Development of a Self-Contained Nucleic Acid Extraction Device to Enable Diagnostic Testing in Resource-Limited Settings

by

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Dissertation

Submitted to the Faculty of the

Graduate School of Vanderbilt University

in partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

in

Biomedical Engineering

May, 2014

Nashville, Tennessee

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ACKNOWLEDGEMENTS

I would like to offer my sincerest thanks to all those people whose, guidance, support, or friendly ear were integral to my completion of this research. First, I thank my advisor, Rick Haselton. His constant support and endless supply of creative insight taught me more than I ever thought possible about research, engineering, and problem solving. I am grateful for the exciting and enlightening research experience I had under his guidance. I also thank my committee members, Franz Baudenbacher, Craig Duvall, Ray Mernaugh, and David Wright for their support, encouragement, and technical expertise during this process. Much of my work would not have been possible without the countless discussions, support, and technical help provided by the colleagues I interacted with on a daily basis. I am especially grateful to the Haselton and Wright Lab members with whom I worked during this research, including Tricia Russ, Josh Trantum, Nick Adams, Amy Creecy, Keersten Davis, Anna Bitting, Mark Baglia, Joe Evans, Laura Albert, Megan Pask, Francesca Solinas, and Elizabeth Dworska. Finally, I am eternally grateful to my friends and family whose unfailing support and encouragement helped me to complete my research. I especially thank my husband Rob Egnatchik, and my parents, Kevin and Juanita Bordelon who constantly made themselves available to share in my struggles and successes throughout this process.

TABLE OF CONTENTS

Page

LIST OF TABLES

Table Page

CHAPTER V

CHAPTER VI

LIST OF FIGURES

Figure Page

CHAPTER III

CHAPTER IV

CHAPTER V

CHAPTER VI

APPENDIX

LIST OF ABREVIATIONS

PCR	polymerase chain reaction
RSV	respiratory syncytial virus
RT-PCR	reverse transcriptase polymerase chain reaction
HEp-2	human epithelial
ELISA	enzyme linked immunosorbant assay
GuSCN	guanidinium thiocyanate
C_t	cycle threshold
PFU	plaque forming unit
TE	tris EDTA
TB	tuberculosis
WHO	World Health Organization
PATH	Program for Appropriate Technology in Health
Tr-DNA	transrenal DNA
i.d.	inner diameter
FEP	fluorinated ethylene propylene

CHAPTER I

INTRODUCTION

This dissertation describes the development of a self-contained biomarker extraction device designed to extract nucleic acids from patient samples in a format suitable for use in low resource settings. The device relies on the dominance of surface tension forces over gravitational forces acting on fluids arrayed sequentially in a length of small diameter plastic tubing. The surface tension holds these processing solutions in place while magnetic beads loaded with captured nucleic acids are pulled down the length of the tubing.

The work is organized into seven chapters. The first chapter summarizes the project's specific aims. The second chapter presents relevant background and significance for the project. The third chapter details the initial proof-of-concept study for developing a low resource nucleic acid extraction device, "Development of a low resource RNA extraction cassette based on surface tension valves," which was published in *ACS Applied Materials and Interfaces* in 2011. The fourth chapter, "A magnetic bead-based method for concentrating DNA from human urine for downstream detection" was published in *PLOS ONE* in 2013, and describes the development of a patient sample-extraction cassette interface for recovering DNA from human urine samples. The fifth chapter, "Automated extraction of nucleic acid and protein biomarkers using surface tension valves," describes the development and testing of an automated biomarker extraction processor and is in the process of being submitted for publication. The sixth chapter evaluates the impact of magnetic bead surface functionalization on extraction simplicity, speed, and nucleic acid recovery and offers suggestions for choosing the ideal surface functionalization. This chapter, "The effects of magnetic bead

surface functionalization on nucleic acid extraction and detection," is in the process of being submitted for publication. The seventh and final chapter provides the project's conclusions and suggestions for future work.

Specific Aim 1: Optimize the RNA capture, wash and elution steps to improve RNA recovery efficiency.

The goal of this aim is to evaluate the RNA-silica capture, processing, and elution steps to improve RNA recovery to levels comparable to the laboratory gold-standard RNA extraction kit. In addition, the advantages of the silica-based RNA capture method are compared to an mRNA-specific, and nucleic acid-sequence specific capture method to evaluate their suitability for use in low resource settings. RNA recovery, total processing time, assay simplicity and toxicity, and available downstream detection modalities are considered for each bead functionality tested. Based on these comparisons the optimal situation for each bead type is identified.

Specific Aim 2: Develop and incorporate a patient sample-extraction cassette interface for sample loading into the small diameter tubing.

The goal of this aim is to develop a simplified interface for incorporating patient samples directly into the small diameter tubing for processing. Nucleic acid-silica adsorption buffer and magnetic beads are lyophilized within the bulb of a polypropylene transfer pipette, which serves as a biomarker capture chamber. The patient sample is collected with the transfer pipette and subsequently introduced into the extraction tubing following biomarker capture. This device eliminates the need for pipettors and disposable pipette tips and reduces the total number of steps required to prepare the sample for introduction into the small diameter tubing of the extraction cassette.

Specific Aim 3: Design a DNA extraction assay to recover DNA from human urine samples in the extraction cassette.

The goal of this aim is to design and test a DNA extraction assay for processing human urine in the low resource extraction cassette. The magnetic beads, small diameter tubing, and DNA capture, wash and elution steps are modified to maximize recovery of spiked *Mycobacterium tuberculosis* DNA from pooled TB negative human urine samples. The patient sample-extraction cassette interface designed in Aim 2 is used to handle and transfer urine samples during processing. The urine sample interface in combination with the small diameter tubing successfully recovered spiked TB DNA biomarkers from human urine samples.

Specific Aim 4: Evaluate an automated extraction cassette processor.

The goal of this aim is to test an automated biomarker extraction cassette processor as an alternative to manual nucleic acid extractions performed in the small diameter tubing. The automated processor was tested on a bank of well-characterized patient sample surrogates prepared by PATH including *Escherichia coli* infected human urine, *Mycobacterium tuberculosis* infected synthetic sputum, and *Plasmodium falciparum* infected human blood culture. Nucleic acid and protein biomarkers were successfully recovered from the sample surrogates.

CHAPTER II

BACKGROUND AND SIGNIFICANCE

Infectious Disease Diagnosis in Low Resource Settings

Accurate and rapid detection of disease biomarkers is essential for efforts to control the spread of infectious diseases [1]. At well-equipped clinics and hospitals, diagnostic technologies include culture of the organism-of-interest, microscopy, antibody-based antigen detection by ELISA, and nucleic acid amplification and detection by PCR. However, often the highest infectious disease burden is found in low-income developing countries where the availability of electricity and clean water is, at best, intermittent. In the poorest of these countries, over 50% of deaths are caused by infectious diseases [2]. Due to the poor infrastructure across these low income countries many patients find it difficult or impossible to reach centralized hospitals and clinics and instead rely on small health posts that serve rural villages and townships. In all of these healthcare settings, basic laboratory supplies may be available, but workers often have little to no training and are incapable of maintaining or repairing sophisticated equipment. Often, infectious samples are handled by workers who have no personal protective equipment [2]. Due to the lack of basic resources, and the difficulty many patients have in reaching the nearest health center or hospital, there is a dire need for rapid, inexpensive, easy-to-use diagnostics that can be safely and effectively utilized under these conditions.

In an attempt to improve healthcare in low resource settings, there is a widespread effort to expand the utility of current clinical diagnostic modalities by adapting them to perform with minimal training, equipment, and other resources [1,3].

Nucleic acid amplification technologies such as PCR are ideal for this effort due to their inherent sensitivity, specificity, and relatively rapid time-to-answer [4]. Significant resources have been poured into adapting PCR-based detection strategies into a self-contained point-of-care format for use in low resource settings [1,5,6]. One or more of these advantages are frequently sacrificed in other diagnostic modalities such as cell culture and ELISA. Cell culture is often highly sensitive and specific, but requires cell growth periods several days to weeks long, highly trained technicians, and large quantities of disposable laboratory supplies. ELISA and other antibody-based methods provide relatively short time-to-result, but offer a lower sensitivity compared to PCR. Antibodies also require consistent cold storage to maintain stability.

Sample Preparation in Low Resource Settings

Patient samples contain inhibitors that directly interfere with nucleic acid amplification and detection during PCR. Therefore, PCR often cannot be performed directly on unprocessed patient samples [7-9]. The removal of PCR inhibitors present in patient samples represents a significant roadblock to the widespread use of PCR as a low resource diagnostic. Sample preparation techniques that remove contaminants such as carbohydrates, proteins, and lipids from a variety of patient sample matrices must be adapted to work under less than ideal conditions. These inhibitors can prevent the amplification or detection of target nucleic acid sequences [7,9-11]. Patient samples frequently contain nucleases which, over time reduce the number of target nucleic acids present in the samples [10]. Failure to consistently extract nucleic acids from complex sample matrices for downstream detection is one of the greatest limitations to the widespread use of genetic diagnostic modalities in low resource settings [1].

In a laboratory setting, there are several effective methods for preparing patient samples for downstream PCR. One traditional laboratory method uses a guanidinium

thiocyanate (GuSCN)-phenol-chloroform cocktail to extract nucleic acids [12]. During the extraction, the mixture separates into an upper aqueous phase containing RNA and a lower organic phase containing DNA. This is an efficient technique for the selective extraction of nucleic acids, however, the protocol requires approximately an hour to obtain a purified sample [13]. Completion of the extraction also requires direct handling of toxic chemicals and specialized equipment. Additionally phenol is a well-known inhibitor of PCR and must be subsequently removed from the sample prior to analysis.

Solid phase nucleic acid extractions are used more frequently than traditional GuSCN-phenol-chloroform separations to extract nucleic acids from patient samples [13]. Silica and chitosan are two examples of solid phases useful for nucleic acid extractions. Both silica and chitosan are used to selectively purify total nucleic acids from patient samples by relying on electrostatic interactions between nucleic acids and the solid phase. For example, in the presence of a strong chaotropic agent such as GuSCN, nucleic acids selectively adsorb to silica surfaces via a salt bridge [14,15]. As a result of this interaction, silica-based surfaces are frequently used to capture nucleic acids from complex samples and subsequently elute them into a pure solution compatible with PCR.

The relative simplicity of solid phase extractions is widely exploited in commercially manufactured kits that consistently extract nucleic acids in as little as 10-15 minutes. Kits such as the Qiagen RNeasy and DNeasy Mini Prep kit and Norgen Urine Extraction kit rely on silica matrices packed into centrifugation columns. Other kits such as the Qiagen MagAttract Mini Prep kit and Dynabeads MyOne silane kit utilize silica-coated magnetic particles that can be easily manipulated in the required wash buffers using a magnetic field, applied either with a robot or by hand. Despite their efficacy in laboratory settings, these commercially available kits are relatively expensive, requiring trained personnel with access to pipettors, large quantities of disposable supplies, and

specialized equipment such as laboratory centrifuges and automated robots. Additionally, all of the kits require multiple pipetting steps that potentially expose the technician to dangerous infectious agents. This is particularly problematic in settings where the technician is untrained and appropriate personal protective equipment is in limited supply.

Recently there has been a growing interest in developing microfluidic technologies for sample preparation [16]. Microfluidic devices overcome many of the limitations of traditional, laboratory-based nucleic acid extraction kits. These devices are self-contained, decreasing the chance for contamination of both the sample and operator. The extractions are automated, reducing the skill required for operation. Additionally, many of these devices are suitable for integration with downstream nucleic acid amplification and detection technologies [17]. Many microfluidic devices rely on the selective adsorption of nucleic acids to a silica surface in the presence of chaotropic salts. Groups have utilized silica-coated microchannels, posts, and embedded microparticles as the solid phase within the microchannels [18-20]. The sample and subsequent washing buffers are then flowed through the microchannels to complete the nucleic acid extraction.

Though many of these technologies boast a high efficiency for nucleic acid extraction, microfluidic devices historically suffer from poor reproducibility and require bulky external equipment for operation [21]. The small diameter of the microfluidic channels results in a limited solid phase surface area available for nucleic acid adsorption, restricting the total sample volume that can be flowed through the chip. This is problematic for processing samples greater than ~500 uL in volume [1].

Recently, several non-microfluidic approaches have been described. Sur and coworkers developed a technique called ‘immiscible phase nucleic acid purification’ by which nucleic acids bound to magnetic particles are pulled from the nucleic acid capture

solution through a single layer of an immiscible hydrophobic liquid [22]. According to this report, the passage of particles through the fluid interface removes loosely bound contaminants. The beads are pulled through the hydrophobic layer directly into an elution buffer. The maximum patient sample volume that can be extracted is limited by the small size of the sample lysis/binding chamber and is ~200 uL. This concept was more recently adapted to a horizontal channel format with multiple extractions performed in parallel on a microtiter plate [23]. However, sample volume is limited to 10 uL in this configuration, and the sample is exposed to the air increasing the likelihood of operator exposure.

Prevalence of Tuberculosis in Low Resource Settings

Improving sample preparation to better enable diagnostics in resource limited settings would positively impact global health and improve the burden of many infectious diseases. Tuberculosis is one major infectious disease whose burden could be significantly reduced by the introduction of a rapid, easy to use diagnostic suitable for use in low resource settings. In 2012 an estimated 8.6 million people developed TB resulting in 1.3 million deaths. It is estimated that one third of the world's population is infected with the latent form of TB, most of these infections occurring in low income countries in South-East Asia, Africa and the Western Pacific [24]. Though the incidence of drug resistant TB disease is increasing, particularly in Eastern Europe and Central Asia, the treatment for greater than 90% of TB cases has been widely available for decades. Despite the availability of treatment, the high TB prevalence and mortality rate remain unacceptably high underscoring the dire need for low resource-appropriate diagnostics [25].

Diagnosis of Tuberculosis in Low Resource Settings

Worldwide, the most common method for diagnosing TB infection is sputum smear microscopy, a technique developed over 100 years ago. As a diagnostic, sputum smear microscopy offers low sensitivity and no means to determine drug susceptibility of the particular strain [25,26]. The smears are often prepared and handled by personnel with poor access to personal protective equipment, increasing the risk of spreading the infection. Despite its status as the most common TB diagnostic, many countries with the highest TB burden have less than 1 microscopy center per 100,000 people [24].

Mycobacterium tuberculosis culture is the worldwide reference standard for TB diagnosis. However, due to the need for well-equipped laboratories and trained technicians, cell culture is not widely available in many of the areas with the highest TB burden. Cell culture results take weeks to obtain, limiting its use in diagnosing patients who do not live in areas where they can conveniently return to health centers for their results, treatment, and monitoring of disease progression [5].

The Cephiad GeneXpert was developed as a self-contained module that performs nucleic acid extraction, amplification, and detection within a single cartridge inserted into the device. The cartridges were designed specifically to extract *Mycobacterium tuberculosis* DNA from sputum samples [1]. To perform the assay, a technician thoroughly mixes the collected sputum sample with a proprietary sample treatment reagent to neutralize any live bacteria and incubates the sample at room temperature prior to inserting the cartridge into the GeneXpert. *M. tuberculosis* lysis, and nucleic acid extraction, amplification, and detection are automatically performed within the GeneXpert without any further user intervention. The GeneXpert has been fully endorsed by the World Health Organization (WHO) for use in low resource settings due to its excellent sensitivity and specificity, as well as its capability to identify multi-drug resistant tuberculosis [27].

Despite the WHO endorsement, the GeneXpert still does not fully meet the requirements for use in low resource settings. The instrument and disposable cartridges are both expensive. Maintenance plans are also costly, and the complexity of the instrument leads to frequent, costly breakdowns. In many cases, clinics and hospitals cannot afford the repairs, and the instrument goes unused. Additionally, sputum is not an ideal patient sample matrix due to the high risk of spreading infection during its collection and subsequent processing. It can also be difficult to obtain a useable sputum sample, particularly from children who are frequently incapable of producing sputum [26].

Transrenal DNA as a Biomarker for Tuberculosis Detection in Urine

Despite the advantages offered by the Cephiad GeneXpert, scientists are still seeking alternative TB biomarkers and diagnostics. One promising biomarker is TB DNA present in human urine, referred to as transrenal DNA (Tr-DNA). The analysis of transrenal DNA (Tr-DNA) for diagnosis of TB is an attractive alternative to traditional diagnostic methods requiring sputum collection. Urine is an ideal patient sample matrix due to the relative ease of collection and a significantly reduced risk for spreading infection. Large volumes of urine can be collected during a single visit, and the use of urine is often more acceptable to the patient [28].

Tr-DNA is thought to originate from cells and organisms that break down within the body. Botezatu et al. injected mice with both radiolabeled DNA and irradiated human cancer cells and detected DNA from both sources in the animals' urine using scintillation counting and PCR, respectively. In the same study, the group detected male DNA in women transfused with male blood as well as women pregnant with male fetuses, suggesting the permeability of the kidney to short 10-150 bp DNA fragments is possible in both mice and humans [29]. The study results suggest that when circulating

nucleic acids present in the bloodstream due to cellular death within the body enter the kidney, there is some level of permeability to the smaller fragments. These fragments are not filtered out, but subsequently pass through the kidney and are excreted in the urine [28-30]. The nucleic acid background in human urine has been shown to vary from ~40-250 ng/mL [31]. Melkonyan et. al. used gel electrophoresis to demonstrate the presence of two populations of human DNA in urine. The first was a population of large (>5000 bp) fragments likely from cells that died within the urinary tract. The second population was generally shorter than 250 bp with the majority ranging from ~10-150 bp [32].

Based on the promise of a noninvasive urine diagnostic, scientists have studied the potential use of Tr-DNA for TB detection. To date, several of these studies have been completed in an attempt to determine the frequency at which TB DNA can be detected. These studies have suggested that human urine contains DNA fragments long enough to be of diagnostic value, and have detected TB-specific sequences in multiple studies [33-35]. Whether TB TR-DNA fragments are present in high enough concentrations to be consistently detectable is controversial despite the handful of studies that examine human urine for such fragments. The majority of studies utilize PCR followed by gel electrophoresis to detect ~100 bp sequences cleaved from the 1350 bp IS6110 insertion sequence of *Mycobacterium tuberculosis* which has been shown to repeat as many as 25 times per genome [36]. Both the sensitivity and specificity of biomarker detection range from 7-100% [26]. In many of the studies, the sample volume and storage conditions are never described, and several different nucleic acid extraction methods are utilized. The most promising study, performed by Aceti et. al. with 13 urine samples, yielded 100% sensitivity and specificity [33]. However, a nucleic acid extraction method is not described, no starting urine volume is provided, and no figures supporting the results are shown. Despite the potential benefit a TB Tr-

DNA biomarker would yield in efforts to control TB in low resource settings, the lack of standardization in urine storage and testing across multiple studies has severely limited its use to date.

Another controversial factor in the use of Tr-DNA for diagnostic purposes is the post-collection sample treatment required to protect the DNA from degradation. Human urine has been shown to have DNase I and II activity which could degrade any DNA biomarkers present in the urine both before and after sample collection [37,38]. Despite the high potential for biomarker degradation, there is no standardized method for urine sample handling prior to biomarker extraction and detection. Several groups have added EDTA to freshly collected urine samples in an attempt to inhibit nuclease activity and prevent nucleic acid degradation. In many studies, the addition of EDTA appears to minimize nuclease activity [39-41]. However the samples are treated differently across the studies, including the use final EDTA concentrations ranging from 10-40 mM. Without a consistent sample handling treatment across multiple studies it is difficult to definitively identify the ideal urine sample handling conditions.

The extreme day-to-day variability of human urine represents another significant roadblock to standardizing Tr-DNA analysis. The composition of human urine varies depending on diet and hydration. A normal adult produces 1-2 liters of urine in a 24 hr period, with a pH range between 4.6 and 8. Typically, 95% of urine is composed of water. The majority of the solid components of urine are urea and creatinine, though there are also varying concentrations of sodium, potassium, magnesium, and calcium. There can also be small amounts of enzymes, carbohydrates and hormones [42]. The high variability of human urine can make it difficult to compare studies performed in separate countries, or even in various settings within a single country.

Nucleic acid extraction using a self-contained cassette

Despite the controversy surrounding the use of Tr-DNA as a reliable biomarker for TB detection, it remains an attractive alternative to more complex diagnostic modalities such as Cephiad's GeneXpert sputum processing. As with many low resource diagnostics, the first major hurdle to developing a suitable TB diagnostic is the sample preparation step [1]. Without an initial nucleic acid extraction step, nucleic acids are not detectable in urine by PCR [43]. A sample preparation device with the flexibility to reliably extract nucleic acid biomarkers from a variety of patient sample matrices would greatly improve the potential impact nucleic acid-based diagnostics could make towards controlling infectious diseases in low resource settings. For example, a technology that could efficiently extract both RNA and DNA biomarkers from a variety of biologically complex sample matrices with differing volumes would be a welcome improvement over expensive, complex technologies that are limited in their flexibility to detect more than a single pathogen.

Functionalized magnetic particles are an ideal tool for achieving the desired flexibility. The particle surface can be functionalized with a variety of surface modifications to selectively capture the biomarker of choice. For example, both DNA and RNA adsorb to silica-coated surfaces, while the poly A tails on mRNA will hybridize to surfaces functionalized with oligo (dT) DNA probes. An additional advantage of using magnetic particles is that they can be easily manipulated using a magnetic field. This ease of manipulation means magnetic beads can be used in a wide variety of formats. This additional flexibility makes magnetic beads the ideal choice for use in a low resource extraction technology.

We have developed a nucleic acid extraction device suitable for operation in low resource settings by taking advantage of the flexibility offered by magnetic beads in addition to the surface tension forces experienced by fluids arrayed in a length of small

diameter tubing. When multiple liquids arrayed in small diameter tubing are separated from one another by small air valves, these liquid/air interfaces remain in place even as magnetic beads are pulled through them with an external magnet [43-46]. This phenomenon allows an inexpensive piece of small diameter plastic tubing to serve as a self-contained device inside which the entire nucleic acid extraction occurs. An external permanent magnet is the only required processing equipment. The process involves three simple steps: 1) Individual processing solutions required for each extraction are pre-arrayed within the small diameter tubing; 2) Nucleic acids are captured onto the surface of magnetic particles within an external chamber; 3) Captured nucleic acids are washed by pulling the magnetic particles through the arrayed wash solutions and eluted in the final chamber.

The flexibility of this design has been demonstrated by extracting viral RNA from nasopharyngeal swab samples [44] and DNA from urine samples [43]. Additionally, the advantages offered by changing of magnetic bead surface functionalization were identified by extracting viral RNA using magnetic beads designed to capture total RNA, mRNA, and respiratory syncytial virus (RSV) N gene mRNA. Coupled with a low resource diagnostic device, the extraction cassette has the potential to improve the quality of healthcare available in poorly equipped areas by providing a user friendly method to quickly and effectively diagnose disease.

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CHAPTER III

DEVELOPMENT OF A LOW RESOURCE RNA EXTRACTION CASSETTE BASED ON SURFACE TENSION VALVES

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ACS Applied Materials and Interfaces 3(6): 2161-2168, 2011

Abstract

Nucleic acid-based diagnostics are highly sensitive and specific, but are easily disrupted by the presence of interferents in biological samples. In a laboratory or hospital setting, the influence of these interferents can be minimized using an RNA or DNA extraction procedure prior to analysis. However, in low resource settings, limited access to specialized instrumentation and trained personnel presents challenges that impede sample preparation. We have developed a self-contained nucleic acid extraction cassette suitable for operation in a low resource setting. This simple design contains processing solutions preloaded within a continuous length of 1.6 mm inner diameter Tygon tubing. Processing solutions are separated by air gaps and held in place during processing by the surface tension forces at the liquid-air interface, viz. surface tension valves. Nucleic acids preferentially adsorbed to silica-coated magnetic particles are separated from sample interferents by using an external magnet to transfer the nucleic acid biomarker through successive solutions to precipitate, wash and elute in the final cassette solution. The efficiency of the extraction cassette was evaluated using quantitative reverse transcriptase PCR (qRT-PCR) following extraction of respiratory syncytial virus (RSV) RNA. RNA was recovered from TE buffer or from lysates of RSV infected HEp-2 cells with 55 and 33% efficiency, respectively, of the Qiagen RNeasy kit. Recovery of RSV RNA from RSV infected HEp-2 cells was similar at 30% of the RNeasy kit. An overall limit of detection after extraction was determined to be nearly identical (97.5%) to a laboratory-based commercially available kit. These results indicate that this extraction cassette design has the potential to be an effective sample preparation device suitable for use in a low resource setting.

Introduction

Recent research has focused on the development of nucleic acid-based detection for low resource settings [1]. Nucleic acid-based detection systems, such as quantitative PCR (qPCR), are particularly attractive technologies for detection of pathogens because of their sensitivity, specificity and relatively rapid time-to-answer. The effectiveness of PCR is dependent on both the quality and quantity of nucleic acid template [2] and the absence of interferents [3]. For example, carbohydrates, proteins, lipids or other unidentified interferents present in clinical samples have all been shown to inhibit PCR and produce false negatives [4-6]. In addition to various interferents, patient samples also contain nucleases, which directly reduce the number of nucleic acid targets present [5].

To minimize false negatives and maximize the efficiency of nucleic acid-based diagnostics, nucleic acids are extracted and concentrated into an interferent-free buffer prior to testing. One classic laboratory method uses a phenol-chloroform cocktail [7]. This method is highly effective, but is not as commonly utilized today because it is time consuming and requires the use of toxic organic chemicals. Several solid phase extraction kits are commercially available to purify DNA or RNA from patient samples. Many of these kits rely on selective nucleic acid binding to silica-coated surfaces in the presence of ethanol and a chaotropic agent such as guanidinium thiocyanate (GuSCN) [8,9]. GuSCN also denatures protein contaminants including nucleases that may be present in the sample [10,11]. These kits are not cost effective for low resource use and often require the use of specialized laboratory equipment, such as a robot or centrifuge, and trained technicians that are unavailable in a low resource setting. Additionally, many involve multiple steps that increase the chance of contamination of both the sample and operator.

Microfluidics is one promising format for low resource nucleic acid-based diagnostics. Recently, there has been a growing interest in expanding microfluidic technologies for sample preparation [1,12]. Many of these devices are suitable for integrating with downstream nucleic acid amplification and detection technologies [13,14]. However, the small surface area of solid phase available for nucleic acid binding and the limited sample volume that can be flowed through the channels limit the total mass of nucleic acid recovered [1], and therefore negatively impact the limit of detection.

We have developed an alternative nucleic acid extraction cassette suitable for operation in a low resource setting. This self-contained extraction cassette is preloaded with processing solutions separated by air gaps, which we refer to as “surface tension valves.” In proof-of-principle RNA extraction studies, RSV infected cells are lysed and viral RNA is selectively adsorbed to silica-coated magnetic particles in the presence of GuSCN and ethanol. Individual processing solutions are preloaded into a single continuous length of Tygon tubing and are separated from one another and held in places by surface tension forces. Removal of interferents is achieved by selective RNA adsorption to silica-coated magnetic particles which are then pulled through each processing solution using an externally applied magnetic field. RNA is eluted from the surface of the magnetic particle in the final solution. This report describes the general characteristics of this approach and compares its performance to laboratory-based commercial kits.

Materials and Methods

Preparation of RSV N gene RNA standards.

Because our laboratories have experience with respiratory syncytial virus (RSV) detection, we have chosen to develop our extraction cassette using RSV RNA. Escherichia coli DH5α transformed with the pGBKT7 vector containing RSV N gene was generously provided by the Crowe Laboratory at Vanderbilt University. E. coli were grown for 18 hours on kanamycin agar plates at 37 °C. A single colony was isolated and transferred into 25 mL of Miller's LB broth with 50 ug/mL kanamycin antibiotics and grown overnight on a rotating rack at 37 °C to an optical density of 0.6-0.8 AU. The plasmid was extracted using a Qiagen Spin Miniprep Kit and linearized using the BssHII restriction enzyme. Linearization was confirmed by running both pre- and post-linearized plasmids on a 1% agarose gel. Linearized plasmid was recovered from the restriction digest by ethanol precipitation. The plasmid was then transcribed into RNA using a T7 MEGAscript transcription kit (Ambion, Austin, TX), and treated with DNase I. The expected RNA length was confirmed on a denaturing 2%-formaldehyde-1.2% agarose gel. The RNA was quantified by UV-Vis spectroscopy.

Preparation of RSV infected and uninfected HEp-2 cell lysates.

Uninfected HEp-2 cell lysates were prepared from a confluent monolayer of HEp-2 cells from a T-150 flask. The cells were harvested by scraping from the T150 flask and centrifuging at 500 x g for 5 minutes. The pellet was resuspended into 8 mL denaturing solution (4 M guanidinium thiocyanate, 25 mM sodium citrate [pH 7.0] 0.5% N-laurosylsarcosine [Sarkosyl], 0.1 M 2-mercaptoethanol) and passed through a pipette tip 10 times. The cell lysates were stored at a concentration of approximately 3×10^6 lysed cells per mL in 1 mL aliquots at -80 °C.

Infected HEp-2 cell lysates were prepared by infecting confluent monolayer of HEp-2 cells in two T150 flasks with RSV strain A_2. After 4 days, one flask was harvested as described above and used to perform RSV RNA extractions from HEp-2 lysates. A plaque assay was performed on the second flask to quantify the concentration of infectious particles. To prepare the assay, cells were scraped from the T150 flask and centrifuged at 500 x g for 5 minutes. The pellet was resuspended into 8 mL of media, and cells were lysed by 3 cycles of freezing in an ethanol and dry ice slurry and thawing in a 37 °C water bath. The cell lysate was centrifuged at 100 x g for 5 minutes, and the supernatant was stored at -80 °C in 1 mL aliquots.

One hundred uL of the lysed cells was serially diluted, and each dilution added in triplicate to a confluent monolayer of HEp-2 cells in a 24-well plate. Plates were incubated at 37 °C for 1 hour. One mL of sterile 0.75% methyl cellulose (w/v) was then added to each well and the plate was placed at 37 °C for an additional 4 days. The infected HEp-2 cells were fixed in 80% methanol at -20 °C for 1 hour, washed 3 times with PBS, and blocked with a 5% milk solution for 1 hour. One hundred fifty µL of 30 µg/mL anti-F protein primary antibody in 5% powdered milk solution was added to each well. After 1 hour, wells were washed 3 times with PBS, and 150 µL of 0.5 µg/mL anti-mouse IgG HRP conjugate secondary antibody (Promega, Madison, WI) in 5% powdered milk solution was added for 1 hour. Wells were washed 5 times with PBS and 150 µL of TrueBlue peroxidase substrate (KLP, Gaithersburg, MD) added for 20 minutes at room temperature. Punctate blue plaques were counted and averaged, and the plaque forming units (pfus) were determined by multiplying by the dilution factor.

Conversion of RSV N gene RNA copy number to pfu/mL reported in clinical literature.

The number of pfu/mL of the RSV infected HEp-2 cell lysates was converted to RSV N gene copies/uL by comparing qRT-PCR and plaque assay results on each half of

a split culture of infected Hep-2 cells harvested 4 days post-infection, the point at which peak titers are reached. The concentration of N gene RNA in copies/uL was determined by RT-PCR using a standard curve following RNA extraction with the RNeasy kit. A calculated extraction efficiency of 18.1% (see section 3, **Figure 4B**) was assumed to determine the total number of RNA copies present in the sample prior to extraction. The number of N gene copies/pfu was approximated by dividing the total number of RNA copies/uL by the number of pfu/mL and determined to be ~10^4 copies/pfu.

Quantitative RT-PCR.

An 82-bp fragment of the RSV N gene was amplified using forward primer 5'-GCTCTTAGCAAAGTCAAGTTGAAATGA-3' and reverse primer 5'-TGCTCCGTTGGATGGTGTATT-3' [15]. Reactions were performed in a 25 µL volume using 5 µL of RNA template and the Clontech one-step RT-PCR kit according to manufacturer's instructions. Thermal cycling consisted of 48 °C for 20 minutes to synthesize cDNA, 95 °C for 3 minutes to inactivate the reverse transcriptase and activate QTaq DNA polymerase, and 40 cycles of 95 °C for 15 s and 60 °C for 60 s using a Rotor-Gene Q thermal cycler (Qiagen, Germantown, MD). Product specificity was confirmed using melting curve analysis and gel electrophoresis. Data was collected and C_t values recorded by Rotor-Gene Q Software (Qiagen, Germantown, MD) and converted to number of copies of RNA per µL using a standard curve.

RNA extraction using prototype capillary extraction cassette.

A prototype extraction cassette (**Figure 1**) was constructed from glass capillary tubes and pipette tips. Glass capillary chambers (2 mm i.d.) were cut from stock tubing into 80 mm lengths, and the ends were flared outward. Six capillary chambers were

aligned linearly on the top of a horizontal aluminum stage using machined aluminum mounts.

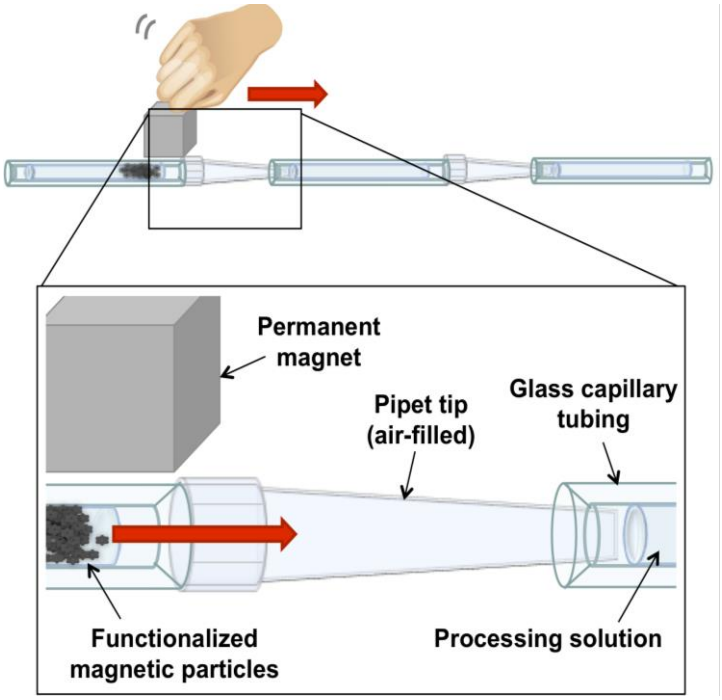

Figure 1. Design of the prototype extraction method showing the processing solutions held in place in glass tubing and separated by air-filled pipette tips. RNA is adsorbed to silica-coated magnetic particles which are pulled left to right through successive processing chambers using an external magnet. Following processing, the RNA is eluted in a final water chamber.

A 1000 µL pipette tip was placed as a spacer in between each capillary chamber with the wide end of the pipette tip around the preceding capillary chamber and the narrow end resting inside the flared region of the next capillary chamber. Thus successive processing chambers were separated from one another by air spacers within the pipette tips. The first capillary chamber was reserved for the RNA sample and was initially left empty. The remaining chambers were preloaded with the processing reagents supplied in the MagAttract RNA Cell Mini M48 kit (Qiagen, Germantown, MD) as follows: 200 µL of “Buffer MW” wash buffer, 200 µL “Buffer RPE” wash buffer, 200 µL “Buffer RPE” wash

buffer, 200 µL RNase/DNase free water, 30 µL RNase/DNase free water heated to 65 °C for elution of RNA. Thirty µL of sample was added to 150 µL of "Buffer RLT" and homogenized by passage through a 20-gauge needle five times. Twenty µL of the MagAttract bead solution (Qiagen, Germantown, MD) was added to the homogenized sample, vortexed, and placed on a rotary mixer for 5 minutes at room temperature. The sample was then pipetted into the first chamber, shown on the left in **Figure 1.** A 2.54 cm cube of grade 40 NdFeB magnet (National Imports, Vienna, VA) was placed adjacent to the first capillary chamber and slowly pulled parallel to the chambers at a rate of ~4 mm/second to pull the magnetic beads through each of the processing chambers. The total pull-through time was ~2 minutes. After reaching the final elution chamber, the beads were held to one side by the magnet and the eluent was collected.

This initial design was used to perform proof-of-principle studies using 14 frozen de-identified nasal wash samples provided by Dr. John Williams' lab (Vanderbilt University Hospital, Nashville, TN). Use of specimens was approved by Vanderbilt University's IRB. At the time of collection, nasal swabs were placed in opti-MEM media (Invitrogen, Oslo, Norway) and frozen at -80 °C. Each sample was characterized by Williams' lab for respiratory syncytial virus (RSV) using RT-PCR after an extraction using Roche Total Nucleic Acid Extraction Kit (Basel, Switzerland). RT-PCR was performed using Roche LC Magna Pure machine (Basel, Switzerland). We obtained samples which tested positive for RSV as determined by a calculated cycle threshold (C_t). We also obtained samples which tested negative for RSV as determined by no calculated cycle threshold value within the cycles that were performed. Seven samples characterized as RSV positive and seven as RSV negative were selected at random. Frozen samples were briefly thawed, divided and refrozen as 100 µL aliquots to facilitate comparison across different RNA extraction methods.

The number of extracted RSV N gene RNA copies/µL was calculated for the 7 RSV positive and 7 RSV negative nasal wash samples after 4 different extraction methods using a standard curve. The recovery efficiency of RNA extraction was compared to the RNeasy Mini kit (Qiagen, Germantown, MD), Dynabeads mRNA Direct kit (Invitrogen, Oslo, Norway) used according to manufacturer's protocols, as well as the MagAttract RNA Cell Mini M48 kit (Qiagen, Germantown, MD) performed manually instead of with the Qiagen M48 BioRobot which was unavailable for these studies. The results were also compared to the calculated copy numbers of RSV N gene RNA detectable in each sample directly amplified by RT-PCR prior to extraction. For each extraction method utilized, RNA was eluted in a 50 µL volume to ensure that RT-PCR analysis was comparable across all extraction methods tested.

RNA extraction from TE buffer and HEp-2 cell lysates using continuous tubing extraction cassette.

The initial prototype design of **Figure 1** was further simplified into a continuous tubing design shown in **Figure 2**.

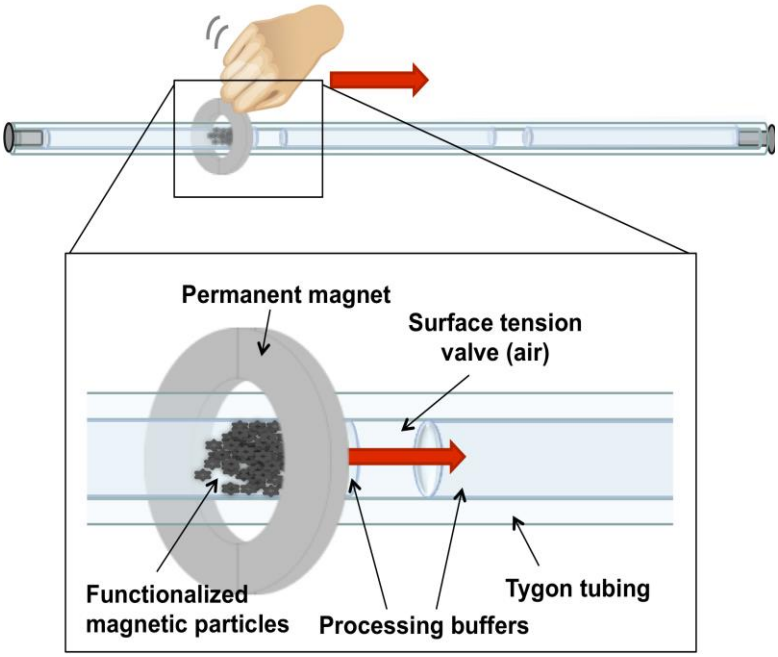

Figure 2. Design of the continuous tubing extraction cassette showing individual processing solutions separated by surface tension valves. An external magnet is used to pull RNA adsorbed to silica-coated magnetic particles through each processing solution. Following processing, the RNA is eluted in a final water chamber.

In this design, 8 processing solutions were preloaded within ~61 cm length of Tygon tubing (1.6 mm i.d.). These solutions were chaotropic wash buffer (300 µL of 4 M guanidine hydrochloride, 25 mM sodium citrate, pH 7.0), two sections containing RNA precipitation buffer (300 µL of 80% ethanol, 5 mM potassium phosphate, pH 8.5,), three sections containing a water wash (100 µL of molecular grade water), and RNA elution (50 µL of molecular grade water). The 50 µL elution volume was chosen so that the RT-PCR input would be comparable to other extraction methods such as the RNeasy kit. Each solution was separated from the next by an air gap ~2 mm in length. Three types of extraction test samples were prepared: 5 µL of RSV N gene standard RNA in TE buffer at a concentration of 1 x 10^6 copies/µL, 20 µL of HEp-2 cell lysates (2 x 10^3 cells/µL) spiked with 5 µL of RNA standard, or 20 µL of RSV infected HEp-2 cell lysates.

Cell lysate samples were homogenized by passage through a 25 gauge needle five times. Prior to extraction, samples were added to 230 µL of RNA-silica binding buffer (230 µL of 2 M guanidine thiocyanate, 25 mM sodium citrate, pH 7.0, 50% ethanol) and 20 µL of silica-coated 1 µm diameter magnetic particles (3 x 10^6 particles/µL) (Bioneer Inc., Alameda, CA) and placed on a rotating mixer for 5 minutes at room temperature. After mixing, each sample was loaded into the tubing, and the tubing ends were capped. The particles were collected in the first chamber by the external magnet and pulled through the surface tension valves and each successive chamber at ~4 mm/second using ~5 cm diameter neodymium ring magnet (Emovendo LLC, Petersburg, WV) as depicted in **Figure 2**. Particles were dispersed in the chaotropic wash and RNA precipitation solutions by rapidly moving the magnet back and forth before being recollected. In the water wash solutions, the particles were moved at ~8 mm/second to minimize RNA loss by elution during the wash. Finally, the particles were dispersed in the final elution chamber and incubated at room temperature for 5 minutes before removal. Although it was utilized in the prototype design, the elution of RNA at 65 °C was not performed in this final design because it would be impractical in most low resource settings. The final chamber contents were collected for RT-PCR analysis. Each RNA extraction was completed in ~15 minutes.

Continuous tubing extraction cassette limit of detection.

The extraction cassette limit of detection was determined by calculating the minimum quantity of target RNA that must be added to RSV negative cell lysates to be detectable by RT-PCR following extraction. This value was compared to the limit of detection calculated for the RNeasy kit. Twenty uL of uninfected HEp-2 cell lysate was spiked with 5 µL of RNA in TE buffer containing 0, 5 x 10^3, 5 x 10^4, 1 x 10^5, 5 x 10^5, 1 x 10^6, and 5 x 10^6 copies of RSV N gene RNA standard and extracted by both methods as

described in section 2.6. After extraction, the RNA was quantified by RT-PCR. The limit of detection was defined as 3 s.d. above the mean value obtained for control extractions containing no RNA.

Post-extraction RNA distribution analysis.

Extraction test samples were prepared using 5 µL of RSV RNA standard in TE buffer added to 230 µL of silica binding buffer. Twenty µL of magnetic particles were added to the sample and mixed for 5 minutes. RNA was extracted using the extraction cassette as described in section 2.6. After extraction, each chamber solution was removed by cutting the tubing with a razor blade and collecting in a separate tube. Each solution was purified with the RNeasy Mini kit according to manufacturer's protocol in order to remove PCR inhibitors. To account for RNA loss during the secondary RNA purification step, a control containing 5 µL of RSV RNA standard was purified from TE buffer using the RNeasy kit. The purified RNA was quantified by RT-PCR analysis and normalized to the TE buffer control to account for loss during this second extraction. RNA remaining on magnetic particles after extraction was determined by recollecting the particles post-elution in 100 µL of nuclease free water. The particles were placed on a rotary mixer for 12 hours at 4 °C. Particles were removed and RNA in solution was purified with the RNeasy Mini kit and quantified by RT-PCR.

Results

RNA extraction from aliquots of frozen nasal wash samples using the prototype extraction cassette, shown in **Figure 1**, recovered on average 510 ± 800 RSV RNA copies per µL (**Figure 3** dark bars). Using aliquots from the same sample, the commercial RNeasy Mini kit, Dynabeads mRNA Direct kit and MagAttract RNA Cell Mini M48 kit recovered 4,400 ± 10,000, 750 ± 1,300, and 940 ± 1,000 copies per µL,

respectively. In an unextracted RSV positive nasal wash sample, 3 ± 3 RSV RNA copies were detected. In samples previously classified as RSV negative, an average of less than 1 copy of RSV RNA was detectable per µL for all methods (**Figure 3**, light bars).

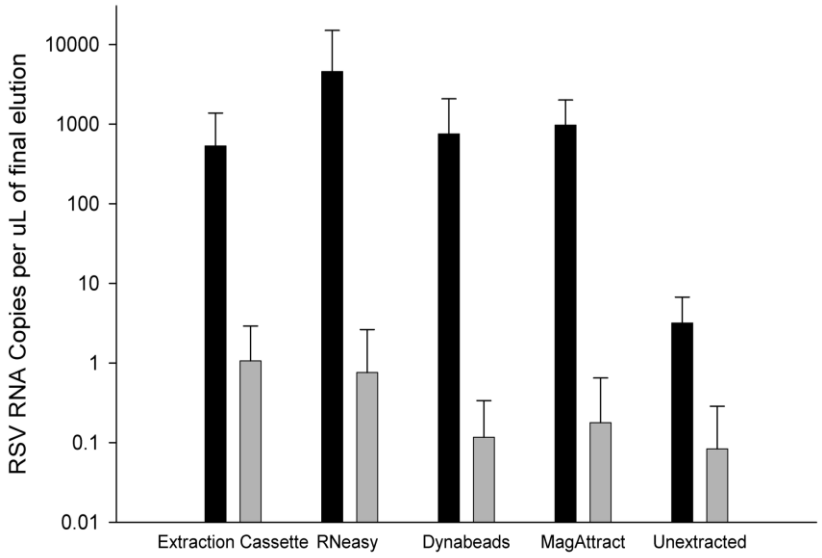

Figure 3. The number of copies of RNA per µL extracted from RSV positive (black bars) and RSV negative (gray bars) nasal wash samples using five extraction methods were compared. Extractions were performed using prototype extraction cassette, RNeasy Mini kit, Dynabeads mRNA Direct kit, and the MagAttract RNA Cell Mini M48 kit (mean ± s.d., n = 7).

Motivated by these encouraging preliminary results using the relatively crude prototype shown in **Figure 1**, a more thorough study was undertaken using a simplified continuous tubing design shown in **Figure 2**. Due to the high sample-to-sample variability and limited number of the frozen nasal wash samples available, further testing was done with RSV infected HEp-2 cell lysates, which unlike the unknown patient samples had no sample-to-sample variation. Using the continuous tubing extraction cassette shown in **Figure 2**, extraction of an RSV N gene standard added to TE buffer

was recovered at an efficiency of 22.5 ± 19% (1.1 ± 0.95 x 10^6 copies) (**Figure 4A**). Recovery efficiency was calculated by dividing the total number of copies extracted by the initial number of copies present in the sample and multiplying by 100%. In TE buffer, the RNeasy kit recovered 41 ± 19% (2.1 ± 0.95 x 10^6 copies). TE buffer does not contain PCR interferents so, as expected, the detection of unextracted standard RNA was 100% (**Figure 4A**, right bar).

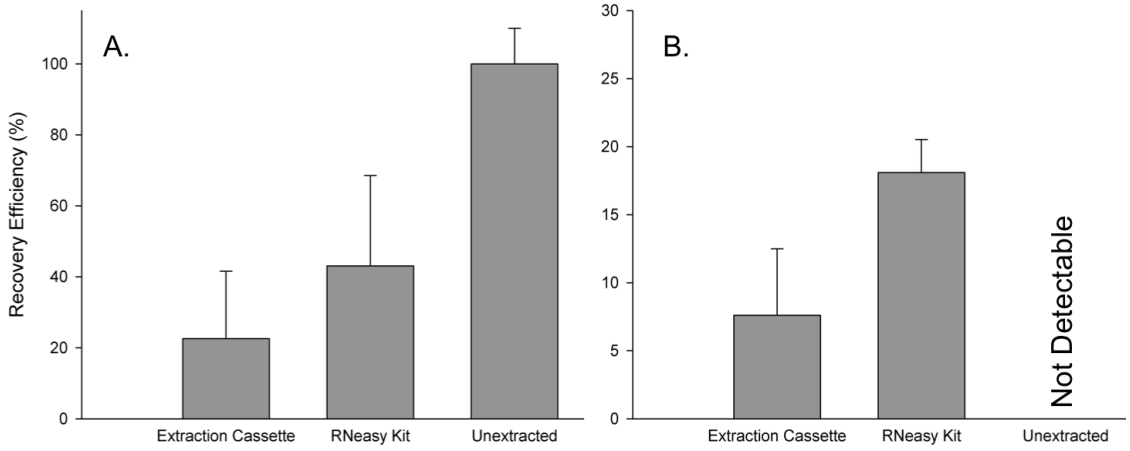

Figure 4. Comparison of the percent of RSV RNA recovered after addition to TE buffer (A) or HEp-2 cell lysates (B) using the extraction cassette (left bars), RNeasy kit (middle bars), or no extraction (right bars) (mean ± s.d., n = 9). The recovery efficiency of the cassette was 55% and 42% of the RNeasy kit from TE buffer and HEp-2 cell lysates, respectively.

In the more complex uninfected HEp-2 cell lysate sample matrix, the recovery efficiency of RNA was 7.6 ± 4.8% (3.8 ± 0.24 x 10^5 copies) using the extraction cassette, and 18.1 ± 2.4% (9.1 ± 1.2 x 10^5 copies) using the RNeasy kit (**Figure 4B**). The spiked cell lysates evidently contained RT-PCR interferents since there was no amplification of the unextracted spiked sample by RT-PCR (**Figure 4B**, right bar).

Using the continuous tubing extraction cassette, RSV RNA extracted from RSV infected HEp-2 cell lysates containing 4.6 x 10^5 pfu/mL recovered 3.6 ± 0.09 x 10^5 RNA copies per µL from the elution chamber compared to 1.2 ± 0.07 x 10^6 copies per µL

using the RNeasy kit (**Figure 5**, black bars). Less than 100 copies/µL was reported in extractions obtained from uninfected cell lysates (**Figure 5**, gray bars), and RNA was not detectable for infected or uninfected cell lysates which were not extracted prior to RT-PCR (C_t > 40) (**Figure 5**, "Unextracted").

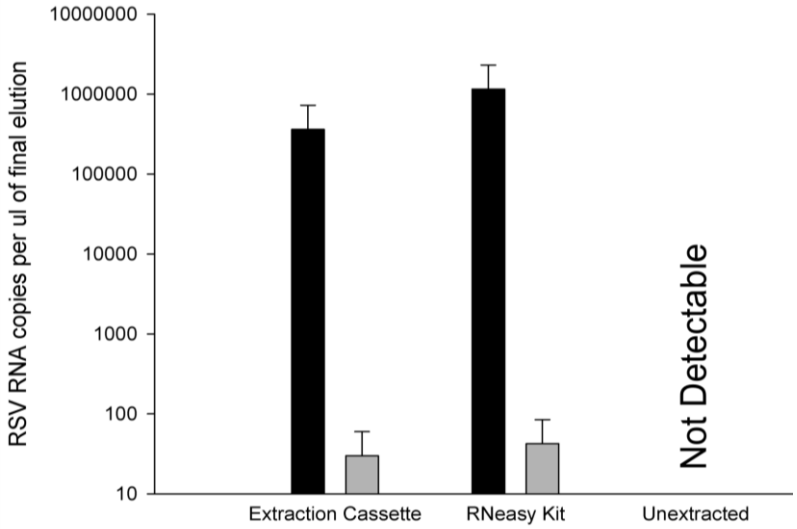

Figure 5. Comparison of RNA extracted from RSV infected (black bars) and uninfected (gray bars) HEp-2 cell lysates using the extraction cassette and RNeasy kit. Unextracted samples failed to report RSV RNA in either sample (mean ± s.d, n = 3).

For all methods, RNA loss during extraction was significant. A post-extraction examination of the distribution of RNA in the processing solutions was successful in accounting for some of this loss. In a separate series of experiments using the continuous tubing extraction cassette, we found that only 59.5% (3.0×10^6 copies) of RNA could be accounted for in a post-processing distribution analysis of RSV N gene standard added to TE buffer (**Figure 6**). Similar to the results found in **Figure 4A**, 28 ± 4.5% ($1.4 \pm 0.23 \times 10^6$ copies) of the RNA was recovered in the elution. Significant RNA was recovered in the water wash solutions, which contained 21.7 ± 4.6% ($1.1 \pm 0.23 \times 10^6$ copies) of the initial RNA. An additional 7.8 ± 3.5% ($3.9 \pm 1.8 \times 10^5$ copies) of the

RNA was recovered from the silica particles after 12 hours of further elution at 4 °C. Less than 2% of the RNA was recovered in the RNA-silica binding, chaotropic wash, and RNA precipitation solutions. The tube wall was also checked for RNA binding by washing with water post-extraction, and no detectable RNA could be recovered (data not shown). Approximately 40.5% (2.0 x 10^6 copies) was either lost or degraded during processing.

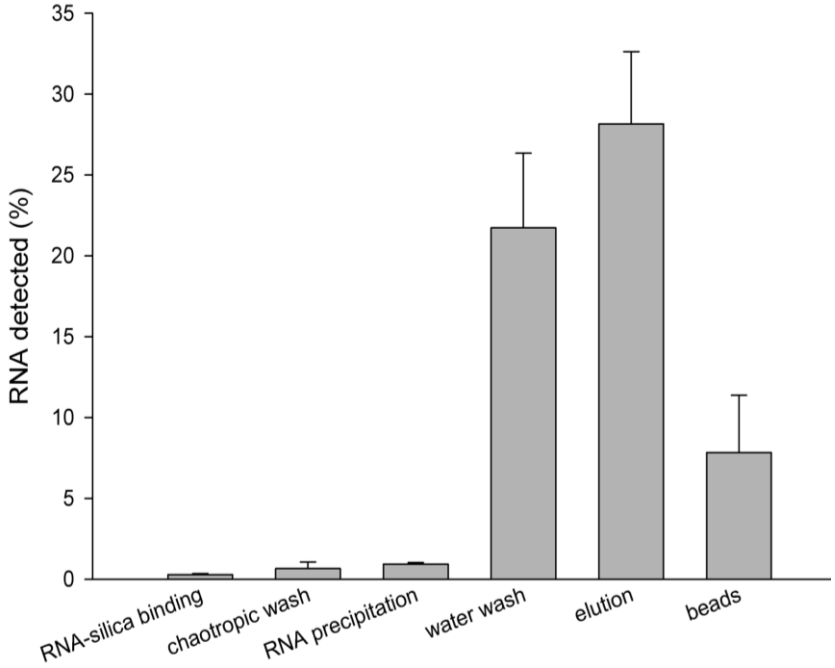

Figure 6. The post-extraction distribution of RNA in each processing solution after RNA extraction from TE buffer is shown. Insignificant amounts were recovered in the first three steps, but the water wash and silica particles contained significant RNA (mean ± s.d., n = 3).

The post-extraction limit of detection was established for the continuous tubing extraction cassette by determining how many RSV RNA copies had to be added to a HEp-2 cell lysate to produce a detectable signal following RNA extraction and RT-PCR. Five thousand copies of RSV RNA spiked into HEp-2 cell lysates (e.g. 5 uL of RNA at 1,000 copies/uL into 20 uL of cell lysate) was the lowest concentration detectable by RT-

PCR after sample extraction using both the continuous tubing extraction cassette and RNeasy kit (**Figure 7**). For the extraction cassette, 197 ± 8.5 copies were reported in the sample containing no RSV RNA, resulting in a 3 s.d. limit of detection of roughly 222 copies. Cell lysates spiked with 5,000 copies prior to extraction reported a value of 228 ± 58.5 copies per PCR reaction. Similarly, 202 ± 9.5 copies were reported in the sample containing no RSV RNA, and 312 ± 26.8 copies from lysates spiked with 5,000 copies and extracted with the RNeasy kit.

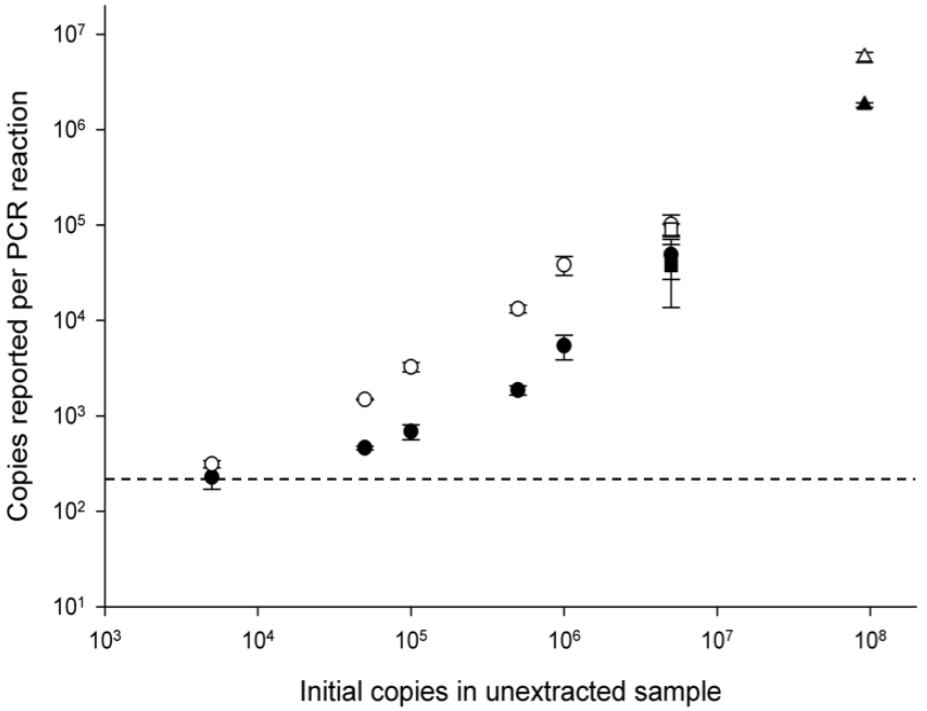

Figure 7. The limit of detection of RNA detectable by RT-PCR after extraction from HEp-2 cell lysates spiked with known amounts of RSV RNA using either the continuous tubing extraction cassette (•) or the RNeasy kit (o) (mean ± s.d, n=3). When a sample containing no copies of RNA was extracted, 197 ± 8.5 RNA copies were detected with the extraction cassette and 202 ± 9.5 copies were detected with the RNeasy kit. The limit of detection is shown for the continuous tubing extraction cassette (dotted line). Comparable data from Figs 4B (black triangle) and 5 (white triangle), which were performed at a single concentration are also included.

Discussion

Preparation of patient samples is necessary to avoid false negative prior to nucleic acid-based testing [6]. Sample preparation techniques mirroring the simple low resource nucleic acid-based diagnostic devices currently being developed are necessary to make diagnosis practical at the point-of-care. Unfortunately, the operation of most existing commercial kits appropriate for RNA extraction and concentration require specialized laboratory equipment and trained laboratory personnel not available in a low resource setting.

In agreement with previous studies [6], we found that without an initial extraction step, only purified RNA in solutions containing no interferents (e.g. TE buffer) can be directly detected by RT-PCR (**Figure 4A**). Direct amplification of viral RNA by RT-PCR prior to RNA extraction failed to detect viral RNA in RSV infected clinical nasal wash samples (**Figure 3**), HEp-2 cell lysates spiked with RSV RNA (**Figure 4B**) and RSV infected HEp-2 cell lysates (**Figure 5)**. Therefore, RSV false negatives are likely to be obtained when the extraction step is omitted prior to RT-PCR, and sample preparation is necessary to make an accurate diagnosis.

A post-extraction limit of detection study was performed using spiked cell lysate samples, and the results suggest that the proposed continuous tubing extraction cassette and the RNeasy kit have a similar limit of detection of ~200 copies per µL. The limit of detection of current clinical diagnostics is ~10^4 pfu/mL [16]. By dividing an RSV infected culture into two parts and measuring pfu/mL by traditional methods and by copies/uL RT-PCR (see section 2.3), it was determined that 10^4pfu/mL corresponds to ~10^5 copies/uL. Therefore, RT-PCR following extraction yields ~50-fold improvement in the limit of detection. In combination with a point-of-care nucleic acid-based diagnostic, the proposed extraction cassette would be ideal in a low resource setting, and with only a two-fold improvement in the extraction process or optimization of the RT-PCR, lower

limits at or below the RSV infectious dose 50 (dose that will infect 50% of subjects, or ~100 copies per µL) [16] are likely achievable.

Currently, there are no commercially available low resource nucleic acid extraction devices for comparison to the proposed method. However, several laboratory-based commercial kits are available, and we compared the proposed low resource method to these approaches. We have utilized a quantitative method for evaluating and directly calculating the efficiency of the extraction cassette in order to simplify comparison of the device to the other methods used in this study. Despite its simplicity, the extraction cassette isolated between 30 and 55% of the Qiagen RNeasy kit. Therefore, using RT-PCR, the Ct values for the extraction cassette would fall within ~1 cycle of the RNeasy kit. As shown in **Figure 4**, ~22.5% of the total RNA input is recovered by the current design under idealized conditions (spiked TE buffer). More complex sample matrices such as cell lysates or nasal wash samples evidently contain components that inhibit RT-PCR and/or make RNA recovery more difficult. All extraction methods tested had lower extraction efficiencies when used to extract RNA from cell lysates. For example, compared to extraction from TE buffer, the recovery from spiked cell lysates using the extraction cassette was reduced by 65%. Similarly, the recovery using the RNeasy kit was reduced by 57%.

The prototype design was tested with a small subset of previously collected de-identified nasal wash samples. These samples were labeled RSV positive or negative during the collection process (not part of this study) using a commercial laboratory RNA extraction process and RT-PCR. The testing of these samples was not designed as a blinded study and served as a simple validation of the basic extraction design. The evaluation of these samples with our prototype device indicated that the basic design performed similarly to commercially available kits (**Figure 3**), but in general recovered less than the other kits tested. All of the extraction methods used correctly classified the

RSV positive and negative samples. However, the amount of RSV RNA present in these samples was quite variable as indicated by the coefficient of variation (s.d./mean) obtained with all of the extraction methods. The coefficients of variation were 157% (extraction prototype), 227% (RNeasy), 173% (Dynabeads), and 106% (MagAttract). This high variation was the primary reason for using the HEp-2 cell lysates as a more controllable clinical sample analogue for further device development and testing. The error obtained with known RNA input is more indicative of variation inherent in the methods themselves. As **Figure 5** indicates, under these more controlled conditions, the coefficient of variation is substantially reduced for both the extraction cassette at 6% and the RNeasy kit at 13%.

The prototype design was refined into the extraction cassette by loading processing solutions into a single length of tubing. The surface tension in the small diameter tubing holds each solution in place, and individual solutions remain undisturbed when magnetic particles pass through the surface tension valves from one solution to the next. This is an agreement with previous studies using a filament-antibody recognition assay which found that high capillary forces held solutions within small diameter capillary tubes even in the presence of a moving filament [17]. Other research groups have analogously demonstrated that an immiscible hydrophobic liquid can effectively separate nucleic acid processing solutions. Work by Sur and coworkers demonstrated that silica-coated magnetic beads are able to carry nucleic acids through a lipophilic barrier floating on the surface of two separate processing solutions to isolate RNA from RT-PCR interferents [18]. Subsequently, Barry and coworkers adapted the idea to separate solutions in a horizontal format, relying on the surface tension forces at the immiscible interface to prevent the processing solutions from mixing while allowing the transport of magnetic beads through the lipophilic barrier [19]. For the development of a low resource RNA extraction device, the use of surface tension valves in a

continuous length of tubing offers several advantages over the reported nucleic acid isolation technologies using lipophilic barriers.

One of the advantages of the extraction cassette design is the inherent flexibility and simplicity which provides a unique format for the development of other sample processing cassettes. The scale of the cassette is similar to that of laboratory-based commercially available kits and can be modified to incorporate relatively large sample volumes. The simple design also makes it suitable for large-scale manufacturing. Additionally, it is possible to adapt the cassette format to perform many other solid phase based assays. By functionalizing the magnetic beads with the appropriate capture moiety and loading the cassette with the appropriate processing solutions, assays could be developed for low resource processing of a variety of biomarkers, including DNA, proteins or carbohydrates. The cassette also has the potential to be coupled with downstream platforms for integrated sample preparation, signal amplification and detection. For example, by simply coupling the end of the extraction cassette tubing onto a thermocycler tube or onto the input port of a thermocycler, it is possible to elute the RNA into a prepared RT-PCR buffer without extra handling or risk of contamination.

The simple format of the extraction cassette also improves the reliability of the device. The utilization of surface tension valves in the continuous tubing design allows the extraction process to be fully self-contained. Individual processing solutions are preloaded into the tubing during manufacturing, eliminating the need for sample handling and pipetting during the extraction process. This is advantageous as it minimizes the potential for contamination of the wash solutions, the extracted RNA, or the operator. The continuous diameter of the tubing minimizes particle loss during sample pull-through by eliminating locations where the particles can become trapped, a limitation of the original prototype design shown in **Figure 1**. The surface tension valves also minimize

interferent carryover by preventing diffusion down the tubing, and separating the water wash into three successive steps also helped to minimize carryover.

Fluid stability and continued separation during processing are key to this design. The surface tension at the valve interface is affected by the surface properties of the tubing and the properties of the air/liquid interface. Consequently, the overall stability of the solution chambers within the cassette is dependent on each of these surface properties [19]. Some preliminary studies have been performed to estimate liquid carryover of processing solution across a valve. Each of the four processing solutions was mixed with sodium fluorescein to create a fluorescein concentration of 2.7 μM. Twenty μL of beads (approximately 5.6 x 10^7 beads) were transported from each of these labeled solutions across a valve into 80 μL of unlabeled water. The fluorescence of the water downstream of the valve was measured and a standard curve used to estimate the volume of solution transferred. The volumes of processing solution carried over for the RNA-silica binding buffer, chaotropic wash buffer, RNA precipitation buffer and water wash were 7.61 μL ± 0.59, 9.88 μL ± 0.27, 5.03 μL ± 0.26, and 5.41 μL ± 0.39, respectively. If we assume that each downstream chamber is perfectly mixed, then the final RNA elution could contain a maximum of ~8.0 x 10^{-4}% ethanol and ~25 nM guanidine salt, two known RT-PCR contaminants. These values are far below the amounts experimentally determined to affect RT-PCR, which we determined to be ~ 1% ethanol or ~ 100 μM guanidine salt (data not shown). Further, ongoing studies are directed at better understanding these properties and the influence of magnetic particle properties such as diameter, density, magnetic susceptibility, and surface chemistry on particle transport through a surface tension valve.

Nucleotide extractions from biologically relevant samples are never 100% efficient, neither in a lab laboratory environment, nor in low resource settings. In fact, the gold standard RNeasy kit only recovered 18.1 ± 2.4% of the RSV N gene RNA

spiked into HEp-2 cell lysates. The performance of the extraction cassette would be improved by reducing the overall loss of RNA during the extraction process. Unlike commercially available extraction kits, all of the required components in this study are based on previously published strategies for RNA extraction. It is likely that further modifications to individual processing solutions will lead to an increase in the recovery efficiency. An estimation of the RNA distribution within each wash chamber allows us to identify variables for optimization (**Figure 6**). Significant quantities of RNA were lost during the water wash steps, which are necessary to remove the ethanol in the absence of centrifugation. For downstream RNA detection by RT-PCR, the ethanol must be removed prior to amplification; however, other nucleic acid-based detection strategies may not be inhibited by the presence of ethanol. In these cases, the water wash chambers could be reduced or eliminated and the recovery efficiency of the extraction cassette improved. Approximately 8% of the RNA still remained on the silica particles after a 5 minute elution in water. By increasing the elution time, the overall yield of the device could be improved by up to 8% in 12 hours, but the total extraction time would be dramatically increased. Future studies will explore potential methods to minimize this loss, including the possibility of direct amplification of the RNA bound to the bead surface without an elution step. Minimal RNA was detected within the RNA-silica binding, chaotropic wash and RNA precipitation solutions. The post-extraction RNA distribution in the processing solutions suggests that RNA may be irreversibly bound to tubing or particle surfaces, degraded during processing, or located on particles that become trapped in the surface tension valves during magnetic pull-through.

The extraction cassette investigated here can potentially be utilized for sample preparation in a low resource setting. It is relatively inexpensive to produce at less than $1.00 per extraction. A rough cost estimate based on current catalog prices of the chemicals and materials required for the continuous tubing design suggests that the

most expensive items are the magnetic particles (about $0.50) and the Tygon tubing (about $0.30). In its current form, the recovery efficiency of this device is acceptable. However, the continuous tubing design can likely be further improved by solution and surface optimization studies. Its major advantages are that, unlike other commercially available methods, it can be performed without a laboratory centrifuge or access to a pipetter and without the skills necessary to operate these laboratory devices. Thus, this approach is an attractive low resource alternative to commercially available methods.

Conclusions

We have shown that our self-contained extraction cassette performs effectively and offers many advantages over similar reported devices and commercially available kits. The simplicity and flexibility of the cassette make it an robust sample preparation tool suitable for use in low resource settings where nucleic acid-based diagnostics must be utilized without specialized equipment and/or trained personnel. The extraction cassette is an ideal format for coupling with downstream nucleic acid amplification and detection modalities. Additionally, the technology is readily adaptable for the isolation of other potential biomarkers of interest, including DNA, proteins or carbohydrates.

Acknowledgments

This work was supported in part by a Vanderbilt University IDEAS Award, NIH R21 EB009235, and N.A. was supported by NIH Training Grant T32 HL007751 from the Heart, Lung, and Blood Institute.

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CHAPTER IV

A MAGNETIC BEAD-BASED METHOD FOR CONCENTRATING DNA FROM HUMAN URINE FOR DOWNSTREAM DETECTION

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PLOS ONE 8: e68369, 2013

Abstract

Due to the presence of PCR inhibitors, PCR cannot be used directly on most clinical samples, including human urine, without pre-treatment. A magnetic bead-based strategy is one potential method to collect biomarkers from urine samples and separate the biomarkers from PCR inhibitors. In this report, a 1 mL urine sample was mixed within the bulb of a transfer pipette containing lyophilized nucleic acid-silica adsorption buffer and silica-coated magnetic beads. After mixing, the sample was transferred from the pipette bulb to a small diameter tube, and captured biomarkers were concentrated using magnetic entrainment of beads through pre-arrayed wash solutions separated by small air gaps. Feasibility was tested using synthetic segments of the 140bp tuberculosis IS6110 DNA sequence spiked into pooled human urine samples. DNA recovery was evaluated by qPCR. Despite the presence of spiked DNA, no DNA was detectable in unextracted urine samples, presumably due to the presence of PCR inhibitors. However, following extraction with the magnetic bead-based method, we found that ~50% of spiked TB DNA was recovered from human urine containing roughly $5x10^3$ to $5x10^8$ copies of IS6110 DNA. In addition, the DNA was concentrated approximately ten-fold into water. The final concentration of DNA in the eluate was $5x10^6$, $14x10^6$, and $8x10^6$ copies/µL for 1, 3, and 5mL urine samples, respectively. Lyophilized and freshly prepared reagents within the transfer pipette produced similar results, suggesting that long term storage without refrigeration is possible. DNA recovery increased with the length of the spiked DNA segments from 10±0.9% for a 75bp DNA sequence to 42±4% for a 100bp segment and 58±9% for a 140bp segment. The estimated LOD was 77 copies of DNA/µL of urine. The strategy presented here provides a simple means to achieve high nucleic acid recovery from easily obtained urine samples which does not contain inhibitors of PCR.

Introduction

Relative to many other types of patient samples, urine samples are easy to obtain. Therefore, they are frequently analyzed for biomarkers of disease. Urine biomarkers include sugars for the diagnosis of diabetes mellitus, proteins for the diagnosis of liver and kidney disorders, and bacteria for the diagnosis of urinary tract infections [1]. In addition, transrenal DNA (Tr-DNA) sequences have been shown to be applicable in pathogen detection. As human cells and microorganisms break down within the body, small nucleic acid fragments are thought to circulate in the blood stream, and subsequently pass through the kidneys and into the urine [2,3]. In healthy individuals, these Tr-DNA fragments have been shown to be less than 250 bp in length [4]. Tr-DNA sequences specific to *Mycobacterium tuberculosis* [5-7], urinary tract infections [8], *Plasmodium* [9], and *Schistosoma mansoni* [10] have been recovered from urine, pointing to Tr-DNA as a promising new biomarker for urinalysis.

The noninvasive collection of urine samples is advantageous in low resource settings where lack of automated equipment and skilled technicians, cultural taboos, or patient age make more invasive sample collection difficult [11]. Compared to blood, sputum, or cheek swab samples, urine sample volume is large, typically 10 to 100 mL, which maximizes the availability of the biomarker of interest. This is a distinct advantage when detecting a biomarker that is expected to be present at relatively low concentrations, like Tr-DNA. However, urine contains inhibitors that interfere with downstream nucleic acid-based detection strategies such as PCR [11,12]. Additionally, isothermal PCR and other nucleic acid-based assays being developed for use in low resource settings are frequently tested using commercial nucleic acid extraction kits that may be inappropriate for use in these settings. Thus, the need for nucleic acid extraction and concentration is a well-recognized roadblock to performing nucleic acid-based detection in low resource settings [11,13,14].

In order for PCR or other nucleic acid-based assays to detect DNA in urine samples, the DNA must be separated from inhibitors contained in the sample. Current methods to extract nucleic acids for nucleic acid-based assays are either not amenable to a large volume patient sample such as urine, are not appropriate to a low resource setting, or both. Commercially-available extraction kits for use in laboratories, such as Exgene Clinic SV (Biomol, GmbH), Norgen Urine DNA Isolation kit (Norgen Biotek Corporation), and QIAmp DNA kit (Qiagen), utilize spin columns to selectively adsorb nucleic acids and remove potential inhibitors to downstream assays. However, these columns require relatively expensive equipment (e.g. centrifuge), disposable laboratory supplies, and trained personnel. Products such as InstaGene Matrix (Bio-Rad) also remove PCR inhibitors from patient samples but dilute the DNA instead of concentrating it. Self-contained microfluidic devices embedded with silica beads or posts have been developed for one-step nucleic acid extractions [15]. However, the small diameter of microfluidic channels restricts the silica surface area available for biomarker adsorption as well as the maximum sample volume that can be flowed through the channels (typically < 500 μL) [14]. This limits the utility of microfluidic devices for processing large volume samples like urine. We report here a means of extracting and concentrating DNA from up to 5 mL urine samples without pipetting and centrifugation steps necessary in commercial DNA extraction kits.

Magnetic beads are an effective tool for extracting and concentrating biomarkers. Using this dispersed solid phase approach, magnetic beads are mixed with large volumes of patient sample to capture biomarkers. The magnetic beads are then recollected magnetically, and the captured biomarkers are released from the bead surface into a more amenable reaction buffer. This has led to their use in a number of bioassay applications [16-18]. The coating on the magnetic bead surface is one of the key factors to this approach. The adsorption of nucleic acids to silica in the presence of

high concentrations of chaotropic salts is used extensively to extract nucleic acids from complex sample matrices into inhibitor-free solutions in bead-based methods as well as in a number of other formats. Additionally, the mobility of magnetic beads allows transfer of adsorbed nucleic acids into smaller volumes and, therefore, leads to more concentrated solutions [19].

The ideal nucleic acid extraction technology for a low resource setting would require minimal processing steps, enable the use of large sample volumes to maximize biomarker recovery, and be self-contained to limit operator exposure to samples during processing [14,20]. We have previously reported the development of a low resource "extraction cassette" for the purification of RNA [21] and protein [22] from small volume patient samples. The extractions were performed in a single length of small diameter tubing pre-arrayed with processing solutions separated from one another by air gaps or mineral oil held in place by surface tension. After biomarker capture on the bead surface, the magnetic beads were pipetted into the small diameter tubing, and an external permanent magnet was pulled along the length of the tubing to wash and release biomarkers into a final buffer for subsequent testing.

In these prior assays, biomarker capture by magnetic beads was performed in a multi-step process using refrigerated reagents added to a centrifuge tube. In this report, we eliminate the need for multiple pipetting steps, refrigerated reagents, and the precise volume transfer to the small diameter tubing. In addition, we have modified the magnetic bead and tubing components of our "extraction cassette" design based on the optimization studies reported by Adams et. al. [23]. The end result is a simple means to collect DNA biomarkers present in large volume urine samples in a transfer pipette bulb and to concentrate the biomarkers into a small volume using our preloaded extraction cassette for subsequent nucleic acid-based detection.

Materials and Methods

Preparation of pooled human urine samples.

Twenty disease-negative urine samples were obtained from the Vanderbilt University Molecular Infectious Disease Laboratory as “on-the-shelf” samples that contained no patient information. An IRB exemption was granted from the Vanderbilt University Institutional Review Board for use of these samples. There were two ways to use the human urine samples. They could either be individually spiked prior to extracting, or they could be pooled and aliquotted prior to spiking. In these studies, the urine samples were pooled allowing us to minimize variability in the concentration of PCR inhibitors that would be expected between individual urine samples. Fifteen milliliters of each sample were pooled, pipetted into 1 mL aliquots, and stored at -80°C. Immediately prior to use, the samples were thawed at room temperature and spiked, in most cases, with a 140 bp synthetic DNA target from the IS6110 sequence of *M. tuberculosis* (Integrated DNA technologies, Coralville, Iowa). The one exception is the sequence length studies described below.

Quantitative PCR.

A 129 bp amplicon of the IS6110 sequence was amplified using forward primer 5’-ACCAGCACCTAACCGGCTGTGG-3’ and reverse primer 5’-CATCGTGGAAGCGACCCGCCAG-3’[7]. Amplification reactions were performed in a 25 µL volume using 5 µL of DNA template and the Qiagen QuantiTect SYBR Green PCR kit (Qiagen, Germantown, MD) according to manufacturer’s instructions. Thermal cycling consisted of 95°C for 15 minutes to activate the Taq DNA polymerase and 40 cycles of 95°C for 15 s, 62°C for 30 s, and 72°C for 30 s using a Rotor-Gene Q thermal cycler (Qiagen, Germantown, MD). Product specificity was confirmed using melting

curve analysis. Data were collected and C_t values recorded by Rotor-Gene Q software and converted to number of DNA copies per µL using a standard curve. The primer efficiency for each standard curve was also determined using the Rotor-Gene Q software.

Preparation of urine collection pipettes.

The urine collection pipettes were prepared by drawing 1 mL of DNA-silica adsorption buffer (4 M guanidine thiocyanate, 25 mM sodium citrate, pH 7.0) containing 6 x 10^8 Dynabeads® MyOne™ Silane magnetic beads (Life Technologies, Grand Island, NY) into the bulb of a 5 mL, fine tipped transfer pipette (Samco Scientific, San Fernando, CA, cat. # 232-20S). The contents of the transfer pipettes were frozen for 2 hours at -80°C, after which the pipettes were transferred to a Labconco bulk tray dryer (Labconco, Kansas City, MO) and lyophilized for ~18 hours. Following lyophilization, the transfer pipettes were stored at room temperature for up to twelve weeks until use.

DNA extraction from pooled human urine samples.

The two components of this magnetic bead-based approach are shown in **Figure 8**. Based on the optimization studies performed by Adams et. al. the previously described "extraction cassette" was modified by using fluorinated ethylene propylene (FEP) tubing in place of Tygon® R-3603 tubing [23]. The tubing was prepared by preloading a 60 cm length of 1.6 mm inner diameter (i.d.) FEP tubing (Saint-Gobain Performance Plastics, Akron, OH) with DNA precipitation buffer (300 µL of 80% ethanol, 5 mM potassium phosphate, pH 8.5), DNA wash solution (300 µL of 70% ethanol), and DNA eluent (50 µL of molecular grade water). Each solution was pipetted sequentially into the tubing and separated from the next by a 4 µL air gap (2 mm in length). A 1 mL human urine sample was spiked with 5 µL of IS6110 DNA to a concentration of 5 x 10^5

copies/μL. Prior to extraction, the samples were drawn into the bulb of the transfer pipette, which was shaken vigorously by hand for 30 s to dissolve the lyophilized salts, then gently for 30 s to allow DNA adsorption to the silica surfaces of the beads. After mixing, the transfer pipette was squeezed to transfer the sample from the bulb to the tip of the pipette. The end of the pipette was then inserted into the preloaded small diameter tubing and the sample was transferred into the tubing (**Figure 8A**). Stainless steel #1 machine screws (McMaster Carr Supply Co, Elmhurst, IL, cat. # 90065A054) were threaded into both ends of the FEP tubing to establish an airtight seal. A 2.5 cm cube-shaped neodymium magnet (Emovendo, Petersburg, WV) adjacent to the DNA-silica adsorption chamber was used to collect the beads. Although one bead is not visible to the naked eye, the quantity of beads used here (6×10^8 beads) is readily perceived as a dark brown clump. The beads were pulled through the air gaps and each successive solution at ~4 mm/s (**Figure 8B**). Beads were dispersed in the DNA precipitation buffer and DNA wash solution by rapidly moving the magnet back and forth along the chamber. Beads were then collected and pulled into the next solution. Finally, the beads were dispersed throughout the eluent for ~10 s, collected, and pulled back into the DNA wash chamber. Each DNA extraction was completed in ~15 minutes.

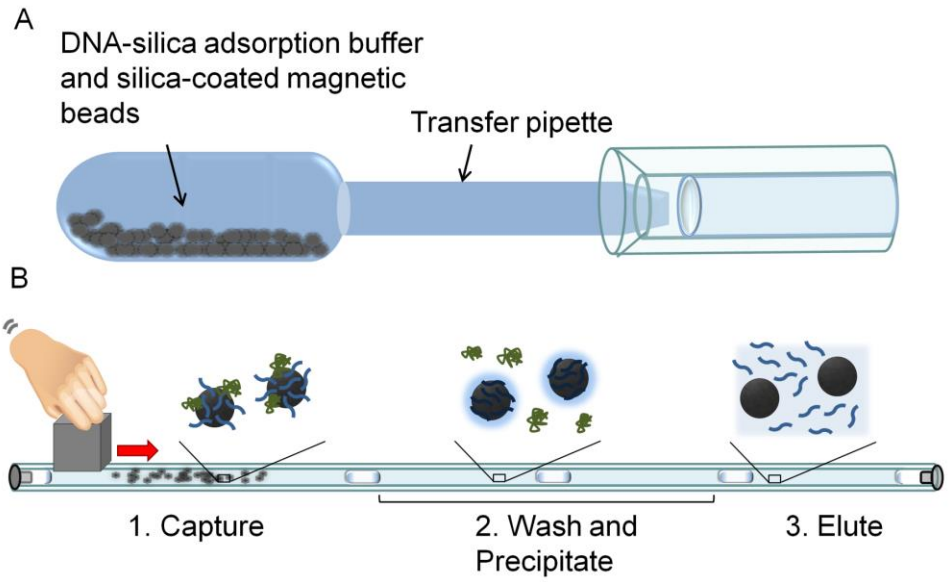

Figure 8. The two components of the magnetic bead-based extraction method: A) DNA is adsorbed to silica-coated magnetic beads previously lyophilized with DNA-silica adsorption buffer in the bulb of a transfer pipette. After mixing, the beads are transferred directly into the small diameter tubing by depressing the bulb. B) The small diameter tubing "extraction cassette" is shown with processing solutions separated by air gaps. DNA adsorbed to silica-coated magnetic beads is pulled through each processing solution by an external magnet and eluted in the final water chamber**.**

DNA recovery was quantified by PCR. To prepare the extracted sample for PCR, the elution chamber was excised from the small diameter tubing using a razor blade, and the eluate was collected by holding the tubing over a 500 µL centrifuge tube. Additional extractions were performed using the commercially available Norgen Biotek urine extraction mini kit (Thorold, Ontario, Canada), performed according to manufacturer's instructions on a 1 mL urine sample. PCR was also performed on 5 µL of an unextracted urine sample containing 5 x 10^5 copies/µL of IS6110 DNA. DNA recovery was calculated by dividing the total number of copies extracted by the initial number of copies present in the sample and multiplying by 100%.

Effect of urine sample volume on final DNA concentration in the eluate.

The recovery of spiked DNA from 1, 3 and 5 mL urine samples, each containing 5 x 10^5 copies/µL of spiked DNA, was determined following magnetic bead-based extraction. Each sample was drawn into the bulb of the lyophilized urine collection pipette, which was shaken vigorously for 30 s to dissolve the lyophilized salts, then gently for 30 s to distribute the magnetic beads for nucleic acid adsorption. To avoid transferring volumes greater than 1 mL into the small diameter tubing and to limit the total length of the small diameter tubing to 60 cm, the magnetic beads were collected to one side of the pipette bulb using a permanent magnet, and all but 1 mL of each sample was transferred into a waste container for disposal. The remaining 1 mL urine sample containing all magnetic beads was then transferred into the small diameter tubing. Finally, the magnetic beads were processed using an external magnet, the eluate collected, and the DNA amplified and quantified by PCR as described above.

Comparison of urine collection pipette to freshly prepared DNA-silica adsorption buffer for DNA capture.

One milliliter pooled human urine samples were extracted as described above by capturing DNA onto silica-coated magnetic beads contained within the urine collection pipette, transferring the sample into the small diameter tubing, and processing the sample using an external magnet. The extractions were performed using urine collection pipettes containing lyophilized DNA-silica adsorption buffer and magnetic beads that had been stored at room temperature for 0, 4, 8, or 12 weeks prior to use. For comparison, the DNA-silica adsorption step was performed in a 2 mL centrifuge tube with freshly prepared DNA-silica adsorption buffer and magnetic beads. One milliliter of DNA-silica adsorption buffer and 6 x 10^8 silica-coated magnetic beads were pipetted into the centrifuge tube followed by a 1 mL DNA-spiked pooled human urine sample. The

contents of the tube were gently shaken for 60 seconds to allow mixing and DNA adsorption to the silica beads. Finally, the sample was transferred to the small diameter tubing by pipetting and processed as described above for DNA extraction. After processing, the recovery of IS6110 DNA from each sample was calculated following PCR.

Effect of spiked DNA concentration of DNA recovery.

To determine the effect of spiked DNA concentration on DNA recovery from human urine samples, multiple concentrations of spiked DNA were extracted and analyzed by PCR. One milliliter human urine samples were spiked with 5 µL of TE buffer containing 0, 5×10^3, 5×10^4, 5×10^5, 5×10^6, or 5×10^8 copies of TB IS6110 DNA. Following extraction the DNA was quantified by PCR.

PCR limit of detection.

The limit of detection was determined for the PCR protocol described above. A 1 mL urine sample containing no TB IS6110 DNA was extracted using the magnetic bead-based method. PCR was performed on the extracted sample, and the number of copies detected in the blank sample was determined by referencing a standard curve run in TE buffer. The PCR LOD was defined to be 3 s.d. above the average number of calculated copies from the extracted unspiked urine sample.

The minimum concentration of DNA that must be spiked into a human urine sample to be detectable in the eluate following extraction with the magnetic bead-based method, given the LOD of the PCR protocol used in this study, was determined. Five microliters of TE buffer containing 0, 5×10^3, 5×10^4, 5×10^5, 5×10^6, or 5×10^8 copies of TB IS6110 DNA were added to each 1 mL urine sample. Following extraction with the magnetic bead-based method, the number of copies detected in the eluate of each

extracted sample was determined by PCR. The approximate number of copies that must be spiked into the initial urine sample to be detectable in the eluate at the PCR LOD was determined as the point which intersects this PCR-concentration curve.

Effect of DNA length on DNA adsorption to silica.

The effect of DNA target length on recovery of DNA using magnetic bead-based extraction was studied to determine the limitations of DNA adsorption to the silica-coated magnetic beads. Human urine samples (1 mL) were spiked with 5×10^8 copies of DNA that was 75, 100, or 140 bp in length. The samples were extracted using the magnetic bead-based extraction, and the recovery of DNA for each sample was determined following PCR amplification by referencing a standard curve. A 67 bp fragment of each target was amplified using forward primer 5'-ACCAGCACCTAACCGGCTGTGG-3' and reverse primer 5'-GTAGGCGAACCCTGCCCAGGTC-3' [7], with cycling conditions identical to those described above.

Adsorption and elution kinetics of silica-coated magnetic beads.

The optimum adsorption and elution times for DNA adsorption to and elution from silica-coated magnetic beads was determined by comparing the recovery of DNA for adsorption or elution times ranging from 30 to 210 s. For adsorption studies, 5×10^8 copies of TB IS6110 DNA were spiked into 1 mL human urine samples and drawn into a transfer pipette bulb containing DNA-silica adsorption buffer and magnetic beads as described above. The pipette was shaken vigorously for 30 s to dissolve the salts, and then gently for an additional 0, 30, 60, or 180 seconds. For elution studies, 5×10^8 copies of TB IS6110 DNA were extracted from 1 mL human urine samples using the magnetic bead-based extraction with an adsorption time of 30 s. After dispersal in the final elution chamber, the beads were incubated at room temperature for 30, 60, 90, or

210 seconds before being collected and removed from the chamber. Each sample was collected and IS6110 DNA was amplified by PCR. The extracted DNA was quantified by referencing a standard curve. The recovery of DNA was determined by dividing the total number of extracted DNA copies by the input number of DNA copies and multiplying by 100%.

Effect of number of magnetic beads on DNA recovery.

The recovery of spiked DNA from 1 mL urine samples was determined following magnetic bead-based extraction performed with different numbers of magnetic beads. For each extraction, a urine collection pipette was prepared with either 12×10^8, 6×10^8, 3×10^8, or 1×10^8 magnetic beads, and the magnetic bead-based extraction was performed on each spiked 1 mL urine sample as described above.

DNA extraction capacity of the silica-coated magnetic beads.

The maximum mass of DNA that could be extracted with the silica-coated magnetic beads was determined by spiking 100 µL of TE buffer with increasing quantities of calf thymus DNA (Sigma Aldrich, St. Louis, MO) and quantifying the mass of DNA extracted. One hundred µL of TE buffer was spiked with 2.5, 5, 7, 10, or 20 µg of calf thymus DNA. Each sample was mixed with 300 µL of DNA-silica adsorption buffer and 6×10^8 silica-coated magnetic beads for 60 s. The DNA was extracted in the small diameter tubing as described above. The extracted DNA was quantified by measuring its absorbance at 260 nm using a NanoDrop ND-1000 spectrophotometer and referencing a standard curve.

Statistical analysis.

All statistical analyses were performed in SigmaPlot 11.0. Analysis of variance (ANOVA) was used to determine statistical significance for data containing 3 or more sample populations, and a T test used for data containing 2 sample populations. A p-value < 0.05 was considered significant.

Results

DNA extraction from pooled human urine samples.

The magnetic bead-based extraction recovered 50 ± 5% of TB IS6110 DNA spiked into 1 mL aliquots of pooled human urine (**Figure 9,** left). The commercially-available Norgen Biotek urine extraction kit recovered 33 ± 6% of spiked DNA (**Figure 9,** middle). No spiked DNA was detectable in the unextracted urine sample (**Figure 9**, right) due to the presence of PCR inhibitors in urine. The primer efficiencies for samples extracted with the magnetic bead-based extraction and Norgen kit were 93% and 96%, respectively. Using the magnetic bead-based extraction, the DNA spiked into urine at an initial concentration of 5×10^5 copies/µL was concentrated to 5×10^6 copies/µL of water as determined by comparison to the PCR standard curve run at the same time.

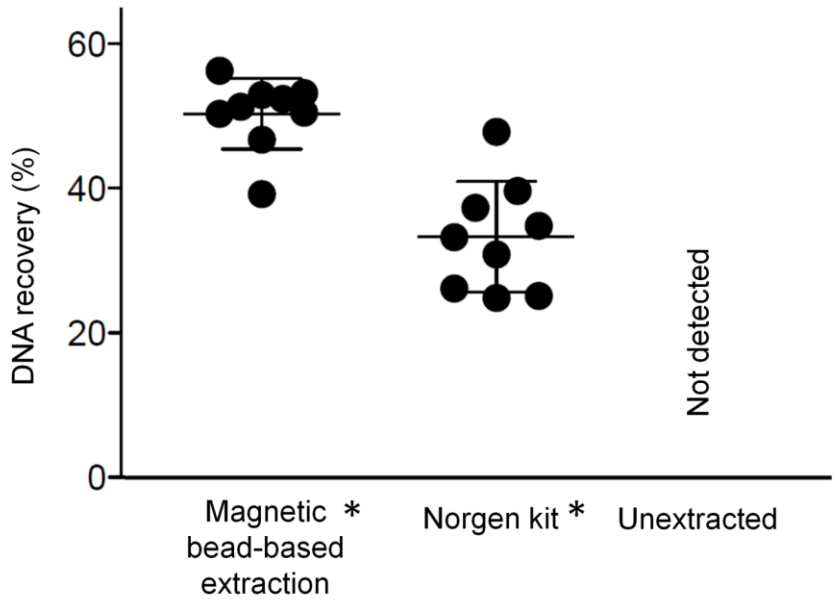

Figure 9. The DNA recovery for the magnetic bead-based extraction method and a commercially available laboratory-based kit are comparable. Though both are significantly higher than the unextracted sample, which is undetectable by PCR, there is no statistical difference between the two extraction methods (mean ± s.d., n = 9); * denotes statistically higher recovery than unextracted sample.

Effect of urine sample volume on final DNA concentration in the eluate.

As shown in **Figure 10**, the final concentration of spiked DNA in the eluate following extraction of the 1 mL urine sample was $5.2 \times 10^6 \pm 7.5 \times 10^5$ copies/µL, corresponding to a recovery of 52%. The DNA concentration was significantly higher in both the 3 mL and 5 mL samples at $13.8 \times 10^6 \pm 16.0 \times 10^5$ and $8.2 \times 10^6 \pm 7.6 \times 10^5$ copies per µL. These corresponded to a total recovery of 46% of spiked DNA from the 3 mL sample and 17% from the 5 ml sample.

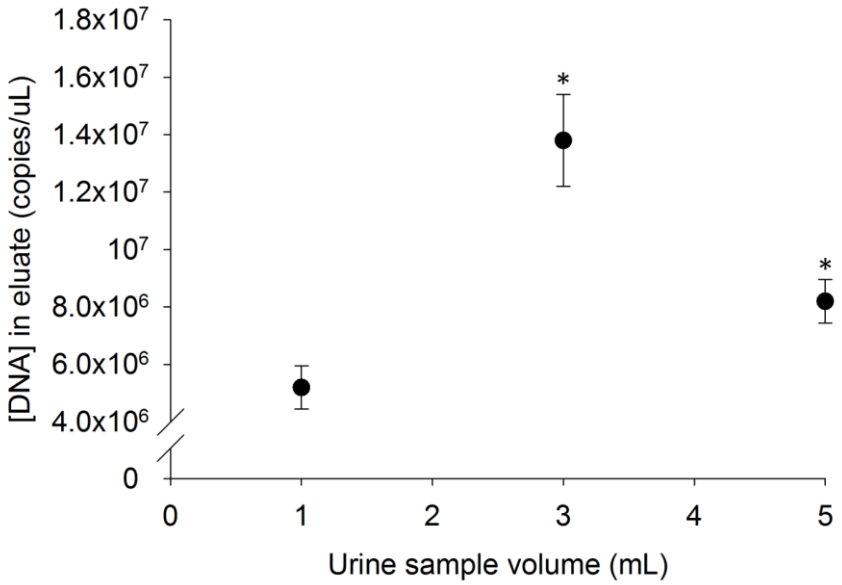

Figure 10. The final concentration of IS6110 DNA in the eluate following magnetic bead-based extraction of 3 and 5 mL spiked urine samples is significantly higher than the IS6110 DNA concentration in the eluate following the extraction of 1 mL samples (mean ± s.d., n = 3); * denotes statistically different from 1 mL sample.

Comparison of DNA capture by urine collection pipette and freshly prepared DNA-silica adsorption buffer.

There was no significant difference in recovery of DNA from the small diameter tubing following DNA capture in the urine collection pipette, which was stored in a lyophilized state, compared to a centrifuge tube containing freshly prepared adsorption reagents. The urine collection pipette method recovered 46 ± 6%, 52 ± 4%, 45 ± 3%, and 42 ± 9% of spiked DNA following urine collection pipette storage for 0, 4, 8, or 12 weeks, respectively, compared to 53 ± 6% of spiked DNA using the freshly prepared DNA-silica adsorption buffer (**Figure 11**).

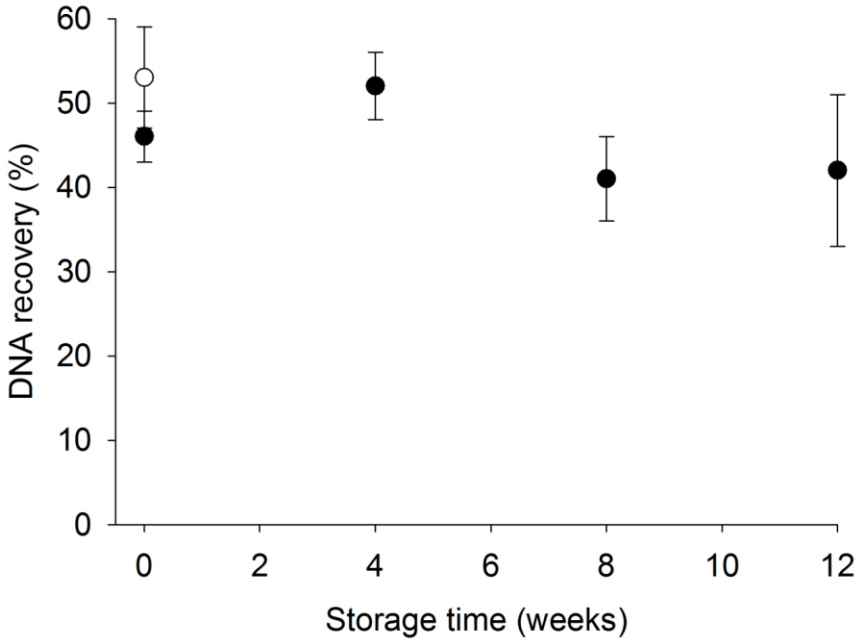

Figure 11. There is no significant difference in DNA recovery using the lyophilized urine collection pipette stored for 0, 4, 8, or 12 weeks (black circles) compared to freshly prepared adsorption reagents (white circle) (mean ± s.d., n = 3).

Effect of spiked DNA concentration on DNA recovery.

As the initial number of copies spiked into the 1 mL human urine sample was increased from 5 x 10^3 to 5 x 10^8, the percentage of DNA recovery from human urine samples did not change significantly over the range tested (**Figure 12**). DNA recovery was 28 ± 42%, 70 ± 68 %, 46 ± 19%, 41 ± 18%, and 50 ± 5% from urine spiked with 5 x 10^3 copies DNA/mL, 5 x 10^4 copies DNA/mL, 5 x 10^5 copies DNA/mL, 5 x 10^6 copies DNA/mL, and 5 x 10^8 copies DNA/mL, respectively.

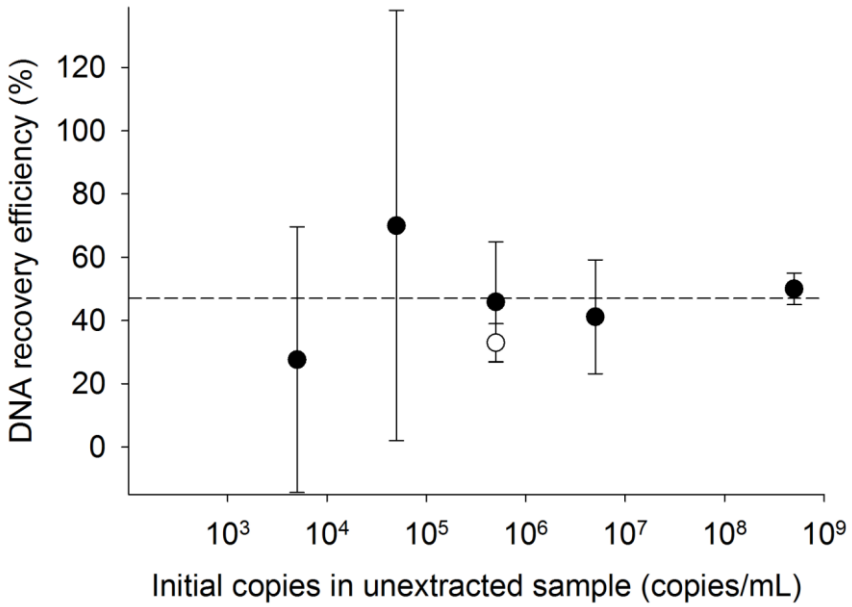

Figure 12. The recovery efficiency of DNA from human urine samples using magnetic bead-based extraction does not change as the number of copies spiked into the 1 mL human urine sample is increased (mean ± s.d., n = 3). The average DNA recovery efficiency of the magnetic bead-based extraction method is shown as a dotted line. The recovery efficiency of the Norgen kit is shown at an initial DNA concentration of 5 x 10^5 copies/mL (white circle).

PCR limit of detection.

The PCR LOD is a reflection of background fluorescence due to primer dimers and other PCR artifacts. Following extraction of a 1 mL human urine sample using the magnetic bead-based method, fluorescence corresponding to 1300 ± 2500 copies of DNA were reported by the PCR reaction. This resulted in a 3 s.d. PCR LOD of roughly 8800 copies (**Figure 13**, dotted line). It was determined that the concentration of IS6110 DNA in the initial human urine samples must be at least 77 copies/µL (**Figure 13**, red circle) to be detectable in the eluate at the calculated PCR LOD.

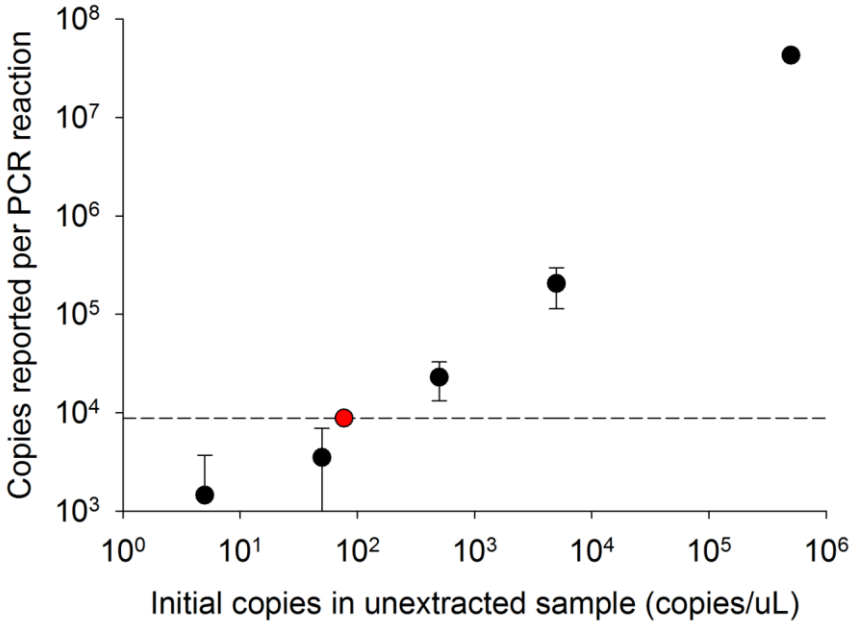

Figure 13. The LOD of the PCR reaction is shown at 8800 copies (dotted line). The lowest initial concentration of IS6110 DNA detectable at the LOD was determined to be 77 copies/µL at the point at which this intersects the PCR-concentration curve (red circle) (mean ± s.d., n = 3).

Effect of DNA length on DNA adsorption to silica.

Increasing the length of target DNA increased the recovery of the magnetic bead-based extraction. As shown in **Figure 14**, when spiked with a 75 bp DNA sequence, the magnetic bead-based extraction recovered 10 ± 0.9% of the sample from 1 mL of human urine. When the length of the DNA sequence was increased to 100 or 140 bases, the recovery increased to 42 ± 4% and to 58 ± 9%, respectively.

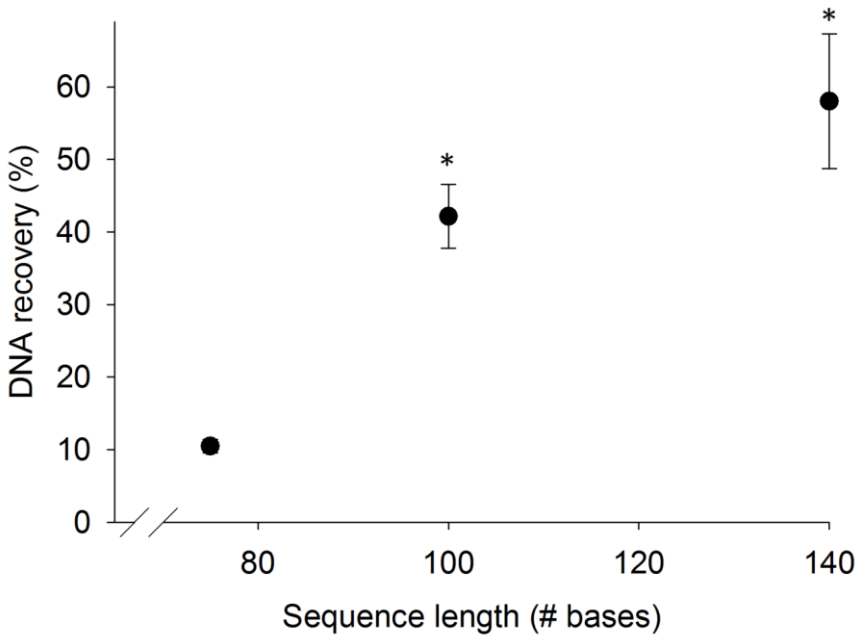

Figure 14. DNA recovery is significantly increased with increasing DNA sequence length (mean ± s.d., n = 3). * denotes statistically different than 75 bp sequence.

Adsorption and elution kinetics of silica-coated magnetic beads.

The percentage of DNA recovered from pooled human urine samples does not increase with increased adsorption or elution times. As shown in **Figure 15**, 54 ± 18% is recovered from extractions with 30 s of adsorption time (white circles), and 44 ± 5% of DNA is recovered from extractions with 30 s of elution time (black circles). As both adsorption and elution times are increased from 30 to 210 s there is no significant increase in the percentage of DNA recovered. In the ideal case, the black and white circles plotted at 30 s would be identical, because they have identical adsorption and elution times. The resulting minimal difference between the two data sets could simply be due to variation in PCR or the fact that the adsorption and elution data sets were collected on two separate days. As the error bars indicate, these points have a high degree of variation which masks any difference between them.

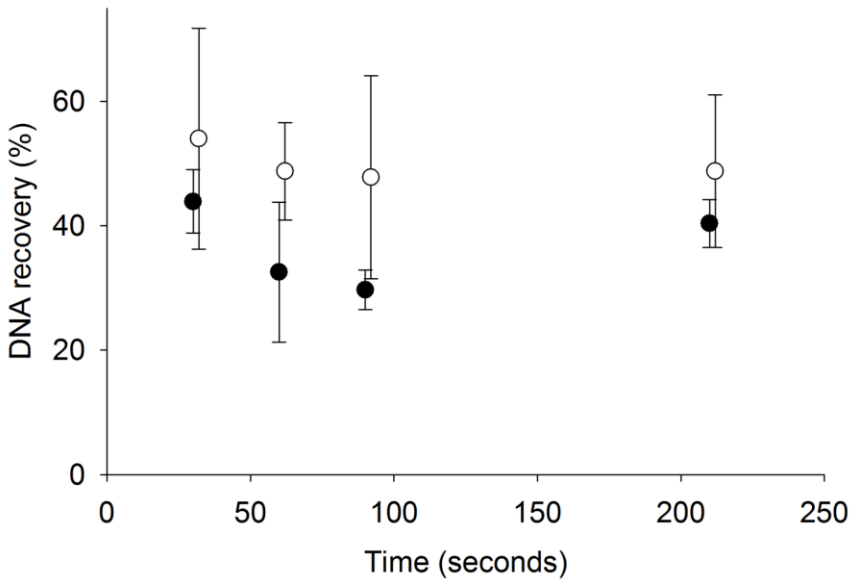

Figure 15. There was no significant change in DNA recovery with increased adsorption (white circles) or elution (black circles) times (mean ± s.d., n = 3). In each experiment, the adsorption time was held at 30 s while the elution time was varied (black circles), and the elution time was held at 30 s while the adsorption time was varied (white circles). To more clearly illustrate the error bars on each data point, the adsorption data was plotted two seconds to the right at each time point.

Effect of number of magnetic beads on DNA recovery.

The percentage of DNA recovered from 1 mL urine samples increases as the number of magnetic beads is increased to 6 x 10^8 beads/mL of urine. After this point, the recovery of DNA does not increase as additional beads are added. As shown in **Figure 16**, 7.6 ± 1% of spiked DNA is recovered from extractions performed with 1 x 10^8 beads/mL, 29 ± 3% from extractions performed with 3 x 10^8 beads/mL, 49 ± 5% from extractions performed with 6 x 10^8 beads/mL, and 45 ± 8% from extractions performed with 12 x 10^8 beads/mL.

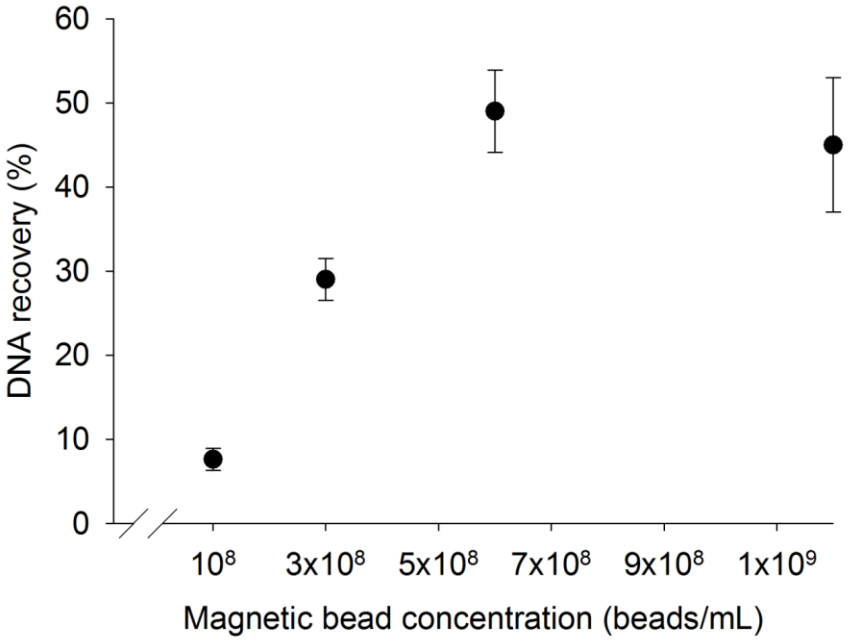

Figure 16. The percentage of DNA recovered from 1 mL urine samples increases as the concentration of magnetic beads increases to 6 x 10^8 beads/mL (mean ± s.d., n = 3).

DNA extraction capacity of silica-coated magnetic beads.

The maximum quantity of total DNA that can be extracted from TE buffer with 6 x 10^8 magnetic beads is 5.5 µg, or ~9 fg of DNA per bead. The mass of extracted DNA increases proportionally with an increase in magnetic beads and then plateaus. As shown in **Figure 17**, 1.8 ± 0.1 µg of DNA was recovered when the extracted sample was spiked with 2.5 µg of DNA. The recovered DNA increased to 4.0 ± 0.3 µg when the sample was spiked with 5 µg of DNA and 4.9 ± 0.2 µg when spiked with 7 µg of DNA. As the quantity of spiked DNA was increased to 10 µg and subsequently to 20 µg, the quantity of extracted DNA was maximized at 5.5 ± 0.2 µg and 5.4 ± 0.1 µg, respectively. Based on the extraction capacity of the beads and an expectation of 250 ng DNA/mL of urine [24], this translates to a minimum of ~2.7 x 10^7 beads.

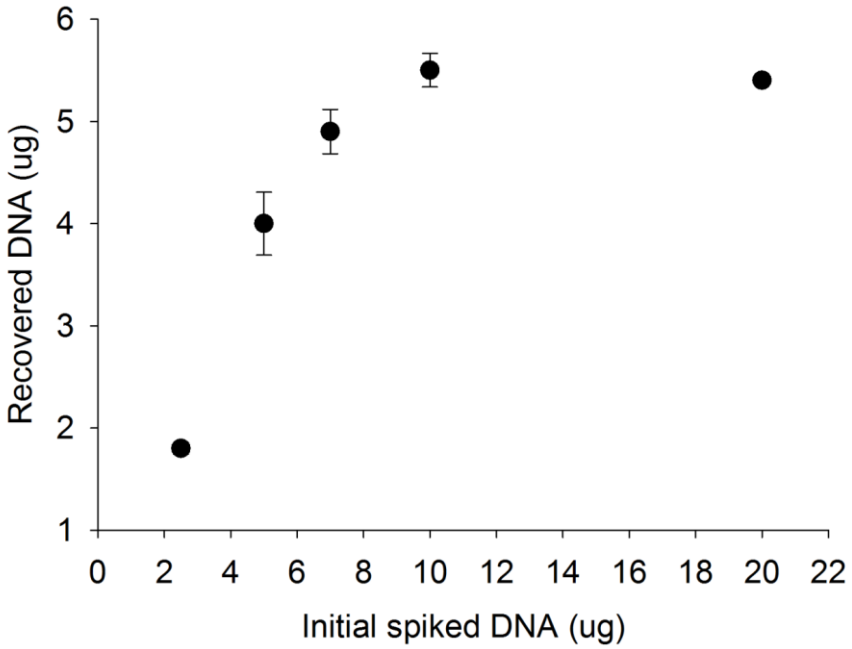

Figure 17. As the mass of spiked DNA is increased the mass of DNA recovered using the magnetic bead-based method increases and then plateaus at 5.5 µg (mean ± s.d., n = 3).

Discussion

When biomarkers are present in low concentrations within patient samples, a large volume sample, such as urine, can maximize the number of biomarkers available for use in a molecular diagnostic test. However, processing large volumes is a challenge, as these large samples are difficult to interface with DNA extraction technologies such as microfluidic devices, particularly in a low resource setting. In our approach, magnetic beads were distributed in a large volume for nucleic acid adsorption and then transferred directly into small diameter tubing. Here, the beads were collected and the adsorbed nucleic acids concentrated in a format compatible with PCR. The magnetic bead-based method for extracting DNA from urine had an average recovery of 50% while concentrating the DNA ten-fold (**Figure 9**). The high primer efficiency for the magnetic bead-based extraction calculated from the PCR reaction (93%) indicated that the extracted DNA was sufficiently separated from PCR inhibitors found in urine

samples. In optimization studies of the device, the elution volume was varied from 25 to 500 µL to determine its effect on DNA recovery. Although increasing the elution volume increased the total recovery of DNA from each sample, the end result was a more dilute final product. We chose a 50 µL elution volume because it yielded the highest final biomarker concentration.

The DNA biomarkers present in up to 5 mL of urine were captured onto the surface of silica-coated magnetic beads pre-lyophilized with the chaotropic salts necessary for nucleic acid-silica adsorption in the bulb of a 5 mL transfer pipette (**Figure 8A**). This design streamlines the concentration of DNA from large volume samples by eliminating the need for additional sample handling and pipetting steps that were required in our previous reports [21,22]. Following adsorption of nucleic acids to the magnetic beads, the transfer pipette was used to directly introduce the sample into the small diameter tubing without additional pipettors or disposable supplies, an advantage in low resource settings. The transfer pipette bulb was large enough to contain the entire sample and allowed the beads to be easily distributed throughout the volume for maximum nucleic acid adsorption. For this study, the 1 mL sample containing the beads was introduced into the tubing resulting in a 60 cm length. However, this step is not necessary, and the overall tubing length can be shortened to ~15 cm by disposing of the majority of the urine sample and transferring only the magnetic beads into the small diameter tubing.

The lyophilized urine collection pipette appears to have some long-term storage advantages, since lyophilized and freshly prepared reagents within the transfer pipette produce similar results (**Figure 11**). This is partially due to the fact that the adsorption buffer consists only of salt and magnetic beads, which would be expected to have a long shelf-life in an unhydrated state but are easily resuspended. The transfer pipettes were prepared in batches of 50 and stored at room temperature for up to 12 weeks prior to

use. Over the 12 week storage period, there was no reduction in device performance (**Figure 11**). The benefits of long term storage may be advantageous in low resource settings where reliable temperature control is not readily available. Furthermore, the composition of the lyophilized contents of the transfer pipette can be modified to target other biomarkers in additional sample matrices such as blood and saliva.

Although 1 mL urine samples were used for the majority of this study, the capacity of the urine collection pipette allows a maximum of 5 mL of urine to be processed with the magnetic bead-based method. This volume is ten-fold greater than the maximum volume that can be processed using a typical microfluidic extraction device. As shown in **Figure 10**, processing larger volumes of urine containing the same starting concentration of spiked DNA as the 1 mL urine sample yields significantly higher DNA concentrations in the eluate, thus improving the likelihood of detecting any biomarkers of interest. This is especially beneficial in cases, such as Tr-DNA, where the expected concentration of biomarkers has not been well characterized but is thought to be low. Since urine can be easily obtained in these volumes, it would be possible to improve detection simply by increasing the sample volume used.

There is a decrease in DNA concentration in the eluate of the 5 mL urine sample compared to the 3 mL urine sample. Because the binding capacity of the beads limits the number of biomarkers that can be captured within a given sample, it is important to consider this when increasing the urine sample volume. The maximum mass of DNA that can be extracted with the magnetic beads was found to be 5.5 µg DNA/6 x 10^8 beads (**Figure 17**). This is ~20 times more DNA than the 250 ng expected in a 1 mL urine sample, or ~4 times more than the 1.25 µg expected in a 5 mL urine sample [24].

Although there is ample DNA capacity on the beads for a pure DNA sample containing the equivalent amount of DNA as a 5 mL urine sample, the additional PCR inhibitors present in the larger urine sample could be one cause of the decreased

concentration. However, the scalability of the magnetic bead-based extraction would allow us to optimize for a 5 mL sample to take advantage of the increased biomarkers in a larger sample. As shown in **Figure 16**, increasing the number of magnetic beads increases DNA recovery up to a point. Additionally, increasing the volumes of wash solutions to remove additional inhibitors may further improve the final DNA concentration in larger samples. With further optimization and a higher capacity transfer pipette, the magnetic bead-based method could be adapted to extract biomarkers from increasingly large urine volumes as high as 20 mL. This drastic increase in volume may require a longer adsorption step, but the opportunity to capture more biomarkers may be worth the trade-off in total processing time.

Two factors limit the general utility of this approach for detection of disease biomarkers excreted in urine. The first is the availability of biomarkers in urine. Relatively little is known about DNA excreted in urine, but there is evidence that a nucleic acid background of 40-250 ng/mL is normally present [24]. Using gel electrophoresis to visualize Tr-DNA extracted from human urine provided by healthy volunteers, Melkonyan et. al determined that the expected size of these DNA fragments in urine is less than 250 bp, with the majority of the fragments being between 10 and 150 bp [4]. With respect to diagnosis of TB, the use of biomarkers in urine is controversial. Though some studies have successfully detected TB DNA in urine [6,7], others have achieved poor results [5,25,26]. This may be due to lack of standardization in sample collection and processing, secondary infections, medication, or differences in patient populations. Active TB infection is the most likely disease state for detection, since in latent TB the bacteria are sequestered within the lungs [27], and one would expect much lower levels of TB DNA biomarker to be available in the blood and urine. Perhaps the biomarker recovery strategy described here will enable more definitive studies of Tr-DNA as a potential TB diagnostic. Additionally, there are other potential applications for which

our technology could be useful, including the extraction of carbohydrates for the diagnosis of TB using LAM, or the extraction of nucleic acid biomarkers for chlamydia, gonorrhea, and prostate cancer [28-30].

DNA segment length affects the binding properties of the silica-coated beads and is a second factor limiting the diagnostic utility of this approach. We found that sequences as short as 75 bp can be extracted from human urine following adsorption in the transfer pipette bulb, and that the recovery of DNA increases as the sequence length is increased. These results agree with previously reported data that suggest the minimum nucleic acid length that can be recovered using silica adsorption is 60 bp, and that the recovery of nucleic acid is lower near this limit [31]. As demonstrated in **Figure 14**, the recovery of 100 and 140 bp DNA sequences is increased approximately four-fold and six-fold, respectively, compared to a 75 bp sequence. Although the recovery of DNA decreases as the sequence length decreases, the longer sequences tested fall within the expected size range for Tr-DNA as reported by Melkonyan et. al [4].

In patient samples exhibiting expected patient variability, the relationship between TB Tr-DNA measurements and clinical diagnosis remains to be determined. In this study, one large stock of pooled human urine samples was used in an effort to minimize sample-to-sample variability of PCR inhibitors and account for the expected difference in nucleic acid concentration between patient samples. A single concentration of synthetic TB IS6110 biomarker was spiked into each sample to create a controlled surrogate patient sample for the validation of our device. Using this approach, we found that DNA recovery from human urine samples remained constant as the number of copies of spiked DNA increased (**Figure 12**). This is an advantage, because when biomarkers are present in low concentrations, the magnetic bead-based method can still be used to effectively extract a high percentage of the biomarkers. As the concentration of spiked DNA was increased, the sample variance decreased. This was an expected

trend since at low copy numbers the signal is more difficult to distinguish from noise, a characteristic not only of the extraction process, but also of the PCR.

After determining the LOD of the PCR reaction used in this study, we found that 77 copies/µL was the minimum concentration of IS6110 DNA that must be present in a 1 mL human urine sample to be detectable by PCR following extraction with the magnetic bead-based method (**Figure 13)**. This reported value is a function of the PCR protocol, the sample preparation method, and the limited sample-to-sample variability of PCR inhibitors present in the pooled human urine samples. There is currently no clinical data available to determine the expected initial human urine biomarker concentration that would be necessary to utilize Tr-DNA samples for TB diagnosis. We expect that if the use of Tr-DNA for clinical diagnosis becomes standardized in the future, our reported biomarker concentration will be useful in this field of study. We also anticipate that the minimum required biomarker concentration would be different for alternative downstream detection strategies.

One of the attractive features of the magnetic bead-based extraction strategy is that it is amenable to low resource settings. First, urine samples are easy to obtain noninvasively and can usually be obtained in much higher volumes than other samples. The use of urine also circumvents the cultural taboos and cross contamination concerns with using blood samples. Second, because of the simplicity and storage capabilities of the urine collection pipette, the magnetic bead-based extraction does not require refrigeration or special handling, both of which are highly desirable in a low resource setting. Third, the reagent costs are relatively low and are estimated to be less than $1 per extraction. Fourth, a short processing time is essential in low resource settings where patients often require an immediate diagnosis. The rapid DNA adsorption and elution from the magnetic beads minimizes the processing time required to achieve maximum DNA recovery (**Figure 15**), thus reducing the overall time-to-diagnosis. The

magnetic bead-based extraction recovers comparable DNA to the Norgen kit and can be completed nearly 3 times faster. Finally, although PCR was used as the nucleic acid detection method to validate the work done in this study, the extracted nucleic acids are expected to be compatible with a variety of downstream detection strategies that may be better suited for use in low resource settings, such as isothermal PCR. One of the long-term goals of this research is to incorporate an isothermal PCR amplification and detection scheme directly into the small diameter tubing to identify biomarkers-of-interest after extraction.

Because there is no comparable low resource urine extraction technology, we chose to validate our magnetic bead-based extraction by comparing its performance to the commercially available laboratory-based Norgen urine extraction kit. Though it performs well, the Norgen kit requires multiple pipetting and centrifugation steps which make it unsuitable for use in a low resource setting. The magnetic bead-based extraction presented here performs similar to or better than the Norgen kit. However, in the field, the magnetic bead-based extraction would be available as a preloaded cassette containing all necessary processing solutions sealed within the tubing. For each extraction, processing would require only a sample collection step within the urine collection pipette followed by the transfer of magnetic beads down the length of the small diameter tubing using an external magnet.

The magnetic bead-based extraction method effectively captures nucleic acids from large volume urine samples and concentrates them for downstream detection. The urine collection pipette in combination with the pre-arrayed solutions in the small diameter tubing achieves high nucleic acid recovery from easily obtainable clinical human urine samples. This approach, therefore, has potential utility for urine-based diagnosis of active TB in low resource settings and other diagnostic applications found to be associated with 60-250 bp DNA fragments excreted in urine.

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CHAPTER V

AUTOMATED EXTRACTION OF NUCLEIC ACID AND PROTEIN BIOMARKERS USING SURFACE TENSION VALVES

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Abstract

Biomarkers oftentimes must be extracted from patient samples to eliminate inhibitors of downstream detection methods. We present an automated biomarker extraction device based on the transfer of biomarker-loaded magnetic beads through a series of processing solutions arrayed in a length of small-diameter tubing and held in place by surface tension valves. The tubing is coiled around a 12.5 cm diameter plastic cassette, and a motor rotates the cassette past a fixed permanent magnet to transfer magnetic beads down the length of tubing, effectively washing and eluting captured biomarkers. The automated extraction device was validated using surrogate patient samples prepared by the Program for Appropriate Technology in Health (PATH) using clinically relevant concentrations of various pathogens corresponding to a low, medium, and high level of infection. Extracted biomarkers included RNA from influenza A infected swabs; DNA from *Escherichia coli* infected urine, *Mycobacterium tuberculosis* (TB) infected sputum, and malaria infected blood; and protein from malaria infected blood. Quantification was completed post-extraction using qRT-PCR, qPCR, and ELISA, respectively. The lowest concentration of pathogen infection was successfully detected in each sample tested. RNA biomarker recovery was at least 7.3 copies/uL from the influenza A sample. DNA biomarker recovery was at least 405 copies/uL from the *E. coli* samples, 0.22 copies/uL from the TB samples, and 167 copies/uL from the malaria samples. Protein biomarker recovery was at least 2.7 pM from the malaria samples. The automated biomarker extraction device was determined to be effective for extracting both nucleic acid and protein biomarkers from complex biological samples.

Introduction

Biomarkers of infectious diseases are typically found in relatively low abundance compared to the host of human biomolecules present in biological samples. Some of the most sensitive and effective biomarker detection methods (i.e., polymerase chain reaction [PCR] for nucleic acids or enzyme-linked immunosorbent assay [ELISA] for proteins) require biomarkers to be captured and extracted from complex samples prior to amplification and detection. Nucleic acid and protein extraction methods are relatively complex, and generally require an extensive number of user steps to be completed. Therefore, there is demand for automated methods for nucleic acid and protein biomarker extraction. Due to the diverse nature of both patient samples and validated biomarkers, there is also demand for an automated extraction device that can recover more than one type of biomolecular target. However, biomarker capture and isolation procedures are generally highly specialized to individual classes of biomarkers. For example, nucleic acid biomarkers are commonly extracted using a silica-coated solid phase and require chaotropic salts and highly concentrated ethanol solutions for processing, whereas proteins are commonly extracted using an antibody-coated solid phase with buffered saline and detergent processing solutions. Consequently, individual biomarker extraction protocols are not easily adapted into a single automated format for extracting multiple biomarker classes from a variety of sample matrices.

The use of functionalized magnetic beads as a platform for solid phase biomarker extraction has facilitated automated extraction of many biomarker classes because magnetic beads enable simple and flexible handling due to their susceptibility to magnetic manipulation. Various research groups have developed automated biomarker extraction assays based on functionalized magnetic beads. In one approach developed by Sur et al., a simple motorized actuator is used to draw magnetic particles

through a single step extraction process [1]. This device is simple to operate and is sealed from external contaminants, while most manual magnetic bead-based extractions require the user to repeatedly open and close the system to the environment to introduce the biomarker-bound beads to the various processing solutions. Multiple nucleic acid isolations were performed with this device using plasma, whole blood, and urine samples, indicating that the device is adaptable for different sample matrices. The device is limited to running one sample at a time and may not perform well with samples that require more than a single-step processing protocol. In another approach developed by Berry et al. [2], antibodies captured on the surface of functionalized magnetic beads are extracted from a simple buffer solution using an automated sliding magnet configuration. The magnet is positioned under an array of processing solutions separated by oil. This device uses a custom made tray to process multiple samples simultaneously. The format is capable of handling sample sizes ranging from 2 μL to 1 mL. However, this open format may be susceptible to environmental contaminants, and was not tested using complex biological matrices. In a more complex approach, a high-throughput robotic instrument was designed to extract nucleic acids from biological samples in a 96-well plate format [3]. The benefits of this design include minimal user interaction after the initial setup and the instrument's high-throughput capabilities with reduced procedure time. However, this instrument contains complex movable parts for solution handling and magnetic bead manipulation, and the plates remain open to the environment within the instrument. Another disadvantage of this approach is the high initial cost of the robotic workstation. Other automated designs based on magnetic beads have been developed that span a wide range of complexity from microfluidic chips to robotic workstations, but none have yet emerged as a universal biomarker isolation technique [4,5]. Despite the success of these approaches, there is a still need for an

automated system capable of extracting multiple biomarker classes from complex samples in diagnostic laboratories.

In this report, we describe a simple yet effective automated device for multiplexed extraction of nucleic acid or protein biomarkers from complex biological samples. This device was based on our previously developed manual biomarker extraction format, which uses functionalized magnetic beads to extract RNA, DNA, and protein biomarkers within small diameter plastic tubing (1.6 mm i.d.) [6-9]. The tubing is pre-loaded with processing solutions separated by air or mineral oil surface tension valves. In this format, the magnetic beads capture the biomarker of interest and are then pulled through the various processing solutions using an external magnet. We describe the adaptation of this versatile format into an automated device by coiling the extraction tubing around a plastic disk and processing the magnetic beads through the various solutions by turning the disk past a fixed permanent magnet using a motor and programmable controller (**Figure 18**). The result is an automated device suitable for extracting nucleic acid or protein biomarkers from complex biological samples with a simple user interface and rapid extraction time.

Materials and Methods

Materials for device fabrication.

The Arduino UNO Rev3 programmable controller was purchased from Arduino (Cat # A000066). The stepper motor drive controller (Cat # STR4), stepper motor (Cat # HT23-401), and power supply (Cat # PS150A24) were purchased from Applied Motion Products. Cylindrical magnets were purchased from SuperMagnetMan (Cat # Cyl0360 N40).

Design and operation of the automated biomarker extraction device.

The automated biomarker extraction device was designed to transport magnetic beads through a series of processing solutions contained within 1.6 mm i.d. tubing by rotating an extraction cassette (i.e., a plastic disk with extraction tubing wrapped around the circumference) past a fixed magnet using a simple stepper motor (**Figure 18**). A roller-drive design was chosen over an axle-drive design to facilitate the loading of multiple independent cassettes onto the device. In this design, individual cassettes are placed onto a rotating shaft that drives the rotation of the cassette past a second shaft containing fixed magnets.

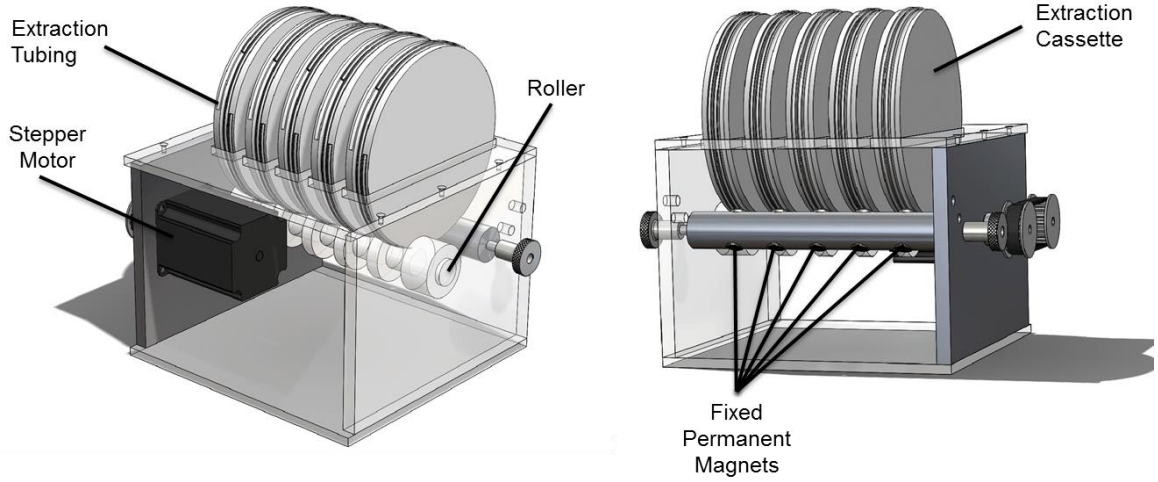

Figure 18. The automated biomarker extraction device is shown. Extraction tubing is wrapped around the outer diameter of each extraction cassette. A stepper motor rotates the roller which drives the rotation of the extraction cassette past an array of fixed permanent magnets for processing.

The Arduino UNO Rev3 programmable controller was used to send logical input commands to the motor controller to regulate the direction, speed, and timing of the drive motor. The motor controller is controlled by the Arduino UNO and modulates the power to the motor from the power supply. The Arduino code was developed with respect to the ratio of the roller drive shaft and extraction cassette diameters, allowing

the operator to simply input desired rotational distance and velocity into the appropriate command line. A device schematic and the default commands used to operate the Arduino UNO controller are provided as supporting information. Two timing gears and a ribbed belt attach the motor to a roller on which the cassettes sit. As the roller rotates, friction between the roller and the cassettes causes the cassettes to rotate past a panel of fixed magnets. The rotation processes magnetic beads through the various solutions within the tubing.

Materials for nucleic acid extraction and detection.

Influenza A, *Esherichia coli* (*E. coli*), *Mycobacterium tuberculosis* (TB), and *Plasmodium falciparum* (malaria) infected sample panels were provided by the Program for Appropriate Technology in Health (PATH). Primers, probes, and reference standard templates were purchased from Integrated DNA Technologies. SuperScript III RT/Platinum TAQ mix was purchased from Life Technologies (Cat # 11732-088). Quanta PerfectA 2x mastermix was purchased from VWR (Quanta Biosciences, Cat # 84010). Dynabeads MyOne Silane magnetic beads (Cat # 37002D) were purchased from Life Technologies. Neodymium magnets were purchased from Emovendo LLC. Filtration swabs (Cat # 5001.02) were purchased from Salimetrics Inc. Ni-NTA Magnetic Agarose Beads (Cat # 36111) were purchased from Qiagen Inc. Antibodies for the *pf*HRP-II ELISA (Cat # ab9206 and ab30384) were purchased from Abcam Inc. 3,3′,5,5′-tetramethylbenzidine (TMB) One solution (Cat # G7431) was purchased from Promega Corporation. Recombinant HRP-II (Cat # AGPF-55) was purchased from Immunology Consultants Laboratory Inc. Hemato-Seal tube sealing compound (Cat # 02-678) was purchased from Fisher Scientific. Fluorinated ethylene propylene (FEP) tubing was purchased from McMaster Carr (Cat # 2129T11 and # 9369T46). Tygon R-3603 tubing

was purchased from Fisher Scientific (Cat # 14-169-1B). All other materials were purchased from Sigma Aldrich or Fisher Scientific.

Optimization of automated extraction cassette processing.

Prior to extraction the extraction tubing was prepared in a 30 cm length of 1.6 mm i.d. FEP tubing according to Bordelon et al [7]. Briefly, the tubing was loaded by pipetting 50 uL nuclease-free water, 300 uL 70% ethanol, and 300 uL DNA precipitation buffer (80% ethanol, 5 mM potassium phosphate, pH 8.0) into one end. Each solution was separated from the next by a 6 mm air valve. A 500 uL human urine sample spiked with 5×10^8 copies of a 140 nucleotide TB IS6110 DNA sequence was combined with 500 uL DNA-silica adsorption buffer (4 M guanidine thiocyanate, 25 mM sodium citrate, pH 7.0) and 6×10^8 silica-coated magnetic beads (20 uL suspension) and placed on a laboratory rotisserie for 5 minutes. Following nucleic acid adsorption, the tube contents were pipetted into the end of the prepared small-diameter tubing and both ends sealed with tube sealing compound. The prepared tubing was processed one of three ways: manually as described by Bordelon and coworkers [7], using a continuous single-speed automated approach, or using a continuous multi-speed automated approach.

For automated processing, the prepared tubing was loaded onto the extraction cassette and placed in the device. During single-speed processing, the extraction device was programmed to rotate the cassette at a single fixed speed to continually pull the beads from one end of the tubing to the other with no mixing. The bead procession rate was set to 5°/second, 10°/second, 15°/second, or 20°/second and the extraction was allowed to proceed until the magnetic beads exited the final chamber. The tubing was removed from the cassette and the elution chamber excised for downstream quantification by PCR using a Rotor-Gene Q thermal cycler.

During multi-speed processing, the extraction device was programmed to rotate the cassette at two alternating speeds. The first was designed to transport the beads down the length of the tubing, and the second to mix the beads within each processing solution. The bead transfer step was set to 10° at a rate of 5°/second, and the bead mixing step was set to 705° at a rate of 150°/second with a 5 second pause to collect the beads before the transfer step was repeated. The extraction was allowed to proceed until the magnetic beads exited the final chamber. The tubing was removed from the cassette and the elution chamber excised for downstream quantification by PCR using a Rotor-Gene Q thermal cycler.

Validated PATH surrogate sample panels.

The efficacy of the automated biomarker extraction device was tested using 5 sets of surrogate patient sample panels prepared and validated by PATH. Each sample panel contained a surrogate patient sample spiked with 3 concentrations of pathogen corresponding to a biologically relevant low, medium, or high level of infection. The sample panels tested are listed in **Table 1**. Each sample panel was received frozen on dry ice and stored at -80°C until needed.

Table 1. Summary of validated PATH surrogate sample panels tested in this study. The sample concentration for Influenza A is provided in approximate expected C_t value for RT-PCR. The concentration is provided in cells/mL for TB and *E. coli* sample panels, and percent parasitemia for malaria samples.

Pathogen	Analyte	Matrix/ Biomarker	Sample Concentration			
			Negative	Low	Medium	High
Influenza A	Virions	Flocked Swab/ RNA	0	35-37 C_t	26-29 C_t	17-21 C_t
TB H37RV	Cells	Sputum/ DNA	0	1 x 10^3 cells/mL	1 x 10^4 cells/mL	1 x 10^5 cells/mL
E. coli	Cells	Urine/ DNA	0	1 x 10^3 cells/mL	1 x 10^4 cells/mL	1 x 10^5 cells/mL
Malaria	Parasite	Blood/ DNA	0	0.001% parasitemia	1% parasitemia	5% parasitemia
Malaria	Parasite	Blood/ Protein	0	0.001% parasitemia	1% parasitemia	5% parasitemia

Extraction of Influenza A RNA from flocked swabs.

Prior to extraction the extraction tubing was prepared in a 15 cm length of 1.6 mm i.d. FEP tubing. The tubing was loaded by pipetting 50 uL nuclease-free water, 300 uL 70% ethanol, and 300 uL RNA precipitation buffer (80% ethanol, 5 mM potassium phosphate, pH 8.0) into one end. Each solution was separated from the next by a 6 mm air valve.

Influenza A virions were eluted from the swab by soaking in 180 uL of sterile PBS for 15 minutes in a 2 mL microcentrifuge tube. The flocked swab was discarded, and the contents of the tube vortexed briefly. The sample was combined with 300 uL RNA-silica adsorption buffer (5.7 M guanidine thiocyanate, 25 mM sodium citrate, 1% 2-mercaptoethanol, pH 7.0) and 6 x 10^8 silica-coated magnetic beads (20 uL suspension) and vortexed thoroughly. After the addition of 300 uL of ethanol, the tube was placed on a laboratory rotisserie for 5 minutes. Following nucleic acid adsorption, the tube contents were pipetted into the end of the preloaded tubing and both ends sealed with tube sealing compound.

The prepared tubing was loaded on the extraction cassette and placed in the device for extraction. The bead transfer step was set to 10° at a rate of 5°/second and the bead mixing step was set to 705° at a rate of 150°/second with a 5 second pause to collect the beads before repeating the transfer step. The extraction was allowed to proceed until the magnetic beads exited the final chamber, resulting in a total assay time of 10 minutes including nucleic acid adsorption. The tubing was removed from the cassette and the elution chamber excised for downstream quantification by RT-PCR using a Qiagen Rotor-Gene Q thermal cycler.

Extraction of E. coli DNA from human urine.

Prior to extraction the extraction tubing was prepared in a 10 cm length of 1.6 mm i.d. FEP tubing. The tubing was loaded by pipetting 50 uL nuclease-free water, 300 uL 70% ethanol, and 300 uL DNA precipitation buffer (80% ethanol, 5 mM potassium phosphate, pH 8.0) into one end. Each solution was separated from the next by a 6 mm air valve.

A 1 mL *E. coli* spiked urine sample was centrifuged for 5 minutes at 10,000 x g in a 1.5 mL microcentrifuge tube and the supernatant was discarded. The *E. coli* cells were lysed by adding 100 uL lysis buffer (50 mM Tris-HCl, 10 mM EDTA, 5% Triton X-100, 100 mM NaCl) and 25 uL lysozyme solution (10 mg/mL). The tube was vortexed briefly and placed on a laboratory rotisserie for 20 minutes. The lysed cells were centrifuged for 5 minutes at 10,000 x g to remove cell debris, and the supernatant was retained for nucleic acid extraction. The sample was combined with 300 uL DNA-silica adsorption buffer (4 M guanidine thiocyanate, 25 mM sodium citrate, pH 7.0) and 6 x 10^8 silica-coated magnetic beads (20 uL suspension) and placed on a laboratory rotisserie for 5 minutes. Following adsorption, the contents of the tube were pipetted into the end of the preloaded tubing and both ends sealed with tube sealing compound.

The prepared tubing was loaded on the extraction cassette and placed in the device for extraction. The bead transfer step was set to 10° at a rate of 5°/second and the bead mixing step was set to 705° at a rate of 150°/second with a 5 second pause to collect the beads before repeating the transfer step. The extraction was allowed to proceed until the magnetic beads exited the final chamber, resulting in a total assay time of 40 minutes including cell lysis and nucleic acid adsorption. The tubing was removed from the cassette and the elution chamber excised for downstream quantification by PCR using a Qiagen Rotor-Gene Q thermal cycler.

Extraction of Mycobacterium tuberculosis DNA from synthetic sputum.

Prior to extraction the extraction tubing was prepared in a 70 cm length of 1.6 mm i.d. FEP tubing. The tubing was loaded by pipetting 50 uL nuclease-free water, 300 uL 70% ethanol, 300 uL RNA precipitation buffer (80% ethanol, 5 mM potassium phosphate, pH 8.0), and 300 uL chaotropic wash buffer (4 M guanidine hydrochloride, 25 mM sodium citrate, pH 7.0) into one end. Each solution was separated from the next by a 6 mm air valve.

A 500 uL TB-infected synthetic sputum sample was combined with TB lysis/binding buffer (4 M GuSCN, 25 mM sodium citrate, 4.9% Triton X-100, 0.2% sodium dodecyl sulfate, pH 7.0) and 6 x 10^8 silica-coated magnetic beads (20 uL suspension) in a 2 mL microcentrifuge tube. The tube was vortexed for 10 minutes on a Fisher Vortex Genie 2 set to speed 4 to lyse the bacterial cells and capture released nucleic acids. Following adsorption, the contents of the tube were pipetted into the end of the preloaded tubing and both ends sealed with tube sealing compound.

The prepared tubing was loaded on the extraction cassette and placed in the device for extraction. The bead transfer step was set to 10° at a rate of 5°/second and the bead mixing step was set to 705° at a rate of 150°/second with a 5 second pause to

collect the beads before repeating the transfer step. The extraction was allowed to proceed until the magnetic beads exited the final chamber, resulting in a total assay time of 20 minutes including cell lysis and nucleic acid adsorption. The tubing was removed from the cassette and the elution chamber excised for downstream quantification by PCR using a Qiagen Rotor-Gene Q thermal cycler.

Extraction of Plasmodium falciparum DNA from human blood culture.

Prior to extraction the extraction tubing was prepared in a 43 cm length of 2.36 mm i.d. FEP tubing. The tubing was loaded by pipetting 50 uL elution buffer (10 mM Tris pH 8.0, 1 mM EDTA, 0.05% Tween-20), 300 uL 70% ethanol, and 300 uL chaotropic wash buffer (80% ethanol, 640 mM GuSCN, 1.6 mM Tris pH 8.0, 160 uM EDTA, 0.08% Triton X-100) into one end. Each solution was separated from the next by a 6 mm air valve.

A 100 uL sample of malaria-infected human blood culture (5% hematocrit) was combined with 300 uL lysis buffer (4 M GuSCN, 10 mM Tris HCl pH 8.0, 1 mM EDTA, 0.5% Triton X-100) and incubated at room temperature for 10 minutes with occasional vortexing to lyse red blood cells. The lysed samples were then filtered using one-fourth of a Salimetrics swab, and the sample was expressed from the swab by using a 5 mL plastic syringe. After the addition of 100 uL of 100% isopropanol and 7.5 x 10^8 silica-coated magnetic beads (25 uL suspension) to the filtered sample, it was incubated for 3 minutes at room temperature. Following nucleic acid adsorption, the tube contents were pipetted into the end of the preloaded tubing and both ends sealed with tube sealing compound.

The prepared tubing was loaded on the extraction cassette and placed in the device for extraction. The bead transfer step was set to 10° at a rate of 4°/second and the bead mixing step was set to 705° at a rate of 80°/second with a 5 second pause to

collect the beads before repeating the transfer step. The extraction was allowed to proceed until the magnetic beads entered the final chamber, at which point the cassette was removed from the extraction device and the beads allowed to elute for 3 minutes. The beads were then removed from the elution chamber using a magnet, and the elution chamber excised for downstream quantification by PCR using the Roche LightCycler 96 thermal cycler. The total assay time was approximately 20 minutes including cell lysis and nucleic acid adsorption.

Extraction of Plasmodium falciparum HRP-II from human blood culture.

Prior to extraction the extraction tubing was prepared in a 24 cm length of 1.6 mm i.d. Tygon R-3603 tubing. The rounded end of a 200 uL PCR tube was cut off, and the top of the cap was punctured with a 27 ½ gauge needle to serve as an air release valve. The modified tube was then inserted into one end of the Tygon tubing. Ten microliters of elution buffer (50 mM potassium phosphate, pH 8.0, 300 mM NaCl, 1 M imidazole, 0.05% Tween-20) was pipetted into the opposite end of the tubing which was then sealed with tube sealing compound. Mineral oil valves (25 uL mineral oil) were added alternately with three 100 uL wash chambers (50 mM phosphate buffer, pH 8.0, 300 mM NaCl, 125 mM imidazole, 0.05% Tween-20) down the length of the tubing using a 27 ½ gauge syringe. Finally, mineral oil was added between the final wash chamber and inserted PCR tube to seal off the tubing to air.

A 100 uL sample of infected blood culture (5% hematocrit) was lysed with 100 uL of lysis buffer (100 mM potassium phosphate pH 8.0, 600 mM NaCl, 250 mM imidazole, 2% Triton X-100) in a 1.5 mL microcentrifuge tube. Following lysis, the sample was filtered through a 1 mL syringe fitted with glass wool to removed lysed cell debris. The filtered sample was mixed with 1.5×10^3 magnetic Ni(II)NTA agarose beads (10 uL suspension) in the modified PCR tube, which was placed on a laboratory rotisserie for

10 minutes to ensure mixing of the beads throughout the sample. After the incubation period, the PCR tube was reinserted into the extraction tubing and the beads were pulled into the adjacent mineral oil valve using a donut-shaped neodymium magnet. Finally, the PCR tube was removed and the extraction tubing sealed with tube sealing compound.

The prepared tubing was loaded on the extraction cassette and placed in the device for extraction. The bead procession step was set to 10° at a rate of 3°/second and the bead mixing step was set to 695° at a rate of 60°/second with a 5 second pause to collect the beads. The extraction was allowed to proceed until the magnetic beads exited the final chamber, resulting in a total assay time of 40 minutes including cell lysis and nucleic acid adsorption. The tubing was removed from the cassette and the elution chamber excised for downstream quantification by ELISA.

Nucleic acid quantification by PCR and RT-PCR.

Nucleic acids extracted from panel samples were quantified by RT-PCR or PCR as appropriate according to protocols adapted by PATH from existing studies [10-13]. The primer/probe sequences and cycling conditions for each individual nucleic acid biomarker are provided in **Table 2**. Influenza A RNA was quantified via RT-PCR using the SuperScript III RT/Platinum Taq mix in 25 uL reaction volumes with a final primer concentration of 800 nM and a final probe concentration of 150 nM. DNA from *E. coli*, TB, and malaria was quantified via PCR using Quanta PerfectA 2x mastermix in 25 uL reaction volumes. Final primer concentrations were 800 nM, 300 nM, and 800 nM for *E. coli,* TB, and malaria, respectively. The final probe concentration was 150 nM for all reactions. All thermal cycling was performed using the Qiagen Rotor-Gene Q or Roche LightCycler 96 thermal cycler as noted above. Rotor-Gene Q or LightCycler software

was used to determine C_t values, and nucleic acids were quantified by referencing a standard curve.

Protein quantification by ELISA.

Extracted *pf*HRP-II protein was quantified by ELISA in 96-well Immulon 2 HB plates according to Davis et. al [9]. Once the plate was developed, absorbance was quantified at 450 nm using a BioTek Synergy H4 microplate reader, and the protein was quantified by referencing a standard curve.

Table 2. The primer and probe sequences and thermal cycling conditions used for the quantification of nucleic acids extracted from validated panel samples are shown.

Influenza A – RT-PCR [10]	Forward Primer (5'→3')	GACCRATCCTGTCACCTCTGAC
	Reverse Primer (5'→3')	AGGGCATTYTGGACAAAKCGTCTA
	Probe (5'→3')	FAM-TGCAGTCCTCGCTCACTGGGCACG-BHQ1
	Cycling Conditions	50°C – 30 minutes 95°C – 2 minutes 45 cycles of: 95°C – 15 seconds 55°C – 30 seconds
TB H37RV – PCR [11]	Forward Primer (5'→3')	GGGTAGCAGACCTCACCTATG
	Reverse Primer (5'→3')	AGCGTAGGCGTCGGTGA
	Probe (5'→3')	FAM-TCGCCTACGTGGCCTTT-MGBQ
	Cycling Conditions	95°C – 3 minutes 45 cycles of: 95°C – 15 seconds 60°C – 60 seconds
***E. coli* – PCR [12]**	Forward Primer (5'→3')	GCAAACTGATGTCTTCCGCAC
	Reverse Primer (5'→3')	CGAGCGGAACAATCAGCAT
	Probe (5'→3')	FAM-TCAGCTGATCAACACCGTTCACGTTGA-TAMRA
	Cycling Conditions	95°C – 3 minutes 45 cycles of: 95°C – 30 seconds 56°C – 60 seconds
Malaria – PCR [13]	Forward Primer (5'→3')	ACATGGCTATGACGGGTAACG
	Reverse Primer (5'→3')	TGCCTTCCTTAGATGTGGTAGCTA
	Probe (5'→3')	FAM-TCAGGCTCCCTCTCCGGAATCGA-BHQ1
	Cycling Conditions	95°C – 3 minutes 45 cycles of: 95°C – 30 seconds 56°C – 60 seconds

Results and Discussion

In this report, we describe the development of an automated device capable of extracting nucleic acid and protein biomarkers from complex samples. The device was designed to move biomarker-bound magnetic beads through a series of processing solutions separated by surface tension valves within 1.6 mm i.d. plastic tubing by rotating the tubing passed a fixed permanent magnet (**Figure 18**). This device features fewer user steps, increased throughput, and enhanced reproducibility when compared to the manual extraction developed in our laboratory [6,7,9]

One significant challenge to automating the process was reproducing the magnetic bead washing steps required during each extraction. This problem was approached with three alternative solutions to determine the optimal extraction cassette processing to maximize biomarker recovery. The first automated program tested was a simple procession of the beads from one end of the tubing to another without dispersing the beads throughout the solution. Using this approach, recovery of a spiked DNA biomarker decreased linearly with increasing cassette rotation speed (**Figure 19A**). The maximum biomarker recovery was $2 \pm 0.09 \times 10^5$ DNA copies, but the recovery was significantly lower than for a manual extraction which recovered $1.2 \pm 0.5 \times 10^7$ copies (**Figure 19B**), indicating that the single-speed extraction process was relatively ineffective due to the lack of bead dispersion in each processing solution.

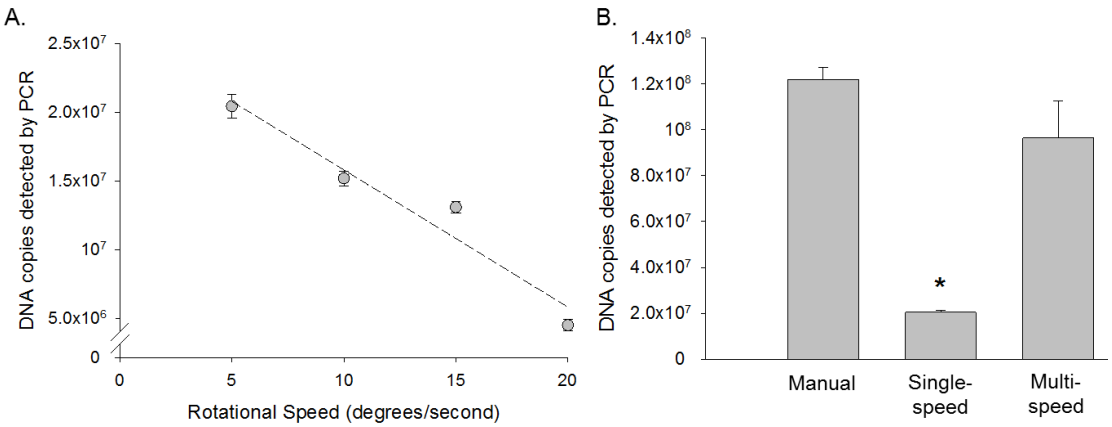

Figure 19. A. DNA biomarker recovery decreases linearly with increasing rotational motor speed when the single-speed extraction process is utilized (n=3, mean ± s.d.). **B.** DNA biomarker recovery is significantly higher using a manual magnetic bead process and a multi-speed automated process compared to the continuous single-speed automated extraction (n=3, mean ± s.d.). * denotes significantly lower DNA recovery compared to manual extraction.

To incorporate mixing steps into the automated program, a more complicated solution was a position-dependent program that repeatedly reversed the motor direction to disperse the beads throughout each chamber. This method depended on precise extraction tubing preparation, and large quantities of magnetic beads were left behind with the slightest deviations in solution volume or position. Additionally, this position-dependent alternative required vastly different programming for each biomarker tested, and was not tolerant of variations in sample volume for a single biomarker. This approach was quickly abandoned in favor of the final method.

Finally, we tested a position-agnostic program based on continuous-direction multi-speed processing. In this approach, the motor spins slowly to move the beads forward ~10° then spins rapidly in the same direction for two complete turns. During the rapid spin, the beads are not entrained by the magnet and become dispersed. The beads are then collected with a short pause step, followed by an additional slow spin. The process repeats until the beads reach the elution chamber. Using this approach, DNA biomarker recovery was 9 ± 2 x 10^7 copies, and was comparable to the manual

extraction process (**Figure 19B**). Variations in tubing contents, sample and wash buffer volumes, and surface tension valve lengths had no effect on biomarker recovery using this position-agnostic multi-speed processing method. The consistently alternating speeds utilized in this program operate independently of extraction cassette alignment, allowing individual extraction cassettes to be added or removed from the device without disrupting the continuous roller. One advantage of this design is that multiplex extractions are enabled.

The automated biomarker extraction device performed well on the sample panels tested, recovering detectable quantities of target biomarker for all high, medium, and low levels of infection. In each sample panel tested there is a decrease in extracted biomarker concentration from high to low infection levels. This was the expected trend because the starting pathogen concentration also decreased significantly from high to low infectivity (**Table 1**). The concentration of recovered RNA from influenza infected swabs was $1.1 \pm 0.6 \times 10^5$, $2.3 \pm 0.4 \times 10^3$, and 7.3 ± 2 copies/uL for high, medium, and low levels of infection, respectively (**Figure 20A**). Approximately $1.8 \pm 0.6 \times 10^4$, $2.5 \pm 0.7 \times 10^3$, and 405 ± 48 copies/uL of DNA biomarkers were detected in high, medium, and low *E. coli* infected urine (**Figure 20B**), 463 ± 472, 69.2 ± 84, and 0.22 ± 0.3 copies/uL in high, medium, and low TB infected sputum (**Figure 20C**), and $1.6 \pm 2 \times 10^6$, $1.2 \pm 1.5 \times 10^4$, and 167 ± 129 copies/uL in high, medium, and low malaria infected blood (**Figure 20D**). Finally, $7.2 \pm 2.7 \times 10^3$, 512 ± 74, and 2.7 ± 1.9 pM HRPII protein biomarker was detected in high, medium, and low malaria infected blood (**Figure 20E**).

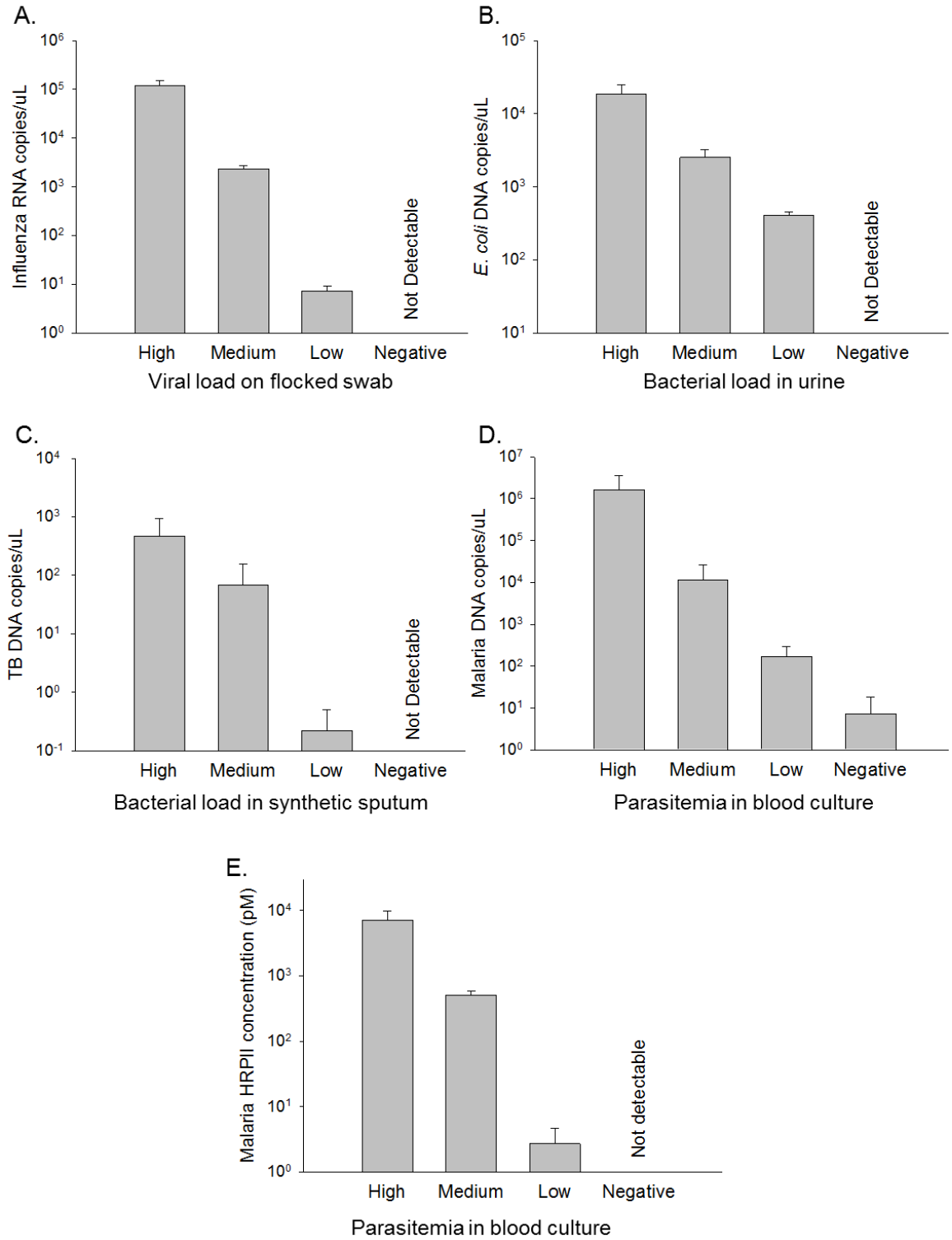

Figure 20. Target biomarkers are detected at biologically relevant high, medium, and low levels of infection for all processed sample panels. **A.** RNA biomarker was detected by RT-PCR following extraction of influenza A infected flocked swabs (n=3, mean ± s.d.). DNA biomarkers were detected by PCR following extraction of **B.** *E. coli* infected human urine (n=3, mean ± s.d.), **C.** TB infected synthetic sputum (n=3, mean ± s.d.), and **D.** malaria infected human blood culture (n=9, mean ± s.d.). **E.** Protein biomarker was detected by ELISA following extraction of *pf*HRPII from malaria infected human blood culture (n=5, mean ± s.d.).

One major hallmark of the automated biomarker extraction device is the flexibility to extract multiple classes of biomarkers using the same device. In this study, the extraction of RNA, DNA, and protein biomarkers is demonstrated, representing a significant advantage over less flexible alternative extraction methods. Theoretically, the automated biomarker extraction device could be used to recover any additional biomarker classes that could be captured on a surface-functionalized magnetic bead, such as whole cells.

In addition to its efficacy, the automated biomarker extraction device was also relatively rapid. Each patient sample surrogate required less than 10 minutes for magnetic bead processing, and total extraction time ranged from 15 to 40 minutes including cell lysis and biomarker capture steps. Additionally, the potential multiplex capabilities of the biomarker extraction device would further increase extraction throughput. Biomarker extraction speed is an important consideration in laboratory sample preparation, particularly when relatively long detection schemes such as PCR and ELISA are utilized.

Conclusions

In conclusion, we successfully developed an automated biomarker extraction device that isolated multiple biomarker classes (RNA, DNA, and protein) from complex surrogate patient samples. The automated format was demonstrated to reduce user processing steps and increase extraction throughput without requiring complex robotics or fluid pumping. Biomarker recovery using the automated device was comparable to the more complex and involved manual extraction. The continuous roller format utilized in this device allows multiple cassettes to be processed simultaneously, and cassettes may be added to or removed from the device without interrupting the processing of other

cassettes. This automated device has the potential to simplify and shorten sample preparation for multiple biomarker classes in diagnostic settings.

Supporting Information

Automated biomarker extraction processor Arduino code

```
int i=0;  // variables to keep count for while loop
int y=0;

//Test Requirements
int rot_1 = 705; //desired rotation for phase 1 (degrees)
int rot_2 = 15; //desired rotation for phase 2 (degrees)
int spd_1 = 150; //desired rotational velocity for phase 1 (deg/sec)
int spd_2 = 5; //desired rotational velocity for phase 2 (deg/sec)
int phase_gap = 5000; //desired delay time between phases (milliseconds)

//Device Specifications
double roller_dia = 0.75; //roller diameter in inches

//Motor Specifications
double step_deg = 0.18; //degrees per step

//Cartridge Specifications
double cart_dia = 5.5; //cartridge diameter in inches

//Calculated Constants
double ratio_eff_gear = cart_dia/roller_dia;
int step_1 = rot_1/step_deg*ratio_eff_gear;
int step_2 = rot_2/step_deg*ratio_eff_gear;
int delay_1 = step_deg/spd_1*1000000/ratio_eff_gear;
int delay_2 = step_deg/spd_2*1000000/ratio_eff_gear;

void setup() {
  pinMode(12, OUTPUT);
}
void loop() {
  while(i < step_1){
  digitalWrite(12, HIGH);   // set the PIN on
  delayMicroseconds(delay_1);   // delay between steps
  digitalWrite(12, LOW);    // set the PIN off
  i++;
  }
  delay(phase_gap);
   while(y < step_2){
  digitalWrite(12, HIGH);   // set the Pin on
  delayMicroseconds(delay_2);    // delay between steps
  digitalWrite(12, LOW);    // set the PIN off
  y++;
  }
  i=0;
  y=0;
  }
```

Automated biomarker extraction processor wiring diagram

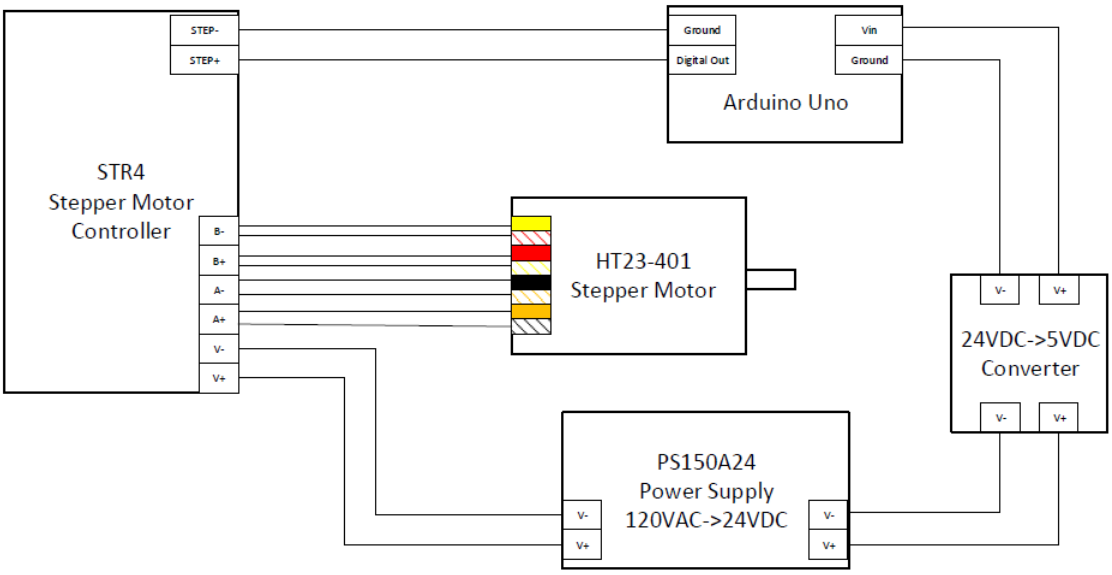

Figure S1. This schematic depicts the connections between individual automated biomarker extraction device components.

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CHAPTER VI

THE EFFECTS OF MAGNETIC BEAD SURFACE FUNCTIONALIZATION ON NUCLEIC ACID EXTRACTION AND DETECTION

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Abstract

Magnetic beads are convenient for extracting nucleic acids from samples preparatory to downstream molecular detection. However, the appropriate bead surface functionality for each situation is not always apparent. Factors such as the desired level of assay simplicity and speed, biomarker specificity, and molecular detection strategy must be considered when choosing the ideal surface functionalization. The advantages and disadvantages of three magnetic bead surface chemistries – silica-coated, oligo (dT) and RSV sequence-specific – were evaluated. Silica-coated and oligo (dT) beads recovered RNA with ~70% efficiency in less than 10 minutes. Silica-coated beads recovered nucleic acids independent of sequence while oligo (dT) beads more selectively recovered mRNA. RSV-specific beads mainly recovered RSV mRNA, but required an incubation time of 3 hours to reach 70% recovery. Following a 10 minute incubation, the RSV-specific beads recovered ~7% of RSV mRNA. Silica-coated bead are well-suited for applications that require nucleic acids other than mRNA, especially when background nucleic acid-tolerant detection such as PCR is to be used. Oligo (dT) beads are ideal when mRNA is to be used as a biomarker. They are rapid and efficient enough to work well with PCR but also enable detection strategies performed directly on nucleic acids hybridized to the bead surface. RSV-specific beads may be too selective to be useful for detection strategies such as PCR. They may be appropriate for detection schemes performed directly on captured sequences that are affected by high concentrations of background nucleic acid.

Introduction

Molecular detection of nucleic acids is an ideal diagnostic modality because of its inherent specificity, sensitivity and rapid time-to-diagnosis. In patient samples, high concentrations of contaminants inhibit molecular detection. Therefore, nucleic acids must first be extracted into a contaminant-free buffer to be detectable [1,2]. Nucleic acid capture using surface functionalized magnetic beads is one widely utilized technique for purifying nucleic acid biomarkers from patient samples. Because magnetic beads can be easily manipulated with a magnetic field, they are a useful alternative to nucleic acid extraction using phase separation or centrifugation columns. Once nucleic acids are captured onto the magnetic bead surface, the beads can quickly and easily be washed and nucleic acids eluted to complete the extraction. For this reason, magnetic bead-based purification is readily adaptable to a variety of formats, including manual extraction performed on a magnetic rack [3], microfluidic chips [4,5] and other self-contained formats [6-8], as well as automated robotic processing [9,10].

The class of nucleic acid biomarkers captured is dependent on the magnetic bead surface functionalization. For example, a silica-coated surface nonspecifically recovers both DNA and RNA, while a DNA probe-coated surface recovers only complementary nucleic acids that hybridize to the probes. Prior to extracting nucleic acids from a sample, a choice must be made as to what surface chemistry will best suit both the desired biomarker and downstream detection modality.

In many cases, the desired biomarkers can be extracted using a variety of surface functionalities. PCR and RT-PCR are tolerant of the high levels of background nucleic acids extracted using silica-coated magnetic particles [3,6,7]. PCR is also used following extraction with oligo (dT) functionalized beads which selectively recover mRNA [11,12] or with sequence-specific beads which recover complementary nucleic acid sequences [13,14]. In many of these cases, it is not always clear why a particular

surface functionality was chosen, because when using PCR, total nucleic acid extraction is likely sufficient to achieve the desired end result.

Conversely, some downstream detection schemes are dependent on the capture of particular nucleic acid sequences or subsets and benefit from a reduction in nonspecific background nucleic acids. Many of these assays rely on hybridization to captured nucleic acids. For example, following extraction with oligo (dT) magnetic beads, captured mRNA has been detected on the bead surface using the isothermal amplification strategy quadruplex priming amplification [15]. The bio-barcode assay developed by Nam et. al. relies on the detection of specific nucleic acid sequences by hybridizing complementary DNA-coated gold nanoparticles to captured nucleic acids [16,17]. Alternatively, when extracting with silica-coated magnetic beads, nucleic acids are precipitated onto the bead surface and unavailable for hybridization. In these applications, choosing the appropriate magnetic bead surface functionality is essential to obtaining the desired results.

In this manuscript we have identified the advantages and disadvantages of three magnetic bead surface functionalities (silica, oligo (dT) and sequence-specific) that extract increasingly specific classes of nucleic acids to enable downstream detection.

Materials and Methods

RSV N gene detection by RT-PCR.

A 277-bp fragment of the RSV N gene was amplified using forward primer 5'-GGAACAAGTTGTTGAGGTTTATGAATATGC-3' and reverse primer 5'-CTTCTGCTGTCAAGTCTAGTACACTGTAGT-3' [18]. PCR reactions were performed in a 20 µL volume using 5µL of RNA template and the Power SYBR Green RNA-to-C_T 1-Step Kit (Applied Biosystems, Grand Island, NY) according to manufacturer's

instructions. Thermal cycling consisted of 48°C for 30 minutes to synthesize cDNA, 95°C for 10 minutes to inactivate the reverse transcriptase and activate QTaq DNA polymerase, and 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds using a Rotor-Gene Q thermal cycler (Qiagen, Germantown, MD). Product specificity was confirmed using melting curve analysis. Data were collected and C_t values recorded by Rotor-Gene Q Software (Qiagen, Germantown, MD) and converted to number of copies of RNA per µL using a standard curve.

Preparation of RSV-specific functionalized magnetic beads.

Dynabeads M-270 Amine magnetic beads (Life Technologies, Grand Island, NY) were functionalized with a 22 base DNA probe specific for the N gene of respiratory syncytial virus (RSV) according to the protocol developed by Hill, et. al. [16]. The thiolated DNA probe consisted of a 9 base A linker sequence followed by 22 bases complementary to the RSV N gene as follows: 5' - /5ThioMC6-D/AAA AAA AAA ATC ATG TAA AAG CAA ATG GAG TA - 3'. The probe sequence was attached to the amine-coated beads by SMCC coupling according to Hill, et. al.

Characterization of RSV-specific and oligo (dT) magnetic beads using fluorescently labeled complementary and scrambled DNA sequences.

A study comparing recovery of complementary and scrambled fluorescent probes was performed to determine whether the laboratory-synthesized RSV-specific beads were comparable to the commercially-available Dynabeads Oligo(dT) magnetic beads (Life Technologies, Grand Island, NY). Twenty-two base DNA sequences complementary to the probes on each bead were purchased with a 5' Cy5 dye to facilitate quantification. In addition, a scrambled control probe was purchased to confirm

that probe hybridization was sequence-specific. The three probe sequences are shown in **Table 3**.

Table 3. Fluorescent DNA probes for determining magnetic bead hybridization specificity of oligo (dT) and RSV-specific magnetic beads.

Probe Name	Sequence
Oligo (dT) complementary probe	5' Cy5 – AAA AAA AAA AAA AAA AAA AAA A 3'
RSV N gene complementary probe	5' Cy5 – TAC TCC ATT TGC TTT TAC ATG A 3'
Scrambled control probe	5' HEX – AGA TGA GAG AAT AGT TTC CAA A 3'

Extraction of complementary probes was adapted from the manufacturer's protocol for mRNA extraction provided with the oligo (dT) beads. To compare fluorescent probe recovery, 5 x 10^8 oligo (dT) or RSV-specific beads were washed with 500 uL of lysis/hybridization buffer (100 mM Tris-HCl, pH 7.5, 500 mM LiCl, 10 mM EDTA, 1% LiDS, 5 mM dithiolthreitol (DTT)) in a 1.5 mL centrifuge tube. The wash was removed while the beads were held to one side of the tube with a magnet. For hybridization, 300 uL of lysis/hybridization buffer was added to the beads along with 400 ng complementary probe and 400 ng scrambled control probe. The beads were then placed on a laboratory rotisserie for 45 minutes to enable fluorescent probe hybridization. Following hybridization, the beads were washed once using 300 uL wash buffer A (10 mM Tris-HCl, pH 7.5, 0.15 M LiCl, 1 mM EDTA, 0.1% LiDS) and once with 300 uL wash buffer B (10 mM Tris-HCl, pH 7.5, 0.15 M LiCl, 1 mM EDTA) according to the manufacturer's instructions. Finally, 50 uL of nuclease-free water was added to the beads, and the hybridized probes were released by heating each tube to 65ºC for 2 minutes. The eluate was collected while the beads were held to one side of the tube with a magnet.

Fluorescent probe recovery was quantified by measuring the fluorescence of each eluate on a NanoDrop 3300 Fluorospectrometer (Thermo Scientific, Inc., Wilmington, DE). The samples were excited using a white light source, and the emission was collected at 665 nm for Cy5 and 556 nm for HEX. The relative fluorescence was obtained for each eluate and converted to concentration in uM using a standard curve. Finally, the hybridization efficiency was calculated by dividing the total number of extracted probes by the total number of input probes and multiplying by 100%.

Preparation of RSV N gene-spiked HEp-2 cell lysates.

HEp-2 cell lysates were prepared from a confluent monolayer of HEp-2 cells grown in a T-150 flask. The cells were harvested by scraping from the flask and centrifuging at 500 x g for 5 minutes. The cell pellet was resuspended into 8 mL denaturing solution (4 M guanidinium thiocyanate, 25 mM sodium citrate [pH 7.0] 0.5% N-laurosylsarcosine [Sarkosyl], 0.1 M 2-mercaptoethanol) and passed through a pipette tip 10 times. The cell lysates were stored at a concentration of approximately $3x10^6$ lysed cells per mL in 1 mL aliquots at -80°C until ready for use. Full length RSV N gene mRNA sequences were synthesized by Bio-Synthesis, Inc. (Lewisville, TX). Spiked HEp-2 cell lysates were prepared by adding 5 uL of RSV N gene mRNA (1×10^6 copies/uL) to 20 uL of HEp-2 cell lysate. The resulting 25 uL samples were used immediately following preparation.

Optimization of magnetic capture hybridization protocol for oligo (dT) and RSV-specific magnetic beads.

The effect of eliminating magnetic bead wash steps from the oligo (dT) and RSV-specific magnetic bead extractions was determined in an effort to further simplify the

extraction procedure. Because the oligo (dT) and RSV-specific beads performed similarly when tested with complimentary nucleic acid biomarkers as described above, the optimization was performed only with oligo (dT) beads. 5 x 10^8 oligo (dT) beads were washed with 500 uL of lysis/hybridization buffer in a 1.5 mL centrifuge tube. After washing, nucleic acid capture was performed by adding 25 uL of RSV N gene-spiked HEp-2 cell lysates with 300 uL of lysis/hybridization buffer to the washed beads and placing on a laboratory rotisserie for 5 min. Following nucleic acid capture, the lysis/hybridization buffer was removed by pipetting while holding the magnetic beads to one side of the tube with a magnet. The beads were then washed according to the manufacturer's protocol with 300 uL of wash buffer A followed by 300 uL of wash buffer B. Alternatively, both wash steps were omitted from the extraction. Finally, 50 uL of nuclease-free water was added to the beads, and the hybridized probes were released by heating the tube to 65°C for 2 minutes. The eluate was collected, and extracted RSV N gene mRNA was quantified by RT-PCR as described above.

RNA extraction capacity of silica-coated, oligo (dT), and RSV-specific magnetic beads.

To ensure the silica-coated, oligo (dT), and RSV-specific beads were compared equally, the RNA extraction capacity was determined for each bead type. In all subsequent experiments, the extraction capacity was equivalent for each bead surface functionality.

The maximum mass of background RNA that could be extracted using the silica-coated magnetic beads was determined by spiking 20 uL of TE buffer with increasing quantities of mouse universal RNA (SABiosciences, Valencia, CA) and quantifying the mass of RNA extracted. Twenty microliters of TE buffer was spiked with 200, 400, 600, 800, or 1000 ng of mouse RNA. The samples were combined with 300 uL of RNA-silica adsorption buffer (5.7 M guanidine thiocyanate (GuSCN), 25 mM sodium citrate, 1% 2-

mercaptoethanol) and 1 x 10^8 Dynabeads MyOne Silane magnetic beads (Life Technologies) and vortexed thoroughly. Three hundred microliters of ethanol was added, and the sample was placed on a laboratory rotisserie for 5 minutes to enable RNA adsorption. Following adsorption, the solution was removed by holding the beads to one side of the tube with a magnet and pipetting out the liquid. The beads were then washed in 300 uL precipitation buffer (80% ethanol, 5 mM potassium phosphate, pH 8.0) followed by a 300 uL 70% ethanol wash. Finally, 50 uL of nuclease-free water was added to the beads to elute captured nucleic acids. The beads were vortexed for 15 seconds, and the eluate was collected. The extracted RNA was quantified by measuring absorbance at 260 nm using a NanoDrop ND-1000 spectrophotometer and referencing a standard curve.

The maximum mass of complementary RNA that could be extracted using the oligo (dT) and RSV-specific magnetic beads was determined by spiking 20 uL of TE buffer with increasing quantities of full length RSV N gene mRNA and quantifying the mass of mRNA extracted. Twenty microliters of TE buffer was spiked with 200, 400, 600, 800, or 1000 ng of mouse RNA. The samples were combined with 300 uL of lysis/hybridization buffer and 1 x 10^8 oligo (dT) or RSV-specific magnetic beads in a 1.5 mL centrifuge tube, vortexed thoroughly, and placed on a laboratory rotisserie for 5 minutes. Following hybridization, the solution was removed by holding the beads to one side of the tube with a magnet and pipetting out the liquid. Finally, 50 uL of nuclease-free water was added to the beads, and they were heated to 65°C for 2 minutes to elute captured mRNA. The extracted RNA was quantified by measuring absorbance at 260 nm using a NanoDrop ND-1000 spectrophotometer and referencing a standard curve.

RNA capture kinetics using silica-coated magnetic beads.

The optimal adsorption time for RNA to silica-coated magnetic beads was determined for extraction of RSV N gene-spiked HEp-2 cell lysates following quantification using RT-PCR. Three hundred microliters of RNA-silica binding buffer and 5 x 10^8 silica-coated magnetic beads were added to 25 uL of RSV spiked HEp-2 cell lysates and vortexed thoroughly. Three hundred microliters of ethanol was added, and the sample was placed on a laboratory rotisserie for 1, 3, 5, 30, 60, 120, or 180 minutes. Following adsorption, the solution was removed by holding the beads to one side of the tube with a magnet and pipetting out the liquid. The beads were then washed in 300 uL precipitation buffer followed by a 300 uL 70% ethanol wash. Finally, 50 uL of nuclease-free water was added to the beads to elute any captured nucleic acids. The beads were vortexed for 15 seconds, and the eluate was collected. Extracted RSV N gene RNA was quantified by RT-PCR as described above.

RNA capture kinetics using oligo (dT) and RSV-specific beads.

The optimal hybridization time for RNA to oligo (dT) and RSV-specific magnetic beads was determined following extraction of RSV N gene-spiked HEp-2 cell lysates and quantification using RT-PCR. For both bead surface functionalities, 6.7 x 10^8 magnetic beads were washed in 500 uL of lysis/hybridization buffer. For hybridization, 300 uL of lysis/hybridization buffer was added to the beads along with 25 uL of N gene-spiked HEp-2 cell lysates. The beads were then placed on a laboratory rotisserie for 1, 3, 5, 30, 60, 120, or 180 minutes. Following hybridization, the solution was removed by holding the beads to one side of the tube with a magnet and pipetting out the liquid. Fifty microliters of nuclease-free water was added to the beads, and the hybridized probes were released by heating the centrifuge tube to 65°C for 2 minutes. The eluate was

collected, and extracted RSV N gene mRNA was quantified by RT-PCR as described above.

Ratio of RSV biomarkers to background RNA following extraction with silica-coated, oligo (dT), and RSV-specific beads.

The quantity of extracted RSV mRNA was compared to a background of tRNA and mRNA for extraction with the silica-coated, oligo (dT), and RSV-specific beads. A mixed RNA sample was prepared by combining 1000 ng background RNA (yeast tRNA, Ambion), 30 ng background mRNA (Influenza A matrix protein gene mRNA synthetically prepared by Bio-Synthesis), and 0.003 ng full length RSV N gene mRNA. This ratio of background nucleic acid, background mRNA, and viral RNA is approximately equivalent to what would be expected in a population of RSV infected cells collected from a patient [19]. The RNA sample was extracted using the methods described above for the silica-coated, oligo (dT), and RSV-specific beads. Extracted RSV N gene mRNA was quantified following RT-PCR as described above. The presence of extracted background DNA was confirmed by measuring the eluate absorbance at 260 nm on a NanoDrop ND-1000 spectrophotometer and comparing the absorbance to a blank sample containing no RNA.

Background mRNA was quantified by amplifying a 106 bp region of the influenza A matrix protein gene using forward primer 5' GACCRATCCTGTCACCTCTGAC 3' and reverse primer 5' AGGGCATTYTGGACAAAKCGTCTA 3' [20]. Twenty-five microliter RT-PCR reactions were prepared using the Power SYBR Green RNA-to-C_T 1-step kit, and cycling conditions were as follows: 30 minutes at 48^0C to synthesize cDNA, 95°C for 10 minutes to inactivate the reverse transcriptase and activate QTaq DNA polymerase, and 40 cycles of 95°C for 15 seconds and 55°C for 60 seconds using a Rotor-Gene Q thermal cycler (Qiagen, Germantown, MD). Product specificity was

confirmed using melting curve analysis. Data were collected and C_t values recorded by Rotor-Gene Q Software (Qiagen, Germantown, MD) and converted to number of copies of RNA per µL using a standard curve.

Self-contained RSV RNA extraction using silica-coated magnetic beads.

The preparation of the self-contained extraction tubing was adapted from our RNA extraction cassette [6]. A 15 cm section of 2.36 mm inner diameter (i.d.) fluorinated ethylene propylene (FEP) tubing (McMaster Carr, catalog # 9369T24) was prepared by pipetting 50 uL of nuclease-free water, 300 uL of 70% ethanol, and 300 uL of RNA precipitation buffer into one end of the tubing. Each solution was separated by pipetting a 6 mm long air valve between each subsequent addition. A 1.5 mL centrifuge tube was prepared with 25 uL of RSV RNA spiked HEp-2 cell lysate, 300 uL of RNA-silica binding buffer, 5×10^8 silica-coated magnetic beads, and 300 uL of ethanol as described above. The tube was placed on a laboratory rotisserie for 5 min. to enable nucleic acid adsorption. Following nucleic acid capture, the contents of the tube were pipetted into the 15 cm piece of tubing, and both ends of the tubing were capped with clay tube sealant (**Figure 21A**). A 2.5 cm cubic neodymium magnet was used to gather the magnetic beads from the RNA-silica binding buffer and pull them through the first air valve into the RNA precipitation buffer. The magnet was then moved rapidly back and forth down the length of the RNA precipitation chamber to disperse the magnetic beads. Beads were collected and pulled through the next air valve into the RNA wash chamber, where they were dispersed by again moving the magnet back and forth. Finally, beads were collected, pulled into the water chamber and dispersed for 10 seconds to elute captured nucleic acids. The beads were then collected and removed from the water chamber. A razor blade was used to excise the water chamber from the remainder of the tubing, and the eluate was collected by holding the excised tubing section over a 0.5

mL centrifuge tube. Extracted RSV mRNA was quantified by RT-PCR as described above.

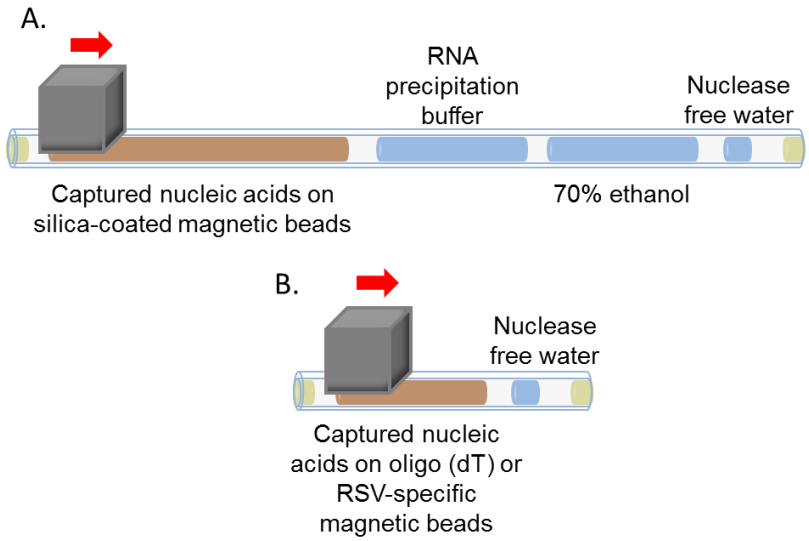

Figure 21. A) Total nucleic acids were captured on silica-coated magnetic beads and extracted by pulling the beads through a series of processing solutions using a permanent magnet. **B)** Sequence-specific nucleic acids were captured on oligo (dT) or RSV-specific beads and extracted by pulling the beads directly into nuclease-free water to elute.

Self-contained RSV RNA extraction using oligo (dT) and RSV-specific beads.

A 10 cm length of 2.36 mm i.d. FEP tubing was prepared by pipetting 50 uL of nuclease-free water into one end. A 6 mm air valve was added to the tubing adjacent to the water chamber. A 1.5 mL centrifuge tube was prepared by washing 6.7 x 10^8 oligo (dT) or 6.7 x 10^8 RSV-specific beads with 500 uL of lysis/hybridization buffer. The wash buffer was removed, and 300 uL of lysis/hybridization buffer and 25 uL of RSV mRNA spiked HEp-2 cell lysates were added to the washed beads. The samples were placed on a laboratory rotisserie for 5 minutes to capture any complementary nucleic acids present in the sample. Following nucleic acid capture, the contents of the tube were pipetted into the small diameter tubing after the 6 mm air valve. Both ends of the tube

were capped with tube sealant (**Figure 21B**). The magnetic beads were collected from the lysis/hybridization buffer using a 2.5 mm cubic neodymium magnet and transported through the air valve into the water chamber. The magnet was rapidly moved back and forth on the outside of the water chamber to disperse the beads. A razor blade was used to excise the water chamber, and the water containing dispersed magnetic beads was collected by holding the excised tubing section over a 1.5 mL centrifuge tube. The tube was then heated to 65^0C for 2 minutes on a heating block. After heating, the eluate was pipetted out of the tube while holding the magnetic beads to one side of the tube with a magnet. Extracted RSV mRNA was quantified by RT-PCR as described above.

Statistical Analysis.

All statistical analyses were performed in SigmaPlot 11.0. Analysis of variance (ANOVA) was used to determine statistical significance for data containing 3 or more sample populations, and a T test used for data containing 2 sample populations. A p-value <0.05 was considered significant.

Results

Characterization of RSV-specific and oligo (dT) beads using fluorescently-labeled complementary and scrambled DNA sequences.

Both the oligo (dT) and RSV-specific beads recovered ~70% of the 22 base complementary fluorescent probe and ~2% of a scrambled fluorescent probe, indicating that the laboratory-synthesized RSV-specific beads were comparable to the commercially-available oligo (dT) beads. The oligo (dT) beads recovered 69 ± 13% of complementary probe 5' Cy5 – AAA AAA AAA AAA AAA AAA AAA A 3', and the RSV-specific beads recovered 74 ± 15% of complementary probe 5' Cy5 – TAC TCC ATT

TGC TTT TAC ATG A 3' (**Figure 22**, black bars). Additionally, the oligo (dT) and RSV-specific beads recovered 2.4 ± 0.3 and 1.9 ± 0.5%, respectively, of a scrambled DNA probe (**Figure 22**, gray bars).

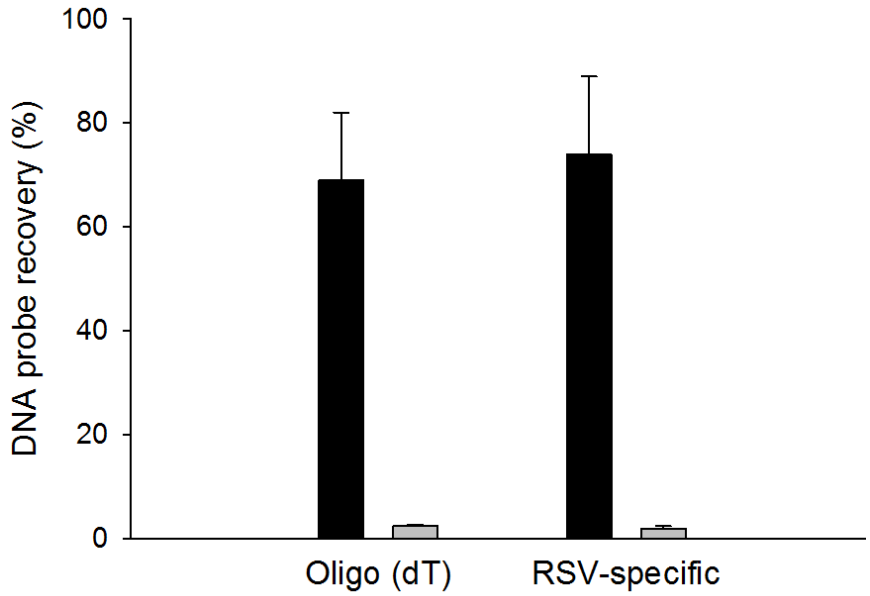

Figure 22. Characterization of oligo (dT) and RSV-specific magnetic beads. The recovery of a complementary DNA probe was equivalent for the oligo (dT) and RSV-specific beads (black bars). Both oligo (dT) and RSV-specific beads recovered less than 3% of a nonspecific scrambled probe (gray bars) (mean ± s.d., n=3)

Optimization of magnetic capture hybridization protocol for oligo (dT) and RSV-specific magnetic beads.

There is a ~50% decrease in mRNA recovery when wash steps are excluded from the oligo (dT) extraction protocol, though this decrease in yield is not statistically significant. When performed according to manufacturer's instructions, the oligo (dT) beads recovered 83 ± 22% of RSV N gene mRNA from 25 uL of spiked HEp-2 cell lysates (**Figure 23**). When no wash steps were included, and the magnetic beads were eluted into water directly following nucleic acid capture, 39 ± 30% of spiked RSV N gene was detectable following RT-PCR.

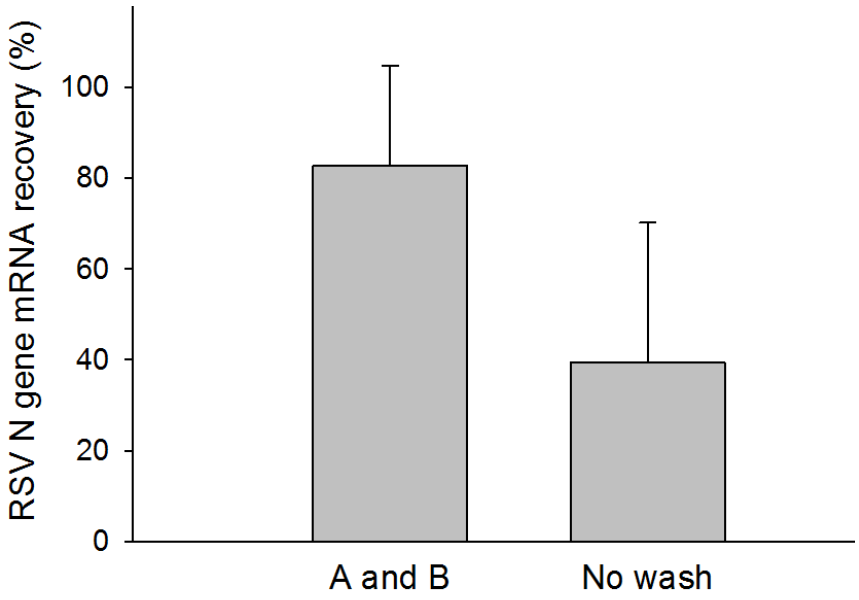

Figure 23. RSV mRNA is detectable by RT-PCR when wash steps are eliminated from the oligo (dT) extraction protocol (mean ± s.d., n=3).

RNA extraction capacity of silica-coated, oligo (dT), and RSV-specific magnetic beads.

The mass of RNA extracted using silica-coated magnetic beads increases to ~400 ng and then plateaus as the mass of spiked RNA is increased to 1000 ng. A similar trend is seen when using oligo (dT) and RSV-specific magnetic beads. As the quantity of full length RSV N gene mRNA is increased from 200 to 1000 ng, the mass of mRNA recovered plateaus at ~300 ng. The maximum quantity of mouse total RNA that can be extracted from TE buffer using 1 x 10^8 silica-coated magnetic beads is ~400 ng or 4 fg/bead (**Figure 24**, black circles). The maximum quantity of full length RSV N gene mRNA that can be extracted using 1 x 10^8 oligo (dT) (**Figure 24**, white circles) and RSV-specific magnetic beads (**Figure 24**, gray circles) is ~300 ng or 3 fg/bead. This corresponds to an oligo (dT) and RSV-specific magnetic bead extraction capacity of ~3800 RSV N gene mRNA copies/bead. To keep the extraction capacity similar when comparing silica-coated, oligo (dT), and RSV-specific beads, a total extraction capacity

of 2000 ng was used for each bead type. Equivalent extraction capacity was achieved using 5 x 10^8 silica-coated magnetic beads and 6.7 x 10^8 oligo (dT) or RSV-specific beads.

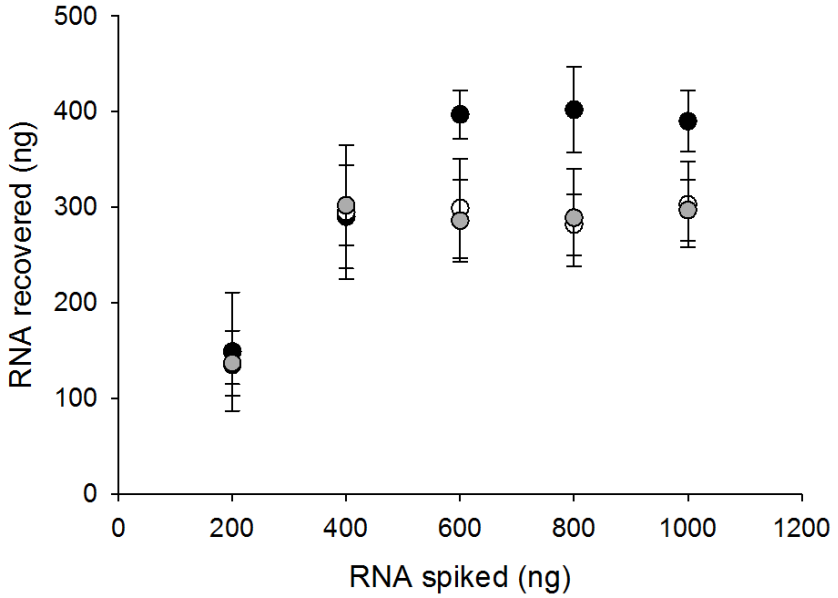

Figure 24. As the mass of spiked RNA is increased, the mass of RNA recovered using silica-coated (black circles), Oligo (dT) (white circles), or RSV-specific beads (gray circles) increases and then plateaus once the RNA extraction capacity of the beads is reached (mean ± s.d., n=3).

RNA capture kinetics using silica-coated, oligo (dT), and RSV-specific beads.

Magnetic bead capture kinetics are dependent on bead surface functionalization. The percentage of RSV N gene recovered from HEp-2 cell lysates remains relatively constant as nucleic acid capture time is increased using the silica-coated and oligo (dT) beads but increases exponentially when using RSV-specific beads. As shown in **Figure 25**, 75 ± 9% of spiked RSV N gene mRNA is recovered using the silica-coated beads (black circles) and 71 ± 26% recovered using the oligo (dT) beads (white circles) following a 1 min nucleic acid capture step. As the nucleic acid capture time is increased from 1 minute to 3 hours this recovery does not increase further.

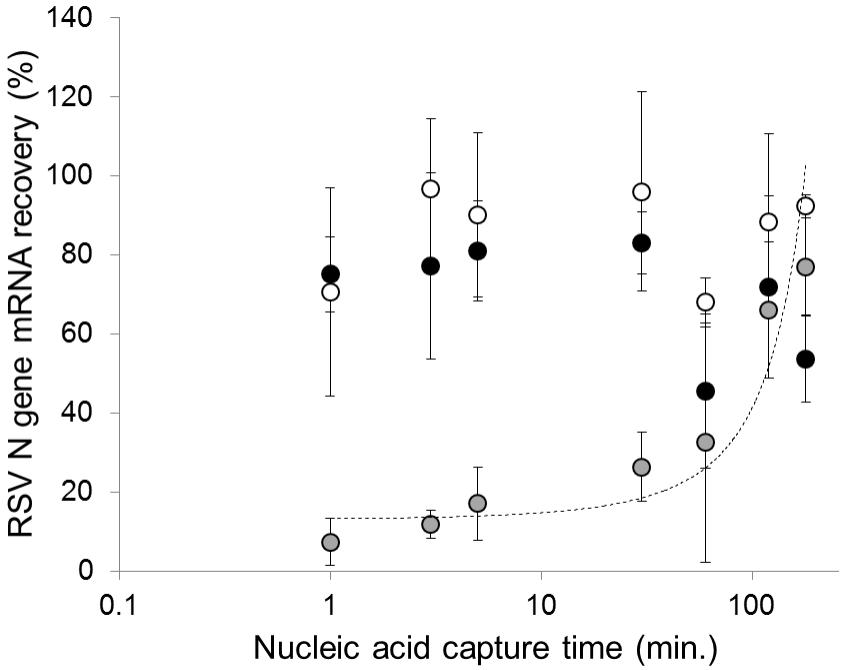

Figure 25. RSV N gene mRNA recovery remains relatively constant as nucleic acid capture time is increased from 1 min to 3 h using silica-coated (black circles) and oligo (dT) (white circles) magnetic beads. RSV N gene mRNA recovery using RSV-specific beads (gray circles) increases exponentially as RNA capture time is increased to 3 hours (dotted line). (mean ± s.d., n=3).

The percentage of RSV N gene recovered using the RSV-specific beads increases exponentially as nucleic acid capture time is increased from 1 minute to 3 hours. After 1 minute, the recovery of RSV N gene is 7.4 ± 6%, and increases to 77 ± 12% after a 3 hour capture step. Following the 3 hour incubation, the recovery of RSV N gene using RSV-specific magnetic beads is similar to the recovery using silica-coated and oligo (dT) beads following a 1 minute capture step.

Ratio of RSV biomarkers to background RNA following extraction with silica-coated, oligo (dT), and RSV-specific beads.

Background yeast tRNA was detectable by absorbance in the eluate following extraction with silica-coated magnetic beads, but not oligo (dT) or RSV-specific beads. As shown in **Figure 26A**, the absorbance measured in eluate collected with silica-coated magnetic beads was 0.016 ± 0.002. The absorbance was 0.002 ± 0.001, and 0.004 ± 0.001 for the oligo (dT) and RSV-specific beads, respectively. These values were equivalent to the blank sample with a measured absorbance of 0.001 ± 0.001.

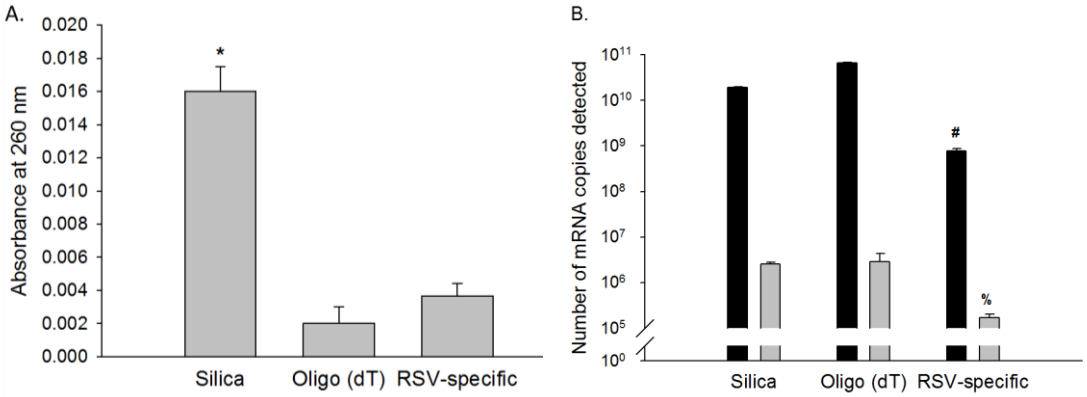

Figure 26. A) Background yeast tRNA recovery was confirmed by measuring absorbance on each eluate. Significant amounts of yeast tRNA were detected in the eluate following extraction with silica-coated magnetic beads. The absorbance on eluate following extraction with oligo (dT) and RSV-specific magnetic beads was below the limit of detection of the spectrophotometer (mean ± s.d., n=3). * denotes detectable quantities of tRNA compared to a blank sample. **B)** The recovery of background mRNA (black bars) and RSV N gene mRNA (gray bars) was measured following RT-PCR. The recovery of background mRNA is significantly lower in the eluate recovered using the RSV-specific beads than the silica-coated and oligo (dT) beads. The recovery of RSV N gene mRNA is comparable for all three bead chemistries tested (mean ± s.d., n=3). # denotes significantly fewer background mRNA copies detected than silica-coated and oligo (dT) beads. % denotes significantly fewer RSV mRNA copies detected than silica-coated and oligo (dT) beads.

A spiked control mRNA was detectable in the eluate from all three bead types, but was significantly lower for the RSV-specific beads than for the silica and oligo (dT) beads. Similarly, the RSV N gene mRNA was detectable in all three eluates, but was 15-fold lower in the RSV-specific bead extraction. As shown in **Figure 26B**, 2 ± 0.05 x

10^{10} copies of spiked control mRNA were detected following extraction with silica-coated magnetic beads, 7 ± 0.3 x 10^{10} copies detected following extraction with oligo (dT) beads, and 8 ± 0.9 x 10^{8} copies detected following extraction with RSV-specific beads. Additionally, 2.5 ± 0.2 x 10^{6}, 2.8 ± 1 x 10^{6}, and 1.7 ± 0.3 x 10^{5} copies of spiked RSV N gene mRNA were detectable following extraction with the silica-coated, oligo (dT), and RSV-specific beads, respectively.

Self-contained RSV RNA extraction using silica-coated, oligo (dT), and RSV-specific beads.

Using the self-contained extraction, ~80% of RSV N gene biomarkers were recovered using silica-coated and oligo (dT) magnetic beads and ~10% were recovered using RSV-specific magnetic beads. Following a 5 minute nucleic acid capture step the silica-coated and oligo (dT) beads recovered 84 ± 48 and 78 ± 38% of spiked RSV N gene mRNA from HEp-2 cell lysates, respectively. The RSV-specific beads recovered 8.9 ± 5.4% of spiked RSN N gene mRNA (**Figure 27**).

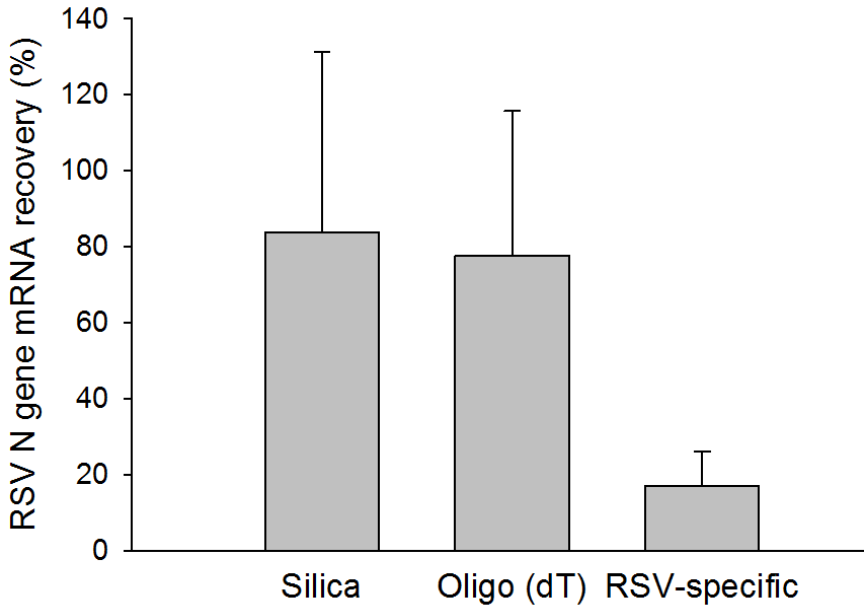

Figure 27. The RSV N gene mRNA recovery from HEp-2 cell lysates is shown for the silica-coated, oligo (dT), and RSV-specific magnetic beads used in the self-contained extraction (mean ± s.d., n=6).

Discussion

In this report, we present a thorough analysis of three magnetic bead functionalities for nucleic acid extraction. Due to their simplicity, speed, flexibility, and compatibility with a variety of downstream detection formats, oligo (dT) beads appear to be the best choice for applications that require purified mRNA. One useful application of the oligo (dT) surface functionality is the detection of pathogens that are actively replicating and producing mRNA [21].

Oligo (dT) beads selectively capture nucleic acid sequences which have poly(A) tails available for hybridization to the poly(T) probes. As a result, samples processed with the oligo (dT) magnetic beads contain mainly mRNA. In an extracted sample containing no background nucleic acids or other potential contaminants, oligo (dT) beads recover over 70% of complementary poly(A) sequence, while recovering less than 3% of a nucleic acid sequence not complementary to the poly(T) probe attached to the

magnetic beads (**Figure 22**). In a more complex sample containing both mRNA and large quantities of background tRNA, the oligo (dT) beads again recovered minimal amounts of the non-complementary sequences (**Figure 26A**). This reduction in background RNA may not be necessary in a background-tolerant diagnostic such as RT-PCR. However, using the oligo (dT) beads, the captured mRNA sequences are available for amplification or detection strategies performed directly on the mRNA sequences while they are still attached to the beads. This advantage enables strategies such as quadruplex priming amplification to be performed on the extracted biomarkers directly on the magnetic bead surface [15].

Total extraction time is an important consideration when preparing a sample for downstream detection. In diagnostics involving RT-PCR, amplification time is frequently greater than 2 hours. In such cases, it is ideal to reduce the total time-to-diagnosis for the patient by reducing other processing steps prior to detection. This is particularly important in settings where the patient has traveled many hours to reach a clinic and may not be willing or able to return for their results. The long 3' poly(A) tails on mRNA rapidly hybridize to the poly(T) probes attached to the oligo (dT) magnetic beads, resulting in a total extraction time less than 10 minutes (**Figure 25**, white circles). The rapid mRNA loading minimizes the total nucleic acid extraction time, which proves useful in minimizing a patient's time-to-diagnosis.

Because the oligo (dT) beads depend solely on nucleic acid hybridization to capture and extract nucleic acids, the hybridization step contains a significantly lower concentration of toxic salts (6 mg lithium chloride/extraction) compared to the silica-based extractions (200 mg GuSCN/extraction). The reduction in toxic chemicals leads to an extraction process that is safer for the user. This is especially useful in settings where the technician has little to no personal protective equipment or where hazardous waste is not managed safely.

The simplest extraction should have a minimum number of processing steps to decrease the likelihood of operator error and risk of contamination. The oligo (dT) bead manufacturer recommends a simple protocol including two magnetic bead wash steps prior to nucleic acid elution. However, the oligo (dT) processing was further simplified by eliminating the wash steps and immediately eluting extracted mRNA following capture. The resulting 50% reduction in recovered mRNA is not statistically significant. More importantly, the self-contained extraction is simplified to a single step (**Figure 23**). In settings where simplicity is the highest priority in a sample preparation process, the one-step extraction may be worth any potential loss in yield. Additionally, the single step makes the oligo (dT) surface chemistry an attractive choice for a variety of simple nucleic acid extraction formats. The oligo (dT) beads were evaluated in our self-contained device, which relies on surface tension valves to separate solutions during processing (**Figure 21B**). The single step oligo (dT) extraction was easily adaptable to the self-contained format, performing similarly to the silica-based extraction (**Figure 27**) and recovering ~70% of spiked RSV mRNA biomarker.

Silica-coated beads are useful when there is no need for a specific subset of nucleic acids, or when mRNA is not the biomarker of interest. The silica-coated magnetic beads are a useful alternative to oligo (dT) beads. They are rapid, easy to use, and nonspecifically capture nucleic acids present in the sample independent of sequence (**Figure 26**). Because silica-based extractions are not selective for specific nucleic acid sequences, they are well-suited for detection strategies that are tolerant of high background nucleic acids, such as PCR and RT-PCR. Furthermore, using these strategies, eluate from a single extraction could potentially be used to detect DNA, RNA, or biomarkers of multiple diseases.

The silica-coated magnetic beads rely on electrostatic interactions to nonspecifically capture nucleic acids when they come into contact with the beads. This

process occurs rapidly, and results in a total extraction time of less than 10 minutes, an advantage in settings where short time-to-diagnosis is important (**Figure 25**, black circles). One disadvantage is that these interactions require the presence of high concentrations of a strong chaotropic agent, in this case 5.7 M GuSCN. In laboratory settings, GuSCN has been relied on for decades as a rapid, dependable method for extracting nucleic acids. However, GuSCN is a potent inhibitor of RT-PCR, and must be washed away during the extraction. Additionally, GuSCN is a highly toxic substance which can be dangerous in settings where the operator does not have the training or personal protective equipment to properly handle and dispose of this chemical. This toxicity should be considered when choosing the appropriate magnetic bead functionalization.

Extractions performed with silica-coated magnetic beads are relatively simple to complete. The silica-coated beads require thorough washing with ethanol to remove traces of GuSCN that could inhibit downstream processes such as RT-PCR. Even with these additional wash steps compared to the oligo (dT) beads, silica-based extractions using magnetic beads have been adapted to a variety of formats due to their relative simplicity compared to alternative methods such as phase separation and centrifugation columns. The silica-based extraction method was successfully adapted to our self-contained tubing format to extract both RNA and DNA from patient samples [6,7]. The silica-coated magnetic beads performed well in the self-contained format shown in **Figure 21A**, recovering ~70% of spiked RSV mRNA biomarker (**Figure 27**).

The RSV-specific magnetic beads are simple to use and capture nucleic acids specific to the species-of-interest (**Figure 22**), but they may require too much processing time to be more advantageous than silica-coated or oligo (dT) beads in many cases. One distinct advantage is that they enable detection strategies that are inhibited by high concentrations of background nucleic acids or that rely on hybridization to the biomarker

while it remains hybridized to the magnetic bead. For example, the bio-barcode assay developed by Nam, et. al. uses DNA-functionalized gold nanoparticles to hybridize to captured nucleic acids and subsequently detect their presence downstream [17].

The RSV-specific magnetic beads recover RSV RNA complementary to the probes attached to the bead surface. There is minimal nonspecific nucleic acid extracted, and background nucleic acids are not recovered at a high enough concentration to be detectable by spectrophotometer (**Figure 26A**). The extraction is performed similarly to the oligo (dT) extraction, and requires a low concentration of toxic salt rather than the high concentration required for silica-based extraction. As a result, the RSV-specific extraction can also be simplified to require no wash steps following nucleic acid capture, reducing the process to a single step. Like the silica-coated and oligo (dT) beads, the RSV-specific beads performed reasonably well when tested in the self-contained tubing format (**Figure 21B**). The recovery was ~15 fold lower than the other beads tested using a 5 minute nucleic acid capture step (**Figure 27**).

Should the sequence-specific beads be chosen, the tradeoff between total extraction time and biomarker yield would be an important consideration. The long extraction time is likely required because the sequence-specific probes must come into contact with a short 25 base complementary sequence from RSV N gene sequences present in the sample. Due to the high selectivity of the probes, RSV-specific capture beads require a binding time approaching 3 hours to maximize biomarker recovery (**Figure 25**). A 3 hour binding time results in a total nucleic acid extraction time longer than many detection strategies, including RT-PCR. Because RT-PCR is typically not affected by background nucleic acids, sequence-specific magnetic beads are probably not an ideal choice for applications which rely on RT-PCR as the final detection strategy.

Conclusions

In choosing the appropriate magnetic bead surface functionality for nucleic acid extractions, the advantages and disadvantages of each must be considered. There are often trade-offs between assay effectiveness, simplicity, speed, and compatibility with the desired downstream detection strategy. Oligo (dT) beads offer the benefits of simplicity, speed, and the flexibility to perform additional detection schemes directly on captured nucleic acids. However, they are not useful when mRNA is not the biomarker of interest. Silica-coated beads are also rapid and simple, though they require a high concentration of toxic salt and additional detection cannot be performed on captured nucleic acids still adsorbed to the bead surface. RSV-specific beads are simple and specific, enabling downstream detection directly on the captured nucleic acids. However, there is a significant trade-off between speed and nucleic acid recovery efficiency which must be considered with these beads.

Acknowledgments

This work was supported in part by the Bill and Melinda Gates Foundation through the Grand Challenges in Global Health Initiative in diagnostics. N.M.A. acknowledges support from the NSF Graduate Research Fellowship (DGE 0909667).

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CHAPTER VII

CONCLUSIONS AND FUTURE DIRECTIONS

This dissertation comprises four manuscripts which describe the development of a biomarker extraction device designed to simplify nucleic acid extractions in resource-limited settings. The self-contained extraction device effectively recovers nucleic acid biomarkers from complex patient samples, enabling downstream molecular detection strategies such as PCR. The self-contained extraction device offers significant advantages over similar reported devices and commercially available extraction kits. These include a simple design and the flexibility to process different biomarkers and patient sample matrices by modifying the tubing contents.

The proof-of-concept design presented in Chapter III relies on silica-coated magnetic particles and surface tension valves to extract RSV RNA biomarkers from complex sample matrices with a recovery efficiency of ~10%. One limitation of this design was the requirement for a multi-step nucleic acid capture process prior to sample introduction into the device. This drawback was addressed through the introduction of a patient sample-extraction tubing interface, described in Chapter IV. The transfer pipette interface was included to enable the extraction of DNA from large-volume human urine samples. Short TB DNA biomarkers were recovered with an efficiency of ~50% and a limit of detection of ~75 DNA copies/uL. This approach has the potential to improve TB diagnosis in resource-limited settings by enabling urine-based biomarker detection, and may also be useful for other diagnostic applications found to be associated with short DNA fragments excreted in urine.

The self-contained extraction device was further improved with the design of an automated extraction processor, presented in Chapter V. A circular extraction cassette

holds the loaded small-diameter tubing, effectively reducing the tubing footprint to a 12.5 cm disk. A continuous processing program rotates each loaded cassette past a fixed permanent magnet to transfer magnetic beads from one end of the tubing to the other. The automated biomarker extraction device extracted detectable quantities of nucleic acid and protein biomarkers from well-characterized patient sample surrogates containing clinically-relevant high, medium, and low concentrations of infectious organisms. The successful detection of influenza A, *E. coli*, TB, and malaria biomarkers extracted with the automated extraction processor demonstrates the flexibility of the self-contained extraction device.

The magnetic beads are one of the major enabling technologies of the surface tension-based extraction device, and three surface functionalities are studied in Chapter VI. When choosing a magnetic bead surface functionality for performing nucleic acid extractions, the advantages and disadvantages of each available magnetic bead type must be considered. There are often trade-offs between assay effectiveness, simplicity, speed, and compatibility with the desired downstream detection strategy. Of the magnetic bead surface functionalities studied, oligo (dT) beads offer the benefits of simplicity and speed. Oligo (dT) beads also enable additional detection schemes performed directly on the bead surface. Silica-coated beads are also useful for rapid, simple nucleic acid extractions, though they require a high concentration of toxic salt. Additionally, adsorbed nucleic acids are not available for downstream detection directly on the bead surface. RSV-specific beads enable simple and specific extractions. However, there is a significant trade-off between assay speed and nucleic acid recovery efficiency which must be considered when choosing these beads.

Future studies should focus on incorporating the patient sample-extraction tubing interface into the automated processor design. In its current iteration, magnetic bead transfer into the small-diameter tubing for automated processing requires pipetting the

entire prepared sample into the tubing using the transfer pipette interface, or manually transferring the magnetic beads down the transfer pipette stem and into the tubing. Eliminating this initial step would decrease user involvement and bring the extraction closer to a fully automated process. Incorporating the patient sample loading into the automated device would require a custom-made device that not only fit seamlessly into the end of the plastic tubing, but was also developed to snap into the circular cassette. This design would allow the magnetic beads to be captured by the fixed magnets and transferred into the tubing using the same process that conveys the beads down the tubing during the extraction.

Validating the improved biomarker extraction device on clinical TB-infected urine samples would further strengthen the promising DNA extraction data presented in Chapter IV. A small retrospective study performed on TB positive urine samples (Appendix A) revealed some limitations of analyzing patient samples collected and stored under uncontrolled conditions. In a prospective study, urine sample collection and storage would be well-controlled, and nuclease activity could be eliminated by the addition of the chelating agent EDTA immediately upon sample collection.

APPENDIX

EXTRACTION OF TRANSRENAL DNA FROM STORED URINE SAMPLES OBTAINED FROM PERU AND SOUTH AFRICA

Introduction

One promising application of the automated extraction cassette processor is the extraction of *Mycobacterium tuberculosis* Tr-DNA biomarkers from patient urine samples. We sought to detect a short segment of the TB IS6110 sequence in the eluates of patient urine samples processed with the automated extraction cassette processor. The study was completed in two separate phases. During the first phase, previously frozen urine samples were shipped to the lab from collaborators at the University of Cayetano in Lima, Peru for processing. During the second phase, a bank of previously frozen urine samples were made available from a collaborator at the University of Cape Town (UCT) in Cape Town, South Africa. Due to regulations in South Africa, the samples could not be shipped to the United States, and were instead processed at UCT.

One critical problem when processing clinical samples is the incorporation of appropriate controls to reduce false negatives. A positive extraction control was included in the device to confirm the automated device was working as expected during each extraction.

Materials and Methods

Incorporation of a positive extraction control.

A control DNA sequence was incorporated into the automated extraction device and co-extracted along with any nucleic acids present in the processed clinical urine samples. A 120 bp sequence from the *Mus musculus* GAPDH gene was selected as the positive control. PrimerBank, an online resource containing primers designed for mouse genes was used to select this sequence. The segment was chosen because the PCR cycling conditions were equivalent to those of the IS6110 TB gene and the two sequences could be amplified and detected during the same PCR run. The incorporation and detection of the positive control sequence are described below.

Urine samples from Lima, Peru.

Frozen urine samples from 20 TB positive and 20 TB negative patients were obtained from the University of Cayetano in Lima, Peru. One milliliter of each sample was provided. All 40 samples were from HIV negative patients. TB infection had been confirmed using both sputum smear microscopy and chest X-Ray. Multiple freeze-thaw cycles were suspected for each sample, though the storage conditions following urine collection were not provided.

Urine samples from Cape Town, South Africa.

The Blackburn Lab at UCT provided 110 previously frozen human urine samples stored in 50 mL centrifuge tubes. The samples were stored at -20°C prior to our arrival, but had been freeze-thawed multiple times. Following an unknown sample storage history, 18 of the samples had been supplemented with 10 mM EDTA to inhibit nuclease activity. The majority of these samples were provided in volumes greater than 10 mL.

However, 6 samples had volumes between 100 uL and 3 mL. These were supplemented with nuclease free water to bring the total volume up to 5 mL for processing.

The Dheda Lab at UCT provided 48 samples that were frozen immediately upon collection and had not been thawed prior to processing. Of the 48 samples, 34 were 1 mL urine samples and 14 were 1 mL serum samples. These 48 samples were supplemented with 4 mL of nuclease free water to bring the volume up to 5 mL for processing.

Preparation of urine collection pipettes for 1 mL Peru samples.

The urine collection pipettes were prepared by drawing 1 mL of DNA-silica adsorption buffer (4M guanidinium thiocyanate, 25 mM sodium citrate, pH 7.0) containing 6 x 10^8 Dynabeads MyOne silane magnetic beads, and 5 x 10^6 copies of a 120 bp segment of the *Mus musculus* GAPDH gene (which served as a positive extraction control) into the bulb of a 5 mL, fine tipped transfer pipette (Samco Scientific, catalog # 232-20S). The contents of the transfer pipette were frozen at -80°C for 2 hours, after which the pipettes were transferred to a Labconco bulk tray dryer and lyophilized for ~18 hours. Following lyophilization, the transfer pipettes were stored at room temperature prior to use.

Preparation of urine collection pipettes for 5 mL South Africa samples.

The urine collection pipettes were prepared by drawing 5 mL of DNA-silica adsorption buffer containing 1.8 x 10^9 silica-coated magnetic beads, and 5 x 10^7 copies of the mouse GAPDH positive extraction control sequence into the bulb of a 15 mL, narrow stemmed transfer pipette (Fisher Scientific, catalog # 13-711-36). The contents of the transfer pipettes were frozen at -80°C for 2 hours, after which the pipettes were

transferred to a Labconco bulk tray dryer and lyophilized for ~36 hours. Following lyophilization, the transfer pipettes were packaged in plastic zip top bags and shipped to South Africa, where they were stored at room temperature for 1-2 weeks prior to use.

Detection of TB IS6110 and mouse GAPDH by PCR.

A 67 bp amplicon of the IS6110 sequence was amplified using forward primer 5'-ACCAGCACCTAACCGGCTGTGG-3' and reverse primer 5'-GTAGGCGAACCCTGCCCAGGTC-3' [1]. A 95 bp amplicon of the mouse GAPDH sequence was amplified using forward primer 5'-AGGTCGGTGTGAACGGATTTG-3' and reverse primer 5'-GGGGTCGTTGATGGCAACA-3' (PrimerBank ID 126012538c1) [2]. The TB IS6110 and mouse GAPDH sequences were amplified in separate tubes during the same PCR reaction.

For analyzing the Peruvian samples, amplification reactions were performed in a 25 uL volume using 5 uL of DNA template and the Qiagen QuantiTect SYBR Green PCR kit according to manufacturer's instructions. Reactions were prepared in 200 uL PCR tubes (Thermo Scientific). Thermal cycling consisted of 95°C for 15 min to activate the Taq DNA polymerase and 35 cycles of 95°C for 30 s, 62°C for 30 s, and 72°C for 60 s using a Rotor-Gene Q thermal cycler. Reaction C_t values were recorded by Rotor-Gene Q software.

For analyzing the South African samples, amplification reactions were performed in a 25 uL volume using 5 uL of DNA template and the Maxima SYBR Green qPCR Master Mix (Thermo Scientific) according to manufacturer's instructions. Reactions were prepared in LightCycler 480 Multiwell Plate 96 white plates (Roche). Thermal cycling consisted of 95°C for 15 min to activate the Taq DNA polymerase and 35 cycles of 95°C for 30 s, 62°C for 30 s, and 72°C for 60 s using a Roche LightCycler 480. Reaction C_t values were recorded by LightCycler software.

Processing of 1 mL Peru samples.

All 40 Peru samples were processed over 4 days. Urine was removed from freezer and thawed over ice immediately prior to use. Once samples were thawed, a 30 cm length of 2.36 mm inner diameter FEP tubing was prepared by preloading DNA eluent (50 uL nuclease free water), DNA wash solution (300 uL 70% ethanol), and DNA precipitation buffer (300 uL 80% ethanol, 5 mM potassium phosphate, pH 8.5). Each solution was pipetted sequentially into the tubing and separated from the next by an 8 uL air valve (4 mm in length).

The 1 mL urine samples were drawn into the bulb of the urine collection pipette, which was shaken vigorously by hand for 30 s to dissolve the lyophilized salts, then gently for 30 s to allow DNA adsorption to the silica surfaces of the beads. After mixing, the transfer pipette was squeezed to transfer the sample from the bulb to the tip of the pipette. The end of the pipette was then inserted into the preloaded small diameter tubing and the sample was transferred into the tubing. Both ends of the tubing were sealed using Hemato-Seal Tube Sealing Compound (Fisher Scientific). The sealed tubing was wrapped around the plastic cassette, which was inserted into the automated processor. The extraction program was run until the magnetic beads exited the elution chamber, at which point the tubing was removed from the plastic cassette, and the eluent was collected in a 0.5 mL microcentrifuge tube. Extracted TB and mouse DNA was detected using the PCR protocol described above.

Preparation of preloaded small diameter tubing for processing Cape Town samples.

A 10 cm length of 2.36 mm inner diameter FEP tubing was prepared by preloading DNA eluent (50 uL nuclease free water), DNA wash solution (300 uL 70% ethanol), and DNA precipitation buffer (300 uL 80% ethanol, 5 mM potassium

phosphate, pH 8.5). Each solution was pipetted sequentially into the tubing and separated from the next by an 8 uL air valve (4 mm in length). Both ends of the tubing were sealed using Hemato-Seal Tube Sealing Compound to prevent liquid evaporation and stored at room temperature until needed. Immediately prior to use, the front end of the tubing (near the DNA precipitation buffer) was opened by cutting off the sealed end with a razor blade.

Processing of 5 mL Cape Town samples.

On the morning of processing, urine samples were removed from the -20°C freezer and thawed at room temperature. Prior to use, the volume of any urine sample less than 5 mL was raised to 5 mL using nuclease free water. Nine samples were prepared and processed simultaneously. First, the 5 mL urine samples were drawn into the bulb of the urine collection pipette, which was shaken vigorously by hand for 30 s to dissolve the lyophilized salts, then allowed to sit for ~2 minutes with intermittent shaking by hand to allow DNA adsorption to the silica surfaces of the beads. Next, the tip of the transfer pipette was inserted into the end of the preloaded small diameter tubing and a 2.5 cm cubic neodymium magnet was used to collect the magnetic beads and transfer them through the pipette stem and into the DNA precipitation buffer. Once the beads were loaded into the small diameter tubing, the transfer pipette was removed and the end sealed with tube sealing compound.

After 9 samples were ready for processing, three sealed tubes were wrapped around each of three extraction cassettes, which were inserted simultaneously into the automated processor. The extraction program was run until the magnetic beads reached the elution chamber, at which point the cassettes were removed from the processor and allowed to rest at room temperature for 2 min. The magnetic beads were removed from the elution chambers using the neodymium magnet, and the eluent was

collected in a 0.5 mL microcentrifuge tube. Extracted TB and mouse DNA was detected using the PCR protocol described above.

Assessing DNase activity of Cape Town urine samples.

The relative DNase activity of all 144 urine samples was determined using the DNaseAlert QC System (Life Technologies Corporation) according to manufacturer's instructions. Due to the inability to obtain this kit in South Africa, it was transported at room temperature, lyophilized, by plane. Briefly, the lyophilized DNaseAlert substrate containing fluorescent DNA probes was resuspended in 1 mL TE buffer. Ten microliters of the DNaseAlert substrate was added to each of the wells of a 96-well plate and mixed with 10 uL of 10X NucleaseAlert Buffer. Finally, 40 uL of urine sample was diluted to 80 uL using nuclease free water and added to each well. Urine samples were prepared and measured in duplicate. The plate was incubated at room temperature for 18 h prior to measuring fluorescence on a Cary Eclipse Fluorescent Spectrometer (Agilent Technologies) using 535/556 nm excitation/emission wavelengths with excitation and emission slit widths of 10 and 5 nm, respectively. Urine sample fluorescent values were compared to a DNase negative control by T-test to confirm the presence of DNase activity.

Gel electrophoresis on selected extracted samples following PCR amplification.

After amplification, all PCR plates were kept at 4°C for up to 48 h prior to polyacrylamide gel electrophoresis (PAGE). Four PAGE gels were prepared in the Mini-PROTEAN Tetra Cell (Bio-Rad Laboratories, Inc.) using 10% acrylamide according to manufacturer's protocol. Prepared gels were allowed to polymerize for 45 minutes prior to use. The Tetra Cell tank was filled with 1X tris-borate-EDTA (TBE) buffer. Four microliters of 6x Orange DNA Loading Dye (Thermo Scientific) was added to the

selected PCR reactions directly into the wells of the PCR plates and mixed well by pipetting. Each reaction was then loaded into the prepared PAGE gels. One lane on each gel was reserved for the O'GeneRuler 50 bp DNA ladder (Thermo Scientific). The gels were then run at 200 V for 90 minutes. The electrophoresed gels were removed from the Tetra Cell tank and incubated for 10 minutes in a 1:10,000 dilution of GrGreen nucleic acid staining solution diluted into 1X TBE buffer. Finally, the gels were visualized using the SynGene GeneSnap UV gel document system.

Results

Peru sample PCR analysis.

The TB IS6110 C_t values were compared for the TB positive and TB negative urine samples. The average C_t value for TB positive urine samples obtained from Peru was 32.3 ± 3.2, while the average C_t value for TB negative samples was 30.4 ± 4.7 (**Figure 28**). There was no significant difference between the two populations.

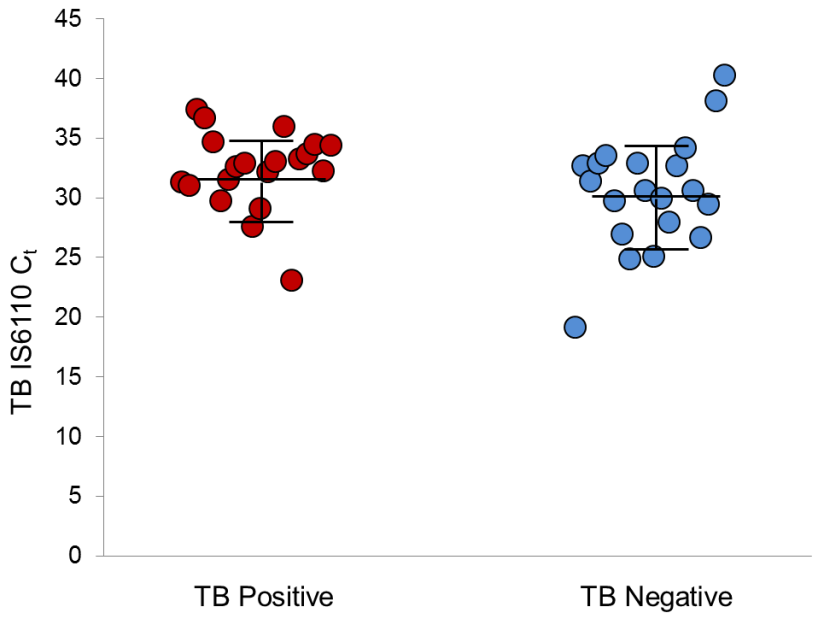

Figure 28. There is no difference in average Ct values for PCR amplified IS6110 in TB positive and negative extracted urine samples (mean ± s.d., n=20).

The mouse extraction control was detected in 39 of the 40 samples tested. The trend was similar for TB positive and negative samples whose TB IS6110 C_t values were compared to the corresponding mouse extraction control C_t value (**Figure 29**).

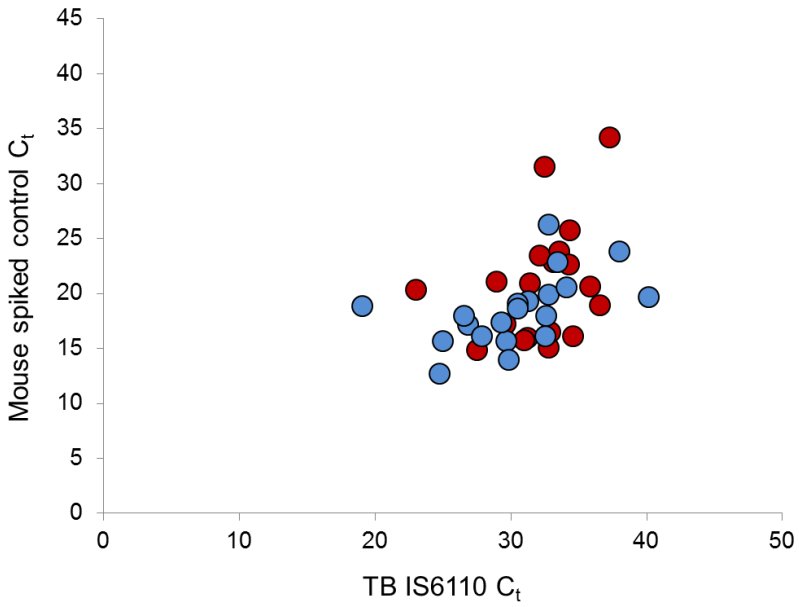

Figure 29. There is no trend difference between TB positive (red circles) and TB negative (blue circles) samples when the TB IS6110 and mouse extraction control C_t values were compared (n=20).

South Africa sample PCR analysis.

The TB IS6110 C_t values were compared for the TB positive and TB negative urine samples processed in South Africa. A total of 67 urine samples (30 TB+; 37 TB-) had no detectable amplification after 35 PCR cycles. The average C_t value for TB positive urine samples was 31.1 ± 3.2, while the average C_t value for TB negative samples was 32.3 ± 2.5 (**Figure 30**). There was no significant difference between the two populations.

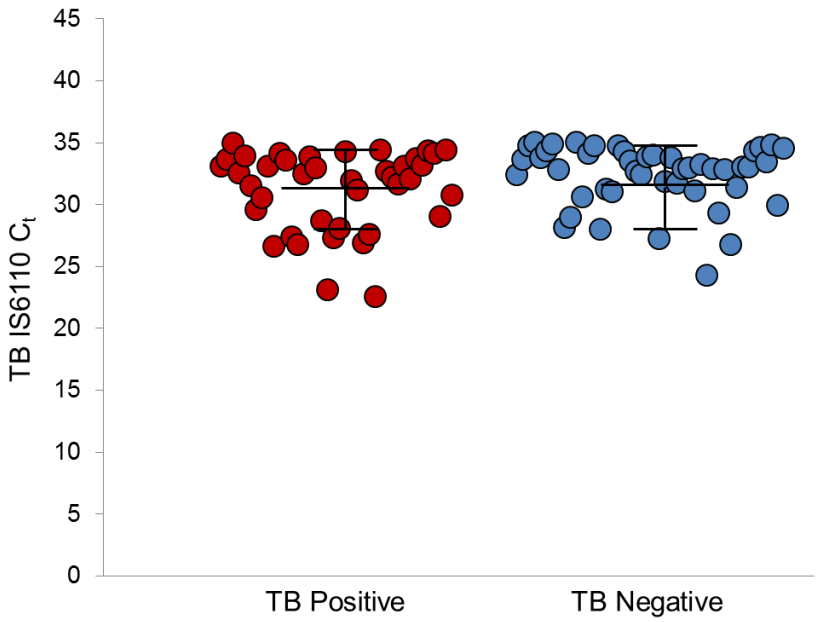

Figure 30. There is no difference in average Ct values for PCR amplified IS6110 in TB positive and negative extracted urine samples (mean ± s.d., n=40 TB+ and 46 TB-).

The mouse extraction control was detected in 92 of the 144 urine samples tested. Of the 92 samples, the TB IS6110 gene was detected in 66 samples (34 TB+, 32 TB-). The trend was similar for TB positive and negative samples whose TB IS6110 C_t values were compared to the corresponding mouse extraction control C_t value.

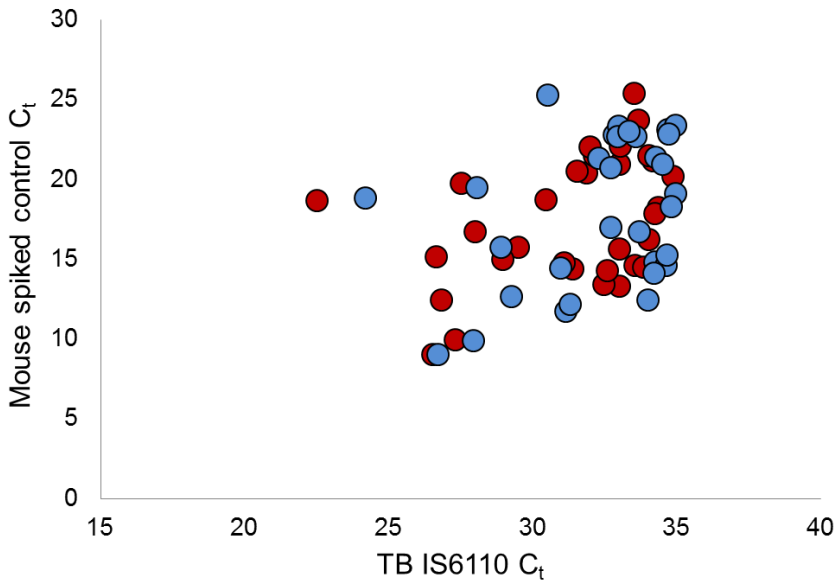

Figure 31. There is no trend difference between TB positive (red circles) and TB negative (blue circles) samples when the TB IS6110 and mouse extraction control C_t values were compared (n=33 TB+ and 31 TB-).

The 52 urine samples whose mouse extraction control did not amplify were eliminated from further analysis. Those urine samples whose TB and HIV status was known were divided into four categories: TB positive/HIV positive, TB positive/HIV negative, TB negative/HIV positive, and TB negative/HIV negative. As shown in **Figure 32**, the average C_t value for each group was 31.5 ± 2.5, 31.8 ± 3.1, 33.6 ± 1.3, and 31.4 ± 3.3, respectively. There was no significant difference among the four populations.

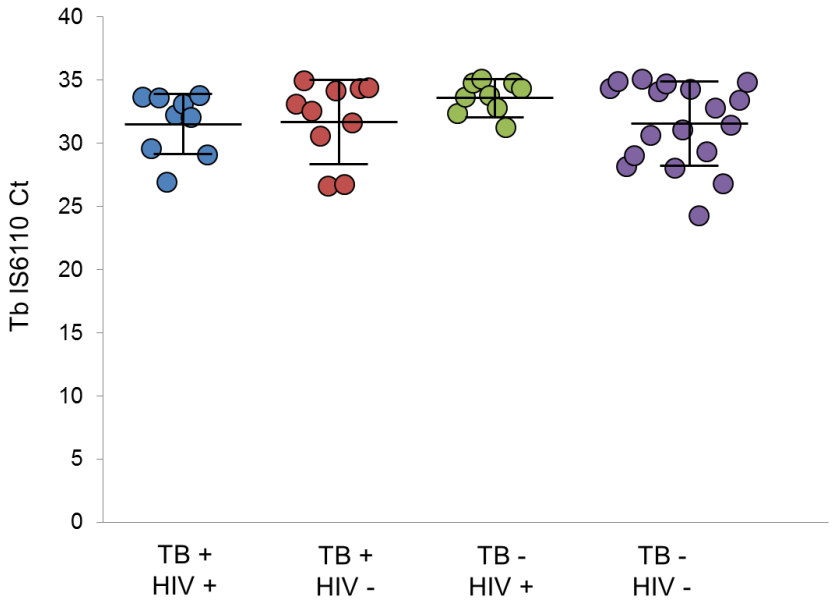

Figure 32. There is no difference in average Ct values for amplified TB IS6110 between TB+/HIV+, TB+/HIV-, TB-/HIV+, and TB-/HIV- extracted urine samples when samples with no detectable mouse extraction control were eliminated from analysis (mean ± s.d., n = 9 TB+/HIV+, 10 TB+/HIV-, 9 TB-/HIV+, and 18 TB-/HIV-).

The samples with the lowest C_t values for the mouse extraction control recovered the highest quantity of spiked GAPDH DNA, and as a result likely obtained the most DNA from the sample. Of the 66 urine samples in which the mouse extraction control and TB IS6110 gene were detected, those with the 33 lowest C_t values were divided into 4 categories and their C_t values averaged. As shown in **Figure 33**, the average C_t values for 5 TB positive/HIV positive, 17 TB positive/HIV negative, 7 TB negative/HIV positive, and 17 TB negative/HIV negative were 29.3 ± 2.7, 31.8 ± 2.8, 33.7 ± 1.7, and 30.9 ± 3.4, respectively. There was no significant difference among the four populations.

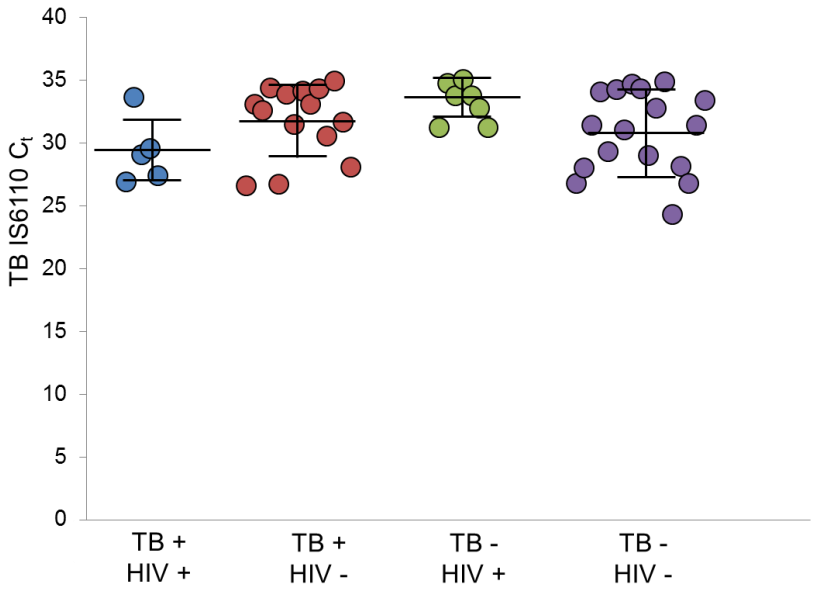

Figure 33. There is no difference in average Ct values for amplified TB IS6110 between TB+/HIV+, TB+/HIV-, TB-/HIV+, and TB-/HIV- extracted urine samples when samples with highest detectable quantities of mouse extraction control were analyzed (mean ± s.d., n = 5 TB+/HIV+, 14 TB+/HIV-, 7 TB-/HIV+, and 17 TB-/HIV-).

Surprisingly, the mouse extraction control was not consistently amplified in the 1 mL urine and serum samples received from the Dheda Lab. These samples were less than 7 days old and had been stored at -80°C since collection. Of the 34 urine samples, the mouse extraction control was detected in only 7, and no TB IS6110 DNA was detected in any of these 7 samples. Of the 14 serum samples, the mouse extraction control was detected in only 9. Of these 9, the TB IS6110 DNA sequence was detected in 2 (one TB positive and one TB negative).

Cape Town urine DNase activity.

DNase activity in the Cape Town urine samples was confirmed by measuring the fluorescence of a DNase-cleavable fluorescent probe that was introduced into each sample. DNase activity was confirmed in 95% of the samples tested (137 out of 144).

The relative DNase activity was 0.295 ± 0.12 for TB positive samples and 0.326 ± 0.20 for TB negative samples (**Figure 34**). There was no significant difference between the two populations.

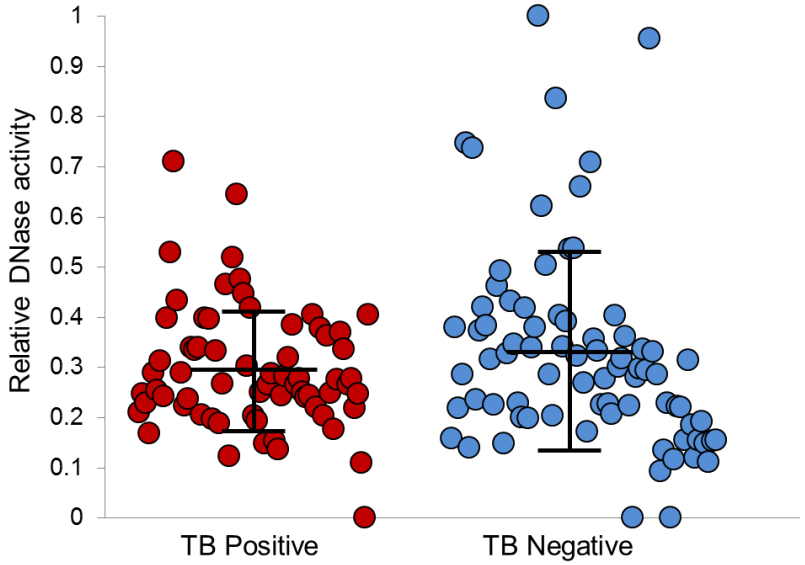

Figure 34. There was no difference in DNase activity between TB positive and TB negative urine samples (mean ± s.d., n = 64 TB+ and 74 TB-).

To determine whether there was a potential trend between low recovery of the mouse extraction control and sample DNase activity, the relative DNase activity for each sample was compared to the C_t value of the corresponding mouse extraction control (**Figure 35**). There was no trend between TB positive or negative samples.

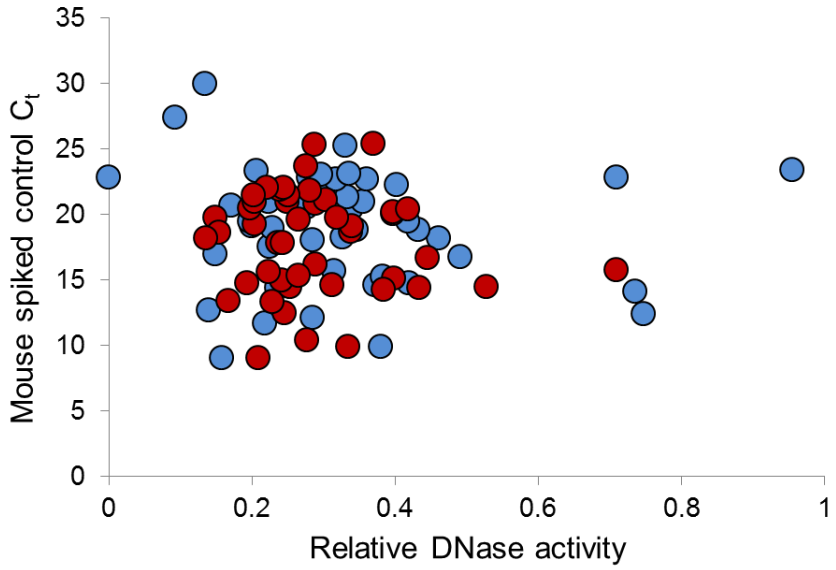

Figure 35. There is no trend difference between TB positive (red circles) and TB negative (blue circles) when the relative DNase activity and mouse extraction control C_t values were compared (n=45 TB+ and 46 TB-).

Polyacrylamide gel electrophoresis on selected PCR products.

The PCR products from 26 randomly selected extracted urine samples were visualized on PAGE gels. The resulting bands from these 26 samples are shown on the four gels in **Figure 36A-D**. There are 3 notable patterns in these samples: 1) a 68 bp band indicating amplification of the IS6110 gene, 2) no visible bands, and 3) 2-6 individual bands indicating the formation of multiple nonspecific products. For example, in **Figure 36A** lane 6, **Figure 36B** lane 5, and **Figure 36C** lane 9, there is a single 68 bp band indicating the presence of TB IS6110 amplicon. These samples are all TB positive. However, in **Figure 36A** lane 5 there is also a single 68 bp band from a TB negative sample. As expected, in some TB negative samples there is no visible product (**Figure 36B** lane 2, **Figure 36C** lane1). However, in others there are multiple bands (**Figure 36A** lane 3, **Figure 36C** lane 6) indicating the presence of nonspecific amplification.

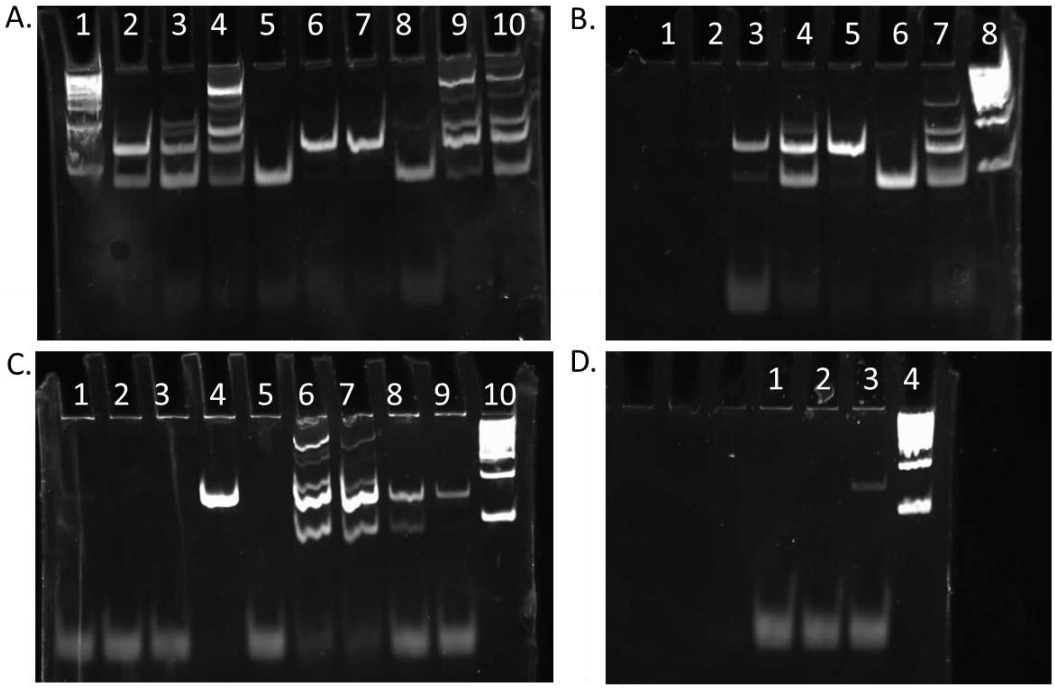

Figure 36. The IS6110 PCR products for 26 extracted urine samples were visualized by polyacrylamide gel electrophoresis. **A).** Lane 1 – 50 bp DNA ladder; Lane 2 – TB+/HIV-; Lane 3 – TB+/HIV+; Lane 4 – TB-; Lane 5 – TB-/HIV-; Lane 6 – TB-/HIV-; Lane 7 – TB+/HIV-; Lane 8 – TB-/HIV-; Lane 9 – No TB status provided; Lane 10 – No TB status provided. **B).** Lane 1 – TB-/HIV-; Lane 2 – TB+/HIV-; Lane 3 – TB+; Lane 4 – TB+; Lane 5 – TB+/HIV+; Lane 6 – TB-/HIV-; Lane 7 – TB+; Lane 8 – 50 bp DNA ladder. **C).** Lane 1 – TB-/HIV-; Lane 2 – TB-/HIV-; Lane 3 – TB+/HIV+; Lane 4 – TB+ control; Lane 5 – TB+/HIV+; Lane 6 – TB-; Lane 7 – TB+/HIV-; Lane 8 – TB-; Lane 9 – TB+/HIV-; Lane 10 – 50 bp DNA ladder. **D).** Lane 1 – TB+/HIV-; Lane 2 – TB-; Lane 3 – TB-/HIV-, Lane 4 – 50 bp DNA ladder.

Discussion

Using the automated biomarker extraction processor followed by PCR, TB IS6110 was not reliably detectable in the 40 samples obtained from Peru. The IS6110 gene was detected at approximately equivalent Ct values for the TB positive and negative urine samples (**Figure 28**). Despite these results, the positive extraction control was detectable in 39 of the samples, indicating a successful extraction was completed for each of these samples (**Figure 29**). Though the results of this initial study were not as expected, we identified several possible reasons why these results were not

ideal. Due to the random cleavage of DNA, it is reasonable to expect that there will be a relatively small number of IS6110 segments that are amplifiable by the PCR primers being used in this study. As a result, it would be ideal to perform the extraction and subsequent amplification on a larger urine volume to increase the total number of biomarkers available for detection. In this study, we were limited to 1 mL urine samples provided by the University of Cayetano.

Additionally, we expect that patients with both active TB and a weakened immune system (i.e. HIV positive patients), or those whose TB has disseminated into the blood stream will have a higher instance of Tr-DNA biomarkers because there are more active organisms throughout the body. The patients in this 40 sample study were all HIV negative, and none had disseminated TB. A larger study that included large urine samples volumes and a wider variety of TB positive patients may improve upon the results obtained in this study.

Repeating the Tr-DNA study on the urine samples stored at UCT provided the opportunity to work with a larger group of samples collected from a broad range of patients including those with TB/HIV coinfections as well as a small group of relatively fresh urine samples that had undergone no freeze-thaw cycles prior to our arrival. However, the majority of the samples had undergone an unknown number of freeze-thaw cycles and their storage conditions were not well documented. Despite these limitations, 104 samples were provided in large enough quantities to work with 5 mL, rather than 1 mL, urine samples. This larger volume would, in theory, contain 5 times more IS6110 biomarkers than similar 1 mL samples. Following extraction and PCR amplification of each urine sample, the IS6110 gene was detected at approximately equivalent C_t values for TB positive and TB negative urine samples (**Figure 30**).

Detection of the mouse extraction control was considered an indication of a successful nucleic acid extraction. Of the 144 processed urine samples, 92 had

detectable quantities of mouse extraction control DNA. Based on these results, we were forced to eliminate 52 samples from analysis (including 36 of the 48 "fresh" urine and serum samples obtained from the Dheda Lab), effectively reducing the pool of samples by 36% and leaving 49 TB+ and 51 TB- samples for analysis. The presence of TB infection did not appear to affect the detection of the mouse extraction control, as there was no difference between the pool of TB positive and TB negative samples when mouse extraction control C_t values were compared to TB IS6110 C_t values (**Figure 31**). Additionally, there is no difference between TB IS6110 C_t values (regardless of HIV coinfection) for TB positive and TB negative samples (**Figure 32**), even when only the samples with the highest mouse extraction control recovery were analyzed (**Figure 33**). Based on these results, the TB IS6110 biomarker was not reliably detected in TB positive samples processed in South Africa.

The presence of DNase in human urine is a significant barrier to detecting partially-degraded DNA biomarkers in a urine sample. Previous studies have suggested that the metal chelator EDTA could be added to urine samples at a concentration ranging from 10-40 mM to inhibit DNase activity immediately upon sample collection [3], however, only 18 of the 144 urine samples had been supplemented with EDTA (after multiple freeze-thaw cycles and long term storage). We confirmed DNase activity in 137 of 144 urine samples processed at UCT, including all 18 which had been previously supplemented with EDTA, and the relative DNase activity was independent of TB infection status (**Figure 34**).

Comparatively higher DNase activity did not appear to affect the recovery of mouse extraction control DNA (**Figure 35**), which suggests the DNA-silica binding buffer, specifically GuSCN, effectively eliminates DNase degradation of nucleic acids once it is added to the sample. This promising result indicates the immediate addition of

a GuSCN-containing buffer upon sample collection may be an effective biomarker preservation strategy in addition to or in place of supplementation with EDTA.

In addition to nucleases potentially reducing available TB biomarkers, an inconsistent PCR reaction may also inhibit reliable biomarker detection. PAGE on 26 amplified PCR products provided some insight into deficiencies in the PCR reaction employed in this study. As shown in **Figure 36**, there are TB positive samples with amplified IS6110 DNA, as well as TB positive and negative samples with no detectable IS6110 DNA. These particular results suggest the PCR reaction amplified IS6110 DNA when present in detectable quantities and did not amplify any product when IS6110 DNA was not present. However, there are also both TB positive and TB negative samples with 1 or more nonspecific bands, indicating the formation of nonspecific PCR products. This inconsistency would likely result in high numbers of false positives for those samples with nonspsecific product amplification and interfere with the identification of true positives.

Future Directions

The problems encountered with these studies clearly demonstrate the limitations posed when performing a retrospective patient sample study. Though we were able to obtain nearly 200 urine samples between collaborators in Peru and South Africa, there was no way to control how those samples had been stored or treated prior to our studies. In fact, with the exception of 34 urine samples received from the Dheda Lab, we did not know how many freeze-thaws the samples had undergone to date.

To further explore the Tr-DNA hypothesis and whether our automated DNA extraction cassette processor is a suitable device for preparing urine samples for Tr-DNA analysis, a prospective study must be completed. Through a prospective study, all aspects of sample analysis can be controlled. This includes the collection, treatment,

and storage of urine samples prior to processing. Preserving any TB biomarkers should begin immediately upon urine collection by inhibiting nuclease activity and properly storing collected samples. Prior studies have demonstrated that the addition of EDTA significantly slows DNA degradation in patient urine, especially when treated samples are stored at -80ºC until needed. A prospective study would test this preservation strategy by adding EDTA immediately to half of a patient sample, while leaving the other half untreated. Both samples could then be extraction and analyzed for the presence of TB biomarkers. With this design, the effects of nuclease inhibition could be compared by examining differences in the frequency of biomarker detection between the treated and untreated samples.

The biomarker detection strategy must also be optimized to ensure a prospective study. In our studies, we utilized primers designed for a 68 bp amplicon and a SYBR green detection scheme. Though short amplicons are desired to increase the chances for detecting extracted Tr-DNA fragments, this also increases the potential for false positives due to nonspecific product formation during the PCR reaction. Additionally, SYBR green is a nonspecific intercalating dye that fluoresces in the presence of any double stranded DNA, regardless of its sequence. Alternatively, a short amplicon coupled with a sequence specific probe, such as a TaqMan probe, could improve both the sensitivity and specificity of the PCR reaction, thus increasing the likelihood for detecting extracted TB biomarkers.

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2. Spandidos A, Wang X, Wang H, Seed B (2010) PrimerBank: a resource of human and mouse PCR primer pairs for gene expression detection and quantification. Nucleic Acids Res 38: D792-799.
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