Magnetic Ionic Liquids as microRNA Extraction Solvents and Additives for the Exponential Amplification Reaction

Miranda N. Emaus and Jared L. Anderson*

Department of Chemistry, Iowa State University, 1605 Gilman Hall, Ames, Iowa 50011, United States

Abstract

The detection of microRNAs (miRNAs) from highly complex matrices has become an area of immense interest as their characterization in biological samples has been utilized for disease diagnosis and body fluid identification. However, conventional northern blotting miRNA detection lacks the sensitivity required to detect circulating miRNAs. Additionally, polymerase chain reaction-based methods for miRNA detection require modified oligonucleotides that are difficult to design. Exponential amplification reaction (EXPAR) is an isothermal amplification method used for miRNA detection that is simple to design but suffers from non-specific amplification that masks low concentration miRNAs. Previous studies have shown that magnetic ionic liquids (MILs) are a promising alternative to traditional nucleic acid extraction methods capable of preconcentrating DNA from complex matrices. In this study, three hydrophobic magnetic ionic liquids (MILs) were investigated as EXPAR additives and miRNA extraction solvents. The addition of MIL to the EXPAR buffer decreased the background signal from non-specific amplification and increased the reaction rate. Reactions containing MIL could detect miRNA at concentration levels down to 10 aM. In comparison, reactions that did not contain MIL could not discriminate 10 fM lethal-7a (let-7a) standards from the no trigger control (NTC). All three MILs extracted miRNA from 2-fold diluted plasma, artificial urine, and artificial saliva with only a 1 min dispersion step. By integrating the miRNA-enriched MIL into the EXPAR buffer, the extraction and detection of femtomolar concentrations of miRNA required only 10 min. In contrast, conventional spin column kits require at least 20 min to isolate miRNA, indicating that a dispersive MIL-based extraction is ideal for high throughput analysis of miRNA.

Keywords: microRNA extraction, ionic liquid, EXPAR, magnetic separation, nucleic acid detection

*Corresponding Author:
Jared L. Anderson
Department of Chemistry
Iowa State University
1605 Gilman Hall
Ames, IA. 50011 USA
andersoj@iastate.edu

1. Introduction

MicroRNAs (miRNA) are short, non-coding RNA fragments (19-25 nucleotides) found in all multicellular organisms and some viruses.[1] By binding to 3' untranslated regions (UTRs) of target messenger RNA, miRNAs can inhibit gene translation leading to research in miRNA replacement therapy to treat cancer.[2,3] Another significant area of miRNA research has involved utilizing differential miRNA expression to diagnose illnesses such as neurological disorders, cardiovascular disease, and type II diabetes.[4] miRNA levels can also be used to classify the origin of cancer metastasis, which is of significant importance in developing treatment programs.[5,6] miRNAs can also be tissue specific and have been found circulating in peripheral blood, saliva, cerebrospinal fluid, urine, breast milk, and semen leading to the possibility for non-invasive biopsies.[7] Moreover, miRNA characterization in biological samples has been explored in forensic serology to identify biological fluids.[8,9] Body fluid identification in forensics is vital to provide context to a crime scene and develop a DNA profile. The application of miRNA in both clinical and forensic fields has great potential to improve patient care and resolve criminal cases. Therefore, miRNA sample preparation and detection methods need to rapidly detect low abundance sequences from complex matrices to ensure accurate and precise results.

miRNA detection is a significant challenge due to their small size and sequence homology. Conventional miRNA detection methods include northern blotting, microarrays, and polymerase chain reaction (PCR)-based amplification.[10] Although miRNA microarrays are ideal for multiplex analysis, northern blotting and microarrays often suffer from low sensitivity limiting their application for low abundance circulating miRNAs.[10,11] Due to the short length of miRNA, traditional reverse transcription (RT) PCR approaches cannot be performed as the unmodified primers are approximately the same length as miRNA, and the extension step cannot

occur. Therefore, PCR-based methods developed for miRNA require stem-loop DNA probes, locked nucleic acid-modified probes, doubly fluorescent labeled TaqMan probes, or poly-adenine tagged oligonucleotide probes to allow extension to occur.[10–12] However, these probes can be challenging to design and expensive to implement. These methods also require thermocycling, thereby limiting their use in point-of-care (POC) applications.

Exponential amplification reaction (EXPAR), an isothermal enzymatic amplification method, has been widely applied towards miRNA detection.[11,13,14] Femtomolar concentrations of miRNA are amplified in minutes using EXPAR, whereas traditional RT-PCR methods require 2-4 h.[13] During the reaction, the target molecule (called a trigger) anneals to an oligonucleotide, referred to as the template. The template contains two regions complementary to the target miRNA separated by a nicking enzyme recognition site. The polymerase extends the sequence after the miRNA anneals to the template. A nicking enzyme cleaves the newly synthesized strand and creates a new miRNA trigger to continue the exponential reaction. Despite having a rapid reaction rate, EXPAR suffers from substantial non-specific amplification originating from non-specific binding of the polymerase to the template.[15,16] Non-specific amplification can prevent the detection of low abundance miRNA targets. Studies have investigated the reduction of non-specific amplification using additives such as tetramethylammonium chloride, bovine serum albumin, single-stranded binding proteins, graphene oxide, and cobalt oxyhydroxide nanoflakes.[16–18] Although some additives reduce non-specific amplification, the rapid rate of background amplification is still a significant problem that limits the use of EXPAR for low abundance miRNAs.

Conventional miRNA purification methods include phenol-chloroform-based extractions and silica-based spin columns.[19–21] Traditional miRNA sample preparation techniques can take

20-60 min to perform, with phenol-chloroform extractions utilizing toxic chemicals to isolate nucleic acids. An ideal miRNA extraction method should not employ volatile chemicals and require minimal equipment to facilitated POC analysis in the field. Recent studies have also reported poor reproducibility between circulating miRNA studies [21–23], and this may be linked to low concentrations of miRNA (~10-300 fM) in samples.[24] Therefore, novel miRNA extraction methods should be explored to preconcentrate miRNAs and improve reproducibility.

Ionic liquids (ILs) and magnetic ionic liquids (MILs) have been reported to possess high DNA and RNA extraction efficiencies.[25–29] ILs are molten salts that exhibit several advantageous features such as negligible vapor pressure at ambient temperatures and tunable chemical structures that can provide different interactions with analytes.[30] MILs are a subclass of ILs that contain a paramagnetic component within their chemical structure, allowing them to respond to an external magnetic field while maintaining many of the physico-chemical properties of ILs.[31–34] ILs and MILs have been employed as PCR additives to decrease the melting temperature and amplify GC-rich DNA sequences [35] and reduce the background signal caused by primer-dimer formation.[36] Recent studies have employed hydrophobic MILs to extract DNA from blood, plasma, and artificial sputum, demonstrating their versatility in extracting nucleic acids from a wide array of matrices.[37–39] Nucleic acid-enriched MILs can also be integrated into custom-designed PCR buffers to desorb DNA from the solvent during the reaction without inhibition allowing for high throughput analysis.[40]

In this study, three hydrophobic MILs were investigated as miRNA extraction solvents and EXPAR additives. As an EXPAR additive, the three MILs significantly increased the amplification rate compared to reactions that did not contain MIL. Most notably, the addition of low volumes of MIL reduced non-specific amplification compared to reactions without MIL. miRNA was

116 successfully extracted from diluted plasma, artificial urine, and diluted artificial saliva by
117 dispersing the trihexyl(tetradecyl)phosphonium ($[P_{6,6,6,14}^+]$)
118 tris(hexafluoroacetylaceto)nickelate(II) ($[Ni(hfacac)_3^-]$), $[P_{6,6,6,14}^+]$
119 tris(hexafluoroacetylaceto)cobaltate(II) ($[Co(hfacac)_3^-]$), and $[P_{6,6,6,14}^+]$
120 tris(hexafluoroacetylaceto)manganate(II) ($[Mn(hfacac)_3^-]$) MILs in the sample. The miRNA-
121 enriched MIL was subsequently added to custom-designed EXPAR buffers for amplification. In
122 total, the extraction and detection of miRNA at femtomolar concentration levels could be achieved
123 within 10 min. Compared to the $[P_{6,6,6,14}^+][Ni(hfacac)_3^-]$ and $[P_{6,6,6,14}^+][Co(hfacac)_3^-]$ MILs, the
124 $[P_{6,6,6,14}^+][Mn(hfacac)_3^-]$ MIL was observed to poorly extract miRNA. These results suggest that
125 the $[P_{6,6,6,14}^+][Ni(hfacac)_3^-]$ and $[P_{6,6,6,14}^+][Co(hfacac)_3^-]$ MILs can be efficient miRNA extraction
126 solvents and excellent EXPAR additives due to the low amount of non-specific amplification
127 facilitated by hydrophobic interactions destabilizing interactions between the Bst polymerase and
128 template. Moreover, the reduction of non-specific amplification allows for the detection of miRNA
129 at attomolar concentration levels. In contrast, conventional EXPAR fails to discriminate a 10 fM
130 standard from the NTC. A commercial miRNA extraction method was found to preconcentrate
131 miRNA allowing the detection of a 10 fM let-7a sample but failed to discriminate the 1 fM sample
132 from the NTC.

133 ## 2. Materials and Methods

134 ### 2.1 Reagents and Materials

135 Ammonium hydroxide (28-30% solution in water), 1,1,1,5,5,5-hexafluoroacetylacetone
136 (99%), creatinine (99+%), manganese(II) chloride tetrahydrate (99+%), and nickel(II) chloride
137 (98%) were purchased from Acros Organics (Morris Plains, NJ, USA). Nuclease-free water was
138 purchased from Ambion (Carlsbad, CA, USA). Anhydrous diethyl ether (99.0%) was purchased

139 from Avantor Performance Materials Inc. (Center Valley, PA, USA). Deoxynucleotides (dNTPs)
140 (10 mM of deoxyribose adenosine triphosphate, deoxyribose cytosine triphosphate, deoxyribose
141 guanine triphosphate, and deoxyribose thymine triphosphate), Bst DNA polymerase, large
142 fragment (8,000 units·mL^{-1}), Nt.BstNBI nicking enzyme (10,000 units·mL^{-1}), 10x ThermoPol
143 reaction buffer, and 10x NEBuffer 3.1 were purchased from New England BioLabs (Ipswich, MA,
144 USA). Trihexyl(tetradecyl)phosphonium chloride (97.7%) was purchased from Strem Chemicals
145 (Newburyport, MA, USA). Ethylenediaminetetraacetic acid (EDTA) (99.4-100.06%), cobalt
146 chloride (97%), and lithium bis[(trifluoromethyl)sulfonyl]imide ([Li^+][NTf_2^-]) were purchased
147 from MilliporeSigma (St. Louis, MO, USA). Apheresis derived pooled human plasma (Na_2EDTA
148 anticoagulant) was obtained from Innovative Research (Novi, MI, USA). SYBR Green I (10,000x)
149 was purchased from Life Technologies (Carlsbad, CA, USA). The miRNA trigger and template
150 (sequences are shown in Table S1) were acquired from Integrated DNA Technologies (Coralville,
151 IA, USA). Optically clear PCR caps, tube strips, dimethylsulfoxide (DMSO) (≥99.7%), N,N-
152 dimethylformamide (DMF) (99.9%), potassium chloride (99.70%), potassium phosphate
153 monobasic (100%), sodium chloride (100.3%), and sodium phosphate dibasic (99.8%) were
154 acquired from Thermo Fisher Scientific (Waltham, MA, USA). Artificial saliva was purchased
155 from Pickering Laboratories (Mountain View, CA, USA). Tris-HCl was obtained from RPI
156 (Mount Prospect, IL, USA). Neodymium rod (0.66 T) and cylinder magnets (0.9 T) were
157 purchased from K&J Magnetics (Pipersville, PA, USA). The miRNeasy Serum/Plasma Advanced
158 kit was purchased from Qiagen (Hilden, Germany). Deionized water (18.2 MΩ cm), obtained from
159 a Milli-Q water purification system, was used to prepare all aqueous solutions (Millipore, Bedford,
160 MA, USA).

161 **2.2 Synthesis of MILs and ILs**

Chemical structures of the three MILs used in this study are shown in Figure 1. These hydrophobic MILs were investigated as miRNA extraction solvents since they are insoluble in aqueous samples such as plasma and urine and, therefore, will respond to a magnetic field.[32] The $[P_{6,6,6,14}^{+}][Ni(hfacac)_3^{-}]$, $[P_{6,6,6,14}^{+}][Co(hfacac)_3^{-}]$, and $[P_{6,6,6,14}^{+}][Mn(hfacac)_3^{-}]$ MILs were investigated as EXPAR additives since previous studies have illustrated that these MILs do not interfere with the amplification efficiency of qPCR assays using optimized reaction buffers.[37,40,41] All MILs [42] and the $[P_{6,6,6,14}^{+}][NTf_2^{-}]$ IL [38] were synthesized as previously reported. The MILs and ILs were stored in a desiccator at room temperature when not in use.

2.3 EXPAR Assays and Conditions

A Bio-Rad CFX96 Touch Real-time PCR (Hercules, CA, USA) was utilized for EXPAR amplification of the let-7a target. The threshold cycle (Cq) was determined using the fluorescence threshold provided by the Bio-Rad CFX Maestro software, and the threshold time was calculated from the threshold cycle. Standard reactions not containing MIL occurred at 55°C. Reactions containing the $[P_{6,6,6,14}^{+}][Ni(hfacac)_3^{-}]$ and $[P_{6,6,6,14}^{+}][Co(hfacac)_3^{-}]$ MILs, as well as the $[P_{6,6,6,14}^{+}][NTf_2^{-}]$ IL, occurred at 55°C. Reactions containing 0.3, 0.5, and 0.7 µL of the $[P_{6,6,6,14}^{+}][Mn(hfacac)_3^{-}]$ MIL occurred at 54°C, 53°C, and 52.6°C, respectively. All reactions occurred for 50 min, and an optical detection step was employed every 30 s to track the reaction in real-time.

The concentration of template, EDTA, dNTPs, Nt.BstNBI, and Bst were optimized to ensure rapid amplification while minimizing non-specific amplification. Amplification of standard reactions that did not contain MIL was achieved using the following assay conditions: 1x Thermopol reaction buffer, 3.1, 1x SYBR Green I, 0.5x NEBuffer 0.2 units·µL^{-1} Nt.BstNBI, 0.08 units·µL^{-1} Bst, 250 µM dNTPs, 100 nM template, and 1.2 mM EDTA. Reactions containing 0.3

or 0.5 µL of either the $[P_{6,6,6,14}^+][Ni(hfacac)_3^-]$ or $[P_{6,6,6,14}^+][Co(hfacac)_3^-]$ MILs required 1x Thermopol reaction buffer, 0.5x NEBuffer 3.1, 1x SYBR Green I, 0.2 units·µL^{-1} Nt.BstNBI, 0.08 units·µL^{-1} Bst, 250 µM dNTPs, 100 nM template, and 4 mM EDTA. Amplification with 0.7 µL of either the $[P_{6,6,6,14}^+][Ni(hfacac)_3^-]$ and $[P_{6,6,6,14}^+][Co(hfacac)_3^-]$ MIL in the EXPAR buffer required 1x Thermopol reaction buffer, 0.5x NEBuffer 3.1, 1x SYBR Green I, 0.2 units·µL^{-1} Nt.BstNBI, 0.08 units·µL^{-1} Bst, 250 µM dNTPs, 100 nM template, and 5 mM EDTA. EXPAR with 0.3, 0.5, or 0.7 µL of the $[P_{6,6,6,14}^+][Mn(hfacac)_3^-]$ MIL in the buffer required 1x Thermopol reaction buffer, 0.5x NEBuffer 3.1, 1x SYBR Green I, 0.2 units·µL^{-1} Nt.BstNBI, 0.08 units·µL^{-1} Bst, 250 µM dNTPs, 100 nM template, and 6 mM EDTA. Reactions containing 0.15-0.45 µL of the $[P_{6,6,6,14}^+][NTf_2^-]$ IL consisted of 1x Thermopol reaction buffer, 3.1, 1x SYBR Green I, 0.5x NEBuffer 0.2 units·µL^{-1} Nt.BstNBI, 0.08 units·µL^{-1} Bst, 250 µM dNTPs, 100 nM template, and 1.2 mM EDTA. All reactions were performed in triplicate.

2.4 miRNA Quantification using Qubit Detection

miRNA was desorbed from the MIL solvent into 10 µL of nuclease-free water at 55°C for 10 min. An aliquot (5 µL) of the aqueous buffer was added to the Qubit miRNA assay (ThermoFisher Scientific), and miRNA was quantified on a Qubit 2.0 fluorometer according to the manufacturer's instructions.

2.5 Capture of miRNA

The general procedure used to extract miRNA was modified from Emaus et al. and is shown in Figure 2.[38] Briefly, a 6 µL aliquot of MIL was dispersed in a 1.0 mL solution of 1 fM-100 pM miRNA and 2 mM Tris buffer (pH 8) for 1 min using a Barnstead/Thermolyne Type 16700 mixer (Dubuque, IA, USA). After recovering the hydrophobic MIL using a 0.7 T rod magnet, the MIL was washed with deionized water. A 0.3 µL aliquot of miRNA-enriched MIL

was placed in an EXPAR tube for amplification and fluorescence detection. For all extractions using human plasma, artificial urine, and artificial saliva, 10 fM of let-7a was spiked into the sample. Artificial urine was prepared as described in Mayrovitz et al (1.9820 g urea, 0.7013 g NaCl, 0.2218 g KH_2PO_4, 0.0568 g Na_2HPO_4, and 0.1697 g creatinine in a 100 mL volumetric flask). [43] All extractions were performed in triplicate.

Extractions with the miRNeasy Serum/Plasma Advanced kit were performed according to the manufacturer's specifications. Briefly, 60 µL of buffer RPL was added to a 200 µL sample and vortexed for 5 s. The sample was subsequently incubated at room temperature for 3 min before 20 µL of buffer RPP was added to the sample. The sample was mixed for 20 s using a vortex and allowed to sit at room temperature for 3 min to allow the precipitate to form. After centrifuging for 3 min, the supernatant was transferred to a new microcentrifuge tube, and 250 µL of isopropanol was added to the sample. The sample was briefly mixed and subsequently added to the RNeasy UCP MiniElute column. The flow-through was discarded after a 15 s centrifuge step. A 700 µL aliquot of buffer RWT was added to the spin column. The sample was centrifuged for 15 s, after which the flow-through was discarded. Prior to another 15 s centrifugation step, 500 µL of buffer RPE was added to the column. The flow-through was discarded again, and 500 µL of 80% ethanol (v/v) was added to the column. The sample was centrifuged for 2 min, and the flow-through was discarded. The spin column was centrifuged for an additional 5 min to dry the sample and ensure that the wash buffers were removed. The spin column was placed in a fresh collection tube, and 20 µL of RNase-free water was added to the spin column. The spin column was incubated for 1 min prior to a 1 min centrifugation step to elute the miRNA.

3. Results and Discussion

3.1 Optimization of EXPAR Assay with MIL

Using EXPAR conditions for standard reactions, introduction of the $[P_{6,6,6,14}^+][Ni(hfacac)_3^-]$, $[P_{6,6,6,14}^+][Co(hfacac)_3^-]$, $[P_{6,6,6,14}^+][Mn(hfacac)_3^-]$ MILs to the buffer was observed to inhibit the reaction with 10 pM let-7a spiked into the buffer. Therefore, the reaction's EDTA concentration was optimized since EDTA is known to chelate Ni^{2+}, Co^{2+}, and Mn^{2+} ions and has been successfully applied to relieve inhibition caused by the MILs.[27,40] The optimal concentration of EDTA was determined to be the concentration at which non-specific amplification and threshold times were minimized. At concentrations below the optimum EDTA concentration, the reaction was inhibited and elevated concentrations of EDTA increased the threshold time due to EDTA chelating the magnesium cofactor. Ultimately, 4.0 mM EDTA was required to integrate 0.3 or 0.5 µL of either the $[P_{6,6,6,14}^+][Ni(hfacac)_3^-]$ and $[P_{6,6,6,14}^+][Co(hfacac)_3^-]$ MIL into the EXPAR buffer. Increasing the volume of MIL to either 0.7 µL of $[P_{6,6,6,14}^+][Ni(hfacac)_3^-]$, 0.9 µL of $[P_{6,6,6,14}^+][Ni(hfacac)_3^-]$, or 0.7 µL of $[P_{6,6,6,14}^+][Co(hfacac)_3^-]$ required 5.0 mM EDTA for uninhibited amplification. EXPAR threshold times in reactions containing 10 aM-100 pM of miRNA with different volumes of $[P_{6,6,6,14}^+][Ni(hfacac)_3^-]$ and $[P_{6,6,6,14}^+][Co(hfacac)_3^-]$ MIL are shown in Figure 3a and 3b, respectively. Attempts to integrate 0.9 µL of $[P_{6,6,6,14}^+][Co(hfacac)_3^-]$ MIL into the EXPAR buffer were unsuccessful as the 1 pM let-7a standard was indistinguishable from the NTC (see Figure S1).

EXPAR with 0.3 µL of $[P_{6,6,6,14}^+][Mn(hfacac)_3^-]$ MIL and 1.2-8.0 mM EDTA in the reaction buffer resulted in a low fluorescence signal when the reaction temperature was maintained at 55°C (see Figure S2). Previous studies have shown that MILs can decrease the melting temperature of short DNA oligonucleotides due to hydrophobic interactions from solubilized MIL and DNA[38], so it was hypothesized that the solubilized Mn-based MIL prevented the miRNA trigger from annealing to the template at 55°C. Generally, EXPAR is performed at 55°C, which is

254 the manufacturer's recommended temperature for the Nt.BstNBI nicking enzyme. The melting
255 temperature of the miRNA trigger to the template is designed to be near the reaction temperature
256 to allow for effective hybridization and easy dissociation after the extension and nicking step.[13]
257 With MIL decreasing the melting temperature of the trigger to the template, the target miRNA
258 may not effectively hybridize to the template preventing the reaction from proceeding. Decreasing
259 the reaction temperature to 54°C permitted amplification with 0.3 µL of $[P_{6,6,6,14}^{+}][Mn(hfacac)_3^{-}]$
260 MIL and 6 mM EDTA in the EXPAR buffer. Increasing the volume of $[P_{6,6,6,14}^{+}][Mn(hfacac)_3^{-}]$
261 MIL in the EXPAR buffer to 0.5 µL still required 6 mM EDTA, but the reaction temperature had
262 to be reduced to 53°C to allow the reaction to proceed. Reactions containing 0.7 µL of
263 $[P_{6,6,6,14}^{+}][Mn(hfacac)_3^{-}]$ MIL still required 6 mM EDTA, but the reaction temperature had to be
264 further reduced to 52.6°C. Increasing the amount of $[P_{6,6,6,14}^{+}][Ni(hfacac)_3^{-}]$ and
265 $[P_{6,6,6,14}^{+}][Co(hfacac)_3^{-}]$ MILs in the reaction decreased the threshold cycle and increased the
266 reaction rate. However, the threshold cycle of the reactions containing 0.7 µL of
267 $[P_{6,6,6,14}^{+}][Mn(hfacac)_3^{-}]$ MIL was higher than reactions containing 0.5 µL of MIL. This may be
268 due to the lower reaction temperature required for amplification with 0.7 µL of
269 $[P_{6,6,6,14}^{+}][Mn(hfacac)_3^{-}]$ MIL in the EXPAR buffer as the activity of the Bst polymerase decreases
270 at reaction temperatures below 65°C.[13,14] Attempts to incorporate 0.9 µL of the
271 $[P_{6,6,6,14}^{+}][Mn(hfacac)_3^{-}]$ MIL required a reaction temperature of 51°C to amplify a 10 pM standard,
272 but the amplification of trigger at low concentrations (100 fM) was unsuccessful (see Figure S3).
273 Failure to amplify low concentrations of miRNA was likely due to the low activity of the Bst
274 polymerase at 50°C (i.e., 35-40%, according to the manufacturer). A summary of amplification
275 conditions used for EXPAR in this study is shown in Table 1.

276 **3.2 MILs as EXPAR Additives**

The addition of MIL to the EXPAR buffer spiked with miRNA had the following significant effects: (1) an increase in the reaction rate (Figure 3) and (2) reduction in non-specific amplification, as shown in Figure 4. Increasing the amount of $[P_{6,6,6,14}^{+}][Ni(hfacac)_3^{-}]$ or $[P_{6,6,6,14}^{+}][Co(hfacac)_3^{-}]$ MIL in the buffer decreased the threshold time significantly compared to standard reactions without MIL. However, the increased reaction rate was not accompanied by an increase in non-specific amplification. A similar effect was previously observed with loop-mediated isothermal amplification (LAMP) and isothermal multiple-self-matching-initiated amplification (IMSA). [36] It was hypothesized that the MILs reduce the background signal by limiting primer-dimer formation. However, non-specific amplification during the early stages of EXPAR is linked to non-specific interactions between the single-stranded template and polymerase.[15,16] Therefore, solubilized MIL may destabilize unprimed interactions between the template and polymerase.

The increased reaction rates and low amount of non-specific amplification were also noted when 0.15-0.45 μL of $[P_{6,6,6,14}^{+}][NTf_2^{-}]$ IL was spiked into the EXPAR buffer, as shown in Figure S4. The background signal was higher with a larger volume of IL added to the buffer. It was previously hypothesized that the solubilized MILs and ILs interact with DNA in a similar fashion to cationic surfactants.[38] At low concentrations, a cationic surfactant experiences hydrophobic interactions with DNA and destabilizes the duplex, whereas concentrations above the surfactant's critical micelle concentration increase the melting temperature of DNA due to electrostatic interactions.[44] The low background associated with adding a hydrophobic IL or MIL to EXPAR suggests that the cation of the IL and MIL is responsible for preventing unprimed interactions between the template and polymerase. Attempts to investigate the effect of adding divalent

transition metal salts (i.e., 4-5 mM $NiCl_2$, 4-5 mM $CoCl_2$, and 6 mM $MnCl_2$) were unsuccessful with and without an equimolar amount of EDTA.

Other EXPAR additives including EDTA, DMSO, and DMF were compared to the $[P_{6,6,6,14}^+][Ni(hfacac)_3^-]$, $[P_{6,6,6,14}^+][Co(hfacac)_3^-]$, and $[P_{6,6,6,14}^+][Mn(hfacac)_3^-]$ MIL additives. The addition of EDTA (1.2-4.0 mM) to the reaction increased the threshold cycle of a 10 pM standard and non-specific amplification as the reaction was slower due to chelation of the Mg^{2+} cofactor (see Figure S5). This suggests that the addition of EDTA to the MIL-EXPAR does not increase the reaction rate, suggesting that EDTA only relieves inhibition by chelating the transition metal component of solubilized MIL. DMSO decreases the melting temperature of DNA by interfering with the hydrogen bonds between complementary sequences.[45] However, adding 5% DMSO to the reaction had little effect on the reaction rate or the amount of non-specific amplification (see Figure S6a), similar to the results of Mok et al.[16] DMF is known to decrease the stability of the DNA duplex like DMSO, ILs, and MILs. However, DMF denatures DNA through hydrophobic interactions.[46,47] When 5% DMF was added to the EXPAR buffer, the background caused by non-specific amplification was not present, similar to the effect caused when the hydrophobic $[P_{6,6,6,14}^+][NTf_2^-]$ IL, $[P_{6,6,6,14}^+][Ni(hfacac)_3^-]$ MIL, $[P_{6,6,6,14}^+][Co(hfacac)_3^-]$ MIL, and $[P_{6,6,6,14}^+][Mn(hfacac)_3^-]$ MIL are used (see Figure S6b).

Sequence homology is a significant challenge to miRNA detection. Therefore, 100 fM let-7c or let-7i DNA was spiked into the EXPAR buffer with a template complementary to let-7a, as shown in Figure S7. Reactions with let-7c and let-7i resulted in an increased threshold time compared to reactions with the complementary let-7a sequence at the optimized reaction temperatures. The increase in threshold time is related to poor hybridization between the let-7c (2 mismatches compared to let-7a) or let-7i (4 mismatches compared to let-7a) sequences and

template since they are not perfectly complementary. With 0.3-0.9 µL of $[P_{6,6,6,14}^+][Ni(hfacac)_3^-]$ MIL or 0.3-0.5 µL of $[P_{6,6,6,14}^+][Mn(hfacac)_3^-]$ MIL in the reaction buffer, the threshold times associated with let-7c and let-7i were not significantly different compared to standard reactions that did not contain MIL. However, there is a longer delay between the threshold time associated with let-7a and the mismatches when the $[P_{6,6,6,14}^+][Ni(hfacac)_3^-]$ or $[P_{6,6,6,14}^+][Mn(hfacac)_3^-]$ MIL were added to the buffer compared to standard reactions. The difference in the threshold times between reactions with the complementary let-7a and mismatch sequence with 0.3-0.7 µL of $[P_{6,6,6,14}^+][Co(hfacac)_3^-]$ MIL in the reaction was less than standard reactions without MIL. Interestingly, reactions with 0.7 µL of $[P_{6,6,6,14}^+][Mn(hfacac)_3^-]$ MIL did not proceed with let-7c or let-7i spiked into the reaction buffer, suggesting that adding Mn(II)-based MILs to an EXPAR buffer would be ideal for the discriminating between miRNA sequences without the need for a secondary, specific detection method such as a lateral flow or modified gold nanoparticles.[48,49] Reactions containing 0.7 µL of $[P_{6,6,6,14}^+][Mn(hfacac)_3^-]$ MIL required the lowest reaction temperature (i.e., 52.6°C) in this study to successfully proceed due to hydrophobic interactions that destabilize interactions between the trigger and template. This destabilization is more significant with a non-complementary trigger and could explain why reactions with 0.7 µL of $[P_{6,6,6,14}^+][Mn(hfacac)_3^-]$ MIL failed to amplify with the non-complementary let-7c and let-7i sequences.

3.3 MILs as miRNA Extraction Solvents

Qubit detection was used to quantify the amount of miRNA extracted by the $[P_{6,6,6,14}^+][Ni(hfacac)_3^-]$ MIL, $[P_{6,6,6,14}^+][Co(hfacac)_3^-]$ MIL, $[P_{6,6,6,14}^+][Mn(hfacac)_3^-]$ MIL, and miRNeasy extraction kit. As shown in Figure S8, the spin column kit extracted more miRNA compared to the three MILs with the $[P_{6,6,6,14}^+][Mn(hfacac)_3^-]$ MIL extracting the least miRNA.

Previous studies have not investigated the ability of Mn(II)-based MILs to extract RNA. However, Mn(II)-based MILs have been shown to poorly extract long and short DNA sequences relative to other transition and lanthanide metal-based MILs.[40,41,50] Spectroscopic studies have shown that nickel(II) and cobalt(II) prefer to interact with the DNA phosphate backbone compared to manganese(II), which interacts more with nucleic acid bases and can destabilize the DNA duplex.[51] The various interactions between DNA and a divalent transition metal may allude to the different extraction behaviors demonstrated by the three hexafluoroacetonate-based MILs.

Extractions of 1 fM-100 pM miRNA trigger were performed with the $[P_{6,6,6,14}^+][Ni(hfacac)_3^-]$, $[P_{6,6,6,14}^+][Co(hfacac)_3^-]$, and $[P_{6,6,6,14}^+][Mn(hfacac)_3^-]$ MILs and miRNA was observed to be successfully desorbed from the MIL during EXPAR, as shown in Figure 5. However, the threshold times associated with the extraction of 10 and 100 pM let-7a were within error when 0.5-0.9 µL of $[P_{6,6,6,14}^+][Ni(hfacac)_3^-]$ and 0.5-0.7 µL of $[P_{6,6,6,14}^+][Co(hfacac)_3^-]$ MIL were added to the reaction buffer. Standard reactions of 10 and 100 pM let-7a with 0.5-0.9 µL of $[P_{6,6,6,14}^+][Ni(hfacac)_3^-]$ or 0.5-0.7 µL $[P_{6,6,6,14}^+][Co(hfacac)_3^-]$ MIL had threshold times less than 4 min. However, threshold times associated with the extraction of 10 and 100 pM let-7a when using 0.5-0.9 µL of either the $[P_{6,6,6,14}^+][Ni(hfacac)_3^-]$ or $[P_{6,6,6,14}^+][Co(hfacac)_3^-]$ MIL were approximately 3 min. This suggests that it takes approximately 3 minutes for miRNA to desorb from the MIL and initiate the reaction.

The expression of miRNAs in biological fluids is a rising interest in the forensic and medical fields. Therefore, the dispersive MIL extraction method was applied to extract 10 fM of let-7a from plasma, artificial urine, and artificial saliva samples. To achieve reproducible extraction of miRNA from plasma and artificial saliva matrices, the sample was diluted 2-fold (50% complex matrix, 50% 2 mM Tris buffer). As shown in Figure 6, miRNA could be extracted

368 from all three matrices. It was noted that the addition of 0.7-0.9 µL of MIL dispersed in spiked
369 plasma, urine, or saliva samples led to higher threshold times compared to when 0.5 µL of MIL
370 was added to the buffer. Previously, adding 0.7 or 0.9 µL of MIL further decreased the threshold
371 cycle. This suggests that adding more MIL to the reaction buffer while increasing the reaction rate
372 also introduces more inhibitors to the reaction. Therefore, volumes less than 0.5 µL of MIL should
373 be added to the reaction to ensure that miRNA can be efficiently desorbed and also limit the
374 amount of inhibitors introduced to the reaction.

375 The MIL-based extractions were benchmarked against a commercial approach using the
376 miRNeasy Serum/Plasma Advanced kit. As shown in Figure 7, introducing a spin column-based
377 extraction allowed for the detection of the 10 fM sample. Previously, the threshold times associated
378 with 10 fM standards could not be discriminated from the NTC. Extractions with the spin columns
379 also were able to discriminate between the 10 and 100 pM samples. However, threshold times
380 associated with the extraction of a 1 fM let-7a sample with the spin column could not be
381 discriminated from the NTC due to non-specific amplification. In comparison, the MIL-based
382 extraction method and custom-designed EXPAR assays allowed for the detection of the 1 fM
383 sample. Lower threshold times were achieved using the $[P_{6,6,6,14}^{+}][Ni(hfacac)_3^{-}]$ and
384 $[P_{6,6,6,14}^{+}][Co(hfacac)_3^{-}]$ MILs as extraction solvents compared to the spin column approach. In
385 addition, the commercial kit takes approximately 20 min to isolate miRNA, whereas the MILs
386 required only 1 min. This suggests that using MILs as a miRNA extraction solvent and directly
387 integrating the miRNA-enriched MIL into the EXPAR buffer is a potential alternative for miRNA
388 sample preparation for point of care applications as less than 15 min is required to extract and
389 detect low concentrations of miRNA with the MIL-based method while conventional miRNA
390 extraction methods require significantly more time and resources.

4. Conclusions

In this study, the $[P_{6,6,6,14}^{+}][Ni(hfacac)_3^{-}]$, $[P_{6,6,6,14}^{+}][Co(hfacac)_3^{-}]$, and $[P_{6,6,6,14}^{+}][Mn(hfacac)_3^{-}]$ MILs were investigated as miRNA extraction solvents and EXPAR additives. It was observed that all three MILs increased the reaction rate and decreased the amount of background signal stemming from non-specific amplification. The addition of MILs to the EXPAR buffer allowed the detection of 10 aM let-7a. In contrast, standard reactions without MIL in the buffer could not discriminate 10 fM let-7a from the NTC due to non-specific amplification. The three MILs were observed to successfully extract miRNA from complex matrices such as plasma, saliva, and urine. However, the $[P_{6,6,6,14}^{+}][Mn(hfacac)_3^{-}]$ MIL was observed to poorly extract miRNA compared to the $[P_{6,6,6,14}^{+}][Ni(hfacac)_3^{-}]$ and $[P_{6,6,6,14}^{+}][Co(hfacac)_3^{-}]$ MILs. Employing MILs as an extraction solvent and subsequently integrating them into a custom-designed EXPAR buffer allows for the extraction, amplification, and detection of attomolar concentrations of miRNA in less than 20 min. In contrast, the overall sample preparation time required for a commercial spin column is approximately 20 min. The MILs also outperformed the commercial miRNA extraction kit at isolating low concentrations of miRNA in the detection of 1 fM let-7a from Tris buffer. As EXPAR additives, the hydrophobic MIL improves the limit of detection 1000-fold compared to traditional EXPAR, permitting the detection of low abundance miRNA. Further studies involving MILs should be performed to evaluate their performance in clinical samples.

Acknowledgments

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575 Table 1: Summary of the optimized conditions used for incorporating MILs and ILs into the
576 EXPAR buffer to achieve successful amplification with minimal non-specific amplification.

MIL or IL Added	Volume of MIL/IL (µL)	EDTA Concentration (mM)	Reaction Temperature (°C)	Detection Range	Sample Preparation Time (min)
None	0	1.2	55.0	100 pM-10 fM	N/A
$[P_{6,6,6,14}^+][Ni(hfacac)_3^-]$	0.3	4.0	55.0	100 pM-10 aM	1
$[P_{6,6,6,14}^+][Ni(hfacac)_3^-]$	0.5	4.0	55.0	100 pM-10 aM	1
$[P_{6,6,6,14}^+][Ni(hfacac)_3^-]$	0.7	5.0	55.0	100 pM-10 aM	1
$[P_{6,6,6,14}^+][Ni(hfacac)_3^-]$	0.9	5.0	55.0	100 pM-10 aM	1
$[P_{6,6,6,14}^+][Co(hfacac)_3^-]$	0.3	4.0	55.0	100 pM-10 aM	1
$[P_{6,6,6,14}^+][Co(hfacac)_3^-]$	0.5	4.0	55.0	100 pM-10 aM	1
$[P_{6,6,6,14}^+][Co(hfacac)_3^-]$	0.7	5.0	55.0	100 pM-10 aM	1
$[P_{6,6,6,14}^+][Mn(hfacac)_3^-]$	0.3	6.0	54.0	10 pM-10 aM	1
$[P_{6,6,6,14}^+][Mn(hfacac)_3^-]$	0.5	6.0	53.0	10 pM-10 aM	1
$[P_{6,6,6,14}^+][Mn(hfacac)_3^-]$	0.7	6.0	52.6	10 pM-10 aM	1
$[P_{6,6,6,14}^+][NTf_2^-]$	0.15	1.2	55.0	1 pM	N/A
$[P_{6,6,6,14}^+][NTf_2^-]$	0.25	1.2	55.0	1 pM	N/A
$[P_{6,6,6,14}^+][NTf_2^-]$	0.35	1.2	55.0	1 pM	N/A
$[P_{6,6,6,14}^+][NTf_2^-]$	0.45	1.2	55.0	1 pM	N/A

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578 N/A: Solvent was not used for miRNA extractions and was only spiked into the reaction.

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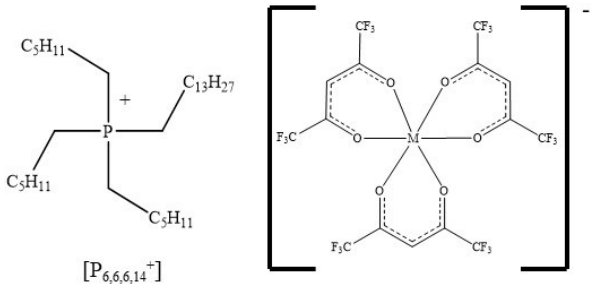

584

585 Figure 1: Chemical structures of the MILs investigated as miRNA extraction solvents and EXPAR

586 additives.

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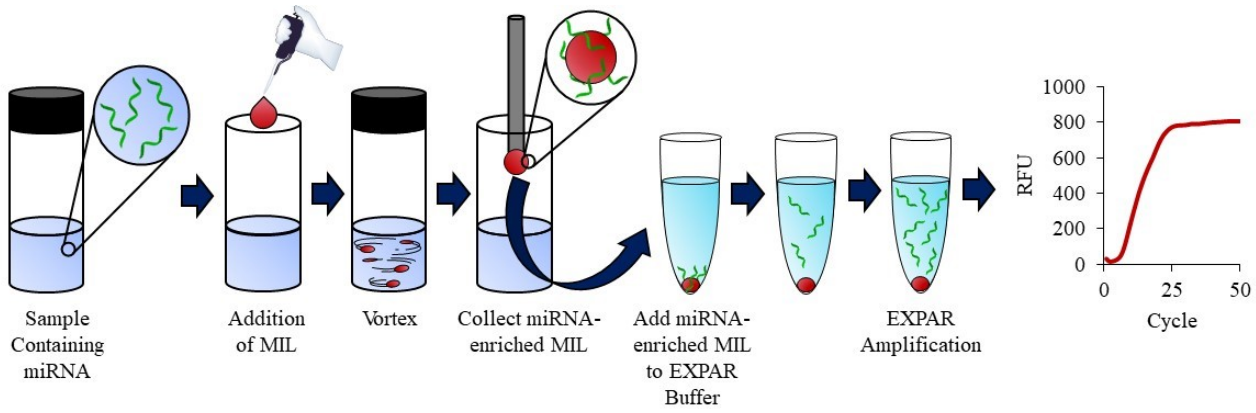
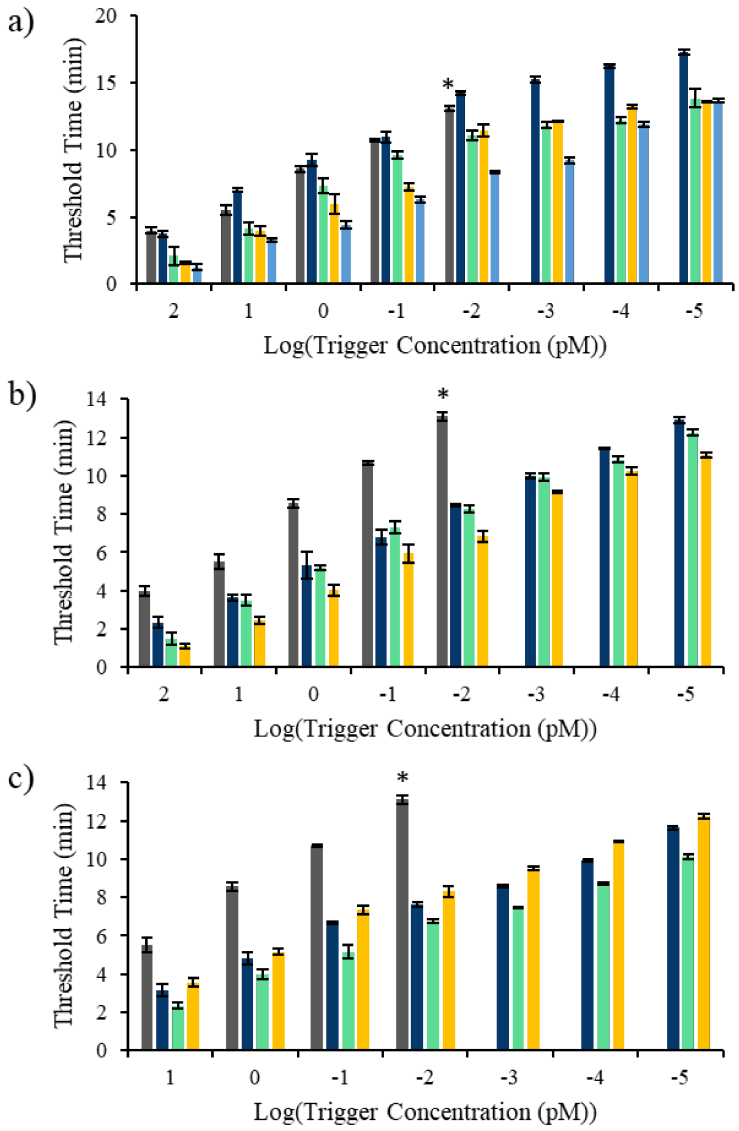


Figure 2: General extraction procedure used to extract miRNA using MILs. miRNA-enriched MIL was added directly to the EXPAR buffer to achieve amplification.



611

612 Figure 3: EXPAR amplification with (blue) 0.3 µL, (grey) 0.5 µL, (yellow) 0.7 µL, and (light blue)

613 0.9 µL of a) $[P_{6,6,6,14}^{+}][Ni(hfacac)_3^{-}]$, b) $[P_{6,6,6,14}^{+}][Co(hfacac)_3^{-}]$, and c) $[P_{6,6,6,14}^{+}][Mn(hfacac)_3^{-}]$

614 MIL in the reaction buffer compared to (orange) reactions that do not contain MIL. *Cq value was

615 indistinguishable from the NTC (n = 3).

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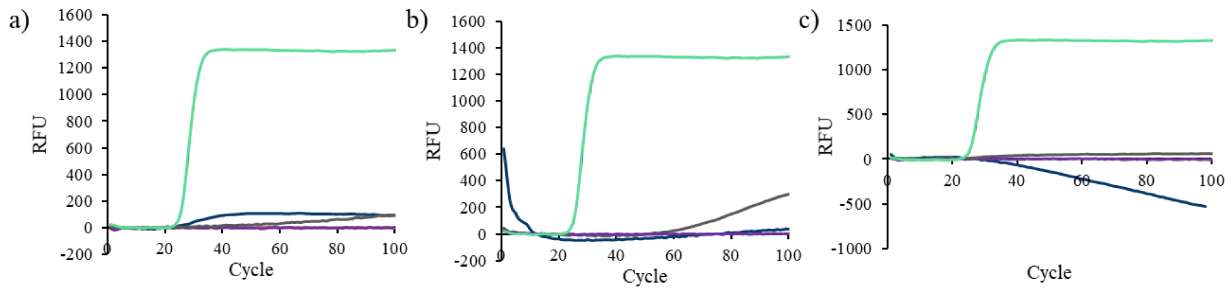
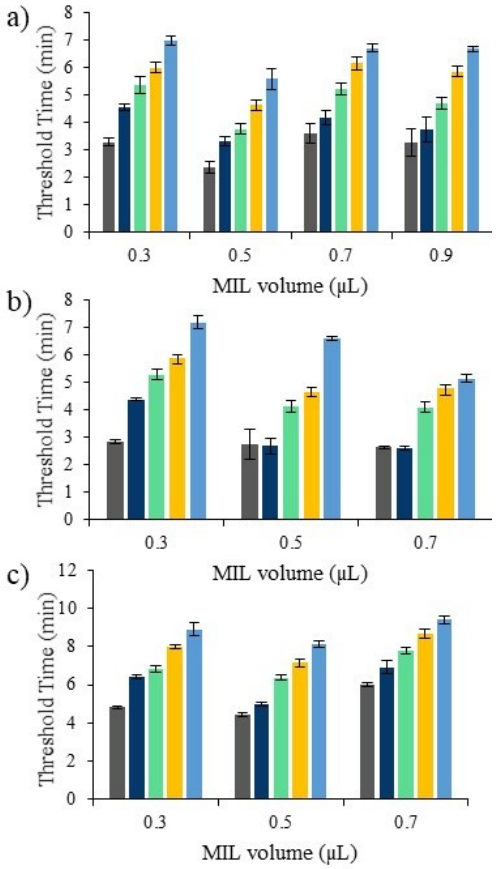


Figure 4: Non-specific amplification generated by EXPAR with (green) 0 µL, (navy) 0.3 µL, (violet) 0.5 µL, (grey) 0.7 µL, and (orange) 0.9 µL using the a) $[P_{6,6,6,14}^{+}][Ni(hfacac)_3^{-}]$, b) $[P_{6,6,6,14}^{+}][Co(hfacac)_3^{-}]$, and c) $[P_{6,6,6,14}^{+}][Mn(hfacac)_3^{-}]$ MILs.



633

634 Figure 5: Threshold times obtained using EXPAR associated with the extraction of (grey) 1 fM,

635 (navy) 10 fM, (green) 100 fM, (yellow) 10 pM, and (light blue) 100 pM of miRNA using the a)

636 $[P_{6,6,6,14}^{+}][Ni(hfacac)_3^{-}]$, b) $[P_{6,6,6,14}^{+}][Co(hfacac)_3^{-}]$, and c) $[P_{6,6,6,14}^{+}][Mn(hfacac)_3^{-}]$ MILs (n = 3).

637 Sample volume: 1.0 mL; volume of MIL dispersed: 6 µL; dispersion time: 1 min.

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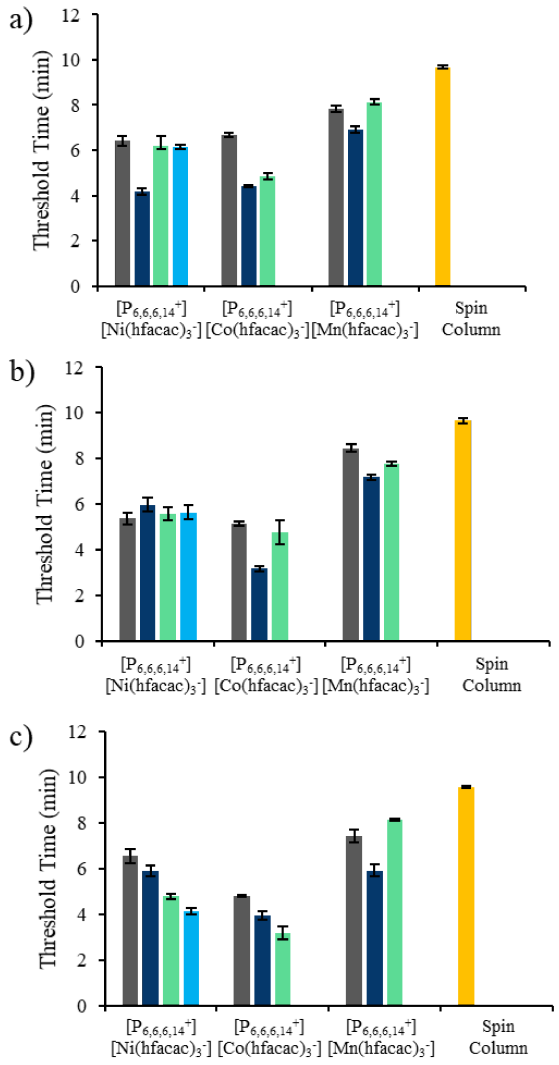

641

642 Figure 6: Threshold times associated with the extraction of 10 fM let-7a from a) 2-fold diluted
643 human plasma, b) 2-fold diluted artificial saliva, and c) artificial urine with (grey) 0.3 µL, (navy)
644 0.5 µL, (green) 0.7 µL, and (light blue) 0.9 µL of miRNA-enriched MIL added to the buffer
645 compared to the miRNeasy spin column kit approach (n = 3). Sample volume: 1.0 mL; volume of
646 MIL dispersed: 6 µL; dispersion time: 1 min.

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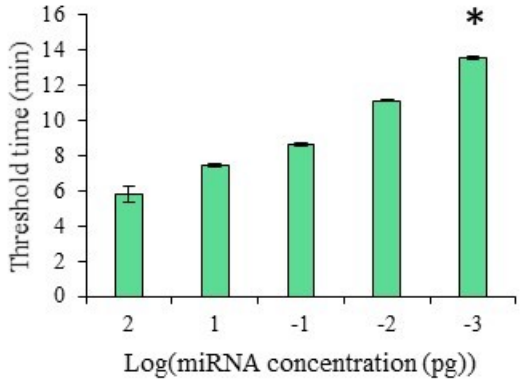
648



649

650 Figure 7: Threshold times associated with the extraction of 1 fM-100 pM let-7a using the

651 commercial miRNeasy Serum/Plasma Advanced Kit (n = 3). *Threshold times were within error

652 of the NTC.