Allelic discrimination between circulating tumor DNA fragments enabled by a multiplex-qPCR assay containing DNA-enriched magnetic ionic liquids

Miranda N. Emaus
Iowa State University, memaus@iastate.edu

Jared L. Anderson
Iowa State University, andersoj@iastate.edu

Follow this and additional works at: https://lib.dr.iastate.edu/chem_pubs

Part of the Analytical Chemistry Commons, and the Medicinal-Pharmaceutical Chemistry Commons

The complete bibliographic information for this item can be found at https://lib.dr.iastate.edu/chem_pubs/1236. For information on how to cite this item, please visit http://lib.dr.iastate.edu/howtocite.html.

This Article is brought to you for free and open access by the Chemistry at Iowa State University Digital Repository. It has been accepted for inclusion in Chemistry Publications by an authorized administrator of Iowa State University Digital Repository. For more information, please contact digirep@iastate.edu.
Allelic discrimination between circulating tumor DNA fragments enabled by a multiplex-qPCR assay containing DNA-enriched magnetic ionic liquids

Abstract
Multiplex amplification of DNA can be highly valuable in circulating tumor DNA (ctDNA) analysis due to the sheer number of potential mutations. However, commercial ctDNA extraction methods struggle to preconcentrate low concentrations of DNA and require multiple sample handling steps. Recently, magnetic ionic liquids (MILs) have been used to extract DNA and were integrated with a quantitative polymerase chain reaction (qPCR). However, in previous studies, DNA could not be preconcentrated from plasma and only one fragment could be amplified per reaction. In this study, MILs were utilized as DNA extraction solvents and directly integrated into a multiplex-qPCR buffer to simultaneously amplify wild-type KRAS, G12S KRAS, and wild-type BRAF, three clinically-relevant genes whose mutation status can affect the success of anti-EGFR therapy. DNA was desorbed from the MIL solvent during a multiplex-PCR without having a significant effect on the amplification efficiency, and allelic discrimination of single-nucleotide polymorphisms could still be achieved. Enrichment factors over 35 for all three sequences were achieved from Tris buffer using the \([\text{N}8,8,8,\text{Bz}^+][\text{Ni(hfacac)}3^-]\) and \([\text{P}6,6,6,14^+][\text{Ni(Phtfacac)}3^-]\) MILs. DNA could still be preconcentrated from 2-fold diluted human plasma using the \([\text{N}8,8,8,\text{Bz}^+][\text{Ni(hfacac)}3^-]\) MIL. Extractions from undiluted plasma were reproducible with the \([\text{P}6,6,6,14^+][\text{Ni(Phtfacac)}3^-]\) MIL although DNA was not preconcentrated with enrichment factors around 0.6 for all three fragments. Compared to commercial DNA extraction methods (i.e., silica-based spin columns and magnetic beads), the MIL-based extraction achieved higher enrichment factors in Tris buffer and plasma. The ability of the MIL-based dispersive liquid-liquid microextraction (DLLME) direct-multiplex-qPCR method to simultaneously achieve high enrichment factors of multiple DNA fragments from human plasma is highly promising in the field of ctDNA detection.

Keywords
DNA extraction, Cell-free DNA, Multiplex PCR, Magnetic ionic liquid

Disciplines
Analytical Chemistry | Medicinal-Pharmaceutical Chemistry

Comments
This is a manuscript of an article posted as Emaus, Miranda N., and Jared L. Anderson. "Allelic discrimination between circulating tumor DNA fragments enabled by a multiplex-qPCR assay containing DNA-enriched magnetic ionic liquids." Analytica Chimica Acta (2020). DOI: 10.1016/j.aca.2020.04.078. Posted with permission.

Creative Commons License
This work is licensed under a Creative Commons Attribution-Noncommercial-No Derivative Works 4.0 License.

This article is available at Iowa State University Digital Repository: https://lib.dr.iastate.edu/chem_pubs/1236
Allelic discrimination between circulating tumor DNA fragments enabled by a multiplex-qPCR assay containing DNA-enriched magnetic ionic liquids

Miranda N. Emaus, Jared L. Anderson

PII: S0003-2670(20)30511-0
DOI: https://doi.org/10.1016/j.aca.2020.04.078
Reference: ACA 237639

To appear in: Analytica Chimica Acta

Received Date: 17 February 2020
Revised Date: 24 April 2020
Accepted Date: 30 April 2020

Please cite this article as: M.N. Emaus, J.L. Anderson, Allelic discrimination between circulating tumor DNA fragments enabled by a multiplex-qPCR assay containing DNA-enriched magnetic ionic liquids, Analytica Chimica Acta, https://doi.org/10.1016/j.aca.2020.04.078.

This is a PDF file of an article that has undergone enhancements after acceptance, such as the addition of a cover page and metadata, and formatting for readability, but it is not yet the definitive version of record. This version will undergo additional copyediting, typesetting and review before it is published in its final form, but we are providing this version to give early visibility of the article. Please note that, during the production process, errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

© 2020 Elsevier B.V. All rights reserved.
DNA Capture using Magnetic Ionic Liquid (MIL) from Plasma

MIL Collection with Rod Magnet

Add MIL to Multiplex Buffer

Desorb and Amplify DNA

Graphs showing HEX and FAM signal over cycles.
Allelic discrimination between circulating tumor DNA fragments enabled by a multiplex-qPCR assay containing DNA-enriched magnetic ionic liquids

Miranda N. Emaus and Jared L. Anderson*

Department of Chemistry, Iowa State University, Ames, Iowa 50011, United States

Abstract

Multiplex amplification of DNA can be highly valuable in circulating tumor DNA (ctDNA) analysis due to the sheer number of potential mutations. However, commercial ctDNA extraction methods struggle to preconcentrate low concentrations of DNA and require multiple sample handling steps. Recently, magnetic ionic liquids (MILs) have been used to extract DNA and integrated into a quantitative polymerase chain reaction (qPCR). However, in previous studies, DNA could not be preconcentrated from plasma in previous studies and only one fragment could be amplified per reaction. In this study, MILs were utilized as DNA extraction solvents and directly integrated into a multiplex-qPCR buffer to simultaneously amplify wild-type KRAS, G12S KRAS, and wild-type BRAF, three clinically-relevant genes whose mutation status can affect the success of anti-EGFR therapy. DNA was desorbed from the MIL solvent during a multiplex-PCR without having a significant effect on the amplification efficiency, and allelic discrimination of single nucleotide polymorphisms could still be achieved. Enrichment factors over 35 for all three sequences were achieved from Tris buffer using the \[[N_{8,8,8,Bz}^+][\text{Ni(hfacac)}_3]\] and \[[P_{6,6,6,14}^+][\text{Ni(Phtfacac)}_3]\] MILs. DNA could still be preconcentrated from 2-fold diluted human plasma using the \[[N_{8,8,8,Bz}^+][\text{Ni(hfacac)}_3]\] MIL. Extractions from undiluted plasma were reproducible with the \[[P_{6,6,6,14}^+][\text{Ni(Phtfacac)}_3]\] MIL although DNA was not preconcentrated with enrichment factors around 0.6 for all three fragments. Compared to commercial DNA extraction methods (i.e., silica-based spin columns and magnetic beads), the MIL-based extraction could achieve higher enrichment factors in Tris buffer and plasma. The ability of the MIL-based dispersive liquid-liquid microextraction (DLLME) direct-multiplex-qPCR method to simultaneously achieve high enrichment factors of multiple DNA fragments from human plasma is highly promising in the field of ctDNA detection.

Keywords: DNA extraction; cell-free DNA; multiplex PCR; magnetic ionic liquid

----------------------------------
Corresponding author:
Jared L. Anderson
Department of Chemistry
Iowa State University
1605 Gilman Hall
Ames, IA 50011 USA
515-294-8356
andersoj@iastate.edu
1. Introduction

Tumor assessment using circulating tumor DNA (ctDNA) can provide a non-invasive option for cancer diagnosis, assessment of residual disease, and treatment response.[1,2] ctDNA originates from primary or metastatic tumor cells that have undergone apoptosis or necrosis.[3,4] The mutational status of certain genes can have significant impact on the success rate of cancer treatment.[2,5] For example, it has been found that mutated KRAS and BRAF genes correlate to a low success rate of anti-EGFR therapy with Cetuximab and Panitumumab.[6,7] The ability to detect and discern ctDNA mutations from wild-type DNA has ultimately led the way for molecular guided therapies. However, detection of ctDNA is challenging especially during the early stages of cancer due to a low abundance of ctDNA.[2,8] In addition, multiple mutations can be associated with a single gene, and mutations associated with KRAS, BRAF, and PIK3CA are often single nucleotide polymorphisms (SNPs), which can cause false positive results due to mishybridization.[1,3,4,9] Therefore, ctDNA detection methods need to be capable of rapidly discerning low concentrations of SNPs from wild-type DNA.

There are significant advantages of detecting multiple ctDNA sequences simultaneously, such as achieving higher sample throughput and reduced analysis costs.[10] Multiplex quantitative polymerase chain reaction (qPCR) methods have been developed to detect DNA fragments simultaneously using fluorescently-tagged probes. Taqman probes consist of a short oligonucleotide complementary to the target sequence with a fluorophore and quencher. When the probe anneals to the target sequence, the DNA polymerase separates the probe from the quencher producing a detectable signal that allows the amplification of DNA to be monitored in real-time.[11,12] However, the development of a multiplex-qPCR method is challenging. Often times, great care is needed to ensure that amplification bias does not occur, competition for PCR
reagents does not prevent amplification of low abundance targets, and that a proper annealing
temperature is used so the Taqman probe anneals to the proper target.[13,14]

DNA extraction is generally the quintessential first step in DNA analysis and is often an
overlooked bottle neck.[15,16] Without a competent DNA extraction, bioassays such as PCR and
DNA sequencing would not be possible due to the high concentration of inhibitors present in
biological and environmental matrices. Traditionally, phenol-chloroform-based methods are used
to extract DNA. However, this method requires toxic chemicals and requires multiple sample
handling steps that limit the practicality of the technique.[17] Commercial DNA extraction kits
typically involve either silica-based spin columns or magnetic beads. These kits are simple to
use, but often poorly extract low concentrations of small ctDNA fragments.[4,18] In addition,
both spin columns and magnetic beads require several sample handling steps and reagents to
isolate DNA. In general, the more steps introduced in the extraction procedure can also increase
the probability of contamination from PCR inhibitors or DNA. Several non-commercial DNA
extraction methods have been designed to overcome the limitations of commercial DNA
extractions. Recently, centrifugation assisted-immiscible fluid filtration (CIFF) was developed
by Juang et al. to extract DNA using glass microparticles.[16] Transfer of the glass
microparticles from the aqueous sample to a fluorinated oil eliminated the need for numerous
wash steps that are commonplace in commercial methods. However, CIFF still requires a manual
DNA recovery step as well as multiple reagents such as chaotropic salts and fluorinated oil that
can inhibit PCR if not otherwise removed. Chitosan microparticles can efficiently extract DNA
using electrostatic interactions.[19] Chitosan-based extractions do not require chaotropic salts or
organic solvents like silica-based methods, and DNA can be desorbed from chitosan
microparticles during PCR.[20] Using the PCR system to desorb DNA removes a sample
handling step from the procedure. However, chitosan microparticles were shown to significantly
decrease PCR efficiency likely due to the adsorption of PCR components to the particles.

Ionic liquids (ILs) have been widely used to extract DNA through hydrophobic and
electrostatic interactions.[21–23] ILs are molten salts with melting temperatures under 100 °C
that possess unique physical properties such as negligible vapor pressures, high thermal stability,
and tunable physiochemical properties.[24–26] Magnetic ionic liquids (MILs) possess similar
properties to ILs.[24,27–29] However, MILs contain a paramagnetic component in either the
anion or cation structure, which allows MIL droplets to be collected on a magnet.[15,30]
Hydrophobic MILs have been applied in dispersive liquid-liquid microextractions (DLLME) to
rapidly extract DNA from complex matrices by dispersing fine droplets of the MIL.[15,31–33]
Originally, DNA was recovered from the MIL phase using a short silica column followed by an
alcohol precipitation step.[15] However, this procedure is highly extensive and time consuming.
Therefore, work was done to shorten the desorption process by integrating the DNA-enriched
MIL into a PCR buffer.[34–36] Thermal desorption of DNA during PCR allows for high
enrichment factors while not having a deleterious impact on the efficiency of the reaction.[35,37]
The MIL-DLLME method coupled to direct-qPCR detection is efficient for extracting and
quantifying short DNA sequences, such as ctDNA fragments. However, current approaches
involving direct-qPCR with MIL solvents have only been applied to singleplex reactions.
Although singleplex reactions are important, the development of a MIL-multiplex-PCR system
to simultaneously detect multiple fragments would greatly improve sample throughput and allow
for the detection and discrimination of SNPs.

In this study, three hydrophobic Ni(II)-based MILs were used as DNA extraction
solvents. The DNA-enriched MILs were integrated into a custom designed multiplex-qPCR
assay to successfully amplify three DNA sequences simultaneously. The efficiency of the
reaction was not affected by the hydrophobic MIL, and allelic discrimination between the three
sequences could be achieved even among the SNPs when the MIL was integrated into the
multiplex-qPCR buffer. The volume of MIL dispersed and total extraction time was optimized
for each MIL. Enrichment factors over 35 were achieved for all three DNA fragments with the
\([\text{N}_{8,8,8,8}\text{Bz}]^{+}[\text{Ni(hfacac)}_3]\) and \([\text{P}_{6,6,6,14}^{+}][\text{Ni(Phtfacac)}_3^{-}]\) MILs. Compared to commercial DNA
extraction methods (employing spin columns and magnetic bead), the MILs exhibited superior
preconcentration of DNA in part due to the low desorption volume required for analysis. In
addition, the \([\text{P}_{6,6,6,14}^{+}][\text{Ni(Phtfacac)}_3^{-}]\) MIL was capable of producing higher enrichment factors
from undiluted plasma compared to commercial kits suggesting that the MIL-based extraction
method could be beneficial in extracting ctDNA from clinical samples.

2. Materials and Methods

2.1 Reagents and Materials

Ammonium hydroxide (28-30% solution in water), 1,1,1,5,5,5-hexafluoroacetylacetone
(99%), 1-phenyl-4,4,4-trifluoro-1,3-butane-1-one (99%), nickel(II) chloride (98%), and
triocytamine (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). Agarose was purchased from Lab Express (Ann Arbor,
MI, USA). Cyanine5 (Cy5) carboxylic acid was purchased from Lumiprobe (Hunt Valley, MD,
USA). Ethylenediaminetetraacetic acid (EDTA) (99.4-100.06%), benzyl bromide (98%),
chloroform (>99.8%), lyophilized plasma from human (4% trisodium citrate), lithium
bis[(trifluoromethyl)sulfonylimide ([Li\textsuperscript+][NTf\textsubscript{2}]), guanidine hydrochloride (>98.0), and magnesium chloride hexahydrate (99.0-102.0%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Proteinase K was purchased from New England Biolabs (Ipswich, MA, USA). Apheresis derived pooled human plasma (Na\textsubscript{2}EDTA anticoagulant) was obtained from Innovative Research (Novi, MI, USA). SYBR Green I (10,000x) was purchased from Life Technologies (Carlsbad, CA, USA). Primers, probes, and oligonucleotides (sequences shown in Table S1) were acquired from Integrated DNA Technologies (Coralville, IA, USA). Modified plasmids (3.9 Kbp) were obtained from Eurofin Genomics (Louisville, KY, USA) and contained an insert consisting of 166 bp wild type KRAS, 166 bp G12S KRAS mutation, or 210 bp wild type BRAF. Optically clear PCR caps, tube strips, Taqman universal PCR master mix, isopropanol (99.9%), and Dynabeads myone silane magnetic beads were acquired from Thermo Fisher Scientific (Waltham, MA, USA). Tris-HCl was obtained 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). Microwell plates (364) were purchased from Corning (Corning, NY, USA). Deionized water (18.2 M\textOmega cm), obtained from a Milli-Q water purification system, was used to prepare all aqueous solutions (Millipore, Bedford, MA, USA).

2.2 MIL Synthesis

Chemical structures of the three MILs are shown in Figure S1. The [NH\textsubscript{4}\textsuperscript+][Ni(hfacac)\textsubscript{3}] salt, [P\textsubscript{6,6,6,14}\textsuperscript+][Ni(hfacac)\textsubscript{3}] MIL, and [P\textsubscript{6,6,6,14}\textsuperscript+][Ni(Phtfacac)\textsubscript{3}] MIL were synthesized and characterized using previously reported procedures.[38,39] The [N\textsubscript{8,8,8,Bz}\textsuperscript+][Br\textsuperscript{−}] salt was synthesized as previously reported and characterized using \textsuperscript{1}H NMR (Varion MR-400, Palo Alto, CA, USA) as shown in Figure S2.[40] The [N\textsubscript{8,8,8,Bz}\textsuperscript+][Ni(hfacac)\textsubscript{3}] MIL was synthesized by stirring equimolar amounts of [N\textsubscript{8,8,8,Bz}\textsuperscript+][Br\textsuperscript{−}] and [NH\textsubscript{4}\textsuperscript+][Ni(hfacac)\textsubscript{3}] in 50 mL of methanol.
overnight. The product was subsequently dried in a vacuum oven and purified using diethyl ether and water. The \([\text{P}_{6,6,6,14}^+][\text{NTf}_2^-]\) IL was synthesized by mixing equimolar amounts of the \([\text{P}_{6,6,6,14}^+][\text{Cl}^-]\) IL and \([\text{Li}^+][\text{NTf}_2^-]\) overnight in 30 mL of methanol. The \([\text{N}_{8,8,8,\text{Bz}}^+][\text{Br}^-]\) IL and \([\text{Li}^+][\text{NTf}_2^-]\) overnight in 30 mL of methanol. The \([\text{P}_{6,6,6,14}^+][\text{NTf}_2^-]\) and \([\text{N}_{8,8,8,\text{Bz}}^+][\text{NTf}_2^-]\) ILs was subsequently purified with diethyl ether and characterized by \(^1\text{H} \text{NMR}\) as shown in Figure S3 and S4, respectively. Elemental analysis results were acquired using a Thermo FlashSmart 2000 CHNS/O Combustion Elemental Analyzer (Thermo Scientific, Waltham, MA, USA). Carbon/hydrogen/nitrogen (CHN) calculated for \([\text{P}_{6,6,6,14}^+][\text{Ni}](\text{Phtfacac})_3^-\): \%C = 62.68, \%H = 7.30, \%N = 0.00; Found: \%C = 62.27, \%H = 7.34, \%N = 0.11. Calculated for \([\text{N}_{8,8,8,\text{Bz}}^+][\text{Ni}(\text{hfacac})_3^-]\): \%C = 49.12, \%H = 5.47, and \%N = 1.25; Found: \%C = 49.54, \%H = 5.39, \%N = 1.37.

2.3 qPCR Assays and Conditions

A 3.9 Kbp plasmid from Eurofin Genomics containing either a 166 bp wild-type \textit{KRAS}, 166 bp G12S \textit{KRAS}, or 210 wild-type \textit{BRAF} insert was individually amplified by PCR. The PCR products were subsequently separated on a 1% agarose gel. Amplified DNA was recovered from the gel using the QIAquick Gel Extraction kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The purified DNA was quantified using a NanoDrop 2000c spectrophotometer (Thermo Scientific, Waltham, MA, USA).

A Bio-Rad CFX96 Touch Real-time PCR (Hercules, CA, USA) was utilized for qPCR amplification of the \textit{KRAS} and \textit{BRAF} targets using the following program: 10 min initial denaturation at 95 °C followed by 40 cycles comprised of a 15 s denaturation step at 95 °C and a 1 min annealing step. After each cycle, an optical detection step was used to track the reaction in real-time. The qPCR products were heated from 65°C to 95°C in 0.5°C increments. Melt curve
analysis of 30 ppm of a 15-mer oligonucleotide to either a complementary sequence and a
sequence containing a 1 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 to
95°C in 0.5°C increments.

The amount of KRAS primers, BRAF primers, wild-type KRAS probe, G12S KRAS probe,
BRAF probe, EDTA, and MgCl₂ was optimized to ensure efficient amplification. Annealing
temperatures were optimized (54-65 °C) to ensure allelic discrimination between all three DNA
sequences, as determined using the Bio-Rad CFX Maestro software. Amplification of target
DNA standards was achieved using the following assay conditions: 1x Taqman universal PCR
mastermix, 1.25 mM MgCl₂, 1 µM forward KRAS primers, 1 µM reverse KRAS primers, 1 µM
forward BRAF primers, 1 µM reverse BRAF primers, 150 nM wild-type KRAS probe, 150 nM
G12S KRAS probe, and 150 nM BRAF probe. The addition of 0.3 µL [P₆,₆,₆,₁₄⁺][Ni(hfacac)₃] MIL to a 20 µL multiplex-qPCR mixture required 1x Taqman universal PCR mastermix, 1.25
mM MgCl₂, 1 µM forward KRAS primers, 1 µM reverse KRAS primers, 1 µM forward BRAF
primers, 1 µM reverse BRAF primers, 150 nM wild-type KRAS probe, 150 nM G12S KRAS
probe, and 150 nM BRAF probe. The addition of 0.3 µL [P₆,₆,₆,₁₄⁺][Ni(Phtfacac)₃⁻] MIL to a 20
µL multiplex-qPCR mixture required 1x Taqman universal PCR mastermix, 1.25 mM MgCl₂, 1
µM forward KRAS primers, 1 µM reverse KRAS primers, 1 µM forward BRAF primers, 1 µM
reverse BRAF primers, 150 nM wild-type KRAS probe, 150 nM G12S KRAS probe, and 150 nM
BRAF probe. qPCR amplification with 0.3 µL of the [N₈,₈,₈,Bz⁺][Ni(hfacac)₃] MIL was achieved
using the 1x Taqman universal PCR mastermix, 2 mM EDTA, 1.25 mM MgCl₂, 1 µM forward
KRAS primers, 1 µM reverse KRAS primers, 1 µM forward BRAF primers, 1 µM reverse BRAF
primers, 150 nM wild-type KRAS probe, 150 nM G12S KRAS probe, and 150 nM BRAF probe
for a final volume of 20 µL. Melt curves of qPCR products were achieved by adding 2x SYBR Green I to the reaction tube containing MIL and 1x SYBR Green I for standard reactions.

The quantitation cycle (Cq) was determined using the fluorescence threshold provided by the Bio-Rad CFX Maestro software and used to determine the amount of wild-type KRAS, G12S KRAS, and wild-type BRAF extracted by the hydrophobic MIL. Standard curves were constructed for wild-type KRAS, G12S KRAS, and wild-type BRAF template with and without MIL present in the multiplex-qPCR buffer. The standard curves were used to determine the concentration of DNA initially present in the reaction. The enrichment factor obtained for each extraction was calculated using equation 1, where $C_{\text{MIL}}$ is the concentration of a one of the DNA fragments extracted by the MIL and $C_{\text{Std}}$ represents the concentration of template in the aqueous sample solution prior to extraction. Preconcentration is achieved when the enrichment factor was above 1.

$$Enrichment\ Factor = \frac{C_{\text{MIL}}}{C_{\text{Std}}}$$  \hspace{0.5em} \text{Equation 1}

2.4 Examining the Capture of Fluorophore during MIL-qPCR

In order to investigate the drop in fluorescence signal upon adding the hydrophobic MIL to the multiplex-qPCR system, 150 nM Cy5 probe was incubated with 0.3 µL of MIL. The static extraction proceeded for 10 min before determining the amount of fluorophore remaining in the aqueous phase using a Biotek Synergy H1 Multi-mode microplate reader (Winooski, VT, USA) using an excitation and emission wavelength of 646 nm and 665 nm, respectively. A 150 nM solution of Cy5 and 0.3 µL of MIL was subjected to qPCR cycling to track the decrease in fluorescence signal throughout the reaction. Prior to thermal cycling, all samples were heated at 50 °C for 2 min and 95 °C for 10 min in order to mimic the multiplex-qPCR procedure.
2.5 Capture of Target DNA

The general procedure used to extract target DNA fragments using DLLME is shown in Fig 1. A 1.0 mL solution of 0.5 fg µL\(^{-1}\) wild-type KRAS, 0.5 fg µL\(^{-1}\) G12S KRAS, and 0.5 fg µL\(^{-1}\) wild-type BRAF targets was prepared in 2 mM Tris buffer (pH 8). An optimized volume of MIL was dispersed using a Barnstead/Thermolyne Type 16700 mixer (Dubuque, IA, USA) for a specific length of time. After dispersing the hydrophobic MIL, DNA-enriched MIL droplets were collected using a rod magnet (B = 0.66 T). The recovered MIL was washed with deionized water, and a 0.3 µL aliquot of DNA-enriched MIL was placed in a qPCR tube for downstream analysis. For all extractions using human plasma, 0.5 fg µL\(^{-1}\) wild-type KRAS, 0.5 fg µL\(^{-1}\) G12S KRAS, and 0.5 fg µL\(^{-1}\) wild-type BRAF was spiked into the plasma.

DNA extractions using the QiaAMP DNA mini kit were performed as specified by the manufacturer. Briefly, 16 units of proteinase K were added to 1 mL 0.5 fg µL\(^{-1}\) wild-type KRAS, 0.5 fg µL\(^{-1}\) G12S KRAS, and 0.5 fg µL\(^{-1}\) wild-type BRAF target. A 1 mL volume of lysis buffer (AL buffer) was added to the sample and allowed to incubate for 10 min at 56 °C. Next, 1 mL of ethanol was added to the sample before placing the solution into a spin column. The column was centrifuged for 1 min at 1.3 × 10\(^4\) rpm. The flow through was discarded and 0.5 mL of wash buffer 1 (AW1 buffer) was added to the column. The sample was centrifuged again for 1 min. The flow through was discarded and 0.5 mL of wash buffer 2 (AW2 buffer) was placed in the column. The column was then centrifuged for 3 min at 1.3 × 10\(^4\) rpm and the flow through discarded. The column was again centrifuged for an additional minute to ensure that the wash buffers were thoroughly removed from the column. Lastly, 20 µL or 200 µL of elution buffer (AE buffer) were added to the column to elute the DNA.
Extractions using the Dynabeads myone silane magnetic beads were performed as recommended by the manufacturer. Briefly, 16 units of proteinase K were added to 0.5 fg µL\(^{-1}\) wild-type \(KRAS\), 0.5 fg µL\(^{-1}\) G12S \(KRAS\), and 0.5 fg µL\(^{-1}\) wild-type \(BRAF\) target and allowed to incubate for 2 min. Afterwards, 350 µL of 6 M guanidine hydrochloride was added to the sample and allowed to incubate for 10 min at 55 °C. Isopropanol (400 µL) and 2 mg of magnetic beads were then added to the sample. The beads were dispersed for 30 s using a vortex and then agitated on an Eppendorf I24R incubator shaker (Eppendorf, Hamburg, Germany) at 100 rpm. The sample was subsequently vortexed again for 30 s and the beads collected on a 0.9 T magnet. The aqueous phase was removed, and the beads were washed three times using 950 µL isopropanol. After the final wash step, the beads were dried using air to ensure that isopropanol did not contaminate the reaction. The beads were suspended in 50 µL 2 mM Tris buffer and vortexed for 2 min to desorb captured DNA.

3. Results and Discussion

3.1 Optimization of Multiplex-qPCR Assay

A multiplex-qPCR system containing no MIL was first optimized to ensure good efficiency and allelic discrimination among the three DNA fragments. The concentration of primers (500-1250 nM), probes (75-350 nM), and MgCl\(_2\) (0-5 mM) was optimized for the standard reaction. The annealing temperature was optimized (54-64°C) in order to discriminate between SNPs. An optimum annealing temperature of 63 °C was chosen as it exhibited good allelic discrimination for the standard reactions, as shown in Figure S5. A standard curve was then generated for each DNA sequence, as shown in Figure S6.
In order to successfully integrate a hydrophobic MIL into the multiplex-qPCR system, the concentration of EDTA was optimized between a range of 0-6 mM for each MIL-multiplex-qPCR. EDTA is capable of chelating solubilized metal ions thereby preventing PCR inhibition caused by the anion component of the MIL.[34,35] The \([\text{P}_{6,6,6,14}^+]\text{[Ni(hfacac)]}_3\) and \([\text{P}_{6,6,6,14}^+]\text{[Ni(Phtfacac)]}_3\) MILs did not require additional EDTA in order to achieve amplification, and the \([\text{N}_{8,8,8,Bz}^+]\text{[Ni(hfacac)]}_3\) MIL only required 2 mM EDTA. However, the high annealing temperature (63 °C) used for the standard reactions significantly inhibited the reaction, as shown in Figure 2. Therefore, the annealing temperature was optimized at 59 °C for the \([\text{P}_{6,6,6,14}^+]\text{[Ni(hfacac)]}_3\) and \([\text{P}_{6,6,6,14}^+]\text{[Ni(Phtfacac)]}_3\) MILs and 62 °C for the \([\text{N}_{8,8,8,Bz}^+]\text{[Ni(hfacac)]}_3\) MIL in order to relieve inhibition but still permit the SNPs to be distinguished from each other. Afterwards, the concentration of MgCl\(_2\) was optimized between 0-5 mM to ensure optimum qPCR efficiency during simultaneous amplification of all three DNA sequences; 2.5 mM MgCl\(_2\) was selected as the optimum concentration of all three MILs examined.

After optimizing the multiplex reaction buffer, allelic discrimination plots and standard curves were developed for the three MILs. As shown in Figure 3, integration of the \([\text{P}_{6,6,6,14}^+]\text{[Ni(hfacac)]}_3\), \([\text{N}_{8,8,8,Bz}^+]\text{[Ni(hfacac)]}_3\), \([\text{P}_{6,6,6,14}^+]\text{[Ni(Phtfacac)]}_3\) MILs into the multiplex-qPCR buffer still allowed for distinct clusters when plotting the endpoint fluorescence signal associated with each probe. The plots show that discrimination between different DNA sequences could be achieved when the MIL was added to the reaction buffer. In addition, the amplification efficiencies fell to between 90-110%, as shown in Figure S7, suggesting that DNA is successfully being duplicated with each cycle.

### 3.2 Investigation into Primer Annealing
Previous studies have suggested that ILs and MILs can decrease the melting temperature (Tm) of DNA fragments.[36,41] In this study, the same phenomenon was observed when optimizing the annealing temperature and performing melt curves of a 15-mer DNA fragment (see Figure 4). This is interesting as slight increases in ionic strength can significantly increase the melting temperature of DNA.[42] The melting temperature also decreased when adding an equimolar amount of \([P_{6,6,6,14}^+]\)[\(\text{NTf}_2^-\)] and \([N_{8,8,8,Bz}^+]\)[\(\text{NTf}_2^-\)] ILs to the buffer, as shown in Figure 4. This finding suggests that the cation is responsible for the decrease in annealing temperature. It is likely that the cation is undergoing hydrophobic interactions with DNA fragments allowing for a decrease in annealing temperature similar to the behavior of cationic surfactants when present at concentrations below the critical micelle concentration.[42]

The melting temperatures of a 1 nt mismatch fragment and 15-mer oligonucleotide were also examined. As shown in Table S2, the \([P_{6,6,6,14}^+]\)[\(\text{Ni(hfacac)}_3^-\)] MIL did not stabilize the mismatch whereas the mismatch was slightly destabilized when the \([P_{6,6,6,14}^+]\)[\(\text{Ni(Phtfacac)}_3^-\)] MIL was added to the buffer. However, in the case of the \([N_{8,8,8,Bz}^+]\)[\(\text{Ni(hfacac)}_3^-\)] MIL, the mismatch was stabilized by adding the MIL into the buffer, which explains the observation that a higher annealing temperature is required in order to differentiate between the wild-type and mutant KRAS fragments.

The melting temperatures of the 89 bp G12S KRAS DNA were examined in order to determine the extent to which the three hydrophobic MILs affect longer DNA sequences. As shown in Figure S8, there was no significant difference between the melting temperatures of DNA when the MIL is present compared to the standard, suggesting that the MIL interacts stronger with shorter DNA fragments. Therefore, melt curves should be able to identify DNA fragments when the MIL is present in the qPCR buffer.[43] As shown in Figure S9, the melt
curves after MIL-DLLME and direct qPCR amplification were within 0.5 °C from the standard. This suggests that the MIL does not alter the DNA sequence during either the extraction or PCR amplification, as has been reported in previous studies.[15,34,35]

3.3 Partitioning of the Hydrophobic Probe to the MIL Phase

When the hydrophobic MILs were added to the reaction buffer, the fluorescence signal drastically decreased compared to the standard. In previous studies, inhibition caused by SYBR Green I partitioning to the MIL phase during qPCR was relieved by adding additional SYBR Green I to the buffer.[35,37] However, increasing the concentration of the Taqman probe generally did not enhance the fluorescence signal. To investigate whether inhibition originates from the fluorophore partitioning to the MIL phase or MIL quenching the signal, a 150 nM solution of Cy5 was incubated with 0.3 µL of each of the three hydrophobic MILs. A six-point standard curve of Cy5 (see Figure S10a) was generated to quantify the amount of Cy5 extracted by the MIL. As shown in Figure S10b, extraction efficiencies of 50.27 ± 0.28%, 26.11 ± 1.68%, and 37.18 ± 1.27% for Cy5 were obtained with the [P_{6,6,6,14}^+][Ni(hfacac)_3^-], [P_{6,6,6,14}^+][Ni(Phtfacac)_3^-], and [N_{8,8,8,Bz}^+][Ni(hfacac)_3^-] MILs, respectively, after sitting at room temperature for 10 min. The effect of PCR cycling on the partitioning of Cy5 to the MIL was also investigated, as shown in Figure S11. Extraction efficiencies plateaued with increasing cycles for the [P_{6,6,6,14}^+][Ni(hfacac)_3^-] and [N_{8,8,8,Bz}^+][Ni(hfacac)_3^-] MILs, suggesting quenching of the fluorophore by MIL that dissolves into the aqueous phase at elevated temperatures used in PCR.[36,44]

3.4 Optimization of MIL-DLLME Method
Cell-free DNA (cfDNA) is generally present at the ng mL\(^{-1}\) level in plasma.[8,45,46] However, ctDNA can comprise less than 0.01% of the total amount of cfDNA depending on the type and stage of cancer.[2,46] Therefore, a clinically relevant concentration of 0.5 fg µL\(^{-1}\) wild-type KRAS, 0.5 fg µL\(^{-1}\) G12S KRAS, and 0.5 fg µL\(^{-1}\) wild-type BRAF fragments was used during optimization in this study.

The volume of MIL dispersed was first optimized to achieve the highest enrichment factors. As shown in Figure S12, optimal volumes for the \([\text{P}_{6,6,6,14}^+][\text{Ni(hfacac)}_3^-]\), \([\text{N}_{8,8,8,Bz}^+][\text{Ni(hfacac)}_3^-]\), and \([\text{P}_{6,6,6,14}^+][\text{Ni(Phtfacac)}_3^-]\) MILs were found to be 6, 8, and 6 µL, respectively. Similar to previously reported studies, lower volumes of MIL were capable of achieving higher enrichment factors compared to larger volumes of MIL, suggesting that extracted DNA is being diluted within the MIL.[32,35,37] Subsequently, the extraction time was optimized. The optimum extraction times for the \([\text{P}_{6,6,6,14}^+][\text{Ni(hfacac)}_3^-]\), \([\text{N}_{8,8,8,Bz}^+][\text{Ni(hfacac)}_3^-]\), and \([\text{P}_{6,6,6,14}^+][\text{Ni(Phtfacac)}_3^-]\) MILs were found to be 2, 2, and 3 min, respectively, as shown in Figure S13.

In order to ensure that the three sequences can still be distinguished from each other after desorbing DNA from the MIL, allelic discrimination plots were generated for all three MILs by extracting only one DNA fragment using the optimized procedure. Distinct clusters were still observed when plotting the fluorescence signals (see Figure S14) suggesting that the desorption of DNA from the MIL does not affect the ability of the reaction to discriminate between the different DNA sequences, even if the sequences are SNPs. As shown in Figure S15, similar enrichment factors were achieved when extracting either one or all three DNA fragments. The mutation load of KRAS was found to be on average 8.4% during stage II/III colorectal cancer and 21.8% during stage IV.[47] Therefore, the MIL-multiplex-qPCR system was evaluated by
performing extractions of 0.5 and 5% G12S \textit{KRAS}. As shown in Fig. S16, when G12S \textit{KRAS} is present at 0.5 fg µL$^{-1}$ (10-fold less compared to wild-type \textit{KRAS} and \textit{BRAF}) the reaction was not significantly affected in the case of all three MILs. However, when the concentration of G12S \textit{KRAS} was 100-fold less than wild-type \textit{KRAS} and \textit{BRAF}, the efficiency was significantly impacted (see Fig. S17) due to the consumption of PCR reagents.[14,48,49] In order to improve the sensitivity, future studies should investigate using a sequence-specific DNA extraction or PCR clamp to limit the amount of wild-type DNA amplified.

3.5 Extractions from a Plasma Matrix

Plasma matrices are highly complex and contain a number of PCR inhibitors such as albumin, immunoglobulin G, and lactoferrin. [50,51] In addition, common anticoagulants such as EDTA or heparin can also inhibit PCR. Therefore, it is necessary to evaluate the performance of a ctDNA extraction procedure in plasma. The extraction of DNA from 10-fold diluted citrate plasma and 10-fold diluted Na$_2$EDTA plasma was evaluated to examine the effect of the anticoagulant on MIL-DLLME. As shown in Figure S18, no significant difference was observed between the citrate and Na$_2$EDTA plasma when using the [P$_{6,6,6,14}^+$][Ni(hfacac)$_3$] MIL. However, this was not the case when comparing the Na$_2$EDTA and citrate plasma with the [N$_{8,8,8,Bz}^+$][Ni(hfacac)$_3$] and [P$_{6,6,6,14}^+$][Ni(Phtfacac)$_3$] MILs, as determined using the Student’s t-test (probability value < 0.05). In particular, there was a sharp decrease in enrichment factor when performing extractions from the 10-fold diluted citrate plasma with the [N$_{8,8,8,Bz}^+$][Ni(hfacac)$_3$] MIL, as shown in Figure S18b. There was also a slight decrease in enrichment factors when performing extractions from a 10-fold diluted citrate matrix with the [P$_{6,6,6,14}^+$][Ni(Phtfacac)$_3$] MIL.
Although performing extractions from a diluted plasma matrix can be beneficial to reduce the matrix effect, a lower concentration of DNA is present. Therefore, different dilutions of Na$_2$EDTA plasma were examined with the [N$_{8,8,8,Bz}$$^+$][Ni(hfacac)$_3$] and [P$_{6,6,6,14}$$^+$][Ni(Phtfacac)$_3$] MILs as they exhibited the best performance in 10-fold diluted plasma. Preconcentration was still achieved in 2 and 4-fold diluted plasma with the [N$_{8,8,8,Bz}$$^+$][Ni(hfacac)$_3$] MIL and 4-fold diluted plasma with the [P$_{6,6,6,14}$$^+$][Ni(Phtfacac)$_3$] MIL, as shown in Figure 5. Enrichment factors of 0.64 ± 0.11, 0.69 ± 0.14, and 0.64 ± 0.09 were achieved for wild-type KRAS, G12S KRAS, and wild-type BRAF from undiluted plasma with the [P$_{6,6,6,14}$$^+$][Ni(Phtfacac)$_3$] MIL, as shown in Figure 5b. The results were not reproducible when attempting to use the [N$_{8,8,8,Bz}$$^+$][Ni(hfacac)$_3$] MIL as an extraction solvent in undiluted plasma. In order to determine whether qPCR inhibitors are co-extracted by the MIL, a standard curve was generated by carrying out extractions from 10-fold diluted plasma for the [P$_{6,6,6,14}$$^+$][Ni(hfacac)$_3$] MIL and 4-fold diluted plasma for the [N$_{8,8,8,Bz}$$^+$][Ni(hfacac)$_3$] and [P$_{6,6,6,14}$$^+$][Ni(Phtfacac)$_3$] MILs. Reaction efficiencies between 90-110% were achieved with the [P$_{6,6,6,14}$$^+$][Ni(hfacac)$_3$] and [P$_{6,6,6,14}$$^+$][Ni(Phtfacac)$_3$] MILs, suggesting that PCR inhibitors are not being co-extracted by this MIL (see Figure S19). However, the [N$_{8,8,8,Bz}$$^+$][Ni(hfacac)$_3$] MIL produced an efficiency of 119.4%, 113.2%, and 149.4% for wild-type KRAS, G12S KRAS, and wild-type BRAF, respectively, when extracting from 4-fold diluted plasma. These high efficiencies suggest that the DNA polymerase activity is being inhibited by a plasma component that was co-extracted by the MIL.[52]

### 3.6 Comparison to Commercial DNA Extraction Procedures

Commercial means of extracting ctDNA utilize silica-based magnetic beads and silica-based spin columns.[18,45] However, these methods require several lengthy sample handling steps (i.e, centrifugation or collection of magnetic beads) that can increase the probability of
contamination. There have also been reports suggesting that commercial-based methods suffer at extracting low concentrations of DNA.[18] Therefore, the enrichment factors achieved using the optimized MIL-DLLME method were compared to commercial magnetic bead and silica columns. As shown in Figure 6, the MIL-DLLME method produced higher enrichment factors when extracting target DNA fragments from 2 mM Tris buffer. Although the columns extracted most of the DNA present in solution, enrichment factors were poor compared to the MIL-DLLME method likely due to the high desorption volume (200 µL) recommended by the manufacturer. However, changing the desorption volume to 20 µL only slightly improved the $E_f$ (see Figure 6). The similar enrichment factors between the two desorption volumes suggests that at lower elution volumes there is not enough buffer to sufficiently elute the DNA.[45] The [N$_{8,8,8,Bz}$]$^+\left[\text{Ni(hfacac)}_3\right]$ and [P$_{6,6,6,14}$]$^+\left[\text{Ni(Phfaca)}_3\right]$ MILs outperformed commercial methods when performing extractions from 4- and 10-fold diluted plasma. The [N$_{8,8,8,Bz}$]$^+\left[\text{Ni(hfacac)}_3\right]$ MIL produced similar enrichment factors to the QiaA MP spin columns with 2-fold diluted plasma. However, extractions using the QiaAMP spin columns and Dynabeads myone silane magnetic beads were not affected by higher concentrations of plasma compared to the MILs, as shown in Figure 5. In addition, extractions from pure plasma were reproducible using the QiaAMP spin columns and Dynabeads myone silane magnetic beads. However, enrichment factors associated with the [P$_{6,6,6,14}$]$^+\left[\text{Ni(Phfaca)}_3\right]$ MIL were significantly higher than both of the silica-based DNA extraction methods as determined using the Student’s t-test (probability value < 0.05).

4. Conclusions

In this study, three MILs were utilized to rapidly preconcentrate clinically-relevant concentrations of ctDNA within 2-3 min. Integrating the MIL into a custom designed multiplex-
qPCR assay allows for a simple method to desorb DNA from the hydrophobic MIL. The addition of the MIL into the multiplex-qPCR buffer did not have a deleterious effect on the amplification efficiency when simultaneously amplifying three DNA fragments, and the fragments could easily be discerned from each other using allelic discrimination plots. However, in order to achieve amplification as well as allelic discrimination, the annealing temperature was lower compared to the standard reaction due to hydrophobic interactions between the MIL and probe. Enrichment factors as high as 42.68 ± 3.63, 38.16 ± 4.30, and 38.78 ± 5.52 were obtained from Tris buffer for the wild-type KRAS, G12S KRAS, and wild-type BRAF, respectively, using the [N₈,₈,₈,B₂⁺][Ni(hfacac)₃⁻] MIL, and preconcentration was still obtainable while dispersing the MIL in 2, 4, and 10-fold diluted plasma. Compared to commercial kits, the MIL-based extraction was better at preconcentrating DNA fragments from a plasma matrix with the [P₆,₆,₆,₁₄⁺][Ni(Phtfacac)₃⁻] MIL capable of achieving enrichment factors over 0.6 for all three DNA fragments from undiluted plasma whereas enrichment factors above of 0.3 and 0.1 were achieved with the QiaAMP spin columns and dynabeads myone silane magnetic beads, respectively. The low enrichment factors from the kits are in part due to the high desorption volumes required to sufficiently desorb DNA compared to the MILs. The ability of MILs to rapidly preconcentrate DNA from plasma is essential in ctDNA analysis especially during the early stages of cancer where there are low abundances of mutant DNA. Therefore, the MIL-DLLME method has potential to be highly beneficial towards ctDNA analysis in clinically-relevant samples.

Acknowledgments
Nabeel Abbasi and Muhammad Qamar Farooq are thanked for their assistance in this study. J. L. A acknowledges funding from the Chemical Measurement and Imaging Program at the National Science Foundation (CHE-1709372).

References


Fig. 1: General procedure used to extract and detect wild-type KRAS, G12S KRAS, and BRAF DNA. DNA-enriched MIL was added to the reaction buffer for multiplex-qPCR detection using three different Taqman probes.
Fig. 2: qPCR curves of (green) wild-type KRAS, (blue) G12S KRAS, (violet) wild-type BRAF generated by spiking 0.3 µL of the $[\text{P}_{6,6,6,14}^*][\text{Ni(hfacac)}_3]$ MIL into the reaction and annealing at 59, 61, and 63 °C.
Fig. 3: Allelic discrimination associated with spiking DNA into the multiplex-qPCR system containing the (a) \([P_{6,6,6,14}^+]\)[Ni(hfacac)\(_3\)], (b) \([N_{8,8,8,8^z}^+]\)[Ni(hfacac)\(_3\)], and (c) \([P_{6,6,6,14}^+]\)[Ni(Phtfacac)\(_3\)] MILs. Het., heterozygous; B, wild-type \(BRAF\); WK, wild-type \(KRAS\); MK, G12S \(KRAS\). Triplicate reactions were performed for each cluster.
**Fig. 4:** Melt curves of a 15-mer DNA oligonucleotide in the presence of (green) $[\text{P}^{6,6,6,14}\text{[Ni(hfacac)]_3}]$, (blue) $[\text{N}^{8,8,8,\text{Bz}}\text{[Ni(hfacac)]_3}]$, (violet) $[\text{P}^{6,6,6,14}\text{[Ni(Phtfacac)]_3}]$, (grey) $[\text{N}^{8,8,8,\text{Bz}}\text{[NTf_2]^-}]$, and (navy) $[\text{P}^{6,6,6,14}\text{[NTf_2]^-}]$ ILs compared to a (black) standard without IL.
Fig. 5: Extractions of (green) wild-type KRAS, (blue) G12S KRAS, and (violet) wild-type BRAF from diluted plasma containing Na$_2$EDTA as an anticoagulant with the (a) [N$_{8,8,8,Bz}$]$^+$[Ni(hfacac)$_3$]$^-$ MIL, (b) [P$_{6,6,6,14}$]$^+$[Ni(Phtfacac)$_3$]$^-$ MIL, (c) QiAMP spin columns, and (d) Dynabeads myone silane magnetic beads. Wild-type KRAS, G12S KRAS, and wild-type BRAF template concentration: 0.5 fg µL$^{-1}$, sample volume: 1.0 mL; [P$_{6,6,6,14}$]$^+$[Ni(hfacac)$_3$]$^-$ volume: 6 µL; [N$_{8,8,8,Bz}$]$^+$[Ni(hfacac)$_3$]$^-$ volume: 8 µL; [P$_{6,6,6,14}$]$^+$[Ni(Phtfacac)$_3$]$^-$ volume: 6 µL; magnetic bead mass: 2 mg. Triplicate extractions were performed under each condition.
Fig. 6: Enrichment factors of (green) wild-type KRAS, (blue) G12S KRAS, and (violet) wild-type BRAF obtained for the optimized MIL-DLLME and commercial methods using the \([\text{P}_{6,6,6,14}^+][\text{Ni(hfacac)}_3^-]\) MIL, \([\text{N}_{8,8,8,Bz}^+][\text{Ni(hfacac)}_3^-]\) MIL, \([\text{P}_{6,6,6,14}^+][\text{Ni(Phtfacac)}_3^-]\) MIL, QiaAMP spin column (desorption volume 200 µL), QiaAMP spin column (desorption volume 20 µL), and Dynabeads myone silane magnetic beads.

Wild-type KRAS, G12S KRAS, and wild-type BRAF template concentration: fg µL⁻¹, sample volume: 1.0 mL; \([\text{P}_{6,6,6,14}^+][\text{Ni(hfacac)}_3^-]\) volume: 6 µL; \([\text{N}_{8,8,8,Bz}^+][\text{Ni(hfacac)}_3^-]\) volume: 8 µL; \([\text{P}_{6,6,6,14}^+][\text{Ni(Phtfacac)}_3^-]\) volume: 6 µL; magnetic bead mass: 2 mg. Triplicate extractions were performed using each extraction method.
Highlights:

- Custom multiplex-qPCR assays were developed to contain magnetic ionic liquid (MIL) solvents
- Circulating tumor DNA (ctDNA) was preconcentrated from plasma matrices with the MILs
- DNA-enriched MIL was added to the multiplex-qPCR assay to desorb captured DNA
- Allelic discrimination and good PCR efficiency was achieved with the MIL-multiplex-qPCR assay
- Commercial kits poorly preconcentrated DNA due to high desorption volumes
Credit Authorization Statement

**Miranda Emaus:** Conceptualization, Methodology, Validation, Formal analysis, Writing original draft. **Jared L. Anderson:** Supervision, Conceptualization, Writing-review and editing, Project administration, Funding.
Conflict of Interest Statement:

The authors declare no conflicts of interest in this work.