BENCHMARKS
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Standardizing Analysis of Circulating MicroRNA: Clinical and Biological Relevance

Nicholas H. Farina,[1,2] Marie E. Wood,[1,3] Scott D. Perrapato,[1,4] Christopher S. Francklyn,[1,2] Gary S. Stein,[1,2] Janet L. Stein,[1,2] and Jane B. Lian[1,2]*

[1] *Vermont Cancer Center for Basic and Translational Research, University of Vermont College of Medicine, Burlington, Vermont 05405*

[2] *Department of Biochemistry, University of Vermont College of Medicine, Burlington, Vermont 05405*

[3] *Department of Medicine, Division of Hematology/Oncology, University of Vermont College of Medicine, Burlington, Vermont 05405*

[4] *Department of Surgery, Division of Urology, University of Vermont College of Medicine, Burlington, Vermont 05405*

ABSTRACT

Circulating microRNAs (c-miRNAs) provide a new dimension as clinical biomarkers for disease diagnosis, progression, and response to treatment. However, the discovery of individual miRNAs from biofluids that reliably reflect disease states is in its infancy. The highly variable nature of published studies exemplifies a need to standardize the analysis of miRNA in circulation. Here, we show that differential sample handling of serum leads to inconsistent and incomparable results. We present a standardized method of RNA isolation from serum that eliminates multiple freeze/thaw cycles, provides at least three normalization mechanisms, and can be utilized in studies that compare both archived and prospectively collected samples. It is anticipated that serum processed as described here can be profiled, either globally or on a gene by gene basis, for c-miRNAs and other non-coding RNA in the circulation to reveal novel, clinically relevant epigenetic signatures for a wide range of diseases. J. Cell. Biochem. 115: 805–811, 2014. © 2013 Wiley Periodicals, Inc.

KEY WORDS: CIRCULATING microRNA; c-miRNA; miRNA; SERUM; PLASMA; qPCR; RNA ISOLATION; BIOMARKER; CLINICAL SAMPLE

The discovery of microRNAs (miRNAs) in the circulation of human patients [Chen et al., 2008; Mitchell et al., 2008] spawned a plethora of studies with a goal to identify novel miRNA biomarkers of disease progression in a wide variety of maladies including cancers, heart disease, and diabetes. There is compelling evidence that circulating miRNAs (c-miRNAs) can be used as effective clinical biomarkers of disease diagnosis, prognosis, and success of treatment [Grasedieck et al., 2013; Williams et al., 2013]. However, when c-miRNA profiles obtained by different laboratories are directly compared for the same disease, there is minimal consistency in miRNA expression [Leidner et al., 2013; Sapre and Selth, 2013]. This conundrum poses a question that must be resolved for the application of c-miRNAs as clinically relevant disease biomarkers: How can c-miRNAs be reproducibly isolated, detected, and analyzed from both archival (banked) and prospectively collected samples?

C-miRNAs are small (18–24 nt) non-coding RNA molecules that are secreted by cells in membrane encapsulated particles that include exosomes [Valadi et al., 2007] or microvesicles [Skog et al., 2008], bound to proteins [Wang et al., 2010], and can be free, not bound to any biomolecule [Wang et al., 2010]. Once secreted, c-miRNAs function as signaling molecules that exchange epigenetic information between cells [Valadi et al., 2007; Skog et al., 2008]. It is hypothesized that the packaging of an individual c-miRNA, being membrane encapsulated, protein-bound, or unbound, determines the cell(s) targeted for specific signals. Additionally, c-miRNAs represent epigenetic changes that can reflect early cellular modifications prior to disease detection, using

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*Correspondence to: Jane B. Lian, Department of Biochemistry, University of Vermont College of Medicine, 89 Beaumont Avenue, Burlington, VT 05405. E-mail: jane.lian@med.uvm.edu

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currently available diagnostic methods, and to changes in cellular activity corresponding to disease progression [Valadi et al., 2007; Chen et al., 2008; Mitchell et al., 2008; Skog et al., 2008].

As potential clinical biomarkers, c-miRNAs appear to be stable as their expression can be detected in archival patient plasma and serum samples stored long-term at −80°C as well as after treatment with RNases [Turchinovich et al., 2011; Bryant et al., 2012; Köberle et al., 2013]. While c-miRNAs in plasma have been detected after up to 8 freeze/thaw cycles without significant changes in expression levels [Mitchell et al., 2008], it is becoming increasingly evident that the stability of c-miRNAs in serum may not be as robust [Köberle et al., 2013]. However, serum is the preferred circulating fraction for c-miRNA detection as plasma contains cellular material that may introduce contaminating cellular miRNAs from apoptotic or lysed cells (e.g., RBCs, platelets) as well as anti-coagulants such as heparin that can inhibit downstream methodologies [Hastings et al., 2012; Kim et al., 2012]. Thus, there is a critical need to develop a protocol for c-miRNA isolation from serum that reduces sample degradation without introducing factors inhibitory to downstream assays. Figure 1 illustrates the initial species of c-miRNA in serum (Fig. 1A) and potential outcomes of c-miRNAs in serum that is handled and processed inconsistently, resulting in differential and incomparable c-miRNA expression levels (Fig. 1B).

Standardization of operating procedures for biomedical research is imperative. The Early Detection Research Network (ERDN) has developed a Standard Operating Procedure for the collection of serum and plasma with guidelines as to the time and temperature for sample processing [Tuck et al., 2009]. Here, we provide an additional standardized method of downstream serum handling for RNA isolation (based on Li and Kowdley [2012]) that will allow for direct comparison of c-miRNAs in archived patient serum with prospectively collected serum. Our protocol rectifies several known issues with detecting c-miRNAs in serum by reducing freeze/thaw cycles, maintaining precise volumes through uniform RNA isolation, and adding a synthetic RNA as a normalization control (as illustrated in Fig. 2). We anticipate that RNA obtained with this method can be readily used for global miRNA profiling studies, next generation sequencing, or qPCR of individual miRNAs.

MATERIALS AND METHODS

HUMAN PATIENT SAMPLES

Human samples were collected from healthy patient volunteers or from men diagnosed with prostate cancer. Blood and serum were collected by a trained professional at Fletcher Allen Health Care following written informed consent to an institutionally approved protocol (CHRMS 04-004 and CHRMS M13-042). All samples were de-identified and handled in accordance with HIPAA regulations.

MOUSE SAMPLES

Animals were housed in an animal facility at The University of Vermont and cared for by a dedicated animal technician as per IUCAC approved protocol (#12-051). All mice used were in the C57Bl/6 background and approximately 10 months of age.

SERUM COLLECTION

Blood was collected from mice via cardiac puncture following an approved IUCAC protocol. Briefly, animals were sacrificed with CO_2, 0.8–1 ml of blood was collected from the beating heart using a 25 5/8

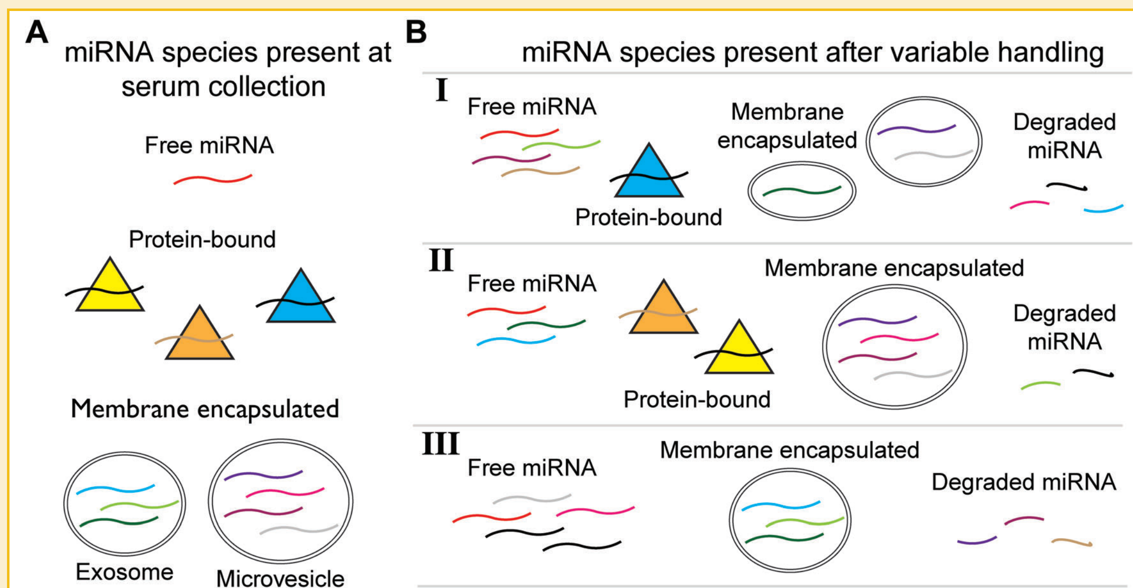

Fig. 1. Potential fates of serum miRNAs based on sample handling. Multiple species of miRNA and miRNA associated particles circulate and can be isolated from serum including free, unbound miRNAs, miRNAs bound to proteins, or membrane encapsulated miRNAs including those in microvesicles and exosomes (A). However, the miRNA complement in serum present after subsequent freeze/thaw cycles likely differs based on the conditions of the subsequent thaws (B). I, II, and III represent potential outcomes of non-standardized sample handling showing differential c-miRNA stability.

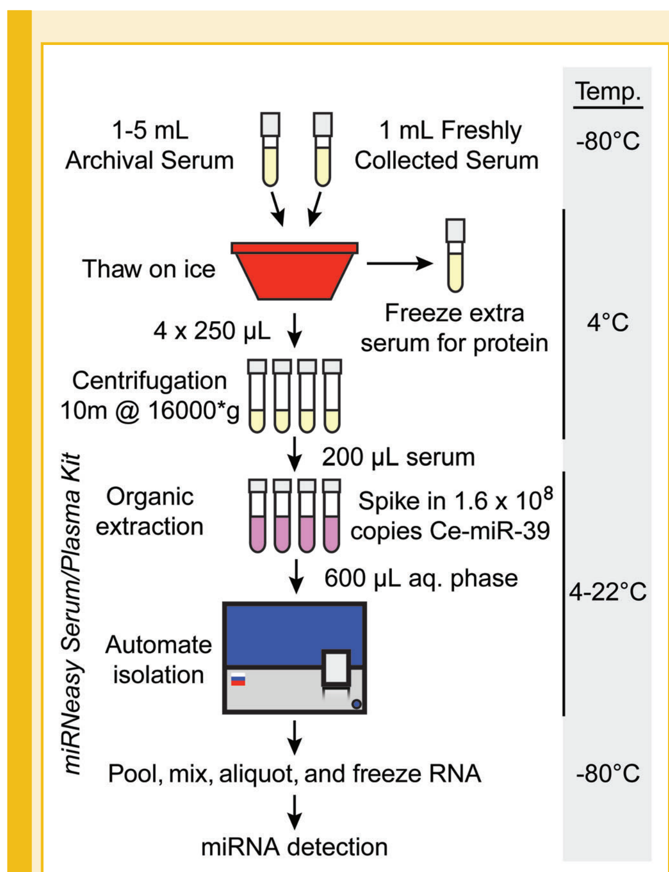

Fig. 2. Sample handling and miRNA isolation schematic. This method is described in detail in the text. Briefly, all serum samples, whether archival or fresh, are stored at −80°C. Serum is slowly thawed on ice and 1 ml divided into four aliquots for RNA isolation. Centrifugation removes cryoprecipitates and exactly 200 μl serum/aliquot is used for RNA isolation using the miRNeasy Serum/Plasma kit (Qiagen) with QIAcube (Qiagen) automation. Following elution, RNA is pooled, divided into aliquots, and frozen for future use.

gauge needle, and blood allowed to clot at room temperature 30–60 min. Following centrifugation for 5 min at 2,040*g* in a bench-top centrifuge, the top translucent yellow phase was carefully removed and serum stored at −80°C until processed for RNA isolation. Blood was collected from human patients according to an institutionally approved protocol. Briefly, 10 ml of blood was collected in four vials, allowed to clot for 10 min at room temperature, and centrifuged for 15 min at 2,000 rpm in a clinical centrifuge. Serum was carefully removed from each vial, pooled, divided into 1 ml aliquots, and stored at −80°C at least overnight until processed for RNA isolation to mimic conditions of archived patient samples.

SAMPLE HANDLING

Serum samples were thawed on ice over 1–2 h and divided into 250 µl aliquots. For those serum samples undergoing multiple freeze/thaw cycles, aliquots were immediately re-frozen and stored at −80°C at least overnight. For the slow second thaw, samples were thawed on ice over 1–2 h. For the fast second thaw, samples were thawed in a 37°C water bath for 30–60 s until just melted and immediately transferred to ice. Once thawed, 250 µl of serum was centrifuged 10 min at 16,000*g* at 4°C to remove cryoprecipitates. Exactly 200 µl of serum was removed and used for RNA isolation.

RNA ISOLATION

We tested miRNA recovery of the *mir*Vana™ PARIS™ (Ambion®-Life Technologies) or miRNeasy Serum/Plasma (Qiagen) kits following manufacturers protocols. RNA was isolated from precisely 200 µl of serum, 1.6×10^8 copies of synthetic ce-miR-39 mimic was spiked-in to each sample prior to addition of chloroform, and preset volumes were used when removing sample containing RNA as described (Table I). The *mir*Vana™ PARIS™ protocol was modified to include a second organic extraction and use of the smaller filter cartridges from the RNaqueous-Micro Kit (Ambion®-Life Technologies). The miRNeasy protocol was modified for automation using a QIAcube (Qiagen) for on-column washes and elution. The minimum recommended elution volume (Table I) for each kit was used to produce the most concentrated RNA.

cDNA SYNTHESIS AND QUANTITATIVE RT-PCR

cDNA was synthesized using the miScript II RT kit (Qiagen) according to the manufacturers instructions with the following modifications for a 10 µl reaction. Five microliters of total serum RNA was incubated with 2 µl 5× HiSpec Buffer, 1 µl Nucleics Mix, 1 µl RNase-Free Water, and 1 µl RT mix at 37°C 1 h followed by enzyme deactivation for 5 min at 95°C. The cDNA was diluted 1:10 in RNase-Free water and miRNA detected using the miScript SYBR® Green PCR kit (Qiagen). Ten microliters reactions were run in 384-well plates on an ABI ViiA7 (Applied Biosystems-Life Technologies). Spiked-in ce-miR-39 was detected with the Ce_miR-39_1 miScript Primer Assay (Qiagen). Specific forward primers (Invitrogen-Life Technologies) for each miRNA were designed as the miRNA sequence and are identical for mouse and human: miR-21-5p 5′-TAGCTTATCAGACTGATGTTGA-

TABLE I. Volumes Used During RNA Isolation

*mir*Vana™ PARIS™ kit		miRNeasy serum/plasma kit	
Reagent/sample	Volume (µl)	Reagent/sample	Volume (µl)
2× Denature Solution	200	QIAzol	1000
Acid-phenol:chloroform 1	400	Chloroform	200
Aqueous phase 1	350	Aqueous phase	600
Acid-phenol:chloroform 2	350	100% EtOH	900
Aqueous phase 2	275	RNase-free water (eluate)	14
100% EtOH	400		
Elution buffer	10		

Consistent volume is critically important to sample reproducibility and comparison as there are no established normalizing factors.

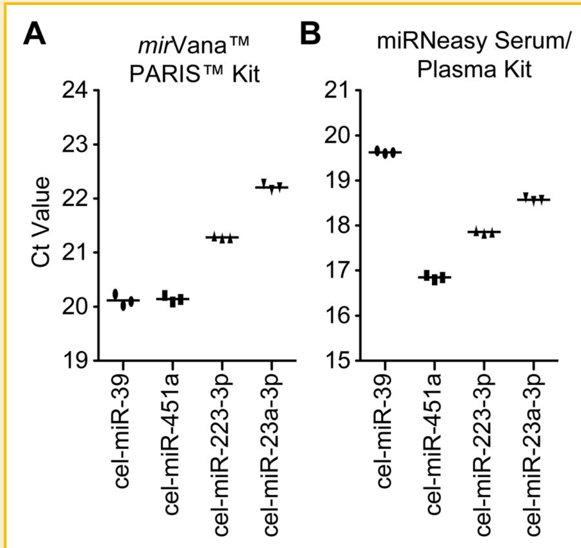

Fig. 3. Serum miRNA isolation efficiency: *mir*Vana™ PARIS™ versus miRNeasy Serum/Plasma. RNA was isolated from the same test mouse serum using the *mir*Vana™ PARIS™ (A) and miRNeasy Serum/Plasma (B) kits. The recovery of spiked-in control cel-miR-39 is similar between kits. However, all three tested miRNAs, miR-451a, miR-223-3p, and miR-23a-3p are detected at lower Ct values indicating higher expression in RNA isolated using the miRNeasy Serum/Plasma kit (compare B to A). Graphs show qRT-PCR miRNA detection, cycle threshold (Ct), and are of three technical replicates.

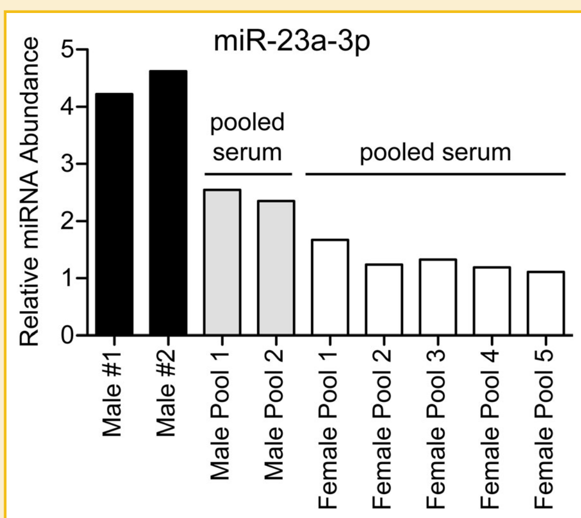

Fig. 4. RNA isolation reproducibility using automation on a QIAcube. RNA was isolated from either individual animals (black bars) or pooled animals (gray and white bars). Levels of miR-23a-3p are consistent between comparable samples (black, gray, or white bars). Individual animals are liter-mates. Pooled serum is from liter-mate animals. Relative miRNA abundance is normalized to levels of spiked-in cel-miR-39.

3′; miR-23a-3p 5′-ATCACATTGCCAGGGATTTCC-3′; miR-93-5p 5′-CAAAGTGCTGTTCGTGCAGGTAG-3′; miR-223-3p 5′-TGTCAGTTTGT-CAAATACCCCA-3′; miR-451a 5′-AAACCGTTACCATTACTGAGTT-3′.

RESULTS

NORMALIZATION REQUIREMENTS FOR CIRCULATING miRNAs

Unlike cellular RNA that can be directly compared to endogenous housekeeping genes, such as U6 and GAPDH, there are no established cell-free RNAs in circulation to which levels of c-miRNAs can be normalized. Many groups have assessed the levels of potential reference genes between diseased and healthy patients [Sanders et al., 2012; Song et al., 2012; Zhu et al., 2012; Chen et al., 2013; Wang et al., 2013]. However, these proposed normalization factors are disease specific and cannot be universally used. Moreover, normalization methods that eliminate the need for endogenous controls, such as Global Means or Quantile normalization, are only suitable for large datasets. Thus, to allow for sample-to-sample comparisons, precise volumes must be used at each step of the RNA isolation (Fig. 2 and Table I). Furthermore, 1.6×10^8 copies of a synthetic miRNA mimic that is only expressed in *Caenorhabditis elegans* and not in mammals is spiked into each serum sample prior to RNA isolation as recommended by the manufacturer for precise quantitation. This technique allows for three concurrent normalization methods: relative miRNA level per serum volume; relative miRNA level as compared to spike-in control; absolute miRNA copy number per serum volume.

RNA ISOLATION: STRINGENT CONTROL OF PARAMETERS TO REDUCE SAMPLE VARIABILITY

Levels of c-miRNA isolated using different methods should not be directly compared as the recovery of c-miRNA varies dramatically [Li and Kowdley, 2012; Page et al., 2013]. Thus, we tested two frequently utilized commercially available miRNA isolation kits: Ambion® *mir*Vana™ PARIS™ and miRNeasy Serum/Plasma kit. The general methodology for both is similar, where RNA from serum is isolated by an organic extraction with phenol/chloroform followed by salt and ethanol washes on a silica membrane. As previously described, the volumes are kept consistent (Table I) and a synthetic control is spiked into the serum prior to RNA isolation (Fig. 2). The recovery of spike-in control RNA between the two kits is within 0.5 Ct of each other indicating similar RNA isolation efficiency (Fig. 3). In agreement with prior work [Li and Kowdley, 2012], all three miRNAs assayed for, miR-451a, miR-223-3p, and miR-23a-3p, are detected at levels more then eightfold higher in RNA isolated using the miRNeasy Serum/Plasma kit as compared to the Ambion® *mir*Vana™ PARIS™ kit (Fig. 3). We anticipate that low abundance miRNAs can be more readily detected in RNA isolated with the miRNeasy Serum/Plasma kit. The ability to detect low abundance miRNAs may allow for the identification of c-miRNA biomarkers of early stage disease.

To further reduce sample-to-sample variability and increase reproducibility of c-miRNA detection, we automated all steps following the organic extraction on a QIAcube. Briefly, 600 μl of the aqueous phase, containing c-miRNAs, along with filter columns, ethanol, buffer RWT, buffer RPE, and RNase-free water are loaded into a QIAcube. All subsequent RNA isolation steps, including ethanol precipitation, membrane binding, and salt washes, are performed and RNA recovered. While this protocol describes the use of a QIAcube (Qiagen), we expect similar robotics to greatly improve sample consistency. We compared c-miRNA expression between individual

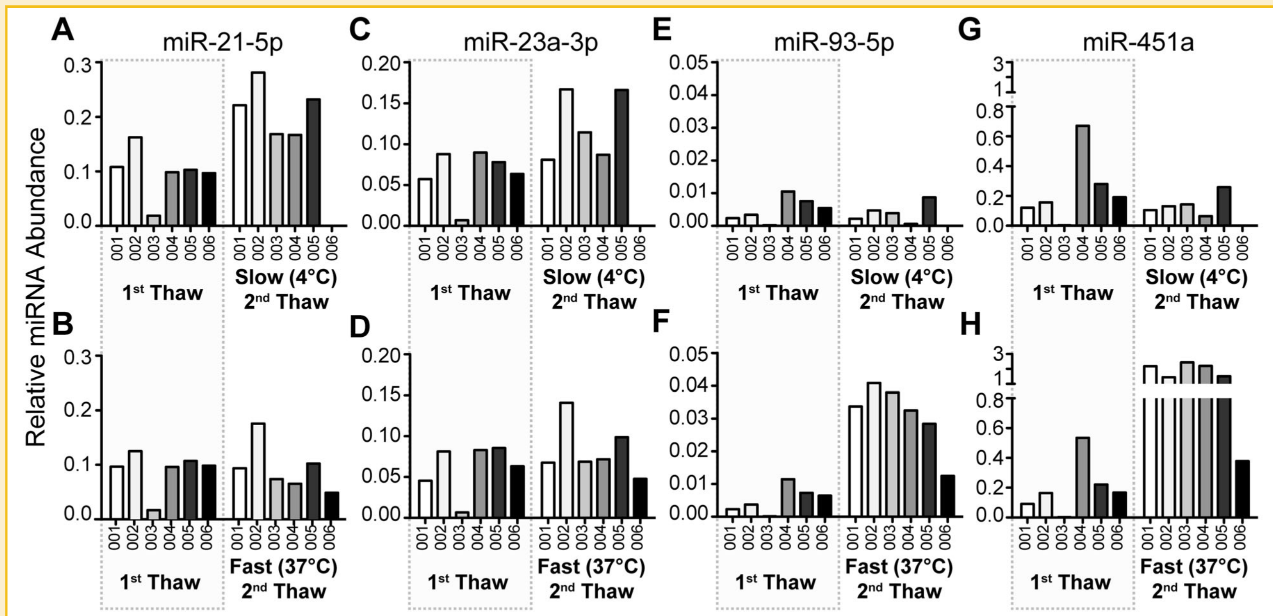

Fig. 5. Multiple freeze/thaw cycles introduce a high degree of variability in c-miRNA detection. The serum levels of four miRNAs, miR-21-5p (A,B), miR-23a-3p (C,D), miR-93-5p (E,F), and miR-451a (G,H) were determined in six healthy human patients. C-miRNA levels varied dramatically when RNA was isolated after two freeze/thaw cycles as compared to RNA isolated from after the first thaw. The speed and temperature at which serum was thawed differentially affected miRNA levels (compare slow (4°C) thaw A, C, E, G to fast (37°C) thaw B,D,F,H). No RNA was recovered from sample 006 when thawed for a second time at 4°C. Relative miRNA abundance is normalized to levels of spiked-in cel-miR-39.

animals and between pooled serum from male or female animals. The levels of detected c-miRNAs are consistent between groups, and varied based on genotype and sex indicating that automation reduced sample variability and may allow for the detection of subtle differences in c-miRNA levels (Fig. 4).

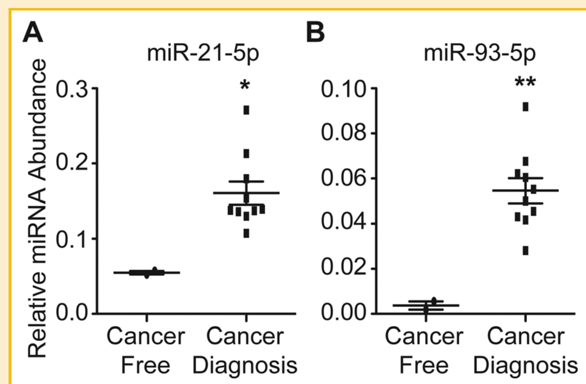

Fig. 6. Method validation: miRNAs associated with prostate cancer are detected at increased levels in diseased human serum. The expression miR-21-5p (A) and miR-93-5p (B) were determined in the serum from 10 men diagnosed with prostate cancer (cancer diagnosis). Both miR-21-5p (A) and miR-93-5p (B) are detected at elevated levels as compared to cancer-free controls. Relative miRNA abundance is normalized to levels of spiked-in cel-miR-39. Error bars are ±SEM, $^{*}P < 0.01$, $^{**}P < 0.001$.

REPEATED FREEZE/THAW CYCLES CAUSE INCONSISTENT C-miRNA DETECTION

Clinical studies designed to identify c-miRNAs as biomarkers will likely compare retrospective archived serum with prospectively collected samples. Consequently, a standard operating procedure must account for the procurement and handling of such samples in a uniform manner. In accordance with the EDRN SOP [Tuck et al., 2009], archived serum is stored at −80°C, requiring at least one freeze/thaw cycle prior to c-miRNA detection. We collected serum from six healthy human patients and stored it at least overnight at −80°C to mimic archived sample conditions. After an initial thaw on ice, each serum sample was divided into 250 μl aliquots. The RNA was immediately isolated from one aliquot (first thaw), as previously described, while the remaining aliquots were stored at −80°C at least overnight. Of the remaining aliquots, one was thawed slowly on ice while one was thawed rapidly at 37°C to assess the effects of differential sample handling on c-miRNA levels.

In agreement with the reduced stability of c-miRNAs in serum as compared to plasma [Köberle et al., 2013; Leidner et al., 2013], both the level and pattern of c-miRNAs change with subsequent freeze/thaw cycles (Fig. 5). Interestingly, the levels of each c-miRNA are not affected in a similar manner, suggesting that stability may be related to the cell-free fraction of the individual c-miRNA, being membrane encapsulated, protein-bound, or unbound. Two c-miRNAs, miR-93-5p and miR-451a, are detected at significantly higher levels when the serum is thawed rapidly at 37°C. One explanation is that these miRNAs are contained within large particles that sediment in the initial centrifugation step to remove cryoprecipitates when serum is

thawed at 4°C that are disrupted or lysed at 37°C, releasing c-miRNAs. Together, our data indicate that inconsistent sample preparation and handling dramatically alters the profile of circulating miRNA in serum leading to irreproducible results.

METHOD VALIDATION

To validate our method, we examined the expression of c-miRNAs in serum collected from men with a positive diagnosis of prostate cancer. Serum from these men was stored at −80°C for various lengths of time (from days to months), modeling a direct comparison of archival with prospectively collected samples, and RNA was isolated accordingly. The expression of two miRNAs with elevated levels in solid tumors of many cancers, including prostate cancer, miR-21-5p [Baer et al., 2013; Mishra et al., 2013] and miR-93-5p [Hamilton et al., 2013] was determined. In agreement with these studies, both miR-21-5p (Fig. 6A) and miR-93-5p (Fig. 6B) are detected at significantly higher levels in men diagnosed with prostate cancer as compared to cancer-free individuals. Thus, when clinical serum samples are analyzed in a consistent manner as outlined here, c-miRNAs that are deregulated in disease states may prove to be effective novel biomarkers in patients at risk for or with early stage disease and can be monitored during disease progression and treatment to assess response to therapies.

DISCUSSION

Consistent and reproducible isolation and detection of miRNAs in circulation of patients currently poses a major problem for the biomedical community. Institutions around the world have extensive tissue and serum banks that provide an existing repository of retrospective samples in which Investigators can identify c-miRNAs as novel disease biomarkers. Moreover, the desire to monitor disease progression and response to treatment necessitates longitudinal studies. Given the clinical need to compare retrospective and prospective samples collected at different times, blood fractions must be stored and processed in a manner that will yield highly confident results. Our results add to the growing body of evidence that improper sample handling likely leads to differential c-miRNA stability and hence, highly variable results. Here, we present a standardized method for isolating miRNAs in circulation from archived and freshly collected serum to reduce technical variability between protocols.

Another problem facing investigators in the design of retrospective studies is how to carefully handle precious, and often irreplaceable, patient samples. Many archived samples are stored in volumes larger than required for a single RNA isolation. Furthermore, future detection of other biomolecules, such as DNA and protein, may be desired from the same sample. Thus, we recommend dividing each serum sample into 1 ml aliquots and re-freezing unused aliquots to −80°C at time of RNA isolation. Proceeding with RNA isolation from at least 1 ml of serum followed by storage of RNA in small aliquots at −80°C for future analysis, eliminates multiple freeze/thaw cycles and precious patient samples are optimally utilized. Importantly for prospective studies, freshly isolated serum should be stored at −80°C at least overnight to allow for direct comparison to archival samples. It is anticipated that RNA isolated using this method is suitable for downstream detection of c-miRNAs using global platforms, such as SYBR and TaqMan-based qPCR profiling, hybridization-based microarrays, and deep sequencing, which offer the most comprehensive picture of c-miRNA levels. Here, we show consistent qPCR detection of individual c-miRNAs from serum. While not directly assessed, other circulating non-coding RNAs likely can also be reproducibly detected and profiled with high efficacy from RNA isolated as described.

CONCLUDING REMARKS

There are many clinical applications and basic experimental questions for which reproducible detection of circulating RNA from human patients can be used. Longitudinal studies that track patients over years, either prior to disease diagnosis or following treatment, may identify c-miRNAs as novel early detection biomarkers, those that can trace response to treatment, or a signature consisting of multiple c-miRNAs that is predictive of disease recurrence. Additionally, deregulated c-miRNAs in diseases provide exciting targets for therapeutics development. Lastly, it will be interesting to investigate the relationship between c-miRNAs and the expression levels of the proteins encoded by the mRNAs targeted by c-miRNAs both in circulation and in diseased cells. Such knowledge can better guide treatment strategies using currently available drugs against these proteins and help design future therapeutics.

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