Video Article

Electricity-Free, Sequential Nucleic Acid and Protein Isolation

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Abstract

Traditional and emerging pathogens such as Enterohemorrhagic *Escherichia coli* (EHEC), *Yersinia pestis,* or prion-based diseases are of significant concern for governments, industries and medical professionals worldwide. For example, EHECs, combined with *Shigella*, are responsible for the deaths of approximately 325,000 children each year and are particularly prevalent in the developing world where laboratory-based identification, common in the United States, is unavailable [1]. The development and distribution of low cost, field-based, point-of-care tools to aid in the rapid identification and/or diagnosis of pathogens or disease markers could dramatically alter disease progression and patient prognosis. We have developed a tool to isolate nucleic acids and proteins from a sample by solid-phase extraction (SPE) without electricity or associated laboratory equipment [2]. The isolated macromolecules can be used for diagnosis either in a forward lab or using field-based point-of-care platforms. Importantly, this method provides for the direct comparison of nucleic acid and protein data from an un-split sample, offering a confidence through corroboration of genomic and proteomic analysis.

Our isolation tool utilizes the industry standard for solid-phase nucleic acid isolation, the BOOM technology, which isolates nucleic acids from a chaotropic salt solution, usually guanidine isothiocyanate, through binding to silica-based particles or filters [3]. CUBRC's proprietary solid-phase extraction chemistry is used to purify protein from chaotropic salt solutions, in this case, from the waste or flow-thru following nucleic acid isolation[4].

By packaging well-characterized chemistries into a small, inexpensive and simple platform, we have generated a portable system for nucleic acid and protein extraction that can be performed under a variety of conditions. The isolated nucleic acids are stable and can be transported to a position where power is available for PCR amplification while the protein content can immediately be analyzed by hand held or other immunological-based assays. The rapid identification of disease markers in the field could significantly alter the patient's outcome by directing the proper course of treatment at an earlier stage of disease progression. The tool and method described are suitable for use with virtually any infectious agent and offer the user the redundancy of multi-macromolecule type analyses while simultaneously reducing their logistical burden.

Video Link

The video component of this article can be found at https://www.jove.com/video/4202/

Protocol

1. Sample Lysis

1. Samples should be in liquid form. If sample is solid, for example food or stool, the sample should be suspended in a liquid media such as water or phosphate buffered saline (PBS).
2. Mix 500 µL of the sample with 500 µL of 6M Guanidine Thiocyanate (pH 6.5) in a 1.5 mL tube.
3. Depress the bulb of an extraction pipette, expelling the ambient air.
4. Pull the entire 1 mL sample into the extraction pipette, over the sorbent material, by allowing the bulb to slowly expand.
5. Invert the extraction tool such that the sorbent beads and sample drain into the bulb.
6. Grind the sorbent beads with the sample in the bulb being careful not to expel the sample out of the extraction pipette.

2. Nucleic Acid Extraction (Figure 1, Panel A)

1. Invert the extraction pipette (opening downward) and expel the sample into the original 1.5 mL tube.
2. Pull the entire 1 mL sample into the extraction pipette, passing over the sorbent, at a moderate rate, being careful to keep the sorbent in the neck of the extraction pipette.
3. Expel the entire sample into the 1.5 mL tube by depressing the bulb.
4. Repeat steps 2.2 and 2.3, passing the sample over the sorbent four more times for a total of 5 passes.

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5. After the fifth pass over the sorbent, expel the entire 1 mL sample into 4mL of the protein extraction buffer (in a 15 mL conical tube), close the tube, and set aside for later use.
6. Wash the isolated nucleic acids by passing 1 mL of 95% ethanol over the sorbent three times, returning the ethanol to its original tube upon final passage.
7. Repeat step 2.6 using a second 1 mL, 95% ethanol wash.
8. Press and release the bulb for 5 minutes, passing ambient air throughout the extraction pipette to dry the nucleic acids/sorbent bed. Wipe residual ethanol from the tip of the extraction pipette using a Kimwipe.
9. Recover the nucleic acids by passing 250 µL of 10 mM Tris (pH 6.8) over the sorbent five times. The Tris buffer can be replaced by any other suitable aqueous solution such as PBS. The resulting solution contains the extracted nucleic acids.

3. Protein Extraction (Figure 1, Panel B)

1. Mix the sample and protein extraction buffer tube from step 2.5 by inversion and pass approximately two to three milliliters of this 5 mL sample over the sorbent of a new extraction pipette.
2. Expel the entire sample into the same 15mL conical tube being careful to keep the sorbent in the neck of the extraction pipette.
3. Repeat steps 3.1 and 3.2, passing the sample over the sorbent 15 times.
4. Wash the sorbent and the bound protein by passing 1 mL of 95% ethanol over the sorbent bed three times, ejecting the ethanol into it's original tube.
5. Repeat step 3.4 using a second 1 mL, 95% ethanol wash.
6. Press and release the bulb for 5 minutes, passing ambient air throughout the extraction pipette to dry the protein/sorbent bed. Wipe residual ethanol from the tip of the extraction pipette using a kimwipe.
7. Recover the protein by passing 250 µL of PBS over the sorbent five times. The PBS buffer can be replaced by any other suitable aqueous solution such as 10 mM Tris (pH 6.8). The resulting solution contains the protein content of the sample.

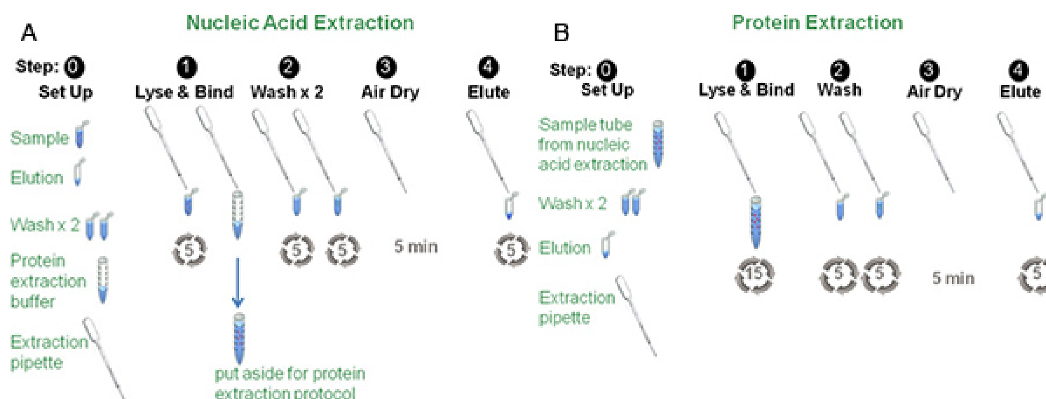

Figure 1.

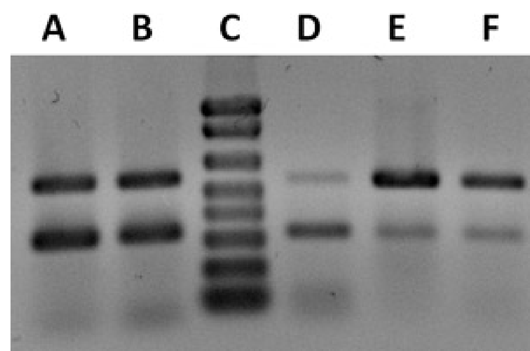

Figure 2.

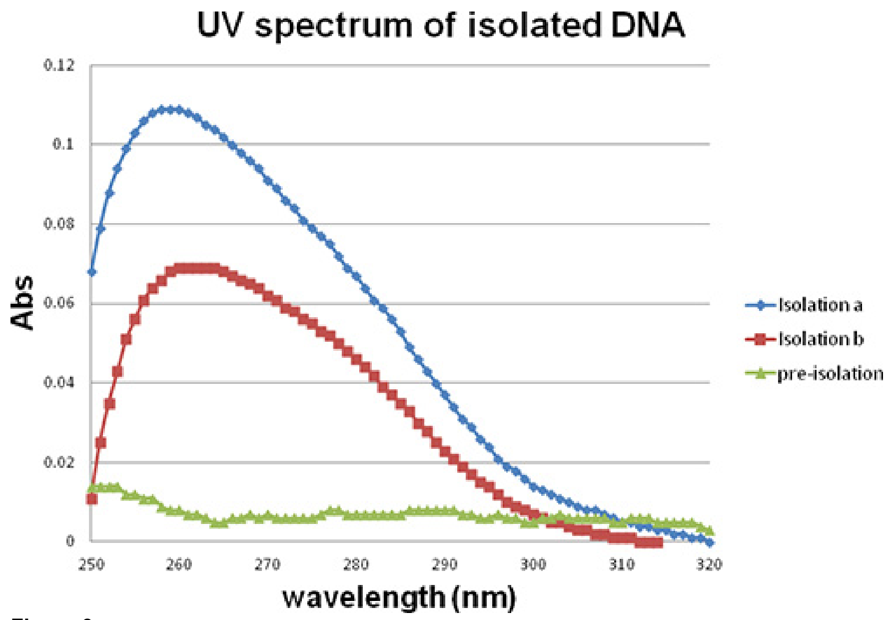

Figure 3.

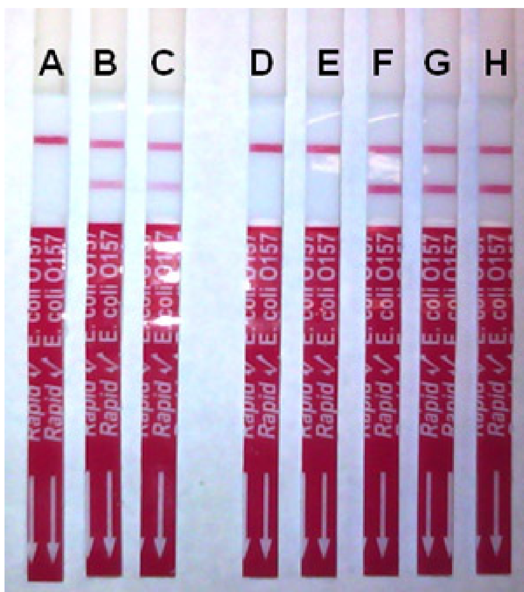

Figure 4.

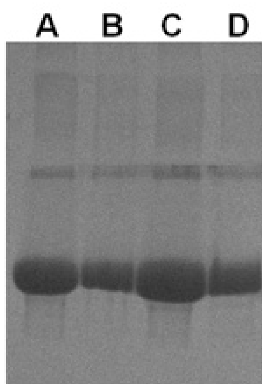

Figure 5.

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Representative Results

Nucleic Acid Isolation: Isolated nucleic acids are PCR amplifiable and free of protein contamination.

Example 1: The recovered nucleic acids are suitable for use in PCR-based applications. For example, **Figure 2** shows the amplification of shiga toxin genes associated with Enterohemorrhagic *E. coli* O157:H7 strain EDL-933 (ATCC # 35150) from a nucleic acid/protein extraction procedure using the extraction pipette. The shiga toxin genes *stx*1 and *stx*2 are found on temperate lambda-like bacteriophage and defective bacteriophage in this and other Enterohemorrhagic *E. coli* strains under the control of the P_R promoter [5 6 7 8]. The activation of the SOS response in *E. coli* leads to prophage induction and/or toxin production [9]. We've utilized a modified version of the multiplexed PCR assay by Feng *et al.*[11] to identify the *stx*1 and *stx*2 genes in both culture and spiked sewage samples. Lanes A and B in **Figure 2** show the results of a PCR amplification of an unprocessed culture sample (lane A) and a processed culture sample (lane B). These results are virtually identical meaning there is no loss of fidelity in the processed or isolated nucleic acids. We next amplified spiked raw sewage (lane D) or isolated nucleic acids from spiked sewage (lanes E & F). These data indicate that the isolated nucleic acids resulted in greater amplification versus the non-isolated nucleic acids from spiked sewage samples. We therefore conclude that either PCR inhibitors naturally found in sewage were removed upon isolation or that the nucleic acids were concentrated upon isolation. A combination of these two hypotheses is also possible.

Ultraviolet spectroscopy was next used to show that the isolated nucleic acids were relatively free of contaminating protein. **Figure 3** shows the UV spectra of two *E. coli* O157:H7 nucleic acid extractions, labeled isolation a and isolation b, in comparison to the normalized spectrum of the bacterial culture, labeled pre-isolation. The UV spectra of the isolated nucleic acids resembles the spectra of pure nucleic acids, having a calculated ratio of absorbance between 260 nm and 280 nm approaching 1.8[10]. These data show that there is very little contaminating protein in the nucleic acid fraction recovered using the technique described.

Protein Isolation: Recovered protein from the nucleic acid/protein isolation experiment is immunoreactive and electrophoretically identical to pure protein.

Example 2: The isolated protein fraction was applied to the RapidChek *E. coli* O157 lateral flow assay strips (SDIX, Inc.). Controls show a positive identification in all samples harboring *E. coli* O157, with or without STX induction (**Figure 4**, lanes B and C, respectively). The data in **Figure 4** also show positive results for those samples harboring *E. coli* O157 that were processed for nucleic acids and then protein using the extraction pipette. These data show that the isolated protein from each sample is immunoreactive thus triggering a positive response.

PCR amplification (as described in Example 1) was performed on the protein samples to show that the isolated protein fraction was devoid of contaminating nucleic acids. The results for these experiments were all negative by gel electrophoresis. Thus, we conclude that the protein content of the sample was separated from contaminating nucleic acids. These results, taken in whole with those described for the nucleic acid isolation step show that the nucleic acids and protein content are isolated in separate fractionation steps.

Example 3: The isolated protein is suitable for electrophoresis [10]. **Figure 5** depicts a coomassie stained 8% acrylamide gel loaded with BSA (**Figure 5**, lanes A & C) and BSA isolated from 6M guanidine isothiocyanate using the extraction pipette and associated protocol. The electrophoretic mobility of the isolated protein is identical to that of the control samples. We thus conclude that the extraction process does not alter the covalent structure of the isolated protein.

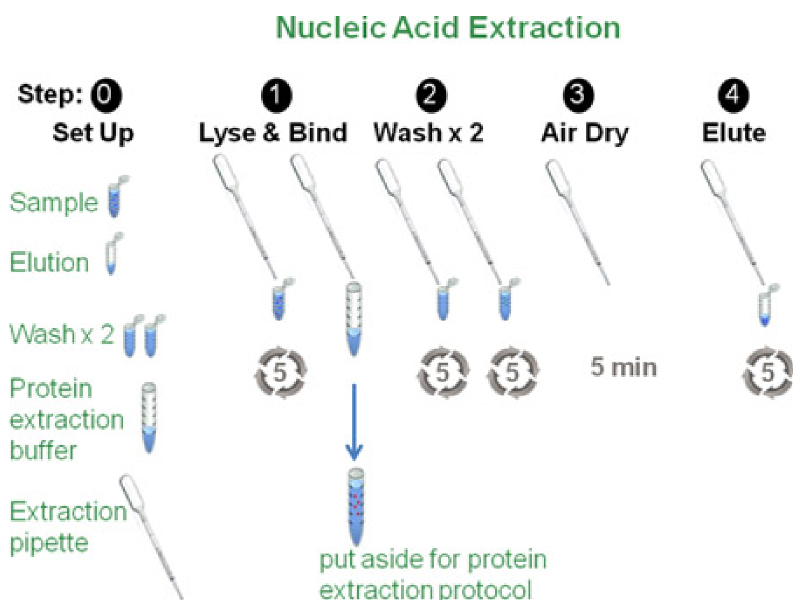

Figure 1, Panel A: Depiction of a typical nucleic acid extraction procedure. The sample is mixed with 6M guanidine thiocyanate in the sample tube and passed over the sorbent five times. After the last passage, the sample is added to the protein extraction buffer. The sorbent and bound nucleic acids are washed twice with ethanol. The sample is then air dried and eluted from the sorbent.

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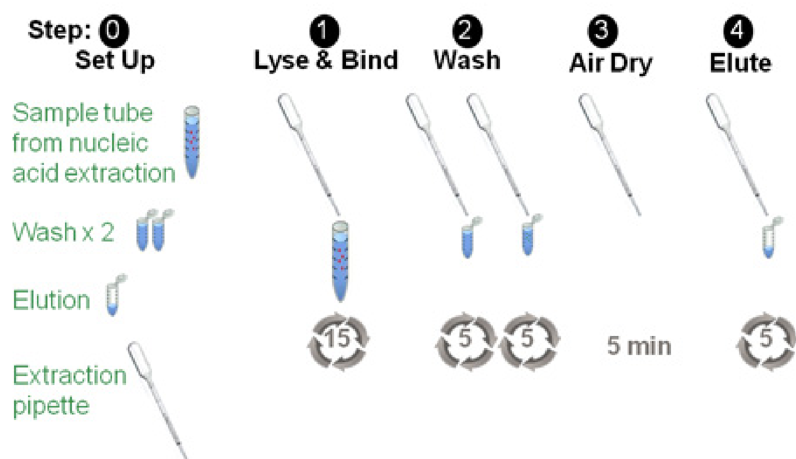

Figure 1, Panel B: Depiction of a typical protein extraction procedure. The sample mixed with protein isolation buffer from step 1, **Figure 1**, panel A is passed over the sorbent of a second extraction pipette 15 times. The remainder of the procedure is identical to that of the nucleic acid extraction protocol.

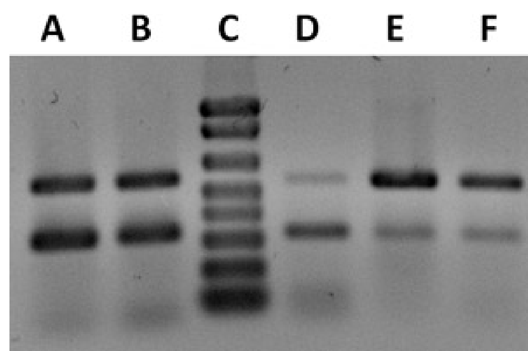

Figure 2: Negative picture of an ethidium bromide stained agarose gel. A multiplexed shiga toxin 1 and 2 gene amplification experiment was performed using culture samples (lanes A and B) or raw sewage samples spiked with *E. coli* O157:H7 (lanes D, E and F). The samples were either directly added to a PCR reaction tube (lanes A and D) or the samples were processed using the isolation process and the extracted nucleic acids were added to the PCR reaction tube (Samples B, E and F). The PCR protocol is a modification of the one described by Feng *et al*[11]. Our modification targeted only those amplified by the *stx*1 (lower band) and *stx*2 (upper band) primer pairs, dropping out all other primer pairs. Lane C is the 1Kb ladder from BioRad.

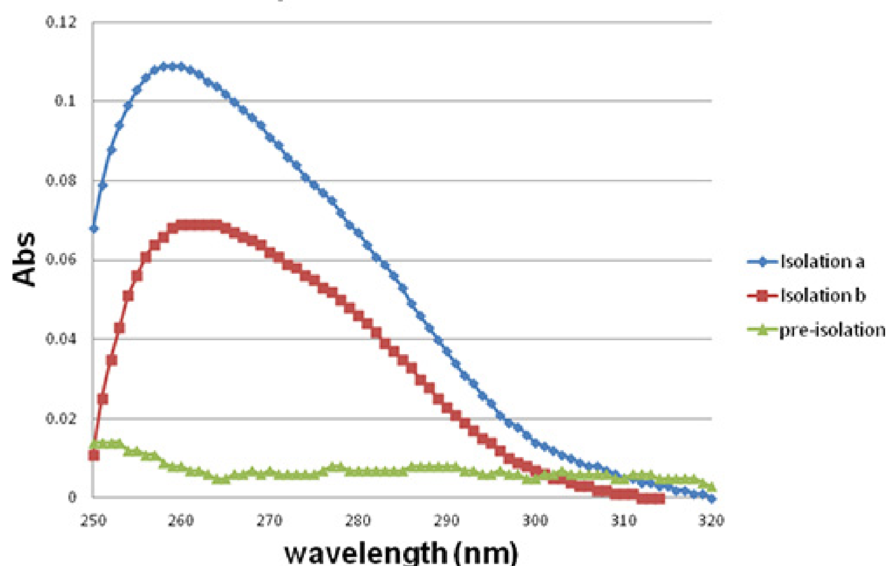

Figure 3: UV spectroscopy results of *E. coli* O157:H7 nucleic acids isolated using the reported extraction pipette. The isolated nucleic acids are labeled isolation a and isolation b. The UV spectrum of the sample was also tested prior to nucleic acid extraction and is labeled pre-isolation. The spectra were obtained using a Hitachi U3010 Spectrophotometer and associated software package.

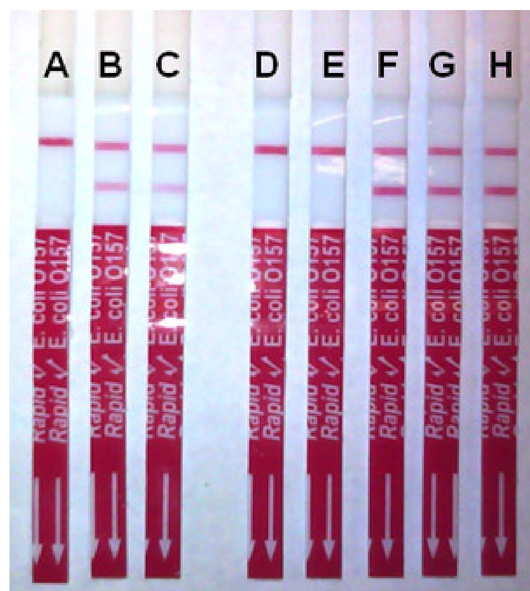

Figure 4: Color photo of RapidChek test strips. The protein content of the samples used in **Figure 2** was isolated using the extraction pipette and tested for immuno-reactivity by lateral flow assay. Lanes A-C are unprocessed controls, that is, cell culture was added to the test strip without nucleic acid or protein extraction. Lane A is *E. coli* BL21 DE3 pLysS as a negative control, Lanes B and C show *E. coli* O157:H7 that was untreated (B), or treated with mitomycin C for 4 hours (C). Lanes D through H show the results of adding the extracted protein to the lateral flow test strips. Lanes D and E were probed using protein extract from *E. coli* BL21 DE3 pLysS either without (D) or with mitomycin C treatment (E) as negative controls. Lanes F-H show the results when extracted protein from *E. coli* O157:H7 was used. Protein was extracted from untreated (F), or from 2 hour (G) or 4 hour (H) Mitomycin C treated cultures. Positive results produce a double red line.

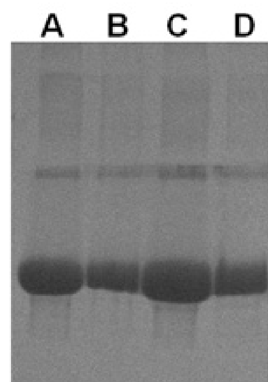

Figure 5: Photograph of a Coomassie stained SDS-PAGE. BSA was isolated from solution using the extraction pipette and described protocols. Lanes A and C are control lanes of 250 µg/mL and 500 µg/mL respectively. Lanes B and D depict recovered BSA from solutions containing the same concentrations as their respective controls.

Discussion

The tool, chemistries and protocol described in this report represent the first known example of an electricity-free, multi-macromolecule extraction system that can be utilized in field-based, point-of-care applications. Both macromolecule types can be applied to a number of downstream diagnostic or analytical devices. For example, the isolated nucleic acids are of PCR quality (**Figure 2 and 3**) while the protein component of the sample is immunoreactive (**Figure 4**). The utilization of this extraction system in austere or resource limited areas of the world could drastically reduce the burden of disease on the populations living there. This extraction system could also aid in disease surveillance programs throughout the world by providing a robust yet rapid and cost effective method for sample processing in austere or remote locations.

One important aspect to the extraction method outlined above is that it can be modified by the user. For example, in cases where sample size is greater or less than the initial 500 µLs (step 1.2), the user can adjust the volumes of the reagents accordingly. That is, the volumetric ratio of the reagents to the sample size should remain constant while the absolute volumes can be altered. Also, adjustments in the number of passages over the sorbent can also be made at the user's discretion. For example, if a larger sample volume is used, passing the sample over the sorbent

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more times than listed (steps 2.4 & 3.3) will increase the likelihood of the macromolecule type (nucleic acid or protein) contacting the sorbent. The increase in passages should increase capture efficiency until sorbent saturation is reached.

We have found that the extraction tool and associated chemistries have some limitations, the most significant of which is that the protein extraction chemistry is inefficient for the recovery of highly glycosylated proteins. In the event that the downstream diagnostic targets a glycosylated protein, this system may not be ideal for identification. Additionally, the upper and lower limits of extraction efficiencies for either nucleic acids or proteins are still being determined, and optimization is underway to maximize recovery efficiencies. Further development of the tool will overcome these current knowledge gaps.

Disclosures

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