the time of collection and during storage.<sup>31</sup> We reasoned that the anticoagulants may differentially affect miRNA stability or detection by interfering with or promoting reactions used for quantitation. Therefore, we evaluated the effect of NaF and KOx on quantitation of miRNAs collected using other blood-draw methods. NaF and KOx were added to frozen samples collected using two of the most common blood-draw methods, that is, serum and plasma collected into EDTA, and miR-16 was measured in these samples (see *Materials and Methods*). It was observed that NaF and KOx increased miR-16 detection twofold in plasma collected using EDTA (*P* = 0.037), and threefold in serum (*P* = 0.014) (Figure 2A). These results indicate that adding NaF and KOx enhances miRNA quantitation even if these reagents are added after the samples are collected using other anticoagulants and stabilizers.

To address how NaF and KOx improve miRNA quantitation, we assessed the effects of NaF and KOx individually on miR-16 quantitation in serum. NaF increased the mean detection of miR-16 by 2.8-fold, and KOx increased sensitivity approximately 3.4-fold (see Supplemental Figure S1, A and B, at <http://jmd.amjpathol.org>). However, the increases lacked statistical significance for each component alone, which suggests that NaF and KOx synergize to effectively increase detection of miRNAs or that they act differently in improvement of quantitation of miRNAs in serum.

### NaF and KOx Increase Detection of Exogenous RNA

We considered that collecting plasma with NaF and KOx can improve detection by either stabilizing the RNA or increasing the amplification efficiency of the miRNAs during the PCR reaction.<sup>29,32</sup> To address the possibility that miRNAs are degraded by plasma ribonucleases,<sup>33</sup> we quantified miRNA stability before miRNA extraction by comparing endogenous miR-16 concentrations with those of a synthetic 22-nucleotide RNA (SYNTH) added during the RNA extraction. It was observed that the quantity of RNA was similar for both SYNTH RNA and endogenous miR-16 for each of the Vacutainer tubes (Figure 2B), which suggests that factors other than RNA stability in serum or plasma account for the differences in the sensitivity of RNA quantitation. Therefore, we tested whether the collection method affects PCR amplification efficiency. No significant changes were detected in the amplification efficiencies induced by the blood stabilizers or anticoagulants (Figure 2C). We concluded from this



**Figure 2.** Fluoride and oxalate improve detection of RNA regardless of origin. **A:** NaF/KOx was added at indicated concentrations to 50  $\mu\text{L}$  serum and plasma samples collected in EDTA. MiR-16 detection at both concentrations was increased ( $P = 0.014$  in serum, and  $P = 0.037$  in EDTA plasma). There was no statistical difference between the two concentrations of NaF/KOx ( $P = 0.827$  for serum, and  $P = 0.275$  for EDTA plasma) ( $n = 3$ ). **B:** Synthetic RNA (SYNTH) was added at 250 fmol/ $\mu\text{L}$  during addition of TRIzol to the serum samples or plasma collected using indicated additives. The effectiveness of SYNTH quantitation was compared with that of endogenous miR-16. **C:** Amplification efficiency of miR-16 and SYNTH in qPCR was measured and compared with that of serum. For miR-16: heparin,  $P = 0.58$ ; heparin plus heparinase,  $P = 0.43$ ; EDTA,  $P = 0.41$ ; citrate,  $P = 0.52$ ; and KOx,  $P = 0.43$ . For SYNTH: heparin,  $P = 0.53$ ; heparin plus heparinase,  $P = 0.49$ ; EDTA,  $P = 0.63$ ; Citrate,  $P = 0.41$ ; and KOx,  $P = 0.47$ . (All  $n = 3$ .)

result that none of the blood collection methods affect PCR amplification of reverse-transcribed miRNAs *per se*. Hence, NaF and KOx may improve miRNA detection by enhancing miRNA yield during extraction by enhancing

the reverse transcriptase reaction or perhaps in general by stabilizing the extracted RNA or the cDNA.

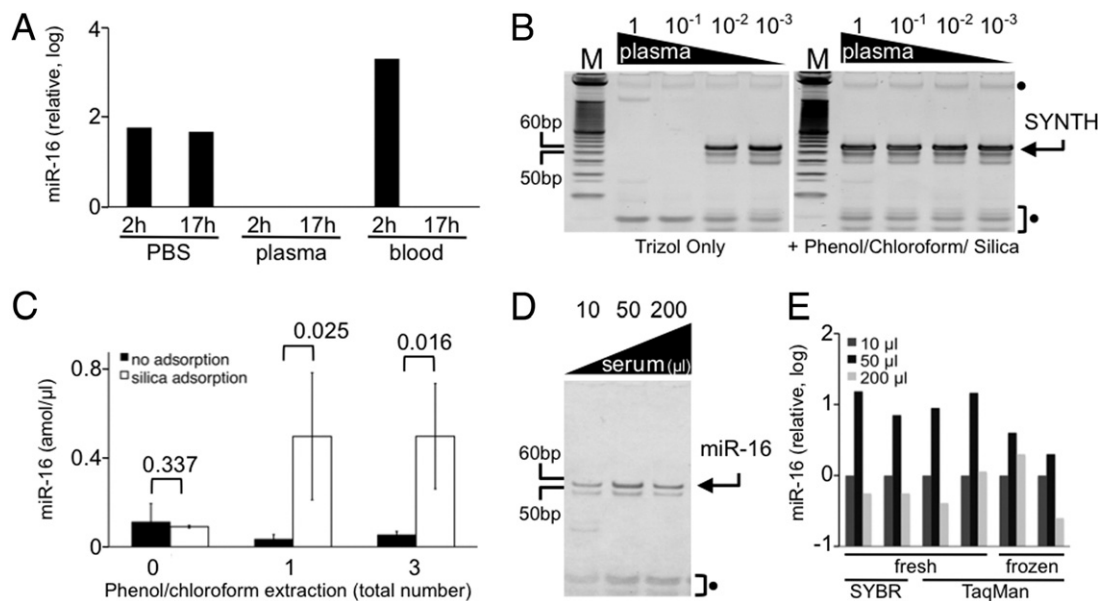
The finding of strong parallels between SYNTH and miR-16 quantitation within each plasma and serum sample supports the convention of using exogenous spiked RNAs as reference molecules for quantifying miRNA in plasma and serum. Alternatively, it was observed that quantitation of SYNTH added in a 4000-fold excess was much less affected by most collection methods (see Supplemental Figure S2 at <http://jmd.amjpathol.org>). This result suggests that high concentrations of RNA can overcome the interference of plasma components on miRNA quantitation and highlights the need to supply spiked RNA at similar concentrations as the endogenous RNA under investigation for accurate reference quantitation.

### *Inhibitors of Polymerases Present in Biological Samples Affect miRNA Detection*

To assess the stability of miRNAs in blood, we added released miRNAs isolated from the media of cultured BC3 cells to PBS, plasma, and whole blood. It was observed that miR-16 could be detected up to 17 hours after addition to PBS but was undetectable in plasma after 2 hours or in whole blood after 17 hours of storage (Figure 3A). This destabilization effect could be due to miRNA degradation in the sample,<sup>34</sup> to components of blood plasma that co-purify with miRNAs and interfere with their detection,<sup>29,35–37</sup> or to both.

Therefore, we tested whether plasma RNA interferes with the detection of SYNTH RNA. SYNTH RNA was supplemented with TRIzol-extracted RNA preparations of plasma collected in EDTA Vacutainer tubes, and SYNTH RNA abundance was quantified (Figure 3B, left panel). It was observed that RNA extracted from blood plasma interfered with SYNTH RNA detection, indicating that inhibitors of reverse transcriptase or PCR are present in RNA preparations extracted using TRIzol alone.

To determine the best method for removing serum and plasma inhibitors of miRNA detection, we applied approaches to improve the purity of the isolated RNA. It was observed that incorporation of a single acidic phenol/chloroform extraction step followed by adsorption of RNA on silica membranes reduced the interference by blood-borne polymerase inhibitors (Figure 3B, right panel) and effected a 4.4-fold increase in miRNA quantitation (Figure 3C). Other treatments such as addition of detergents<sup>38</sup> or ribonuclease inhibitors did not improve miRNA detection in serum or plasma (see Supplemental Figure S3 at <http://jmd.amjpathol.org>), which suggests that components other than ribonucleases or other proteins were responsible for the interference. We concluded that enrichment of small RNAs using TRIzol and phenol/chloroform extractions and silica adsorption effectively remove blood-borne reverse transcriptase and/or PCR inhibitors that prevent accurate quantitation of miRNAs in blood plasma.



**Figure 3.** Removal of blood plasma and serum components that inhibit detection of circulating MiRNA using organic extraction and silica-based RNA enrichment. **A:** Particulate miR-16 released from cells and added to plasma or whole blood is not detectable within 17 hours. Particulate RNAs released from BC3 cells was incubated in PBS, plasma, or blood for the indicated time, and miR-16 was quantified using TaqMan RT-qPCR. Portions of miR-16 in 2-hour blood samples are likely of whole-blood origin. **B:** Phenol/chloroform extraction and silica absorption of RNA remove inhibitors of miRNA detection. RNA was isolated from plasma collected in EDTA using TRIZOL only (**left panel**) or TRIZOL followed by one extraction of phenol/chloroform and enrichment of small RNAs on a silica membrane (**right panel**), and cDNA was added to a standard PCR reaction to quantify SYNTH RNA. RNA was added at the same concentration used in a standard RT-qPCR reaction (indicated by “1” above the gel), and 10-fold dilutions thereof ( $10^{-1}$ ,  $10^{-2}$ ,  $10^{-3}$ ). **C:** Enhanced detection of miR-16 in plasma extracted via phenol/chloroform and silica absorption. Plasma preparations were extracted using indicated numbers of phenol/chloroform extractions, followed by absorption to silica, or were directly assessed using RT-qPCR. **D** and **E:** Low volumes of plasma or serum effect greater detection of miRNAs. Indicated volumes of serum extracted using TRIZOL were subjected to end-point PCR for miR-16, and were separated using PAGE. Solid circles indicate amplification-independent products. MiR-16 was quantified in EDTA plasma immediately after collection or after storage at  $-80^{\circ}\text{C}$ .

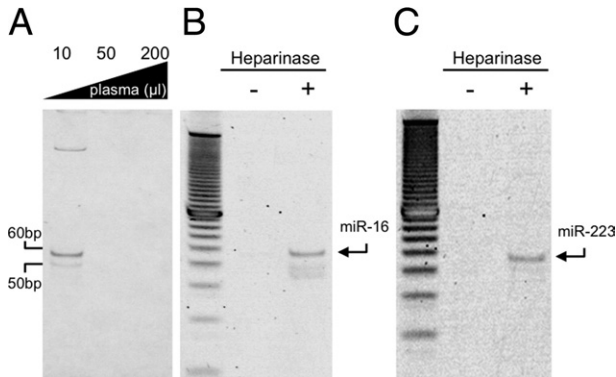
### Plasma Volume Affects miRNA Detection and Quantitation

The presence of inhibitors of miRNA detection in blood suggests that the greater the plasma or serum starting material used to extract the miRNA, the greater the abundance of co-purified blood-borne inhibitors of RT-PCR. Thus, we tested whether dilution of starting material affects the efficiency of detection. To do so, we quantified miRNA extracted from 10, 50, or 200  $\mu\text{L}$  fresh serum or plasma. It was observed that 50  $\mu\text{L}$  serum improved detection of miRNA by end-point PCR (Figure 3D) and yielded an 11-fold increase in the sensitivity of miRNA detection using SYBR Green or TaqMan qPCR (Figure 3E), perhaps reflecting a balance between miRNA and inhibitor abundance. To test whether similar concentration effects also apply to stored samples, we tested plasma collected in EDTA Vacutainer tubes from patients infected with HIV and Kaposi’s sarcoma-associated herpesvirus and stored for several months. It was observed that, similar to fresh samples, detection of miR-16 abundance was about threefold more sensitive at 50  $\mu\text{L}$  than at 10 or 200  $\mu\text{L}$  (Figure 3E). These data are consistent with the counterintuitive idea that using more blood for detecting miRNA results in less efficient detection than using less blood. Because this effect was observed in both plasma and serum, the latter of which is collected in the absence of additives, we concluded that inhibitors of miRNA detection are inherent in blood rather than introduced by chemicals used for collection. Of im-

portance, by reducing the starting material, inhibitors were presumed to be diluted below a threshold of interference. Thus, careful titration of starting material yields more accurate miRNA quantitation.

### Heparinase Treatment of Plasma Increases miRNA Detection

Heparin is an endogenous component of blood and is one of the original anticoagulants used in medicine. In some instances such as when analyzing historical samples or evaluating the blood of patients receiving heparin regimens because of deep venous thrombosis, stroke, or pulmonary embolism or during organ transplantation or heart surgery, heparinized plasma may be the only source of miRNA. In these cases, it is important to evaluate the effect of heparin on miRNA quantitation. We observed that miRNA quantified from heparinized plasma gave a poor yield (Figure 1), consistent with RT-PCR inhibition by heparin.<sup>39</sup> In testing whether reducing the starting volume could improve detection, we observed that using less starting material enabled detection of miR-16 from heparinized blood. However, greater dilutions were required to effect similar detection as in plasma collected using other methods (compare Figures 3D and Figure 4A). Therefore, we tested whether miRNA detection can be improved by digesting heparin with lyase heparinase I before RT-PCR.<sup>24,40</sup> This treatment enabled detection and quantitation of previously

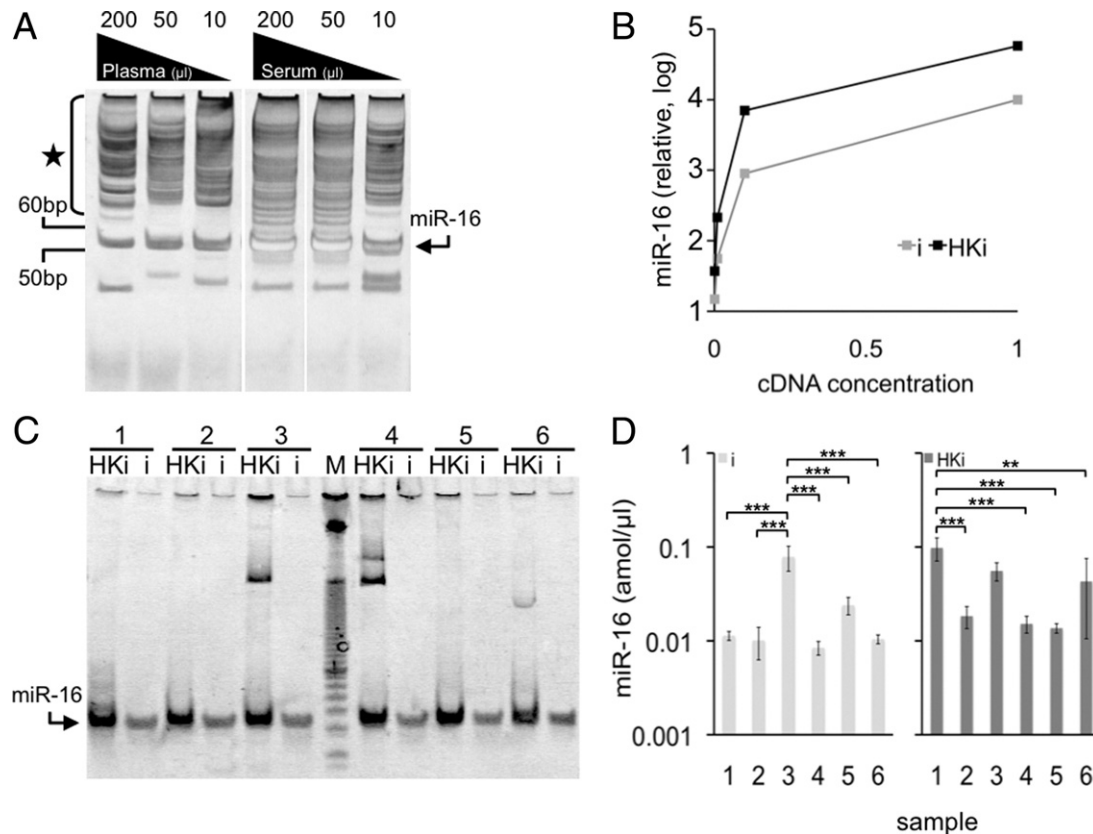


**Figure 4.** Heparin interference with detection of miRNA is relieved via heparinase treatment. Plasma was collected in Vacutainer tubes containing sodium heparin, and indicated volumes of plasma were assessed using end-point PCR (A). RT-PCR was performed after treatment with heparinase I (+) or on RNA without heparinase treatment (-). PCR products for miR-16 (B) and miR-223 (C) were assessed using PAGE.

undetectable miRNAs (Figure 4, B and C). We concluded that heparinase can increase detection of miRNAs in heparinized plasma. These results suggest that, when possible, heparin tubes should not be used in miRNA analysis. Alternatively, heparinase treatment provides an approach to detection of miRNAs when use of heparin is unavoidable or when heparin is present in previously collected samples.

### Mutant TaqDNA Polymerase Improves Quantitation of miRNA

An alternative approach to avert interference from blood-borne inhibitors of RT-PCR is to use different polymerases<sup>29,35-37,41</sup>. We tested enzymes reported to be more resistant to inhibitors: Phusion, Phire, and Hemo KlenTaq. Hemo KlenTaq is a mutant Taq DNA polymerase that has 100-fold lower sensitivity to blood inhibitors than does wild-type Taq.<sup>29</sup> Our analysis indicates that Hemo KlenTaq amplified miR-16, more efficiently than did Phusion (Figure 5A; see also Supplemental Figure S4A at <http://jmd.amjpathol.org>), and standard Taq DNA polymerase yielded low or no detectable PCR products in the same samples (Figure 3D). However, initial attempts to adopt these enzymes for qPCR of miRNAs using TaqMan failed (see Supplemental Figure S4B at <http://jmd.amjpathol.org>). It is likely that for Hemo KlenTaq, quantitation was compromised by production of multiple spurious bands in addition to the correct band (Figure 5A; see also Supplemental Figure S4 at <http://jmd.amjpathol.org>). We tested whether this lack of specificity is a general property of Hemo KlenTaq by evaluating the purity of amplifying miR-16 released from BeWo cells in culture. We detected only the correct miR-16 PCR product. However, supplementing such PCR reactions with blood plasma cDNA was sufficient to give rise to the



**Figure 5.** Mutant TaqDNA polymerase, Hemo KlenTaq improves the sensitivity of miRNA detection. **A:** Hemo KlenTaq (HK) was used to detect miR-16 (arrows) in 200, 50, and 10  $\mu$ l plasma and serum (see Figure 3C). Additional PCR products are indicated by a star. **B:** TaqMan qPCR of dilutions of reverse-transcribed miR-16 in serum or NaF/KOx plasma and using an intact Taq polymerase (i) plus Hemo KlenTaq (HKi) as indicated. **C:** PAGE of miR-16 amplified with HKi or i from six individuals (1 to 6). **D:** Absolute quantitation of miR-16 amplification products. \*\* $P < 0.01$ ; \*\*\* $P < 0.001$  (Tukey-Kramer multiple comparisons test).

extra products regardless of attempts to purify cDNA further before PCR amplification (see Supplemental Figure S5 at <http://jmd.amjpathol.org>). This finding suggests that the complexity or other properties of blood cDNA interfere with exclusive amplification of the miRNAs of interest and cause nonspecific cDNA amplification. This phenomenon may be a consequence of lack of the editing and proofreading 5'->3' exonuclease domain in Hemo KlenTaq.<sup>32</sup>

### Complementing Enzyme Cocktail for Effective Amplification of Products

To overcome the limitations of Hemo KlenTaq, we tested whether the reduced proof-reading activity of Hemo KlenTaq could be complemented by an intact Taq polymerase. It was observed that Hemo KlenTaq in combination with intact Taq polymerases amplified specific PCR products suitable for qPCR using SYBR Green or TaqMan (Figures 5, B and C; see also Supplemental Figure S6A at <http://jmd.amjpathol.org>). Of importance, the complementing Taq polymerases produced, on average, approximately 30-fold more amplification products from plasma miR-16 than did the intact polymerase alone (see Supplemental Figure S6B at <http://jmd.amjpathol.org>), which suggests that overcoming blood-borne inhibitors of Taq polymerase using Hemo KlenTaq yields an overall increase in detection sensitivity and specificity of circulating miRNAs.

### Mir-16 Quantitation Sensitive to Method of Detection

To test whether overcoming endogenous inhibitors with complementing polymerases provides proportionally

higher miRNA quantitation, we analyzed circulating miRNAs in the blood of six healthy individuals (see Supplemental Figure S7 at <http://jmd.amjpathol.org>). Five of the six individuals tested demonstrated similar miR-16 plasma concentrations regardless of the approach used, thus validating the use of complementing enzymes for improved detection of circulating miRNAs. With each approach, one individual consistently exhibited higher plasma miR-16 abundance than did the others (Figure 5D). However, another individual in either approach exhibited higher miR-16 concentrations, which suggests that quantitation of circulating miR-16, which is used as a reference miRNA or a biomarker of some cancers,<sup>16-19</sup> depends on the PCR conditions. These results suggest that differences in plasma composition among individual donors can yield different miRNA measurements.

### Discussion

This study demonstrates that inherent differences in biological samples and the methods used to collect and analyze them can dramatically affect the detection and quantitation of miRNAs. The implications of the work are that without consideration of the variables we have identified, miRNA quantitation of human samples may not be reliable for the purpose of biomarker development. Our results suggest that failure to detect plasma miRNAs may be due to polymerase inhibitors rather than to the absence of miRNA. Such inhibitors may include hemoglobin,<sup>42</sup> lactoferrin,<sup>36</sup> and IgG,<sup>43</sup> which can co-purify with nucleic acids.<sup>37</sup> This limitation can be overcome with concomitant use of two complementing Taq polymerases: Hemo KlenTaq, which is resistant to blood-borne inhibitors, in combination with another intact poly-

**Table 1.** Effectiveness of Various Treatments in miRNA Detection

Treatment/approach	Sensitivity*	Extra time per sample	Cost (%) <sup>†</sup>	Sensitivity/cost <sup>‡</sup>	Figure
Choice of anticoagulant and stabilizer					
NaF/KOx	2.7	0	99	2.7	1
EDTA	1.1	0	99	1.1	1
Citrate	1.7	0	100	1.7	1
Heparin	0.3	0	101	0.3	1
Serum	1	0	100	1.0	1
NaF/KOx after collection	3.6 <sup>§</sup>	Seconds	100	3.6	2A
Starting volume (μL)					
10	1	0	100	1.0	3, D and E; 4A
50	11 <sup>¶</sup>	0	100	11	3, D and E; 4A
200	0.7	0	100	0.7	3, D and E; 4A
Silica/phenol/chloroform	4.4	11 minutes <sup>  </sup>	181	2.4	3, B and C
Hemo KlenTaq/GoTaq	30	Seconds	101	30	5
Heparinase	2 <sup>**</sup>	1 Hour	130	1.5	1 and 4

\*Approximate fold difference in detected abundance compared with serum miR-16.

<sup>†</sup>Material cost per sample, calculated as percent of total cost of serum miR-16 detection using TaqMan, in \$US, based on list price of materials used in the study, which was \$9.25 for triplicate TaqMan.

<sup>‡</sup>Increase in sensitivity returned per material investment.

<sup>§</sup>2.4-Fold when supplemented to EDTA plasma.

<sup>¶</sup>Threefold in frozen samples.

<sup>||</sup>Extra total time spent on adsorption per sample is gained by elimination of the chilling step for precipitation of RNA (1+ hour) using the standard method. However, there is an increase in active labor, which, depending on the number of samples processed, may be substantial.

<sup>\*\*</sup>Fold of plasma collected in heparin. Ranges from statistically nonsignificant changes (Figure 1) to detecting signal only after heparinase treatment (Figure 4).



merase that has effective proofreading ability. In addition, the study demonstrates that diluting out inhibitors from blood samples also provides salient improvements in miRNA detection (Table 1). Additional purification of plasma or serum miRNA preparations, using organic extraction and silica adsorption to remove inhibitors, also increased the detection, albeit at greater cost in labor and funds.

The inability to detect specific miRNAs in plasma or serum, in many cases, reflects the low abundance of particular miRNAs in the circulation. For example, the release of some miRNAs from cells into blood is limited or selective.<sup>18,44,45</sup> Furthermore, depending on the nature of the complex that circulating miRNAs are associated with,<sup>18,34,46–52</sup> some miRNAs may be more stable than others<sup>34</sup> to the degradation by plasma ribonucleases or may be more amenable to amplification by polymerases. When usual techniques fail, the improvements outlined herein enable quantitation of miRNAs with low abundance and are expected to increase the repertoire of miRNAs that can be analyzed as potential biomarkers of disease.

The mechanism by which some of the approaches, for example, use NaF/KOx, improve detection is unclear. It is possible that NaF effects higher cDNA stability during the reverse transcriptase reaction, rather than stabilizing input miRNA, because classic experiments identified NaF as an inhibitor of RNase H,<sup>53</sup> an enzyme that degrades RNA and DNA hybrid substrates. However, KOx may promote miRNA detection by reducing calcium, an inhibitor of Taq, in the blood sample.<sup>41</sup>

Of interest, the absolute quantity of circulating miR-16 measured in the blood of some individuals differed depending on the enzyme used. These differences raise the possibility that factors including diet,<sup>54</sup> exercise,<sup>55</sup> circadian rhythms,<sup>56</sup> and seasons,<sup>57,58</sup> which alter the blood chemistry, might ultimately affect miRNA detection and quantitation. The disparate effectiveness of miRNA detection in an individual's plasma or serum may be indicative of other physiologic and pathogenic properties of the blood including endogenous heparin concentrations<sup>59</sup> or familial disease.<sup>60</sup> Our results demonstrate that such variations in blood chemistry can affect detection of miRNAs and must be considered and neutralized to accurately and efficiently assess miRNA abundance in blood serum and plasma.

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