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Methodological Improvements in the mRNA Profiling Assays for Incorporation into DNA Casework Workflows

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METHODOLOGICAL IMPROVEMENTS IN THE MRNA PROFILING ASSAYS FOR INCORPORATION INTO DNA CASEWORK WORKFLOWS

by

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ABSTRACT

Currently, DNA profiling is the gold standard to identify an individual. However, determining body fluid origin is important in criminal investigations, offering additional information surrounding the circumstances of a crime. However, crime labs can only definitively identify blood and semen and presumptively saliva using techniques that consume time and sample and do not simultaneously identify all forensically relevant body fluids. This causes many crime labs to want to bypass body fluid identification altogether. Therefore, advances into more definitive molecular-based body fluid methods are necessary. One such technique is mRNA profiling because it provides a highly sensitive and specific approach to definitively identifying all relevant body fluids in parallel. Although advancements have been made, improvements to mRNA profiling methodologies still need to be researched such as 1) possible mRNA recovery from established DNA workflows and 2) possible integration of mRNA profiling into an upfront male DNA screening assay for triaging sexual-assault evidence likely to contain male DNA and reduce/eliminate a significant bottleneck in the standard DNA workflow of microscopic sperm identification. This study was designed to address these two issues by evaluating a novel way to recover RNA, for body fluid identification, from the waste fractions of a PrepFiler™ DNA extraction, and from the DNA extracts directly. Next, this study aimed to provide a relatively quick molecular-based approach for screening sexual-assault evidence. It involves extraction of RNA using the Dynabeads™ mRNA DIRECT™ Kit, while saving the extraction waste fractions for downstream male-DNA quantitation and STR profiling. The RNA

is then used in a rapid and sensitive 1-step combined reverse transcription-HRM assay to positively detect the presence of sperm. Both non-conventional co-extraction methods successfully addressed current body fluid identification challenges and allowed for easy integration into existing workflows when single sourced, mixture and mock casework samples were analyzed.

To my fiancé James

and to my family and friends

for all of their unconditional love and support throughout this journey.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

µL	Microliters
Amp	Amplification
B	Blood
cDNA	Complementary DNA
CE	Capillary Electrophoresis
DFSC	Defense Forensic Science Center
DNA	Deoxyribonucleic acid
ESR	The Institute of Environmental Science and Research
HRM	High Resolution Melt
ID	Identification
MB	Menstrual Blood
Min	Minutes
mL	Milliliters
mRNA	Messenger RNA
NCFS	National Center for Forensic Science
ng	Nanograms
PCR	Polymerase Chain Reaction
PolyA	Polyadenylated
PRM2	Protamine 2

PSA	Prostate-specific antigen
RFD	RNA from DNA
RFU	Relative Fluorescence Unit
RNA	Ribonucleic acid
RT-HRM	Reverse Transcription-High Resolution Melt
S	Saliva
SAKs	Sexual Assault Kits
SE	Semen
Sec	Seconds
SEMG1	Semenogelin 1
STR	Short Tandem Repeat
SMART	Science, Mathematics and Research for Transformation Scholarship Program
VS	Vaginal Secretion
Y-STR	Y-chromosome short tandem repeat

CHAPTER ONE: INTRODUCTION

Routinely, evidence gathered from a crime scene, believed to contain human biological material, is analyzed for human genomic DNA to identify the contributor(s) of a stain. Although DNA profiling is the gold standard [1] it has also become increasingly important that crime labs determine the origin of the stain to aid investigators in better understanding the circumstances surrounding a crime. In circumstances such as sexual-assaults, it is important to not only identify those involved but to prove the presence of male specific body fluids such as semen and even more specifically sperm. In some crime labs, evidence goes through a preliminary screening process to confirm the presence of specific body fluids, but they are often not confirmatory for all relevant body fluids such as saliva, vaginal secretions, menstrual blood, and skin. These techniques are also very time and sample consuming, do not allow simultaneous detection of all forensically relevant body fluids, and are not easily integrated into DNA workflows [2, 3]. Therefore, some crime labs are aiming to bypass body fluid identification altogether and go straight into DNA analysis, which can be detrimental in court if the evidence is misrepresented [4].

Currently, crime labs use chemical or enzymatic assays to presume the presence of some forensically relevant body fluids [1, 3, 5-7]. These include presumptive body fluid testing for blood (Phenolphthalein, Luminol, Hemastix® (Sirchie Youngsville, NC) [1, 3, 5-7]), semen (acid phosphatase, and ABAcard® p30 (Abacus Diagnostics® West Hills, CA) [1, 3, 5-8]), and saliva (Phadebas® Amylase test (Magle Life Sciences Cambridge, MA), SALIgAE® (Abacus Diagnostics®), RSID™ Saliva Kit (Independent Forensics Lombard, IL) [1, 3, 5-7]), but most often these tests are not sensitive enough and can produce false-positives in the presence of other

body fluids. Although there are confirmatory tests for blood (ABAcard® HemaTrace® (Abacus Diagnostics®) or RSID™-blood test (Independent Forensics Lombard, IL) [1, 3, 5-7]) and semen (microscopic examination with Christmas Tree staining [1, 6, 8-10]), they are very time consuming (particularly microscopic identification of sperm), use a lot of sample and do not allow for the positive identification of saliva, vaginal secretions or menstrual blood [1, 3, 5-7].

Depending on the nature of the crime, a positive identification of male specific body fluids such as spermatozoa, semen or seminal fluid can play a key role in determining the circumstances surrounding a crime. When a victim believes he or she has been sexually assaulted a medical professional generally uses a sexual assault kit (SAK) to collect evidence that could have been left behind. These kits vary, but often include swabs and collection envelopes for any loose biological materials or undergarments [11]. In sexual assault crimes involving sexual intercourse, whether oral, vaginal or anal, it is important to establish the body fluids that are present on pieces of evidence. Often times, SAKs are first screened for the presence of these body fluids and if there is not a positive identification, further testing might not be performed [6, 11].

Current male DNA screening methods include testing for the presence of seminal acid phosphatase, an enzyme present in semen [6, 7], or a rapid immunochromatographic assay for the presence of prostate-specific antigen (PSA) that is found in semen [8, 9]. Although these tests are able to rapidly screen SAK evidence, they are often times used presumptively because they do not confirm the presence of spermatozoa and have a tendency to produce false positives when in the presence of some vaginal specific enzymes or proteins [1, 6, 8, 9]. Currently, the only confirmatory tests performed in crime labs to determine the presence of human spermatozoa is the microscopic analysis of sperm cells using a Christmas tree stain. Microscopic examination of

sperm entails an analyst physically counting the total number of sperm present on a slide that has been stained with dyes for better visualization of single cells (Nuclear Fast Red and picroindigocarmine, hematoxylin-eosin [12] or SPERM HY-LITER™ (Independent Forensics) [13]. This method is by no means rapid, sometimes subjective, and must often times be used in combination with the presumptive tests when little to no sperm is present or are difficult to distinguish amongst larger epithelial cells to ensure the presence of male DNA or that the semen donor is vasectomized (azoospermic) [6, 14, 15].

Consequently, a major bottleneck with microscopic sperm identification is that it causes major backlogs in the processing of SAKs as well as other sexual-assault related evidence [14]. Because a large amount of a crime lab's backlog includes SAKs and other sexual assault-related evidence that has been received but has not yet been processed [11, 16], the federal government addressed the problem and made funding available for research into addressing the backlog. Under the Sexual Assault Forensic Evidence Reporting Act of 2013, DNA evidence specifically from a SAK or related sexual-assault crime must be processed quickly while still following proper protocol to ensure quality analysis [17]. However, because some crime labs want to move away from traditional body fluid ID, there is a significant need for more rapid molecular-based techniques in order to provide critical sample information in a more timely manner [2, 3, 18].

In recent years, there have been many advancements in the development of molecular-based body fluid assays that aim to improve upon currently used body fluid identification methods. Some such studies include the use of tissue-specific epigenomic modifications to study gene expression patterns through DNA methylation [5, 19, 20], the exploration of tissue-specific post-translational modifications using protein-biomarkers [21], and the use of body fluid or

tissue specific messenger RNA transcripts, better known as mRNA profiling [1-7, 10, 18, 19, 22-28]. Amongst these methods, mRNA profiling has been well studied in regard to body fluid identification and shows great promise for integration into current DNA workflows [25, 29, 30].

RNA, specifically messenger RNA, is transcribed directly from DNA and plays an active role in controlling gene expression that further directs protein synthesis. In certain cells, specific genes are expressed, turned on, and others are turned off allowing for the synthesis of certain proteins. The fluids and tissues that are of interest in forensics, such as semen, blood, menstrual blood, vaginal secretions, saliva, and skin [1-4, 10, 22-29, 31, 32] contain a mixture of multiple cell types that collectively encompass the multicellular transcriptome. It is this multicellular transcriptome that can be targeted for mRNA profiling because it is unique to each body fluid or tissue. Although, in biochemical applications, RNA is generally known to be highly degradative due the 2'-OH in ribose and the presence of ribonucleases, it has been found to be highly stable in biological evidence [1, 2, 6, 33]. Once mRNA is reversely transcribed back into its original complementary DNA (cDNA) transcript, the transcripts can be amplified using techniques (PCR amplification, capillary electrophoresis (CE)) and equipment that are used routinely in operational crime laboratories, thus reducing the need for additional training or specialized equipment. One such method uses multiplex reverse transcription-polymerase chain reaction (RT-PCR) followed by CE [2, 4, 22-29], to detect the amplified product once fluorescently-labeled primers are incorporated (Not yet published; Table 3) to target each mRNA sequence. They are detected much like how short tandem repeats (STRs) are detected in DNA profiling. Another method that is not typically used for mRNA profiling but has been well studied for its use in variant detection is High Resolution Melt (HRM) analysis [29, 34, 35]. Recent research has proven the ability to monitor a gene-specific marker in real time with the use of non-

fluorescently labeled primers and a saturating intercalating fluorescent dye [34, 35] such as Eva Green that preferentially binds to double stranded DNA. Because the primers are unlabeled, in contrast to CE based methods that use fluorescently labeled primers [22, 26, 27, 31, 32, 34, 35], it allows a lower cost alternative that provides the same or even greater sensitivity. When a reverse transcription combined HRM assay is performed, the simultaneous conversion of mRNA to cDNA followed by a traditional PCR reaction can be monitored in real-time. Followed by a final melting stage, the level of fluorescence can once more be monitored in real-time and the temperature at which the two strands dissociate 50% can be detected. The real-time software takes the negative first derivative of fluorescence (F) in relation to temperature (T)(-dF/dT) [34, 35] and produces a unique melt-curve that is amplimer (also referred to as amplicon) sequence and assay specific. In a single-plex assay containing one gene-specific primer there is a single melting curve, but multiplex assays are also possible that can allow for multiple melting curves to be detected simultaneously [26, 27, 34, 35].

While the application of RNA in body fluid identification has shown to be robust, it is essential that DNA and RNA can be co-extracted from the same portion of stain, thus reducing the effect stain composition may have on analysis and can easily be adapted to current DNA workflows. Recent studies into DNA/RNA co-isolation and co-extraction techniques have proven that both RNA and DNA can be extracted with the use of a single stain [1, 7, 22, 25, 27, 30-33, 36] but do not integrate well into current DNA workflows. A study performed by the Institute of Environmental Science and Research (ESR), New Zealand, has recently evaluated the ability to recover RNA from saved extraction waste fractions using the DNA IQ system [18, 30]. Although recovery efforts allow for ideal integration into current DNA workflows, the DNA IQ system is not directly applicable to all crime labs in the United States. With the ability to

integrate RNA recovery into current DNA workflows, mRNA profiling can be applied in other ways that would not require any additional modifications, and therefore, validation of existing DNA methods and also uniquely in ways that could result in reductions in evidence backlogs. This includes research not only into other possible ways to recover RNA without interrupting the flow of DNA analysis but to also provide a possible upfront sperm detection that would help to rapidly screen SAK evidence while eliminating the major bottle neck of microscopic sperm ID. Furthermore, the detection of sperm provides a strong indication of sexual intercourse taking place while also indicating the presence of y-typeable male DNA to better identify male contributor(s).

Therefore, the first goal of this study was to evaluate the ability of another commonly used DNA extraction procedure to recover RNA using extraction waste fractions. Most crime labs aim to streamline their DNA evidence workflows to include a more automated system to increase productivity and reduce possible errors [14, 37]. As a result, they will typically use commercially available kits. These may include the Promega DNA IQ kit, QIAGEN EZ1 Investigator kit, or the Applied Biosystems™ PrepFiler™ Forensic DNA Extraction Kit. Although all three of these kits listed are able to be performed manually or through automation [37] a validation study determined that PrepFiler™ was able to extract more total DNA when compared to the other kits [38]. In 2009, the PrepFiler™ Forensic DNA Extraction Kit was validated and implemented into crime labs across the country for the isolation of genomic DNA using a wide range of biological samples [38]. As previously stated, the kit allows for both a manual and automated DNA extraction using magnetic beads that bind DNA through an “optimized multicomponent surface chemistry” permitting inhibitors to be washed away. Per protocol, the flow through during these bead washing steps is normally discarded [39]. This

study investigates the ability of the PrepFiler™ Forensic DNA extraction kit to extract DNA while simultaneously saving the extraction wash step waste fractions to check for future presence of co-extracted RNA.

Throughout the study, several body fluid samples were manually extracted according to protocol [39] and the individual waste fractions were saved and purified using the PureLink® RNA Mini Kit [40]. mRNA body fluid markers were targeted using a multiplex reverse transcription-PCR assay [unpublished, 10-plex primer set used in-house] and detected using capillary electrophoresis. By saving the first lysate waste fraction, all forensically relevant body fluids including blood, saliva, menstrual blood, vaginal secretions and semen were able to be detected. A major advantage to this method is the ability to save the waste fraction without altering the manual DNA extraction and does not seem to hinder the quality or quantity of the DNA extracted. In addition, it was discovered that body fluid identification was also possible without purification, using mRNA present in the primary DNA extract itself. Analysts would therefore have the ability to take a small aliquot of their DNA extract and perform the traditional mRNA profiling analysis process as previously described. Both methods, whether saving the waste fractions of a DNA extraction, or using the DNA extract itself, allow for possible RNA recovery and downstream mRNA profiling applications.

The second goal of this study was to address another major challenge in current DNA workflows by developing a rapid upfront male DNA screening method to triage sexual assault evidence while integrating a sperm ID using mRNA profiling. Recently, a rapid male screening assay was described that could provide an upfront detection of male DNA using a rapid lysis followed by DNA quantitation to identify the presence of male DNA [41]. Although this method can provide a triage for sexual assault evidence, it does not provide the confirmatory

identification of sperm which is a major bottleneck previously discussed. Another quantitation-based method has been developed that aims to eliminate the need for a microscopic sperm identification and infers the presence of sperm from the detection of a significant amounts of male DNA in the sperm fraction of differential extractions [42]. While useful, it does not incorporate a definitive identification of sperm which may be a challenge for some laboratories. Thus, this study aims to integrate mRNA profiling into an upfront detection for male DNA, providing not only a rapid way to screen sexual-assault evidence for the presence of male DNA, but to uniquely provide a definitive identification of sperm, thus reducing or eliminating the challenging and time-consuming microscopic identification of sperm.

Since the co-extraction method developed in this work allowed for successful detection of all forensically relevant body fluids using purified extraction waste, it was a possibility that a similar method could be used to not only detect male DNA, but also permit recovery of male DNA profiles as well as sperm identification using mRNA profiling. The current work paired a rapid RNA extraction with a 1-step combined RT-HRM assay for the detection of the sperm specific protein Protamine 2 (PRM2) [10]. Figure 8 represents the potential work flow for this rapid male DNA screening assay and the extraction protocol, much like the assays previously discussed, allows for the analyst to save the lysis buffer waste fraction for downstream DNA analysis. The Dynabeads® mRNA Direct kit (Invitrogen® by ThermoFisher) allows for the simple but rapid isolation of pure, intact polyadenylated (polyA) mRNA while using a very small amount of sample. The rapidly lysed cells release the mRNA and covalently bind to oligo $(dT)_{25}$ beads that bind to the polyA tails present in all eukaryotic mRNAs [43]. Like in the previous study, the normally discarded waste fractions were saved and provided an upfront detection of male DNA, indicated by a male quant. Next, RT-HRM analysis was performed using the Power

SYBR® Green RNA-to-C_T™ 1-Step Kit (Applied Biosystems™ Foster City, CA) to analyze mRNA extracts for detection of sperm using PRM2. Successful detection of sperm was represented by the presence of a single distinct PRM2 melt peak at a T_m of 82˚C. Experimental analysis of single donor semen samples and vaginal-semen mixture samples, extracted using the mRNA Direct kit, supported the detection of sperm at the expected melting temperature with no cross-reactivity with other body fluids. Once samples were successfully screened for male DNA and analyzed for the presence or absence of sperm, the saved lysis buffer/lysate waste fraction was able to be used for downstream male DNA profiling. The mRNA Direct assay allowed for a simultaneous rapid male DNA detection and upon successful screening, detection of sperm and male Y-STRs in as little as 1 µL of semen, with no further purification needed.

The goal of this study was to provide methodological improvements to mRNA profiling for better integration into DNA workflows. Although more work needs to be performed to ensure this method is compatible with an automated DNA extraction, the successful recovery of RNA in both purified DNA extraction waste and un-purified DNA extracts, permitted successful integration of mRNA profiling into established DNA workflows without altering DNA recovery. Next, to improve SAK backlogs and reduce or eliminate the bottleneck of microscopic sperm ID, it was important to provide an upfront detection of male specific DNA while allowing for subsequent sperm detection and or male DNA profiling. By performing a rapid mRNA extraction, the detection of sperm could be determined relatively quick using HRM analysis and by saving the extraction waste, the analyst could screen the sample for male DNA and use the recovered DNA for male DNA profiling without having to perform additional extractions. Current work is underway for a semen and sperm duplex RT-HRM assay that would allow for

the detection of male DNA in low-level sperm samples or in the absence of sperm, as is the case for vasectomized males.

CHAPTER TWO: METHODOLOGY

Body Fluid Sample Preparation

All body fluids were collected from anonymous volunteers using procedures approved by the University of Central Florida's Institutional Review Board and informed consent was obtained from each donor. All dried 50 µL blood stains (10 mL, Bioreclamation, Westbury, NY), unless otherwise stated, were deposited onto sterile cotton swatches from a vacutainer containing EDTA, dried at room temperature and stored at -46˚C until needed. Freshly ejaculated semen was collected (10 donors; 3 mL, BIOIVT, Nassau, NY) (2 donors; 3 mL, Lee Biosolutions, Maryland Heights, MO) in 15 mL conical tubes and stored at -20˚C. Once thawed, 50µL of liquid semen was pipetted onto sterile cotton swabs or sterile cotton swabs were soaked directly in the liquid. Mixture samples used in the Rapid Male DNA Screening Assay were made by depositing 10, 5, 2 and 1 µL of liquid semen onto a tip-sized dried vaginal secretion swab. All mixture samples were dried at room temperature overnight and stored at -20˚C until needed. Buccal swabs (saliva) were collected by swabbing the inside of the donor's mouth. Semen free vaginal secretions and menstrual blood were collected using sterile cotton swab, dried overnight at room temperature and stored at -20˚C until needed. All other stains used in RNA/DNA co-extractions were made by pipetting 25, 10, 5, and 1 µL of un-preserved blood collected directly from finger-pricks, freshly ejaculated semen, and liquid saliva, were pipetted onto sterile cotton swatches, dried overnight at room temperature and stored at -20˚C until needed.

All mock casework stains and swabs used in RNA/DNA co-extractions were previously prepared and stored at -46˚C until needed. Fifty microliters of liquid semen, saliva and blood

were deposited onto denim, polyester and paper swatches and incubated at room temperature for 1 week, 6 months and 1 year. Fifty microliters of liquid semen, saliva, and blood were deposited on cotton swatches and incubated at 37˚C and 56˚C for 1 week to 1 month. Vaginal secretions were collected on sterile cotton swabs and stored at room temperature, 37˚C, and 56˚C for 1 week and 1 month. Mixture samples were prepared by pipetting liquid body fluid (semen or saliva) onto previously dried body fluid swabs dried at room temperature and stored at -20˚C until needed.

PrepFiler™ Manual DNA Extraction

Total DNA was extracted from various semen, saliva, blood, menstrual blood, and vaginal secretions using the PrepFiler™ Forensic DNA Extraction Kit (Applied Biosystems™ by ThermoFisher, Foster City, CA) following the manufacturer's recommended protocol [39]. Once the PrepFiler™ lysis buffer and PrepFiler ™ Magnetic Particles were briefly incubated (10 minutes at 37˚C) to remove any precipitates, 300 μL of PrepFiler™ Lysis Buffer and 3 μL of 1 M DTT (5 μL for semen samples) were added to a PrepFiler™ spin tube containing the stain or swab. The samples were incubated using a thermal shaker set at 70˚C and 900 rpm for 40 minutes to lyse the cells. The stain or swab was added to a PrepFiler™ filter column, placed back into the original PrepFiler spin tube, and centrifuged at 14,000 rpm for two minutes. The filter columns were discarded, and the tubes were incubated at room temperature for 5 minutes.

Next, 15 μL of magnetic beads, followed by 180 μL of isopropanol, was added to the lysate and incubated (25˚C and 1,000 rpm for 10 minutes) to allow the DNA to bind to the beads. Then the tubes were placed onto the PrepFiler™ magnetic stand (See Figure 1 to ensure proper

magnetic strip alignment) until the beads formed a pellet against the back of the tube or after approximately 2 minutes for opaque liquids. The lysate remaining in the tubes (about 500 µL) was removed and saved in a new 2 mL tube as "waste fraction 1." The beads were washed three times, 1) 600 µL of wash buffer A, 2) 300 µL of wash buffer A and, 3) 300 µL of wash buffer B and collected as individual waste fraction or as a combined waste fraction. All waste fractions were stored at -20˚C until purified. After the beads were allowed to air-dry with the tube cap open for 7-10 minutes, 50 µL of elution buffer was added to the tubes and incubated (70˚C and 900 rpm for 5 minutes) to elute the DNA off of the beads. DNA extracts were used right away or stored at 5˚C.

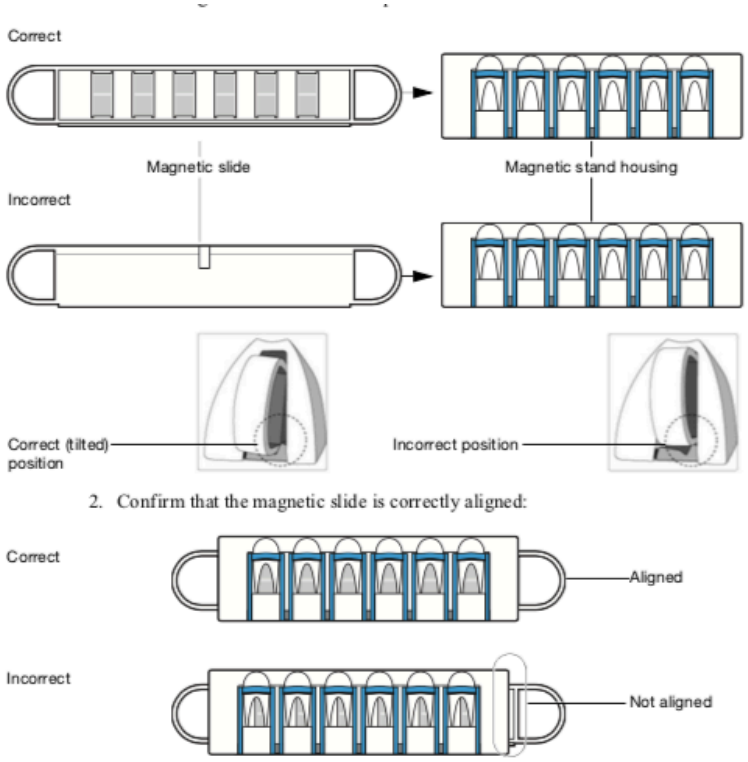

Figure 1 Illustration depicting the proper usage of the PrepFiler magnetic stand [https://www.thermofisher.com/order/catalog/product/4463351.] [39]

RNA Purification of PrepFiler™ DNA Waste Fractions

All individual and combined "waste fractions" were purified using the PureLink® RNA Mini Kit (Invitrogen™ by ThermoFisher) and thawed, prior to purification. A PureLink® Lysis buffer and ß-mercaptoethanol (Calbiochem® by Millipore Sigma, St. Louis, MO) mixture was made based on the total volume of waste being purified (Table 1). An equal amount of lysis buffer to waste volume was combined with 1 µL of ß-mercaptoethanol per 100 µL of lysis buffer and added to the waste fractions. Then, an equal volume of 100% ethanol was added to the waste and the entire volume of lysate was flowed through a PureLink® filter cartridge (centrifuged for 15 sec. at 14,000 rpm). With each flow through, the collection tube was discarded, and the cartridge was placed in a new collection tube. Table 1 and Table 2 list the number of flow-through steps necessary for each volume of waste. Next, the membrane was washed three times with 1) 650 µL of wash buffer I and, 2) 500 µL of wash buffer II twice (centrifuging for 15 sec. at 14,000 rpm) replacing the collection tube each time. The membrane filter cartridge was dried by centrifuging at 12,000 x g for 1 minute and placed in a new 1.5 mL recovery tube. Finally, 30 µL of RNase-free water was added to the center of the membrane and centrifuged at 12,000 x g for 2 min to elute the purified RNA off of the membrane.

Table 1 The contents, total volume and number of purification flow through steps required for Waste Fractions 1 through 4

Waste Fraction	Contents	Total Volume	Number of Flow Through Steps
1	Lysis Buffer, DTT, Isopropanol	500 µL	3
2	Wash Buffer A (mostly ethanol)	600 µL	3
3	Wash Buffer A (mostly ethanol)	300 µL	2
4	Wash Buffer B (mostly ethanol)	300 µL	2

Table 2 Flow through steps needed when all of the waste fractions are combined together

Volume of Waste (µL)	**Number of Flow Through Steps**
200	1
300	2
500	3
600	3
650	3
750	4
1,000	5
1500	7

RNA Extraction for Rapid Male DNA Identification Assay

All single sourced and mixture samples were extracted using the Dynabeads™ mRNA Direct™ Purification kit (Invitrogen™ by Thermo Fisher). All reagents were stored at 5˚C and the Lysis/Binding buffer, Dynabeads™ oligo $(dT)_{25}$ and Washing Buffer A and B were brought to room temperature prior to use to remove any precipitates in the solutions. A small piece of sample (Figure 2) and 100 µL of Lysis/Binding Buffer were added to a sterile 1.5 mL tube and incubated at 70˚C and 750 rpm for 10 minutes. The swab was added to a spin basket, centrifuged at 1300 rpm for 3 minutes then discarded. Next, 10 µL of Dynabeads™ oligo $(dT)_{25}$ were added

to the lysate and incubated at room temperature for 5 min. The samples were added to a DynaMag-2™ magnet and once a pellet formed against the tube walls, or after about two minutes for opaque liquids, the lysate was removed and saved for further DNA analysis. The beads were washed three times with 1) 600 µL of Wash Buffer A twice, then 2) 300 µL of Wash Buffer B. Finally, 10 µL of 10 mM Tris-HCl (elution buffer) was washed across the beads, incubated at 65˚C and 350 rpm for 2 min, and removed for further RNA analysis.

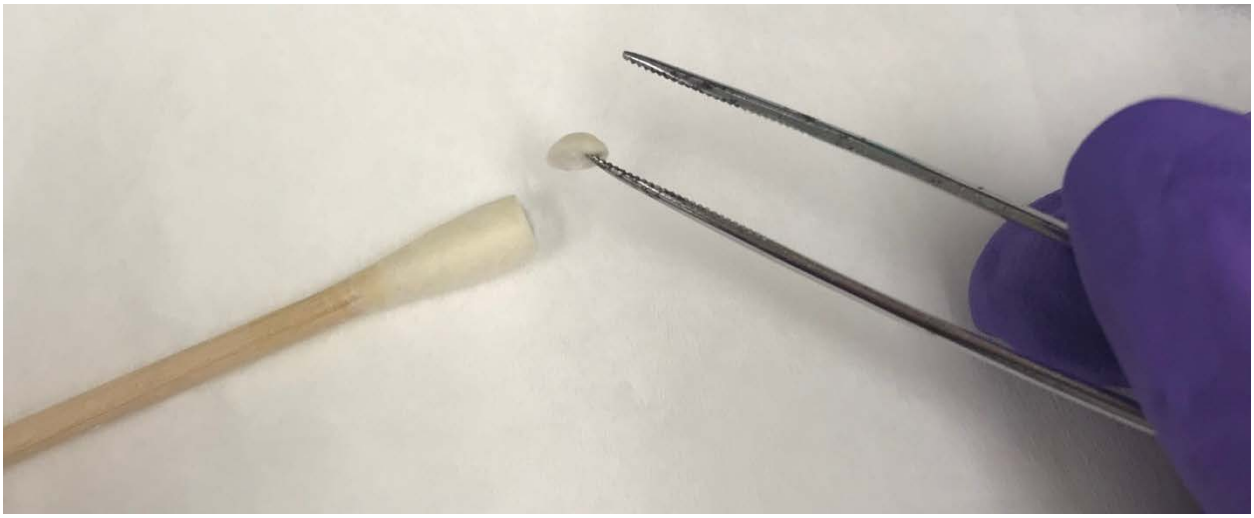

Figure 2 Image of the sample size used in the mRNA Direct RNA extraction

DNase Treatment of All RNA Extracts

All RNA extracts were DNase-treated using the TURBO™ Dnase kit (Invitrogen™ by ThermoFisher) to remove DNA remaining in the sample. 1 µL of TURBO DNase™ Buffer (per 1 µL of extract) and 1 µL of Turbo DNase™ was added to the samples. Samples were incubated at 37˚C for 30 minutes and then 75˚C for 10 minutes. All DNase-treated RNA extracts were stored at -20˚C until further analysis was performed.

cDNA Synthesis of Purified Waste and DNA From RNA Aliquots

All DNase-treated RNA extracts were reverse transcribed using the SuperScript™ IV (SS4) Vilo™ Master Mix kit (Invitrogen™ by Thermo Fisher). A 20 µL reaction mix containing 4 µL of SS4™ Vilo™ Master Mix, 8 µL of nuclease-free water and 8 µL of sample was added to 0.2 µL MicroAmp™ tubes (Applied Biosystems™ by ThermoFisher). Samples were reverse-transcribed using the following cycling program: one cycle at 25˚C, 10 min; 50˚C, 10 min; 85˚C 5 min using a GeneAmp® PCR System 9700 (Applied Biosystems™ by ThermoFisher). All RT products were stored at -20˚C until needed.

cDNA Amplification of Purified Waste and RNA From DNA Aliquots

All cDNA samples were amplified using the AmpliTaq™ Gold 360 Master Mix Kit (Applied Biosystems™ by ThermoFisher). An 11.5 µL reaction mix containing 6.25 µL of AmpliTaq™ Gold® 360 Master Mix, 1.25 µL of 360 GC Enhancer, 1.25 µL of an in-house CE 10-plex primer mix Table 3), 2.75 µL of RNase-free water and 1 µL of reverse transcribed cDNA was added to 0.2 µL MicroAmp™ tubes (Applied Biosystems™ by ThermoFisher). The following PCR program was performed using a GeneAmp® PCR System 9700 (Applied Biosystems™ by ThermoFisher): 95˚C, 15 min; followed by 33 cycles at 94˚C, 30 sec; 55˚C, 90 sec (+0.2˚C/cycle); 72˚C, 45 sec and ending at 72˚C, 30 min.

Table 3 10-Plex body fluid marker Primer Mix prepared in-house for Multi-plex PCR

Concentration (pmol/µL)	Marker	Body Fluid ID	Product Size (bp)	Primer	Volume added to Primer Mix (µL)
200	ANK1 [29]	Blood	113	R2, FF	5
100	ALAS2 [27, 28]	Blood	134	R, FF	0.45
100	PRM2 [26-28]	Sperm	91	R2, F2V	0.25
100	SEMG1 [28, 29]	Semen	86	R3, FN	0.3
200	HTN3 [26-28]	Saliva	227	R, FN	4.5
200	STATH [26-28]	Saliva	181	R, FN	6.0
100	CYP2B7P1 [24]	Vaginal Secretions	198	R, FF	1.5
100	MMP10 [27-29]	Menstrual Blood	168	R, F2F	0.75
100	LEFTY2 [28]	Menstrual Blood	126	R, FP	4
100	LCE1C [23]	Skin	60/62	R2, FP	0.3
	H_2O				**53.9**

Capillary Electrophoresis and Data Analysis

of cDNA From Purified Waste and DNA From RNA Aliquots

Capillary electrophoresis was performed using an ABI 3130 Genetic Analyzer (Applied Biosystems™ by ThermoFisher). An 11.5 µL reaction mix containing 9.7 µL Hi-Di™ formamide (Applied Biosystems™ by ThermoFisher), 0.3 µL GeneScan™ 500 LIZ™ dye Size Standard (Applied Biosystems™ by ThermoFIsher) and 1 µL of sample was added to a 96-well plate and covered with a plate septa. Samples were injected through a 4-capillary 36 cm array with POP-7™ polymer (Applied Biosystems™ by ThermoFisher), using run ModuleG5_General and FragmentAnalysis36_POP7 template (18 sec injection, 1.2 kVolts, 60˚C, 1200 sec run time). Samples were analyzed using 3130 Genetic Analyzer Data Collection Software v3.0 (Applied Biosystems™ by ThermoFisher).

High Resolution Melt (HRM) analysis for Rapid Male DNA Screening Assay

All RNA extracts were screened for the presence of sperm using the Power SYBR® Green RNA-to-C_T™ 1-Step Kit (Applied Biosystems™). An 18µL single-plex reaction mix containing 10 µL of PowerSYBR® Green RT PCR mix, 2 µL of 10 µM PRM2 (sperm) forward and reverse primers (primers were custom designed by Invitrogen, Table 4), 0.16 µL of 10X RT enzyme mix, 3.84 µL of RNase-free water and 2 µL of DNased mRNA extract, was added to a 96-well plate. The plate was sealed using an Optical Adhesive Film and analyzed on a 7500 Real-Time PCR instrument (Applied Biosystems™) using the HID Real-Time PCR Analysis Software. A modified reduced-time Power SYBR® run protocol was used: reverse transcription (48˚C, 15 min), followed by a hot start to activate the Taq polymerase (95˚C, 10 min), 40 cycles of PCR (95˚C, 15 sec; 60˚C, 30 sec), ending with the melt curve stage (95˚C, 15 sec; 60˚C, 15 sec; 95˚C, 15 sec).

Table 4 Primer Design for PRM2 forward and reverse primers used in 1-Step Reverse Transcription-HRM single plex assay

Primer	Sequence (5’ to 3’)	Primer Length
PRM2-F-HRM	(DNA) GGC GCA AAA GAC GCT CC	17
PRM2-R-HRM	(DNA) GCC CAG GAA GCT TAG TGC C	19

DNA Quantification

All DNA extracts were quantified using the Quantifiler™ Trio DNA Quantification kit (Applied Biosystems™ by ThermoFisher) according to the manufacture’s protocol. A set of Quantifiler™ Trio DNA standards were prepared ranging from 0.005-50 ng/µL of DNA. A 20 µL reaction mix containing 8 µL of Quantifiler™ Trio Primer mix, 10 µL of Quantifiler™ Trio Reaction mix and 2 µL of sample or standard was dispensed into designated wells of a 96-well

plate. The plate was sealed using a MicroAmp™ Optical Adhesive Film (Applied Biosystems™ by ThermoFisher) and analyzed in real-time using the HID Real-Time PCR Analysis Software v1.2 on a 7500 Real-Time PCR instrument (Applied Biosystems™ by ThermoFisher). Total human and male DNA was quantified with respect to the small autosomal (QSY7 target and NFQ-MGB quencher) and T.Y. target (FAM target, NFQ-MGB quencher). The samples were amplified using the following program: 95˚C hot start for 2 minutes followed by 40 cycles of 95˚C 9 sec; 60˚C 30 sec. The total amount of human and male DNA in each sample was analyzed in ng/µL with respect to a standard DNA curve (R value $\geq$ 0.99). All DNA samples were diluted to a target DNA concentration of 0.5 ng/µL, if necessary, using DNA Suspension Buffer (Tris 10 mM, EDTA 0.1 mM, pH 8.0) (TEKnova, Hollister, CA).

DNA Amplification

All DNA extracts extracted using the PrepFiler™ Forensic DNA Extraction Kit were amplified using the GlobalFiler™ PCR Amplification Kit (Applied Biosystems™ by ThermoFisher) according to the manufactures standard protocol. The 6-dye, short tandem repeat (STR) multiplex assay kit amplifies human genomic DNA at a target DNA concentration of 0.5 ng/µL allowing for the detection of 21 autosomal STR loci, 1 Y-STR, 1 insertion/deletion polymorphic marker on the Y chromosome, and Amelogenin (sex determining marker) [44]. An 11.5 µL total reaction mix containing 3.75 µL of GlobalFiler™ Reagent mix, 1.25 µL of GlobalFiler™ Primer mix, 6.5 µL of DNA Suspension Buffer (TEKnova), and 1 µL 0.5 ng/µL DNA extract was added to 0.2 µL MicroAmp™ tubes (Applied Biosystems™ by ThermoFisher). All samples were amplified using the GeneAmp® PCR System 9700 (Applied Biosystems™ by

ThermoFisher) under the following program: 95˚C, 1 min in max ramp mode; 29 cycles 94˚C, 10 sec; 59˚C, 90 sec; 60˚C, 10 min.

DNA waste fractions saved from the mRNA Direct™ RNA extraction were amplified with the Yfiler™ Plus PCR Amplification Kit (Applied Biosystems™) according to the manufacture's reduced volume protocol [45]. All DNA extracts were diluted to a Y-target concentration of 0.5 ng/µL prior to amplification. An 11.5 µL reaction mix containing 5 µL of Yfiler™ Plus master mix, 2.5 µL of Yfiler™ Plus Primer mix, 4 µL of DNA suspension buffer and 1 µL of 0.5 ng/µL DNA extract was added to 0.2 µL MicroAmp™ tubes. All samples were amplified using a 9700 Applied Biosystems™ Thermal Cycler under the following program: hot start to activate the Taq polymerase (95˚C, 1 min), followed by 30 cycles of denaturing and annealing (94˚C, 5 sec; 61.5˚C, 1 min), ending with a final extension (60˚C, 22 min).

Capillary Electrophoresis and Data Analysis

Electrophoresis was performed on all amplified DNA products to separate the DNA fragments by size and color using a 6-dye fluorescent system. Each dye correlated to a single color (6-FAM, blue; VIC, green; NED, yellow; TAZ, red; SID, purple; LIZ, orange), excited by a laser and detected as a relative fluorescent unit. Electrophoresis was performed using an ABI 3130 Genetic Analyzer (Applied Biosystems™ by ThermoFisher) and the fluorescence data was captured using the 3130 Genetic Analyzer Data Collection Software v3.0 (Applied Biosystems™ by ThermoFisher). For all DNA products amplified with the GlobalFiler™ PCR Amplification Kit (Applied Biosystems™ by ThermoFisher), an 11 µL total reaction volume containing 9.6 µL Hi-Di formamide (Applied Biosystems™ by ThermoFisher), 0.4 µL GeneScan™ 600 LIZ™ dye

Size Standard v2.0 (Applied Biosystems™ by ThermoFisher) and 1 µL of sample or GlobalFiler™ Allelic Ladder (Applied Biosystems™ by ThermoFisher) was added to a 96-well plate and covered with a plate septa. Samples were injected through a 4-capillary 36 cm array with both POP-7™ or POP-4™ polymer (Applied Biosystems™ by ThermoFisher), under the run module GlobalFiler_10sec.inj or J6_POP4_GF3 and the FragmentAnalysis36_POP7 (10 sec injection, 1.2 kVolts, 60˚C, 1500 sec run time) or HIDFragmentAnalysis36_POP4 template (2 sec injection, 1.5 kVolts, 60˚C, 1500 sec run time). Samples amplified using the Yfiler™ Plus PCR amplification kit were plated as previously described with the exception of a Yfiler™ Plus Allelic Ladder. All samples amplified using the Yfiler ™ Plus Amplification kit were electrophoresed using the following 3130*xl* protocol: Injected through a 4-capillary 36 cm array with POP-4™ polymer, run module J6_POP4_General and HIDFragmentAnalysis36_POP4 template (5 sec injection, 3 kVolts, 60˚C, 1500 sec run time). All signals were detected by the 3130 Genetic Analyzer Data Collection Software v3.0 and analyzed using the GeneMapper™ ID-X software as individual electropherograms.

CHAPTER THREE: RESULTS

INTEGRATION OF MRNA PROFILING INTO AN ESTALISHED DNA WORKFLOW

To evaluate the capability of recovering RNA from an established DNA extraction, a number of initial validation studies were performed. First, the DNA extraction waste fractions were saved and purified to removed impurities such as PCR inhibitors and extraneous DNA. All purified waste was analyzed using multiplex-PCR followed by CE that had the ability to detect, via specific mRNA biomarkers, the presence of saliva, semen, blood, vaginal secretions menstrual blood and skin (not specifically studied). All of these body fluid types are routinely encountered in biological evidence and are potential sources of DNA. The PrepFiler™ DNA extraction involves multiple wash steps, each being potential sources for RNA recovery. An initial waste fraction validation was performed to locate the best chances for RNA recovery by analyzing the successful detection of multiple single sourced body fluid samples. Next, a sample size validation study was performed to determine the success rate of detecting body fluids when increasingly smaller amounts of body fluid are present. To determine if RNA could be recovered by other means, the DNA extract itself was analyzed with respect to its ability to successfully detect body fluids. Upon successful detection of all body fluids without the need for purification, an initial analysis of different extract volumes was performed and allowed for a direct comparison between the two methods. Finally, mock casework samples were analyzed to ensure both methods were capable of detecting body fluids exposed to forensically relevant circumstances.

Initial Waste Fraction Validation

To determine if RNA recovery was possible without modifying the PrepFiler™ DNA extraction, the saved waste fractions were purified both individually and combined. To analyze the all relevant body fluids using PCR, a 10-plex primer mix containing markers for semen [PRM2 and SEMG1], blood [ANK1 and ALAS2], vaginal secretions [CYP2B7P1], saliva [STATH and HTN3] and menstrual blood [MMP10 and LEFTY2]) and skin (LCE1C) (Table 1) was used. Figure 3 represents a positive detection of all corresponding body fluids if they were present in a mixture. Only body fluid markers observed above the 50 RFU threshold were considered to be detected. To initially test the extraction waste fractions, two sets of single sourced saliva, blood, semen, vaginal secretions, and menstrual blood samples were analyzed in regard to their corresponding body fluid markers. Upon initial testing it was observed that whole blood and semen swabs/stains overwhelmed the beads during the extraction process, causing beads to be pulled into the saved waste fractions. Therefore, all initial waste fraction testing was performed using ½ of a 50 µL blood stain and ½ or ¼ of a swab for all other body fluids. First, individual waste fractions were analyzed separately to 1) determine if RNA could be recovered in the waste of the extraction and 2) determine which waste fraction was consistently able to detect the correct body fluid markers for each sample. Table 5 summarizes the total number of body fluid specific markers detected for each corresponding body fluid in each waste fraction after purification. Body fluids were detected in all of the waste fractions. However, waste fraction 1 successfully detected all of the body fluid markers when ½ stains and swabs were tested. Similar levels of detection were observed for waste fraction 1 when ¼ sized swabs were tested for more proteinaceous body fluids (vaginal secretions and semen).

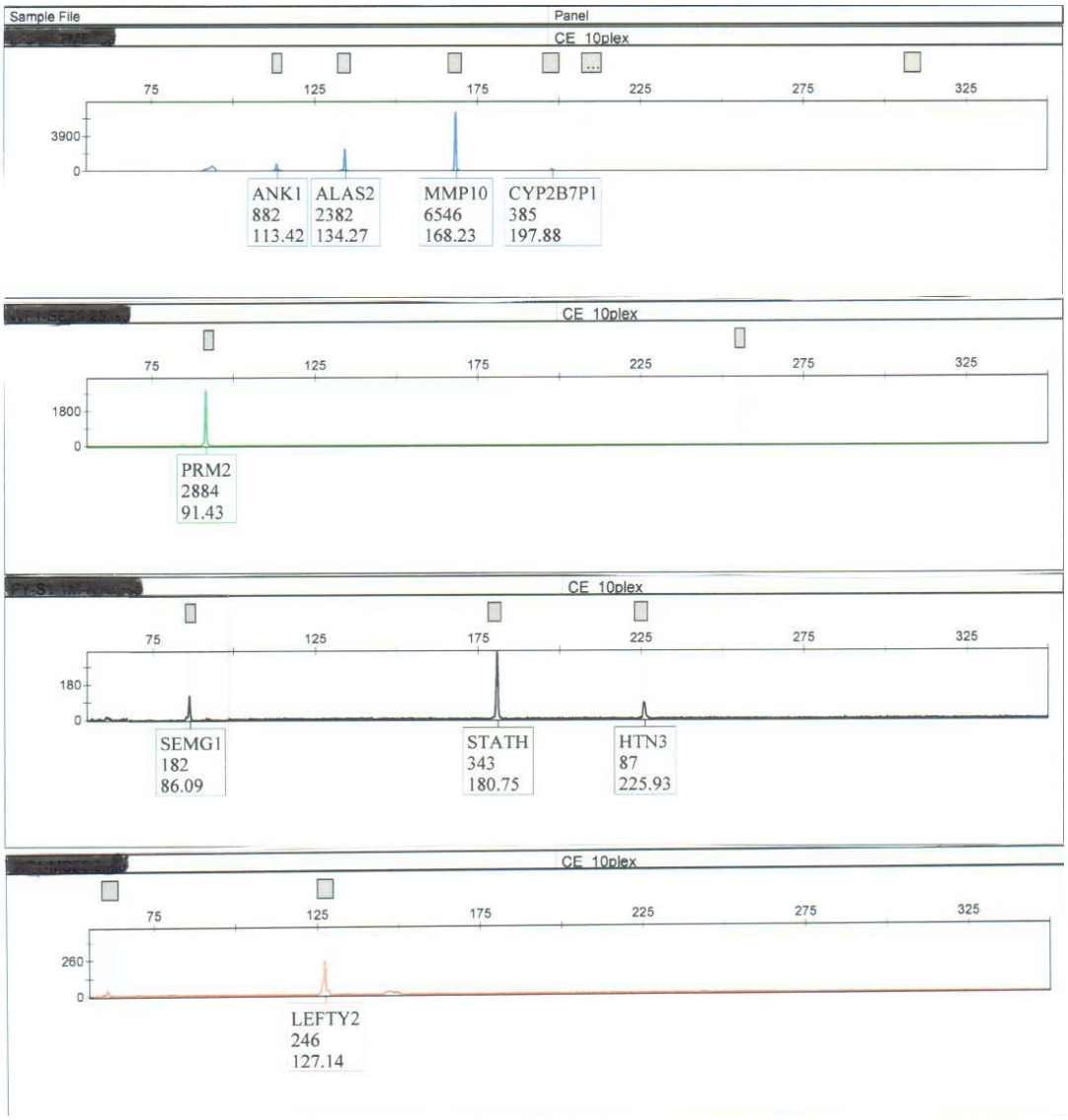

Figure 3 Electropherogram of all possible body fluid markers detected using an in-house CE 10-plex primer mix.

Blue channel (FAM) - ANK1 and ALAS2 - blood markers, MMP10 – menstrual blood, CYP2B7P1 – vaginal secretions. Green channel (VIC) - PRM2 – sperm marker. Yellow channel (NED, shown in black) - SEMG1 – seminal fluid, STATH and HTN3 - saliva. Red channel (PET) - LEFTY2 – menstrual blood. Below each peak is the name, the RFU value and the base pair size of the detected gene product. X-axis: base pair (bp) size; Y-axis: relative fluorescence units (RFUs).

Table 5 Detection of saliva, vaginal secretions, semen, menstrual blood, and blood mRNA body fluid markers in individual extraction waste fractions after purification.
The detection of a body fluid marker is indicated by a colored cell (saliva-blue, vaginal secretions- green, semen-yellow, menstrual blood-pink, blood-red). The cell is colored gray when no corresponding body fluid markers were detected for the total number of samples tested. The total number of markers detected in each sample out of the total number of samples tested are listed in each cell. a) contains the results for ½ sized stains or swabs; b) table contains the results from ¼ sized swabs.

a)

Body Fluid Detection in Individual Extraction Waste Fractions						
Body Fluid	Marker	Swab Size	Waste Fraction			
			1	2	3	4
Saliva	STATH	1/2	2/2	1/2	0/2	1/2
	HTN3		2/2	0/2	1/2	1/2
Vaginal	CYP2B7P1		2/2	2/2	0/2	2/2
Semen	PRM2		2/2	0/2	0/2	0/2
	SEMG1		2/2	0/2	0/2	0/2
Menstrual Blood	MMP10		2/2	1/2	1/2	0/2
	LEFTY2		2/2	0/2	0/2	0/2
Body Fluid	Marker	Stain Size	Waste Fraction			
			1	2	3	4
Blood	ANK1	1/2	2/2	0/2	0/2	0/2
	ALAS2		2/2	0/2	0/2	0/2

b)

Body Fluid Detection in Individual Extraction Waste Fractions						
Body Fluid	Marker	Swab Size	Waste Fraction			
			1	2	3	4
Vaginal	CYP2B7P1	1/4	1/2	1/2	0/2	1/2
Semen	PRM2		2/2	2/2	1/2	1/2
	SEMG1		2/2	0/2	0/2	0/2

Next, the waste fractions were combined to determine 1) if RNA recovery was still possible when aliquots of waste were purified and 2) to determine if RNA recovery is more or less successful when compared to separate waste fractions. Two sets of each body fluid for each aliquot size were analyzed with the exception of the ¼ sized semen swabs (four sets of donors for the 1500 µL and 750 µL aliquots). Aliquots of 1500 µL, 1000 µL, 750 µL and 500 µL were purified and the results can be seen in Table 6. The 1500 µL and 1000 µL aliquots were able to identify all single sourced body fluids similar to waste fraction 1.

Table 6 Detection of saliva, vaginal secretions, semen, menstrual blood, and blood mRNA body fluid markers in aliquots of combined waste fractions after purification.
The detection of a body fluid marker is indicated by a colored cell (saliva-blue, vaginal secretions- green, semen-yellow, menstrual blood-pink, blood-red). The cell is colored gray when no corresponding body fluid markers were detected for the total number of samples tested. The total number of markers detected in each sample out of the total number of samples tested are listed in each cell. a) contains the results for ½ sized stains or swabs, and b) contains the results from ¼ sized swabs.

a)

Body Fluid Detection in Combined Extraction Waste Fractions						
Body Fluid	Marker	Swab Size	Volume of Combined Waste Fraction			
			1500 µL	1000 µL	750 µL	500 µL
Saliva	STATH	1/2	1/2	2/2	1/2	1/2
	HTN3		2/2	2/2	2/2	1/2
Vaginal	CYP2B7P1		2/2	2/2	3/3	1/4
Semen	PRM2		2/2	2/2	2/2	2/2
	SEMG1		1/2	1/2	1/2	1/2
Menstrual	MMP10		2/2	2/2	2/4	1/2
	LEFTY2		2/2	1/2	2/4	0/2
Body Fluid	Marker	Stain Size	Volume of Combined Waste Fraction			
			1500 µL	1000 µL	750 µL	500 µL
Blood	ANK1	1/2	2/2	2/2	2/4	1/2
	ALAS2		1/2	1/2	1/4	0/2

b)

Body Fluid Detection in Combined Extraction Waste Fractions						
Body Fluid	Marker	Swab Size	Volume of Combined Waste Fraction			
			1500 µL	1000 µL	750 µL	500 µL
Vaginal	CYP2B7P1	1/4	2/2	2/2	2/2	1/2
Semen	PRM2		4/4	2/2	3/4	2/2
	SEMG1		2/4	1/2	2/4	0/2

However, to achieve similar results as waste fraction 1, the number of flow through steps were increased, causing an increase in resources and time. As the volume of waste used was decreased, the success rate for body fluid identification also decreased. The 500 µL aliquot, the same volume of waste purified for waste fraction 1 separately, did not allow for consistent body fluid identification of saliva, vaginal, menstrual blood or blood samples. This is not unexpected as the combined waste contains essentially diluted waste since not as much RNA is recovered from subsequent waste fractions. Although RNA recovery is achievable when the waste fractions

were combined, body fluid identification was more consistently successful when waste fraction 1 was purified separately. Therefore, all further validation studies included analysis of waste fraction 1 only.

Initial Sample Size Validation

To determine the optimal sample size that would allow for all forensically relevant body fluids to be successfully identified, the following sample sizes were tested: 1) dried saliva, vaginal, semen, menstrual blood swabs - ½ , ¼ and 1/8 swabs; 2) dried 50 µL blood, semen and saliva stains - ½, ¼, and 1/8 stains; and 3) whole 25, 10, 5, and 1 µL blood, semen and saliva stains were analyzed. Each sample/swab size for each body fluid were analyzed with two different donors with the exception of liquid saliva and semen stains (4 different saliva donors and 3 different semen donors). These sample types were more challenging, and evaluation of additional donors was necessary. The results can be seen in Table 7 and lists all of the body fluid makers that were detected for each sample size. A positive identification of all ½ sized saliva, vaginal secretions, semen, and menstrual blood swabs and blood stains were observed, which is consistent with the results from the initial waste fraction validation study. Additionally, semen and vaginal secretions were correctly identified for all swab sizes that were tested. Liquid semen and saliva stains were more variable, being identified in only 50 and 33 percent of the ½ and ¼ 50 µL stains, respectively. Additional donors were tested to ensure the variable detection was genuine and not due to possible sample issues. Un-preserved blood, semen and saliva were available in liquid form for variable stain size testing. Blood was the only body fluid detectable when 25 µL of liquid was deposited as a stain.

Table 7 Detection of saliva, vaginal secretions, semen, menstrual blood, and blood mRNA body fluid markers in various sample sizes.

The detection of a body fluid marker is indicated by a colored cell (saliva-blue, vaginal secretions-green, semen-yellow, menstrual blood-pink, blood-red). The cell is colored gray when no corresponding body fluid markers were detected for the total number of samples tested. The total number of markers detected in each sample out of the total number of samples tested are listed in each cell. a) contains the results for all swab sizes and 50 µL stain sizes, b) contains the results from specific volumes of liquid dried as a stain.

a)

Body Fluid Detection in Various Sample Sizes				
Body Fluid	Marker	Dried Swab		
		1/2	1/4	1/8
Saliva	STATH	2/2	1/2	0/2
	HTN3	2/2	1/2	1/2
Vaginal	CYP2B7P1	2/2	2/2	2/2
Semen	PRM2	2/2	2/2	2/2
	SEMG1	1/2	0/2	1/2
Menstrual	MMP10	2/2	1/2	2/2
	LEFTY2	2/2	0/2	0/2
Body Fluid	Marker	Dried 50 µL Stain		
		1/2	1/4	1/8
Saliva	STATH	2/4	0/4	0/4
	HTN3	2/4	2/4	0/4
Blood	ANK1	2/2	2/2	2/2
	ALAS2	2/2	2/2	2/2
Semen	PRM2	1/3	1/3	1/3
	SEMG1	0/3	0/3	0/3

b)

Body Fluid	Marker	Liquid Fluid Dried as Stain			
		25 µL	10 µL	5 µL	1 µL
Saliva	STATH	1/4	1/4	0/4	0/4
	HTN3	1/4	1/4	0/4	0/4
Blood	ANK1	2/2	0/2	0/1	0/2
	ALAS2	0/2	0/2	0/1	0/2
Semen	PRM2	1/3	1/3	0/3	0/3
	SEMG1	0/3	0/3	0/3	0/3

Unfortunately, saliva and semen were only detectable in one out of the total number of 25 µL samples tested. Although all body fluids on swabs were able to be correctly identified, more analysis needs to be performed to ensure mRNA profiling can be performed with liquid body

fluid stains using this method. Based on these results, the optimal sample size for saliva, and menstrual blood was determined to be ½ of a swab, ¼ of a semen and vaginal secretion swab, and ½ of a 50 µL blood, saliva and semen stain. For this to be applied in casework, labs would need to evaluate their own standard sample size because the body fluid of origin would be unknown, and the sample size would typically be consistent amongst all evidence being processed. For the purpose of this study, further validation studies included an analysis of the sample sizes with respect to each body fluid type previously mentioned and any other sample sizes not included would need to be assessed.

RNA from DNA

Although RNA was successfully recovered and analyzed using purified DNA-extraction waste, it was not successful for all samples. A possible explanation for some of these differences was that some of the RNA may be retained on the silica-based magnetic beads and therefore eluted into the DNA extract itself rather than being present in the waste fractions as originally expected. Therefore, DNA extracts were evaluated in order to determine if RNA was present and could be used without the need for additional purification as was performed with waste fraction testing. Without modifications to the DNA workflow, an aliquot of the 50 µL DNA extract (48 µL after DNA quantification and 33 µL after amplification) was DNase treated, amplified using the original 10-plex primer mix and electrophoresed, consistent with the purified waste fractions. Initial testing was performed using 25 µL (half the total elution volume) of saliva samples previously extracted from the sample size validation study. As can be seen in Table 8, saliva body fluid markers were detected for some samples that were previously undetectable using the

Table 8 Comparison of body fluid markers detected in saliva, vaginal secretions, semen, menstrual blood, and blood samples when extraction waste fractions are purified vs. when aliquots of un-purified DNA extracts are analyzed.

The detection of a body fluid marker is indicated by a colored cell (saliva-blue, vaginal secretions- green, semen-yellow, menstrual blood-pink, blood-red). The cell is colored gray when no corresponding body fluid markers were detected for the total number of samples tested. The total number of markers detected in each sample out of the total number of samples tested are listed in each cell. a) contains the results for all ½ swab and ½ 50 µL stain sizes, b) contains the results from specific volumes of liquid dried as a stain. RFD = RNA recovered from DNA extracts

Comparison of Body Fluid Markers Detected for Two RNA Recovery Methods				
Body Fluid	Marker	Volume of DNA Extract	Dried Swab	
			Purified	RFD
Saliva	STATH	10 µL	2/2	2/2
	HTN3		2/2	2/2
Vaginal	CYP2B7P1		2/2	2/2
Semen	PRM2		2/2	1/2
	SEMG1		0/2	0/2
Menstrual Blood	MMP10		2/2	2/2
	LEFTY2		2/2	2/2
Body Fluid	Marker	Volume of DNA Extract	Dried 50 µL Stain	
			Purified	RFD
Saliva	STATH	25 µL	2/4	4/4
	HTN3		2/4	3/4
Blood	ANK1	10 µL	2/2	2/2
	ALAS2		2/2	1/2
Semen	PRM2		1/3	1/3
	SEMG1		0/3	0/3

Comparison of Body Fluid Markers Detected for Two RNA Recovery Methods										
Body Fluid	Marker	Volume of DNA Extract	25 µL Stain		10 µL Stain		5 µL Stain		1 µL Stain	
			Purified	RFD	Purified	RFD	Purified	RFD	Purified	RFD
Saliva	STATH	25 µL	1/4	3/4	1/4	4/4	0/4	3/4	0/4	2/4
	HTN3		1/4	3/4	1/4	3/4	0/4	2/4	0/4	2/4
Blood	ANK1	10 µL	2/2	2/2	0/2	2/2	0/1	1/1	0/2	1/2
	ALAS2		0/2	2/2	0/2	2/2	0/1	0/1	0/2	0/2
Semen	PRM2	10 µL	1/3	2/3	1/3	1/3	0/3	0/3	0/3	1/3
	SEMG1		0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3

purified method. Specifically, the saliva body fluid markers, HTN3 and STATH, were not detected in purified waste fraction 1 for the 5 µL and 1 µL saliva stains. The RNA from DNA method (referred to as RFD subsequently) was able to detect 50-75% more saliva markers in these samples when compared with the purification method. Although preliminary testing was performed using 25 µL of DNA extract, it was determined that that volume may not be realistic for operational laboratories to use since they would need to retain some extract for additional testing. As a result, the volume of DNA extract was lowered to 10 µL for testing of the previously extracted size variable samples. Again, Table 8 compares the number of body fluid markers detected when the purified method and the RNA from DNA method was used with only 10 µL of DNA extract. When both methods were compared, the 10 µL RFD volume, in all cases except two, resulted in the detection the same or increased numbers of body fluid markers. PRM2 in one semen sample and ALAS2 in one blood sample were undetected with the RFD method while previously detected using the waste purification method. In order to ensure that these negative results were not due to the reduced volume, all further validation studies were performed using 15 µL of DNA extract for the RFD method.

Mock Casework Validation

Although the results indicate that the developed co-extraction method can successfully recover RNA from single sourced body fluid samples, it was important to determine if the same results would be obtained from casework-type samples that are not always found in ideal conditions. To determine if this method was suitable for use with mock casework samples, studies were performed using body fluids deposited onto different substrates, exposed to varying

environmental temperatures and two-fluid admixed body fluid samples. Fifty microliters of liquid saliva, semen and blood were deposited on forensically relevant textiles (denim, polyester and paper) and incubated at room temperature for 1 month, 6 months, and 1 year. Next, the same body fluids were deposited onto cotton swatches and exposed to harsh temperatures (37˚C and 56˚C) for 1 week and 1 month. Vaginal swabs exposed to room temperature, 37˚C and 56˚C for 1 week and 1 month were also analyzed. Finally, to test the methods success rate for detecting two body fluids simultaneously, two-person mixtures were analyzed that contained various amounts of liquid semen or saliva deposited onto dried body fluid stains or swabs. All samples were extracted using two different donors if possible and the results are summarized in Table 9. When body fluids were deposited on denim, there was not enough RNA recovered in the waste fraction to successfully identify semen or saliva under any time point, which can be seen in Table 9a. Although blood was detectable, it was not identified in half of the samples tested. In comparison, the RFD method was able to provide some additional marker detection. ANK1 markers were detectable on all 6-month blood on denim samples but was unable to detect a single marker in the 1-year denim samples, previously detectable using the purified waste. The lack of detection with these samples could be due to fibers, invisible to the naked eye, remaining in the lysate. Although, most fibers were removed using a waste basket, some fibers remained and bound to the magnetic beads making the beads unable to stick to the walls of the tube. This effected the amount of DNA as well as RNA recovered. This sample problem was not observed with paper or polyester. Interestingly, blood and saliva were detected more often in the DNA extract than in the waste fraction when the body fluids were deposited on paper and polyester. Semen was detectable, in almost every case for both methods and was the most successfully detected body fluid amongst the three tested on polyester and paper.

Table 9 Comparison of body fluid markers detected in mock casework samples using purified extraction waste fractions and 15 µL of DNA extract.

The detection of a body fluid markers is indicated by a colored cell (saliva-blue, vaginal secretions- green, semen-yellow, menstrual blood-pink, blood-red). The cell is colored gray when no corresponding body fluid markers were detected for the total number of samples tested. The total number of markers detected in each sample out of the total number of samples tested are listed in each cell. (a) shows the results for body fluids deposited on denim, polyester and paper, over time and compares detection when the purified waste and RFD methods were used. (b) shows the results for body fluids deposited on cotton swatches or from swabs that were exposed to increased temperatures over time and compares detection when the purified waste and RFD methods were used.

a)

Substrate	Exposure	Time	Purified		RFD		Purified		RFD		Purified		RFD	
			Blood				Semen		Semen		Saliva		Saliva	
			ANK1	ALAS2	ANK1	ALAS2	PRM2	SEMG1	PRM2	SEMG1	STATH	HTN3	STATH	HTN3
Denim	Room Temperature	1 M	1/2	0/2	1/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2
		6 M	1/2	0/2	2/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2
		1 Y	1/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2
Polyester	Room Temperature	1 M	1/2	1/2	2/2	0/2	2/2	1/2	2/2	2/2	0/2	0/2	2/2	2/2
		6 M	0/2	0/2	2/2	0/2	1/2	0/2	2/2	0/2	0/2	0/2	2/2	0/2
		1 Y	0/1	0/1	1/1	0/1	2/2	0/2	2/2	0/2	0/2	0/2	0/2	0/2
Paper	Room Temperature	1 M	0/2	0/2	1/2	1/2	2/2	0/2	2/2	1/2	0/2	0/2	0/2	1/2
		6 M	0/2	0/2	2/2	1/2	2/2	0/2	2/2	0/2	0/2	0/2	1/2	0/2
		1 Y	0/2	0/2	0/2	0/2	2/2	0/2	2/2	0/2	0/2	0/2	0/2	0/2

b)

Exposure	Time	Purified		RFD		Purified		RFD		Purified		RFD		Purified	RFD
		Saliva		Saliva		Semen		Semen		Blood		Blood		Vaginal	Vaginal
		STATH	HNT3	STATH	HNT3	PRM2	SEMG1	PRM2	SEMG1	ANK1	ALAS2	ANK1	ALAS2	CYP2B7P1	CYP2B7P1
37°C	1 W	0/2	0/2	0/2	0/2	2/2	0/2	2/2	0/2	1/2	0/2	2/2	0/2	1/2	1/2
	1 M	0/2	0/2	0/2	0/2	2/2	0/2	2/2	0/2	2/2	1/2	1/2	0/2	0/2	1/2
56°C	1W	0/2	0/2	0/2	0/2	1/2	0/2	2/2	0/2	2/2	1/2	1/2	1/2	1/2	1/2
	1M	0/2	0/2	0/2	0/2	2/2	0/2	2/2	0/2	2/2	2/2	1/2	1/2	0/2	1/2
Room Temperature	1 W													0/2	1/2
	1 M													0/2	1/2

A similar trend was observed for saliva samples exposed to increased temperatures over time (Table 9b). Saliva was not identified in any of the samples tested. However, unlike with the substrate samples, blood was detected in all of the samples exposed at 56˚C and all samples except a 1-week sample exposed at 37˚C when the waste was tested. Semen was less variable and was able to be detected in all temperatures over time using the RFD method as well as the purified waste method with the exception of one sample exposed to 56˚C for 1 week. Additionally, half of the vaginal samples across all temperatures over time were successfully identified using the RFD method, whereas one 37˚C 1-week and one 56˚C 1-week vaginal sample was detected using the purified waste method. Because blood and semen could be identified in most all cases, it can be said that both body fluids can be successfully identified using both methods when exposed to extreme temperatures over time. Although both RNA and DNA are expected to be highly degraded for these sample types, body fluid identification and autosomal STR typing were not negatively effected overall.

Finally, to determine if this method was able to positively identify two different body fluids simultaneously, liquid semen and saliva was deposited in various amounts on different dried body fluid swabs. The results of the mixture analysis can be seen in Table 10 and compares the two RNA recovery methods. When semen was deposited onto dried vaginal secretions, semen was detected in half of the tested samples in as little as 1 µL of liquid body using the purified waste method. Vaginal secretions were not detected in these samples when either method is used. Body fluid detection was more successful for the other mixtures when liquid semen was deposited on dried blood, saliva and menstrual blood swabs.

Table 10 Comparison of body fluid markers detected in two-body fluid mixtures when extraction waste fractions are purified and when 15 µL of DNA extract is used.

The detection of a body fluid markers is indicated by a colored cell (saliva-blue, vaginal secretions- green, semen-yellow, menstrual blood-pink, blood-red). The cell is colored gray when no corresponding body fluid markers were detected for the total number of samples tested. The total number of markers detected in each sample out of the total number of samples tested are listed in each cell a) shows the results for the mixtures where liquid semen was deposited onto a different dried body fluid swab and compares both RNA recovery methods; b) shows the results for the mixtures where liquid saliva was deposited onto a different dried body fluid swab and compares both RNA recovery methods

a)

Dried Body Fluid	Volume of liquid Body Fluid (µL)	Body Fluid 1				Body Fluid 2			
		Purified		RFD		Purified		RFD	
Vaginal- Semen	Marker	CYP2B7P1		CYP2B7P1		PRM2	SEMG1	PRM2	SEMG1
	10	0/2		0/2		1/2	0/2	0/2	0/2
	5	0/2		0/2		1/2	0/2	0/2	0/2
	1	0/2		0/2		1/2	0/2	1/2	0/2
Blood- Semen	Marker	ANK1	ALAS2	ANK1	ALAS2	PRM2	SEMG1	PRM2	SEMG1
	10	2/2	1/2	0/2	0/2	1/2	0/2	0/2	0/2
	5	1/2	0/2	0/2	0/2	1/2	0/2	1/2	0/2
	1	0/2	0/2	1/2	0/2	0/2	0/2	1/2	0/2
Saliva-Semen	Marker	STATH	HNT3	STATH	HNT3	PRM2	SEMG1	PRM2	SEMG1
	10	0/2	1/2	0/2	0/2	1/2	0/2	0/2	0/2
	5	1/2	1/2	0/2	0/2	1/2	0/2	1/2	0/2
	1	2/2	0/2	0/2	0/2	1/2	0/2	1/2	0/2
Menstrual Blood-Semen	Marker	MMP10	LEFTY2	MMP10	LEFTY2	PRM2	SEMG1	PRM2	SEMG1
	10	1/2	0/2	1/2	0/2	2/2	0/2	1/2	0/2
	5	1/2	1/2	0/2	0/2	2/2	0/2	0/2	0/2
	1	1/2	1/2	1/2	1/2	1/2	0/2	2/2	0/2

b)

Dried Body Fluid	Volume of liquid Body Fluid (µL)	Body Fluid 1				Body Fluid 2			
		Purified		RFD		Purified		RFD	
Vaginal- Saliva	Marker	CYP2B7P1		CYP2B7P1		STATH	HNT3	STATH	HNT3
	10	1/2		1/2		1/2	1/2	1/2	1/2
	5	1/2		1/2		0/2	0/2	0/2	0/2
	1	1/2		1/2		0/2	0/2	0/2	0/2
Blood- Saliva	Marker	ANK1	ALAS2	ANK1	ALAS2	STATH	HNT3	STATH	HNT3
	10	2/2	1/2	2/2	1/2	2/2	2/2	2/2	2/2
	5	1/2	1/2	1/2	0/2	1/2	2/2	0/2	0/2
	1	2/2	1/2	2/2	1/2	1/2	0/2	1/2	1/2
Menstrual Blood-Saliva	Marker	MMP10	LEFTY2	MMP10	LEFTY2	STATH	HNT3	STATH	HNT3
	10	1/2	0/2	1/2	0/2	0/2	0/2	1/2	1/2
	5	1/2	1/2	1/2	1/2	0/2	0/2	1/2	1/2
	1	1/2	1/2	2/2	1/2	0/2	0/2	0/2	0/2

In some cases, both body fluids were identified, and semen especially was identified in at least half of all samples tested. Next, when liquid saliva was deposited onto various dried body fluid swabs, both body fluids (except in the case of the 5 µL and 1 µL vaginal-saliva samples) were able to be detected when both RNA recovery methods were taken into account. Dried blood and liquid saliva mixtures were the most consistently successful samples allowing for a positive identification of both body fluids with as little as 1 µL of saliva. In most cases, a successful identification of both body fluids was possible in one of the two donor sets tested for a particular variable. Unfortunately, RNA recovery for both methods was too variable to conclude whether mRNA profiling can successfully identify two-body fluid mixtures when liquid semen or saliva are deposited onto various dried swabs.

DNA Analysis

All samples were extracted, quantified, amplified and electrophoresed to ensure the DNA workflow was not affected by integrating mRNA profiling into the workflow. As can be seen in Table 11 a total number of 294 biological samples were extracted and amongst them, an average of 3,705 ng of DNA was recovered, allowing for complete STR detection in all preliminary validation studies. Likewise, when as little as 1 µL of liquid body fluid or 1/8 of a 50 µL stain (Figure 4 and Figure 5) was extracted using the co-extraction method, complete profiles were obtained. Although DNA recovery was consistently reliable for these types of samples, problems arose when body fluids were deposited on various substrates. When body fluids were deposited onto denim, abnormally low-levels of DNA were recovered

Table 11 The number of biological samples and their average DNA recovery using the PrepFiler™ DNA Extraction Kit for validation of RNA recovery in purified extraction waste and aliquots of DNA extract

Validation Study	Variable	# of Samples Extracted	Avg. DNA Conc. (ng/µL)	Avg. Total DNA (ng)
Waste Fraction	Separate Waste	14	154.0	7700
	Combined Waste	63	104.3	5215
Sample Size	Swabs/Stains	86	48.8	2440
Mock Casework	Substrate Deposition	53	6.0	300
	Temperature Exposure	36	81.0	4050
	Mixtures	42	50.5	2525
Total		294	74.1	3705

(average of 52 ng total yield of DNA) due not only to invisible fibers left in the lysate but also due to extraction inhibition caused by the indigo dyes in denim. Once the PrepFiler™ magnetic particles were added, they adhered to the fibers causing the beads to accumulate at the bottom of the tube, making lysate removal difficult. Although problems with the extraction were encountered, full profiles were obtained for all samples that contained fibers. As can be seen in Figure 6, even though 2 ng total yield of DNA was obtained (0.3 ng input into amp when the recommended input is 0.5 ng) from a semen sample deposited on denim and exposed to room temperature for 1 year, a complete STR profile was still obtained. It should be noted that the alleles at CSF1PO, D7S280 and SE33 locus have observably low RFU signals but are still above the 50 RFU threshold. This reduction in peak heights for some of the larger loci (i.e. ski slope effect) may be due to the fact that this was a low template sample. Full profiles were obtained for all other samples that were deposited on paper or polyester.

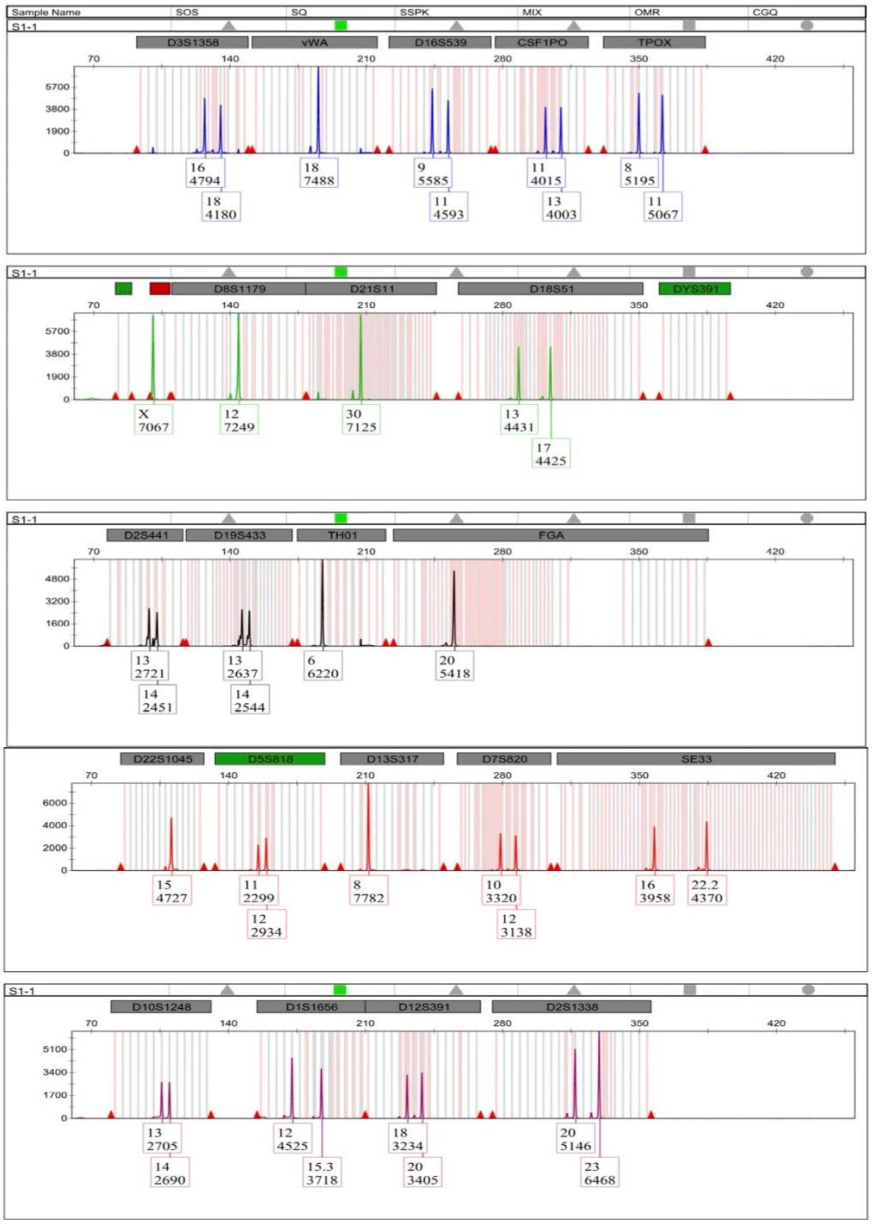

Figure 4 Autosomal STR profile from a 1 µL female saliva stain.
Complete 24 loci GlobalFiler™ profile of DNA recovered from a 1 µL saliva stain using a PrepFiler™ manual DNA extraction. Loci separated by dye color: Blue Channel (6-FAM), Green Channel (VIC), Yellow Channel (NED - shown in black), Red Channel (TAZ), Purple Channel (SID) purple; Channel Orange (LIZ-not shown). Below each peak is the allele call, and the RFU value. X-axis: base pair (bp) size; Y-axis: relative fluorescence units (RFUs).

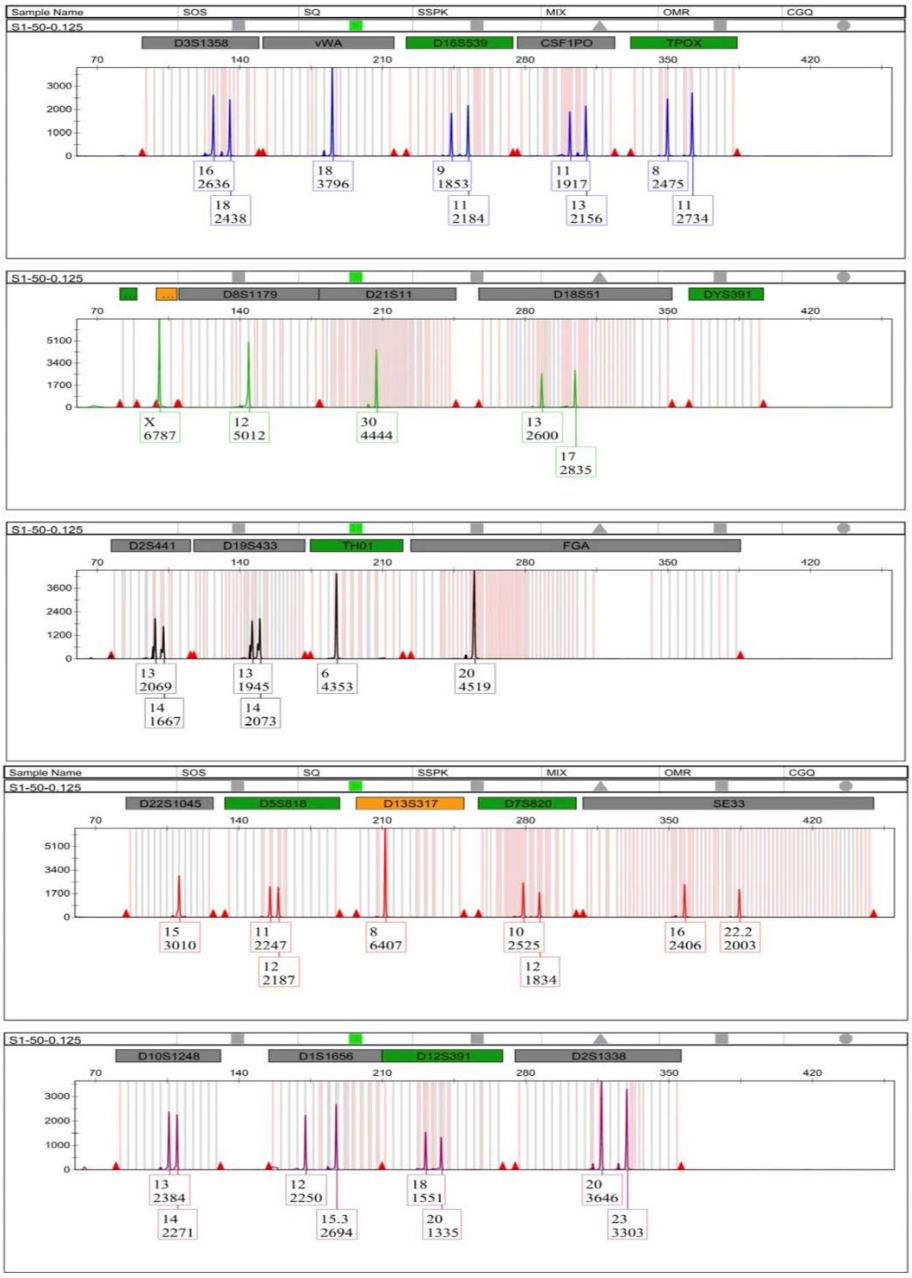

Figure 5 Autosomal STR profile of a 1/8th sized portion of a female 50 µL saliva stain. Complete 24 loci GlobalFiler™ profile of DNA recovered from a 1/8 sized portion of a 50 µL saliva stain using a PrepFiler™ manual DNA extraction. Loci separated by dye color: Blue Channel (6-FAM), Green Channel (VIC), Yellow Channel (NED - shown in black), Red Channel (TAZ), Purple Channel (SID) purple; Channel Orange (LIZ-not shown). Below each peak is the allele call, and the RFU value. X-axis: base pair (bp) size; Y-axis: relative fluorescence units (RFUs).

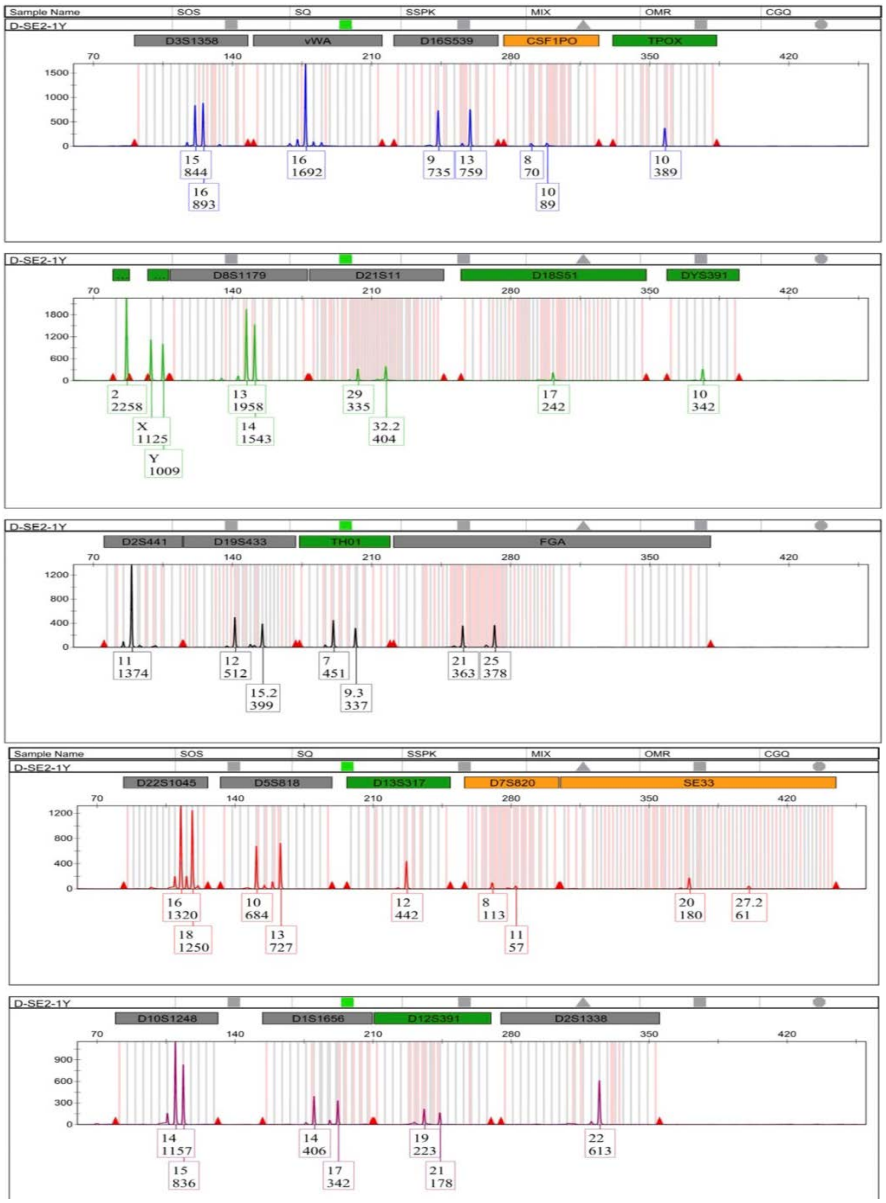

Figure 6 Autosomal STR profile of a semen stain deposited on denim and incubated at room temperature for one year.

Complete 24 loci GlobalFiler™ profile of DNA recovered from a semen stain deposited on denim and incubated at room temperature for one year using a PrepFiler™ manual DNA extraction. A max input of 0.3 ng of DNA was input into the amp (0.5 ng recommended). Loci separated by dye color: Blue Channel (6-FAM), Green Channel (VIC), Yellow Channel (NED - shown in black), Red Channel (TAZ), Purple Channel (SID) purple; Channel Orange (LIZ-not shown). Below each peak is the allele call, and the RFU value. X-axis: base pair (bp) size; Y-axis: relative fluorescence units (RFUs).

CHAPTER FOUR: RESULTS

RAPID MALE SPERM IDENTIFICATION 1-STEP RT-HRM ANALYSIS

To address another major problem in current DNA workflows, a novel rapid male DNA screening assay was developed to provide not only a way to triage sexual assault evidence but to possibly eliminate the need for microscopic sperm ID. The assay paired a rapid lysis-based RNA extraction with a 1-step combined RT-HRM. A rapid-RNA lysis was used to quickly extract RNA from sample types typically found in SAKs (eg. vaginal swabs), while consuming very little sample (tip-sized portion). The extraction permitted the waste fractions to be saved, as studied previously, for male DNA screening and possible male profiling. This was a similar process as previously discussed, however the process involved extracting RNA and recovering DNA from the waste fractions. The RNA extraction was paired with a High-Resolution Melt assay to provide a rapid detection and reduced cost alternative to CE based methods. When combined with a reverse transcription reaction, gene-specific markers are able to be amplified in real-time and detected with respect to their melting temperatures and distinct melt peak. To assess the capability of integrating sperm identification by mRNA profiling into this rapid upfront male DNA screening assay, an HRM analysis method first needed to be developed and optimized. An initial performance and sensitivity test were performed to ensure accurate detection of sperm using a 1-step combined RT-HRM assay to detect PRM2. Once optimized, body fluid samples were extracted using the rapid-RNA lysis-based method and tested for the presence of sperm using the RNA recovered from the extraction. To assess the performance of

the assay, single-sourced semen, vaginal secretions, menstrual blood, blood and saliva samples were extracted while saving the lysate waste. Once diluted and successfully screened for male DNA, indicated by a male quantitation value, the RNA extracts were examined for the presence of sperm. If there was an indication of male DNA and the presence of sperm, the waste samples were brought forward for possible male DNA profiling. Finally, because sexual assault evidence is the target sample type for this assay, vaginal-semen mixtures were analyzed to ensure the assay was just as robust in the presence of multiple body fluids.

Sensitivity and Initial Primer Testing

Based on previous work with HRM, PRM2 was selected for its amplification efficiency and sperm specificity [unpublished work, Ballantyne laboratory]. Initial HRM assay testing was performed to determine the melting temperature (T_m) of PRM2, which was determined to be 82.5 ($\pm$ 0.5 ˚C). To test the sensitivity of PRM2 using the reduced-time Power SYBR® 1-step RT-HRM assay, diluted RNA extracts from single-sourced semen samples from 2 different individuals with known concentrations (extracted using a standard organic RNA extraction, not the rapid lysis method) were serially diluted from 2 ng-0.1 ng of total RNA for set 1, and 2 ng - 0.125 ng of total RNA for an additional two sets (different donors used in each set). The results from the sensitivity testing are provided in Table 12. For set 1, PRM2 was not detected past 0.8 ng of input RNA. However, in set 2, PRM2 was detectable with as little as 0.125 ng of input RNA. For set 3, PRM2 was undetectable with 0.5 ng or less of input RNA. Therefore, despite some variation, PRM2 (i.e. sperm) was detected in as little as 0.5-0.13 ng of total RNA.

Table 12 Melting temperatures of serially diluted single sourced semen RNA extracts to test the sensitivity of the Power SYBR 1-Step reduced time assay

The detection of PRM2 is represented by a melting temperature (T_m) of ~82˚C and an orange colored cell. Cells colored in gray and a melting temperature ~64˚C represent that the sample did not detect PRM2.

Set 1	**2 ng**	**1.6 ng**	**1.2 ng**	**0.8 ng**	**0.4 ng**	**0.2 ng**	**0.1 ng**
C_T	30.4	30.1	31.4	31.4	38.4	N/A	37.1
T_m	**82.2**	**82.2**	**82.4**	**82.4**	64.6	64.6	65.4

Set 2	**2 ng**	**1 ng**	**0.5 ng**	**0.25 ng**	**0.125 ng**
C_T	34.1	35.5	36.4	37.0	35.9
T_m	**82.1**	**82.5**	**82.5**	**82.6**	**82.6**

Set 3	**2 ng**	**1 ng**	**0.5 ng**	**0.25 ng**	**0.125 ng**
C_T	35.6	36.2	36.8	N/A	N/A
T_m	**82.5**	**82.6**	**82.6**	64.4	64.24

Single-Source Samples

Next, analysis was performed to determine if it was possible to 1) analyze extract waste for male DNA for the triage of sexual-assault evidence and 2) integrate mRNA profiling into a male sperm detection assay to eliminate the need for microscopic sperm ID. To simulate the screening process (Figure 7), all sample waste fractions, saved from the rapid-RNA lysis-based

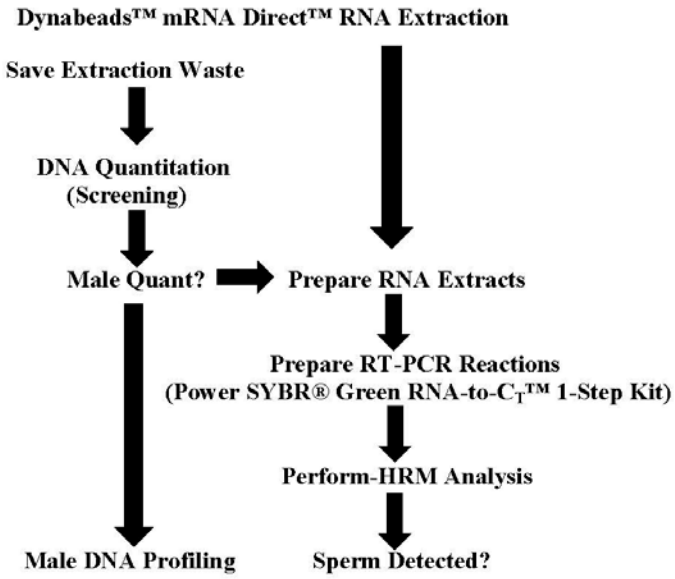

Figure 7 Suggested Rapid Male DNA Screening Assay workflow

extraction, were quantified to determine if male DNA was present and was indicated by an observed male quantitation value. Fifteen single-sourced non-vasectomized male semen swabs, 1 vasectomized male semen swab, 8 vaginal secretion swabs, and 4 menstrual blood, saliva, and blood samples were extracted using the rapid-lysis RNA extraction and the first waste fraction was saved for DNA analysis. For the purpose of the study, all samples were brought forward for subsequent HRM analysis to test the capability of the assay. PRM2 was successfully detected at the expected melting temperature for all tested semen samples and were undetected in the vasectomized male semen, vaginal secretion, saliva, blood and menstrual blood samples that were also tested (Table 13), thus demonstrating the specificity of the developed assay.

Table 13 Single sourced non-vasectomized male semen, vasectomized male semen, vaginal secretions, menstrual blood, saliva and blood samples analyzed for the presence of PRM2 using a rapid male sperm 1-step RT-HRM assay

The detection of PRM2 in single-sourced body fluid (Semen-orange, Vaginal secretions-green, menstrual blood-pink, saliva-blue, and blood-red) samples is represented by an orange colored cell. When PRM2 was undetected the cell is colored gray. The total number of PRM2 specific peaks detected at ~82˚C out of the total number of samples are listed in each cell.

Body Fluid	PRM2 Detected at ~82˚C (+/- 0.5˚C)
Semen	15/15
Semen (Vasectomized)	0/1
Vaginal	0/8
Menstrual blood	0/4
Saliva	0/4
Blood	0/4

As can be seen in Figure 8 (right), a single orange melt curve can be seen at ~82˚C, representing a positive detection of sperm-specific PRM2, compared to the melt curve (Figure 8 (left)) of a vasectomized male (azoospermic) semen sample, representing no detection of PRM2. Next, those samples tested positive for male DNA and/or sperm, were then subjected to downstream DNA analysis. As described in the proposed workflow for the Rapid Male Sperm Screening Assay (Figure 8), the lysate waste from the RNA extraction was saved and quantified. Once diluted to ensure DTT did not inhibit PCR, Y-STR profiling was performed. All DNA waste extractions were amplified using the Yfiler™ Plus PCR Amplification kit, which detects up to 27 potential Y-STR loci. All 27 loci were detectable in 14 out of the 15 single sourced semen samples.

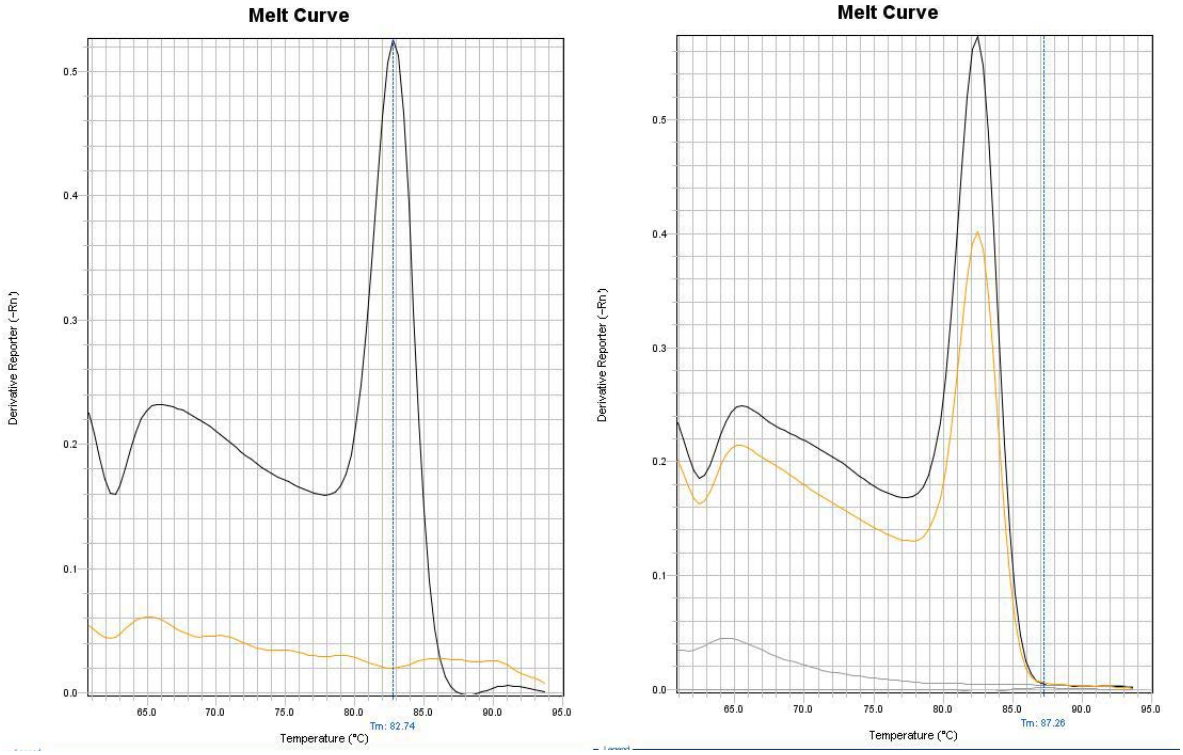

Figure 8 High Resolution Melt graphs for two different semen donors extracted using the rapid lysis-based RNA extraction.

Graph 1 (left) represents a vasectomized male semen sample; Graph 2 (right) represents a non-vasectomized male semen sample. Black curve-semen positive control sample; Gray curve-extraction blank and negative control; Orange curve- semen sample. The X-axis represents the temperature in Celsius and the Y-axis represents the negative first derivative of fluorescence with respect to temperature (-dF/dT).

Table 14 shows the amount of DNA input into the amp for each sample (0.15 ng to the targeted amount of 0.5 ng of DNA input). Those samples that do not reach the recommended amp input have a greater chance for locus drop-out. Semen sample 25 (SE25) can be considered low-level, with respect to male DNA, because only 0.15 ng of total DNA was amplified and as a result, only 26 out of 27 alleles were detectable. Due to the Y-chromosome's haploid nature, male-specific DNA may not yield a complete profile but often times the total amount of DNA is sufficient for traditional STR profiling. Therefore, being able to also amplify autosomal STRs is

important. In the case of SE25 a complete Y-STR profile was not obtained, but there was sufficient total DNA present in the sample to detect a complete autosomal STR profile (Figure 9). Based on the results thus far, single sourced evidence was successfully screened for the presence of male DNA, was able to detect sperm in non-vasectomized male semen samples and offered the ability to obtain a male DNA profile without disrupting the flow of analysis.

Table 14 Amount of amplified male DNA with respect to the number of alleles detected for single sourced samples successfully screened for PRM2

The total amount of male DNA amplified to target 27 Y-STRs. The number of alleles detected out of the total detectable alleles is listed in the cells. Samples that positively detected PRM2 during HRM analysis are represented by an orange colored cell.

Sample	Total Male DNA amplified (ng)	Number of alleles present
SE5	0.3	**27/27**
SE15	0.2	**27/27**
SE16	0.5	**27/27**
SE25	0.15	26/27
SE26	0.5	**27/27**
SE460	0.25	**27/27**
SE461	0.4	**27/27**
SE462	0.5	**27/27**
SE463	0.3	**27/27**
SE464	0.5	**27/27**
SE324	0.5	**27/27**
SE325	0.5	**27/27**
SE326	0.5	**27/27**
SE327	0.3	**27/27**
SE328	0.5	**27/27**

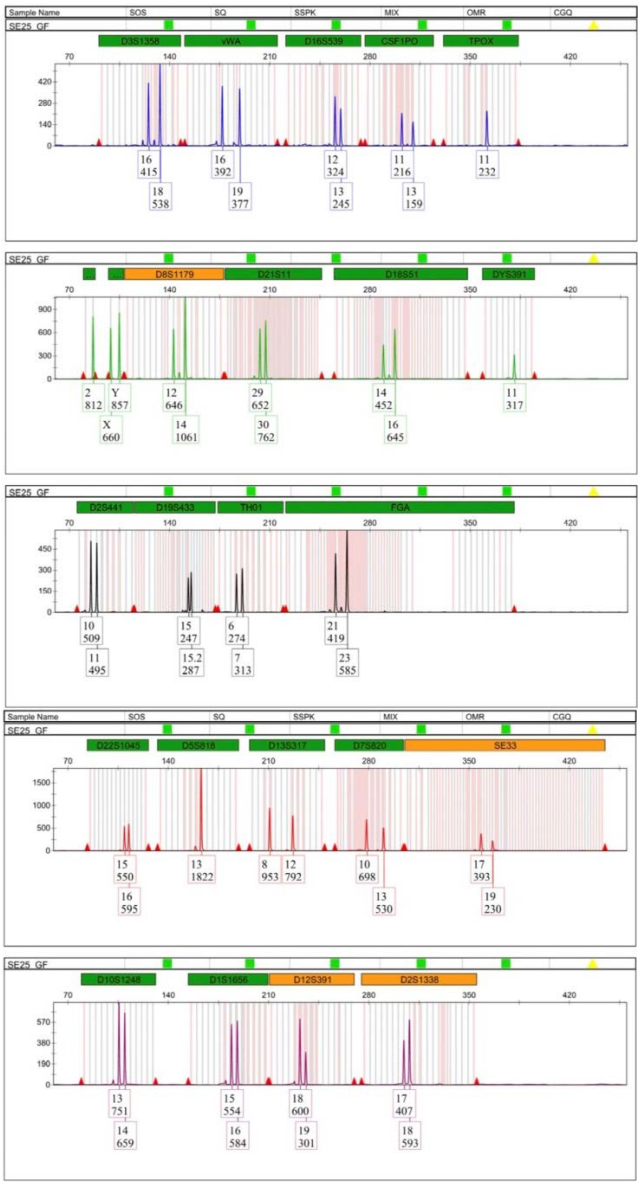

Figure 9 Autosomal STR profile obtained from the extraction waste fraction of a semen sample extracted with rapid RNA lysis-method.

Complete 24 loci GlobalFiler™ profile when 0.15 ng of DNA is amplified and recovered from the waste fraction of a tip-sized semen sample extracted with the Dynabeads® mRNA Direct® kit. Loci separated by dye color: Blue Channel (6-FAM), Green Channel (VIC), Yellow Channel (NED - shown in black), Red Channel (TAZ), Purple Channel (SID) purple; Channel Orange (LIZ-not shown). Below each peak is the allele call, and the RFU value of the detected gene product. X-axis: base pair (bp) size; Y-axis: relative fluorescence units (RFUs).

Mixture Samples

To examine the capability of the screening assay to detect male DNA in sexual-assault related evidence, admixed vaginal-semen mixtures were analyzed. Several vaginal-semen mixtures, each with different male-female donors, were made by depositing 10, 5, 2 and 1 µL of liquid semen onto dried vaginal swabs. Once each sample was extracted using the Dynabeads™ mRNA Direct™ mini kit and the lysis waste saved, the waste was screened for male DNA. As expected, although there were variations between donors, as the volumes of semen decreased so did the amount of male DNA detected. Next, HRM analysis was performed to identify the presence of sperm in each mixture. As can be seen in Table 15, PRM2 was detectable in all of the 10 and 5 µL semen deposits, five out of six of the 2 µL semen deposits and four out of the six 1 µL semen deposits.

Table 15 Detection of sperm in vaginal-semen mixtures with different amounts of semen deposited onto dried vaginal swabs using the rapid male sperm assay

Each cell lists the number of samples in which sperm was successfully detected out of the total number of mixtures analyzed with respect to the amount of semen deposited for each mixture.

	Semen 10 µL	**Semen 5 µL**	**Semen 2 µL**	**Semen 1 µL**
Vaginal Donor	6/6	6/6	5/6	4/6

Upon melt curve analysis, there was a single peak at the expected melting temperature for the mixtures represented in Figure 9. It should be noted that this assay is not necessarily quantitative, and the height of a taller melt peak does not mean there has a greater quantity of RNA in the sample when compared to a smaller peak. In Figure 10a a vaginal-semen mixture

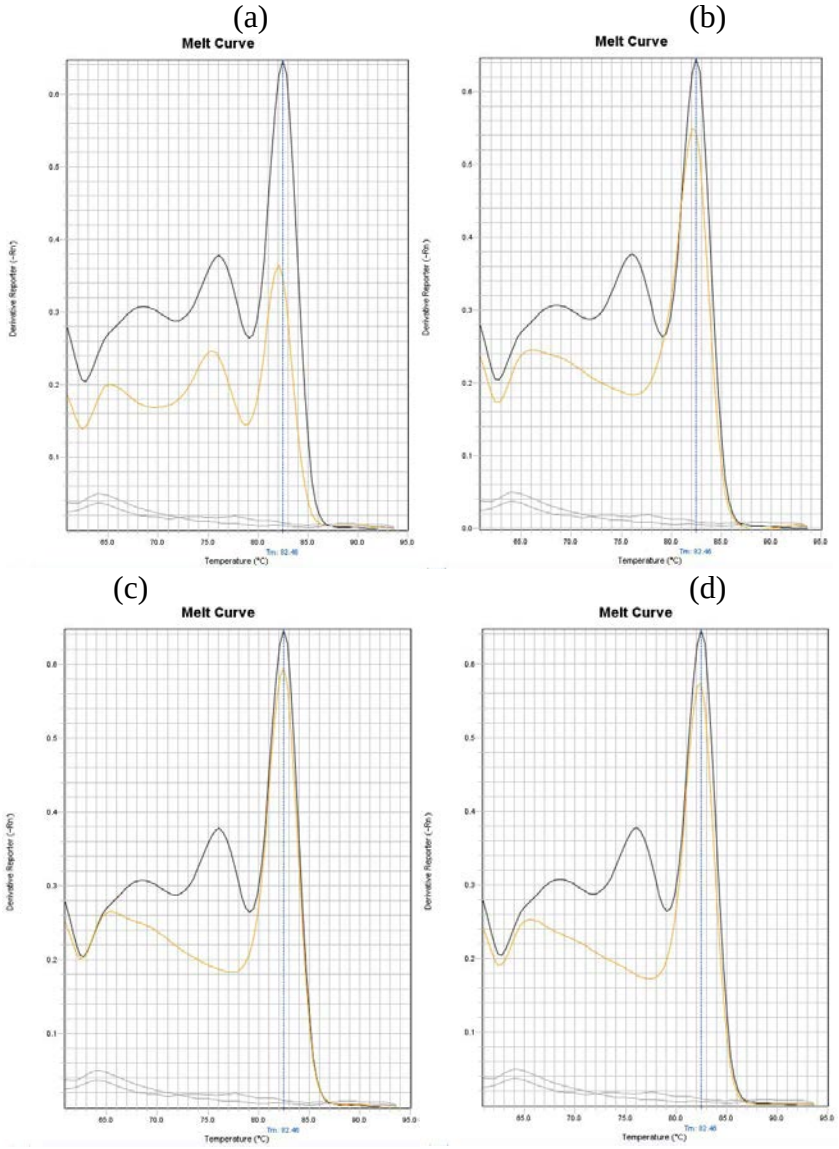

Figure 10 High Resolution Melt graphs for vaginal secretion-semen mixtures extracted using the rapid lysis-based RNA extraction.

a) Vaginal-semen mixture with 10 µL of semen deposited onto a dried vaginal swab; b) vaginal-semen mixture with 5 µL of semen deposited onto a dried vaginal swab; c) vaginal-semen mixture with 2 µL of semen deposited onto a dried vaginal swab vasectomized male semen sample; d) vaginal-semen mixture with 1 µL of semen deposited onto a dried vaginal swab. Black curve-semen positive control sample; Gray curve-extraction blank and negative control; Orange curve- semen sample. The x-axis represents the temperature in Celsius and the y-axis represents the negative first derivative of fluorescence with respect to temperature (-dF/dT).

containing 10 µL of semen has a comparatively lower peak height than a vaginal-semen mixture containing 1 µL of semen (Figure 10d). Based on these results, the sensitivity of this HRM assay can possibly detect sperm in as little as 1 µL of semen but is 100% successful with as little as 5 µL, when vaginal secretion-specific RNA is present. Based on the successful detection of sperm in mixtures containing as little as 1 µL of semen, a mixture sensitivity test was performed. Volumes of semen ranging from 1.25-0.16 µL were deposited onto dried vaginal swabs. The vaginal-semen donors were kept the same for this analysis. As can be seen in Table 16, while variations between donors was observed, PRM2 was detectable in as 0.15 µL of semen using this assay.

Table 16 Detection of Sperm specific PRM2 body fluid marker in vaginal-semen body fluid mixtures with 0.15-1.25 µL of semen to determine the limit of detection of the 1-Step RT-HRM assay

The detection of PRM2 is represented by a melting temperature (T_m) of ~82˚C and an orange colored cell. Cells colored in gray represent samples that did not detect PRM2.

Vaginal Donor	Semen Donor	Volume of Semen (µL)	C_T	T_m
VS8	**SE461**	1.25	32.9	**82.1**
		0.625	36.8	**82.3**
		0.3125	37.0	**82.3**
		0.1563	N/A	
VS40	**SE462**	1.25	35.5	**81.8**
		0.625	34.3	**82.3**
		0.3125	35.3	**82.5**
		0.1563	34.8	**82.7**
VS37	**SE463**	1.25	N/A	
		0.625	N/A	
		0.3125	N/A	
		0.1563	N/A	

As a result, the HRM assay has the capability to detect sperm in mock sexual-assault evidence and in evidence that may contain very little semen in the presence of other body fluids.

Next, to determine if DNA analysis was possible in these types of samples, mixtures successfully screened for male DNA and/or tested positive for sperm were subjected to male DNA profiling. Table 17 lists the six different vaginal-semen mixture samples that were analyzed with respect to the total amount of DNA inputted into the amp. The specific samples that detected sperm are colored in orange and those that tested negative are in gray. As expected, the samples negative for sperm also had a comparably low DNA yield. A majority of these low-level DNA samples obtained a complete male profile. Based on previous work with single-sourced semen samples, it is possible to positively detect sperm in a sample and unsuccessfully detect a complete Y-STR profile in the DNA waste. Figure 11 represents the melt-curve for a sample in which sperm was successfully detected and 0.07 ng of DNA was amplified from the extraction waste fraction. There was not enough male DNA present in the waste to obtain a complete male profile (Figure 12). The overall goal of this assay is not to obtain a male profile but to be able to successfully screen samples for male DNA and possibly detect the presence of sperm. As a result, this rapid male screening assay can successfully screen mock sexual-assault evidence for male DNA using the waste from a rapid- lysis- based RNA extraction. This assay has the additional benefit of being able to detect sperm and obtain a male-profile with no modifications to the DNA workflow.

Table 17 Number of Y-STR alleles detected in the RNA extraction waste fractions of vaginal-semen mixtures with various amounts of semen and previously screened for sperm
The total amount of male DNA amplified to target 27 Y-STRs. The number of alleles detected out of the total detectable alleles is listed in the cells. Samples that positively detected PRM2 during HRM analysis are represented by an orange colored cell. Samples in which PRM2 was not detected are represented by an orange cell.

Vaginal Donor	Semen Donor	Volume of Semen (µL)	Total Male DNA amplified(ng)	Alleles Present
VS4	SE460	10	0.3	**27/27**
		5	0.3	**27/27**
		2	**0.07**	18/27
		1	**0.07**	23/27
VS41	SE26	10	0.5	**27/27**
		5	0.5	**27/27**
		2	0.5	**27/27**
		1	0.5	**27/27**
VS42	SE25	10	**0.1**	16/27
		5	**0.05**	14/27
		2	**0.01**	5/27
		1	**0.003**	5/27
VS8	SE461	10	0.5	**27/27**
		5	0.45	**27/27**
		2	0.25	**27/27**
		1	**0.1**	**27/27**
VS37	SE463	10	0.5	**27/27**
		5	0.25	**27/27**
		2	**0.1**	**27/27**
		1	**0.1**	26/27
VS40	SE462	10	0.5	**27/27**
		5	0.5	**27/27**
		2	0.35	**27/27**
		1	**0.05**	**27/27**

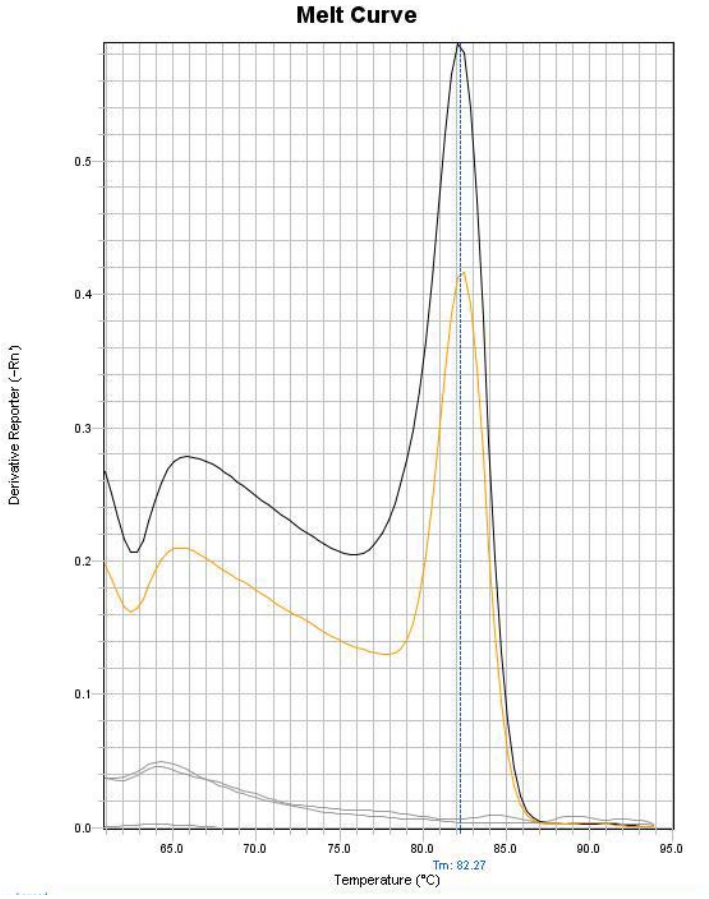

Figure 11 High Resolution Melt graphs for a vaginal-semen mixture extracted using the rapid lysis-based RNA extraction.

1 µL of semen deposited onto dried vaginal swab and extracted using the rapid RNA lysis method. Black curve-semen positive control sample; Gray curve-extraction blank and negative control; Orange curve- semen sample. The x-axis represents the temperature in Celsius and the y-axis represents the negative first derivative of fluorescence with respect to temperature (-dF/dT).

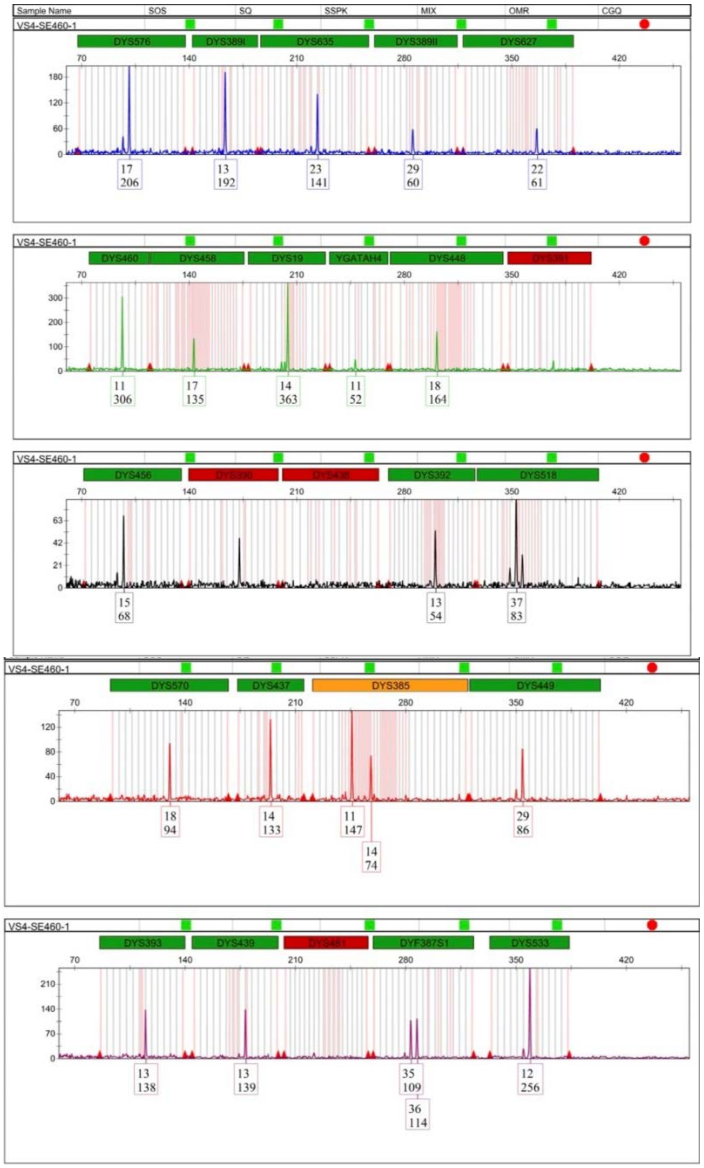

Figure 12 Y-STR profile obtained from the waste fraction of a vaginal-semen mixture containing 1 µL of semen extracted with the rapid RNA lysis method.

Incomplete 25 loci YFiler™ profile when 0.07 ng of DNA is amplified and recovered from the waste fraction of a vaginal-semen mixture containing 1 µL of semen and extracted with the Dynabeads® mRNA Direct® kit. Dropout observed at the DYS391, DYS390, DYS438, and DYS481 Loci. Loci separated by dye color: Blue Channel (6-FAM), Green Channel (VIC), Yellow Channel (NED - shown in black), Red Channel (TAZ), Purple Channel (SID) purple; Channel Orange (LIZ-not shown). Below each peak is the allele call, and the RFU value of the detected gene product. X-axis: base pair (bp) size; Y-axis: relative fluorescence units (RFUs).

CHAPTER FIVE: CONCLUSIONS

The main goal of this study was to provide improvements to mRNA profiling methodologies for better integration into DNA workflows. The traditional body fluid identification methods, still currently used by crime labs, are laborious, do not allow for definitive identification of all forensically relevant body fluids and are not easily integrated into DNA workflows. Therefore, crime labs want to bypass body fluid identification altogether. This could possibly underrepresent the evidence and be detrimental in court proceedings. It is important for investigators to determine the origin and location of a stain/tissue because it can provide additional information surrounding the circumstances of a crime and locate potential sources of DNA. Although many molecular-based methods for the identification of body fluids have been studied, mRNA profiling has the most promise for potential integration into established DNA workflows. Because mRNA profiling works by targeting the multicellular transcriptome unique to body fluids of forensic interest, it is a sensitive and specific way to potentially identify all relevant body fluids in biological stains. It also has the added benefits of using modern DNA technologies, making it easily integrated into DNA workflows. While advancements have been made, more research is needed to be done to improve mRNA profiling.

This study aimed to improve two challenges effecting DNA workflows. The first was to provide a possible RNA recovery method that did not interfere with established DNA workflows and the second was to integrate mRNA profiling into an upfront male DNA screening assay that not only permitted a triage for sexual-assault evidence, by indicating the presence of potential

male DNA, but could also potentially reduce or eliminate a significant bottleneck in the standard DNA workflow of microscopic sperm identification. The first study was able to provide two unique methods for recovering RNA, without modifying an established DNA extraction, using the waste from DNA extractions and the DNA extract itself. Both of these methods permitted the identification of blood, semen, saliva, vaginal secretions and menstrual blood when mRNA profiling was performed using multiplex-PCR followed by capillary electrophoresis. Additionally, both methods had no effect on DNA recovery, allowing for detection of complete STR profiles for the samples analyzed. The second study provided a method for rapidly screening sexual assault evidence with an upfront male DNA detection and subsequent sperm and Y-STR detection. This method paired a rapid-lysis RNA extraction with a sensitive 1-step combined reverse transcription-HRM assay for the detection of sperm. The extraction allows for the waste fraction to be saved and quantified for an upfront detection of male-DNA, indicated by a male quant value. Then the RNA extracts can be analyzed for the presence of sperm using the HRM assay. Once samples were successfully screened for male DNA and/or detected for sperm, the waste fraction could be sent forward for male profiling.

In the first study, several validation studies were performed to determine if mRNA profiling was possible amongst various waste fractions and aliquots of DNA extract, sample sizes, and forensically relevant evidence types. Body fluid detection was possible in all waste fractions saved from a PrepFiler™ manual DNA extraction. However, the first lysis buffer/lysate waste fraction once purified with the PureLink® RNA mini kit, permitted consistent body fluid detection for all forensically relavent sample types. It was also discovered that RNA could be

recovered without purification of waste fractions, using the DNA extract itself. It was believed that mRNA also binds to the silica-based beads, during the extraction, and are eluted off of the beads along with DNA. As little as 10 µL of DNA extract provided successful identification of all relevant body fluids under normal conditions. To evaluate if either method allowed for preferential body fluid identification, all validation studies were performed using both RNA recovery methods.

In terms of sample size, half and quarter sized swabs worked best for detecting saliva/menstrual blood and semen/vaginal secretions respectively. This was consistent amongst both RNA recovery methods, with the exception of a single semen sample that was unidentified using the RNA from DNA. Body fluid identification was much more variable when it came to stain testing. Blood was successfully identified in half 50 µL stains and the equivalent 25 µL stain using both methods. However, RNA from as little as 1 µL of blood was only successfully recovered using the RNA from DNA. Likewise, more saliva makers were successfully identified in the RNA from DNA than from the waste fraction. Semen was the least successfully identified body fluid across both methods when deposited as a stain. Next, the methods were analyzed when mock casework samples were tested. Body fluids were deposited as stains on denim, polyester and paper were left out at room temperature for up to a year. Denim samples posed a problem during the extraction process and resulted in decreased DNA recovery and very few successful body fluid identifications, mainly blood, across all time points. Blood and saliva stains on polyester were successfully identified across most time points using the RNA from DNA and although the results varied, they were more successfully identified on paper using the RNA from

DNA as well. Semen was successfully identified using both methods on both polyester and paper. When the same body fluids were deposited on cotton and exposed to higher temperatures, saliva was unidentifiable in all cases using both methods. However, semen and blood were more successfully identified even when exposed at 56˚C for 1 month using either method. Finally, both methods were evaluated with body fluid mixtures. It can be said that when making mixture samples the liquids being deposited onto swabs are very viscous and the total volume does not always get absorbed into the swab. As a result, both methods allowed for possible identification of one or both of the two-body fluids when mixed, but the results were variable and further analysis needs to be performed to validate if these methods can identify more than one body fluid when present in samples.

Although there were false negative results for some body fluids, this method did not produce any false positive identifications. Therefore, the detection of a specific allele can be considered a positive identification whereas no detection might mean that although body fluid identification was unsuccessful further analysis optimization (i.e. lowering analysis RFU thresholds) could improve the number of false negative results. Based on these results, mRNA profiling can be successfully integrated into a DNA workflow using both the DNA extraction waste, once purified, and also directly from the DNA extract itself without the need to purify. Integration of this method does not influence the recovery of DNA and allows for mRNA profiling simultaneously or when necessary. Although the method is variable amongst sample types, it has the ability to provide investigators with additional information in terms of body fluid identification.

In the second study, the use of the DynaBeads™ mRNA Direct™ Kit allowed for a quick way to extract mRNA from very little sample as well as the ability to save the extraction waste for an upfront male DNA detection. The crude RNA extracts were easily integrated into a highly sensitive 1-step combined RT-HRM assay for the detection of sperm. Single-sourced body fluid samples were successfully screened for the presence of male DNA and sperm could be successfully detected using the PRM2 biomarker. Upon HRM analysis, samples containing sperm had a single distinct melt peak around a T_m of 82˚C, which was absent when PRM2 was not detected. The HRM assay provides a quick and sensitive way to detection of sperm without the need to perform a laborious microscopic sperm ID. The analyst then has the ability to move forward with DNA analysis using the remaining unused sample or using the waste fraction from the RNA extraction that was already performed. Because the purpose of this assay was to screen sexual-assault evidence, it was important to determine if vaginal-semen mixtures containing low levels of semen could be successfully screened for male DNA and subsequently detect sperm and a male DNA profile. Although variable, male DNA was detected in all extraction waste samples and PRM2 was detectable in all vaginal-semen mixtures containing 10-5 µL of semen. These sample waste fractions were also able to provide enough male DNA to detect a complete Y-STR profile. Although the assay was capable of detecting male DNA in vaginal-semen mixtures containing as little as 1 µL of semen the waste fraction did not always allow for complete Y-STR detection. Samples that were successfully screened for PRM2 but did not recover enough DNA in the waste fraction, can always be re-extracted using traditional methods because very little sample was used for the screening assay.

Although this study proved that mRNA profiling can be fully integrated into existing DNA workflows, more optimization to both methods needs to be done. Many crime labs use an automated DNA extraction process and cannot simply remove the waste fractions throughout the process. The PrepFiler™ kit was designed for both a manual and automated DNA extraction, and further validation studies need to be performed to determine if RNA can be recovered when an automated extraction is performed. This may lead to more consistently successful body fluid detection for those more difficult sample types. Another future study that may improve RNA recovery in extraction waste, is to evaluate a reduced volume purification protocol. By reducing the elution volume, the recovered RNA could in turn be more concentrated allowing for better detection. Finally, although the HRM assay was to designed to provide a sperm ID, it is the hope that more body fluid markers can be integrated into the assay. Current work is underway into a duplex 1-step RT-HRM assay that can identify both seminal fluid and sperm. Although sperm is indicative of semen, sperm is not always present in semen (azoospermic males). With the ability to detect both seminal fluid and sperm, there would be no need to perform microscopic sperm ID or semen specific tests such as PSA [8, 9]. The results provided in this study along with further validation and optimization of the methods, prove the promise of integrating mRNA profiling into DNA workflows. These advantages prove beneficial for forensic casework and provide alternative methods for body fluid identification.

REFERENCES

[1] Butler, John. "Advanced Topics in Forensic DNA Typing: Methodology." *Elsevier*: (2012): 1-18. Print.

[2] Juusola, Jane, and Jack Ballantyne. "Messenger RNA profiling: a prototype method to supplant conventional methods for body fluid identification*." Forensic Science International*. 135 **(**2003):85-96. Print.

[3] Carr, Sophie. "Forensic Science: The Future of Body Fluid Identification." *Measurement + Control*. 42 (2009): 310-13. Print.

[4] Hanson, Erin, and Jack Ballantyne. "RNA Profiling for the identification of the Tissue Origin of Dried Stains in Forensic Biology." *Forensic Science Review*. 22(2010): 145-57. Print.

[5] Harbison, SA., and RI Fleming. "Forensic body fluid identification: state of the art." *Research and Reports in Forensic Medical Science*. 6 (2016): 11-23. Print.

[6] Virkler, Kelly, and Igor Lednev. "Analysis of body fluids for forensic purposes: From laboratory testing to non-destructive rapid confirmatory identification at a crime scene." *Forensic Science International.* 188 (2009): 1-17. Print.

[7] Ja Hyun An, Hwan Young Lee, et al. "Body fluid Identification in forensics." *The Korean Society for Biochemistry and Molecular Biology: BMB Reports*. (2012): 545-53. Print.

[8] Yokota, Makoto, et al. "Evaluation of prostate-specific antigen (PSA) membrane test for forensic examination of semen." *Legal Medicine.* 3 (2001): 171-76. Print

[9] Hochmeister, Manfred, et al. "Evaluation of prostate-specific anigen (PSA) membrane test assays for the forensic identification of seminal fluid." *Journal of Forensic Science.* 44.5 (1999): 1057-60. Print.

[10] Bauer, M., and Patzelt, D. "Protamine mRNA as molecular marker for spermatozoa in semen stains." *International Journal of Legal Medicine.* 117.3 (2003): 175-79. Print.

[11] U. S. Department of Justice. "National Best Practices for Sexual Assault Kits: A Multidisciplinary Approach" *National Institute of Justice*. (2017): 1-140. Print.

[12] Allery, JP., et al. "Cytological detection of spermatozoa: comparison of three staining methods." *Journal of Forensic Science*. 46.2 (2001): 349-51. Print.

[13] Old, Jennifer, Martersteck, C., and A. Kalinina. "Developmental Validation of SPERM HY-LITER Experss" (Lombard, IL) *Independent Forensics.*Print.

[14] Hochendoner, Sara. "Streamlining the DNA Process through Implementation of Automation and Information Technologies." *U.S. Department of Justice* (2011): 1-269. Print.

[15] Department of Forensic Science. "FBS07- Microscopic Examination of Spermatozoa by Christmas Tree Stain." *District of Columbia.* (2014). 1-6. Print.

[16] Sacco, Lisa, and Nathan James. "Backlog of Sexual Assault Evidence: In Brief." *Congressional Research Service*. (2015): 1-11. Print.

[17] Senate Judiciary. "Sexual Assault Forensic Evidence Reporting Act." *S. 80.* (2013).

[18] Lindenberg, Alexander, Maaskant, Petra, and Titia Sijen. "Implementation of RNA profiling in forensic casework." *Forensic Science International: Genetics.* 3 (2012): 159-166. Print.

[19] Sijen, Titia. "Molecular approaches for forensic cell type identification: On mRNA, miRNA, DNA methylation and microbial markers." *Forensic Science International: Genetics*. 18 (2015): 21-32. Print.

[20] Vidaki, A., Daniel, B. and Denise Syndercombe Court. "Forensic DNA Methylation profiling-Potential opportunities and challenges." *Forensic Science International: Genetics*. 7 (2013): 499-507. Print.

[21] Legg, Kelvin, et al. "Discovery of highly specific protein markers for the identification of biological stains." *Electrophoresis* 35.22 *(*2014): 3069-78. Print.

[22] Haas, Cordula, Hanson, Erin, and Jack Ballantyne. "Capillary Electrophoresis of a Multiplex Reverse Transcription-Polymerase Chain Reaction to Target Messenger RNA Markers for Body Fluid Identification." *Methods Molecular Biology.* 830 (2012): 169-183. Print.

[23] Hanson, Erin, Haas, Cordula, et al. "Specific and Sensitive mRNA biomarker for the identification of skin in 'touch DNA' evidence." *Forensic Science International: Genetics.* 6.5 (2012): 548-558. Print.

[24] Hanson, Erin, and Jack Ballantyne. "Highly specific mRNA biomarkers for the identification of vaginal secretions in sexual assault investigations." *Science and Justice.* 53 (2013): 14-22. Print.

[25] Haas, C., Hanson, E., Morling, N., Jack Ballantyne. "Collaborative EDNAP exercises on messenger RNA/DNA co-analysis of body fluid identification (blood, saliva, semen) and STR profiling". *Forensic Science International: Genetics*. 3 (2011): e5-e6. Print.

[26] Juusola, Jane, and Jack Ballantyne. "Multiplex mRNA profiling for the identification of body fluids." *Forensic Science International.* 152 (2005): 1-12. Print.

[27] Juusola, Jane, and Jack Ballantyne. "mRNA Profiling for Body Fluid Identification by multiplex Quantitative RT-PCR." *Journal of Forensic Science.* 52.6 (2007): 1252-62. Print.

[28] Roeder, Amy, and Cordula Haas. "mRNA profiling using a minimum of five mRNA markers per body fluid and a novel scoring method for body fluid identification." *International Journal of Legal Medicine.* 127 (2013): 707-721. Print.

[29] Fang, R., Manohar, et al., "Real-time PCR for the detection of tissue and body fluid specific mRNAs." *International Congress Series.* 1288 (2006): 685-87. Print.

[30] Bowden, Anna, Fleming, and SallyAnn Harbinson. "A method for DNA and RNA co-extraction for use on forensic samples using the Promega DNA IQ system." *Forensic Science International: Genetics.* 5 (2011): 64-68. Print.

[31] Alvarez, Michelle, Jane Juusola, and Jack Ballantyne. "An mRNA and DNA co-isolation method for forensic casework samples." *Analytical Biochemistry.* 335.2 (2004): 289-298. Print.

[32] Parker, Charley, Hanson, Erin, and Jack Ballantyne. "Optimization of dried stain co-extraction methods for efficient recovery of high quality DNA and RNA for forensic analysis. *Forensic Science International: Genetics.* 3 (2011): 309-310. Print.

[33] Schweighardt, Andrew, et al. "Evaluation of Commercial Kits for Dual Extraction of DNA and RNA from Human Body Fluids." *Journal of Forensic Sciences***.** 60 (2015): 157-65. Print.

[34] Hanson, Erin, and Jack Ballantyne. "Multiplex high resolution melt (HRM) messenger RNA profiling assays for body fluid identification." *Forensic Science International: Genetics.* 4 (2013): 125-126. Print.

[35] Hanson, Erin, and Jack Ballantyne. "Rapid and inexpensive body fluid identification by RNA profiling-based multiplex High Resolution Melt (HRM) analysis." *F1000Research.* 2.281 (2014): 1-23. Print.

[36] Grabmüller, Melanie, Madea, Burkard, and Cornelius Courts. "Comparative evaluation of different extraction and quantification methods for forensic analysis." *Forensic Science International: Genetics.* 16 (2015): 195-202. Print.

[37] Brevnov, Maxim, et al. "Automated Extraction of DNA from Forensic Sample Types Using the PrepFiler Automated Forensic DNA Extraction kit." *JALA* 14 (2009): 294-302. Print.

[38] Brevnov, Maxim, et al. "Developmental Validation of the PrepFiler Forensic DNA Extraction Kit for Extraction of Genomic DNA from Biological Samples." *Journal of Forensic Science.* 54.3 (2009): 599-607. Print.

[39] Applied Biosystems™. "PrepFiler™ Forensic DNA Extraction Kit: User Guide." ThermoFisher, Foster City, CA. (2008): Catalog # 4390932 Rev. B. Print.

[40] Life Technologies. "PureLink® RNA Mini Kit" ThermoFisher, Foster City, CA. (2012): Catalog # 12183018 Rev A. Print.

[41] Applied Biosystems™. "Novel Y-screen assay for sexual assault samples using the Quantifiler Trio DNA Quantification Kit." ThermoFisher, Foster City, CA. (2015): 1-6. Print.

[42] Alderson, Gerry, et al. "Inferring the presence of spermatozoa in forensic samples based on male DNA fractionation following differential extraction." *Forensic Science International: Genetics*. 36 (2018): 225-232. Print.

[43] Life Technologies. "DynaBeads® mRNA Direct™ kit: For the isolation of pure mRNA directly from crude samples." ThermoFisher, Foster City, CA. (2012): Catalog # 610111 Rev 009. Print.

[44] Applied Biosystems™. "GlobalFiler™ PCR Amplification Kit: User Guide." ThermoFisher, Foster City, CA. (2016: Catalog # 4476135 Rev E. Print.

[45] Applied Biosystems™. Yfiler™ Plus PCR Amplification Kit User Guide. ThermoFisher, Foster City, CA. (2019): Catalog # 4484678 Rev D. Print.