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Capture, Concentration and Detection of Salmonella in Foods Using Magnetic Ionic Liquids and Recombinase Polymerase Amplification

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Abstract

We have previously investigated the extraction and concentration of bacteria from model systems using magnetic ionic liquid (MIL) solvents, while retaining their viability. Here, we combine MIL-based sample preparation with isothermal amplification and detection of *Salmonella*-specific DNA using Recombinase Polymerase Amplification (RPA). After initial developmental work with *Serratia marcescens* in water, *Salmonella* Typhimurium ATCC 14028 was inoculated in water, 2% milk, almond milk or liquid egg samples and extracted using one of two MILs, including: trihexyl(tetradecyl)phosphonium cobalt(II) hexafluoroacetylacetonate ([P66614+][Co(hfacac)3–]) and trihexyl(tetradecyl)phosphonium nickel(II) hexafluoroacetylacetonate ([P66614+][Ni(hfacac)3–]). Viable cells were recovered from the MIL extraction phase after the addition of modified LB broth, followed by a 20 min isothermal RPA assay. Amplification was carried out using supersaturated sodium acetate heat packs and results compared to those using a conventional laboratory thermocycler set to a single temperature. Results were visualized using either gel electrophoresis or nucleic acid lateral flow immunoassay (NALFIA). The combined MIL-RPA approach enabled detection of *Salmonella* at levels as low as 103 CFU mL-1. MIL-based sample preparation required less than 5 min to capture and concentrate sufficient cells for detection using RPA, which (including NALFIA or gel-based analysis) required approximately 30 - 45 min. Our results suggest the utility of MILs for the rapid extraction and concentration of pathogenic microorganisms in food samples, providing a means for physical enrichment that is compatible with downstream analysis using RPA.

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Article

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Capture, Concentration and Detection of *Salmonella* in Foods Using Magnetic Ionic Liquids and Recombinase Polymerase Amplification

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ABSTRACT: We have previously investigated the extraction and concentration of bacteria from model systems using magnetic ionic liquid (MIL) solvents, while retaining their viability. Here, we combine MIL-based sample preparation with isothermal amplification and detection of *Salmonella*-specific DNA using Recombinase Polymerase Amplification (RPA). After initial developmental work with *Serratia marcescens* in water, *Salmonella* Typhimurium ATCC 14028 was inoculated in water, 2% milk, almond milk or liquid egg samples and extracted using one of two MILs, including: trihexyl(tetradecyl)phosphonium cobalt(II) hexafluoroacetylacetonate ($[P_{66614}^+][Co(hfacac)_3^-]$) and trihexyl(tetradecyl)phosphonium nickel(II) hexafluoroacetylacetonate ($[P_{66614}^+][Ni(hfacac)_3^-]$). Viable cells were recovered from the MIL extraction phase after the addition of modified LB broth, followed by a 20 min isothermal RPA assay. Amplification was carried out using supersaturated sodium acetate heat packs and results compared to those using a conventional laboratory thermocycler set to a single temperature. Results were visualized using either gel electrophoresis or nucleic acid lateral flow immunoassay (NALFIA). The combined MIL-RPA approach enabled detection of *Salmonella* at levels as low as 10^3 CFU mL^{-1}. MIL-based sample preparation required less than 5 min to capture and concentrate sufficient cells for detection using RPA, which (including NALFIA or gel-based analysis) required approximately 30 - 45 min. Our results suggest the utility of MILs for the rapid extraction and concentration of pathogenic microorganisms in food samples, providing a means for physical enrichment that is compatible with downstream analysis using RPA.

Introduction: *Salmonella* is a ubiquitous, gram-negative bacterium that is widespread in the environment and is a contaminant in various foods, food ingredients, and in industrial food processing environments.[1] Infection typically results from ingestion of tainted food products, including consumption of contaminated poultry, eggs and dairy products. The Centers for Disease Control and Prevention (CDC) estimates that nontyphoidal *Salmonella* spp. are responsible for 1.2 million cases of illness, 19,000 hospitalizations and nearly 380 deaths in the United States annually, resulting in an economic burden greater than 3.4 billion USD.[2] Therefore, rapid, streamlined and field-deployable methods for detection of *Salmonella* spp. and other foodborne pathogens are crucial for ensuring the safety and quality of the foodstream.

Current detection techniques used by the food industry include standard culture methods and polymerase chain reaction (PCR), due to the selectivity, reliability and regulatory acceptance of these techniques. However, culture-based methods for *Salmonella* may require between several days to more than a week, depending on the sample.[3,4] To address these time-to-result issues, the food industry relies PCR as a means for rapid screening of food samples for contamination. Although PCR has gained wide acceptance in the food industry over the past 20 years, the thermal cyclers needed for this approach can be expensive and most systems remain bench-bound or have limited portability.[5]

Recently, recombinase polymerase amplification (RPA) has demonstrated promise as an alternative means for the rapid detection of microbial pathogens and viruses.[6] Unlike PCR, which relies on high heat to denature and separate duplex DNA (dsDNA), RPA accomplishes this at lower temperatures using an enzyme, recombinase.[7] While RPA maintains several similarities to PCR, including exponential amplification of target sequences, a major difference between the two detection methods is the temperature profile used for amplification. RPA is an isothermal process, which obviates the need for a thermal cycler, allowing use of simple, small and inexpensive heating devices. The optimal temperature of RPA has been reported to be 37 – 42 °C, but amplification of specific products has been demonstrated at temperatures ranging from 25 – 45 °C.[8] Suitable product amplification can be achieved in less than 20 min with RPA, as time-consuming ramping between separate denaturation, annealing and extension temperatures is not required.[9] Together, these attributes make RPA advantageous for use in resource-poor environments.

Although RPA products can be visualized using gel electrophoresis, gels require specialized equipment and are time-consuming, taking upwards of 35 min to get results. As an alternative, disposable, colorimetric lateral flow devices can be used for rapid (<10 min) amplicon detection. These paper- or nitrocellulose-based devices rely on capillary action and therefore have no requirement for power. Nucleic acid lateral flow immunoassays (NALFIA) like those used in this work with RPA are typically based on a sandwich-type assay.[10] Like RPA itself, these NALFIA devices are portable, economical and simple to use outside of laboratory settings – characteristics that make them ideal for use in the field. While nucleic acid amplification techniques are highly specific, their successful

use in foods may be limited by low pathogen levels.[11] Without a suitable means for capture, concentration and purification of bacteria prior to downstream analysis ("extraction" in purely chemical terms), the detection of pathogens in contaminated food samples may suffer from lack of reproducibility or from poor sensitivity due to inhibitory substances carried over from the sample matrix. Cultural enrichment is commonly used prior to detection, allowing dilution of food matrix-associated assay inhibitors, recovery of stressed or injured cells and generation of a detectable threshold of cells. However, key drawbacks include increased assay time, outgrowth of target organisms by competitive microflora and loss of information on initial pathogen load.[3] While filtration- and centrifugation-based sample preparation techniques can enable physical enrichment of cells, clogging of filters or co-isolation of particles or debris that may interfere with assay performance can be problematic.[11] Magnetic techniques can also be used for capture, concentration and purification of cells from complex food matrices, including the use of magnetic microbeads or nanoparticle substrates functionalized with pathogen-specific antibodies. Other means for functionalizing substrates for cell capture include the use of cationic charge, which is non-selective, or those that depend on semi-selective cell-ligand interactions, such as lectins, antimicrobial peptides or antibiotics.[12] In these approaches, functionalized magnetic beads or nanoparticles are dispersed throughout a sample slurry where they encounter and bind to bacteria. A magnetic field is then applied for physical isolation of the cell-enriched sorbent.[11] Drawbacks to particulate magnetic sorbents such as microbeads may include aggregation, diffusion- or suspension-based limitations or poor access to microscopic physical niches where bacteria may be present. These issues may result in lower extraction efficiencies and/or clogging of microfluidic systems. These problems can be addressed, but not completely avoided, through the use of functionalized nanoparticles.[11]

Magnetic ionic liquids (MILs) are paramagnetic molten salts comprised of organic/inorganic cations and anions that exhibit melting points at or below 100 °C. Similar to conventional ionic liquids (ILs), MILs possess negligible vapor pressures at ambient temperatures and tunable physicochemical properties including viscosity, solvent miscibility, and solvation capabilities.[13,14,15] Owing to their tunable chemical structures and susceptibility to magnetic fields, MILs have been applied for the analysis of hormones in biological fluids,[16] acidic pharmaceuticals and endocrine disrupters,[17] and the extraction and preservation of nucleic acids.[18,19] Very recently, MILs were also investigated as solvents for the preconcentration of viable bacteria for culture and PCR-based detection.[20] By dispersing the hydrophobic MIL in an aqueous suspension of *Escherichia coli* K12, viable cells were rapidly extracted and concentrated for downstream analysis using qPCR. However, the ability of MIL solvents to extract industrially-relevant foodborne pathogens, such as *Salmonella* Typhimurium, in complex food matrices was not tested. In this study, we report development of a method for the preconcentration and detection of *S.* Typhimurium that capitalizes on the rapid and cell-compatible extraction capabilities of MIL solvents and the portability, simplicity and rapidity of a RPA detection platform.

Experimental Section

Magnetic Ionic Liquids: The structures of the three MIL solvents examined in this work are shown in Fig. 1, panel a. Synthesis and characterization of the MILs was performed as previously described.[15] MIL solvents were purified by liquid-liquid extraction with acetonitrile/hexane and dried *in vacuo*. MILs were kept for long-term storage in capped glass vials and MILs were stored in a dessicator for at least 24 h prior to use.

Bacteria and Culture Conditions: *Serratia marcescens* (originally from Carolina Biological Supply Company, Burlington, NC, USA) was sourced from a teaching lab at Iowa State. *Salmonella enterica* subspecies *enterica* ser. Typhimurium ATCC 14028 and *Escherichia coli* ATCC 25922 were from the American Type Culture Collection (ATCC, Manassas, VA, USA). Overnight cultures (10 mL) of *S. marcescens* were grown at 25 °C in 250 mL glass Erlenmeyer flasks containing Luria Bertani (LB) broth (Becton, Dickinson and Company [BD], Franklin Lakes, NJ, USA) supplemented with 1% (wt/vol) glycerol to accelerate production of the red pigment prodigiosion.[21]. Flasks were incubated with shaking at 190 rpm in a Shel Lab Shaking Incubator (Sheldon Manufacturing, Inc., Cornelius, OR, USA). *S.* Typhimurium and *E. coli* were grown in 14 mL polystyrene round-bottom tubes (Corning Inc., Corning, NY, USA) containing 10 mL Tryptic Soy Broth (TSB) (BD), and incubated at 37 °C. Organisms were enumerated using Tryptic Soy Agar (TSA) plates (BD).

Pasteurized Liquid Food Products: Two-percent milk (Hy-Vee Reduced Fat Milk), almond milk (Hy-Vee All Natural Original) and a liquid egg product (Hy-Vee 99% Real Egg) were purchased from a local grocery store (Hy-Vee, Ames, IA) for evaluation of MIL-based capture in liquid food products. All foods were evaluated before the "Sell by", "Best if used by" or "Use by" dates listed on their packaging.

MIL-Based Extraction of Viable Bacteria: A representative schematic for the MIL-based extraction of bacteria is depicted in Fig. 1, panel b. A 1 mL aliquot of diluted cell suspension, artificially spiked milk, almond milk or liquid egg product was added to a 2 mL or 4 mL screw cap glass vial. A small volume of MIL (e.g., 15 µL) was added and vortexed vigorously for 30 s to create a cell-capturing microdroplet dispersion. With some samples (e.g. the egg product, due to viscosity and foaming), a magnet was applied externally to concentrate the cell-enriched MIL, although this step was not necessary with some samples, as the hydrophobic, denser-than-water MIL droplets were able to sink to the bottom of the extraction vial. After gravity-based deposition or magnetic extraction, the aqueous phase was then discarded and the MIL was subjected to a brief wash step using 1 mL of nuclease-free water (Integrated DNA Technologies, Coralville, IA, USA), to ensure adequate removal of residual bacteria that were not captured by the MIL microdroplets. Recovery of viable cells from the MIL extraction phase was achieved through a "back-extraction" step that involved vortexing the cell-enriched MIL in 1 mL or 200 µL of a nutritive medium comprised of tryptone (20 g/L; "2x tryptone") and NaCl (10 g/L; "1x NaCl") for 2 min. After back-extraction, captured bacteria were detected using microbiological culture or RPA. Prior to RPA, the cell-enriched back-extraction media was heated at 100 °C for 10 min for cell lysis and release of target nucleic acids. The MIL-RPA method was compared to a

commercial nucleic acid sample preparation approach using the PrepMan Ultra Sample Preparation Reagent (PMU; Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions.

Plating and Enumeration: Following back-extraction, aliquots of the cell-enriched modified LB media were serially diluted in 0.1% peptone water. A 10 µL aliquot of each dilution was applied to the appropriate lane on square, gridded TSA plates as described previously.[22] The track plates were then tilted to an angle of~80° for 15 min to allow the deposited liquid to travel toward the opposite end of the plate. Plates were incubated for 48 h at 25 °C (*S. marcescens*) or for 24 h at 37 °C (*S.* Typhimurium). Colonies were manually counted for determination of the number of CFUs in each sample. The enrichment factor (EF) for the MIL-based method was calculated using Equation 1, where C_{MIL} represents the concentration of bacteria in suspension following MIL-based extraction and C_S is the concentration of bacteria in the initial sample.

$$E_F = \frac{C_{MIL}}{C_S} (1)$$

Recombinase Polymerase Amplification: RPA TwistAmp Basic and TwistFlow *Salmonella* were purchased from TwistDx (Cambridge, UK). RPA was carried out according to the manufacturer's instructions and results were visualized using either gel electrophoresis (TwistAmp Basic kit) or a chromatographic lateral flow assay (TwistFlow *Salmonella* kit). Using the TwistAmp Basic kit, a 340 bp region of the putative dienelactone hydrolase gene (DLH) was amplified using the following primers: (Forward primer) 5'-GCC GGG CAG CRA TTA TTC TGC ATG AA-3'and (Reverse primer) 5'-TGG CGT ATA CGG GAA CCG TAA TAG CA-3'. An *in silico* analysis of this primer set using the Primer-Blast tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi)[23] indicated that within the *Salmonella enterica* I database (NCBI taxonomy ID: 59201), the primer set matched several subspecies I serotypes, including the top three disease-causing serotypes identified in the most recent Centers for Disease Control and Prevention (CDC) *Salmonella* Surveillance Report.[24] These include *S.* Enteritidis (225 hits within the *S. enterica* I database), *S.* Typhimurium (57 hits) and *S.* Newport (21 hits). Because the Primer-Blast software does not accommodate degenerate bases (the DLH primer set contains an "R", which indicates either an "A" or a "G" in this position), it is expected that additional *Salmonella* serovars will be detected with this primer set. An *invA* target, using primers described by Liu et al. was also investigated for use in gel electrophoresis-based experiments.[25] For both assays, primers were diluted with nuclease free water from a 100 µM stock of mixed primers to a working concentration of 10 µM. Because the DLH primer set resulted in higher amplicon production, it was used in subsequent experiments.

For the TwistFlow *Salmonella* kit (also targeting the *invA* gene), a master mix containing primer in rehydration buffer and nuclease-free water was prepared. Sample DNA was obtained from the MIL back-extraction, or using the PMU approach as per manufacturer's instructions. For each kit, master mix, plus 1 µL of sample DNA was added to the lyophilized RPA reagents contained in a PCR tube, where the entire volume was mixed using a pipette. Following this, 2.5 µL of 280 mM magnesium acetate was added to initiate amplification. Sample tubes were inverted vigorously 10 times, vortexed for 10 s, followed by centrifugation for 5 s to draw the sample to the base of the tube. This mixing process was repeated after 4 min of incubation, and after completion of incubation. For both the TwistAmp Basic and TwistFlow *Salmonella* kits, reactions were incubated using a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA) at 40 °C for 20 min. *S.* Typhimurium was tested for each RPA kit, with *E. coli* serving as a negative control.

Gel Electrophoresis: After heating, RPA products generated using the TwistAmp Basic kit were mixed with 10 µL of 6X bromophenol blue/xylene cyanol FF loading dye and loaded on a 1% agarose gel stained with either SYBR Safe DNA Gel Stain (Thermo Fisher Scientific) or GelRed (Biotium, Fremont, CA, USA). Using 1X TBE as the running buffer, samples were electrophoresed using a Mupid-2Plus Submarine Electrophoresis System (Mupid Co., Ltd., Tokyo, JP), for 35 min at 100 V. Bands were visualized using either a Safe Imager 2.0 Blue Light Transilluminator (Thermo Fisher Scientific), or an Azure Biosystems c300 imaging system (Azure Biosystems, Dublin, CA, USA) at 302 nm with a 20 s exposure time.

Lateral Flow Assay: Single-tube amplification products generated using the TwistFlow *Salmonella* kit were added directly to a nucleic acid lateral flow immunoassay (NALFIA) disposable cartridge (Ustar Biotechnologies (Hangzhou) Ltd., Hangzhou, CN). The NALFIA relies on visual detection of a test band facilitated by the extension of biotin and 6-carboxyfluorescein (6-FAM) labeled primers during RPA. The amplification product is visible by eye as a result of aggregation of streptavidin-conjugated gold nanoparticles, which bind to the biotin-labeled 5' end of the double-stranded amplicon. The terminal 6-FAM group of the amplicon is also selectively captured by the anti-FAM antibody, which is embedded in the test line on the lateral flow strip. A control line consisting of biotin-conjugated BSA exhibits strong affinity for any remaining streptavidin-conjugated gold nanoparticles and can be visualized for a valid assay. Generation of a red band at the test position indicates successful amplification of the double-stranded product, sandwiched between the bound anti-FAM antibody and the streptavidin-conjugated gold nanoparticles. A positive result was recorded if both the control and test bands were identified within 10 min, whereas detection of only the control band indicated a negative result.

Results and Discussion

Improved MIL Extraction Conditions for Gram-Negative Bacteria: To begin our investigation of the MIL capture process for bacteria other than the previously-reported *E. coli* K-12, we selected the non-pathogenic *Serratia marcescens*. *S. marcescens*, a gram-negative bacterium in the same family as *Salmonella*, produces the reddish-orange pigment prodigiosin, allowing its unambiguous visual detection when concentrated and facilitating its use as a model gram-negative bacterium in development of pre-analytical sample preparation methods.[26] Since the chemical structure of the MIL has profound implications on its extraction behavior,[18,27] three MILs were studied for bacterial extraction (Fig. 1a). By vigorously dispersing a small volume of MIL (e.g., 15 µL) in an aqueous suspension of *S. marcescens* (1×10^3 CFU mL^{-1}), cells were extracted into the resulting MIL microdroplets and cell-enriched microdroplets were harvested based on either MIL

density (~1.3 g mL^{-1} for the Ni(II) MIL) or through manipulation with an external magnetic field (**Fig. 1b**).

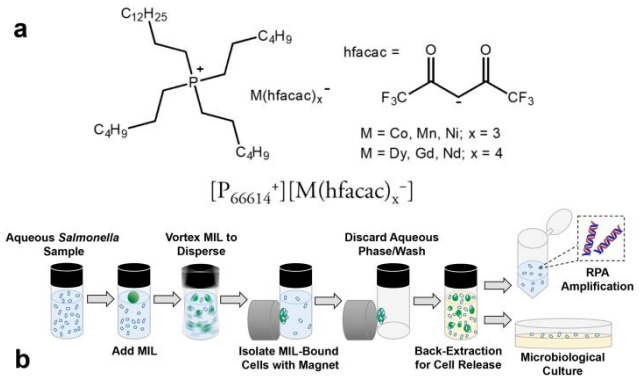

Figure 1. (a) Structures of MILs evaluated in this study; **(b)** Schematic for the extraction and preconcentration of *Salmonella* Typhimurium from aqueous samples, followed by downstream analysis using RPA amplification and microbiological culture detection methods. Panel b adapted from Clark et al., 2017.[20]

After the cell-enriched MIL was rinsed with deionized water, bacteria were recovered from the extraction phase using Luria-Bertani nutrient broth (LB, per L: 10 g tryptone, 5 g yeast extract, 10 g NaCl), plated, and incubated at 25 °C for 48 h prior to enumeration. Of the three MILs studied, the Ni(II) and Co(II) MILs yielded comparable colony forming units (CFUs), whereas no growth was detected after extraction using the Dy(III) MIL (Fig. 2, inset). Despite possessing identical cation moieties ([P_{66614}^+]) and ligands (hfacac), the extraction of *S. marcescens* by MILs strongly depended on the identity of the metal component. This phenomenon has also been observed when using similar MIL solvents for the preconcentration of nucleic acids from aqueous solution.[28]

To maximize the recovery of viable bacteria from the MIL extraction phase, several LB-based back-extraction media varying in ionic strength and nutrient composition were investigated (Fig. 2). For these experiments, suspensions of *S. marcescens* (1×10^3 CFU mL^{-1}, in 1 mL 0.1% peptone) were vortexed for 30 s with 15 µL of the Co(II) MIL, then resuspended for a 2 min back-extraction into 1 mL of either water (control), LB medium or 7 variations of the basic LB medium recipe. Back-extraction using deionized water resulted in the lowest quantity of bacteria recovered from the MIL while the greatest quantity of cells was obtained with a nutrient-rich tryptone medium supplemented with NaCl (2x T, 1x NaCl, Fig. 2). Back-extracted samples were diluted 100-fold prior to plating to ensure that countable dilutions within the statistically valid range of 25 – 250 CFU were obtained. Our results show that *S. marcescens* cells were physically enriched by the MIL to levels between 5 and 6 times higher than their initial concentration. Apart from the higher ionic strength of the best back-extraction media, which has previously been shown to assist in the recovery of gram-negative bacteria,[20] it is conceivable that the hydrophobic MIL solvent imposes stress on the cell in a process that is attenuated by transferring the bacteria to a supportive nutrient media.[29] Because the 2x T, 1x NaCl back-extraction medium provided the highest recoveries for *S. marcescens*, it was selected for use in subsequent experiments.

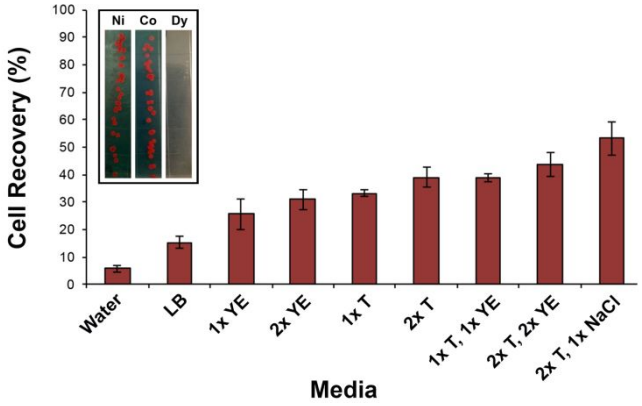

Figure 2. Recovery of *S. marcescens* extracted with Co(II) MIL as a function of back-extraction medium composition. Percentage of initial cell load recovered from the Co(II) MIL extractant using aqueous back-extraction media of different ionic composition is shown. A suspension of *S. marcescens* was prepared and captured with the Co(II) MIL as described in the text, then back-extracted into water (control), LB medium or 7 variations of the basic LB medium recipe. Back-extraction media used: **1X YE** (5 g L^{-1} yeast extract); **2x YE** (10 g L^{-1} yeast extract); **1x T** (10 g L^{-1} tryptone); **2x T** (20 g L^{-1} tryptone); **1x T, 1x YE** (10 g L^{-1} tryptone, 5 g L^{-1} yeast extract); **2x T, 2x YE** (20 g L^{-1} tryptone, 10 g L^{-1} yeast extract); **2x T, 1x NaCl** (20 g L^{-1} tryptone, 10 g L^{-1} NaCl). Average cell recoveries for three separate experiments are shown. **Inset:** Representative cell growth on track plates obtained following the extraction of *S. marcescens* using Ni(II), Co(II) and Dy(III) MILs.

Next, we investigated our MIL-based method for the preconcentration of *S.* Typhimurium, using the 2x T, 1x NaCl back-extraction medium. As with *S. marcescens*, similar recoveries of viable *Salmonella* were observed for the Ni(II) and Co(II) MILs, resulting in enrichment factors of approximately 12, which is comparable to previous enrichment factors for the MIL-based extraction of *E. coli*.[20] The work reported here represents the first use of MILs as solvents for the preconcentration of viable pathogenic bacteria from aqueous suspensions. Because Ni(II) and Co(II) results were similar, subsequent experiments toward coupling MIL-based bacterial extraction with molecular detection using RPA were performed using the [P_{66614}^+] [Ni(hfacac)$_3^-$] MIL.

Combining MIL-based Extraction with *Salmonella*-Targeted RPA: In an effort to identify improved approaches for food analysis, we investigated the feasibility of coupling our MIL-based method for capture and concentration of *S.* Typhimurium from aqueous media with the speed and simplicity of RPA analysis. Briefly, after preconcentration and recovery of *S.* Typhimurium from an aqueous sample using the Ni(II) MIL, the back-extraction suspension was heated for 10 min at 100 °C to lyse the bacteria and release their nucleic acids for downstream RPA analysis. Initially, we compared two primer sets for the amplification of nucleic acids from *S.* Typhimurium: primers described by Liu et al.[25] targeting the *invA* gene, which codes for a protein involved in invasion of host intestinal epithelia by *Salmonella*, and primers identified *via* comparative genomic analysis and amplifying a 340 bp

region of a putative dienelactone hydrolase gene (DLH, this study). Although both primer sets successfully amplified DNA from the bacteria recovered from the MIL phase, gel electrophoresis results demonstrated that the DLH-targeted primers yielded bands with higher fluorescence intensities (Fig. S1). As a control, *E. coli* ATCC 25922 (10^5 CFU mL^{-1}) was extracted using the Ni(II) MIL, lysed and examined *via* DLH-based RPA. No amplicon was detected, indicating good selectivity of the DLH primers for *Salmonella* (Fig. S1).

Evaluating Use of a Power-free Heat Source for *Salmonella* -Targeted RPA: A major limitation of many nucleic acid amplification methodologies is their reliance on electricity to power a heat source such as a thermal cycler in PCR or a heat block for isothermal methods. In an effort to circumvent this limitation, we examined supersaturated sodium acetate heat packs - a small, portable consumer-grade novelty product used in handwarmers and earmuffs (Cristalheat, www.xUmp.com) - as a power-free means for driving RPA. In initial work, we measured the internal temperature of a template-free RPA reaction tube sandwiched between two activated heat packs using an OPTOCON FOTEMP1-4 fiber optic temperature monitoring system (Optocon AG, Dresden, Germany). Data were collected using the FOTEMP Assistant software, exported to Microsoft Excel and plotted in Prism graphing software (Prism 7 for Mac OS X, v. 7.0d, GraphPad Software, La Jolla, CA) (Fig. 3). The temperature of the RPA mixture increased rapidly after heat pack activation, reached equilibrium between 42 °C and 44 °C (Fig. 3a) and remained within optimal RPA temperature range for up to 40 min. We then tested the performance of heat pack-driven RPA using the DLH primer set and MIL-extracted *S.* Typhimurium. Intense amplicon bands were seen for sodium acetate-driven DLH-RPA with DNA isolated using either the PrepMan Ultra Sample Preparation Reagent (PMU-SA) or with MIL-extracted cells (MIL-SA) (Fig. 3b). Although a band was seen with the sodium acetate-heated no-template control (NTC-SA), it is expected that use of lateral flow-based detection would enable differentiation of legitimate amplicons from spurious NTC bands sometimes seen on agarose gels with RPA[30,31] These results highlight the utility of sodium acetate heat packs as a viable, power-free means for amplifying nucleic acids from microbial pathogens using RPA.

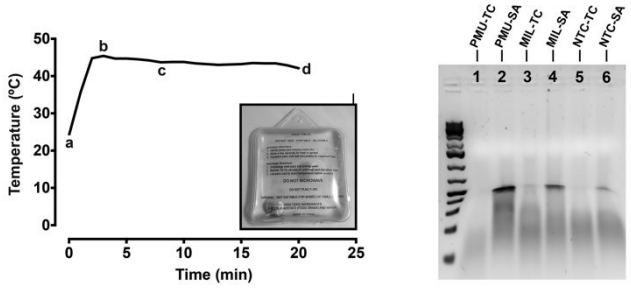

Figure 3. Evaluation of sodium acetate heat pack for power-free incubation of RPA reactions. In order to assess the utility of sodium acetate heat packs (inset) for portable and power-free incubation of RPA reactions, we measured the internal temperature of a template-free RPA reaction tube sandwiched between two activated heat packs using a fiber optic temperature monitoring system as described in the text. **Lefthand panel:** Sodium acetate heat packs were able to provide near-optimal RPA reaction temperatures (reported range, 37 °C – 42 °C) over typical amplification times. Temperatures at various points along the time-temperature curve are 25.2 °C (initial temperature, point a), 45.4 °C (point b), 43.7 °C (point c) and 42.1 °C (point d). **Righthand panel:** Comparison of DLH-RPA reactions driven with a thermal cycler (TC) or with sodium acetate heat packs (SA), using bacterial DNA obtained using the PrepMan Ultra Sample Preparation Reagent (PMU) or *via* MIL-based extraction (MIL).

Comparison of Methods for Amplicon Detection, Further Improvement of MIL Approach: All elements of a detection assay (cell capture, release of nucleic acids, amplification of target DNA and product detection) may impact the quality of the final result. With this in mind, we evaluated different methods for nucleic acid release in conjunction with further improvement of the MIL-based workflow (larger sample vial size, smaller back-extraction volume) and two approaches for amplicon detection (gel electrophoresis, nucleic acid lateral flow immunoassay [NALFIA]). For direct comparison of methods for release of nucleic acids prior to RPA, we compared heating of the cell-enriched MIL to 100°C (with modifications, as described below) with use the commercial reagent PMU. For both approaches, aqueous samples were inoculated with *S.* Typhimurium at concentrations ranging from 10^3 to 10^6 CFU mL^{-1}, followed by DLH-targeted RPA.

The PMU method was used according to the manufacturer's instructions. Briefly, cells from a liquid suspension were lysed in 200 µL of PMU reagent, followed by heating (15 min, 100°C) and centrifugation to separate cellular debris from the DNA-containing supernatant. The PMU method facilitated consistent detection down to 10^4 CFU mL^{-1}, while detection at lower levels was inconsistent (Fig. 4 gel image, lane 1). PMU was not evaluated for lysis of MIL-captured cells, as this would have introduced additional assay elements, namely use of a chemical lysis reagent and a centrifugation step. Based on observations of liquid behavior during vortexing, we hypothesized that slight modifications to our established MIL-RPA approach might result in process improvements. By decreasing the sample vial size from 4 mL to 2 mL and decreasing the back-extraction volume from 1 mL to 200 µL, we found we could lower our detection limit to 10^3 CFU mL^{-1}, presumably due to enhanced contact between the MIL and back extraction solution when a smaller sample vial was used and the increased concentration of bacteria in the smaller back-extraction volume (Fig. 4 gel image, lane 3). These slight modifications enabled us to improve the sensitivity of our streamlined "capture, concentrate, heat and amplify" MIL-RPA process, without the addition of further assay elements.

Without these process modifications, the limit of detection for our MIL-RPA approach was 10^4 CFU mL^{-1} for gel electrophoresis-based visualization of *Salmonella* DLH amplicons.

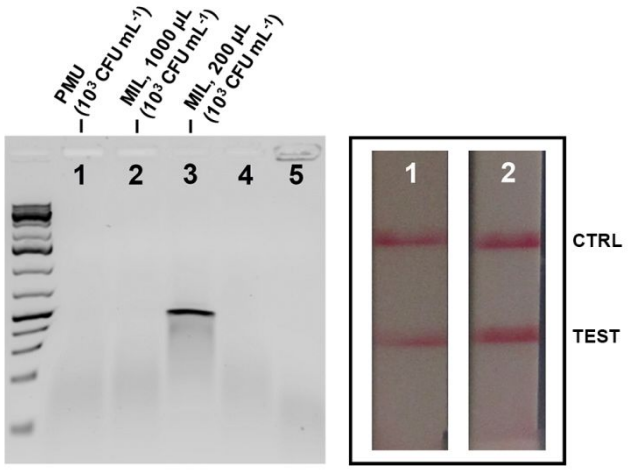

Figure 4. MIL-RPA with Ni(II) MIL. The combined MIL and RPA approach was visualized using gel electrophoresis (35 min) or lateral flow (10 min). MIL-based extraction was compared with concurrent extraction using PrepMan Ultra Sample Preparation Reagent (PMU). For gel electrophoresis (**lefthand panel**), PMU LOD was consistently identified as 10^4 CFU mL^{-1}, however, bands were inconsistently present at lower levels (lane 1), or were less defined than those for the Ni(II) MIL at the same level (lane 3). Lanes 4, 5 represent no template controls (NTC). With lateral flow (**right-hand panel**) detection was achieved as low as 10^4 CFU mL^{-1} for both PMU (strip 1) and the MIL (strip 2).

Because conventional gel electrophoresis is inherently laboratory-bound and time consuming, we sought to evaluate the use of a rapid, portable alternative for amplicon detection. Due to its simplicity and portability, NALFIA is often used in resource-limited environments, or in non-laboratory settings,[10] as it does not require electricity or laboratory equipment. Using the Ni(II) MIL for preconcentration and extraction of aqueous samples of *S.* Typhimurium at concentrations ranging from 10^3 to 10^6 CFU mL^{-1}, RPA was carried out using the TwistFlow *Salmonella* kit from TwistDx. Test results were determined using a 5 min NALFIA step. Initially, the combined MIL-RPA-NALFIA approach facilitated detection at levels as low as 10^5 CFU mL^{-1}. Since the detection limits using NALFIA were 2 log higher than with gel electrophoresis, we investigated whether metal ions released from the hydrophobic MIL phase (e.g., Ni^{2+})[28] during back-extraction had any influence on the outcome of the NALFIA step. For these experiments, PMU samples were spiked with levels of $NiCl_2$ ranging from 0.2 mM to 2 mM. Visible control and test bands were observed for all $NiCl_2$ samples, suggesting that Ni^{2+} potentially released from the MIL did not inhibit the NALFIA. It is important to note that the NALFIA targets the *invA* gene, using primers and conditions developed by the manufacturer. Our choice of the dienelactone hydrolase gene target for the gel electrophoresis experiments may also have contributed to differences in observed detection limits due to differences in amplification efficiency between the two primer sets. In order to improve detection limits, the back-extraction volume was decreased to 200 µL, resulting in detection limits for *S.* Typhimurium as low as 10^3 CFU mL^{-1} with the Ni(II) MIL (Fig. 4). The PMU method provided detection of *Salmonella* at levels as low as 10^4 CFU mL^{-1} (Fig. 4), but once again requires the use of a benchtop centrifuge that is incompatible with pathogen analysis in the field or in resource limited settings.

MIL-RPA for detection of *Salmonella* in Liquid Food Samples: In order to examine the application of the combined MIL-RPA method in a practical setting, it was applied for the detection of *S.* Typhimurium in food samples including milk (2% milk fat) and almond milk. *S.* Typhimurium cells were inoculated into 1 mL samples at 10^5 CFU mL^{-1} and extracted using the Ni(II) MIL under improved conditions. The combined approach enabled detection of S. Typhimurium at 10^5 CFU mL^{-1} (Fig. 5a). The PMU method did not consistently detect *S.* Typhimurium in spiked 2% milk samples. To explore whether dilution of samples (and of potentially interfering substances such as fats and proteins) might lead to improvements in both PMU and MIL samples, samples were diluted with 0.1% peptone water (PW) in some experiments. Interestingly, and for unknown reasons, PW appeared to reduce the efficacy of both PMU extraction of cell DNA and MIL-based cell capture, with the diluted MIL sample not yielding a detectable band (Fig 5a, lane 6). These results suggest examination of alternate diluents (i.e. molecular-grade water) in future experiments to explore whether such inhibition may be avoided and if a more appropriate dilution medium may represent a viable approach for reducing sample complexity, if needed. Because *Salmonella* spp. have been especially problematic in eggs, with large outbreaks occurring in 2010 (almost a half a billion eggs recalled)[32] and 2018 (almost 207 million eggs recalled)[33], the MIL-based preconcentration method was applied to liquid egg samples spiked with concentrations of *S.* Typhimurium ranging from 10^3 to 10^5 CFU mL^{-1}. The foamy nature of the liquid egg sample initially caused challenges in recovering a sufficient volume of MIL for downstream detection bacteria. However, ~1 min exposure of the sample to a 0.66 T rod magnet to the base of the 2 mL glass vial facilitated collection of the cell-enriched MIL solvent, enabling detection limits as low as 10^4 CFU mL^{-1} (Fig. 5b). The commercial PMU method was concurrently compared to the MIL-based approach and exhibited an identical detection limit using RPA and gel electrophoresis. However, the PMU method resulted in less intense bands than those from the MIL-based extraction method (Fig. 5b). The Ni(II) MIL was also applied for preconcentration of *S.* Typhimurium in liquid egg samples coupled to RPA and NALFIA readout. The combined MIL-RPA-NALFIA approach facilitated detection at levels as low as 10^4 CFU mL^{-1} in inoculated liquid egg samples. Once again, this method was concurrently compared with the use of PMU, which maintained similar detection levels (Fig. 5c) but required a centrifugation step that is not compatible with field sampling or on-site analysis.

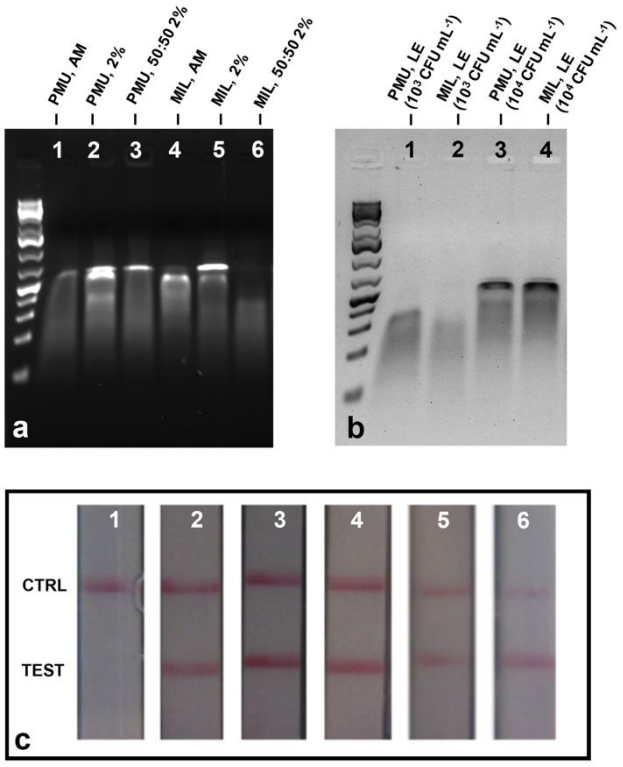

Figure 5. RPA-based detection of *Salmonella* Typhimurium in liquid food products. The combined MIL and RPA approach was applied to almond milk, milk (2% milk fat), 50:50 dilution of milk (2% milk fat: 0.1% peptone water) and liquid egg product. Results were visualized using gel electrophoresis (35 min) or lateral flow (10 min). MIL-based extraction was compared with concurrent extraction using PrepMan Ultra Sample Preparation Reagent (PMU). For *Salmonella*-spiked almond milk (**Panel a, lanes 1, 4**), milk (2% milk fat) (**Panel a, lanes 2, 5**) and 50:50 milk dilution samples (**Panel a, lanes 3, 6**) using gel electrophoresis and either extraction method (PMU or MIL), detection was achieved at 10^5 CFU mL^{-1}. For liquid egg samples spiked with *S.* Typhimurium (**Panel b**), PMU and MIL LOD was 10^4 CFU mL^{-1} (**Panel b, lanes 3, 4**). Using lateral flow (**Panel c**), for *Salmonella*-spiked liquid egg samples, LOD was identified 10^4 CFU mL^{-1} for both PMU (**Panel c, 3, 4**) and MIL-extracted samples (**Panel c, 5, 6**). Strips 1 and 2 are the no template control (NTC) and internal control, respectively.

Conclusions: We report further improvement of our previously described method for capture of gram-negative bacteria and the first use of this approach for capture and concentration of a human pathogen from aqueous suspensions, including at-risk foods. Post-capture growth on non-selective media suggested a lack of cytotoxicity with this approach. We coupled capture and concentration of *S.* Typhimurium to RPA, a rapid isothermal method for DNA amplification, and found that we could drive RPA reactions using inexpensive and regenerable sodium acetate heat packs, eliminating the need for an external power source. *Salmonella* RPA amplicons could be detected in <10 min using a simple chromatographic readout. Our approach is simple, streamlined and amenable to analyses in the field or in other resource-limited environments.

Apart from their magnetic properties, the MILs used here have other advantageous characteristics useful for the analysis of aqueous foods or food suspensions. Unlike other recently reported MILs, which have reported room temperature densities that are on par with that of water[34], the Ni(II) and Co(II) MILs used here had densities of ~1.3 g mL^{-1}, which lie between the densities of glycerol (1.26 g mL^{-1}) and corn syrup (~1.4 g mL^{-1}). Because the MILs used in this study are both hydrophobic and denser than water, they are well-suited for analysis of aqueous solutions such as liquid foods or food suspensions, and can be collected through either simple density-based sedimentation or with application of an external magnet. For automated and high-throughput applications in the food industry, it is possible that use of a strong electromagnet for post-extraction collection of MILs could result in fast and uniform capture of cell-enriched MILs. It may also be possible to minimize the costs and environmental impacts of high-throughput use of MILs in food testing by developing methods capable of recycling MILs for multiple rounds of cell capture. The approach described here bridges the disciplines of food science, materials science and chemistry, providing new tools for rapid and efficient extraction of viable cells in support of pathogen detection efforts. Future work will focus on extending MIL-RPA to additional foodborne pathogens, including the gram-positive pathogen *Listeria monocytogenes* and assessment of any negative impacts that MIL-based capture may have on bacterial physiology, such as injury or antimicrobial activity, with the development of approaches for mitigation of these impacts, should they occur.

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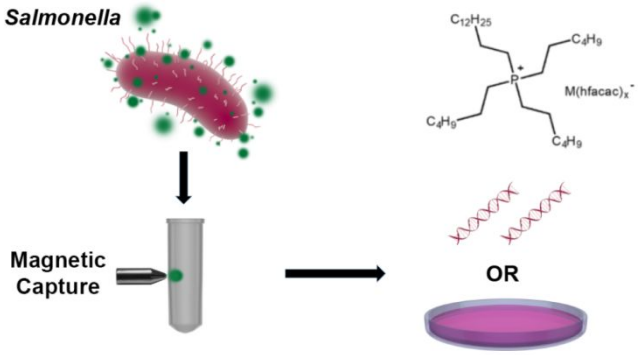

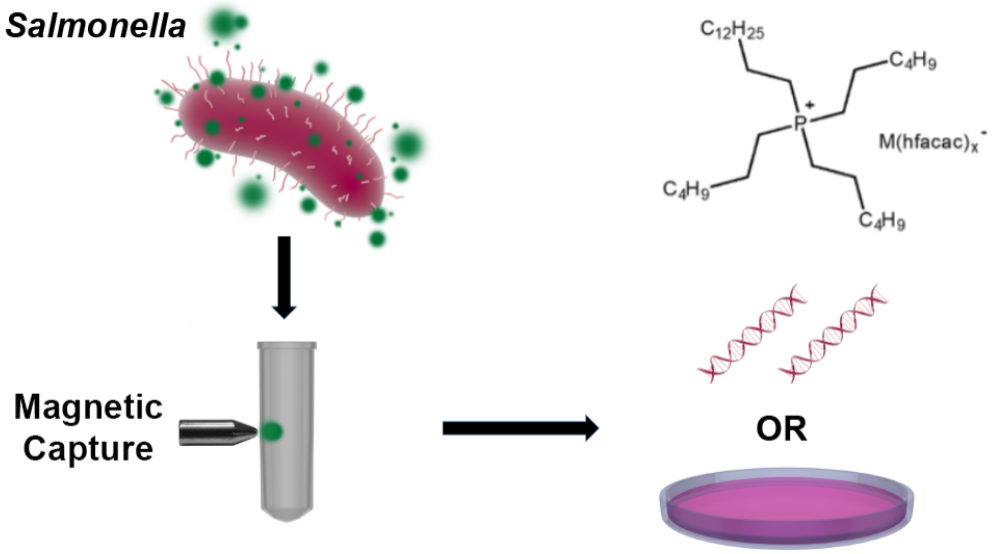

Magnetic ionic liquid (MIL)-based extraction and concentration of *Salmonella* from liquid foods was combined with rapid detection of this pathogen using Recombinase Polymerase Amplification (RPA). Our results demonstrate the utility of MILs for pre-analytical sample preparation of foods in support of rapid molecular diagnostics for the food industry.

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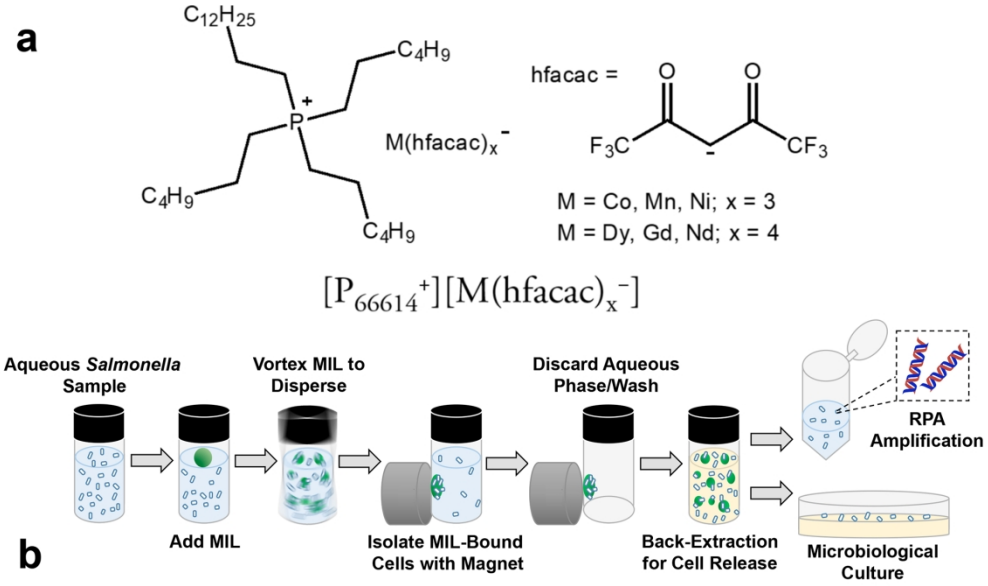


Figure 1. (a) Structures of MILs evaluated in this study; (b) Schematic for the extraction and preconcentration of *Salmonella* Typhimurium from aqueous samples, followed by downstream analysis using RPA amplification and microbiological culture detection methods. Panel b adapted from Clark *et al.*, 2017.[20]

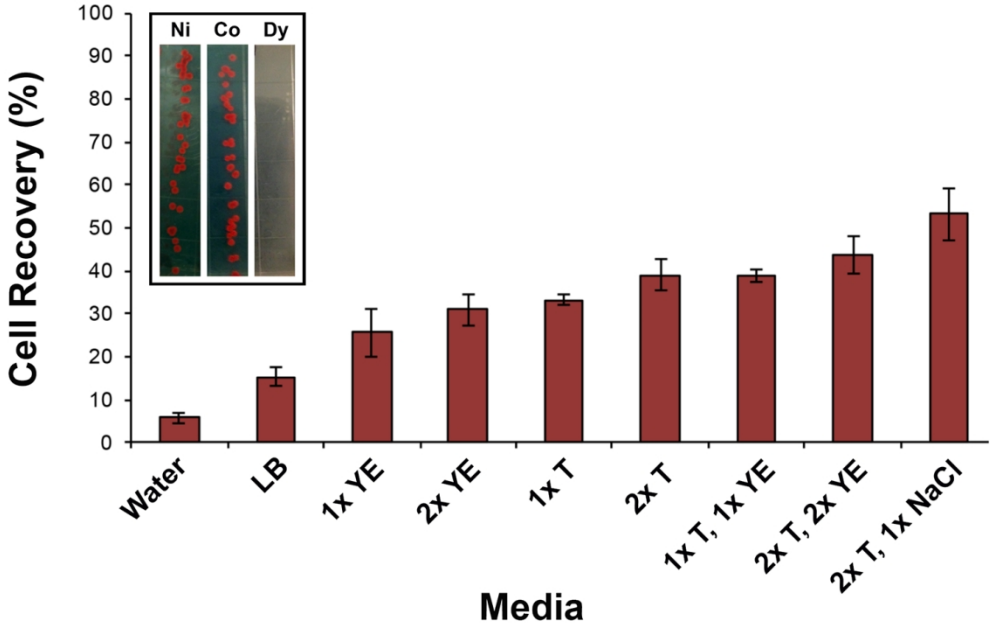


Figure 2. Recovery of *S. marcescens* extracted with Co(II) MIL as a function of back-extraction medium composition. Percentage of initial cell load recovered from the Co(II) MIL extractant using aqueous back-extraction media of different ionic composition is shown. A suspension of *S. marcescens* was prepared and captured with the Co(II) MIL as described in the text, then back-extracted into water (control), LB medium or 7 variations of the basic LB medium recipe. Back-extraction media used: **1X YE** (5 g L^{-1} yeast extract); **2x YE** (10 g L^{-1} yeast extract); **1x T** (10 g L^{-1} tryptone); **2x T** (20 g L^{-1} tryptone); **1x T, 1x YE** (10 g L^{-1} tryptone, 5 g L^{-1} yeast extract); **2x T, 2x YE** (20 g L^{-1} tryptone, 10 g L^{-1} yeast extract); **2x T, 1x NaCl** (20 g L^{-1} tryptone, 10 g L^{-1} NaCl). Average cell recoveries for three separate experiments are shown. Inset: Representative cell growth on track plates obtained following the extraction of *S. marcescens* using Ni(II), Co(II) and Dy(III) MILs.

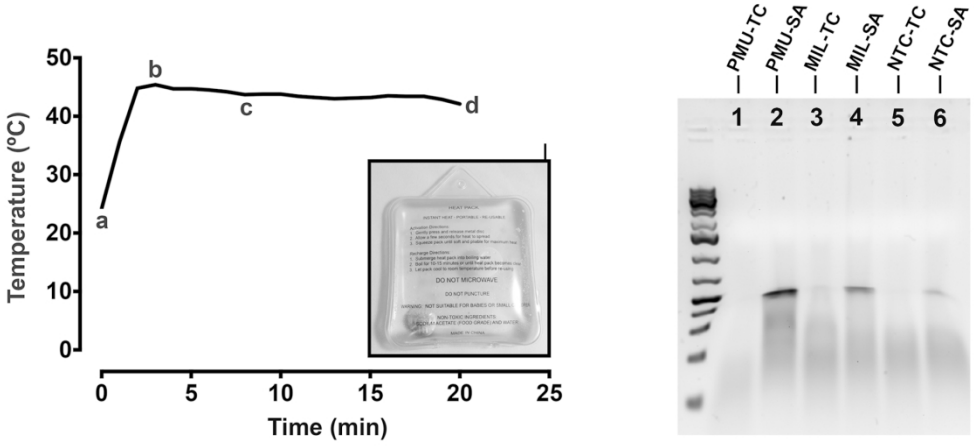
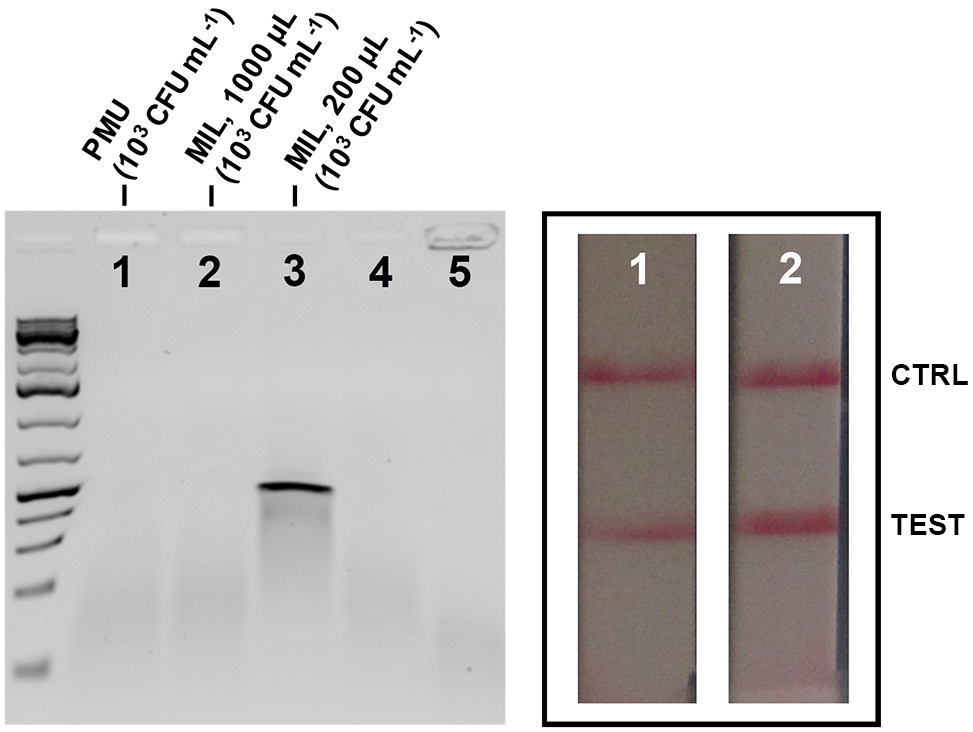


Figure 3. Evaluation of sodium acetate heat pack for power-free incubation of RPA reactions. In order to assess the utility of sodium acetate heat packs (inset) for portable and power-free incubation of RPA reactions, we measured the internal temperature of a template-free RPA reaction tube sandwiched between two activated heat packs using a fiber optic temperature monitoring system as described in the text. **Lefthand panel:** Sodium acetate heat packs were able to provide near-optimal RPA reaction temperatures (reported range, 37 °C – 42 °C) over typical amplification times. Temperatures at various points along the time-temperature curve are 25.2 °C (initial temperature, point a), 45.4 °C (point b), 43.7 °C (point c) and 42.1 °C (point d). **Righthand panel:** Comparison of DLH-RPA reactions driven with a thermal cycler (TC) or with sodium acetate heat packs (SA), using bacterial DNA obtained using the PrepMan Ultra Sample Preparation Reagent (PMU) or *via* MIL-based extraction (MIL).



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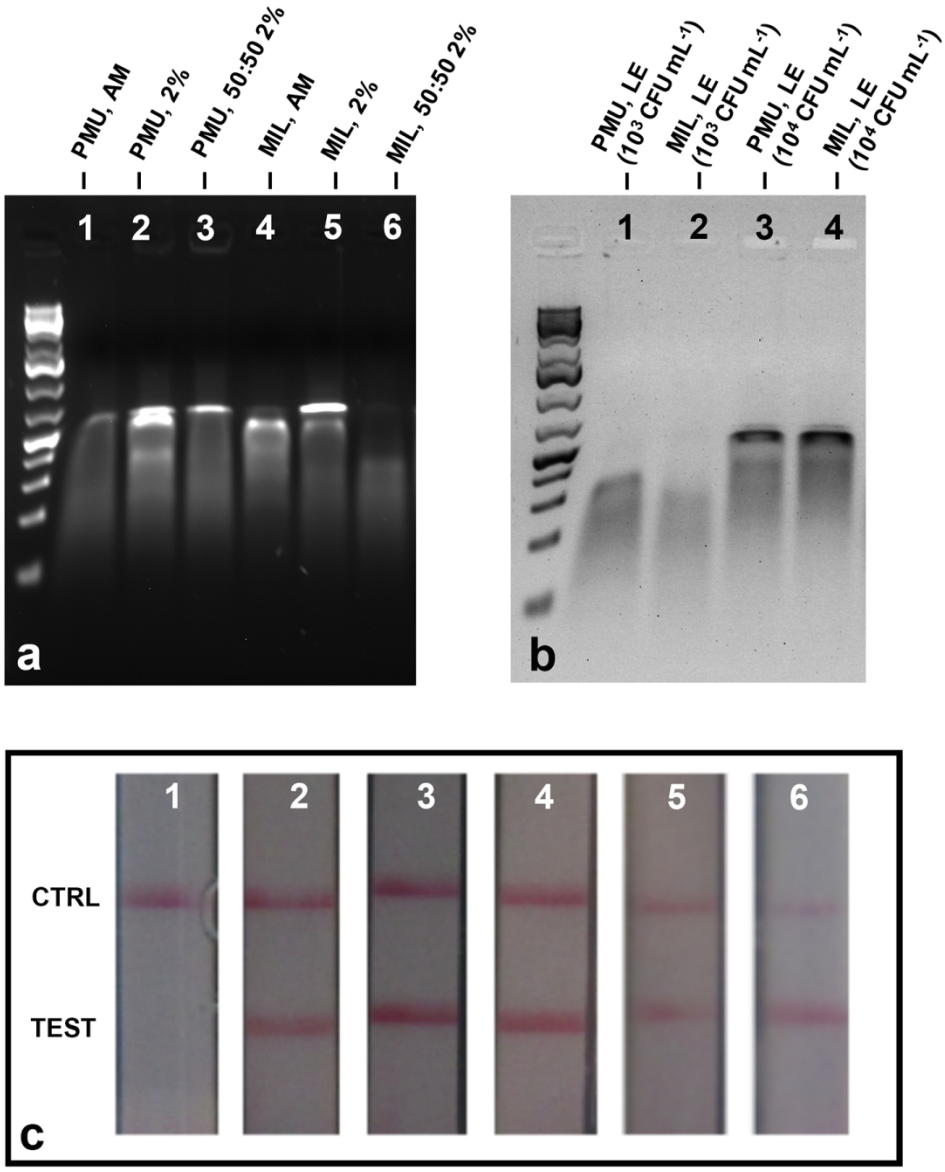

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