DEVELOPMENT OF A NOVEL VIRAL RNA EXTRACTION METHOD

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Thesis

Submitted to the Faculty of the

Graduate School of Vanderbilt University

in partial fulfillment of the requirements

for the degree of

MASTER OF SCIENCE

in

Biomedical Engineering

December, 2009

Nashville Tennessee

Approved:

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ACKNOWLEDGEMENTS

I would like to thank the National Institute of Health providing the financial resources to complete my research.

I am grateful for all the support and guidance provided by my advisor Dr. Rick Haselton. I am indebted to members of the Haselton lab and the members of Dr. David Wright's lab, this work would not have been possible without their help.

Finally, I would like to thank my family and friends. I would especially like to thank my parents Kerry and Julie Klemm, my sister Kimberly and my aunt and uncle Kathy and Paul Baker for all their patience, support, and encouragement.

TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF ABBREVIATIONS

Abbreviation	Abbreviation For
C_t	Cycle Threshold
DNA	Deoxyribonucleic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
IF	Immunofluorescence
LCR	Ligase Chain Reaction
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
RNA	Ribonucleic Acid
RSV	Respiratory Syncytial Virus
RT	Reverse Transcriptase

CHAPTER I

INTRODUCTION

Need for Point-of-Care Viral Detection

An accurate diagnosis is critical for quickly implementing an effective response to an invasive pathogen. There is a need for a fast and reliable detection method which can discriminate among a wide variety of pathological agents. There are several effective antiviral medications but their effects are acute and it is extremely critical for the efficacy of the antiviral to be administered in the early stages of infection, therefore rapid and early diagnosis of a virus could lead to better treatment. The primary use for point-of-care viral detection is the selection of pathogen-specific treatments, but correct identification of specific pathogen(s) can also help minimize the spread of infection and lead to more effective monitoring of long-term complications.

Another important use for rapid virus detection in particular is in the control of over-prescription of antibiotics. Viral infections are one of the most common reasons patients seek medical care, but the misdiagnosis of viral pathogens as bacterial has led to the over-prescription of antibiotics (McGowan and Tenover, 1997). Two major complications can arise from this misdiagnosis. First, the virus is not effectively treated because antibiotics have no effect on a virus. Secondly, treatment with unnecessary antibiotics exacerbates the ever growing list of antibiotic resistance pathogens. Thus point-of-care detection methods

which are inexpensive, simple, reliable, and sensitive would have a great impact on patient care.

In the following pages, traditional and genome-based viral detection methods are reviewed. Pitfalls and limitations of these methods are highlighted. In addition to these topics, the extraction of nucleic acids from patient samples in both a laboratory and in a clinical setting is discussed.

Traditional Virus Detection Methods

Currently there are several viral detection methods available. Conventional methods include the current gold standard using cell culture methods. Additional methods include serology and antigen detection and diagnostic methods which include immunofluorescence (IF), and enzyme-linked immunosorbent assay (ELISA).

Cell Culture

Cell culture is considered the gold standard for the detection of most viruses, principally because it can provide characterization and identification of a viable viral pathogen. It is performed by replicating or amplifying the viral pathogen in a suitable host system. Host systems include laboratory animals or an incubated egg but it is most often a culture of cells. Although it is both sensitive and specific, the procedure is expensive, labor intensive, requires a high level of expertise, and the results could take days to weeks. For example,

one study showed the diagnosis of a clinical sample for respiratory syncytial virus (RSV) by cell culture took up 16 days (Ahluwalia et al., 1987).

Serology

This method relies on the body's immune response to invasion by a foreign body. Serology characterizes the antibodies circulating in the blood. Antibody response varies, but typically beginning at the onset of an infection there is a rise in antibody titer which continues until the infection has cleared. There several serology techniques including ELISA, latex agglutination, precipitation, complement-fixation and immunofluorescence. The technique implemented is dependent upon the antibody to be detected. Serology is often used when other methods of virus detection are not possible or when too much viral shedding has occurred. The specificity and sensitivity varies according to the technique used. Disadvantages of serology include poor correlation with disease, high background titers, and cross-reactivity. Serology was also shown to have limited diagnostic value when testing for RSV in children younger than 6 months (Brandenburg et al., 1997).

Immunofluorescence

Immunofluorescence (IF) allows for direct fluorescent antigen labeling or indirect visualization of viral antigens by the use of fluorescently labeled monoclonal or antispecies antibodies. This technique is relatively rapid and generates results in as little as 1-2 hours (Madeley and Peiris, 2002). IF is most

commonly used to test nasopharyngeal aspirate specimens for respiratory viral infections such as respiratory syncytial virus (RSV), parainfluenza viruses, influenza A and B and adenovirus. In order to analyze and obtain useable results from the specimen, there must be an adequate number of cells and the specimen must be of high-quality. Therefore, IF requires highly skilled personnel to work. Low sensitivity also remain a major issue with IF. The method showed a detection of only 19% for respiratory viruses when the viral load was below 10^6 copies per mL (Kuyper et al., 2006).

Enzyme- Linked Immunosorbent Assay

Another common viral detection method that utilizes antibodies is enzyme-linked immunosorbent assay (ELISA). There are several different types of ELISA including indirect ELISA, sandwich ELISA, and competitive ELISA. A typical ELISA begins by the passive absorption of a monoclonal or polyclonal capture antibody to the surface of a well in a microtiter plate, typically a 96-well plate. A solution containing the antigen is then added to each well of the microtiter plate, followed by several washing and blocking steps. A detection molecule is then added to each well, this molecule is often a monoclonal enzyme-linked antibody that is different than the capture antibody. If the target virus is present an antibody sandwich is formed and any uncoupled antibody is removed in a rinse step. Finally, the enzyme substrate is added which is converted by the enzyme linked to the detection antibody to elicit a chromogenic, fluorogenic, or electrochemical signal. ELISA-based systems are often used for point-of-care

detection of RSV, influenza, and herpes simplex virus. However there are major drawbacks. For example, the monoclonal antibodies must be matched pairs otherwise they may hinder the binding onto the antigen, the conjugation of the enzyme to the antibody limits restricts the amplification and therefore limits the sensitivity of the assay, and the enzyme itself can reduce the immunoreactivity of the antibody. Other limitations of antigen detection include false positives, long term antigen shedding, and narrow detection window. ELISA has also been shown to have lower sensitivity but higher specificity than IF (Takimoto et al., 1991).

Limitations of Traditional Virus Detection Methods

Despite the wide use of traditional virus detection methods, they still have significant limitations. Although cell culture produces conclusive and unambiguous results it requires days to perform. While serology, IF, and ELISA may be faster to perform they are much lower in sensitivity and specificity. Also a major pitfall of any antibody-based detection method is the inability to detect virus infection at an early stage.

Genome-Based Virus Detection Methods

An alternative to antibody-based detection is genome-based methods. Genome-based virus detection methods can potentially take full advantage of the ever expanding knowledge of viral genomics. These methods such as polymerase chain reaction (PCR), ligase chain reaction (LCR), molecular

beacons and DNA microarrays offer potential advantages over traditional methods. The presence and viability of a pathogen can be addressed without the need for culturing the organism, the tests are less time consuming so they report results faster, and it offers much higher sensitivity over antibody-based detection methods. Genomic detection also can potentially more fully characterize a viral infection by identifying the subtype, genotype, variants, mutants, and genotypic resistance patterns.

Polymerase Chain Reaction

There are three broad classifications of nucleic acid amplification methods and these include target amplification, probe amplification and signal amplification (Whelen and Persing, 1996). Polymerase Chain Reaction (PCR) is a widely used clinical tool for target amplification. It can be used for the direct detection of pathogen-specific DNA. It is an extremely sensitive tool which can be used to detect molecules with a copy number as low as 1 molecule per milliliter, but typically detection is within the range of 10 to 100 molecules per milliliter (Palmer et al, 2003). Reverse transcriptase can be added to the reaction to convert RNA to DNA, thus allowing for the use of PCR as an effective tool for the detection of viral RNA. Also multiplex PCR assays allow for the testing of multiple viral pathogens simultaneously (Létant et at., 2007).

The PCR process can be complex, for instance, primer design is a critical component for effectively amplifying the target region. Primers are designed to flank the gene of interest and software is available to help with this process. The

process starts by annealing the DNA to separate the strands to allow for binding of the primers. Then in the presence of activated enzyme such as *Taq* polymerase and excess nucleotide bases, amplification of the target DNA then occurs through the repeated heating and cooling cycles (Saiki et al., 1988). In traditional PCR, an agarose or polyacrylamide gel electrophoresis combined with a dye or fluorescent marker that binds to double-stranded DNA is used to detect the final PCR product. Real time PCR allows for quantitative analysis of the final product by the measuring the increasing fluorescence during the PCR process of an intercalating dye added to the initial PRC mix, which binds to double-stranded DNA (Bustin, 2004).

Although there are many attractive features of PCR, it also has a number of disadvantages. The highly sensitive nature of PCR creates an elevated risk of contamination, environmental contaminates or contamination from a previous sample which can easily introduce variation into PCR results. The presence of DNase or RNase can destroy the target of interest and can also skew PCR results. Therefore, to avoid these potential issues, PCR should be performed by a skilled technician in isolated areas which are specifically dedicated to PCR preparation. PCR also requires a large investment in equipment which makes it extremely costly to perform. PCR is extremely useful in research settings but it is less useful for point-of-care detection.

Ligase Chain Reaction

Ligase Chain Reactions (LCR), similar to PCR, is another technique to detect and amplify a target sequence. However unlike PCR, which amplifies the target DNA, LCR instead amplifies the probe. It is described as an oligonucleotide probe-based assay. In this approach ligation of two sets of oligonucleotide pairs is triggered by hybridization to the target DNA (Widemann et al., 1994). The probes are designed to so that the junction between the 3' end of upstream and downstream primers coincides with the nucleotide that distinguishes one type of target from another. A positive reaction occurs only when the appropriate target is present and is indicated by the ligation of the two sets of oligonucleotides. If the target sequence matches the two adjacent oligonucleotides the ligated probes serve as templates and lead to a 2-fold amplification in the number of templates. This is similar to, but less robust than the cycling process of PCR. However, if there is a mismatch at the pair junction there is no ligation between the probes, and the oligonucleotides must be ligated in order to be detected. A single base-pair mismatch can prevent ligation of the oligonucleotides, making LCR highly sensitivity for detecting single base-pair changes or mutations. LCR has been shown to be as sensitivity as PCR for the detection of viruses (Marshall et al., 1994). Detection strategies for LCR include gel electrophoresis or coupling with PCR.

Molecular Beacons

Molecular beacons are single-stranded oligonucleotide probes used for the detection of specific nucleic acids. They have been designed to take advantage of the stem-and-loop structure of single-stranded oligonucleotides. The probe is located in the loop region, and is designed to be complementary to the target DNA. The loop sequence is flanked by a sequence containing a fluorophore on one end and a sequence containing a quencher on the other. The two flanking regions anneal to form the stem portion of the beacon, and this quenches the fluorphore. However, in presence of target DNA the beacon forms a more stable structure by the hybridization of the target to the probe region, this opens up the beacon and separates the fluorophore from the quencher thus allowing the fluorophore to fluoresce (Tyagi and Kramer, 1996).

Molecular beacons can be used in conjunction with PCR, however there are uses for molecular beacons independent of PCR. For example, viral RNA detection has been performed using 100 μm gold-clad filaments with covalently attached molecular beacons (Perez, Haselton, and Wright, 2009). The DNA probe used was specifically designed to target an RSV gene end-intergenic start sequence, and due to the presence of multiple copies of this region it creates multiple targets for each strand of RNA. The DNA functionalized filament was then placed in a series of microcapillary tubes containing 200 μL solutions. The first tube contained viral RNA and was followed by wash solutions. The analysis was done measuring the fluorescence of the filament using a flatbed microarray scanner.

DNA Microarrays

PCR, LCR, and molecular probes methods all require either specifically identified primers or probes to operate, meaning they all require a priori knowledge of the identity of the virus being tested. DNA microarrays offer a diagnostic system which allows for screening samples without such specific a priori knowledge and they offer high levels of sensitivity, specificity, automation, and throughput capacity, all with reduced sample volume (Ivnitski et al., 2003).

There are several techniques used to design DNA microarrays. Generally a glass or silicone chip is used to covalently bond hundreds of oligonucleotides designed from various viral genomes. For example, one approach allowed for the potential screening of hundreds of viruses simultaneously by utilizing 70-mer long oligonucleotides designed from over 140 sequence viral genomes (Wang et al., 2002). Hybridization of the target DNA to the oligonucleotide probes is then detected using fluorphores. Since the specific viral genome location on the microarray is known, the identity of the virus can be determined.

Major Roadblock of Genome-Based Virus Detection Methods

The complexity and reproducibility of the extraction of nucleic acids from patient samples remains one of the greatest limitations to genome-based virus detection methods. Patient samples such as blood, urine, stool, cerebrospinal fluid, throat swabs, nasal washes, and nasal swabs contain contaminates which block the effectiveness of genomic detection methods. For instance, it has been shown that high concentrations of carbohydrates present in clinical samples can

inhibit the results of PCR (Monteiro et al., 1997). Also one of the biggest barriers for the implementation of microarrays in clinical settings is the complexity of the sample pretreatment and need for a method which is coupled and codependent in terms of sample volume, time, and reagent consumption (Lichtenberg, Rooij, and Verpoorte, 2002). In addition to containing contaminants which inhibit genome-based detection, patient samples can also contain DNases and RNases, which over time, reduces the number of any target viral nucleic acids present in the samples.

There are several ways to approach the issue of handling contaminants in patient samples. One strategy is to identify and remove the contaminant, but this method would not be efficient considering that patient samples most likely contain several contaminants and the identification and extraction of each would be difficult and time consuming. Another strategy adds a substance to the patient sample to remove the contaminant, but this only works if the contaminant could either be cleaved or absorbed and if the substance being added does not interfere with testing. A third option is to extract target viral DNA or RNA from the patient sample and place it in a contaminant-free buffer. Although this is a time-consuming and labor intensive process, this is currently the strategy pursued by most laboratories.

Commercially Available RNA Extraction Kits/Reagents

There are numerous commercially available methods used specifically to isolate RNA from animal cells and tissue samples. The most popular of these

are the Invitrogen Dynabead mRNA Direct kit, Invitrogen TRIzol, the Qiagen RNeasy Mini kit, and the Qiagen MagAttract RNA Cell Mini M48 kit. Each of these is briefly described below.

Invitrogen Dynabeads mRNA Direct Kit

The Invitrogen Dynabead kit (Oslo, Norway) is designed to isolate mRNA from crude lysates of cells and tissue. It utilizes magnetic beads with a covalently bound short sequence of oligo-dT on the surface, and relies on the hybridization of the polyA tail of mRNA to the bead-bound oligo-dT for extraction. The lysate is mixed with the provided lysis/binding buffer and then added to the dynabeads. Then the beads are washed with a series of wash buffers, also provided in the kit. And finally the isolated mRNA is extracted from the beads using the elution buffer.

Invitrogen TRIzol

TRIzol (Invitrogen, Oslo, Norway) is a monophasic solution of phenol and guanidine thiocyanate used for isolating total RNA, DNA and proteins from cell and tissue samples. TRIzol disrupts cells and cellular components without disrupting the integrity of the nucleic acids (Simms, Cizdziel, and Chomczynski, 1993). The lysate and TRIzol after centrifugation and the addition of chloroform separates into an aqueous phase, which contains the RNA, and an organic phase, which contains DNA and proteins. Isopropanol is used to precipitate the RNA and ethanol is used to precipitate DNA. Although it is an effective reagent

for isolating, phenol interferes with PCR amplification and therefore necessitates removal of the phenol. Another disadvantage of using TRIzol is that it contains toxic substances such as phenol and chloroform.

Qiagen RNeasy Mini Kit

The Qiagen RNeasy Mini kit (Germantown, MD), which is based on silica-membrane technology, is designed for the purification of total RNA from cells and tissues. The sample with lysis buffer is added to the RNeasy Mini spin column, which contains a silica-membrane that binds RNA. Spin or vacuum technology paired with wash buffers are then used to eliminate contaminants. The purified RNA is extracted from the membrane in a final elution step. This procedure is very rapid, in fact, a purification can be obtained in a matter of minutes which is ideal for a clinical setting. However, it still requires the use of lab equipment such as a centrifuge or a vacuum pump which are not typically available in a physician's office.

Qiagen MagAttract RNA Cell Mini M48 Kit

Similar to the RNeasy Mini Kit, the Qiagen MagAttract RNA Cell Mini M48 kit (Germantown, MD) is intended for the purification of total RNA from cells. Silica coated magnetic beads are used to bind nucleic acid contained in cell lysates. Then, similar to the Invitrogen Dynabeads kit, the beads are treated with a series of wash buffers to eliminate contaminates. Then finally the RNA is eluted from the silica beads with an elution buffer. One of the major drawbacks

of using the MagAttract RNA M48 kit is that it is intended to be used in conjunction the Qiagen BioRobot M48 workstation. It can be used without the assistance of the BioRobot, but the manual instructions are not optimized for this.

Ideal Purification for Patient Samples in a Clinical Setting

The best extraction technique would be simple, safe, fast, cheap, and effective. It needs to be simple so that it does not require the use of a skilled technician in order to get the desired results. Since it will be used within a clinical setting it should not be hazardous. Time is an important factor and the preparation of a patient sample should not take longer to perform than the detection analysis. Integration of the extraction strategy into a point-of-care device would be difficult if the process was costly therefore it is important that it remains inexpensive. Most important the method needs to isolate enough target to run the desired virus detection test.

An ideal viral detection device allows for the patient sample to be prepared and analyzed in a clinical setting, giving accurate and reliable results in short amount of time, without requiring the use of skilled technicians, and without requiring the need for expensive laboratory equipment. No existing kit or reagent fulfills all of the requirements. For example, TRIzol may be a simple and cheap technique but the trade-off is toxicity, because it requires the handling of potentially hazardous chemicals. Of the kits listed, the RNeasy Mini kit is the fastest extraction however, it requires the use of a centrifuge or vacuum which increases both the complexity and cost of using this kit for extractions. The

magnetic separation techniques such as Dynabead and MagAttract kits, offer certain advantages over the other standard separation procedures. Magnetic separation can be extremely cost effective because of the possible recovery and reuse of the magnetic particles (Safarik and Safarikova, 2004). The disadvantage of using the Dynabead or MagAttract kits are that they require precise pipetting and handling of several solutions, making them less attractive for clinical use. The kits listed are designed to be used by skilled lab technicians, and although they may be the quickest and simplest method for performing extraction of genomic target from patient samples in a laboratory, they are not the best method for performing extractions in a clinical setting.

Components of Proposed Magnetic Pull-through Capillary RNA Extraction

The goal of this thesis is to design a simple extraction method that is more appropriate for use in a clinical setting. The proposed RNA extraction method uses a modified magnetic separation technique to perform the extraction. The components of this proposed method are sketched in Figure 1. The basic idea of this approach combines magnetic entrapment of silica-coated beads and surface tension confinement of processing solutions. In this approach, the sample is combined with a lysis buffer and mixed with silica-coated magnetic beads. Six sequential capillary chambers aligned horizontally, with air spacers in between each capillary. The first capillary chamber is initially empty while the other five chambers are pre-loaded with processing solutions. The sample mixed with the

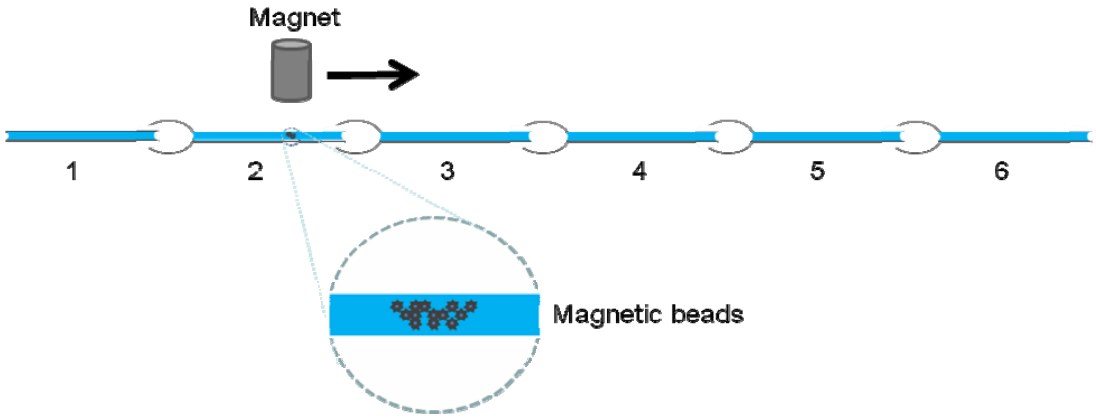

Figure 1. Capillary chambers were arranged sequentially on a horizontal stage. Sample lysate with lysis buffer and magnetic beads are added to the first chamber. As a magnet is moved over the exterior of the capillaries the magnetic beads are pulled through the chambers and transferred to chamber 6. This process washes, rinses and elutes RNA from the sample lysate.

lysis buffer and beads is loaded into the empty first chamber. The 2nd, 3rd, and 4th chambers are filled with wash buffers. The 5th chamber is filled with a rinse buffer, and the final chamber is filled with an elution buffer. After the sample is added to the first chamber a magnet is placed on the outside of the capillary tube. The beads are attracted to the magnet, and as the magnet is pulled down over the wash capillaries, then over the rinse capillary, and then finally over the elution capillary the beads follow the magnet. Because of the small capillary diameter (~2mm) the processing solutions do not follow the magnetic beads. The spacers between each capillary allow for movement of the beads without removal of the buffer from the capillary chamber because the solutions are held in place by surface tension. Figure 2 shows the movement of the beads across a spacer. This allows for sufficient washing of contaminants from the beads and

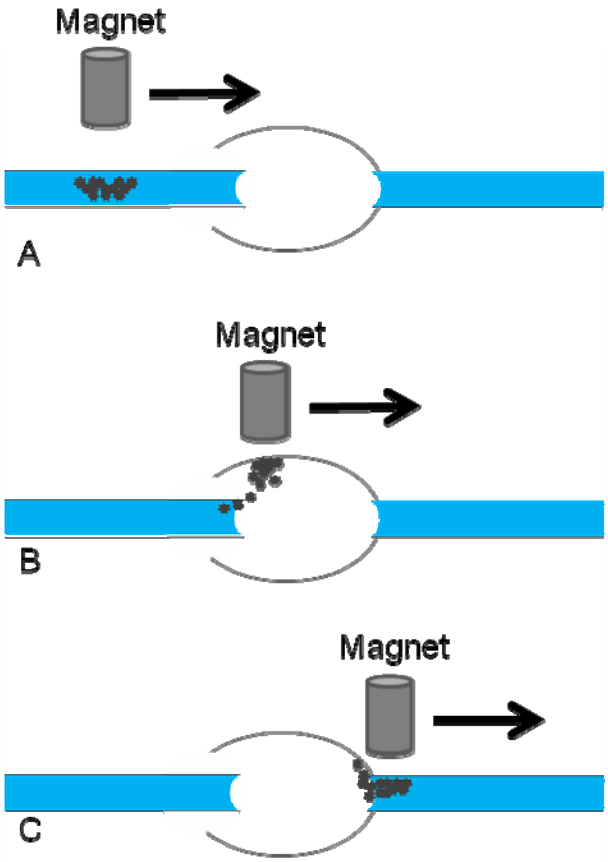

Figure 2. The beads follow the magnet as the magnet is moved from left to right. Blue represents the processing solutions and the air spacers between them allow for movement of the beads from one capillary to the next without transferring solutions. The retention of the solution is due to surface tension holding the solution within the capillary chamber.

for the extraction of viral RNA present in the patient sample. This method does not require a skilled technician to perform it and it does not require any additional equipment such as a centrifuge. It only requires a magnet. This method also does not require pipetting or handling of hazardous chemicals. Finally, the time required to perform this extraction is minimal since it basically removes all liquid

handling steps. The design has the potential to dramatically improve viral RNA extractions from patient samples in a clinical setting.

Overall Study Design

The next section provides additional details of the proposed magnetic pull-though capillary RNA extraction method and compares its performance to three commercially available extraction kits: Invitrogen Dynabead mRNA Direct kit, the Qiagen RNeasy Mini kit, and the Qiagen MagAttract RNA Cell Mini M48 kit. Each kit is used to extract viral RNA from patient samples known to contain respiratory syncytial virus (RSV).

CHAPTER II

DEVELOPMENT OF A NOVEL VIRAL RNA EXTRACTION METHOD

Abstract

The complexity and reproducibility of the extraction of nucleic acids from patient samples remains one of the greatest limitations to genome-based virus detection methods. This report describes magnetic pull-through capillary RNA extraction method which uses a magnetic to pull magnetic silica beads through a series of capillary chambers separated by air spacers to isolate RNA from clinical samples. The pull-through method and the extraction obtained from this approach is compared to three commercially available RNA extraction kits: Qiagen RNeasy Mini Kit, Qiagen MagAttract RNA Cell Mini M48 kit, Invitrogen Dynabeads mRNA Direct kit. RT-PCR was used to calculate cycle threshold and the number of RNA copies per µL. Clinical samples known to be positive for RSV had a copy number of 3 copies per µL for raw sample, 4,577 copies per µL for extractions performed using RNeasy kit, µL 755 copies per µL for extractions performed using Dynabeads kit, 973 copies per µL for extractions performed using MagAttract kit, and 531 copies per µL for extractions performed using the magnetic pull-through capillary method.. The proposed magnetic pull-through capillary method is simpler, less time consuming, and requires less additional equipment than most commercially available RNA extraction kits. This platform may prove attractive for point-of-care settings.

Introduction

An accurate diagnosis is critical for quickly implementing an effective response to an invasive pathogen. There is a need for a fast and reliable detection method which can discriminate among a wide variety of pathological agents. There are several effective antiviral medications but their effects are acute and it is extremely critical for the efficacy of the antiviral to be administered in the early stages of infection, therefore rapid and early diagnosis of a virus could lead to better treatment. The primary use for point-of-care viral detection is the selection of pathogen-specific treatments, but correct identification of specific pathogen(s) can also help minimize the spread of infection and lead to more effective monitoring of long-term complications.

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The complexity and reproducibility of the extraction of nucleic acids from patient samples remains one of the greatest limitations to genome-based virus detection methods. Patient samples such as blood, urine, stool, cerebrospinal fluid, throat swabs, nasal washes, and nasal swabs contain contaminates which block the effectiveness of genomic detection methods. For instance, it has been shown that high concentrations of carbohydrates present in clinical samples can inhibit the results of PCR (Monteiro et al., 1997). Also one of the biggest barriers for the implementation of microarrays in clinical settings is the complexity of the sample pretreatment and need for a method which is coupled and codependent in terms of sample volume, time, and reagent consumption (Lichtenberg, Rooij, and Verpoorte, 2002). In addition to containing contaminants which inhibit genome-based detection, patient samples can also contain DNases and RNases, which over time, reduces the number of any target viral nucleic acids present in the samples.

There are several ways to approach the issue of handling contaminants in patient samples. One strategy is to identify and remove the contaminant, but this method would not be efficient considering that patient samples most likely contain several contaminants and the identification and extraction of each would be difficult and time consuming. Another strategy adds a substance to the patient sample to remove the contaminant, but this only works if the contaminant could either be cleaved or absorbed and if the substance being added does not interfere with testing. A third option is to extract target viral DNA or RNA from the patient sample and place it in a contaminant-free buffer. Although this is a time-

consuming and labor intensive process, this is currently the strategy pursued by most laboratories.

The best extraction technique would be simple, safe, fast, cheap, and effective. It needs to be simple so that it does not require the use of a skilled technician in order to get the desired results. Since it will be used within a clinical setting it should not be hazardous. Time is an important factor and the preparation of a patient sample should not take longer to perform than the detection analysis. Integration of the extraction strategy into a point-of-care device would be difficult if the process was costly therefore it is important that it remains inexpensive. Most important the method needs to isolate enough target to run the desired virus detection test.

An ideal viral detection device allows for the patient sample to be prepared and analyzed in a clinical setting, giving accurate and reliable results in short amount of time, without requiring the use of skilled technicians, and without requiring the need for expensive laboratory equipment. No existing kit or reagent fulfills all of these requirements. This paper provides details of a proposed magnetic pull-though capillary RNA extraction method and compares its performance to three commercially available extraction kits: Invitrogen Dynabead mRNA Direct kit, the Qiagen RNeasy Mini kit, and the Qiagen MagAttract RNA Cell Mini M48 kit.

Materials and Methods

Clinical Samples

Clinical samples were obtained from Dr. John William's lab (Vanderbilt University Hospital, Nashville, TN). A total 840 de-identified nasal swabs that had been placed in opti-MEM media (Invitogen, Oslo, Norway) and frozen at -80°C were available for this study. Each sample was previously characterized for respiratory syncytial virus (RSV) using RT-PCR. They were characterized after an extraction using Roche Total Nucleic Acid Extraction Kit (Basel, Switzerland), and real-time RT-PCR was performed using Roche LC Magna Pure machine (Basel, Switzerland). We obtained samples which tested positive for RSV and positive samples were determined to be samples that had a calculated cycle threshold (C_t). We also obtained samples which tested negative for RSV and negative samples were determined to be the ones which did not have a calculated cycle threshold (C_t) within the cycles that were performed.

Real time RT-PCR

Rt-PCR was used to estimate the number of viral copies in the test solutions. Real time RT-PCR was performed using a Rotor-Gene Q (Qiagen, Germantown, MD). Reactions were done in a 25 µL volume using 0.5µL of either raw clinical samples or samples that had RNA extractions performed using various different kits, 12.5 µL of 2X One-Step qRT-PCR Buffer plus SYBR (Clontech, Mountain View, CA), 0.5 µL of 50X QTaq DNA Polymerase Mix (Clontech, Mountain View,

CA), 0.4µL of 60X qRT Mix (Clontech, Mountain View, CA), 200nM left and right RSV primers, and RNase-free water. The protocol consisted of RT followed by a three-step PCR. RT was performed at 48°C for 20 minutes followed by an initial QTaq DNA polymerase activation step of 95°C for 3 minutes and 40 cycles at 95°C for 15 seconds to denatures, 60°C for 60 seconds to anneal and extend, and fluorescence measurements were made after each cycle. Specificity of the amplification was confirmed using melting curve analysis. Data were collected and recorded by [insert software name] and expressed as a function of threshold cycle (C_t). The primer set used was RSV forward primer 5'-GCTCTTAGCAAAGTCAAGTTGAAATGA-3' and RSV reverse primer 5'-TGCTCCGTTGGATGGTGTATT-3'. The primers were purchased from Biosearch Technologies (Novato, CA). Data was collected and recorded by Rotor-Gene Q Software (Qiagen, Germantown, MD) and expressed as a function of threshold cycle (C_t) and number of copies of RNA per µL. Every RT-PCR trial was performed with two standard samples from the standard curve, and copy number was obtained using a standard curve to relate C_t to starting copy. Every RT-PCR test was performed in triplicate and the results were recorded as an average.

PCR Standard Curve for RSV

E. coli transformed with the RSV N-gene cloned into pcDNA3.1(-) vectors was received from the Crowe Lab (Vanderbilt University, Nashville, TN). *E. coli* was grown overnight in Miller's LB Broth (Invitrogen, Oslo, Norway) at 37°C on a

rotating rack. The next day, growth was confirmed by the turbidity of the broth and the plasmid was extracted using a Qiagen Spin Miniprep Kit (Germantown, MD). The concentration of extracted plasmid DNA was then calculated using UV-Vis spectroscopy. The purified plasmid was linearized using *Pvu I* restriction enzymes. Linearization was confirmed by running both pre- and post-linearized plasmids on a 1% agarose gel. Linearized plasmid was purified using ethanol precipitation. The plasmid was then transcribed into RNA using a T7 MEGAscript transcription kit (Ambion, Austin, TX). Appropriate RNA length was confirmed on a denaturing agarose-formaldehyde gel. The N-gene RNA was then quantified using UV-Vis spectroscopy. N-gene RNA was separated into aliquots and stored at -80°C. Ten-fold standard curve dilutions were made in TE buffer (Ambion, Austin, TX) ranging from 10^7 copies per µL to 10^1 per µL, and RT-PCR was performed to create a standard curve comparing the number of RNA copies per µL to cycle threshold (C_t).

Comparison of the extraction kits on patient samples

RT-PCR was performed on 7 positive and 7 negative clinical samples. Comparison were made of the cycle threshold (C_t) and number of copies of RNA per µL based on the RT-PCR results performed on patient samples before RNA extraction (raw sample) and after RNA extractions using the RNeasy Mini kit (Qiagen, Germantown, MD), MagAttract RNA Cell Mini M48 kit (Qiagen, Germantown, MD), Dynabeads mRNA Direct kit (Invitrogen, Oslo, Norway), and the magnetic pull-though capillary method.

RNA Extraction using Magnetic Pull-Through Capillary Method

The magnetic pull-though capillary method was used to isolate RNA from sample lysates. Glass capillary chambers were cut from ¼ inch stock tubing into 80 mm lengths, and the ends were flared outward. The exterior diameter of the chamber is 6mm and the interior diameter of the chambers is 2mm. Six capillary chambers were aligned linearly on the top of a horizontal aluminum stage using machined aluminum mounts. 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 sitting the in flared region of the next capillary chamber. The arrangement of the chambers and spacers is depicted in Figure 3. The MagAttract RNA Cell Mini M48 kit magnetic beads and buffers (Qiagen, Germantown, MD) were used in conjunction with the magnetic pull-though capillary system. The 1st capillary chamber was initially left empty. Chambers 2 to 6 were pre-filled with the processing reagents supplied by Qiagen. The 2nd chamber was filled with 200 µL of MW wash buffer (Qiagen, Germantown, MD). The 3rd and 4th chambers were each with 200 µL of RPE wash buffer (Qiagen, Germantown, MD). The 5th chamber was filled with 200 µL of RNase-free water. The 6th and last chamber was filled with 30 µL RNase-free water at 65º C. Thirty µL of sample lysate was added to 150 µL buffer RLT (Qiagen, Germantown, MD) and vortex. The lysate was then passed though a 20-gauge needle 5 times. Twenty µL of the MagAttract bead solution (Qiagen, Germantown, MD) was added to the lysate and buffer RLT, the mixture was then

vortexed, and placed on a mixer for 5 minutes at room temperature. Two hundred µL of the lysate, buffer RLT, and MagAttract bead sample was then loaded into the first capillary chamber. A 2.54cm cube of grade 40NdFeB magnet (National Imports, Vienna, VA) was placed on top of the first capillary chamber then the magnet was slowly pulled at a rate of ~4mm per second sequentially along all the spacers and capillary chambers, all the way to the 6th and final chamber, the total pull-through time was just under 2 mintues. Once the magnet was on top of the 6th chamber, the elution was collected. An illustration of the movement of the beads across an air spacer is shown in Figure 4. The magnet used generates a magnetic field between 100 and 500mT and has a magnetic field gradient of 45Tm^{-1} (Kuhn, Hallahan, and Giorgio, 2006).

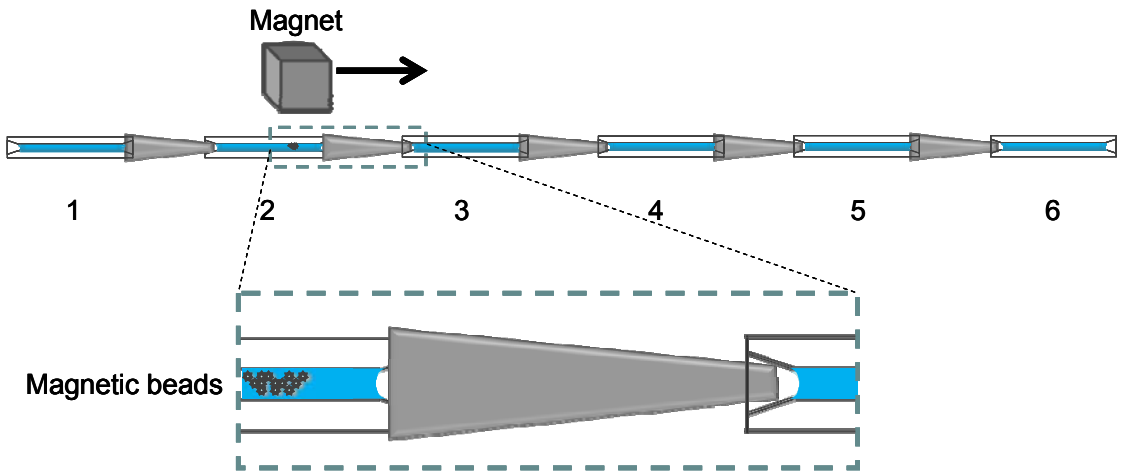

Figure 3. Capillary chambers were arranged sequentially on a horizontal stage. Sample lysate with lysis buffer and magnetic beads are added to the first chamber. As a magnet is moved over the exterior of the capillaries the magnetic beads are pulled through the chambers and transfers the beads to chamber 6. This process washes, rinses and elutes RNA from the sample lysate.

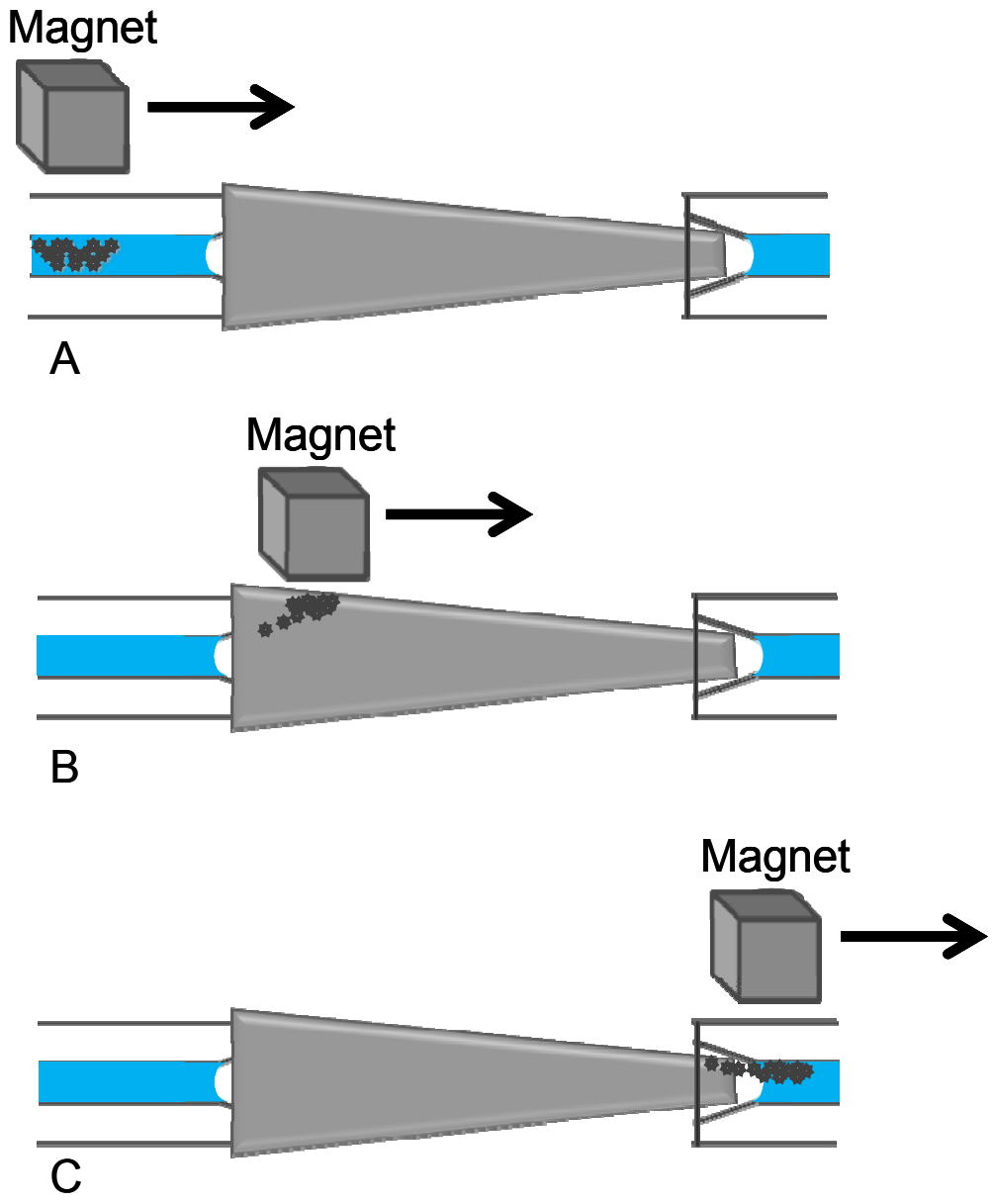

Figure 4. The beads follow the magnet as the magnet is moved from left to right. The small capillary diameter which creates high surface tension and the air spacers allow for movement of the beads from one capillary to the next without transferring buffer from one capillary chamber to the next.

RNA Extraction using RNeasy Mini Kit

The RNeasy Mini kit (Qiagen, Germantown, MD) was used to isolate RNA from sample lysates following the manufacturer's instructions. Briefly, 100 µL of sample lysate was added to 600 µL buffer RLT (Qiagen, Germantown, MD) and then vortexed. The lysate was then passed though a 20-gauge needle 5 times. Then one volume of 70% ethanol was added, then vortexed. Seven hundred µL of the sample was added to the RNeasy spin column (Qiagen, Germantown, MD). The spin column was centrifuged at 10,000 rpm for 15 seconds and the flow-though was discarded. Seven hundred µL buffer RW1 (Qiagen, Germantown, MD) was added to the spin column, the spin column was centrifuged at 10,000 rpm for 15 seconds, and the flow-though was discarded. Five hundred µL buffer RPE (Qiagen, Germantown, MD) was added to the spin column, centrifuged at 10,000 rpm for 15 seconds, and the flow-though was discarded. Five hundred µL Buffer RPE was added to the spin column, centrifuged at 10,000 rpm for 2 minutes, and then the flow-though was discarded. For the elution of the RNA from the spin column,50 µL RNase-free water was added to the spin column and then centrifuged at 10,000 rpm for 1 minute and the flow-though was collected.

RNA Extraction using MagAttract RNA Cell Mini M48 Kit

The MagAttract RNA Cell Mini M48 kit (Qiagen, Germantown, MD) was used to isolate RNA from sample lysates. The extraction was performed

manually instead of with the assistance of the Qiagen BioRobot M48 workstation (Germantown, MD). One hundred µL of sample lysate was added to 600 µL buffer RLT (Qiagen, Germantown, MD) and then vortexed. The lysate was then passed though a 20-gauge needle 5 times. Sixty µL of the MagAttract bead solution (Qiagen, Germantown, MD) was added to the 700 µL of the lysate mixed with lysis buffer, vortexed, and then placed on a mixer for 5 minutes at room temperature. The beads were separated on a magnet, supernatant discarded and 900 µL of MW wash buffer (Qiagen, Germantown, MD) was added and the bead were re-suspended and vortexed. The beads were separated on a magnet, supernatant discarded and 900 µL of RPE wash buffer (Qiagen, Germantown, MD) was added and the beads were re-suspended and vortexed, and this was repeated once more. The beads were separated on a magnet, supernatant discarded and 1 mL RNase-free water was added and pipette 3 times over the beads without moving the beads from the magnet. For elution of the RNA from the magnetic beads 50 µL of RNase-free water at 65º C was added, then beads were re-suspended and vortexed. The beads were separated on a magnet and the elution was collected.

RNA Extraction using Dynabeads mRNA Direct Kit

The Dynabeads mRNA Direct Kit (Invitrogen, Oslo, Norway) was used to isolate RNA the sample lysates following the manufacturers instructions. Briefly, 100 µL of sample lysate was added to 600 µL buffer RLT and then vortexed. The lysate was then passed though a 20-gauge needle 5 times. Two hundred

and fifty µL Dynabeads (Invitrogen, Oslo, Norway) were placed in 1.5 mL eppendorff tube and placed on a magnet, supernatant was removed, and 250 µL lysis/binding buffer (Invitrogen, Oslo, Norway) was added. The beads were separated on a magnet, supernatant was removed, 700 µL of the lysate was added. The beads were vortexed and placed on a mixer for 5 minutes at room temperature. The beads were separated on a magnet, supernatant discarded and 1 mL of washing buffer A (Invitrogen, Oslo, Norway) was added. The beads were re-suspended and then vortexed. The beads were separated on a magnet, supernatant discarded and 1 mL of washing buffer B (Invitrogen, Oslo, Norway) was added and the beads were re-suspended and vortexed., and this was repeated once more. For the removal of the RNA from the beads 50 µL of 10mM Tris-HCL elution buffer (Invitrogen, Oslo, Norway) was added, the beads were re-suspended, vortexed then incubated at 65º C for 2 minutes. The beads were separated on a magnet and the elution was collected.

RNA Extraction kit efficiency test

Extraction methods were compared under idealized lab conditions. Extracted RSV N-gene RNA was diluted in TE buffer (Ambion, Austin, TX) to 10^5 copies per µL. Comparison were made of the threshold cycle (C_t) and number of copies of RNA per µL based on the RT-PCR results performed on the RSV N-gene RNA run without RNA extraction (raw sample) and after RNA extractions using the RNeasy Mini kit (Qiagen, Germantown, MD), MagAttract RNA Cell Mini

M48 kit (Qiagen, Germantown, MD), and the magnetic pull-though capillary method.

Spiked negative clinical samples

The RSV N-gene RNA was added to negative nasal swab samples as means to control the viral concentration in an environment that otherwise resembles a clinical sample. Three spiked negative clinical samples containing 10^5 copies of RNA per µL each were made by adding 20 µL of RSV N-gene RNA to 180 µL of a negative clinical sample. RT-PCR was used to determine the cycle threshold (C_t) of spiked samples before and after extraction using RNeasy Mini kit. Comparison were made of the cycle threshold (C_t) and calculated number of copies of RNA per µL based on the RT-PCR results performed on the spiked negative clinical samples tested without RNA extraction (raw sample) and after RNA extractions using the RNeasy Mini kit (Qiagen, Germantown, MD).

Results and Discussion

Comparison of extraction under ideal conditions

A comparison of viral RNA extraction from TE buffer found that the RNeasy Mini kit was the most efficient with a recovery of 37.8% ± 17.3%, Qiagen MagAttract was second with 27.0% ± 4.8%, and the magnetic pull-through was the least efficient with a recovery of 11.7% ± 4.8%. These results are shown in Figure 5, the raw data for the threshold cycle can be found in Appendix Table 1,

the raw data for the number of copies can be found in Appendix Table 2, and the number of copies normalized to the original can be found in Appendix Table 3. The efficiency of the all extraction methods including the commercial kits was

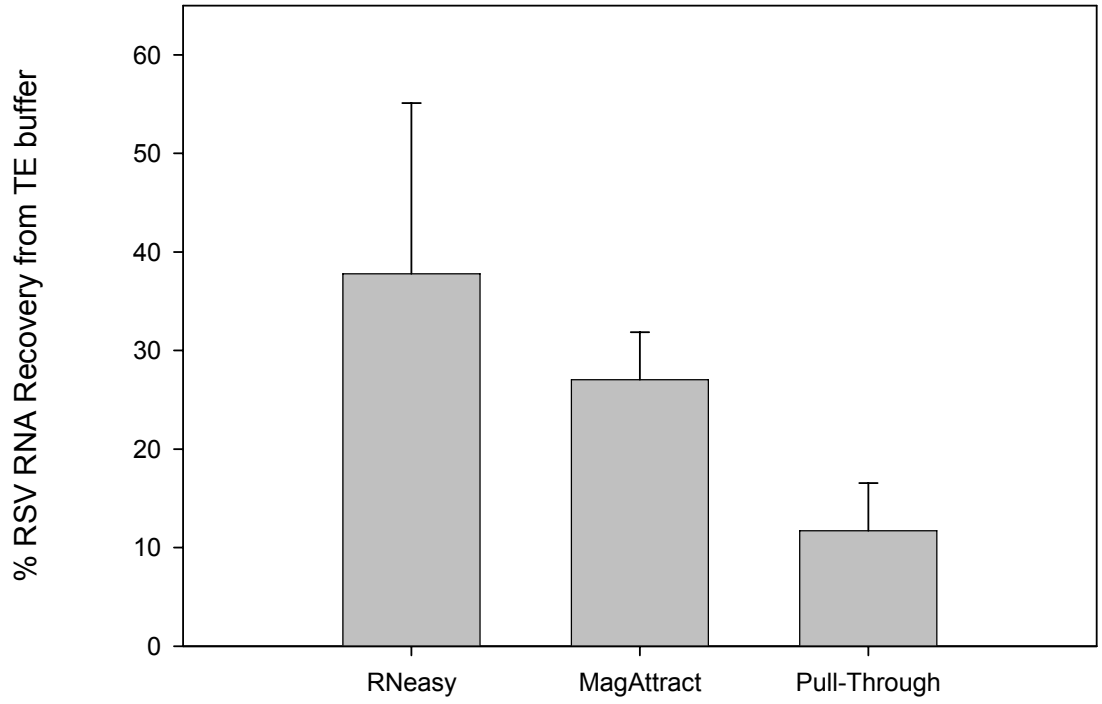

Figure 5. Comparisons were made of the number of copies of RNA per µL before and after extraction from TE buffer. The percent recovery was calculated by dividing the copy number values for each extraction method by the copy number obtained before extraction. Tests were run in triplicate and shown as means ± standard deviation (N=3).

lower than expected especially considering that the RNA was being extracted from TE buffer and not from a clinical samples containing potential contaminants. The lower performance of the MagAttract kit as compared to the RNeasy kit is

not surprising considering that particular kit is designed to work most optimally with the use of the Qiagen BioRobot M48 workstation, and since the extraction was performed manually it was expected that the extraction would not be as effective. Although the magnetic pull-through capillary method did not perform as well as the commercial kits did, the results show that this method extracts a significant fraction of viral RNA which is readily detected by RT-PCR.

Extraction from negative clinical samples

The number of RSV RNA copies per µL, as measured by RT-PCR, of un-isolated negative clinical sample spiked with 10^5 RSV RNA was calculated to be 11,000 ± 1,500 copies per µL. The same negative clinical sample spiked with RSV RNA and isolated using the RNeasy Mini kit showed a much higher copy number of 27,000 ± 7,020 copies per µL. The cycle threshold data is listed in Appendix Table 4 and the copy number data can be found in Appendix Table 5. The data was normalized to the original copy number present in the spiked negative clinical sample, and this data is listed in Appendix Table 6. The results for the percent recovery of RSV RNA from spiked negative clinical samples are shown in Figure 6. The percent recovery before extraction was 11% ± 1% RSV RNA and after extraction was 28% ± 7% RSV RNA. The lower copy number and low percent recovery of the raw sample shows the negative effects which contaminants within patient samples have on viral detection methods such as RT-PCR. The over 2-fold increase in copy number and percent recovery after extraction confirms that running an extraction is necessary to rid the sample of

contaminants which affect the results of RT-PCR. The comparison of RSV RNA recovery from TE buffer and from spiked negative clinical samples after extraction using the RNeasy Mini kit is shown in Figure 7. There is a higher percent recovery when extracting from TE buffer than from a sample which

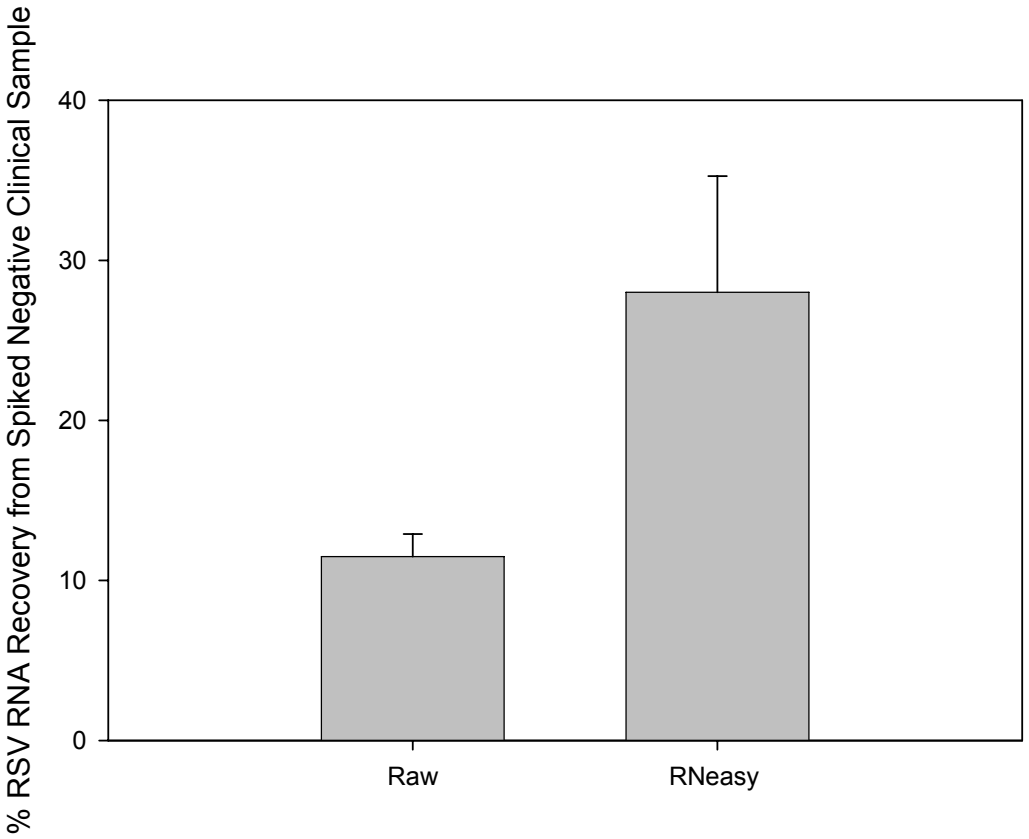

Figure 6. Comparisons were made of the number of copies of RNA per µL before and after extraction using RNeasy Mini kit from negative clinical samples spiked with RSV N-gene RNA. The percent recovery was calculated by dividing the copy number values for before and after extraction by the copy number known to be in the sample. Tests were run in triplicate and copy number was calculated and shown as means ± standard deviation (N=3).

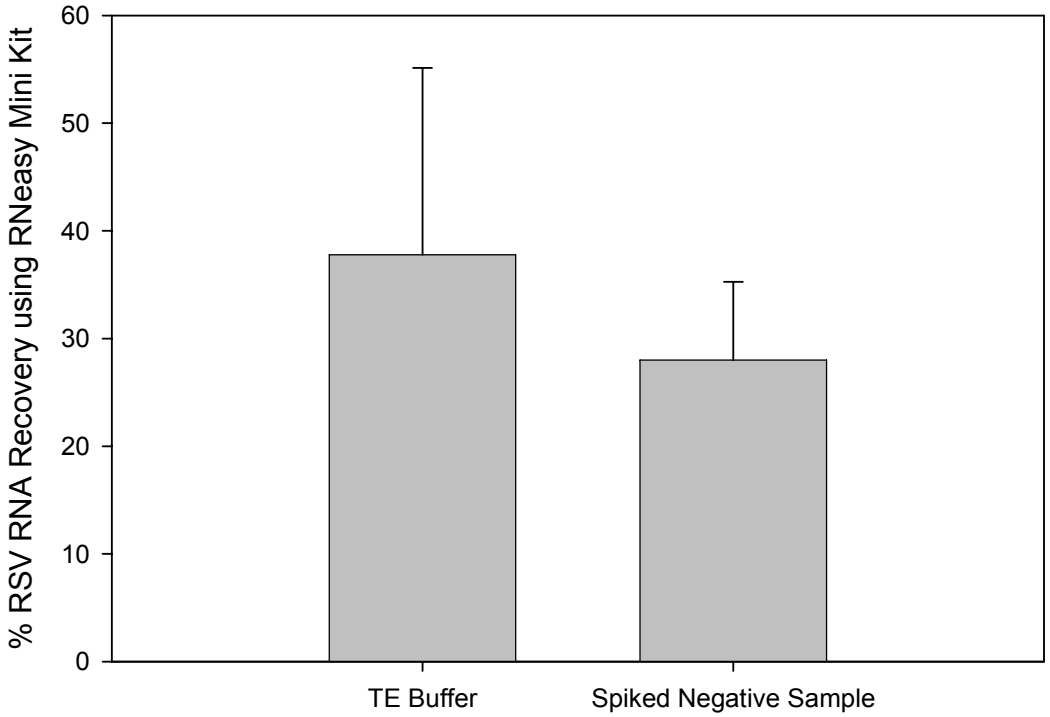

Figure 7. Comparison of RSV RNA recovery from TE buffer and from spiked negative clinical samples after extraction using the RNeasy Mini kit. The percent recovery was calculated by dividing the copy number values for before and after extraction by the copy number known to be in the sample. Tests were run in triplicate and copy number was calculated and shown as means ± standard deviation (N=3).

contains containments, so although extraction is necessary to remove containments it does not remove all containments which affect RT-PCR results.

Comparison of the extraction kits on patient samples

The number of RSV RNA copies per µL, as measured by RT-PCR, of un-extracted RSV positive clinical samples was calculated to be 3 ± 3 copies per µL. The same RSV positive clinical samples after extraction was shown to be 4,400 ± 10,000 copies per µL for extractions done using RNeasy Mini kit, µL 750 ± 1,300 copies per µL for extractions done using Dynabeads Direct kit, 940 ± 1,000 copies per µL for extractions done using MagAttract kit, and 510 ± 800 copies per

µL for extractions done using the magnetic pull-through capillary method. The number of RSV RNA copies per µL, as measured by RT-PCR, of un-isolated RSV negative clinical samples was calculated to be 0.1 ± 0.2 copies per µL and the same RSV positive clinical samples after running extractions was shown to be 0.7 ± 1.8 copies per µL for extractions done using RNeasy Mini kit, µL 0.1 ± 0.2 copies per µL for extractions done using Dynabeads Direct kit, 0.1 ± 0.4 copies per µL for extractions done using MagAttract kit, and 1.0 ± 1.8 copies per µL for extractions done using the magnetic pull-through capillary method. These results are shown in Figure 8. The data for RSV positive clinical sample cycle threshold is in Appendix Table 7, data for RSV negative clinical sample cycle threshold is in Appendix Table 8, data for RSV positive clinical sample copy number is in Appendix Table 10, data for RSV negative clinical sample copy number is in Appendix Table 11. There is high variation in the average data for each kit tested but this variation is largely due to the variation of the clinical samples. Although there is also variation amongst the extraction methods and to show this variation a comparison of the cycle threshold (C_t) of the positive clinical samples was made and the samples were ranked in order of lowest value to highest. A listing of the ranking for before and after extraction with the various methods is shown in Appendix Table 9.

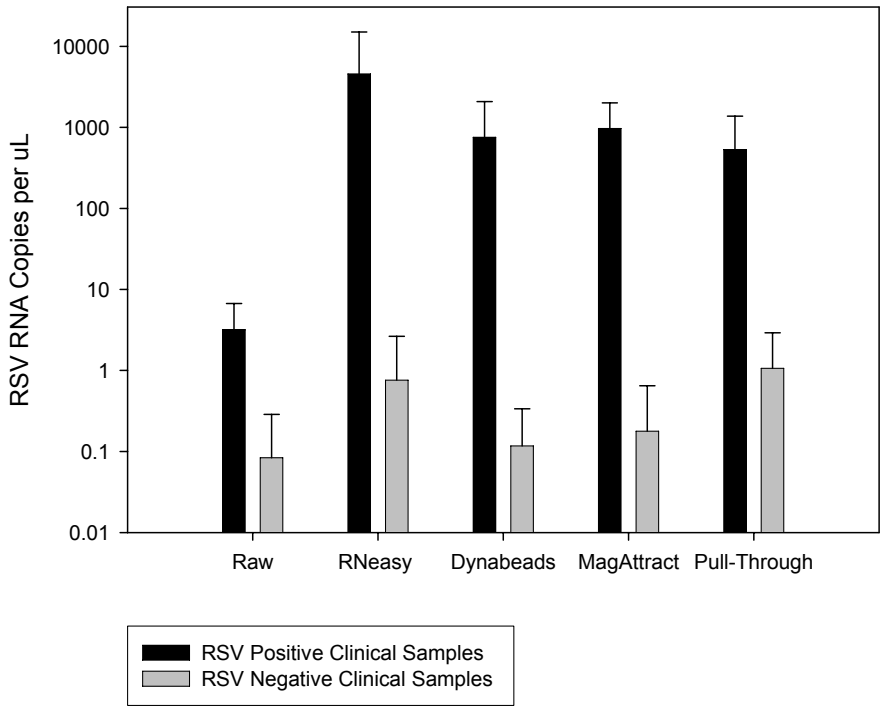

Figure 8. Comparisons were made of the number of copies of RNA per µL before and after extraction from positive clinical samples (black bars) and negative clinical samples. Extractions were performed on 7 positive and 7 negative clinical samples using RNeasy Mini kit, Dynabeads mRNA Direct kit, MagAttract RNA Cell Mini M48 kit, and the magnetic pull-through capillary method. Tests were performed in triplicate and results are shown as means ± standard deviation (N=7). Note the y log-scale, which tends to mask the high standard deviations are greater than they appear.

The best extraction technique for use in a physician's office would be simple, safe, fast, cheap, and effective. The data shows the magnetic pull-though capillary method performs similarly to several frequently used commercial kits. The steps for extraction, time necessary and the equipment needed are listed in Appendix Table 12 for the RNeasy kit, Appendix Table 13 for the Dynabead kit, Appendix Table 14 for the MagAttract kit, and Appendix Table 15

for the magnetic pull-through capillary method. Comparisons of the kits are shown in Appendix Table 16. The magnetic pull-through capillary methods does not require as much sample or buffer volume per extraction. The magnetic pull-through capillary method has the fewest steps compared with the commercially available kits. The majority of the time and the extra equipment needed for the magnetic pull-through capillary method are used in the disruption and homogenization of the clinical sample. Greater optimization of the disruption and homogenization could eliminate most of the time necessary to run the extraction and could eliminate the use of a 20-gauge needle and syringe, vortex, and a rotating mixer, which would remove most of the extra equipment. Comparing the performance and the steps, time, and equipment needed for performing the extraction the magnetic pull-through capillary method would be the best for use in a clinical setting. Although the trade-off is a lower efficiency performance, it is the quickest, contains the least number of steps, and requires little additional equipment.

Conclusion

In summary, the current magnetic pull-through capillary RNA extraction method is useful in the extraction of RNA from clinical samples. It is simpler, less time consuming, and requires less additional equipment. This design has the potential to be useful in a clinical setting. Further optimization of the magnetic pull-through capillary RNA extraction method is necessary to improve extraction efficiency.

APPENDIX

Table 1. Threshold cycle (Ct)) based on the RT-PCR results were calculated for three samples containing RSV N-gene RNA that were performed without RNA extraction (raw sample) and after RNA extractions using the RNeasy Mini kit, MagAttract RNA Cell Mini M48 kit, and the magnetic pull-though capillary method. Tests were run in triplicate.

Efficiency Test of Isolation Methods (Threshold Cycle (C_t))				
Sample #	**Raw**	**RNeasy**	**MagAttract**	**Pull-Through**
Sample 1	14.62	17.09	16.37	18.78
Sample 2	14.58	16.11	16.88	17.84
Sample 3	14.58	15.51	16.65	17.37
Average	14.59	16.24	16.63	18.00
Standard Deviation	0.03	0.79	0.25	0.72

Table 2. The number of copies of RSV RNA per µL based on the RT-PCR results were calculated for three samples containing RSV N-gene RNA that were performed without RNA extraction (raw sample) and after RNA extractions using the RNeasy Mini kit, MagAttract RNA Cell Mini M48 kit, and the magnetic pull-though capillary method. Tests were run in triplicate.

Efficiency Test of Isolation Methods (RSV RNA Copies per µL)				
Sample #	**Raw**	**RNeasy**	**MagAttract**	**Pull-Through**
Sample 1	1000000	200000	320000	66000
Sample 2	1000000	380000	220000	120000
Sample 3	1000000	560000	270000	160000
Average	1000000	380000	270000	115333
Standard Deviation	0	180000	50000	47173

Table 3. The number of copies of RSV RNA per µL based on the RT-PCR results were calculated for three samples containing RSV N-gene RNA that were performed without RNA extraction (raw sample) and after RNA extractions using the RNeasy Mini kit, MagAttract RNA Cell Mini M48 kit, and the magnetic pull-though capillary method. The copies numbers for the samples after RNA extractions were normalized to the raw sample ran without RNA extraction, and the results were averaged. Tests were run in triplicate.

Efficiency Test of Isolation Methods (Normalized to Raw/Original)				
Sample #	**Raw**	**RNeasy**	**MagAttract**	**Pull-Through**
Sample 1	1	0.20	0.32	0.07
Sample 2	1	0.38	0.22	0.12
Sample 3	1	0.55	0.27	0.16
Normalized Average	1	0.38	0.27	0.12
Normalized Standard Deviation	0	0.17	0.05	0.05

Table 4. Threshold cycle (Ct)) based on the RT-PCR results were calculated and averaged for three samples containing RSV N-gene RNA that was added to negative nasal swab samples as means to control the viral concentration in an environment that otherwise resembles a clinical sample. Tests were run in triplicate.

Spiked Negative Samples (Cycle Threshold (C_t))		
Sample ID	**Raw**	**RNeasy**
1-06-171	32.80	29.61
1-06-174	33.28	30.50
2-06-048	33.55	31.29
Average	33.21	30.47
Standard Deviation	0.38	0.84

Table 5. The number of copies of RSV RNA per µL based on the RT-PCR results were calculated and averaged for three samples containing RSV N-gene RNA that was added to negative nasal swab samples as means to control the viral concentration in an environment that otherwise resembles a clinical sample. Tests were run in triplicate.

Spiked Negative Samples (RSV RNA Copies per µL)			
Sample ID	**Original**	**Raw**	**RNeasy**
1-06-171	100000	13000	35000
1-06-174	100000	11000	27000
2-06-048	100000	10000	21000
Average	100000	11333	27667
Standard Deviation	0	1528	7024

Table 6. The number of copies of RSV RNA per µL based on RT-PCR results for samples before and after extraction contain RSV N-gene RNA that was added to negative nasal swab clinical samples was normalized to the original copy number placed in the sample.

Spiked Negative Samples (Normalized to Original)			
Sample ID	**Original**	**Raw**	**RNeasy**
1-06-171	1	0.13	0.36
1-06-174	1	0.11	0.27
2-06-048	1	0.10	0.21
Normalized Average	1	0.11	0.28
Normalized Standard Deviation	0	0.01	0.07

Table 7. Threshold cycle (Ct)) based on the RT-PCR results were calculated and averaged for seven RSV positive clinical samples. Tests were run in triplicate.

RSV Positive Clinical Samples (Cycle Threshold (Ct))						
Sample ID	**William's Lab**	**Raw**	**RNeasy**	**Dynabead**	**MagAttract**	**Pull-Through**
1-06-012	21.86	34.09	22.49	27.07	26.73	26.93
1-06-018	23.25	35.78	23.20	21.49	27.59	26.32
1-06-088	22.83	30.78	25.62	32.94	35.66	24.63
2-06-025	19.84	31.14	18.12	22.36	23.06	24.11
2-06-027	23.82	32.33	23.03	27.73	25.62	26.20
1-06-159	20.79	36.54	23.12	28.30	22.03	21.98
1-06-163	23.68	32.98	25.73	26.25	23.16	31.27
Average	22.30	33.38	23.04	26.59	26.26	25.92
Standard deviation	1.52	2.21	2.53	3.85	4.63	2.90

Table 8. Threshold cycle (Ct)) based on the RT-PCR results were calculated and averaged for seven RSV negative clinical samples. Tests were run in triplicate. The – indicates that the sample did not come up before the 40th cycle, therefore no cycle threshold was calculated.

Virus Negative Samples (Cycle Threshold (Ct))						
Sample ID	**William's Lab**	**Raw**	**RNeasy**	**Dynabead**	**MagAttract**	**Pull-Though**
1-06-014	-	-	-	-	-	-
2-06-010	-	35.12	-	-	-	-
4-06-011	-	-	39.19	-	-	-
1-06-090	-	39.11	36.19	35.04	-	31.84
1-06-097	-	-	31.62	36.34	33.81	32.39
4-06-027	-	-	-	-	-	-
1-06-157	-	-	-	-	-	-
Average	-	37.12	35.67	35.69	33.81	32.11
Standard deviation	-	2.82	3.81	0.92	0.00	0.39

Table 9. Ranking of positive clinical samples based on calculated cycle threshold (Ct) values.

RSV Positive Clinical Samples Ranking Based on (Cycle Threshold (Ct))						
Sample ID	**William's Lab**	**Raw**	**RNeasy**	**Dynabead**	**MagAttract**	**Pull-Through**
1-06-012	3	5	2	4	5	6
1-06-018	5	6	5	1	6	5
1-06-088	4	1	6	7	7	3
2-06-025	1	2	1	2	2	2
2-06-027	7	3	3	5	4	4
1-06-159	2	7	4	6	1	1
1-06-163	6	4	7	3	3	7

Table 10. The number of copies of RSV RNA per µL based on the RT-PCR results were calculated and averaged for seven RSV positive clinical samples. Tests were run in triplicate.

RSV Positive Samples (RSV RNA Copies per µL)					
Sample ID	**Raw**	**RNeasy**	**Dynabead**	**MagAttract**	**Pull-Through**
1-06-012	1	1600	90	110	98
1-06-018	0.3	1000	3100	64	140
1-06-088	8	220	2	850	420
2-06-025	6	26000	1800	2200	590
2-06-027	3	1100	59	220	150
1-06-159	0.2	1100	41	2200	2200
1-06-163	2	210	150	1000	6
Average	2.93	4461	749	949	515
Standard deviation	3.26	10214	1307	1016	807

Table 11. The number of copies of RSV RNA per µL based on the RT-PCR results were calculated and averaged for seven RSV negative clinical samples. Tests were run in triplicate.

Virus Negative Clinical Samples (RSV RNA Copies per µL)					
Sample ID	**Raw**	**RNeasy**	**Dynabead**	**MagAttract**	**Pull-Though**
1-06-014	0.00	0.00	0.00	0.00	0.00
2-06-010	0.54	0.00	0.00	0.00	0.00
4-06-011	0.00	0.04	0.00	0.00	0.00
1-06-090	0.04	0.28	0.57	0.00	4.37
1-06-097	0.00	5.00	0.25	1.25	3.07
4-06-027	0.00	0.00	0.00	0.00	0.00
1-06-157	0.00	0.00	0.00	0.00	0.00
Average	0.08	0.76	0.12	0.18	1.06
Standard deviation	0.20	1.87	0.22	0.47	1.85

Table 12. List of steps, total time and extra supplies needed to perform an extraction using the Qiagen RNeasy Mini kit on a sample prior to performing RT-PCR.

Qiagen RNeasy Mini Kit		
Steps	**Time (s)**	**Extra Supplies needed**
Disruption and homogenization		
1 Add 600uL Buffer RLT to cells	10	Pipet, vortex
2 Pass lysate through a 20-gauge needle (5x)	25	Syringe, 20-gauge needle
3 Add 1 volume 70% ethanol	10	Pipet
RNA isolation		
4 Add 700uL sample to RNeasy spin column	10	Pipet,
5 Centrifuge at 10,000rpm	15	Centrifuge
6 Discard flow-through	10	Pipet
7 Add 700uL Buffer RW1	10	Pipet, vortex
8 Centrifuge at 10,000rpm	15	Centrifuge
9 Discard flow-through	10	Pipet
10 Add 500uL Buffer RPE	10	Pipet, vortex
11 Centrifuge at 10,000rpm	15	Centrifuge
12 Discard flow-through	10	Pipet
13 Add 500uL Buffer RPE	10	Pipet, vortex
14 Centrifuge at 10,000rpm	120	Centrifuge
15 Place RNeasy spin column in new collection tube	10	
16 Add 35-50 uL RNase-free water	10	Pipet, vortex
17 Centrifuge at 10,000rpm	60	Centrifuge
Total	**360** s	
	6 min	

Table 13. List of steps, total time and extra supplies needed to perform an extraction using the Invitrogen Dynabeads mRNA Direct kit on a sample prior to performing RT-PCR.

Invitrogen Dynabeads mRNA Direct Kit		
Steps	**Time (s)**	**Extra Supplies needed**
Preparation of Dynabeads Oligo(dT)25		
1 Resuspend Dynabeads	60	Vortex
2 Transfer 250uL of beads from stock tube	10	Pipet
3 Place on magnet (when suspension is clear)	30	Magnet
4 Remove supernatant	10	Pipet
5 Remove from magnet	2	
6 Add 250ul Lysis/Binding Buffer	10	Pipet, vortex
Preparation of Lysate from Cultured Cells/ Cell Suspensions		
7 Add 1250ul Lysis/Binding Buffer to lysate	10	Pipet
8 Pass lysate through a 20-gauge needle (5x)	25	Syringe, 20-gauge needle
9 Centrifuge to reduce foam	10	Centrifuge
Direct mRNA Isolation Protocol		
10 Place beads on magnet	30	Magnet
11 Remove Lysis/Binding Buffer from beads	10	Pipet
12 Remove from magnet	2	
13 Add sample lysate	10	Pipet, vortex
14 Incubate with continuous mixing at RT	300	Rotating mixer
15 Place on magnet	120	Magnet
16 Remove supernatant	10	Pipet
17 Add 1mL Washing Buffer A	10	Pipet, vortex
18 Place on magnet	30	Magnet
19 Remove supernatant	2	Pipet
20 Add 1mL Washing Buffer A	10	Pipet, vortex
21 Place on magnet	30	Magnet
22 Remove supernatant	2	Pipet
23 Add 1mL Washing Buffer B	10	Pipet, vortex
24 Place on magnet	30	Magnet
25 Remove supernatant	2	Pipet
26 Add 25uL of 10mM Tris-HCL (Elution Buffer)	10	Pipet, vortex
27 Incubate at 65-80 C	120	Heating block
28 Place on magnet	10	Magnet
29 Remove supernatant	30	Pipet
30 Transfer supernatant to new Rnase-free tube	10	Pipet
Total	**955** s	
	16 min	

Table 14. List of steps, total time and extra supplies needed to perform an extraction using the Qiagen MagAttract RNA Cell Mini M48 kit on a sample prior to performing RT-PCR.

Qiagen MagAttract RNA Cell Mini M48 Kit		
Steps	**Time (s)**	**Extra Supplies needed**
Disruption and homogenization		
1 Add 720uL Buffer RLT to cells	10	Pipet, vortex
2 Pass lysate through a 20-gauge needle (5x)	25	Syringe, 20-gauge needle
RNA Isolation Protocol (manual, w/o use of BioRobot)		
3 Add 60uL bead solution	10	Pipet, vortex
4 Incubate with continuous mixing at RT	300	Rotating mixer
5 Separate on magnet	10	Magnet
6 Discard supernatant	10	Pipet
7 Add 900uL MW Wash Buffer	10	Pipet, vortex
8 Separate on magnet	10	Magnet
9 Discard supernatant	10	Pipet
10 Add 900uL RPE Wash Buffer	10	Pipet, vortex
11 Separate on magnet	10	Magnet
12 Discard supernatant	10	Pipet
13 Add 900uL RPE Wash Buffer	10	Pipet, vortex
14 Separate on magnet	10	Magnet
15 Discard supernatant	10	Pipet
16 Add 1mL H2O, pipet 3x	10	Pipet
17 Discard supernatant	10	Pipet
18 Add 50-200uL RNase free water at 65C	10	Pipet, heat block
19 Separate on magnet	10	Magnet
20 Collect the eluate	10	Pipet
Total	**505** s	
	8 min	

Table 15. List of steps, total time and extra supplies needed to perform an extraction using the magnet pull-through capillary RNA extraction method on a sample prior to performing RT-PCR.

Magnet Pull-Through Capillary RNA Extraction Method		
Steps	**Time (s)**	**Extra Supplies needed**
Disruption and homogenization		
1 Add 150uL Buffer RLT to cells	10	Pipet, vortex
2 Pass lysate through a 20-gauge needle (5x)	25	Syringe, 20-gauge needle
RNA Isolation Protocol (manual, w/o use of BioRobot)		
3 Add 30uL bead solution	10	Pipet, vortex
4 Incubate with continuous mixing at RT	300	Rotating mixer
5 Load 200uL of lysate into the first chamber	10	Pipet
6 Load 200uL of MW Wash buffer into the second chamber	10	Pipet
7 Load 200uL of RPE Wash Buffer into the third chamber	10	Pipet
8 Load 200uL of RPE Wash Buffer into the fourth chamber	10	Pipet
9 Load 200uL of RNase-free water into the fifth chamber	10	Pipet
10 Load 30uL of RNase-free water into the sixth chamber	10	Pipet
11 Drag magnet from first to last chamber	60	Magnet
12 Collect the elute	10	Pipet
Total	**475** s	
	8 min	

Table 16. Comparison of the number of steps, total time and extra supplies needed to perform an extraction using the Qiagen RNeasy Mini kit, Invitrogen Dynabeads mRNA Direct kit, Qiagen MagAttract RNA Cell Mini M48 kit, and the magnet pull-through capillary RNA extraction method on a sample prior to performing RT-PCR.

Isolation Method	Total Time (mins)	No. of Steps	Extra Instrumentation
Raw sample, no isolation	0	0	
Qiagen RNeasy Mini Kit	6	17	Pipet, syringe, 20-gauge needle, vortex, centrifuge
Invitrogen Dynabeads mRNA Direct Kit	16	30	Pipet, syringe, 20-gauge needle, vortex, centrifuge, heating block, rotating mixer, magnet
Qiagen MagAttract RNA Cell Mini M48 Kit	8	20	Pipet, syringe, 20-gauge needle, vortex, heating block, rotating mixer, magnet
Magetic Pull-Through Capillary Method	8	12	Pipet, syringe, 20-gauge needle, vortex, rotating mixer, magnet

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