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Sequence-specific preconcentration of a mutation prone *KRAS* fragment from plasma using ion-tagged oligonucleotides coupled to qPCR compatible magnetic ionic liquid solvents

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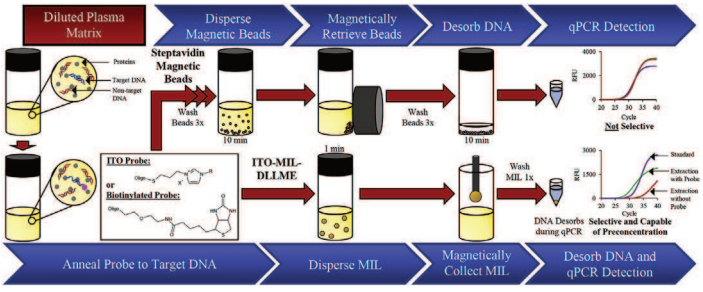
HIGHLIGHTS

- Ion-tagged olignonucleotides (ITO) were synthesized to anneal to KRAS fragment.
- Magnetic ionic liquids (MIL) were developed to exhibit high ITO loading efficiency.
- Preconcentrated DNA was analyzed by directly adding MIL to qPCR.
- qPCR amplification efficiency was not affected extracting from diluted-plasma.
- ITO-MIL approach outperformed steptavidin magnetic beads from diluted plasma.

GRAPHICAL ABSTRACT



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ABSTRACT

Circulating tumor DNA (ctDNA) is a source of mutant DNA found in plasma and holds great promise in guiding cancer diagnostics, prognostics, and treatment. However, ctDNA fragments are challenging to detect in plasma due to their low abundance compared to wild-type DNA. In this study, a series of ion-tagged oligonucleotides (ITO) were synthesized using thiol-ene click chemistry and designed to selectively anneal target DNA. The ITO-DNA duplex was subsequently captured using a hydrophobic magnetic ionic liquid (MIL) as a liquid support. Extracted target DNA was quantified by adding the DNA-enriched MIL to the quantitative polymerase chain reaction (qPCR) buffer to streamline the extraction procedure. Clinically relevant concentrations of the mutation prone *KRAS* fragment, which has been linked to colorectal, lung, and bladder cancer, were preconcentrated using the ITO-MIL strategy allowing for enrichment factors as high as 19.49 ± 1.44 from pure water and 4.02 ± 0.50 from 10-fold diluted plasma after a 1 min extraction. Preconcentration could only be achieved when adding the ITO probe to the sample validating the selectivity of the ITO in the capture process. In addition, the amplification efficiency of qPCR was not affected when performing extractions from a diluted-plasma matrix demonstrating that the ITO-MIL approach coupled to direct-qPCR can be used to quantitate DNA from complex matrices. In comparison, commercially available steptavidin-coated magnetic beads were observed to lose selectivity when performing extractions from a 10-fold diluted plasma matrix. The selectivity of the ITO-MIL method, coupled with the ability to rapidly preconcentrate clinically relevant concentrations of target DNA from 10-fold diluted plasma, suggests that this method has the potential to be applied towards the extraction of ctDNA fragments from clinical samples.

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1. Introduction

Detection of low levels of circulating tumor DNA (ctDNA) holds great promise in guiding cancer diagnostics, prognosis, and treatment [1,2]. ctDNA is believed to originate from tumor cells that have undergone apoptosis or necrosis resulting in the release of tumor DNA into the bloodstream [3]. However, there is a low abundance of ctDNA in plasma, especially in the early stages of cancer (i.e., less than 0.01%), and the presence of high levels of wild-type DNA can increase the probability of false-positive results due to primer mishybridization or mask present ctDNA [4]. In addition, ctDNA sequences such as *KRAS* and *EGFR* are prone to single nucleotide polymorphisms (SNPs) and differentiation of mutant ctDNA fragments from wild-type DNA is imperative to guiding cancer treatment [2,5,6]. Therefore, to distinguish SNPs from wild-type DNA, a sequence-specific DNA extraction method is often required to preconcentrate target ctDNA fragments.

There are several approaches to achieve sequence-specific ctDNA extraction and preconcentration. Synchronous coefficient of drag alteration (SCODA) is a novel microfluidic method for ctDNA preconcentration and analysis [7]. SCODA uses an oligonucleotide-functionalized electrophoresis gel to preconcentrate DNA sequences when exposed to a rotating electric field. Although SCODA is successful at preconcentrating low concentrations of ctDNA mutations with limits of detection of 0.001% mutation abundance without PCR amplification, the technique is expensive and has limited sample throughput [8]. Commercially-available streptavidin magnetic beads have been widely used in the extraction of ctDNA [3,9]. In this approach, target DNA anneals to a biotinylated DNA probe followed by the probe-target complex binding to streptavidin coated magnetic beads, which are collected using an external magnet. Differential strand separation at critical temperature (DISSECT) is another magnetic bead-based extraction procedure that utilizes a dual-biotinylated probe conjugated to streptavidin-coated magnetic beads to extract both wild-type and mutant ctDNA fragments [10]. With DISSECT, the desorption temperature is controlled so that only the mutant DNA desorbs from the magnetic beads while wild-type DNA remains hybridized to the probe. DISSECT is highly sensitive and can detect 1 mutant allele in the presence of 10,000 wild-type fragments. However, magnetic beads are prone to aggregation and sedimentation, which decreases the amount of DNA extracted [11,12]. The ideal sequence-specific DNA extraction method should rapidly and selectively isolate SNPs from complex media. However, current methods such as SCODA and DISSECT suffer from high economic costs, sedimentation, and time-consuming purifications steps. Therefore, alternative extraction procedures should be investigated to improve sample throughput, selectivity, and detection limits.

An attractive alternative to traditional bead-based approaches is the use of paramagnetic liquid extraction solvents such as magnetic ionic liquids (MILs). MILs are a subclass of ionic liquids (ILs) that contain a paramagnetic component in either the cation or anion. MILs exhibit a number of interesting properties including negligible vapor pressure at room temperature, high thermal stability, magnetic susceptibility, and tunable physiochemical properties [13–15]. These properties enable the use of MILs as extraction solvents in a wide range of applications, including the sequence-specific extraction of DNA [16,17]. In particular, strategies that couple ion-tagged oligonucleotide (ITO) probes and MILs represent an economical, particle-free alternative towards sequence-specific DNA extraction with low background DNA co-extraction and high extraction efficiencies. ITOs are designed to form a duplex with a single-stranded DNA target and can be captured by the MIL solvent through various interactions, such as hydrophobic interactions. ITOs are typically synthesized via the thiol-ene click reaction between a 3' thiol-modified oligonucleotide and an allylimidazolium IL.

Quantitative polymerase chain reaction (qPCR) is an important tool in DNA analysis capable of rapidly amplifying and quantifying small amounts of DNA. The reaction can be monitored in real-time using fluorescent probes such as SYBR Green or FAM, eliminating the lengthy electrophoretic separation step required in end-point PCR. However, qPCR is highly susceptible to inhibition and requires an initial DNA purification step in order to obtain accurate quantification [18]. Current sequence-specific DNA extraction procedures often require a time-consuming extraction step (i.e., up to 60 min) [19]. Extraction times can often be dramatically reduced by employing dispersive liquid-liquid microextraction (DLLME) as opposed to static extraction methods. DLLME involves dispersing the extraction solvent into small droplets which significantly increases the surface area of the extraction phase. DLLME methods can take as little time as 1 min, and the high agitation rate prevents sedimentation of the extraction solvent [20–22]. MILs have recently been employed as DLLME solvents in order to circumvent the numerous centrifugation steps required to sediment traditionally employed organic solvents [23]. The paramagnetic properties of MILs can be exploited to rapidly collect droplets using a magnet resulting in more efficient extraction procedures.

One major bottleneck in DNA extraction procedures is the recovery step. Commercial magnetic beads recommend a 10 min thermal desorption step which ultimately reduces sample throughput. One approach to overcoming this bottleneck is introducing the extraction phase to the qPCR buffer and using elevated temperatures of qPCR to desorb target DNA to the reaction. However, introducing magnetic beads or chitosan microparticles to the reaction buffer decreases the amplification efficiency making quantification unreliable [24]. It has been shown that MILs can be directly added to PCR by designing a buffer capable of relieving any inhibition caused by the MIL with minimal effect on the amplification efficiency [25]. The use of direct-MIL-qPCR streamlines the extraction procedure by removing time-consuming DNA recovery steps.

Although previously reported ITO-MIL approaches have provided a selective DNA extraction method capable of extracting large amounts of DNA from cell lysate compared to commercially available magnetic bead-based methods, the ITO-MIL strategy has only been applied to high concentrations of target DNA (282 pM) and required lengthy extraction and desorption steps [16,26]. To overcome the aforementioned disadvantages of previously reported ITO-MIL methods, a dispersive extraction method was developed to rapidly preconcentrate low concentrations (3.3 fM) of the mutation prone *KRAS* oncogene fragment from plasma. DNA-enriched MIL was subsequently incorporated into the qPCR buffer to further increase sample throughput. The extraction of albumin and DNA by three hydrophobic, manganese(II)-based MILs consisting of trihexyl(tetradecyl)phosphonium ($[P^{+}_{6,6,6,14}]$) tris(hexafluoroacetylaceto) manganate(II) ($[Mn(hfacac)^{-}_{3}]$), trioctylbenzylammonium ($[N^{+}_{8,8,8,Bz}]$) $[Mn(hfacac)^{-}_{3}]$, and $[N^{+}_{8,8,8,Bz}]$ bis(hexafluoroacetylaceto)phenyltrifluoroacetylacetomanganate(II) ($[Mn(hfacac)_2(Phtfacac)^{-}]$) were evaluated. MILs were specifically designed to provide minimal background DNA and protein extraction while maintaining qPCR compatibility. ITOs were designed to contain a 20 nt oligonucleotide complimentary to the *KRAS* amplicon. In addition, four ITOs containing either alkyl or aromatic moieties and different anions were investigated as DNA extraction probes. Once the ITO annealed to the target sequence, the ITO-DNA duplex was preconcentrated in as little as 1 min followed by collection of MIL droplets using an external magnet. Furthermore, DNA was desorbed from the MIL during qPCR thereby avoiding lengthy desorption steps.

Incorporation of the DNA-enriched MIL into the qPCR buffer did not significantly affect the amplification efficiency when extracting *KRAS* target from pure water or 10-fold diluted plasma. The ITO-MIL-DLLME method obtained enrichment factors as high as 19.49 ± 1.44 from pure water, and selectively preconcentrated DNA from a diluted plasma matrix indicating its potential for the analysis of ctDNA from clinical samples.

2. Materials and methods

2.1. Reagents and materials

Manganese(II) chloride tetrahydrate (98.0–101.0%) was purchased from Alfa Aesar (Ward Hill, MA, USA). Ammonium hydroxide (28–30% solution in water), 1,1,1,5,5,5-hexafluoroacetylacetone (99%), 1-phenyl-4,4,4-trifluoro-1,3-butanedione (99%), and trioctylamine (97%) were purchased from Acros Organics (Morris Plains, NJ, USA). Anhydrous diethyl ether (99.0%) was purchased from Avantor Performance Materials Inc. (Center Valley, PA, USA). Trihexyl(tetradecyl)phosphonium chloride (97.7%) was purchased from Strem Chemicals (Newburyport, MA, USA). Ethylenediaminetetraacetic acid (EDTA) (99.4–100.06%), bovine serum albumin (BSA) (≥96%), allyl bromide, 1-bromooctane (99%), benzylimidazole (99%), triethylamine (≥99.5%), LC-MS grade acetonitrile (≥99.9%), lyophilized plasma from human (4% trisodium citrate), deoxyribonucleic acid sodium salt from salmon testes (stDNA), and magnesium chloride hexahydrate (99.0–102.0%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). SYBR Green I (10,000x) was purchased from Life Technologies (Carlsbad, CA, USA). Urea (>99%) and tris(2-carboxyethyl)phosphine (TCEP) (>98%) were purchased from P212121 (Ypsilanti, MI, USA). Ammonium persulfate (APS) (≥98.0%), 40% acrylamide, bis-acrylamide solution 29:1, SsoAdvanced Universal SYBR Green Supermix, and a *KRAS*, human PrimePCR™ SYBR green assay (120 base pair amplicon; additional information can be found on Bio-Rad's website) were purchased from Bio-Rad Laboratories (Hercules, CA, USA). Thiolated, biotinylated, and unmodified oligonucleotides (sequences shown in Table S1) were purchased from Integrated DNA Technologies (Coralville, IA, USA). PCR caps, tube strips, sodium chloride, and Dynabeads Myone Steptavidin C1 magnetic beads were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Tris-HCl was purchased from RPI (Mount Prospect, IL, USA). Neodymium rod (0.66 T) and cylinder magnets (0.9 T) were purchased from K&J Magnetics (Pipersville, PA, USA) and used to collect dispersed MIL droplets or magnetic beads. Deionized water (18.2 MΩ cm), obtained from a Milli-Q water purification system, was used to prepare all aqueous solutions (Millipore, Bedford, MA, USA).

2.2. Instrumentation

ITO characterization was performed using an Agilent 1260 Infinity high performance liquid chromatograph (HPLC) with a diode array detector coupled to an Agilent 6230B accurate mass time-of-flight (TOF) mass spectrometer with an electrospray source. A Zorbax Extend C_{18} column (50 mm × 2.1 mm i.d. × 1.8 μm particle size) purchased from Agilent Technologies was used for the separation and characterization of ITOs. The column was equilibrated for 20 min at 0.2 mL min^{-1} with a mobile phase composition of 95:5 A:B where mobile phase A was 5 mM triethylammonium acetate (pH 7.4) and B was LC-MS grade acetonitrile. In order to prevent non-volatile imidazolium salts and urea from entering the mass spectrometer, LC eluent was diverted to the waste for the first 8 min. Gradient elution was performed using the following program: 5% B for 5 min, gradient increase 5%–19.4% B from 5 to 17 min, increased 19.4%–35% B from 17 to 18 min, held at 35% B from 18 to 20 min, increased 35% B to 100% B from 20 to 30 min, and held at 100% B from 30 to 33 min. The nebulizing gas was set to 35 psi. The drying (N_2) gas flow rate was 9 L min^{-1} with a temperature of 350 °C and a capillary voltage of 4000 V. Spectra were acquired from 100 to 3000 m/z with a scan rate of 1 spectrum sec^{-1}.

HPLC separations were performed on an Agilent Technologies 1260 system with variable wavelength detection (Santa Clara, CA, USA) to investigate the capability of the investigated MILs to extract DNA and protein as well as to examine loading of the DNA-ITO duplex to the MIL phase. A TSKgel DEAE-NPR anion exchange column (35 mm × 4.6 mm i.d. × 2.5 μm particle size) with a TSKgel DEAE-NPR guard column (5 mm × 4.6 mm i.d. × 5 μm particle size) was obtained from Tosoh Bioscience (King of Prussia, PA, USA) and used to examine the DNA and protein extraction ability of the three MILs. When separating and detecting stDNA, the column was equilibrated with a mobile phase composition of 50:50 A:B (i.e, mobile phase A: 20 mM Tris-HCl (pH 8) and mobile phase B: 1 M NaCl and 20 mM Tris-HCl (pH 8)). Gradient elution was achieved from 50% mobile phase B and ramping to 100% B from 0 to 10 min. In the separation of 20 bp DNA, the column was first equilibrated with mobile phase A for 20 min followed by gradient elution (0%–50% mobile phase B) from 0 to 10 min and increased to 100% B from 10 to 15 min. In order to separate BSA, the column was first equilibrated with mobile phase A for 20 min followed by gradient elution (0%–50% B) from 0 to 15 min and increased to 100% B from 15 to 20 min. A flow rate of 0.5 mL min^{-1} was used for all HPLC separations. DNA and albumin were detected at 260 and 280 nm, respectively.

Denaturing polyacrylamide gel electrophoresis (PAGE) was performed using a Mini Protean 3 electrophoresis system from Bio-Rad Laboratories with an ECPS 3000/150 power supply from Pharmacia (Stockholm, Sweden). An 18% polyacrylamide gel was prepared using 7 M urea to separate the ITOs from unreacted oligonucleotides. A 30 min pre-run was performed at 200 V and 150 W to equilibrate the gel and improve band resolution. Once the sample was loaded, the gel was run at 200 V and 150 W for approximately 1 h with an ice bath to cool the electrophoresis tank.

A Bio-Rad CFX96 Touch Real-time PCR was utilized for qPCR amplification of the *KRAS* target using the following program: 2 min initial denaturation at 95 °C followed by 40 cycles comprised of a 5 s denaturation step at 95 °C, a 30 s annealing step at 60 °C, and an optical detection step. Melt curves of qPCR products were achieved by starting at 65 °C for 5 s and increasing to 95 °C in 0.5 °C increments. Melt curve analysis of the ITO to a complementary sequence and sequences containing a 1 or 2 nucleotide (nt) mismatch was achieved using the following program: initial 5 min denaturation step at 90 °C, 10 min annealing step at 20 °C, and a ramp from 20 °C for 5 s and increasing to 95 °C in 0.5 °C increments.

2.3. MIL and ITO synthesis

Chemical structures of the three MILs are shown in Fig. 1(**1**–**3**). The $[NH_4^+][Mn(hfacac)_3^-]$ salt and $[P_{6,6,6,14}^+][Mn(hfacac)_3^-]$ MIL were synthesized and characterized using a previously reported procedure [27]. The $[N_{8,8,8,Bz}^+]$ cation was synthesized as previously reported and characterized using NMR, as shown in Fig. S1 [28]. The $[N_{8,8,8,Bz}^+][Mn(hfacac)_3^-]$ MIL was synthesized by stirring equimolar amounts of $[N_{8,8,8,Bz}^+][Br^-]$ and $[NH_4^+][Mn(hfacac)_3^-]$ in 30 mL of methanol overnight. The $[NH_4^+]$ hexafluoroacetylacetonate ($[hfacac^-]$) and $[NH_4^+]$ phenyltrifluoroacetylacetonate ($[Phtfacac^-]$) salts were prepared by dissolving either hexafluoroacetylacetone (hfacac) or phenyltrifluoroacetylacetone (Phtfacac) in 30 mL of ethanol with equimolar amounts of $[NH_4^+][OH^-]$. The product was subsequently dried for 5 h in a vacuum oven. The $[NH_4^+][Mn(hfacac)_2(Phtfacac)^-]$ salt was synthesized by reacting 2 molar

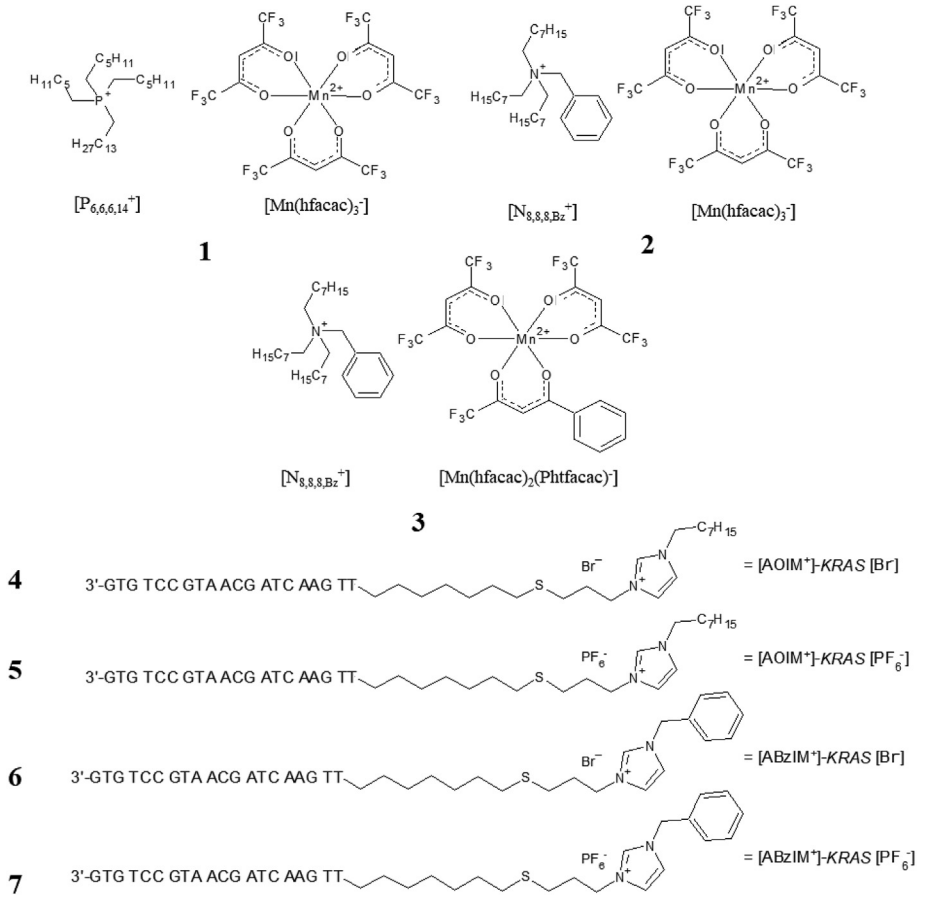

Fig. 1. Chemical structures of the manganese(II)-based hydrophobic MILs (**1**–**3**) and octyl- and benzyl-imidazolium-based ITO (**4**–**7**) structures used for all experiments.

equivalents of $[NH_4^+][hfacac^-]$, 1 molar equivalent of $[NH_4^+][Phtfacac^-]$, and 1 molar equivalent of $MnCl_2$ overnight in ethanol. The $[N_{8,8,8,Bz}^+][Mn(hfacac)_2(Phtfacac)^-]$ MIL was synthesized by stirring equimolar amounts of $[N_{8,8,8,Bz}^+][Br^-]$ and $[NH_4^+][Mn(hfacac)_2(Phtfacac)^-]$ salts in 30 mL of methanol overnight. All three MILs were purified using diethyl ether and water and subsequently dried overnight in a vacuum oven. The MIL solvents were stored in a desiccator when not in use. Elemental analysis results were acquired using a PE 2100 Series II combustion analyzer (PerkinElmer Inc., Waltham, M.A.). Carbon/hydrogen/nitrogen (CHN) calculated for $[N_{888Bz}^+][Mn(hfacac)_2(Phtfacac)^-]$: %C = 54.26, %H = 5.89, %N = 1.24; Found: %C = 53.47, %H = 5.75, %N = 1.56. Calculated for $[P_{66614}^+][Mn(hfacac)_3^-]$: %C = 48.67, %H = 6.17; Found: %C = 50.15, %H = 6.16. Calculated for $[N_{888Bz}^+][Mn(hfacac)_3^-]$: %C = 49.29, %H = 5.49, and %N = 1.25; Found: %C = 50.22, %H = 5.45, %N = 1.80.

Chemical structures of the four ITOs used in this study are shown in Fig. 1(**4**–**7**). ITOs were prepared according to previously reported methods [16]. By reacting the $[AOIM^+]$-*KRAS* $[Br^-]$ and $[ABzIM^+]$-*KRAS* $[Br^-]$ ITOs with an equimolar amount of KPF_6, the $[AOIM^+]$-*KRAS* $[PF_6^-]$ and $[ABzIM^+]$-*KRAS* $[PF_6^-]$ ITOs were prepared. All ITOs were characterized using HPLC-TOF MS, as shown in Table S2 and Fig. S2.

2.4. Background DNA and protein Co-extraction by MILs

To investigate the protein extraction capabilities of the three MILs, a 20 μL volume of MIL was added to a 1 mL solution of 1 mg mL^{-1} BSA and manually agitated for 30 s. After dispersing the MIL, 20 μL of the aqueous solution was subjected to anion exchange chromatography for quantitative analysis. To ensure low background DNA co-extraction by the MIL, two different DNA sequences were examined involving the addition of a 20 μL volume of MIL to 50 ng μL^{-1} stDNA, or 18.3 ng μL^{-1} 20 bp DNA. The solution was manually agitated for 30 s. Subsequently, 20 μL of the aqueous solution was subjected to anion exchange separation for analysis.

2.5. Examining DNA-ITO duplex loading to MIL

To determine the optimum ITO and MIL pair, a 1 μL aliquot of MIL was added to a 60 μL solution containing 25 mM NaCl and 1 ng μL^{-1} ITO-DNA duplex solution. The solution was then incubated for 10 min at room temperature. Subsequently, 20 μL of the aqueous phase was subjected to anion exchange chromatographic analysis.

2.6. Annealing and capture of target DNA

The general procedure used to anneal target *KRAS* template to the ITO and subsequent extraction using DLLME is shown in Fig. 2. A 1 mL solution of 25 mM NaCl, 2×10^4 copies μL^{-1} of *KRAS* template, and an optimized amount of ITO was prepared in a 5 mL screw cap glass vial. A 10 μL aliquot was then removed and used as a standard. The DNA solution was heated to 90 °C using a Fisher Isotemp 2322 water bath (Rochester, MN, USA) for 2 min to melt the DNA duplex and then cooled to 30 °C for 8 min to anneal the target DNA to the ITO. Subsequently, an optimized volume of MIL was dispersed using a Barnstead/Thermolyne Type 16700 mixer (Dubuque, IA, USA) for an optimal amount of time. MIL droplets were collected using a rod

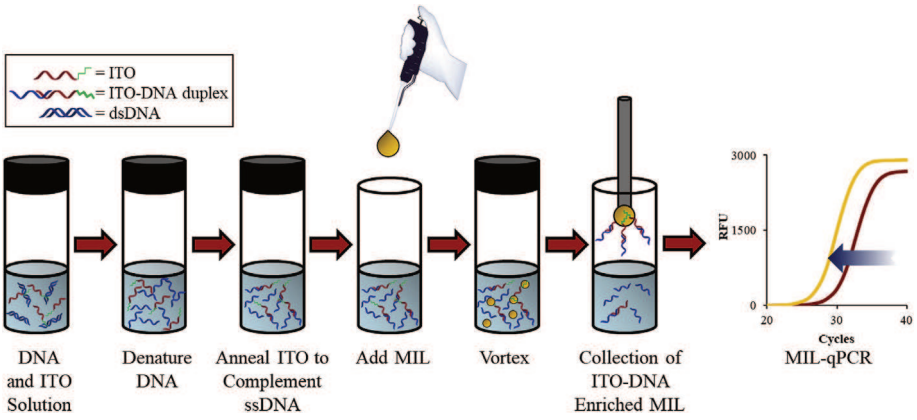

Fig. 2. General extraction procedure used to capture target *KRAS* DNA. DNA-enriched MIL was added to the reaction buffer for qPCR detection.

magnet (B = 0.66 T) and subsequently washed with deionized water (18.2 MΩ cm). A 0.3 μL aliquot of DNA-enriched MIL was then placed into a qPCR tube for downstream analysis.

Sequence-specific DNA extractions using Dynabeads Myone Steptavidin C1 beads were performed according to the manufacturer's instructions. A 1 mL solution of 25 mM NaCl, 2×10^4 copies μL^{-1} of template, and 332 fM biotinylated probe was prepared in a 5 mL screw cap glass vial. A 10 μL aliquot of this solution was used as a standard. The sample solution was heated for 5 min at 90 °C and then cooled on ice for 5 min. The magnetic beads (i.e., 10 μg) were washed three times with 5 mM Tris-HCl (pH 7.5), 0.5 mM EDTA, and 1 M NaCl prior to adding magnetic beads to the sample solution. The sample was agitated using a New Brunswick Scientific incubator shaker (Edison, NJ, USA) for 10 min at 250 rpm. Subsequently, the beads were collected using an external magnet (B = 0.9 T) and washed three times with 5 mM Tris-HCl (pH 7.5), 0.5 mM EDTA, and 1 M NaCl. The beads were then suspended in 20 μL of H_2O and heated at 90 °C for 10 min to desorb captured DNA.

2.7. qPCR amplification

The addition of 0.3 μL $[P^+_{6,6,6,14}][Mn(hfacac)^-_3]$ MIL to a 20 μL qPCR mixture required 1x SsoAdvanced Supermix, 1x PrimePCR assay mix, 4 mM EDTA, and additional 1x SYBR Green I. qPCR amplification with 0.3 μL of the $[N^+_{8,8,8,Bz}][Mn(hfacac)^-_3]$ MIL was achieved using the 1x SsoAdvanced Supermix, 1x PrimePCR assay mix, 6.25 mM $MgCl_2$, 4 mM EDTA, and an additional 0.4x SYBR Green I for a final volume of 20 μL. The addition of 0.3 μL $[N^+_{8,8,8,Bz}][Mn(hfacac)_2(Phtfacac)^-]$ MIL to a 20 μL qPCR mixture required 1x SsoAdvanced Supermix, 1x PrimePCR assay mix, 2.5 mM $MgCl_2$, 2 mM EDTA, and additional 1x SYBR Green I.

The threshold cycle (Cq) was determined using the fluorescence threshold provided by the Bio-Rad CFX Maestro software and used to determine the amount of the *KRAS*-ITO duplex extracted by the hydrophobic MIL. A standard curve was constructed for *KRAS* template (see Fig. S3) and to determine the concentration of DNA extracted using the ITO-MIL-DLLME procedure. The enrichment factors (E_f) obtained for each extraction were calculated using equation (1), where C_{MIL} is the concentration of DNA extracted using the MIL and C_{Std} represents the concentration of target DNA initially present in the sample.

$$E_f = \frac{C_{MIL}}{C_{Std}} \quad (1)$$

3. Results and discussion

3.1. qPCR conditions to mitigate the inhibitory effects of MILs

In order to remove tedious sample handing steps and increase sample throughput, DNA-enriched MIL was incorporated into the qPCR buffer in which DNA is capable of desorbing from the MIL due to the elevated temperatures required for the reaction. However, it has been previously shown that hydrophobic MILs can dissolve under the elevated temperatures required for PCR [29]. Solubilized MIL components can inhibit PCR amplification; nevertheless, qPCR inhibition can be relieved through the addition of EDTA, BSA, additional $MgCl_2$, additional Tris-HCl, and additional SYBR Green I.

The inhibition of qPCR due to the addition of $[P^+_{6,6,6,14}][Mn(hfacac)^-_3]$, $[N^+_{8,8,8,Bz}][Mn(hfacac)^-_3]$, and $[N^+_{8,8,8,Bz}][Mn(hfacac)_2(Phtfacac)^-]$ MILs was mitigated by titrating 0–8 mM $MgCl_2$, 0–8 mM EDTA, and 0-1x SYBR Green I into the buffer. Incorporation of 0.3 μL of $[P^+_{6,6,6,14}][Mn(hfacac)^-_3]$ MIL to the qPCR buffer required 4 mM EDTA and 1x SYBR Green I, as previously reported [25]. The addition of 0.3 μL of the $[N^+_{8,8,8,Bz}][Mn(hfacac)^-_3]$ MIL to the qPCR buffer was optimized to require 4 mM EDTA, 6.25 mM $MgCl_2$, and 0.4x SYBR Green I. Inhibition caused by 0.3 μL of the $[N^+_{8,8,8,Bz}][Mn(hfacac)_2(Phtfacac)^-]$ MIL required 4 mM EDTA, 2.5 mM $MgCl_2$, and 1x SYBR Green I in order to achieve amplification. Addition of EDTA to the MIL-qPCR buffer chelates solubilized anion providing relief to qPCR inhibition. Without additional SYBR green in solution, the fluorescence signal remains low likely due to the partitioning of SYBR Green I to the hydrophobic MIL phase [30].

The addition of excess oligonucleotides to a PCR reaction can either accelerate the reaction or lead to the formation of primer-dimers [31–34]. Therefore, to investigate the effect of the ITO on the reaction, 4×10^6 copies of ITO were spiked into the qPCR buffer. However, as shown in Fig. S4, spiking ITOs into the qPCR did not affect the Cq nor did the melt curve indicate the presence of primer-dimers. In this case, it appears that the addition of ITO did not affect the reaction likely due to the low concentration of the probe.

Examination of the melting temperature (T_m) can be used to differentiate SNPs [35]. Therefore, the T_m of the qPCR product was examined to investigate whether the MIL-based DNA extraction or direct qPCR amplification altered the DNA sequence. Fig. S5 shows that the T_m of extracted target DNA was comparable to the *KRAS* standard (±0.5 °C) suggesting that the sequence was not altered due to the MIL. This result is in agreement with previous MIL-based DNA amplification studies [29].

3.2. MIL and ITO screening

An ideal MIL solvent should be capable of capturing the ITO-DNA duplex while not extracting background DNA and plasma components. However, plasma contains 35–80 mg mL^{-1} of protein making it an extremely challenging and complex matrix due to the fact that plasma proteins can inhibit qPCR amplification [18,36–38]. Furthermore, plasma contains high levels of background DNA that presents a challenge for targeted analysis. Therefore, to prevent co-extraction of background DNA, manganese(II)-based MILs were investigated as they have been previously shown to poorly extract DNA [16,25]. In addition, manganese(II) binds poorly to albumin, which makes up over 50% of the total protein content of plasma [39–41]. As shown in Fig. 3, the addition of aromatic moieties to either the cation or anion component of the MIL was shown to reduce the amount of BSA extracted. The three MILs were also tested to examine the extraction efficiency of short (20 bp) and long (20 Kbp) DNA fragments. All three MILs extracted less than 2% of either DNA sequence, as shown in Fig. 3.

Previously, it was reported that DNA-ITO duplexes interact with the MIL solvent primarily through hydrophobic interactions [16]. Exchanging the [Br^-] anion of the ITO with a more hydrophobic anion, such as [PF_6^-], has the potential to facilitate stronger interactions with the MIL solvent and possibly improve loading efficiency. As shown in Fig. 4, 56.94 ± 1.61% of the target was extracted by the $[P^+_{6,6,6,14}][Mn(hfacac)_3^-]$ MIL using the [$AOIM^+$]-*KRAS* [PF_6^-] ITO while only 48.18 ± 3.89%, 45.98 ± 3.32%, and 38.59 ± 4.90% was loaded using the [$AOIM^+$]-*KRAS* [Br^-], [$ABzIM^+$]-*KRAS* [Br^-], and [$ABzIM^+$]-*KRAS* [PF_6^-] ITOs, respectively. In addition, incorporation of aromatic moieties into the ITO and MIL structures facilitated π-π stacking interactions and provided a modest increase in the amount of ITO loaded to the MIL. As shown in Fig. 4, 65.94 ± 9.55% of the [$ABzIM^+$]-*KRAS* [Br^-] ITO-DNA duplex was loaded onto the $[N^+_{8,8,8,Bz}][Mn(hfacac)_3^-]$ MIL whereas only 26.13 ± 3.44%, 37.83 ± 1.28%, and 20.17 ± 2.04% was loaded using the [$AOIM^+$]-*KRAS* [Br^-], [$AOIM^+$]-*KRAS* [PF_6^-], and [$ABzIM^+$]-*KRAS* [PF_6^-] ITOs, respectively. Using the $[N^+_{8,8,8,Bz}][Mn(hfacac)_2(Phtfacac)^-]$ MIL as an extraction solvent, 16.56 ± 3.36% [$AOIM^+$]-*KRAS* [Br^-], 42.05 ± 0.72% [$AOIM^+$]-*KRAS* [PF_6^-], 53.69 ± 2.27% [$ABzIM^+$]-*KRAS* [Br^-] ITO, and 25.10 ± 4.91% [$ABzIM^+$]-*KRAS* [PF_6^-] ITO was loaded onto the MIL solvent, as shown in Fig. 4.

3.3. Optimization of the ITO-MIL-DLLME method

Cell-free DNA (cfDNA) is present in relatively high concentrations (1–100 pM) in blood, plasma, and serum [42]. However, certain tumor mutations can comprise less than 0.01% of the total amount of cfDNA [43]. Therefore, a clinically relevant concentration of 2×10^4 copies μL^{-1} (33 fM) of target *KRAS* fragments was used during optimization. A 1 mL sample volume was selected in order to maintain the minimum sample volume capable of being dispersed.

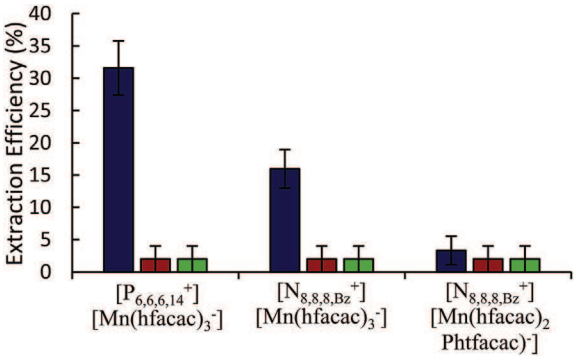

Fig. 3. Extraction efficiency of BSA (blue), stDNA (red), and 20 bp DNA (green) using the $[P^+_{6,6,6,14}][Mn(hfacac)_3^-]$, $[N^+_{8,8,8,Bz}][Mn(hfacac)_3^-]$, and $[N^+_{8,8,8,Bz}][Mn(hfacac)_2(Phtfacac)^-]$ MILs. BSA concentration: 1 mg mL^{-1}, stDNA concentration: 50 ng μL^{-1}, 20 bp DNA concentration: 18.3 ng μL^{-1}; sample volume: 1 mL; agitation time: 30 s; MIL volume: 20 μL. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

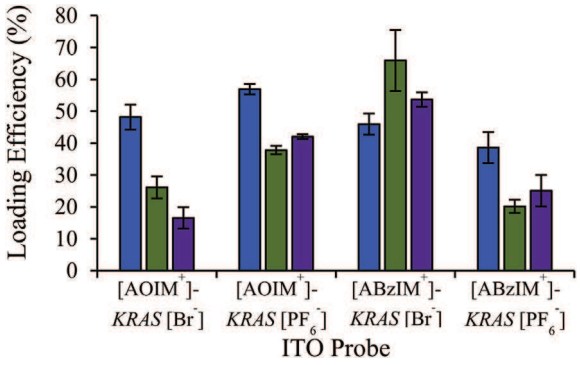

Fig. 4. Loading efficiencies of the ITO-DNA duplex to the MIL phase using the [$AOIM^+$]-*KRAS* [Br^-], [$AOIM^+$]-*KRAS* [PF_6^-], [$ABzIM^+$]-*KRAS* [Br^-], and [$ABzIM^+$]-*KRAS* [PF_6^-] ITOs and $[P^+_{6,6,6,14}][Mn(hfacac)_3^-]$ (blue), $[N^+_{8,8,8,Bz}][Mn(hfacac)_3^-]$ (green), and $[N^+_{8,8,8,Bz}][Mn(Phtfacac)(hfacac)_2^-]$ (purple) MILs. ITO concentration: 1 ng μL^{-1}; *KRAS* complement: 1 ng μL^{-1}; NaCl concentration: 25 mM; sample volume: 60 μL; MIL volume: 1 μL; extraction time: 10 min. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

The amount of ITO versus the amount of DNA present in the solution was first optimized. The sample was initially heated to 90 °C to denature the DNA duplex followed by a cooling step to 30 °C. After annealing the ITO to the target DNA, 6 μL of MIL was added to the solution. The solution was vortexed for 1 min, and the DNA-enriched MIL was recovered from the aqueous solution using a rod magnet (B = 0.66 T) and subjected to qPCR analysis. As shown in Fig. S6, a 10-fold excess of either [$AOIM^+$]-*KRAS* [PF_6^-] and [$ABzIM^+$]-*KRAS* [Br^-] ITO was found to be optimum for the $[P^+_{6,6,6,14}][Mn(hfacac)_3^-]$, $[N^+_{8,8,8,Bz}][Mn(hfacac)_3^-]$, and $[N^+_{8,8,8,Bz}][Mn(hfacac)_2(Phtfacac)^-]$ MILs. Target DNA can either reanneal to the complimentary sequence or anneal to the ITO. Therefore, to increase the probability that target DNA anneals to the ITO, an excess amount of ITO was needed. However, high concentrations of ITO may result in the MIL extracting unhybridized ITO instead of the desired ITO-DNA complex.

The volume of MIL dispersed in the solution was also optimized for all three MILs. A volume of 8 μL of $[P^+_{6,6,6,14}][Mn(hfacac)_3^-]$ MIL was found to be optimum while dispersing 6 μL and 4 μL of $[N^+_{8,8,8,Bz}][Mn(hfacac)_3^-]$ and $[N^+_{8,8,8,Bz}][Mn(hfacac)_2(Phtfacac)^-]$ MILs, respectively, produced the highest extraction efficiencies, as shown in Fig. S7. Larger volumes of MIL generally resulted in lower E_f as DNA can be diluted within the MIL [44].

Additionally, the extraction time was optimized to achieve the highest E_f in the shortest amount of time. As shown in Fig. S8, an extraction time of 3 min was optimum for the $[P^+_{6,6,6,14}][Mn(hfacac)_3^-]$ MIL while 1 min was optimum for the $[N^+_{8,8,8,Bz}][Mn(hfacac)_3^-]$ and $[N^+_{8,8,8,Bz}][Mn(hfacac)_2(Phtfacac)^-]$ MILs. After the optimum times for the $[P^+_{6,6,6,14}][Mn(hfacac)_3^-]$ and $[N^+_{8,8,8,Bz}][Mn(hfacac)_3^-]$ MILs, a sharp decrease in the amount of DNA extracted was observed.

3.4. Comparison to commercial sequence-specific magnetic beads

Streptavidin magnetic beads have been used to capture specific ctDNA mutations from clinical plasma samples [3,9,10]. A

significant drawback to using magnetic beads is their propensity to aggregate and sediment, which is especially problematic when extraction of low concentrations of target DNA requires the beads to be suspended for long periods of time. MILs are uniquely capable of overcoming these issues as they can be easily dispersed into fine droplets that remain suspended in solution for extended periods of time [45]. Due to these properties, MILs have the potential to provide unique advantages over commercial magnetic beads in sequence-specific DNA extractions. The ITO-MIL-DLLME procedure was compared to the commercial Dynabeads Myone streptavidin C1 magnetic beads. As shown in Fig. 5a, extractions performed using the $[P^{+}_{6,6,6,14}][Mn(hfacac)^{-}_{3}]$ and $[N^{+}_{8,8,8,Bz}][Mn(hfacac)^{-}_{3}]$ MILs produced E_f values of 19.49 ± 1.44 and 16.44 ± 2.21 outperforming the commercial streptavidin Dynabeads, which provided a respectable E_f of 9.73 ± 0.42 from pure water. When examining the amount of DNA co-extracted by the MIL and magnetic beads, the optimized extraction was performed without either the ITO or biotinylated probe. As shown in Fig. 5a, a low E_f from pure water was obtained without ITO and biotinated probe present in solution. The limited co-extraction of DNA by the MIL solvent or magnetic beads indicates that DNA extracted can be attributed to the ITO or the biotinylated probe.

When comparing the extraction of *KRAS* target from 10-fold diluted plasma using the three MILs and the commercial magnetic beads, the $[ABzIM^{+}]$-*KRAS* $[Br^{-}]$ ITO and $[N^{+}_{8,8,8,Bz}][Mn(hfacac)^{-}_{3}]$ MIL produced the highest E_f, as shown in Fig. 5b. The $[N^{+}_{8,8,8,Bz}][Mn(hfacac)_2(Phtfacac)^{-}]$ MIL experienced only a two-fold decrease in E_f, which may be linked to the low BSA extraction efficiency associated with this MIL. However, extractions using the magnetic beads from 10-fold diluted plasma produced an E_f of only 1.30 ± 0.07, possibly due to the biotinylated probe interacting with plasma proteins [46]. This is supported by the fact that the E_f obtained using streptavidin-coated magnetic beads, with or without the biotinylated probe, were within error (i.e., 1.25 ± 0.18). As shown in Fig. 5b, low E_f values were achieved when the ITO was not present in solution suggesting that the ITO plays a dominant role in preconcentrating DNA from the plasma solution. However, selectivity was lost when performing extractions with the steptavidin-coated magnetic beads from 10-fold diluted plasma.

In order to determine whether the ITO-MIL-DLLME-qPCR method could be used for accurate quantification, calibration curves for each MIL were constructed by performing a series of five extractions at different concentration levels followed by the determination of amplification efficiency using equation (2). Ideally, the amplification efficiency should range between 90 and 110%

$$Efficency = \left(10^{\left(-\frac{1}{slope}\right)} - 1\right) * 100\% \quad (2)$$

indicating that DNA is successfully duplicated with each cycle and that quantification can be achieved [47]. It was observed that all three MILs did not significantly alter the amplification efficiency when incorporated into the qPCR buffer. The $[P^{+}_{6,6,6,14}][Mn(hfacac)^{-}_{3}]$ MIL produced an efficiency of 100.1%, while the $[N^{+}_{8,8,8,Bz}][Mn(hfacac)^{-}_{3}]$ MILs exhibited an efficiency of 107.9%, and the $[N^{+}_{8,8,8,Bz}][Mn(hfacac)_2(Phtfacac)^{-}]$ MIL enabled an efficiency of 107.9%, as shown in Fig. 6. Calibration curves from 10-fold diluted plasma were also constructed for all three MILs to determine if quantification could be achieved from a more complex medium. As shown in Fig. 7, the amplification efficiencies associated with the extraction of *KRAS* from 10-fold diluted plasma fell between 90 and 110% with the addition of the $[P^{+}_{6,6,6,14}][Mn(hfacac)^{-}_{3}]$, $[N^{+}_{8,8,8,Bz}][Mn(hfacac)^{-}_{3}]$, and $[N^{+}_{8,8,8,Bz}][Mn(hfacac)_2(Phtfacac)^{-}]$ MIL to the qPCR buffer resulting in efficiencies of 106.3%, 109.3%, and 103.5% respectively. Amplification efficiencies obtained from the ITO-MIL-DLLME procedure in pure water and diluted plasma indicate that there is little inhibition attributed to adding manganese(II)-based MILs to the qPCR buffer and that this method can be used for quantification purposes.

3.5. Selectivity of the ITO-MIL-DLLME method

The detection of low abundance ctDNA is of particular importance for early cancer detection, prognosis, and treatment monitoring [48]. However, several common ctDNA fragments are SNPs that complicate ctDNA detection [2]. In order to investigate the selectivity of the ITO probes for the complementary sequence and 1–2 nt mismatch, melt curve analysis was performed. If the oligonucleotides are not complimentary, the T_m will decrease indicating instability of the hybridization [49]. Therefore, to examine the selectivity of the ITOs towards target DNA, the T_m of the ITO to a complementary oligonucleotide (20 nt), 1 nt mismatch (20 nt), and 2 nt mismatch (20 nt) was examined. Fig. S9 shows that the T_m significantly decreased when examining the hybridization of the ITO to the 1 nt mismatch, and a T_m could not be determined when

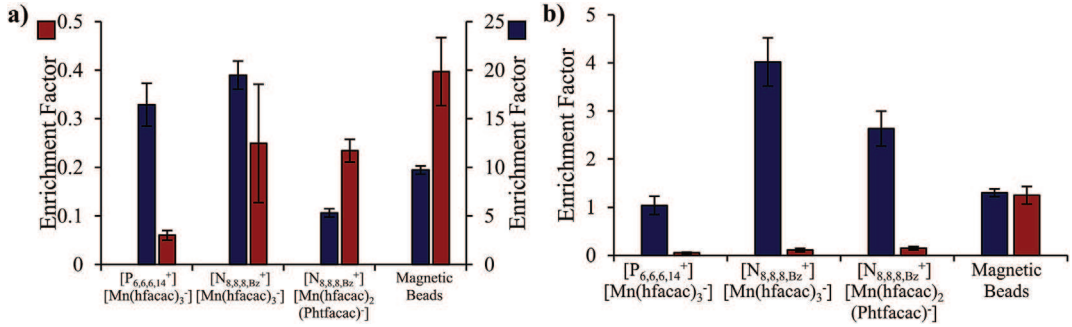

Fig. 5. Enrichment factors obtained for the sequence-specific extraction of KRAS target using the $[P^{+}_{6,6,6,14}]$ $[Mn(hfacac)^{-}_{3}]$ MIL, $[N^{+}_{8,8,8,Bz}][Mn(hfacac)^{-}_{3}]$ MIL, $[N^{+}_{8,8,8,Bz}]$ $[Mn(hfacac)_2(Phtfacac)^{-}]$ MIL, and Dynabeads Myone Steptavidin C1 magnetic beads from pure water (a) and 10-fold diluted plasma (b) with (blue) and without (red) ITO or biotinylated probe. $[P^{+}_{6,6,6,14}]$ $[Mn(hfacac)^{-}_{3}]$ MIL conditions: KRAS template concentration: 2×10^4 copies μL^{-1}, amount of $[AOIM^{+}]$-*KRAS* $[PF^{-}_{6}]$ ITO relative to DNA: 10x, NaCl concentration: 25 mM, sample volume: 1.0 mL, MIL volume: 8 μL; extraction time: 3 min $[N^{+}_{8,8,8,Bz}][Mn(hfacac)^{-}_{3}]$ MIL conditions: KRAS template concentration: 2×10^4 copies μL^{-1}, amount of $[ABzIM^{+}]$-*KRAS* $[Br^{-}]$ relative to DNA: 10x, NaCl concentration: 25 mM, sample volume: 1.0 mL, MIL volume: 6 μL: extraction time: 1 min $[N^{+}_{8,8,8,Bz}]$ $[Mn(hfacac)_2(Phtfacac)^{-}]$ MIL conditions: KRAS template concentration: 2×10^4 copies μL^{-1}, amount of $[ABzIM^{+}]$-*KRAS* $[Br^{-}]$ relative to DNA: 10x, NaCl concentration: 25 mM, sample volume: 1.0 mL, MIL volume: 4 μL; extraction time: 1 min. Dynabeads Myone Steptavidin C1 magnetic beads conditions: KRAS template concentration: 2×10^4 copies μL^{-1}, concentration of biotinylated probe: 332 fM, NaCl concentration: 25 mM, mass of magnetic beads: 10 μg; sample volume: 1.0 mL; extraction time: 10 min; agitation rate: 250 rpm; desorption time: 10 min; desorption volume: 20 μL. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

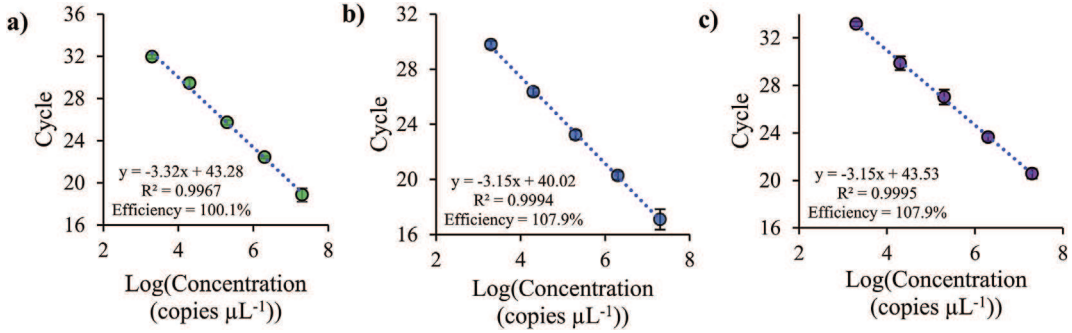

Fig. 6. Standard curves generated using the optimized MIL-DLLME method for the (a) $[P^{+}_{6,6,6,14}]$ [Mn(hfacac)$_3^-$], (b) $[N^{+}_{8,8,8,Bz}]$[Mn(hfacac)$_3^-$], (c) $[N^{+}_{8,8,8,Bz}]$ [Mn(hfacac)$_2$(Phtfacac)$^-$] MILs as extraction solvents. $[P^{+}_{6,6,6,14}]$ [Mn(hfacac)$_3^-$] MIL conditions: *KRAS* template concentration: 2×10^4 copies μL^{-1}, amount of [AOIM$^+$]-*KRAS* [PF_6^-] relative to DNA: 10x, NaCl concentration: 25 mM, sample volume: 1.0 mL, MIL volume: 8 μL; extraction time: 3 min $[N^{+}_{8,8,8,Bz}]$[Mn(hfacac)$_3^-$] MIL conditions: *KRAS* template concentration: 2×10^4 copies μL^{-1}, amount of [ABzIM$^+$]-*KRAS* [Br$^-$] relative to DNA: 10x, NaCl concentration: 25 mM, sample volume: 1.0 mL, MIL volume: 6 μL: extraction time: 1 min $[N^{+}_{8,8,8,Bz}]$ [Mn(hfacac)$_2$(Phtfacac)$^-$] MIL conditions: *KRAS* template concentration: 2×10^4 copies μL^{-1}, amount of [ABzIM$^+$]-*KRAS* [Br$^-$] ITO relative to DNA: 10x, NaCl concentration: 25 mM, sample volume: 1.0 mL, MIL volume: 4 μL; extraction time: 1 min.

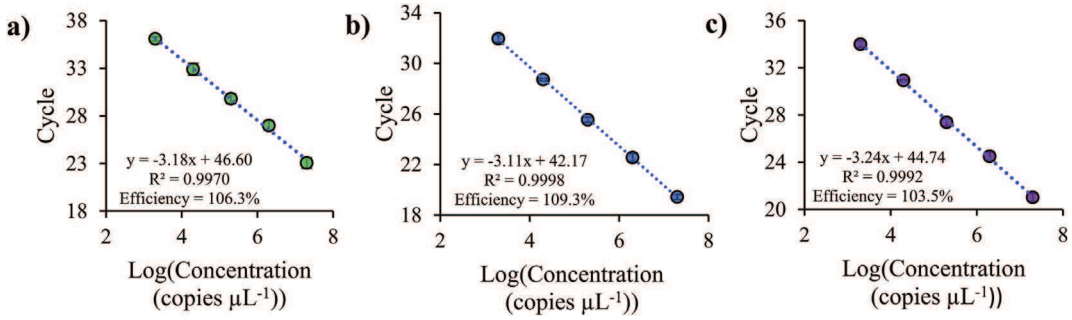

Fig. 7. Standard curves generated using the optimized MIL-DLLME method from 10-fold diluted plasma for the (a) $[P^{+}_{6,6,6,14}]$ [Mn(hfacac)$_3^-$], (b) $[N^{+}_{8,8,8,Bz}]$[Mn(hfacac)$_3^-$], (c) $[N^{+}_{8,8,8,Bz}]$ [Mn(hfacac)$_2$(Phtfacac)$^-$] MILs as extraction solvents. $[P^{+}_{6,6,6,14}]$ [Mn(hfacac)$_3^-$] MIL conditions: *KRAS* template concentration: 2×10^4 copies·μL^{-1}, amount of [AOIM$^+$]-*KRAS* [PF_6^-] ITO relative to DNA: 10x, NaCl concentration: 25 mM, sample volume: 1.0 mL, MIL volume: 8 μL; extraction time: 3 min $[N^{+}_{8,8,8,Bz}]$[Mn(hfacac)$_3^-$] MIL conditions: *KRAS* template concentration: 2×10^4 copies·μL^{-1}, amount of [ABzIM$^+$]-*KRAS* [Br$^-$] ITO relative to DNA: 10x, NaCl concentration: 25 mM, sample volume: 1.0 mL, MIL volume: 6 μL: extraction time: 1 min $[N^{+}_{8,8,8,Bz}]$[Mn(hfacac)$_2$(Phtfacac)$^-$] MIL conditions: *KRAS* template concentration: 2×10^4 copies·μL^{-1}, amount of [ABzIM$^+$]-*KRAS* [Br$^-$] ITO relative to DNA: 10x, NaCl concentration: 25 mM, sample volume: 1.0 mL, MIL volume: 4 μL; extraction time: 1 min.

hybridizing the [AOIM$^+$]-*KRAS* [PF_6^-], [ABzIM$^+$]-*KRAS* [Br$^-$], and [ABzIM$^+$]-*KRAS* [PF_6^-] ITOs to the 2 nt mismatch.

Background cfDNA in cancer patients is approximately the same length as ctDNA (i.e, about 166 bp) and is present in high concentrations, typically 0–1000 ng mL^{-1} for cancer patients with mutation abundances less than 0.01% [4,8,50]. To examine the effect of background DNA on the ITO-MIL-DLLME method, stDNA was sheared to around 150 bp and spiked into the aqueous sample. stDNA was sheared using a sonication method involving 30 s cycles (30s of sonication followed by a 30 s rest period) for 1 h and verified by gel electrophoresis, as shown in Fig. S10a. Fig. S10b shows that the E_f did not significantly decrease when performing extractions from a sample solution containing 2×10^4 copies μL^{-1} of target *KRAS* and 1000 ng mL^{-1} of sheared stDNA (mutation abundance of 0.009%) indicating that clinically-relevant concentrations of background cfDNA do not have a significant effect on the extraction of low-abundance target sequences.

4. Conclusions

Circulating tumor DNA is difficult to isolate due to the presence of SNPs and large amounts of background DNA and proteins in human plasma. In this study, three hydrophobic MILs were designed and synthesized to function as liquid supports in the capture of the target ITO duplex without co-extraction of background DNA or protein. The fine dispersion of MIL solvent facilitated the formation of droplets with high surface area to capture the target ITO duplex without sedimentation. After collecting the MIL droplets on a magnetic rod, the MILs were directly added to qPCR using specially designed buffers that mitigated inhibition from MILs during the thermal cycling process. Incorporation of the manganese(II)-based MILs to the qPCR buffer did not significantly affect the amplification efficiency, even when extractions were performed from diluted human plasma, indicating no significant inhibition. Target *KRAS* DNA was preconcentrated from pure water and 10-fold diluted plasma in as little as 1 min using the $[P^{+}_{6,6,6,14}]$[Mn(hfacac)$_3^-$], $[N^{+}_{8,8,8,Bz}]$[Mn(hfacac)$_3^-$], and $[N^{+}_{8,8,8,Bz}]$ [Mn(hfacac)$_2$(Phtfacac)$^-$] MILs. Compared to commercially-available streptavidin magnetic beads, the ITO-MIL-DLLME approach using the $[N^{+}_{8,8,8,Bz}]$[Mn(hfacac)$_3^-$] and $[N^{+}_{8,8,8,Bz}]$[Mn(hfacac)$_2$(Phtfacac)$^-$] MILs produced a higher E_f when extracting target DNA from diluted plasma. The magnetic beads were unable to selectively preconcentrate target DNA from 10-fold diluted plasma while the ITO-MIL procedure maintained selectivity towards the ITO-DNA duplex. The ability of the ITO-MIL system to selectively preconcentrate low concentrations of target DNA from diluted plasma indicates the promise that the ITO-MIL-DLLME method has in the detection of ctDNA fragments from clinical samples.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aca.2019.04.005.

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