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DNA Sample in Blood, Plasma, or Saline

Anneal ITO or DTO Probe to Target DNA

Capture Target-Probe DNA Duplex with MIL

Desorb DNA

qPCR Detection

Desorb in high ionic strength

Or Desorb in qPCR buffer

ITO Probe:

With probe

Without probe

ITO

DTO

2-fold diluted plasma

10-fold diluted blood

Saline

RFU

Cycles

0 500 1000 1500 2000 2500

24 28 32 36 40
Selective hybridization and capture of KRAS DNA from plasma and blood using ion-tagged oligonucleotide probes coupled to magnetic ionic liquids

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Abstract

Detection of circulating tumor DNA (ctDNA) presents several challenges due single-nucleotide polymorphisms and large amounts of background DNA. Previously, we reported a sequence-specific DNA extraction procedure utilizing functionalized oligonucleotides called ion-tagged oligonucleotides (ITOs) and disubstituted ion-tagged oligonucleotides (DTOs). ITOs and DTOs are capable of hybridizing to complementary DNA for subsequent capture by a magnetic ionic liquid (MIL) through hydrophobic interactions, π-π stacking, and fluorophilic interactions. However, the performance of the ITOs and DTOs in complex sample matrices has not yet been evaluated. In this study, we compare the amount of KRAS DNA extracted using ITO and DTOs from saline, 2-fold diluted plasma, 10-fold diluted plasma, and 10-fold diluted blood. We demonstrate that ITO/DTO-MIL extraction is capable of selectively preconcentrating DNA from diluted plasma and blood without additional sample preparation steps. In comparison, streptavidin-coated magnetic beads were unable to selectively extract DNA from 10-fold diluted plasma and 10-fold diluted blood without additional sample clean-up steps. Significantly more DNA could be extracted from 2-fold diluted plasma and 10-fold diluted blood matrices using the DTO probes compared to the ITO probes, likely due to stronger interactions between the probe and MIL. The ability of the DTO-MIL method to selectively preconcentrate small concentrations of DNA from complex biological matrices suggests that this method could be beneficial for ctDNA analysis.

Keywords: DNA extraction, Cell-free DNA, PCR, Ionic liquid, Magnetic separation
1. Introduction

Circulating tumor DNA (ctDNA) is fragmented tumor-derived DNA found in blood originating from tumor cells that have undergone apoptosis or necrosis.[1] Detection of ctDNA from blood has massive potential to supplement or replace invasive tissue biopsies for cancer diagnosis, treatment, and the monitoring of residual disease.[2] However, during the early stages of cancer or after cancer treatment, ctDNA fragments are present in low amounts relative to wild-type DNA with mutation abundances potentially less than 0.01%.[3] Large amounts of background DNA can mask low abundance mutant fragments causing false negative results.[2] Furthermore, ctDNA fragments are prone to single-nucleotide polymorphisms (SNPs).[4] The KRAS gene is particularly prone to SNPs, typically around codon 12 and 13 in exon 2, which can impair the guanosine triphosphatase activity of the KRAS protein resulting in cellular proliferation.[5,6] The presence of certain KRAS SNPs have also been correlated to the success of anti-EGFR therapy making it crucial to distinguish SNPs from wild-type DNA.[7,8] Therefore, ctDNA analysis requires sequence-specific detection in order to distinguish low concentrations of SNPs from complex matrices.

There are several polymerase chain reaction (PCR) methods for sequence-specific amplification and detection.[2,9] However, large amounts of background DNA may result in false positives due to mishybridization or mask target DNA fragments.[10] Wild-type DNA can be co-amplified with ctDNA when using similar primer sets. PCR blockers, such as peptide nucleic acids (PNAs) or locked nucleic acids (LNAs), can limit amplification of wild-type DNA.[11–13] However, PNAs and LNAs are expensive, and their ability to discriminate between the wild-type and mutant fragment relies heavily on the positioning and sequence of the
Co-amplification at lower denaturation temperature (COLD) PCR can also limit co-amplification of wild-type DNA by carefully optimizing the annealing temperature to ensure that wild-type DNA remains a duplex during the annealing and extension step.[16] While COLD-PCR is useful for selectively amplifying known and unknown mutations, the critical temperature of the mutant fragment must be lower than the wild-type DNA for selective amplification, which prevents approximately 30% of all mutations from being detected.[17] In addition, all PCR methods require highly pure DNA for amplification to occur, and blood and plasma components such as IgG or hemoglobin can inhibit PCR.[18,19] Therefore, sequence-specific DNA extraction step is needed in order to limit the co-extraction of non-target DNA and PCR inhibitors.

Sequence-specific DNA extractions can supplement PCR amplification by preventing large amounts of background DNA from contaminating the reaction. Commercial methods for such extractions often rely on streptavidin-coated magnetic beads and biotinylated oligonucleotides.[1,20,21] Target DNA can be captured using streptavidin-coated magnetic beads due to streptavidin’s exceptional affinity for biotin ($K_d = 4 \times 10^{-14} \text{ M}$).[22] However, streptavidin-coated magnetic beads are prone to aggregation and sedimentation, which can reduce extraction efficiencies and clog microfluidic devices.[23,24] Other sequence-specific extraction methods include the use of oligonucleotide-modified monoliths; however, these procedures can currently achieve picomolar detection limits where most ctDNA fragments are present at femtomolar concentrations or lower.[25–27] Oleoyl-modified oligonucleotides have also been used for sequence-specific DNA extractions. DNA can be selectively hybridized to the oleoyl-modified oligonucleotide and be contained within reverse micelles of diauroylphosphatidylcholine and 1-hexanol.[28] It was demonstrated that over 60% of the target
DNA was extracted using the oleoyl-modified oligonucleotides whereas only 6% and 4% of the 1 nt and 2 nt mismatch fragments, respectively, were extracted. However, a lengthy extraction step (i.e., 3 h) is required to form the reverse micelles as significant agitation can disrupt the interface between the aqueous and organic phases. In addition, this method has never been used to extract clinically-relevant concentrations of ctDNA nor has it been applied to a complex matrix.

A recent and promising alternative to commercial sequence-specific DNA extraction approaches is the use of magnetic ionic liquids (MILs).[29] MILs are a subclass of ionic liquids (ILs) that contain a paramagnetic component in either the cation or anion allowing them to be dispersed as droplets and be rapidly collected using an external magnet.[30,31] Hydrophobic manganese(II)-based MILs, in particular, have been found to poorly extract DNA with extraction efficiencies less than 2%.[29,32,33] A series of DNA extraction probes termed ion-tagged oligonucleotides (ITOs) have been designed to contain an imidazolium headgroup that interacts with a manganese(II)-based MIL solvent support through hydrophobic interactions, π-π stacking, and fluorophilic interactions.[29,34] Recent efforts have focused on improving loading of the ITO-DNA duplex to the MIL support though the use of disubstituted ion-tagged oligonucleotides (DTOs), which contain two imidazolium headgroups capable of interacting with the MIL through hydrophobic interactions.[34] Another recent study developed a dispersive ITO-MIL method that drastically decreased the sample preparation time by dispersing the MIL using a vortex and incorporating DNA-enriched MIL into the qPCR buffer, allowing for a 19-fold enrichment of target DNA.[33]

Despite significantly higher loading efficiencies associated with the DTO, both the ITO and DTO probes extract similar amounts of DNA under optimized extraction conditions.[34]
However, it still remains unclear as to whether the DTO and ITO probes exhibit similar DNA extraction performances from complex matrices such as plasma and whole blood. In addition, there has been little optimization of the desorption step in the ITO/DTO-MIL procedure, as previous methods have relied on either a time-consuming 10 min desorption step at 90 °C or desorption of DNA during qPCR amplification, which is restricted by the temperature program and buffer conditions required for PCR.[29,33–35] This study examines the sequence-specific extraction of KRAS DNA from 10-fold diluted plasma, 2-fold diluted plasma, and 10-fold diluted whole blood using both ITO and DTO probes. In addition, the desorption time and ionic strength of the desorption solution were optimized to ensure maximum recovery of target DNA from the MIL. The ITO/DTO-MIL method was found to be selective when performing extractions from saline (154 mM NaCl), 10-fold diluted plasma, 2-fold diluted plasma, and 10-fold diluted blood. However, the DTO probes were significantly more advantageous in extractions from 2-fold diluted plasma and 10-fold diluted whole blood whereas the ITO probes extracted more DNA from saline compared to DTOs. These results suggest that the DTO-MIL procedure is highly versatile and capable of selectively preconcentrating femtomolar levels of target DNA from very complex samples that contain high amounts of protein and background DNA whereas the ITO probe excels when performing extraction from samples with elevated ionic strength.

2. Methods and Materials

2.1 Materials and Reagents

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). Manganese(II) chloride tetrahydrate (98.0-101.0%) was purchased from Alfa Aesar (Ward Hill, MA, 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). Ammonium persulfate (APS) (≥98.0%), allyl bromide, ethylenediaminetetraacetic acid (EDTA) (99.4-100.06%), 1-bromooctane (99%), benzylimidazole (99%), triethylamine (≥99.5%), sodium octylsulfate ([OS⁻]) (>95%), Tween 20, LC-MS grade acetonitrile (ACN) (≥99.9%), lyophilized plasma from human (4% trisodium citrate), potassium hexafluorophosphate ([PF₆⁻]) (≥99%), 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). Proteinase K was purchased from New England Biolabs (Ipswich, MA, USA). Tris(hydroxymethyl)aminomethane (Tris) hydrochloride (HCl), urea (>99%) and tris(2-carboxyethyl)phosphine (TCEP) (>98%) were purchased from P212121 (Ypsilanti, MI, USA). SsoAdvanced Universal SYBR Green Supermix (2x), 40% acrylamide, bis-acrylamide solution 29:1, tetramethylethylenediamine (TEMED), and KRAS, a human PrimePCR™ SYBR green assay (120 base pair amplicon), were purchased from Bio-Rad Laboratories (Hercules, CA, USA). Cyclic disulfide-modified, thiol-modified, biotinylated, and unmodified oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA, USA). PCR caps, tube strips, sodium chloride, fresh human whole blood, and Dynabeads Steptavidin M-270 magnetic beads were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Neodymium rod (0.66 T) and cylinder magnets (0.9 T) were purchased from K&J Magnetics (Pipersville, PA, USA). 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 Synthesis and characterization of DTOs, ITOs, and MILs
The trihexyl(tetradecyl)phosphonium ([P_{6,6,6,14}^+] ), tris(hexafluoroacetylaceto)manganate(II) ([Mn(hfacac)_{3}^-]), [P_{6,6,6,14}^+] ), tris(phenyltrifluoroacetylaceto)manganate(II) ([Mn(Phtfacac)_{3}^-]), trioctylbenzylammonium ([N_{8,8,8,Bz}^+] ), [Mn(hfacac)_{3}^-], and [N_{8,8,8,Bz}^+] )

bis(hexafluoroacetylaceto)phenyltrifluoroacetylacetomanganate(II) ([Mn(hfacac)_{2}(Phtfacac)'^-]) MILs were synthesized and characterized as previously reported.[33,36–38] MIL structures are shown in Figure 1. When not in use, all four MILs were stored at room temperature in a desiccator.

The 1-allyl-3-octylimidazolium bromide ([AOIM]^+[Br^-]), 1-allyl-3-decylimidazolium bromide ([ADIM]^+[Br^-]), and 1-allyl-3-benzyllimidazolium bromide ([ABzIM]^+[Br^-]) ILs were synthesized as previously reported.[29] In addition, all ITOs and DTOs were also synthesized as previously reported.[29,34] Briefly, 4 nmol of the thiol-modified oligonucleotide or 2 nmol of the cyclic disulfide-modified oligonucleotide was reduced using 40 nmol of TCEP. Subsequently, 400 nmol of either the [AOIM]^+[Br^-], [ADIM]^+[Br^-], or [ABzIM]^+[Br^-] salt (30% v/v ACN) was reacted with the reduced oligonucleotide under nitrogen (N_2) at 365 nm UV light for approximately 120 min. A NotePal X-slim Cooler Master (New Taipei City, Taiwan) was used to cool the reaction to prevent solvent evaporation. The product was isolated using polyacrylamide gel electrophoresis (PAGE) and recovered by crushing the gel and eluting with water.

ITOs and DTOs were characterized using reversed-phase ion-pair liquid chromatography coupled to time-of-flight mass spectrometry (TOF-MS) on an Agilent 1260 Infinity high performance liquid chromatograph (HPLC) with a diode array detector coupled to an Agilent 6230B TOF mass spectrometer with an electrospray source. ITOs and DTOs were separated on a
50 mm × 2.1 mm i.d. × 1.8 µm particle size Zorbax Extend C₁₈ column purchased from Agilent Technologies (Santa Clara, CA, USA). Mobile phase A contained 5 mM triethylammonium acetate (pH 7.4) and mobile phase B was ACN. The column was equilibrated for 20 min at 0.2 mL min⁻¹ at 5% B. Gradient separation of ITO products was performed using the following program: 5% B from 0 to 5 min, increased from 5% to 19.4% B from 5 to 17 min, increased 19.4 to 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. After each separation, the column was equilibrated at 5% B for 7 min prior to subsequent injections. The LC eluent was diverted to waste for the first 8 min to prevent non-volatile imidazolium salts and urea from entering the mass spectrometer. Nebulizing gas was set to 35 psi, and the drying gas (N₂) flow rate was 9 L min⁻¹ at 350 °C using a capillary voltage of 4000 V. Spectra were acquired from 100 to 3000 m/z with a scan rate of 1 spectrum sec⁻¹. ITO and DTO structures are shown in Figure 2. The extracted ion chromatograms and mass spectra of the [AOIM⁺]₂-KRAS 2[Br⁻], [ADIM⁺]₂-KRAS 2[Br⁻], and [ABzIM⁺]₂-KRAS 2[Br⁻] DTOs are shown in Figure S1 (supplementary information).

2.3 Loading efficiency of ITOs and DTOs to the hydrophobic MIL

The stability of the interaction between the ITO/DTD-O-DNA duplex and MIL was determined by anion exchange chromatography using an Agilent 1260 HPLC with variable wavelength detection. First, 2 ppm of the DNA extraction probe was hybridized with 2 ppm of its complementary sequence in 25 mM NaCl by heating at 90 °C for 5 min then cooling to 4 °C for 5 min. The ITO/DTD-O-DNA duplex was incubated for 10 min at room temperature with 1 µL of MIL. After this, 20 µL of the aqueous solution was injected onto a 35 mm × 4.6 mm i.d. × 2.5 µm TSKgel DEAE-NPR anion exchange column with a 5 mm × 4.6 mm i.d. × 5 µm TSKgel DEAE-NPR guard column from Tosoh Bioscience (King of Prussia, PA). Mobile phase A
consisted of 20 mM Tris-HCl (pH 8) and mobile phase B was 20 mM Tris-HCl and 1 M NaCl (pH 8). The column was equilibrated with mobile phase A at 0.5 mL min$^{-1}$ for 20 min prior to injection. Gradient elution was performed with the following program: increased from 0 to 50% B from 0 to 10 min, increased to 100% B from 10 min to 15 min, held at 100% B from 15 min to 20 min, decreased to 0% B from 20 min to 22 min, held at 0% B from 22 min to 30 min.

### 2.4 qPCR conditions

Quantitative PCR (qPCR) was achieved using 1x Sso Supermix and 1x PrimePCR assay mix with a final volume of 20 µL. Addition of 0.3 µL $[^{P_{6,6,6,14}}][{\text{Mn(hfacac)}}_3]$ MIL to the qPCR buffer required 1x Sso Supermix and 1x PrimePCR assay mix, 4 mM EDTA, and an additional 1x SYBR Green I to relieve inhibition caused by the MIL. Direct MIL-qPCR amplification using the $[^{N_{8,8,8,8,8,8}}][{\text{Mn(hfacac)}}_3]$ MIL required 1x Sso Supermix and 1x PrimePCR assay mix, additional 6.25 mM MgCl$_2$, 4 mM EDTA, and an additional 0.4x SYBR Green I. A 20 µL qPCR containing 0.3 µL of the $[^{N_{8,8,8,8,8,8}}][{\text{Mn(hfacac)}}_3](\text{Phtfacac})^{-}$ MIL required 1x Sso Supermix and 1x PrimePCR assay mix, an additional 2.5 mM MgCl$_2$, 2 mM EDTA, and an additional 1x SYBR Green I. Amplification was achieved on a Bio-rad CFX 96 qPCR using the following cycling protocol: 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. Quantification cycles (Cq) were determined using the Bio-Rad CFX Maestro software. A standard curve was constructed for the KRAS template, as shown in Figure S2, and used to determine the amount of target extracted using the ITO-MIL procedure. Enrichment factors (E$_f$) were determined using equation 1 where C$_{\text{MIL}}$ is the amount of DNA extracted by 0.3 µL of MIL and C$_{\text{std}}$ is the amount of DNA present in a 0.3 µL of the solution prior to the extraction.
2.5 Extraction of target DNA

Static extractions were used to selectively extract high concentrations of target DNA using previously published methods, with slight modifications.[29,34] Briefly, $7.2 \times 10^7$ copies $\mu L^{-1}$ (112 pM) of target DNA was hybridized to either $3.6 \times 10^8$ copies $\mu L^{-1}$ of DTO or $7.2 \times 10^8$ copies $\mu L^{-1}$ of ITO by heating the solution at 90 °C for 5 min to denature double-stranded DNA followed by cooling to 4 °C for 5 min. After hybridization, 1 $\mu$L of MIL was added to the sample for 10 min at room temperature. Subsequently, the MIL was washed with water to remove non-specifically adsorbed DNA and inhibitors from the surface of the MIL. Target DNA was subsequently desorbed in 50 $\mu$L of 25-400 mM NaCl at 90 °C for 1-10 min.

Dispersive extractions were achieved using the previously optimized method with a DTO probe to target ratio of 5:1 and ITO probe to target ratio of 10:1.[33,34] Briefly, 4, 6, or 8 $\mu$L of the $[N_{8,8,8,Bz}^+][Mn(Phtfacac)(hfacac)_2]^-$, $[N_{8,8,8,Bz}^+][Mn(hfacac)_3]^-$, and $[P_{6,6,6,14}^+][Mn(hfacac)_3]^-$ MILs, respectively, were dispersed in a 1 mL solution containing $2 \times 10^4$ copies $\mu L^{-1}$ (33 fM) of KRAS DNA using a vortex (Barnstead International, Dubuque, IA). After a 1 or 3 min dispersion, the MIL was collected using a 0.66 T rod magnet, and 0.3 $\mu$L of the recovered MIL was added to qPCR buffer.

Extractions using the M-270 streptavidin-coated magnetic beads were performed as recommended by the manufacturer. Prior to every extraction, 10 $\mu$g of magnetic beads were washed three times with 5 mM Tris-HCl, 0.5 mM EDTA, and 1 M NaCl (pH 7.5). All DNA extractions consisted of 10:1 biotinylated probe to target ratio in 25 mM NaCl. The sample was initially heated at 90 °C for 5 min then cooled to 4 °C for 5 min to anneal the biotinylated probe.
to the target. Samples were subsequently agitated at 60 rpm or 250 rpm for extractions of 7.2×10⁷ and 2×10⁴ copies µL⁻¹ KRAS, respectively, on an orbital shaker for 10 min to disperse the beads and minimize sedimentation. The beads were collected using a 0.9 T magnet and washed three times with a solution consisting of 5 mM Tris-HCl, 0.5 mM EDTA, and 1 M NaCl (pH 7.5). DNA was desorbed from the beads in 20 µL water by heating to 90 °C for 10 min.

Target KRAS DNA was spiked into plasma and whole blood matrices. Plasma samples were diluted using water whereas whole blood was diluted in saline in order to prevent settling of cellular debris prior to the extraction. The amount of total genomic DNA in whole blood was determined using the QIAmp DNA mini-prep kit following procedures suggested by the manufacturer.

2.6 Statistical analysis

The Student *t*-test was used to determine whether the DTO or ITO probe was extracting significantly more DNA. Selectivity was also determined using the Student *t*-test by comparing the extractions with and without a DNA extraction probe. Probability values (p-values) were determined from the *t*-test results, and a significance level of 0.05 was chosen. Therefore, if the p-value is less than 0.05, the two data sets were considered statistically different.

3. Results and Discussion

3.1 Synthesis of novel DTO probes

Since ITO and DTO probes can interact with the MIL through hydrophobic interactions, incorporation of longer alkyl chain substituents within the probe would be expected to improve the amount of DNA loaded to the MIL. Previously, an [AOIM⁺][Br⁻] ITO specific to a 261 bp DNA sequence was capable of achieving a modest loading efficiency of 48±4% with the
[P_{6,6,6,14}][\text{Mn(hfacac)}_3] \text{MIL}; however, increasing the alkyl chain by just two carbons significantly improved the loading efficiency to 75±5%.[34] In this study, a novel DTO containing two decyl-imidazolium headgroups was synthesized to further improve the loading efficiency of the probe to the MIL. Attempts to synthesize a DTO with alkyl chains longer than ten carbon atoms resulted in the allylimidazolium cation reacting with only one of the two thiol groups, as shown using MS (Figure S3). The incomplete reaction maybe due to the imidazolium salt exhibiting surfactant-like properties and forming aggregates with the cyclic disulfide-modified oligonucleotide.[39]

In order to identify the optimum ITO and DTO for loading target DNA to the MIL, the loading efficiency of four ITOs and seven DTOs to three hydrophobic MILs was studied, as shown in Figure 3. The [AOIM\textsuperscript{+}]-KRAS [PF\textsubscript{6} - ] ITO (56.94±1.61%) provided the highest loading efficiency to the [P_{6,6,6,14}][\text{Mn(hfacac)}_3] \text{MIL} whereas there was no significant difference in loading efficiencies observed for the [ADIM\textsuperscript{+}]-KRAS 2[Br\textsuperscript{-}] (87.65±2.21%) and [ADIM\textsuperscript{+}]-2-KRAS 2[OS\textsuperscript{-}] DTOs (83.56±5.06%). For the [N_{8,8,8,Bz}][\text{Mn(hfacac)}_3] \text{MIL}, the [ABzIM\textsuperscript{+}]-KRAS [Br\textsuperscript{-}] ITO (65.94±9.55%) produced the highest loading and there was no significant difference in loading for the [ADIM\textsuperscript{+}]-KRAS [Br\textsuperscript{-}] (86.28±4.39%), [ADIM\textsuperscript{+}]-KRAS [PF\textsubscript{6} - ] (94.28±2.47%), and [ABzIM\textsuperscript{+}]-KRAS [Br\textsuperscript{-}] DTOs (89.2±2.68%). Based on the Student \(t\)-test, there was no significant advantage in loading of the DNA extraction probe to the [N_{8,8,8,Bz}][\text{Mn(hfacac)}_2(\text{Phtfacac})\textsuperscript{-}] MIL using either the [AOIM\textsuperscript{+}]-KRAS 2[Br\textsuperscript{-}] (56.91±7.67%) or [ADIM\textsuperscript{+}]-KRAS 2[Br\textsuperscript{-}] DTOs (60.83±3.95%). Among the ITOs and DTOs examined, the [ABzIM\textsuperscript{+}]-KRAS [Br\textsuperscript{-}] ITO (53.69±2.27%) produced the highest loading efficiency using the [N_{8,8,8,Bz}][\text{Mn(hfacac)}_2(\text{Phtfacac})\textsuperscript{-}] MIL.
It was previously reported that ITOs containing the [OS:] anion exhibited superior loading to the MIL solvent compared to [Br⁻], bis[(trifluoromethyl)sulfonyl]imide, or perfluorobutanesulfonate anions suggesting that the anion plays an important role in facilitating the capture of the ITO by the MIL.[34] Therefore, replacing the [Br⁻] anion within the DTO structure with the amphiphilic [OS:] anion should improve the loading of the DTO probe to the MIL. However, introducing the [OS:] anion to the DTO did not significantly improve the loading efficiency. Utilizing the [PF₆⁻] anion to facilitate fluorophilic interactions between the DTO and MIL also did not improve the loading of the probe to the MIL. These results suggest that the anion component of the DTO plays a less important role in facilitating interactions between the DTO and the MIL compared to the ITO probe. Interestingly, the [ABzIM⁺][KRAS]₂[Br⁻] DTO showed relatively low loading to the [P₆,₆,₆,₁₄⁺][Mn(Phtfacac)₃⁻] (17.21±1.70%) and [N₈,₈,₈,Bz⁺][Mn(hfacac)₂(Phtfacac)⁻] (14.23±0.91%) MILs. The [ABzIM⁺][KRAS]₂[Br⁻] DTO was effective at loading to the [N₈,₈,₈,Bz⁺][Mn(hfacac)₃⁻] MIL and the [ABzIM⁺]-KRAS [Br⁻] ITO exhibited the highest loading efficiencies for the [N₈,₈,₈,Bz⁺][Mn(hfacac)₃⁻] and [N₈,₈,₈,Bz⁺][Mn(hfacac)₂(Phtfacac)⁻] MILs. These results suggest that exploiting π-π stacking interactions with the DTOs is effective when the aromatic moieties are only in the cation.

3.2 Distinguishing SNPs from target DNA using ITO and DTO probes

In blood plasma samples, especially with late stage cancer patients, there are large amounts of non-target background DNA fragments (ranging from 0-100 pM) that can potentially be co-extracted.[27] In addition, ctDNA fragments are prone to SNPs and require great care to ensure that only the desired sequence is detected. To evaluate the effect interfering DNA has on the annealing of target DNA to the extraction probe, extractions from samples containing 7.2×10⁷ copies μL⁻¹ of 20 nt complement, 1 nt mismatch, or 2 nt mismatch fragments (i.e., 1:1
ratio with target DNA) were performed using the \([P_{6,6,6,14}^+]\text{[Mn(hfacac)}_3^-]\) MIL and streptavidin coated magnetic beads. Without interfering fragments present, the \([\text{ADIM}^+]_2\text{-KRAS 2[Br]}\) and \([\text{ADIM}^+]_2\text{-KRAS 2[OS]}\) DTOs extracted the most DNA (target capture Cq of 21.13±0.30 and 21.28±0.05, respectively). The addition of a 20 nt complementary sequence to the sample resulted in a higher target capture Cq indicating that less of the 120 bp target was extracted, as shown in Figure S4. These results suggest that the complementary fragment competed with the 120 bp amplicon for the probe causing lower DNA recoveries. The addition of either 1 or 2 nt mismatch fragments to the sample solution did not significantly increase the target capture Cq when using the \([\text{AOIM}^+]_2\text{-KRAS 2[Pf]}\), \([\text{ABzIM}^+]\text{-KRAS [Br]}\), \([\text{AOIM}^+]_2\text{-KRAS 2[Br]}\), \([\text{AOIM}^+]_2\text{-KRAS 2[OS]}\), \([\text{ADIM}^+]_2\text{-KRAS 2[Br]}\), and \([\text{ADIM}^+]_2\text{-KRAS 2[OS]}\) extraction probes. This suggests that the 1 nt and 2 nt mismatch fragments are not interfering with the annealing of target DNA to the probe.

The streptavidin-coated magnetic beads also experienced an increase in target capture Cq when performing extractions in the presence of a 20 nt complement. However, the target capture Cq also increased when performing extractions with 1 nt and 2 nt mismatch fragments spiked in the sample. This increase suggests that the biotinylated probe is annealing to the 1 and 2 nt mismatch fragment and, therefore, reducing the amount of target DNA that can be captured.

### 3.3 Optimization of the desorption step

Although there has been a significant amount of work performed to improve the loading efficiency of the ITO-DNA duplex to the MIL, few studies have evaluated the desorption step. Therefore, the desorption time and ionic strength of the desorption solution were optimized to improve the recovery of DNA from the \([P_{6,6,6,14}^+]\text{[Mn(hfacac)}_3^-]\), \([N_{8,8,8,8,Bz}^+]\text{[Mn(hfacac)}_3^-]\), and \([N_{8,8,8,8,Bz}^+]\text{[Mn(hfacac)}_2\text{(Phtfacac)}^-]\) MILs. As shown in Figure S5, an optimum desorption time
of 4 min was achieved for the \([P_{6,6,6,14}^+][\text{Mn(hfacac)}_3^-]\) and \([N_{8,8,8,\text{Bz}}^+][\text{Mn(hfacac)}_2(\text{Phtfacac})^-]\) MILs, and an optimum desorption time of 4 min and 8 min was achieved for the \([N_{8,8,8,\text{Bz}}^+][\text{Mn(hfacac)}_3^-]\) MIL with the ITO and DTO probe, respectively. Less DNA was recovered after longer desorption times likely due to DNA adsorbing to the polypropylene tube.[40] Therefore, 0.05% Tween 20 was added to the desorption solution in an effort to minimize adsorption. A 4 min desorption time was chosen as optimum due to the high amount of DNA recovered using all three MILs, while still maintaining a short desorption time. In addition, the presence of NaCl greatly promoted the desorption of DNA, as shown in Figure S6. The optimum concentration of NaCl was 100 mM for the \([P_{6,6,6,14}^+][\text{Mn(hfacac)}_3^-]\) MIL, 200 mM for the \([N_{8,8,8,\text{Bz}}^+][\text{Mn(hfacac)}_3^-]\) MIL, and 50 mM for the \([N_{8,8,8,\text{Bz}}^+][\text{Mn(hfacac)}_2(\text{Phtfacac})^-]\) MIL.

There was no significant difference between the amount of DNA detected when ITO and DTO were used to extract DNA if desorbing at higher ionic strength. This suggests that electrostatic interactions between the ITO or DTO and the MIL may also play a role in the capture of the ITO/DTO-DNA duplex.

Recent studies have exploited the elevated temperatures required for PCR to desorb DNA from the MIL solvent during the reaction.[32,33,41] Adding DNA-enriched MIL to the PCR buffer is highly beneficial as it reduces the number of sample handling steps, allowing for higher sample throughput and minimizes the possibility of contamination. Interestingly, despite similar target capture Cqs being achieved with the DTO and ITO probes using 25 to 400 mM NaCl as a desorption solution, lower target capture Cqs and higher \(E_t\) were achieved using the ITOs when desorbing in 1x Sso Supermix, as shown in Figures S7 and S8.

### 3.4 Sequence-specific DNA extractions from complex matrices
Several reported sequence-specific DNA extraction procedures isolate total DNA prior to an extraction due to the complexity of the sample matrix.[20,42] However, this is often very time consuming, and an ideal sequence-specific DNA extraction should be capable of selectively extracting DNA from complex sample matrices. Previously, the ITO-MIL procedure has been applied towards bacterial cell lysate (1.53×10⁸ E. coli cells),[29] 10-fold diluted plasma,[33] and plant cell lysate.[35] However, it has been unclear if either the ITO or DTO probe can extract more DNA from complex matrices. When comparing the extraction of KRAS from 10-fold diluted plasma using the DTO or ITO probe, more DNA was detected using the [AOIM⁺]-KRAS [PF₆⁻] ITO with the [P₆,₆,₆,₁₄⁺][Mn(hfacac)₃] MIL and the [ADIM⁺₂]-KRAS 2[Br⁻] DTO probe with the [N₈,₈,₈,Bz⁺][Mn(hfacac)₃] MIL, as shown in Figure 4. There was no significant difference observed between the [ADIM⁺₂]-KRAS 2[Br⁻] DTO and [ABzIM⁺]-KRAS [Br⁻] ITO probes with the [N₈,₈,₈,Bz⁺][Mn(hfacac)₃(Phtfacac)⁻] MIL in 10-fold diluted plasma. At femtomolar concentration-levels of target DNA, similar Eᵣ was achieved using either the [ADIM⁺₂]-KRAS 2[Br⁻] DTO or [ABzIM⁺]-KRAS [Br⁻] ITO probes with the [N₈,₈,₈,Bz⁺][Mn(hfacac)₃] MILs, as shown in Figure 5. The [AOIM⁺]-KRAS [PF₆⁻] ITO was capable of preconcentrating significantly more DNA from 10-fold diluted plasma compared to the [ADIM⁺₂]-KRAS 2[Br⁻] DTO probe with the [P₆,₆,₆,₁₄⁺][Mn(hfacac)₃] MIL.

Selective preconcentration of 2×10² and 2×10³ copies µL⁻¹ (0.33 and 3.3 fM, respectively) of KRAS from 10-fold diluted plasma was achieved using the ITO and DTO probes, as shown in Figure S9. However, based on the Student t-test there was no significant difference in Eᵣ using the DTO or ITO at these concentrations suggesting that both probes function similarly at enriching low concentrations of DNA. When performing extractions of 2×10² copies µL⁻¹ of KRAS from 10-fold diluted plasma, the [N₈,₈,₈,Bz⁺][Mn(hfacac)₃] and
[\text{N}_{8,8,8,Bz}^+][\text{Mn(hfacac)}_2(\text{Phtfacac})^-]\text{MILs exhibited slight preconcentration of DNA suggesting that the selectivity of the ITO/DTO-MIL method worsens as the concentration of target DNA decreases. Interestingly, extractions of either 7.2\times10^7 or 2\times10^4 \text{ copies mL}^{-1} \text{ KRAS DNA from 10-fold diluted plasma using streptavidin-coated magnetic beads were not selective. It is possible that free biotin naturally found in plasma blocks binding sites on the beads or that the biotinylated probe is binding to plasma proteins.}[43–45]

Previous studies have shown that imidazolium ILs that contain longer alkyl chain substituents (i.e., octyl chain length) interact more with proteins.[46] In the case of the ITO/DTO-procedure, the longer alkyl chains are used to improve loading of the probe-DNA duplex to the hydrophobic MIL; however, it is unclear if certain ITOs or DTOs strongly interact with plasma proteins instead of the MIL. Therefore, extractions from 10-fold diluted plasma were performed using the [\text{ADIM}^+]_2-\text{KRAS} 2[\text{Br}^-] and [\text{ABzIM}^+]_2-\text{KRAS} 2[\text{Br}^-] DTOs with the [\text{N}_{8,8,8,Bz}^+][\text{Mn(hfacac)}_3^-] \text{MIL and the [ADIM}^+]_2-\text{KRAS} 2[\text{Br}^-] and [\text{ADIM}^+]_2-\text{KRAS} 2[\text{OS}^-] \text{DTOs with the [P}_{6,6,6,14}^+][\text{Mn(hfacac)}_3^-] \text{MIL. These DTOs were chosen because they exhibited similar loading efficiencies and, therefore, should extract similar amounts of DNA. As shown as Figure S10, the target capture Cqs associated with the extractions using the [\text{ADIM}^+]_2-\text{KRAS} 2[\text{Br}^-] and [\text{ABzIM}^+]_2-\text{KRAS} 2[\text{Br}^-] DTOs with the [\text{N}_{8,8,8,Bz}^+][\text{Mn(hfacac)}_3^-] \text{MIL and the [ADIM}^+]_2-\text{KRAS} 2[\text{Br}^-] and [\text{ADIM}^+]_2-\text{KRAS} 2[\text{OS}^-] \text{DTOs with the [P}_{6,6,6,14}^+][\text{Mn(hfacac)}_3^-] \text{MIL were within error. The target capture Cqs suggest that the different DTO probes suffer from similar matrix effects due to the plasma components.}

Diluting the sample matrix can be beneficial as it decreases the concentration of PCR inhibitors and reduces the amount of protein aggregates. However, diluting the sample matrix also decreases the amount of DNA making it more challenging to detect low abundance
mutations. Therefore, ITO/DTO-MIL extractions from 2-fold diluted plasma were investigated. Selective extractions from 2-fold diluted plasma were achieved for all three MILs, as shown in Figure S11. However, extractions using the DTO probe produced lower target capture Cqs compared to extractions using the ITO probes, possibly due to stronger interactions between the probe and hydrophobic MIL. The streptavidin-coated magnetic beads were capable of selective extraction in 2-fold diluted plasma whereas previously the extractions with and without the biotinylated probe in 10-fold diluted plasma were within error of one another. This may be linked to the significant amount of protein aggregation that occurs during the annealing step with 2-fold diluted plasma. Protein precipitation could limit the amount of biotinylated probe that interacts with soluble plasma proteins and allow for higher DNA recoveries.[43]

In most ctDNA sample preparation methods, ctDNA is extracted from plasma instead of whole blood due to the high probability of contaminating the sample with genomic DNA from circulating cells.[47,48] To ensure that plasma is cell-free, a lengthy double centrifugation step is required.[47,48] In addition, the process of generating plasma from blood removes circulating cell surface-bound DNA from analysis, which can aid cancer diagnosis and monitoring.[49] Therefore, the ITO/DTO-MIL method was applied towards the extraction of KRAS from 10-fold diluted whole blood to examine if selective extraction could be achieved. When performing ITO/DTO-MIL extractions from 10-fold diluted blood at picomolar levels of DNA, selective extraction was achieved using the \([\text{P}_{6,6,6,14}^+]\)[Mn(hfacac)\(_2\)] and \([\text{N}_{8,8,8,Bz}^+]\)[Mn(hfacac)\(_2\)] MILs, as shown in Figure 6. However, selective extraction could not be achieved using the \([\text{ABzIM}^+]\)-KRAS [Br\(^-\)] ITO with the \([\text{N}_{8,8,8,Bz}^+]\)[Mn(hfacac)\(_2\)(Phtfacac)] MIL or the magnetic beads. Selective extraction using the magnetic beads could only be achieved when diluting the blood 40-fold, as shown in Figure S12. It was also possible to achieve selective extraction using the
streptavidin-coated magnetic beads by proteinase K treatment at 56 °C for 15 min prior to annealing the probe to target DNA, as shown in Figure 7. In this procedure, the sample was also filtered to remove cellular debris and precipitated proteins before the magnetic beads were added. It was found that the \([\text{N}_{8,8,8,\text{Bz}}^+][\text{Mn(hfacac)}_2(\text{Phtfacac})^-]\) MIL was unable to selectively extract target DNA due to the saline solution used to dilute whole blood, as shown in Figure S13. Extractions from saline using the \([\text{P}_{6,6,6,14}^+][\text{Mn(hfacac)}_3^-]\) or \([\text{N}_{8,8,8,\text{Bz}}^+][\text{Mn(hfacac)}_3^-]\) MILs resulted in higher target capture Cqs when compared to extractions performed from pure water, possibly due to the higher ionic strength increasing the melting temperature of the target DNA. The ITO probes were capable of extracting more DNA from saline compared to the DTO, but all extractions from saline with the \([\text{P}_{6,6,6,14}^+][\text{Mn(hfacac)}_3^-]\) or \([\text{N}_{8,8,8,\text{Bz}}^+][\text{Mn(hfacac)}_3^-]\) MILs were selective.

When performing extractions from clinically-relevant concentrations of ctDNA, selective extraction using either the ITO or DTO probes was achieved with the \([\text{P}_{6,6,6,14}^+][\text{Mn(hfacac)}_3^-]\) and \([\text{N}_{8,8,8,\text{Bz}}^+][\text{Mn(hfacac)}_3^-]\) MILs, as shown in Figures 7. However, the DTO preconcentrated significantly more DNA compared to the ITO in whole blood matrices possibly due to stronger interactions between the probe and MIL during the extraction and washing steps. When performing extractions at \(2 \times 10^2\) and \(2 \times 10^3\) copies µL\(^{-1}\) (mutation abundance of 0.009% and 0.09%, respectively) with the \([\text{P}_{6,6,6,14}^+][\text{Mn(hfacac)}_3^-]\) and \([\text{N}_{8,8,8,\text{Bz}}^+][\text{Mn(hfacac)}_3^-]\) MILs, selective preconcentration of target DNA could still be achieved using the \([\text{ADIM}^\text{+}][\text{KRAS} 2[\text{Br}^-]\) DTO, as shown in Figure S14. However, extractions of \(2 \times 10^2\) copies µL\(^{-1}\) \(\text{KRAS}\) from 10-fold diluted blood resulted in slight preconcentration when the DTO probe was not present, again suggesting that the DTO-MIL method is prone to co-extraction under conditions of low target...
DNA concentration. This effect could possibly be limited by designing the MIL to further limit DNA extraction.

4. Conclusions

The ability of the ITO/DTO-MIL method to selectively extract DNA from 2-fold diluted plasma, 10-fold diluted plasma, and 10-fold diluted whole blood has been demonstrated. The desorption time and ionic strength of the desorption solution were optimized to ensure the maximum amount of DNA was desorbed from the MIL in a short amount of time (i.e., 4 min), with higher ionic strength significantly facilitating the desorption of DNA from the hydrophobic MIL. ITO probes were successful at extracting more DNA in saline compared to DTO probes, but DTO probes outperformed ITOs by extracting more DNA from complex biological matrices, including 2-fold diluted plasma and 10-fold diluted blood. However, there was no significant difference in enrichment factor when performing extractions from 10-fold diluted plasma with either the ITO or DTO. In comparison, commercially-available streptavidin-coated magnetic beads only exhibited selectivity in 2-fold diluted plasma and 40-fold diluted whole blood without labor-intensive sample clean-up steps. The DTO-MIL method represents a simple two-step extraction procedure to selectively preconcentrate low concentrations of DNA from increasingly complex matrices, suggesting that this method has the potential to be highly valuable in the field of ctDNA detection and analysis.

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Figure Legends:

**Fig. 1** Chemical structures of the (1) \([P_{6,6,6,14}^+][Mn(hfacac)_3^-]\), (2) \([P_{6,6,6,14}^+][Mn(Phtfacac)_3^-]\), (3) \([N_{8,8,8,Bz}^+][Mn(hfacac)_3^-]\), and (4) \([N_{8,8,8,Bz}^+][Mn(hfacac)_2(Phtfacac^-)]\) MILs examined in this study.

**Fig. 2** Chemical structures of the imidazolium-based ion tags associated with the (A–B) ITOs and (C–E) DTOs examined in this study.

**Fig. 3** Loading efficiencies of the ITO and DTO probes to the (a) \([P_{6,6,6,14}^+][Mn(hfacac)_3^-]\), (b) \([N_{8,8,8,Bz}^+][Mn(hfacac)_3^-]\), and (c) \([N_{8,8,8,Bz}^+][Mn(hfacac)_2(Phtfacac^-)]\) MILs. ITO or DTO concentration: 2 ng \(\mu\)L\(^{-1}\); \(KRAS\) complement: 2 ng \(\mu\)L\(^{-1}\); NaCl concentration: 25 mM; sample volume: 60 \(\mu\)L; MIL volume: 1 \(\mu\)L; extraction time: 10 min.

**Fig. 4** Target capture Cq associated with the static extraction using the (blue) \([ADIM^+][2-KRAS\ 2[Br^-]]\) DTO, (green) highest loading ITO, (orange) biotinylated probe, and (grey) without a DNA extraction probe from 10-fold diluted plasma. ITO/DTO-MIL method: \(KRAS\) template concentration: 7.2\(\times\)10\(^7\) copies \(\mu\)L\(^{-1}\), amount of \([ADIM^+]-KRAS\ 2[Br^-]\) DTO relative to DNA: 5:1.
amount of [AOIM$^+$]-KRAS [PF$_6$] ITO relative to DNA: 10:1, sample volume: 50 µL, MIL volume: 1 µL; extraction time: 10 min, desorption time: 4 min, desorption volume: 50 µL.

Dynabeads M-270 Steptavidin magnetic beads conditions: KRAS template concentration: 2×10$^4$ copies µL$^{-1}$, concentration of biotinylated probe: 332 fM, NaCl concentration: 25 mM, extraction time: 10 min; agitation rate: 250 rpm; desorption time: 10 min; desorption volume: 50 µL.

**Fig. 5** Enrichment factors from the dispersive extraction using the (blue) [ADIM$^+$]-2-KRAS [Br$^-$] DTO, (green) highest loading ITO, (orange) biotinylated probe, and (grey) without a DNA extraction probe from 10-fold diluted plasma. [P$_{6,6,6,14}^+$] [Mn(hfacac)$_3$] MIL conditions: amount of [ADIM$^+$]-KRAS 2[Br$^-$] DTO relative to DNA: 5:1, amount of [AOIM$^+$]-KRAS [PF$_6$] ITO relative to DNA: 10:1, NaCl concentration: 25 mM, sample volume: 1.0 mL, extraction time: 3 min. [N$_{8,8,8,Bz}^+$][Mn(hfacac)$_3$] MIL conditions: amount of [ADIM$^+$]-KRAS 2[Br$^-$] DTO relative to DNA: 5:1, amount of [ABzIM$^+$]-KRAS [Br$^-$] relative to DNA: 10:1, NaCl concentration: 25 mM, sample volume: 1.0 mL, extraction time: 1 min. Dynabeads M-270 Steptavidin magnetic beads conditions: concentration of biotinylated probe: 332 fM; extraction time: 10 min; agitation rate: 250 rpm; desorption time: 10 min; desorption volume: 20 µL.

**Fig. 6** Target capture Cq obtained from static extraction using the (blue) [ADIM$^+$]-2-KRAS 2[Br$^-$] DTO, (green) highest loading ITO, and (orange) biotinylated probe compared to extractions (grey) without a DNA extraction probe from 10-fold diluted blood. ITO/DTO-MIL method: KRAS template concentration: 7.2×10$^7$ copies µL$^{-1}$, amount of [ADIM$^+$]-KRAS 2[Br$^-$] DTO relative to DNA: 5:1, amount of [AOIM$^+$]-KRAS [PF$_6$] ITO relative to DNA: 10:1, sample volume: 50 µL, MIL volume: 1 µL; extraction time: 10 min, desorption time: 4 min, desorption volume: 50 µL. Dynabeads M-270 Steptavidin magnetic beads conditions: KRAS template concentration: 7.2×10$^7$ copies µL$^{-1}$, concentration of biotinylated probe: 7.2×10$^8$ copies µL$^{-1}$; extraction time: 10 min; agitation rate: 250 rpm; desorption time: 10 min; desorption volume: 50 µL.

**Fig. 7** Enrichment factor produced from the dispersive extraction of KRAS target using the (blue) [ADIM$^+$]-2-KRAS 2[Br$^-$] DTO, (green) highest loading ITO, and (orange) biotinylated probe compared to extractions (grey) without a DNA extraction probe from 10-fold diluted blood. [P$_{6,6,6,14}^+$] [Mn(hfacac)$_3$] MIL conditions: amount of [ADIM$^+$]-KRAS 2[Br$^-$] DTO relative to DNA: 5:1, amount of [AOIM$^+$]-KRAS [PF$_6$] ITO relative to DNA: 10:1, NaCl concentration: 25 mM, sample volume: 1.0 mL, extraction time: 3 min. [N$_{8,8,8,Bz}^+$][Mn(hfacac)$_3$] MIL conditions: amount of [ADIM$^+$]-KRAS 2[Br$^-$] DTO relative to DNA: 5:1, amount of [ABzIM$^+$]-KRAS [Br$^-$] relative to DNA: 10:1, NaCl concentration: 25 mM, sample volume: 1.0 mL, extraction time: 1 min. Dynabeads M-270 Steptavidin magnetic beads conditions: concentration of biotinylated probe: 332 fM, extraction time: 10 min; agitation rate: 250 rpm; desorption time: 10 min; desorption volume: 20 µL.
A = [AOIM\(^+\)]-KRAS
B = [ABzIM\(^+\)]-KRAS
C = [AOIM\(^+\)]\(_2\)-KRAS
D = [ADIM\(^+\)]\(_2\)-KRAS
E = [ABzIM\(^+\)]\(_2\)-KRAS
Magnetic Beads
\[ \text{Cq} = \text{Magnetic Beads} \]
Magnetic Beads

![Graph showing comparison of [P,0,6,1r] and [N,3,8,Br] with Magnetic Beads. The graph plots the E_r values for each compound against the presence of magnetic beads.]
Highlights:

- Mono- and disubstituted ion-tagged oligonucleotides used as DNA probes
- Magnetic ionic liquid (MIL) solvents used to capture oligonucleotide probes
- KRAS DNA was captured using probes and MIL from blood and plasma samples
- Disubstituted oligonucleotide probes captured more DNA from diluted plasma and blood
- Conventional streptavidin-coated magnetic beads were only selective after sample clean up steps
Conflict of Interest Statement:

The authors declare no conflicts of interest in this work.