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Development and Evaluation of a Combinatorial RT-qPCR Multiplex for Forensic Body Fluid Identification

Carolyn A. Lewis
Virginia Commonwealth University

DEVELOPMENT AND EVALUATION OF A COMBINATORIAL RT-qPCR MULTIPLEX FOR FORENSIC BODY FLUID IDENTIFICATION

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Integrative Life Sciences at Virginia Commonwealth University

by

Carolyn Anne Lewis
B.S. in Forensic Science, Virginia Commonwealth University, 2015

Principal Investigator: Sarah Seashols-Williams, Ph.D.
Associate Professor & Graduate Program Director
Department of Forensic Science

Virginia Commonwealth University
Richmond, Virginia
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Carolyn Anne Lewis

APPROVED BY:

Sarah Seashols-Williams, Ph.D.
Committee Chair
Department of Forensic Science

Baneshwar Singh, Ph.D.
Committee Member
Department of Forensic Science

Catherine Connon, Ph.D.
Committee Member
Department of Forensic Science

Tracey Dawson Green, Ph.D.
Committee Member
Department of Forensic Science

Edward Boone, Ph.D.
Committee Member
Department of Statistical Science and Operations Research

DEDICATION

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ABBREVIATIONS

α	Alpha
µL	Microliter
µM	Micromolar
AP	Acid phosphatase
BFID	Body fluid identification
BV	Bacterial vaginosis
CART	Classification regression tree
CE	Capillary electrophoresis
cDNA	Complementary DNA
Cq	Quantification cycle
DNA	Deoxyribonucleic acid
dsDNA	Double-stranded DNA
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
fg	Femtogram
GTF	Glucosyltransferase enzyme
HCl	Hydrochloric acid
hsa	Human specific antigen
HTS	High-throughput sequencing
IDT	Integrated DNA Technologies, Inc.
IRB	Institutional Review Board
ISR	Intergenic spacer region

KM	Kastle-Meyer
KPICS	Kernechtrot–Picroindigocarmine stain
LAMP	Loop-mediated isothermal amplification
LNA	Locked nucleic acid
M	Molar
MB	Menstrual blood
mg	Milligrams
min	Minutes
MIQE	Minimum information for publication of qPCR experiments
miRNA or miR	MicroRNA
mL	Milliliter
mM	Millimolar
mm	Millimeter
mRNA	Messenger RNA
NaCl	Sodium chloride
ng	Nanograms
NGS	Next-generation sequencing
NIJ	National Institute of Justice
nM	Nanomolar
nm	Nanometer
NTC	No template control
PCR	Polymerase chain reaction
pg	Picogram

PSA	Prostate-specific antigen
qPCR	Quantitative PCR
RB	Reagent blank
rpoB	RNA polymerase β-subunit gene
rRNA	Ribosomal RNA
RT	Reverse transcription
RT-qPCR	Reverse transcription-qPCR
RT-RB	Reverse transcription-reagent blank
RNA	Ribonucleic acid
rpm	Revolutions per minute
SD	Standard deviation
SDS	Sodium dodecyl sulfate
sec	Seconds
SF	Seminal fluid
Spp.	Several species of the same genus
STR	Short tandem repeat
SWGDAM	Scientific Working Group on DNA Analysis Methods
TE	Tris-EDTA buffer
U	Enzyme unit
UV	Ultraviolet
VCU	Virginia Commonwealth University
VF	Vaginal fluid

GLOSSARY

MIQE terms

qPCR:	amplification of a target sequence measured in real time through the use of fluorescent dyes
RT-qPCR:	transcribing an RNA sequence into DNA before amplification
Endogenous reference gene:	Gene used for normalization
Hydrolysis probe:	complementary to the target sequence composed of a reporter dye and quencher that fluoresces as it degrades during amplification
Cq:	quantification cycle
Run:	a single qPCR plate
Reaction:	all components in a single well
Technical replicates:	multiple reactions of the same sample within a run

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Co-extraction:	isolating/purifying DNA and RNA simultaneously from a sample
Differential expression:	method of analyzing data from genes that require normalization; reported as ΔCq
DNase-treatment:	removal of DNA from a sample to ensure no genomic contamination during RNA experiments
Neg RT or RT-RB:	negative control for RT reactions (no RNA input)
NTC:	no template control for qPCR reactions (no DNA or cDNA input)
qPCR standard:	synthetic sequence of gene of interest used to validate specific amplification and optimize primer and probe concentrations in a qPCR assay
Reagent Blank (RB):	negative control for DNA extraction that contains all reaction components and either liquid reagents or a blank cotton swab (no DNA or RNA input); ran alongside a batch of samples to ensure no contamination at the extraction step
Transcription:	biological process in which DNA is converted to RNA
Translation:	biological process in which RNA is converted into a protein

ABSTRACT

DEVELOPMENT AND EVALUATION OF A COMBINATORIAL RT-qPCR MULTIPLEX FOR FORENSIC BODY FLUID IDENTIFICATION

By Carolyn Anne Lewis, Ph.D.

Virginia Commonwealth University, 2021

Body fluid identification is essential in the forensic biology workflow that assists DNA analysts in determining where to collect DNA evidence. Current presumptive tests lack the sensitivity and specificity molecular techniques can achieve; therefore, molecular methods, such as microRNA and microbial signatures, have been extensively researched in the forensic community. Limitations of each method suggest combining molecular markers to increase discrimination efficiency of multiple body fluids from a single assay. While microbial signatures have been successful in identifying fluids with high bacterial abundances, microRNAs have shown promise in fluids with low microbial abundance. A disadvantage of RNA analysis in forensic casework is RNA extraction; however, several reports have demonstrated that microRNAs co-extract with DNA, increasing implementation potential.

This project synergized on the benefits of microRNAs and microbial DNA to identify body fluids using DNA extracts. First, microRNA detection in DNA extracts was confirmed, demonstrating that RNA extraction and DNase-treatment are not necessary. A reverse transcription (RT)-qPCR duplex targeting miR-891a and let-7g was validated, with significantly different relative expression observed between blood and semen. Lastly, a qPCR multiplex targeting 16S rRNA genes of *Lactobacillus crispatus*, *Bacteroides uniformis*, and *Streptococcus salivarius*, was designed to identify vaginal/menstrual secretions, feces, and saliva, respectively.

The developed classification regression tree model that classified five body fluids with 94.6% overall accuracy, providing proof of concept that microRNAs and microbial DNA can identify multiple body fluids at the quantification step of the current forensic DNA workflow.

KEYWORDS: forensic serology, body fluid identification, microRNA, 16S rRNA gene, microbial analysis, reverse transcription-quantitative PCR (RT-qPCR), multiplex primer validation, microRNA co-extraction, classification regression tree analysis

CHAPTER 1:

INTRODUCTION

The analysis of deoxyribonucleic acid (DNA) is one of the most powerful and individualizing tools in forensic science. The standard DNA analysis workflow consists of evidence collection, body fluid identification, DNA extraction, human DNA quantification, and short tandem repeat (STR) analysis, in which STRs are amplified by polymerase chain reaction (PCR) then detected via capillary electrophoresis (CE).[1] The resulting STR profile is interpreted by at least one analyst, and the finalized written report is returned to the individual who originally submitted the evidence to the laboratory. Each step of this process is important and must be documented in detail for the results to be presented during a court case.

CURRENT METHODS IN FORENSIC SEROLOGY

Purpose and Importance

Forensic serology is defined as the detection or identification of biological fluids in relation to crime scene evidence. Body fluid identification (BFID) has long been the first essential step in the forensic DNA analysis workflow as it plays a crucial role in story corroboration of witnesses, defendants, and/or suspects.[2] It can also be useful for developing investigative leads and/or crime scene reconstruction. Equally important, it allows a DNA analyst to determine the best location to swab or cut to obtain a DNA profile from an evidentiary sample.[1,2] There are two classifications of serological tests used in forensic casework—presumptive and confirmatory tests. Presumptive tests are often more sensitive, meaning they can detect a body fluid when less is present; however, there are often false positives due to their high sensitivity and reaction mechanisms.[2] Confirmatory tests are more specific to the intended body fluid but often require more sample for the method to produce a result.[2] It is important to note the language resulting from presumptive and confirmatory testing—presumptive tests only *detect*, indicating that there is a possibility that the body fluid is present, while confirmatory tests

identify, meaning there is no doubt that the body fluid is present on the evidence.[3] A presumptive test can be followed by a confirmatory test for higher confidence before moving forward with DNA analysis;[1,3] however; most forensic laboratories only perform confirmatory testing when the presence of semen is suspected.

Screening or Enhancement Methods

Enhancement methods are presumptive screening tools that can be used by investigators at the crime scene to locate biological stains not visible to the naked eye. They can also be used in the crime lab during evidence processing to determine the best location for swabbing/cutting for serology testing and DNA analysis. Most commonly used is an alternate light source (ALS), which is any device that emits wavelengths of light that are outside of the visible range, such as ultraviolet light or 400-450 nm wavelength light with the use of an orange filter.[4] The use of ALS allows a large area to be examined and is non-destructive; therefore, downstream serology testing and DNA analysis is not affected. Most commercially available devices, such as the Wood's Lamp, are designed for semen detection and are based on the fluorescence of flavins and choline when exposed to short wavelengths of light (315-400 nm).[5–7] Limitations of these devices include that they must be used in a dark environment, which may not be possible at the time of evidence collection, and difficulty visualizing stains on dark or highly absorbent fabrics.[6] An important limitation of using an ALS is the fluorescence of other flavin-containing substances, including soaps, lotions, food, and other human body fluids, such as saliva and urine.[4,6,7] There are newer devices equipped for use in daylight, and some are capable of detecting multiple body fluids at different wavelengths, but fabric type and body fluid specificity are still problematic.[8,9]

Luminol is a blood enhancement technique that can be used both at the crime scene and in the laboratory. Red blood cells transport oxygen throughout the body via the hemoglobin protein, which is composed of four heme groups that exhibit peroxidase-like activity. If blood is present, its enzymatic-like activity reacts with the luminol reagents to produce a blue chemiluminescent glow.[10] This peroxidase-like activity of hemoglobin is the basis for all presumptive blood tests, so luminol has the dual-purpose advantage of enhancing and detecting blood simultaneously. Although luminol is a sensitive an popular blood detection method, it must be used in the dark, and false positives are commonly observed on items containing materials that oxidize under similar reaction conditions, such as copper or bleach.[10,11] Luminol itself is non-destructive; however, spraying too much on an area may dilute any biological material present and could make downstream analysis more difficult.[12] Fluorescein is a comparable alternative to luminol, but it has the advantages of working in lighted environments and longer reaction persistence.[13] Sensitivity, ease of use, cheap reagent cost, and commercially available dissolvable products, such as Bluestar® Forensic tablets, have all contributed to the widespread use of chemiluminescent enhancement techniques to detect bloodstains at crime scenes.[4,10,11,13,14]

Chemical-Based Presumptive Tests

Once biological material is located, catalytic enzyme-based tests are often performed if it is suspected that blood, semen, or saliva is present. These catalytic tests result in a visible color-change upon enzymatic (or enzymatic-like) activity between the reagents and some highly abundant component of the body fluid in question.[4] Although historically used, presumptive tests for urine, vaginal fluid, and feces are rarely performed because they often result in false positives/negatives, and results are of less probative given the high sample consumption.[15,16]

Laboratory presumptive tests for blood rely on the same reaction mechanism as luminol but result in a color change instead of chemiluminescence. The Kastle-Meyer (KM) test is most common due to its high sensitivity, simplicity, and cheap reagent cost.[10,17,18] A reduced pH indicator (phenolphthalein) is first applied to the substrate followed by an oxidizing reagent (hydrogen peroxide). A positive result is recorded if the colorless substrate turns pink after the addition of hydrogen peroxide. The reagents are strategically added in a specific order in attempt to reduce false positives. For example, if the pink color change is observed prior to the addition of hydrogen peroxide, then the test is inconclusive, which could be due to the sample or substrate containing chemical oxidants.[4,10,11] There are some vegetable peroxidases, such as horseradish, that will remain colorless until hydrogen peroxide is added thus resulting in a false positive.[10,11] A combined P-TMB test (phenolphthalein-tetramethylbenzidine) can be used to increase confidence that blood may be present.[19] Since the tests work in different pH environments, the TMB reagents can be added after the KM test without consuming additional evidence sample. Since the reaction mechanisms of both tests are the same, false positives can still occur with oxidizing substrates.[18,20] Commercially available products, such as HemaStix® (Lynn Peavey Company, Lenexa, KS) provide DNA laboratory examiners with a quick presumptive method and provides investigators with a portable option for blood detection at the crime scene.[10]

The acid phosphatase (AP) test, also known as Kaye's test, is the most common presumptive test for semen detection. AP is a lysosomal enzyme that hydrolyzes phosphates at acidic pH levels and is secreted from the prostate at much higher levels than other human body fluids.[21] The enzyme catalyzes the production of α-naphthol from α-Naphthyl phosphate, which then reacts with either a blue or red dye resulting in a time-dependent purple-pink or orange color change, respectively.[22–24] The test is best performed by directly applying the reagent to the

stain in question, but an indirect blotting method can be performed if analyzing a large area.[25–27] Specific cutoff times for a positive semen result vary among laboratories, but generally exposure times are 15-30 sec to no longer than 2 min; however, it has been demonstrated that it may take longer, sometimes up to 15 min, to detect diluted semen stains, and that important presumptive information could be lost if using too short of a cutoff.[28,29] Sexual assault evidence often contains vaginal/semen mixtures, so many efforts have been made to quantify AP in post-coital vaginal swabs to ensure prostatic AP is being detected and not that of vaginal fluid.[30–37] Short reaction cutoff times are an effort to reduce false positives for prostatic AP, but it can consequently reduce sensitivity of seminal fluid detection.[31–33] Importantly, prostatic AP is detectable in azoospermatic seminal fluid, which can be useful in cases where a suspect has naturally low sperm count or has been vasectomized.[38] As with other presumptive tests, a confirmatory test can be performed if a positive result is obtained.[1,3]

Saliva detection relies on the abundance of salivary α-amylase—an enzyme that breaks down starch into smaller molecules.[39] Since α-amylase is also produced in the pancreas, the methods are considered presumptive due to documented false positives with other human body fluids.[39] The original starch-iodine test results in blue color change when α-amylase breaks down starch upon the addition of orange/yellow iodine solution.[40] The more popular Phadebas® test (Phadebas Inc, Cambridge, MA) relies on the same enzymatic activity, but the starch is covalently attached to a blue dye that becomes visible in the presence of α-amylase.[41] These tests can be performed in-tube for a swab cutting or using a paper substrate for larger evidence items;[41–43] however, it has been reported that Phadebas® paper is more sensitive in α-amylase detection, resulting in decreased saliva specificity.[43] Several other commercially-available products for saliva detection have been developed that rely on similar chemistry with proprietary

reagents, such as SALiGAE® (Abacus Diagnostic, Inc., West Hills, CA).[41] Regardless of which α-amylase detection method is used, all results are presumptive for saliva, and confirmatory testing can be performed in order to rule out the presence of other body fluids.

Immunochromatographic Tests and Microscopic Examination

Immunological serological assays are based on human specific antigen-antibody reactions that occur on a portable immunochromatographic strip or card, which allows for testing to occur in the laboratory or at the crime scene.[4,16] Like presumptive tests, these assays also rely on some highly abundant fluid component but utilize a different reaction mechanism, similar to an at-home pregnancy test. If the suspected body fluid component (antigen) is present, it binds to dye-labeled monoclonal antibodies, and the mobile antigen-antibody complex migrates up an absorbent substrate that has a test and control line.[2] The test region contains immobilized antigen-specific antihuman antibodies that trap the mobile complex, where a visible pink colored band is observed for a positive result.[2] A control zone containing immobilized anti immunoglobulin antibodies captures the antibody-dye conjugates that are unable to bind to the test area, which serves as a positive control. If the control line is absent, then the test result is inconclusive.[2,4]

Immunological assays for blood and saliva typically target the same component as chemical-based tests (hemoglobin and α-amylase, respectively); however, the p30 protein, also known as prostate specific antigen (PSA) is the targeted component for seminal fluid detection. PSA is a prostatic glycoprotein secreted in seminal plasma that assists in liquifying semen ejaculate.[22] Detection of p30 was originally considered confirmatory for semen identification as it was thought to only be present in human semen; however, the majority of the forensic community now considers it presumptive since there has been reported cross-reactivity with

other human body fluids, such as breast milk and male urine.[44–46] Although both are technically presumptive, it is still common for forensic biologists to perform both AP and p30 tests to increase confidence that semen is present, especially in sexual assault cases where post-coital swabs are examined. Two reports evaluating semen detection in post-coital samples found that p30 was detectable up to 27 hours after intercourse, whereas AP was detectable up to 14 hours.[34,47] Therefore, if an analyst only performed the AP test on those particular samples more than 14 hours after the assault, a false negative could result.

Commercial companies have streamlined the use of immunochromatographic strips in forensic casework. The Rapid Stain Identification (RSID™) kits (Independent Forensics, Lombard, IL) are seemingly the most marketable products developed for forensic use. These include field and laboratory kits for semen, blood, saliva, urine, or a multi-fluid kit that tests for blood, semen and saliva.[48] Abacus Diagnostics, Inc. also has been successful in commercializing portable immunochromatographic tests, such as the ABAcard® p30 for semen, which is reportedly more sensitive than the semen RSID™ kits.[49–51] The ABAcard® HemaTrace test for blood is also reported as more sensitive than the blood RSID™ kits; however, false positives can occur with higher primates and ferret blood.[50] The SERATEC® PSA Semiquant test has been reported as an alternative semi-quantitative presumptive method with comparable sensitivity to the AP test and ABAcard® p30.[52] Regardless of the presumptive method chosen by a forensic analyst, a confirmatory test is still necessary to undoubtedly conclude that a body fluid is present.

The most universally valued confirmatory method still in practice in forensic laboratories today is the microscopic examination of sperm cells, in which the unique morphology of human sperm allows for human semen identification.[53] Traditional sperm cell visualization methods include phase-contrast microscopy or brightfield microscopy with Kernechtrot–

Picroindigocarmine (KPICS) stained-cells.[54,55] KPICS, also known as the Christmas tree stain, includes two dyes: Nuclear Fast Red (Kernechtrot) that stains sperm cell heads red and inside nuclear material pink-purple, and picroindigocarmine stains the epithelial membranes, cytoplasmic material, and sperm tails green.[56] While tails are readily displaced during staining, positive sperm cell results rely on the sperm head to remain in-tact.[57] Unfortunately, microscopic examination is time consuming and labor-intensive, sometimes leading to overlooked sperm cells; therefore, several efforts have been made to overcome these limitations.[54,57] The SPERM HY-LITER™ kit (Independent Forensics) uses a human sperm-specific mouse monoclonal antibody paired with a fluorescent dye so that only human sperm cells fluoresce, which allows for easier and faster microscopic visualization.[57] However, non-specific staining and increased background fluorescence in some circumstances limit the widespread use of this method.[57] The KPICS SpermFinder™ (NicheVision Forensics, LLC, Akron, OH) takes a different approach by using an algorithm-based program to locate KPICS stained sperm cells; however, this process still requires a qualified, trained analyst to review and confirm the data.[54] Another disadvantage of sperm cell visualization, in general, is that it requires the presence of sperm cells in the ejaculate, which would not occur if an individual has naturally low sperm count or has been vasectomized.[53] Even with its limitations, microscopic examination of sperm cells remains the most popular confirmatory test in practice among forensic laboratories.

PROPOSED METHODS FOR BODY FLUID IDENTIFICATION

Serological Assay Requirements and Forensic Considerations

There are some important factors to consider when developing a serological test for forensic use. The assay must be cost effective and easily implemented, meaning that it requires minimal training for analysts and that the results can easily be interpreted and communicated to a

jury. It is also important that the test require minimal sample input and is non-destructive to the sample DNA so that it can be further processed for downstream human identification.[3] While current serological methods meet some of these desired characteristics, there is no all-inclusive body fluid test. This can become especially problematic in terms of sample consumption; for example, if an analyst suspects that both saliva and blood are present on a swab, three cuttings of that swab would be needed (one for each serology test and one for DNA extraction). In this event, the two cuttings for blood and saliva tests are consumed and cannot be used downstream, and only a small portion of the evidentiary swab is remaining to be stored for any possible retesting in the future. All these factors contribute to the desired balance of sensitivity and specificity of a comprehensive assay, requiring minimal sample input to identify multiple human body fluids preferably simultaneously. As genetic technology has advanced over the years, forensic scientists have considered transitioning towards molecular-based techniques to supplement or replace current serology testing.[4,16]

There are three main areas that can be targeted in molecular methods for BFID—the proteome, the transcriptome, and the genome, and each has been the subject of research within the forensic community. The proteome refers to the protein composition of a body fluid; body fluid-specific proteins have been identified and studied in the forensic research. Unfortunately, protein instability and costly instrumentation/personnel training limit the potential broader impact of proteomic applications for forensic science.[58–60] Alternately, the transcriptome refers to ribonucleic acid (RNA) molecules transcribed from the genome, such as messenger RNA (mRNA) or microRNAs (miRNAs). Finally, the genome, refers to either microbial DNA or human DNA and/or its epigenetic patterns, such as DNA methylation. DNA methylation has potential for both BFID and forensic phenotyping, such as age prediction.[61] The most successful

efforts of DNA methylation for BFID have been for semen identification using tissue-specific DNA methylation regions (tDMRs).[62–65] However, its forensic casework potential is limited by inter-person variation, difficulty interpreting body fluid mixtures, and time-consuming analysis that involves non-traditional methods, such as bisulfite sequencing or restriction enzyme digestion.[62,66–69]

The chemistry or methodology of a molecular-based serological assay remains an important consideration for forensic implementation. There are two general approaches regarding genomic molecular markers for BFID—discovery and application. Discovery refers to the search for new body fluid-specific markers or the confirmation of those in the literature, whereas application refers to the utilization of molecular markers that have been identified during discovery or that have been previously reported in the literature. The discovery approach typically involves *de novo* sequencing or the use of microarrays to screen for large numbers of targets simultaneously.[70–72] Since these methods are currently not part of the standard forensic DNA workflow, potentially informative molecular targets are often evaluated using instruments frequently validated for forensic casework, such as qPCR or CE.[73–75] Additionally, body fluid specificity of markers can vary depending on the chemistry or platform used because of differences in assay sensitivity; for example, markers discovered through high-throughput sequencing (HTS) may not exhibit body fluid specificity when applied to a qPCR assay.[76] As sequence-based analysis becomes increasingly popular in forensic genomics, sequencing approaches for forensic BFID have been proposed,[77,78] but as of now, most reported methods rely on qPCR or CE detection chemistries regardless of molecular target type.

Microbial DNA for Body Fluid Identification

The use of microbial DNA for forensic applications stemmed from the Human Microbiome Project, which explored the diversity of microbial communities within the human body as well as between individuals.[79,80] The determination that there are approximately 38 trillion bacterial cells present within the human body suggested to forensic researchers that these cells may still be detectable even when human DNA or RNA is scarce or degraded.[79,81] Microbial analysis has been applied to bioterrorism and infectious agent detection for years,[82–85] and multiple efforts have been made to standardize or validate analysis methods to rapidly respond in situations that pose threat to public health or national security.[86–88] With the advancement of next-generation sequencing (NGS) technologies over the last 15 years, microbial forensics has expanded to human identification,[89] body fluid identification,[70,90] and postmortem interval estimation.[91]

Most reports that address microbiome based BFID rely on 16S rRNA gene sequencing, as this is a gold standard in microbiome analysis.[90,92] The 16S rRNA gene codes for a ribosomal subunit that is specific to prokaryotes. The gene sequence is conserved within the same genus and species but has nine hypervariable regions (V1-V9), in which both characteristics allow for microbial taxonomical classification.[92] In clinical and environmental microbiology research, the RNA polymerase β-subunit gene (*rpoB*) gene has been suggested as an alternative to 16S for the discovery of novel bacteria and for better species differentiation within a genus.[93–95] Additionally, the 16S-23S rRNA intergenic spacer region (ISR) has been considered a suitable marker for species differentiation.[96–98] The use of other target genes has also been successful in BFID research, such as the *rpoB* gene of *Bacteroides* for feces,[78,99] glucosyltransferase (GTF)

genes of *Streptococcus* for saliva,[100–102] and the 16S-23S ISR of *Lactobacillus* for vaginal fluid.[103,104]

Compared to other forensically relevant body fluids, the vaginal and saliva microbiomes have been the most described/characterized by microbiologists; therefore, most attempts at microbial BFID for forensic applications involve methods that predict vaginal fluid, saliva, and/or feces (Table 1.1). The first forensic report for microbial BFID was in 2009 by Nakanishi et al.,[101] in which they identified saliva by amplifying GTF genes of *S. salivarius* and *S. mutans* via PCR and visualizing via gel electrophoresis. Nakanishi et al.[102] later developed a loop-mediated isothermal amplification (LAMP) assay for saliva identification that targets the GTF gene of *S. salivarius*. In 2013, the same research group proposed a qPCR assay that included three *Bacteroides* species, in which they concluded that amplification of *rpoB* in *B. uniformis* and *B. vulgatus* was the most successful for feces identification.[99] Zou et al.[78] also was successful at identifying feces when targeting the *rpoB* gene in *B. uniformis* and the α-1-6-mannanase gene of *B. thetaiotamicron*. Three other PCR-based attempts at saliva identification using various Streptococcus genes have been proposed, where the most recent uses direct PCR combined with an immunochromatographic strip for portable detection of saliva.[75,105,106]

Important to note is that each of these proposed microbial assays targets multiple genes and/or species to predict a single body fluid (Table 1.1), and although they might be more specific and/or sensitive than current serological methods, they do not necessarily meet the desired characteristics of a comprehensive molecular assay in terms of forensic implementation and cost. For example, saliva detection using α-amylase may be quicker and more cost effective in some cases than a PCR-based approach. Microbial assays are more appealing for body fluids that forensic biologists do not currently have serology tests for, such as vaginal fluid; therefore,

Table 1.1—Summary of reported microbial targets for the forensic identification of vaginal fluid, saliva and/or feces. The left column demonstrates which bacteria are most abundant in which fluid, and research group columns are author recommendations of which genera and/or species should be used for successful identification of the target body fluid (different genes are indicated by lettered superscripts, spp.=primers were designed using a conserved region of multiple species of the same genus, VF=vaginal fluid, MB=menstrual blood).

		Research Group													
BF with Highest Microbe Abundance	**Microbial Target**	Nakanishi et al.[101] 2009	Fleming et al.[103] 2010*	Nakanishi et al.[102] 2011	Nakanishi et al.[99] 2013	Hsu et al.[105] 2012	Akutsu et al.[118] 2012	Giampaoli et al.[107] 2012	Giampaoli et al.[119] 2014‡	Doi et al.[120] 2014	Choi et al.[123] 2014*	Zou et al.[223] 2016	Jakubowska et al.[124] 2016*	Jung et al.[75] 2018	Won Lee et al.[106] 2018
Vaginal fluid	*Lactobacillus* spp.									X[a]					
	L. crispatus		X[c]				X[a]	X[a]	X[a]		X[a]	X[a]	X[c]		
	L. gasseri		X[c]					X[a]	X[a]		X[a]				
	L. jensenii						X[a]					X[a]			
	L. iners														
	L. gasseri/L. johnsonii												X[c]		
	Atopobium vaginae						X[a]								
Saliva	*Veillonella atypica*										X[a]				
	Streptococcus spp.					X[a]									
	S. salivarius	X[b]		X[b]				X[a]	X[a]		X[a]			X[g]	X[g]
	S. sanguinis													X[c]	X[c]
	S. mutans	X[b]						X[a]	X[a]						
	Staphylococcus aureus							X[a]	X[a]						
	Neisseria subflava													X[h]	
Feces	*Enterococcus faecalis*							X[e]	X[e]						
	Bacteroides uniformis				X[d]							X[d]			
	B. thetaiotamicron				X[f]							X[f]			
	B. vulgatus				X[d]										
	Predicted or Targeted BF(s)	**Saliva**	**VF**	**Saliva**	**Feces**	**Saliva**	**VF**	**VF**	**VF**	**VF**	**Saliva & VF/MB**	**VF & Feces**	**VF**	**Saliva**	**Saliva**

*=integrated assay that uses additional molecular markers to identify other forensically relevant body fluids not presented
‡ = an expanded inter-laboratory study of the same qPCR assay from Giampaoli et al.2012
a=16S rRNA
b=GTF enzymes
c=16S-23S ISR
d=*rpoB*
e=23S rRNA
f=α-1-6-mannanase
g=methoionine aminopeptidase
h=aspartate semialdehyde dehydrogenase

the most successful attempts at a forensic microbial BFID assay are ones that use *Lactobacilli* to identify vaginal and/or menstrual secretions.

Extensive clinical microbiology research on the vaginal microbiome has demonstrated that *Lactobacilli*—specifically *L. crispatus*, *L. gasseri, L. jensenii*, and *L. iners*—dominate the vaginal cavity.[107–111] *Lactobacilli* are the primary source of lactic acid in the vagina, which is necessary to maintain a healthy, low-pH environment.[112] It has been proposed that the high levels of starch in the human diet contribute to increased glycogen and therefore increased presence of *Lactobacilli*.[113] Imbalance of vaginal microbiota can physiologically affect an individual, such as complications with conception and/or pregnancy and increased risks of acquiring sexually-transmitted infections.[114] Bacterial vaginosis (BV) is an often-asymptomatic infection commonly observed among young, sexually-active adults that is associated with decreased lactobacilli abundances and increased abundances of *Gardnerella vaginalis* and *Atopobium vaginae.*[115,116] Specifically, inverse relationships between *L. gasseri* and *A. vaginae* and between *L. gasseri* and *L. iners* have been reported.[117] Therefore, potentially increased levels of *A. vaginae* and *L. iners* and decreased levels of *L. gasseri* in individuals with BV could affect results of an assay that targets only *L. iners* or *L. gasseri*. For these reasons, it has been suggested to include at least one BV marker in microbial forensic assays to prevent potential false negatives for vaginal fluid, especially in sexual assault cases.[118]

The first microbial forensic qPCR assay was reported in 2012 by Giampaoli et al.,[107] in which rRNA genes of six bacterial species were amplified to predict vaginal secretions (Table 1.1). Success of this later termed "ForFLUID kit" was confirmed in 2014 during an inter-laboratory evaluation study.[119] The ForFLUID kit includes primer sets for species highly abundant in saliva and feces to eliminate the possibility that a sample contains saliva or feces and

increase confidence that vaginal fluid is present.[119] As previously mentioned with other body fluids, it has been proven difficult to identify vaginal fluid by detecting a single *Lactobacillus* species.[70,120] Doi et al.[120] took a different approach and addressed this by designing qPCR primers flanking the conserved region of 16S at the genus level of the four most abundant *Lactobacillus* species (*L. crispatus*, *L. gasseri, L. jensenii*, and *L. iners*). *Lactobacillus* DNA was relatively quantified to a human-specific primer set, allowing for the simultaneous confirmation of sample source species.[120] This would be a cheaper method to implement into forensic casework with only two primer sets utilized (compared to six), but the ForFLUID kit could provide more information regarding body fluid mixtures if additional research was performed for that purpose.

The most significant concern with microbial BFID is the high levels of inter-person variation and population differences. Thus, numerous studies have focused on specific populations for characterization of specific microbial markers.[78,108,109,111,121,122] Other studies have focused on incorporating additional molecular markers, such as mRNA or DNA methylation sites, as a way to account for these differences and identify multiple body fluids simultaneously.[103,123,124] In 2010, Fleming et al.[103] proposed the first integrated 11-plex assay amplifying the 16S-23S ISR of *L. crispatus* and *L. gasseri* and nine mRNA markers that could identify blood, menstrual blood, seminal fluid, saliva, and vaginal secretions using a CE platform. A similar approach was taken by Jakubowska et al.,[124] in which vaginal material was identified via end-point PCR of the 16S-23S ISR of *L. crispatus* and *L. gasseri/L. johnsonii* and four mRNA markers. Unfortunately, these methods require RNA isolation and reverse transcription, which would consume evidence sample and add extra steps/cost to the DNA analysis workflow. The only other integrated molecular approach known was reported by Choi et

al.[123] in 2014, which was a CE-based PCR multiplex targeting microbial DNA and tDMRs to identify blood, semen, saliva, and vaginal/menstrual secretions. With this method, the 16S rRNA genes of *L. crispatus* and *L. gasseri* were amplified to identify vaginal/menstrual secretions, while *Veillonella atypica* and *S. salivarius* were used to identify saliva (Table 1.1).[123] Semen and blood were differentiated through DNA methylation; however, the authors stated that additional tDMRs should be considered to reduce the potential for false negatives since semen identification relies on the presence of a single tDMR peak and the absence of three tDMR peaks in the electropherogram.[123] Furthermore, methylation analysis requires time-consuming restriction enzyme digestion, and when considering forensic implementation, the use of DNA endonucleases in a casework laboratory is not ideal.

In summary, research efforts in microbial BFID have been successful for fluids with high bacterial composition, while fluids with low microbial abundance, such as blood and semen, require additional molecular markers for accurate identification. The only integrated molecular assays that have been proposed combine microbial DNA with mRNA or DNA methylation analysis, and the limitations of each suggest that incorporating other molecular markers may increase confidence in identifying forensically relevant body fluids.

microRNAs for Body Fluid Identification

Early attempts at forensic BFID using the transcriptome focused solely on mRNA because its biological function and tissue specificity have been well-described for many years.[125,126] To that end, there have been many successful studies that demonstrate body fluid specificity using mRNA.[127–132] However, mRNA analysis has not been widely accepted in the United States likely due to conflicting research regarding its susceptibility to degradation, the added workflow requirement of an RNA extraction, as well as several other well-known

laboratory challenges associated with mRNA.[125–127,133–140] Conversely, mRNA *has* been validated for casework in other countries. For example, the European DNA Profiling Group (EDNAP) performed a series of collaborative studies between 2011 and 2014 to form a consensus on mRNA analysis prior to any casework implementation.[141–144] Limitations of mRNAs for forensic BFID will be discussed further in a later section. However, there is no doubt that all of the extensive mRNA research efforts have laid the foundation for microRNA research by describing the benefits and limitations of applying RNA analysis to forensic BFID.

microRNAs are short non-coding transcripts (~18-24 nucleotides) that assist in cell regulatory processes by binding the 3' untranslated region of its complementary mRNA to either signal for mRNA degradation or to stop translation.[145,146] microRNAs were first discovered and characterized in roundworms (*Caenorhabditis elegans*).[147–150] The first miRNA, *lin-4*, was discovered in 1993 by Lee et al.,[148] and by 2001, miRNAs were being proposed as their own class of small RNAs due to their regulatory functional roles in *C. elegans.*[147,149] Since their initial discovery, miRNAs have been steadily investigated within various research fields and are constantly being uploaded to sequence databases. In miRBase, there are now over 38,000 documented miRNAs among 271 organisms, with approximately 2,000 reported as human-specific.[151] The biological functions of miRNAs allow for consistent expression in all human tissues and for tissue-specificity, as regulatory processes differ between cell types.[146,152–154] This concept was first (and is still being) explored in clinical research towards the identification of biomarkers that can be used for the diagnosis and treatment of common human diseases, including cancer, neurodegenerative or immune-related disorders.[155–160] Expression profiles of miRNAs have also been characterized in normal human tissues,[157] which quickly became of interest to forensic scientists researching RNA-based BFID.

In 2009, Hanson et al.[161] was the first to investigate miRNA expression differences in forensically relevant dried body fluids; this step was crucial as clinical sample types are vastly different from evidentiary samples. Although no miRNAs were determined to be body fluid-specific after reverse transcription (RT)-qPCR analysis of 452 human miRNAs, unique differential expression patterns between body fluids were observed using a panel of nine markers.[161] The authors also suggested that a discriminant statistical approach may be best suited for classification of body fluids based on miRNA data analysis.[161] This is concordant with more recent studies that frequently utilize discriminant methods for this purpose.[162,163] Altogether, these findings have demonstrated the potential benefits and limitations of using miRNAs in BFID; however, the discovery phase of miRNAs is in its infancy compared to that of bacteria. Since new miRNAs are constantly being discovered, there is not yet a consensus on what biomarkers and/or endogenous reference genes should be used in the forensic community. Furthermore, there have only been a few sequencing-based approaches for discovery of miRNA markers in forensically relevant body fluids,[76,77,164,165] and most of these rely on microarrays or RT-qPCR targeted approaches evaluating previously discovered miRNA markers.[73,74,166,167]

As mentioned earlier, miRNA body fluid specificity may differ between detection platforms and/or amplification chemistries. For example, Zubakov et al. 2010 reported the first microarray screening of 718 human miRNAs that identified 11 as potentially body fluid-specific; however, only four of these were confirmed in subsequent RT-qPCR analysis.[74] Alternately, Courts and Madea et al. performed an 800-miRNA microarray screening in blood and saliva and validated six differentially expressed miRNAs (three per fluid) via RT-qPCR.[168,169] While many research groups have aptly demonstrated the use of differentially expressed miRNAs to distinguish between multiple body fluids as a suitable method for BFID. There is significant

variability in the forensic literature regarding sample preparation, body fluid and/or normalization markers, and data analysis methods, detection chemistries, etc.[73,74,76,161,166,170–177] It should be noted that these differences are technique-based rather than biological—meaning implementation is prohibited by lack of consensus within the forensic community rather than invalidity of any proposed method or markers.

miRNA Detection Chemistries

Multiplexing miRNAs is more difficult than other types of molecules because their short length makes traditional PCR primer design challenging. This has been addressed in some studies that utilize other amplification methods, such as rolling circle amplification (RCA)[178,179] and/or LAMP.[179,180] However, it is important to keep in mind forensic implementation potential. There are two forensically-validated PCR detection platforms that are commonly used in forensic laboratories and could therefore easily be used for miRNA detection—CE and qPCR.

Several reports have shown success using CE detection of miRNAs for BFID because its size-based fragment separation allows for multiple amplicons to be detected using the same fluorescent dye.[129,170,173,175] With this, more targets can be analyzed at once compared to qPCR, which is limited by the number of optical channels in the instrument's detection system.[1] The biggest disadvantage of CE methodology for BFID is that it is the last step of the hands-on workflow, and BFID should occur as early as possible in the workflow to assist analysts with decisions regarding DNA processing.[3] On the other hand, qPCR is performed earlier in the forensic DNA workflow and relies solely on fluorescent signal and not fragment size. Most qPCR instruments in forensic laboratories contain five optical channels and therefore can detect up to four targets in a well (one used for passive reference dye). Although multiplexing

capability is limited, the high sensitivity and sequence-specificity of RT-qPCR have made it one of the most popular miRNA analysis methods.

Figure 1.1 illustrates two primary RT-qPCR chemistries for miRNA detection. One approach is the use of a two-step RT method that adds a poly(A) tail to any RNAs in a sample, which are then reverse transcribed using a universally-tagged oligo dT reverse transcription primer.[181,182] The complementary DNA (cDNA) is then amplified with a miRNA sequence-specific forward primer and universal reverse primer, and double-stranded DNA (dsDNA) products are detected with an intercalating dye (Figure 1.1-A). The use of an intercalating dye does not allow for multiplexing;[182] however, since all miRNAs are converted to cDNA, the same RT reaction can be used to analyze multiple targets, which reduces the number of RT reactions required depending on how many miRNAs are evaluated.

An alternative strategy is the use of a miRNA-specific stem-loop RT primer so that only the miRNA of interest is reverse transcribed. The synthesized cDNA strand then has enough nucleotides for hydrolysis-probe qPCR chemistry to occur, in which the forward primer and hydrolysis probe are miRNA-specific while the reverse primer is universally complementary to the RT primer[182–184] (Figure 1.1-B). The stem-loop method is reportedly far more specific to the miRNA target than the linear poly(A) tailing strategy,[184,185] whereas the polyadenylation method is reportedly more sensitive.[177] This technique has a huge advantage in terms of multiplexing capability. However, primer design is difficult due to the short length of miRNA sequences.[185] Since RT and qPCR primer sequences in commercial RT-qPCR kits are often proprietary, numerous reports have outlined methodology designing primer and probes specific to stem-loop RT-qPCR chemistry.[184,186–189] These reports allow other research groups to not only design primers for other miRNAs but also replicate studies confirming the technique and/or reported

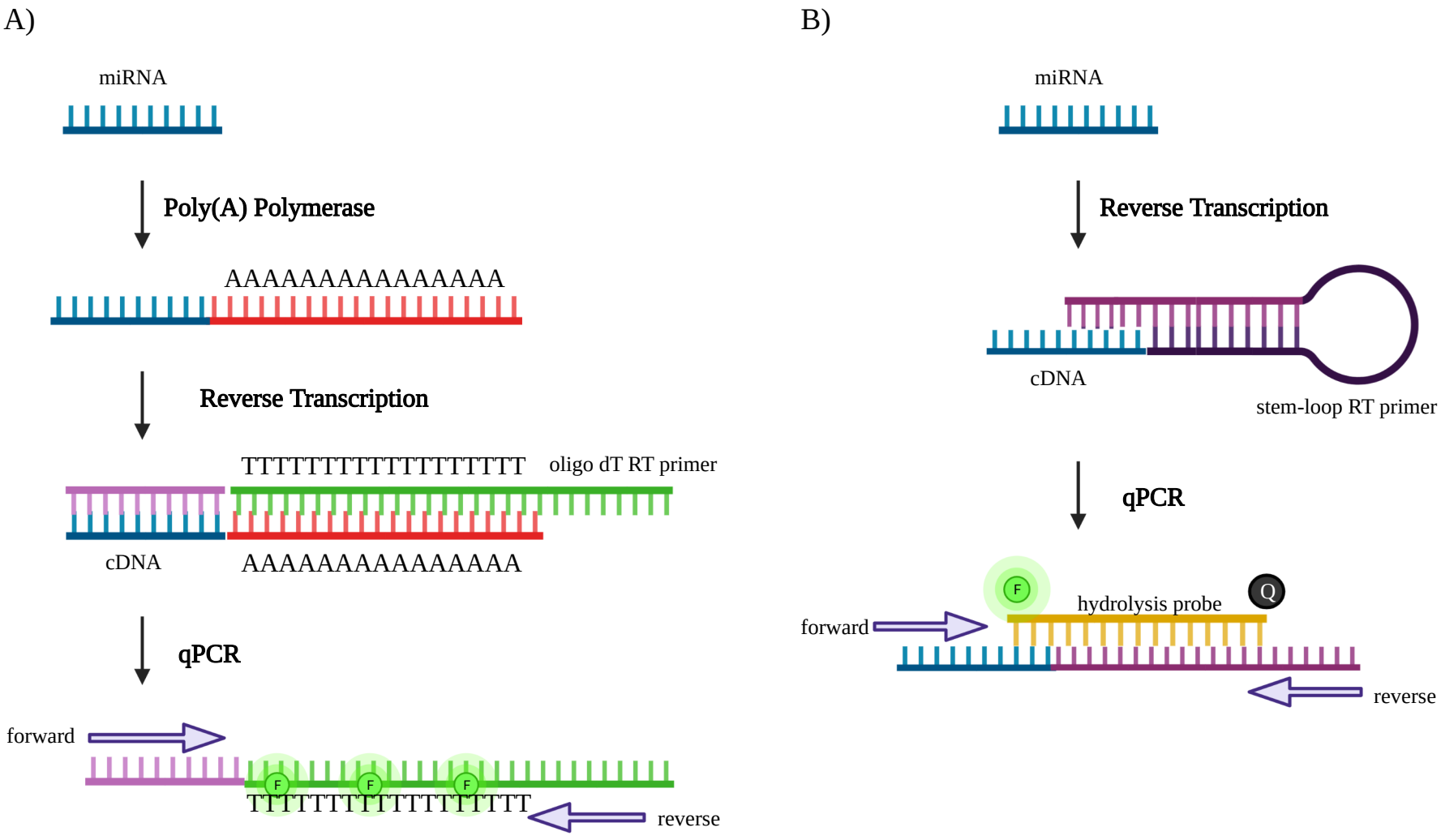

Figure 1.1—RT-qPCR chemistries for microRNA detection. The left (A) demonstrates the addition of a poly(A) tail and a complementary oligo dT RT primer to synthesize cDNA, which is amplified and detected using a dsDNA intercalating dye during qPCR. The right (B) demonstrates the use of a miRNA-specific stem-loop RT primer to initiate cDNA synthesis for hydrolysis probe-based qPCR.

markers used by other groups, which is important for forensic implementation of a new methodology.

In summary, the selection of RT-qPCR chemistry/detection is an important consideration for implementation of a proposed miRNA panel for forensic BFID. Generally, a stem-loop RT method would be most cost effective if targeting only a few miRNAs that could be multiplexed at the RT step, as the RT step is the most expensive step (especially if a separate RT is required for each miRNA). If analyzing a large number of miRNA targets via qPCR, a poly(A) tailing RT method may be more cost effective since multiple miRNAs can be targeted from the same RT reaction.

Previous and Ongoing Research in the Seashols-Williams' Laboratory

The Seashols-Williams' laboratory at VCU has been conducting research on miRNAs for forensic BFID since 2012. We initially evaluated commercially available RNA isolation kits and optimized the best performing method for miRNA extraction. We then performed the first HTS-based approach for discovery of miRNAs in forensically relevant body fluids, in which we sequenced the entire small-RNA transcriptome of urine, feces, blood, menstrual blood, vaginal fluid, semen, sweat, and saliva to identify candidate normalization and body fluid-specific miRNA markers.[76] Following that, the use of let-7g and let-7i as novel endogenous reference genes that could dually-normalize miRNA expression between fluids was confirmed via RT-qPCR.[76] Other studies have since supported let-7g as a potential normalization marker.[77,170,190] Since our HTS results were not always concordant with RT-qPCR results in terms of body fluid specificity, we investigated various miRNA markers from the literature as well as others we previously identified as candidates during HTS.

Once we were confident with our selection of BFID miRNA targets, we performed a full developmental validation of an RT-qPCR miRNA panel (miR-200b-3p, miR-320c, miR-10b, miR-1246, miR-26b, miR-891a, let-7g and let-7i) that can distinguish between saliva, feces, urine, blood, menstrual blood, and semen with high accuracy through quadratic discriminant analysis. These studies included evaluating a large population (50 donors of each body fluid), variation within donors over time, and body fluid-specificity in human organ/tissue and other non-human species. We also performed limit of detection study based on a standard curve that was internally developed/validated for accurate copy number quantification of miRNAs. We then used the lower limits of detection to compare sensitivity of commercially available RT-qPCR methods (Table 1.2).

As noted earlier, two major concerns in the forensic community with mRNA analysis are that an RNA extraction method is often required, which would consume evidence, and that mRNA is easily susceptible to degradation, which is not ideal considering the often compromising nature of forensic samples.[139,140,191] For these reasons, we explored miRNA stability in blood, urine, semen, and saliva stains after various heat, chemical, or environmental treatments that would be similar to those encountered in forensic samples.[192] Our results were concordant with other reports that have demonstrated miRNA stability in stored or degraded RNA samples.[190,193–195]

One limitation of our reported miRNA panel was the inability to identify vaginal fluid, even after performing additional RT-qPCR screening and testing vaginal miRNA markers reported in the literature. Furthermore, we initially evaluated the final miRNA panel in RNA extracts, which did not address the concern of RNA isolation—further limiting its potential for forensic implementation. Biomedical research studies had reported the ability of RNA to co-

Table 1.2—Lower limit of detection for individual microRNAs across three commercially available RT-qPCR kits. Values represent how many copies/µL of synthetic RNA oligo were detected below 36 cycles. Sensitivities were similar across miRNAs, but higher sensitivities were observed using TaqMan® chemistry compared to qScript™.

	let-7g-5p	**let-7i-5p**	**miR-200b-3p**	**miR-144-3p**
qScript™	10^5	10^4	10^5	10^6
TaqMan® Advanced	10^4	10^4	not tested	not tested
TaqMan® MicroRNA	10^3	not tested	not tested	not tested

extract with DNA, but there have only been a few forensic reports that specifically addressed miRNA detection in DNA extracts. Therefore, the first objective of this thesis was to ensure miRNAs could be detected using isolation methods commonly utilized in forensic casework. Successful results would indicate that DNA extracts are suitable for both microbial and miRNA analyses during the remainder of the project.

STATEMENT OF THE PROBLEM AND RESEARCH OBJECTIVES

Statement of the Problem

The ability to accurately identify body fluids is important in forensic casework, as it can assist in crime reconstruction and story corroboration. Being able to do this early in the workflow without consuming limited evidentiary sample is a hurdle that forensic research aims to overcome. Unfortunately, since the early 2000s, the major focus in the forensic community has been the advancement of DNA typing methods rather than the implementation of new serological tests.[196,197] Meanwhile, a plethora of body fluid research has been performed, but additional validation studies are necessary to be in accordance with developmental validation and method introduction guidelines outlined by the Scientific Working Group on DNA Analysis Methods (SWGDAM).[3,198] In 2018, the National Institute of Justice's Technology Working Group published operational requirements stating that sufficient scientific and technological evaluations for BFID research exist, but there is still a need for policy/protocol development and dissemination/training.[199] Furthermore, SWGDAM proposed a Body Fluid Identification Working Group in July 2019 that would review the operational value and integration potential of current and proposed methods to streamline screening workflows.[200] Extensive research efforts and the recent development of a body fluid-specific working group clearly demonstrate the desire to improve existing and/or implement new serological techniques in forensic casework.

Research Objectives and Study Design

Recognition of the advantages and disadvantages of each molecular method has led to the proposition that combining molecular markers would increase discrimination efficiency of multiple body fluids from a single assay.[103,123] To our knowledge, there has never been a molecular approach that analyzes microbial DNA and miRNAs, concurrently, to identify forensically relevant body fluids.

This project synergizes on the benefits of utilizing both miRNAs and microbial DNA to characterize blood, menstrual blood, semen, saliva, feces, and vaginal fluid from a single DNA extract. The first objective (described in Chapter 2) explores miRNA detection in DNA extracts from nucleic acid isolation methods commonly used in forensic casework (Figure 1.2). The effect of DNase-treatment and body fluid specificity is also evaluated as proof of concept that DNA extracts are suitable for subsequent miRNA analysis in this project. The findings from Chapter 2 provided critical preliminary data for a successful NIJ proposal designed to perform developmental evaluations of our previously validated miRNA panel in DNA extracts.

Chapters 3 and 4 describe the primary novel objective of this body of work—the development of a combinatorial RT-qPCR assay (Figure 1.3). A microbial qPCR multiplex is first designed in Chapter 3, in which body fluid specificity for feces, saliva, and vaginal/menstrual secretions is demonstrated. The final objective of incorporating miRNA markers for discrimination between blood and semen is investigated in Chapter 4.

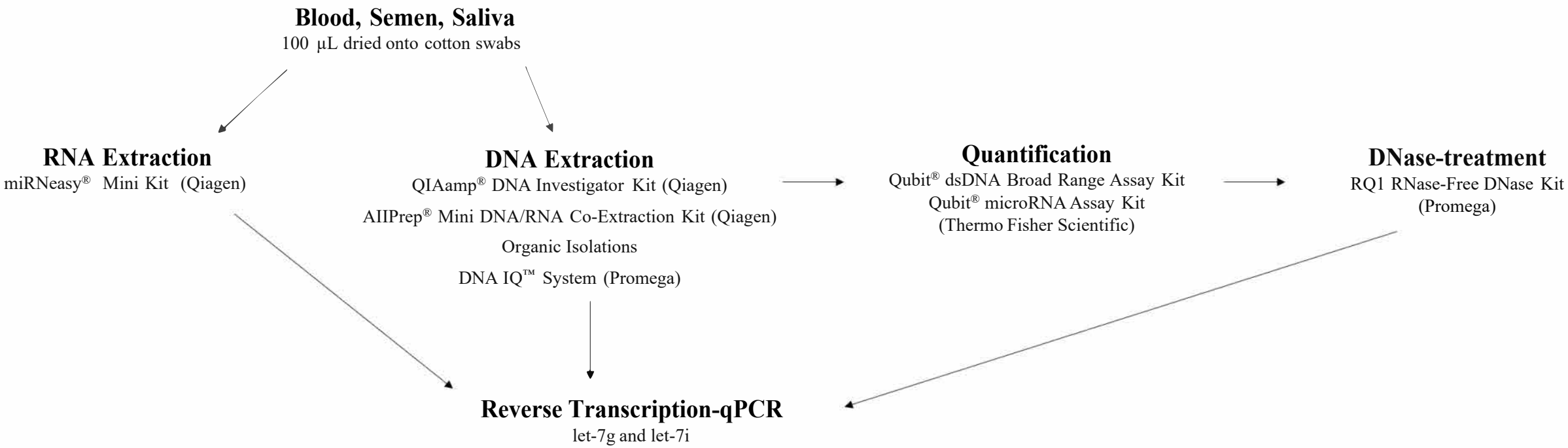

Figure 1.2—Experimental design for evaluating microRNA and DNA co-extraction. Blood, semen, and saliva (n=3 donors of each) were evaluated using commercially available nucleic acid isolation methods, and miRNAs let-7g and let-7i were detected via RT-qPCR before and after DNase-treatment**.**

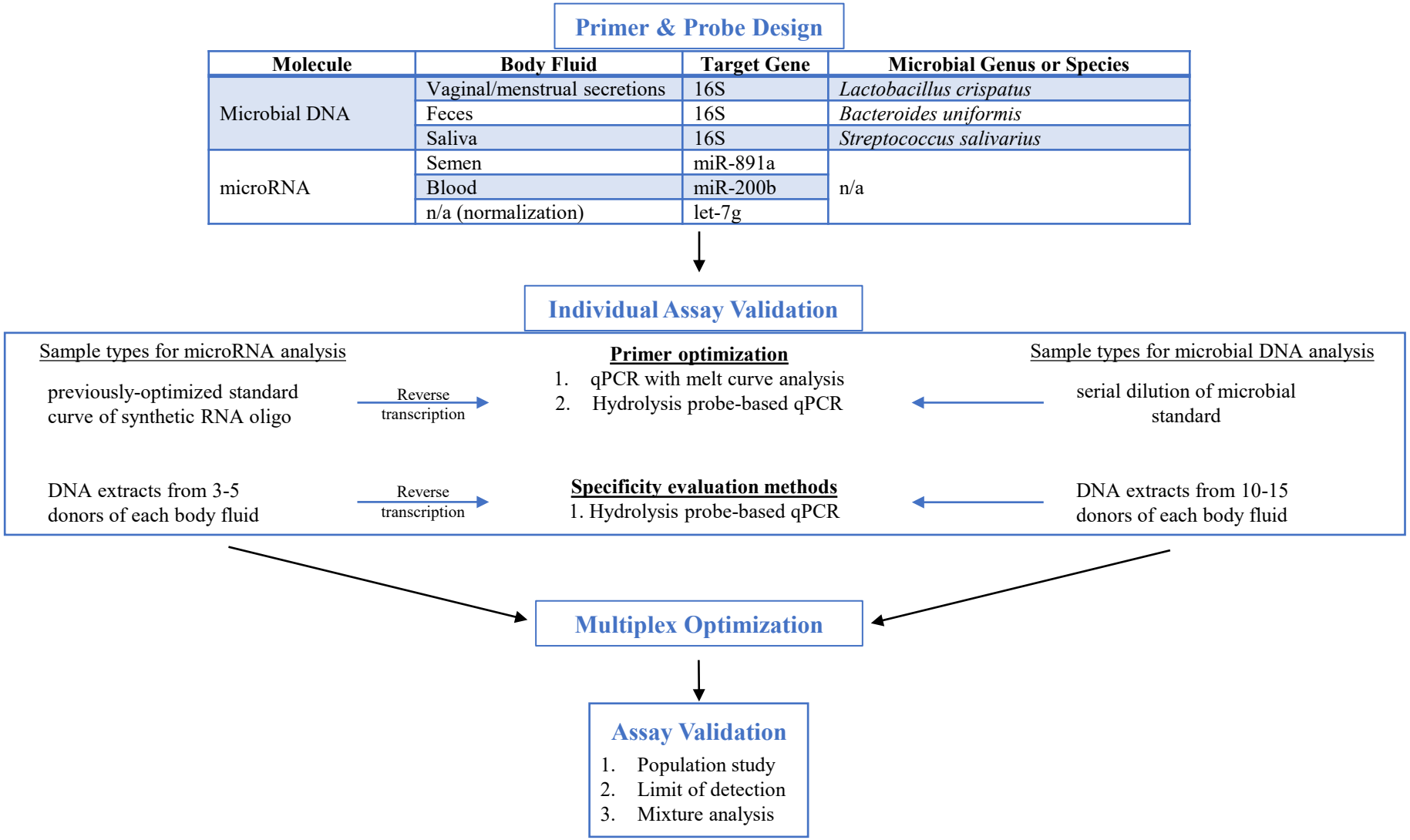

Figure 1.3—Experimental design of a combinatorial RT-qPCR multiplex assay for forensic body fluid identification. Primer optimization and body fluid specificity were evaluated in each analysis (microRNA and microbial DNA) before performing multiple assay developmental validation studies.

CHAPTER 2:

Detection of microRNAs in DNA extractions for forensic biological source identification

This chapter was published in the Journal of Forensic Sciences in 2019:

Lewis CA, Layne TR, Seashols-Williams SJ. Detection of microRNAs in DNA Extractions for Forensic Biological Source Identification. *Journal of Forensic Sciences*. 2019;64(6):1823-1830. doi:10.1111/1556-4029.14070

INTRODUCTION

Context clues are critical in the reconstruction of a crime and pivotal for courtroom testimony; especially with biological evidence, the identification of a biological fluid can be a particularly informative component of crime reconstruction. Recent efforts by forensic researchers have focused on molecular-based methods for body fluid identification in order to overcome the well-documented flaws with current serological techniques used in forensic casework.[138,191] Nucleic acid-based methods, such as miRNA analysis, can be more sensitive and specific as they are amplification-based, as opposed to the current methods that rely on catalytic enzyme activity or immunological affinity.[138] Other benefits of these molecular-based methods are that they typically utilize instrumentation that is commonly seen in a forensic laboratory, and they could potentially reduce the amount of hands-on time.

miRNAs are a class of small, non-coding RNA molecules that are 18–25 nucleotides in length.[182] They regulate gene expression by binding the 3' untranslated region of target mRNA to either signal for degradation of the mRNA or stop translation of the message.[145,152,169] Research has shown that miRNAs have the potential to identify forensically relevant body fluids, where a pair of oligonucleotides can be used to detect a specific miRNA sequence in high abundance in a body fluid. For example, the forensic literature has thus far demonstrated that miR-891a-5p is consistently differentially expressed in semen as opposed to other body fluids, while miR-200b-3p may be highly expressed in blood more than in other body fluids.[76,171,176,201] Furthermore, miRNAs let-7g and let-7i are highly conserved among species and have shown similar abundance within and across multiple body fluids, allowing these miRNAs to be used as endogenous reference markers during analysis.[76,153] Aside from cellular function, miRNAs also

show potential for forensic applications because their short length contributes to their long-term stability and resistance to degradation.[193]

miRNA analysis still requires a separate RNA extraction and therefore consumes valuable sample.[202] Previous work has proposed or evaluated DNA/RNA co-extraction methods for RNA detection using one or more of the typically discarded washes, but a complete evaluation of the most common forensic DNA isolation and purification methods has not been undertaken in a single study.[203–206] The previous reports showing miRNA detection from silica column DNA extractions did not address the exact mechanism of coextraction.[207,208]

The overall purpose of this study was to determine if miRNAs are detectable in both silica column and other DNA extracts using a variety of common extraction methods and to compare the results to those obtained from traditional RNA extracts to evaluate any overall detection differences. The experimental design for the first, exploratory portion of the project was previously illustrated in Figure 1.1. Secondarily, we evaluated global miRNA detection and analyzed differential expression of the miRNA panel previously described[76] using DNA extracts of multiple body fluids. Although these were not the primary goals of this work, any additional supporting data would strengthen the argument that miRNAs can be a useful molecular technique for forensic body fluid identification.

MATERIALS AND METHODS

miRNA Detection in Blood, Semen, and Saliva DNA Extracts

Sample Collection & Preparation

Liquid donations of blood and saliva from three individuals were collected according to VCU's approved IRB Human Subjects Research Protocol (HM20002931). Saliva was deposited into sterile collection cups before aliquoting, and venous blood was collected into a Vacutainer

containing EDTA (Beckton, Dickinson & Company, Franklin Lakes, NJ). Seminal fluid of three healthy individuals was purchased from Lee Biosolutions (Maryland Heights, MO) and stored at -20°C before aliquoting. Equal volumes (100 µL) of each were dried onto sterile cotton swabs; blood and saliva were also dried onto indicating FTA mini cards (Whatman, Pittsburgh, PA). Samples were stored at room temperature for at least one week prior to nucleic acid isolation.

DNA and RNA Isolation

One swab each of blood, semen, and saliva from three different donors was isolated using each of the following methods: organic isolations, QIAamp® DNA Investigator Kit (Qiagen, Hilden, Germany), and AllPrep® DNA/RNA Mini Kit (Qiagen). FTA purifications and DNA IQ™ extractions were performed on the blood and saliva of three donors.

Organic extractions were performed using 400 µL of stain extraction buffer (1 M Tris-HCl, ddH2O, 5 M NaCl, 0.5 M EDTA, 10% SDS, pH=8.0) and 15 µL of Proteinase K (20 mg/mL). Semen samples were incubated with 25 µL of 1M DTT. A phenol:chloroform:isoamyl alcohol (25:24:1) method was carried out followed by a Microcon® Y-100 (Millipore, Billerica, MA) precipitation with a final elution volume of 50 µL.[209] The extracts were stored at -80°C until quantification.

The QIAamp® DNA Investigator Kit was performed according to the manufacturer's protocol, including the addition of 20 µL of 1M DTT to semen samples and 50 µL elution volume for all samples. The AllPrep® DNA/RNA Mini Kit was also performed according to the manufacturer's protocol, with the following modifications: the addition of 500 µL Buffer RLT and 7.14 µL of 14.3 M β-mercaptoethanol to each swab with a 2-hour incubation at 56°C at 900 rpm. Residual liquid was drained from each swab using a spin basket centrifuged at 5,000 rpm for 5 min. After lysis, both DNA and RNA purification protocols provided by the manufacturer

were followed with an elution volume of 50 µL for RNA fractions and 100 µL for DNA fractions.

The DNA IQ™ extractions were performed according to Promega's DNA IQ™ System-Small Sample Casework Protocol[210] with the following modifications: the addition of 300 µL of lysis buffer with an incubation at 56°C for 30 min, the addition of 8 µL of DNA IQ™ resin, and a 10-min incubation at 56°C before eluting with 50 µL of DNA IQ™ Elution Buffer. The first two wash steps listed in the protocol were individually saved and purified using the ReliaPrep™ RNA Cell MiniPrep System (Promega) according to manufacturer's protocol beginning with the addition of 100% isopropanol. For consistency, an elution volume of 50 µL was used.

FTA purifications were performed on blood and saliva by adding 4 mm punches to 50 µL of FTA Solution (Whatman). The punches were washed twice with 100 µL of FTA Solution and once with 100 µL of 6% fresh ammonium hydroxide, with 15-min incubations at room temperature during each wash step before discarding the solution. The punches were incubated at room temperature with 100 µL of TE. Once the TE was removed, the punches were dried for 2 hours at 60°C and then re-suspended in 7 µL of RNase-free water before storage.

Total RNA was isolated as an RNA positive control from each sample using the miRNeasy® Mini Kit (Qiagen) per the manufacturer's protocol with an elution volume of 50 µL. Reagent blanks were carried out as negative controls for each extraction method except FTA, in which a blank FTA paper punch was used.

Nucleic Acid Quantification and DNase-Treatment

DNA and RNA extracts were quantified in all extracts using the Qubit® 2.0 Fluorometer (Thermo Fisher Scientific). DNA was quantified with the Qubit® dsDNA Broad Range Assay Kit (Thermo Fisher Scientific), and RNA was quantified using the Qubit® microRNA Assay Kit

(Thermo Fisher Scientific). Both methods were carried out according to the manufacturer's protocol using a sample volume of 2 µL per assay. A small volume of each sample (8 µL) was treated with the RQ1 RNase-Free DNase Kit (Promega) following the protocol provided by the manufacturer, and then all samples (untreated and DNase-treated) were stored at -80°C until cDNA synthesis.

RT-qPCR

The qScript™ microRNA Quantification System (Quanta Biosciences, Inc., Gaithersburg, MD) was utilized for RT-qPCR. The poly(A) tailing reaction was carried out according to the manufacturer's protocol using a 7 µL input volume of DNA or RNA extract, 2 µL of Poly(A) Tailing Buffer (5X) with a reduced volume of 0.6 µL of Poly(A) Polymerase for a total reaction volume of 9.6 µL. Samples were incubated for 40 min at 37°C followed by 70°C for 5 min to complete the Poly(A) tailing reaction. Immediately after incubation, 9 µL of microRNA cDNA Reaction Mix and 1 µL of qScript™ Reverse Transcriptase were added to the 9.6 µL of Poly(A) tailing reaction for a total reaction volume of 19.6 µL. First-strand cDNA synthesis was performed by incubation at 42°C for 20 min followed by 85°C for 5 min, and cDNA was stored at -20°C until qPCR.

qPCR was performed according to protocol in quarter volume reactions: 6.25 µL of 2X PerfeCTa SYBR Green SuperMix (Quanta Biosciences), 0.25 µL (2.5 µM) PerfeCTa microRNA Assay (Quanta Biosciences) and Universal Primer (UP: 5'-ATGGCGGTAAGTCCAGATACG-3'), 3.75 µL of nuclease-free water, and 2 µL of cDNA reaction for a total reaction volume of 12.5 µL. MicroRNA targets are listed in Table 2.1 with their respective primer sequences. Thermal cycling parameters on the Applied Biosystems™ 7500 Real-time PCR System (Thermo Fisher Scientific) were set at: 95°C for 2 min, followed by 40 cycles of 95°C for 5 sec, 60°C for

Table 2.1—microRNA sequences used in the evaluation of microRNA and DNA co-extraction. Information regarding body fluid-specific and normalization microRNA RT-qPCR targets for forensic body fluid identification are listed (excluding the Exiqon 752-microRNA panel).

Function	Target Gene	Accession Number	Sequence (5'-3')	qPCR Forward Primer Sequence (5'-3')
Normalization	hsa-let-7g-5p	MIMAT0000414	UGAGGUAGUAGUUUGUACAGUU	CCGAGCTGAGGTAGTAGTTTGTAC
	hsa-let-7i-5p	MIMAT0000415	UGAGGUAGUAGUUUGUGCUGUU	CGTTCTGAGGTAGTAGTTTGTGCT
Body fluid specificity	hsa-miR-200b-3p (blood & menstrual sec)	MIMAT0000318	UAAUACUGCCUGGUAAUGAUGA	ACTGCCTGGTAATGATGAAAAA
	hsa-miR-1246 (menstrual sec)	MIMAT0005898	AAUGGAUUUUUGGAGCAGG	GCAATGGATTTTTGGAGCA
	hsa-miR-320c (feces)	MIMAT0005793	AAAAGCUGGGUUGAGAGGGU	AAAGCTGGGTTGAGAGGGT
	hsa-miR-10b-5p (urine)	MIMAT0000254	UACCCUGUAGAACCGAAUUUGUG	CGTACCCTGTAGAACCGAATTTGT
	hsa-miR-26b-5p (saliva)	MIMAT0000083	UUCAAGUAAUUCAGGAUAGGU	CGCTTCAAGTAATTCAGGATAGGT
	hsa-miR-891a-5p (semen)	MIMAT0004902	UGCAACGAACCUGAGCCACUGA	CGAACCTGAGCCACTGAAA

15 sec, and 70°C for 34 sec (data collection), with a final extension step of 95°C for 15 sec, 60°C for 1 min, and 95°C for 15 sec. Each miRNA target was amplified in triplicate technical replicates for each sample with NTCs and RT-RB controls on each plate. Raw data was analyzed at a threshold of 0.01 within SDS software, v1.3.1 (Thermo Fisher Scientific) and exported into Microsoft Excel.

miRNA Global Detection Comparison Between DNA and RNA Extracts

Three blood extracts from the same donor (one RNA control, one organic, and one QIAamp® DNA Investigator) were selected for miRNA global detection analysis with the microRNA Ready-to- use PCR Human Panel I+II, V4.M (Exiqon Inc., Woburn, MA), which includes 752 miRNAs across two 384-well plates. Reverse transcription was carried out using the miRCURY LNA™ Universal RT microRNA PCR system (Exiqon) according to manufacturer's recommendation of 40 ng RNA input per sample. UniSp6 and cel-miR-39-3p from the accompanying RNA Spike-in kit (Exiqon) were added to the RT reaction as internal controls. The RT reactions were set-up with 8 µL of 5X Reaction buffer, 18 µL of nuclease-free water, 4 µL of enzyme mix, 2 µL of synthetic RNA spike-in, and 8 µL of template total RNA. First-strand cDNA synthesis was performed by incubating samples at 42°C for 60 min followed by 95°C for 5 min to inactivate the reverse transcriptase. cDNA was stored at -20°C until qPCR.

qPCR was conducted on the Applied Biosystems™ QuantStudio™ 6 Flex Real-time PCR instrument (Thermo Fisher Scientific) with 2,000 µL of 2X ExiLENT SYBR Green master mix (Exiqon), 1,977 µL of nuclease-free water, 3 µL of ROX passive reference dye (30 nM final concentration), and 19.8 µL of cDNA per plate, with 10 µL of cDNA/master mix added to each well. Thermal cycling parameters were set at: 95°C for 10 min, followed by 40 cycles of 95°C for 10 sec, 60°C for 60 sec with a ramp-rate of 1.6°C/sec[5]. Positive and negative controls were

included in each set of panel plates. Cycle thresholds and background subtraction for each reaction were manually set using the QuantStudio™ Real-Time PCR Software v1.3 (Thermo Fisher Scientific). Data was normalized following the manufacturer's protocol for GenEx qPCR software v6.1 (MultiD Analyses AB, Göteborg, Sweden), and data for miRNAs detected in all three extraction methods were exported to Microsoft Excel.

Body Fluid Identification using a miRNA Panel

Previous work in our laboratory outlined an RT-qPCR method for body fluid identification capable of distinguishing six forensically relevant biological fluids (venous blood, menstrual blood, seminal fluid, urine, feces, and saliva). The panel targets six body fluid-specific miRNAs and two endogenous reference miRNAs (Table 2.1) that relies on a ΔCq dual-normalization method to adjust for differences in both RNA quantity and variable miRNA expression between biological fluids.[76] The described method was validated using RNA extracts; therefore, for proof of concept, we tested the miRNA panel on DNA extracts of menstrual secretions, seminal fluid, saliva and blood (three donors of each). Saliva, semen, and blood were collected as described above, and menstrual secretions were collected on sterile cotton swabs by the donor and returned in swab boxes using informed consent and approved VCU IRB protocols (HM20002931). The same three donors of blood were used in this portion of the study; however, donations of saliva, semen, and menstrual blood were collected from individuals different from those in the extraction portion of the project because samples from previous donors had been consumed. The saliva, semen and menstrual samples were extracted on the QIAcube (Qiagen) using the QIAamp® DNA Investigator kit and eluted in 50 µL, while the blood samples were manually extracted using the same protocol and elution volume. The RT-qPCR workflow was performed as described above using Quanta Biosciences reagents, followed by calculating

differential expression for the panel miRNAs. This was calculated by subtracting the average Cq value of let-7g and let-7i from the Cq value of the target miRNA ($\Delta Cq = Cq_{(target)} - Cq_{(avg\ let\text{-}7g\ \&\ let\text{-}7i)}$). The differential expression data (ΔCq values) were then used to predict the body fluid following the decision matrix previously described.[76]

Statistical Analysis

Statistical analyses were performed in R v3.4.2 (R Foundation for Statistical Computing, Vienna, Austria) or JMP® v14.2.0 (SAS Institute, Cary, NC). Normal distribution and equal variance were confirmed for all sample sets using quantile-quantile plots and Levine's test, respectively. Student's *t*-tests were applied (two-tailed distribution, equal variance) in two-group comparisons (DNase-treatment, DNA IQ™ washes, and Exiqon panel evaluations). In multi-group comparisons, a one-way ANOVA test was performed with a Tukey's HSD pairwise comparison. The data are presented as averages ± SD. A value of $p<0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

miRNA Detection in Blood, Semen, and Saliva DNA Extracts

miRNA and DNA Quantification

Organic and QIAamp® DNA Investigator kit extractions resulted in the highest DNA yields as expected (Table 2.2); however, no further DNA analyses were conducted, as that was not the purpose of this work. FTA purifications were not quantified, as it is not a common practice among forensic laboratories.

Previous work in our laboratory has shown Qubit® microRNA assays to be the most reliable and consistent miRNA quantification method as compared to other UV-spectroscopy or

Table 2.2—Qubit® dsDNA broad range assay quantification results for various nucleic acid extraction methods. Three donors of blood, semen, and saliva were evaluated using each isolation method.

	DNA Concentration (ng/µL)					
Sample ID	**miRNeasy® Mini**	**DNA Investigator**	**Organic**	**AllPrep® DNA fraction**	**AllPrep® RNA fraction**	**DNA IQ™**
Blood 1018	< 1	1.60	6.08	< 1	< 1	< 1
Blood 1019	< 1	1.78	6.84	< 1	< 1	< 1
Blood 1020	< 1	1.15	7.81	< 1	< 1	< 1
Saliva 1007	< 1	4.41	3.77	1.15	< 1	< 1
Saliva 1011	< 1	13.6	8.03	5.82	< 1	1.94
Saliva 1017	1.03	16.1	12.5	4.2	< 1	1.95
Semen 3545	< 1	23.3	44.6	8.25	< 1	N/A
Semen 4032	1.03	20.5	10.5	11.5	< 1	N/A
Semen 4646	< 1	< 1	1.21	< 1	< 1	N/A

Bioanalyzer (Agilent Technologies, Santa Clara, CA) analysis of low quantity samples.[76] Organic DNA extractions yielded the highest miRNA concentrations for all body fluids, followed by QIAamp® DNA Investigator and the remaining methods (Table 2.3). Regardless of the extraction method used, quantifiable RNA was detected in all samples except for FTA purifications. A study in the forensic literature comparing RNA extraction methods found that the AllPrep® DNA/RNA Mini Kit had the lowest RNA recovery but also had the highest DNA recovery performance, concluding that the kit was more designed for DNA analysis and that small RNAs <200 bp may be lost.[202] Our results were concordant in that RNA concentrations were relatively low in the RNA fraction as compared to the DNA fraction, indicating that small RNAs are potentially washed through the column. Nevertheless, some miRNA analysis methods do not require quantification as the data is normalized to endogenous reference genes, so low quantities of RNA may not affect the overall differential expression patterns of miRNAs as long as they remain in detectable quantities.

DNase-Treatment

Some studies include DNase-treatment as a step in a proposed RNA analysis protocol, while others have included it as a type of negative control or do not specify any DNase-treatment being performed.[203–205,211] We observed that DNase-treated extracts were less than or equal to one cycle different from untreated extracts for all isolation methods. A paired Student's *t*-test indicated that there is no significant difference between miRNA detection in DNase-treated and untreated extracts (p-value <0.05) (Figures 2.1 and 2.2) in the two miRNAs assessed. Given that the DNase-treatment was found to be insignificant in miRNA detection differences, observed Cq values are reflective of miRNA detection rather than amplification of genomic DNA; regardless, DNase-treatment was continued throughout the remainder of the study. Therefore, DNase-

Table 2.3—Qubit® microRNA assay quantification results for various nucleic acid extraction methods. Three donors of blood, semen, and saliva were evaluated using each isolation method.

	RNA Concentration (ng/μL)					
Sample ID	**miRNeasy® Mini**	**DNA Investigator**	**Organic**	**AllPrep® DNA fraction**	**AllPrep® RNA fraction**	**DNA IQ™**
Blood 1018	2.46	4.29	24.8	0.597	0.268	< 0.25
Blood 1019	2.57	6.77	28.7	0.749	0.336	< 0.25
Blood 1020	1.96	2.99	36	0.5	0.327	< 0.25
Saliva 1007	1.11	29.7	15	3.02	1.21	1.83
Saliva 1011	5.51	53.8	31.7	20.7	5.17	7.34
Saliva 1017	5.01	74.1	57.7	15	3.62	7.44
Semen 3545	< 0.25	> 75	> 75	36.4	3.36	N/A
Semen 4032	4.13	> 75	43.4	47.8	4.25	N/A
Semen 4646	0.308	1.25	1.99	0.496	< 0.25	N/A

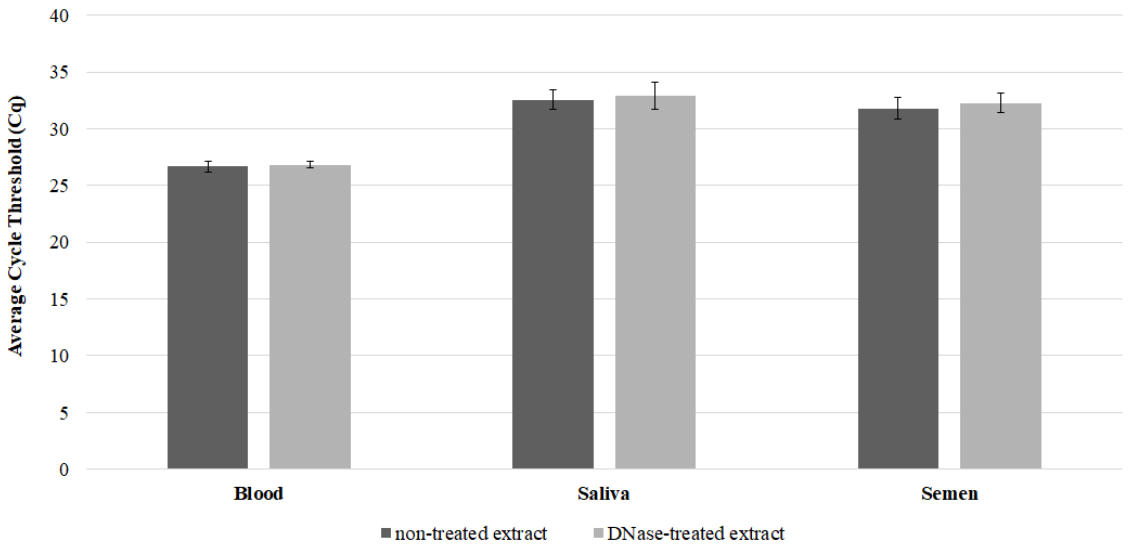

Figure 2.1—Detection of let-7g before and after DNase-treatment. DNase-treatment did not have a statistically significant impact on let-7g detection in blood, semen, or saliva extracted using the QIAamp® DNA Investigator Kit (n=3 donors, average of triplicate technical replicates, error bars represent 1 SD).

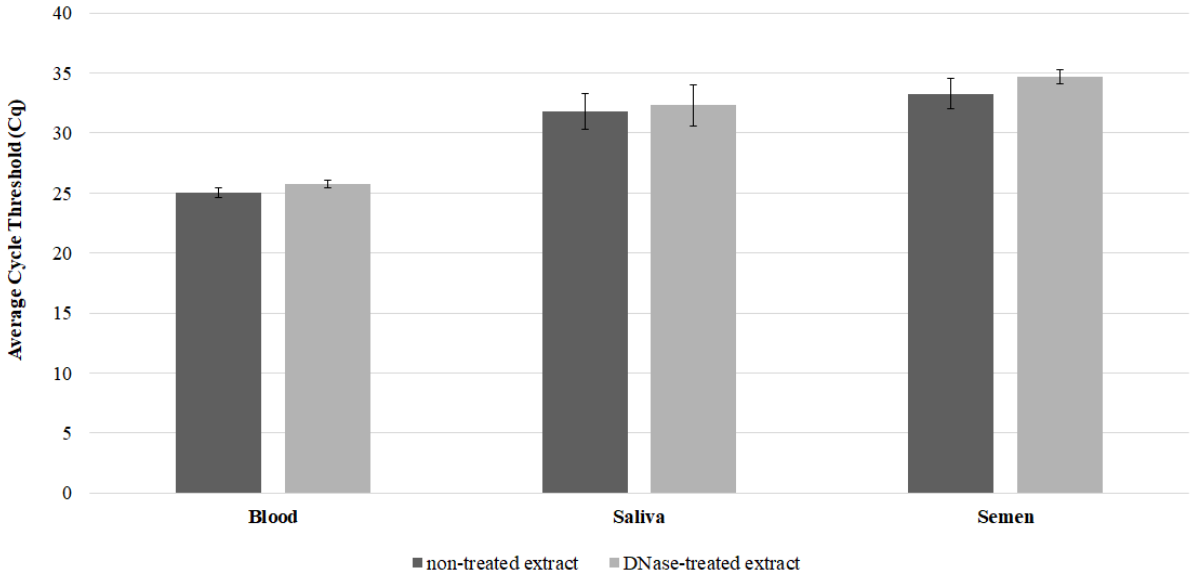

Figure 2.2—Detection of let-7i before and after DNase-treatment. DNase-treatment did not have a statistically significant impact on let-7i detection in blood, semen, or saliva extracted using the QIAamp® DNA Investigator Kit (n=3 donors, average of triplicate technical replicates, error bars represent 1 SD).

treatment can be considered an optional step upon validation in individual labs. This may benefit any forensic DNA laboratory that does not want to purposefully introduce DNases into a low-copy DNA analysis environment.[205]

RT-qPCR Analysis

No results were obtained from FTA purifications of saliva (data not shown), and any observed Cq from FTA purifications of blood were greater than 30 cycles; therefore, we concluded that FTA extractions are not conducive for miRNA co-analysis. No significant differences in let-7g detection between RNA and DNA extracts were detected in semen through RT-qPCR analysis, but significant differences between control RNA extracts and some DNA extraction methods were observed in blood and saliva (Figure 2.3). As expected, the same detection patterns were observed for let-7i in semen, blood, and saliva (Figure 2.4). Though significant differences were observed in miRNA detection among DNA isolation methods, variation within any given DNA isolation method was low. Thus, if a given DNA isolation method was used for casework consistently within a laboratory, comparisons between samples could be confidently made. It should be of note that this portion of the study utilized a small sample set of three donors with only two miRNA markers; thus, it would be essential to validate all markers in a larger population before implementation into casework analysis.

Interestingly, quantification results did not always correlate with detection differences among extraction methods. For example, DNA IQ™ extracts resulted in much lower RNA concentrations using the Qubit® microRNA fluorescent method than the QIAamp® DNA Investigator kit did. However, similar detection levels of let-7g and let-7i were observed in both blood and saliva during RT-qPCR analysis. We hypothesize that this could be due to quantifying samples before DNase-treatment, as presence of genomic DNA may have affected the Qubit®

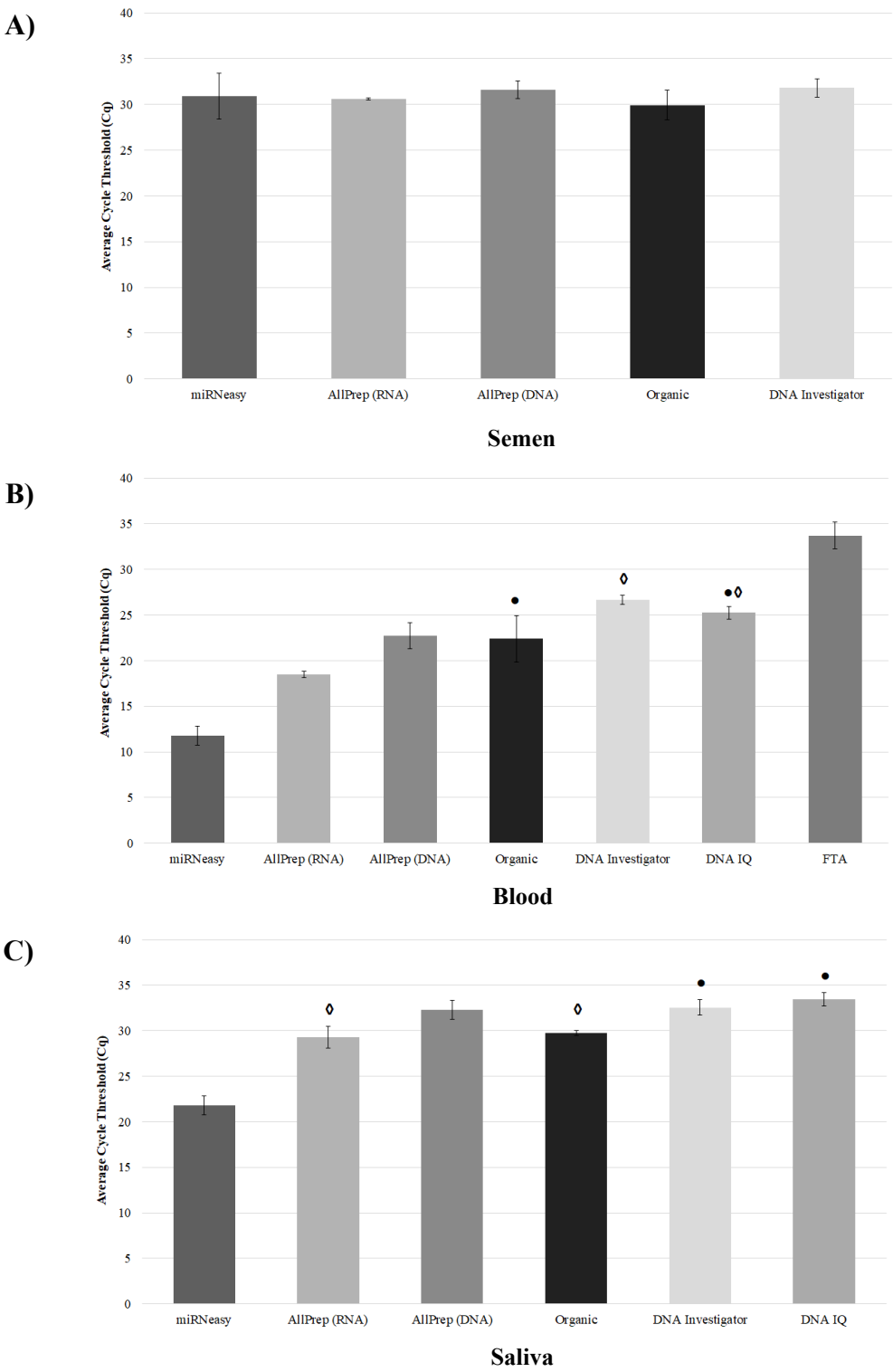

Figure 2.3—let-7g detection among various nucleic acid extraction methods in semen (A), blood (B), and saliva (C). There was no statistically significant difference between RNA and DNA isolation methods in semen samples. In blood and saliva extracts, statistically significant differences were observed between all methods except for those with shared symbols (ANOVA with a TukeyHSD test, $p<0.05$) (n=3 donors, average of triplicate technical replicates, error bars represent 1 SD).

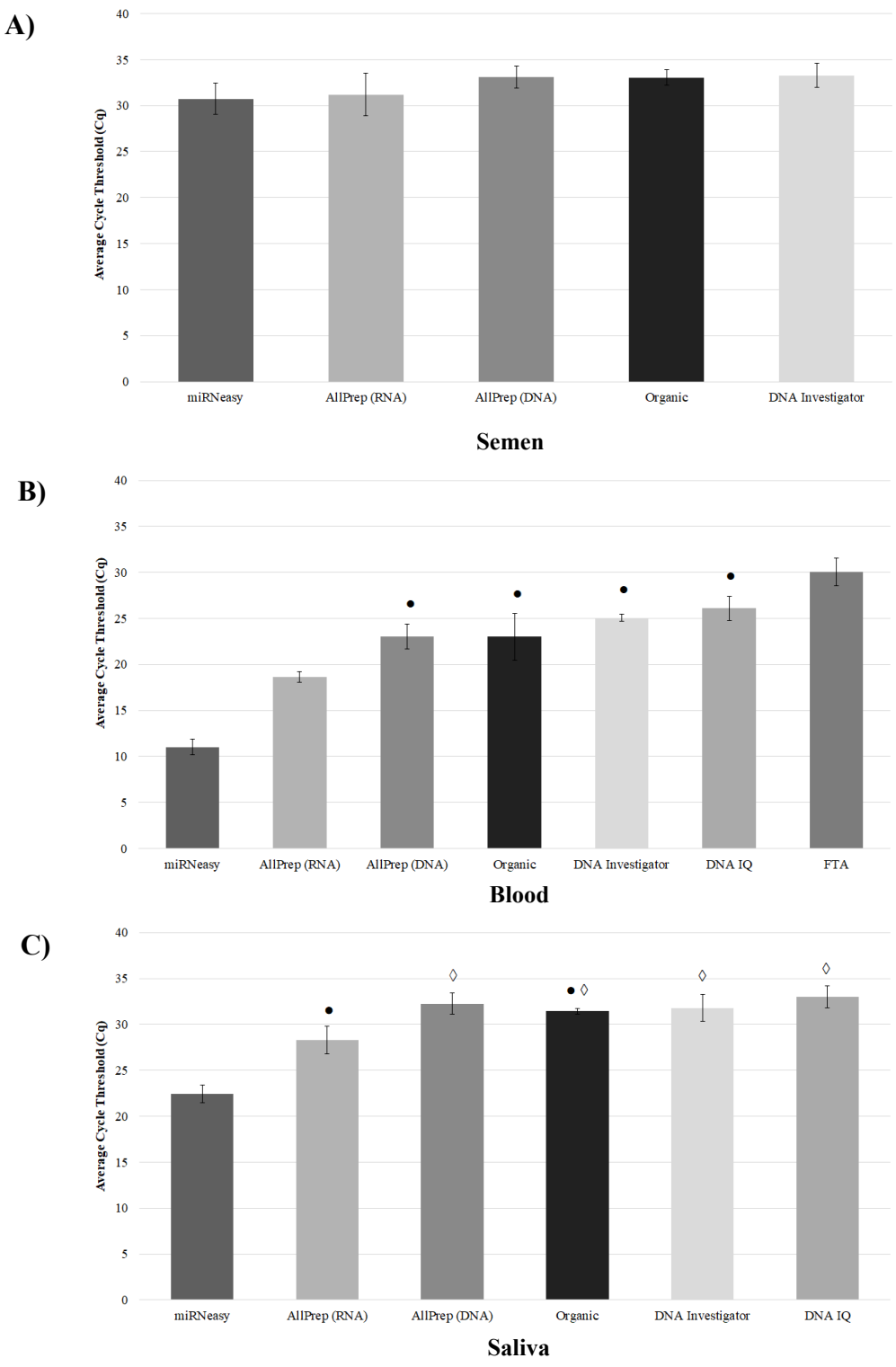

Figure 2.4—let-7i detection among various nucleic acid extraction methods in semen (A), blood (B), and saliva (C). There was no statistically significant difference between RNA and DNA isolation methods in semen samples. In blood and saliva extracts, statistically significant differences were observed between all methods except for those with shared symbols (ANOVA with a TukeyHSD test, $p<0.05$) (n=3 donors, average of triplicate technical replicates, error bars represent 1 SD).

assay. In addition, quantification is based on total RNA or miRNAs as a body of molecules, so RNA concentrations are not likely to be reflective of individual miRNA abundance.

A previous report used the typically discarded lysate from the DNA IQ™ extraction process for messenger RNA analysis.[203] We evaluated the presence of miRNAs in the DNA IQ™ buffer lysate as well as the subsequent wash step (also referred to as Wash 1 and Wash 2, respectively). We found that similar levels of let-7g and let-7i were detected in the DNA IQ™ extract, and purifications of both washes (Figures 2.5 and 2.6). Because purification of the washes did not significantly increase miRNA yield or detection capabilities and required an additional purification step that would increase the amount of time spent per sample, we see no need to perform the additional work when the DNA extract itself will yield similar results. This conclusion supports the earlier work of Omelia et al.,[206] which concluded that miRNAs are predominantly found in the DNA extract itself rather than the subsequent wash steps.

There are clinical reports suggesting simultaneous extractions of DNA and RNA, but few are focused on forensically relevant sample sizes or low molecular weight DNA.[212–215] Based on the data we obtained and the available literature, we hypothesize that miRNAs are co-extracted with DNA because of the extraction chemistries chosen for forensic laboratories. Most forensic labs employ two types of extraction methods, silica-based or organic. Although the chemistries of these two methods are different, neither preferentially isolate one nucleic acid over the other. For example, in silica-based extraction methods, all nucleic acids are adsorbed to silica, but the washes allow for preferential binding of DNA or RNA. In order to preferentially bind RNA, one would decrease the pH during the washes because silica binds RNA under an acidic pH.[216,217] Alternatively, organic extractions utilize the polarity of nucleic acids for a phase separation under certain chemical conditions. More specifically, phenol-chloroform methods allow for

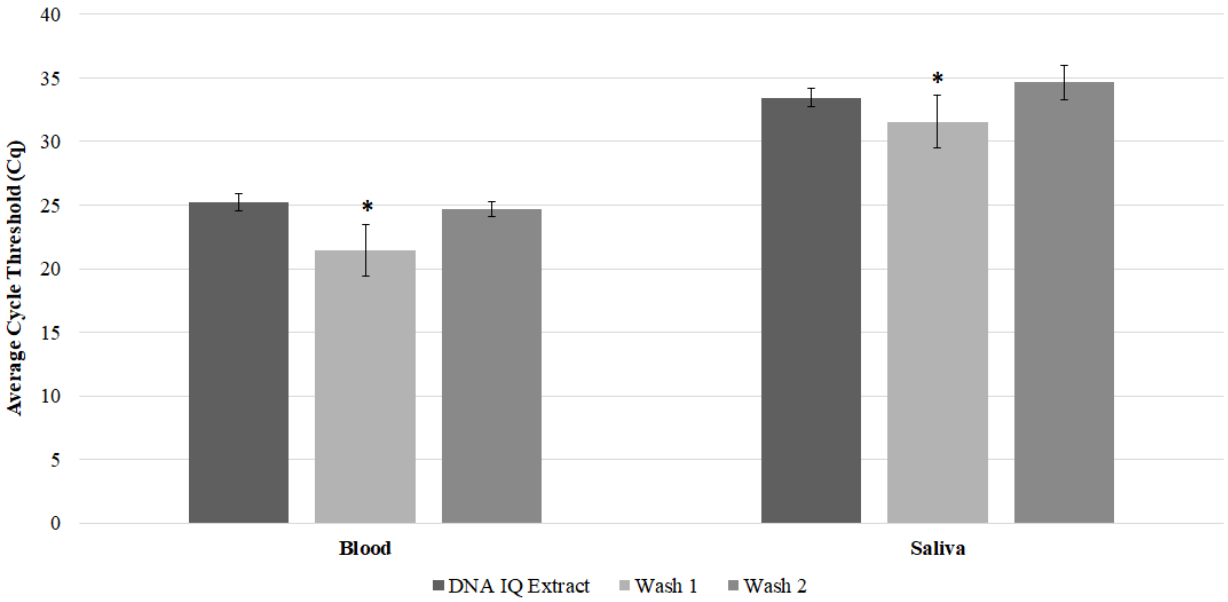

Figure 2.5—let-7g detection in DNA IQ™ extracts and subsequent washes. There was a significant difference in let-7g detection in Wash 1 of blood and saliva from DNA IQ™ extracts. No significant difference was observed between the DNA IQ™ extract and Wash 2 (n=3 donors, average of triplicate technical replicates, *indicates statistically significant difference, p-value<0.05, error bars represent 1 SD).

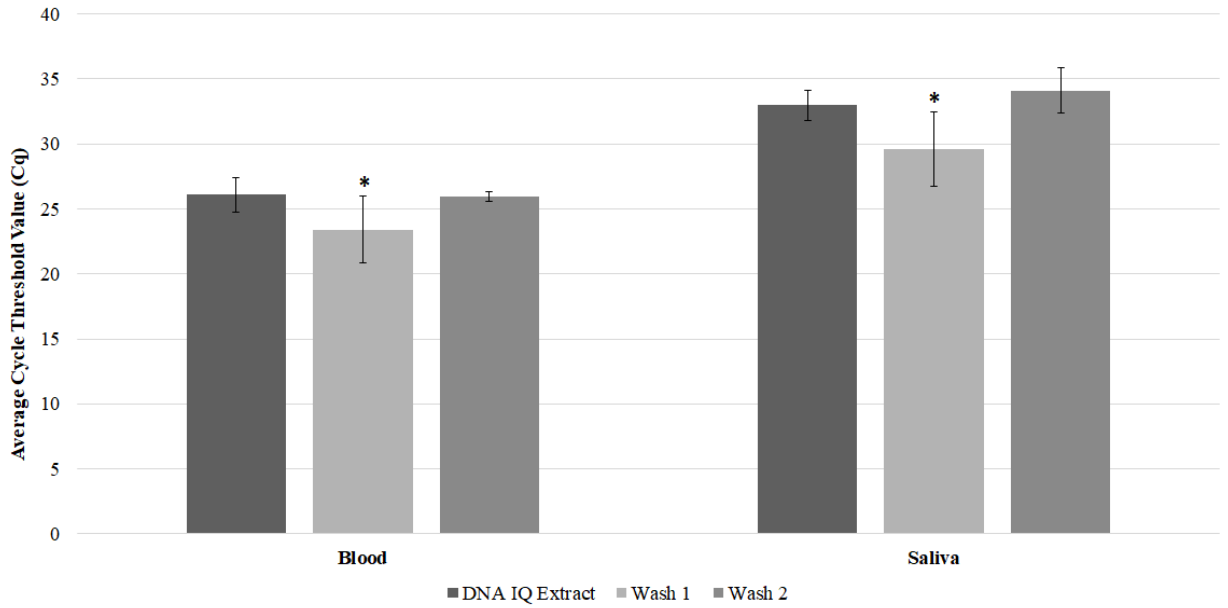

Figure 2.6—let-7i detection in DNA IQ™ extracts and subsequent washes. There was a significant difference in let-7i detection in Wash 1 of blood and saliva from DNA IQ™ extracts. No significant difference was observed between the DNA IQ™ extract and Wash 2 (n=3 donors, average of triplicate technical replicates, *indicates statistically significant difference, p-value<0.05, error bars represent 1 SD).

separation of nucleic acids from proteins; however, various ratios of phenol-chloroform will affect the quantities of DNA and RNA obtained. The ratio of phenol-chloroform and a neutral pH allows for DNA and RNA to be extracted simultaneously in the aqueous phase. Since RNA and DNA molecules are both negatively charged, it is expected that they would react similarly under these conditions. The 1:1 ratio of phenol to chloroform under a neutral pH allows for low molecular weight-DNA to remain in the inorganic or aqueous layer,[218] which is often the method utilized in forensic labs due to isolate degraded DNA from evidence samples. Therefore, as we observed, miRNAs are simultaneously extracted using the DNA extraction methods used in forensic laboratories.

miRNA Global Detection Comparison Between DNA and RNA Extracts

After the initial exploration of our two target miRNAs (let-7g and let-7i) among various DNA extraction methods, we were interested in miRNA global detection differences between RNA and DNA extracts and between DNA extraction methods. We compared miRNA detection in 752 miRNAs using Exiqon's miRCURY LNA™ Human panels I&II on blood from the same donor. We chose to compare the RNA control to the QIAamp® DNA Investigator kit and organic isolation methods. These two DNA extraction methods were chosen because they had the highest RNA yield, and the Exiqon RT protocol recommended a 40 ng RNA input. Out of the total 752 miRNAs evaluated, 228 human miRNAs were consistently detected in all three extraction methods. An unpaired Student's *t*-test indicated that there was a significant difference in the global miRNA detection among RNA and DNA isolation methods ($p<0.0001$), which was expected based upon our initial findings. However, there was no significant difference in global miRNA detection between the QIAamp® DNA Investigator kit and the organic extraction method (Figure 2.7). This provides further evidence that miRNAs are detectable at similar levels in

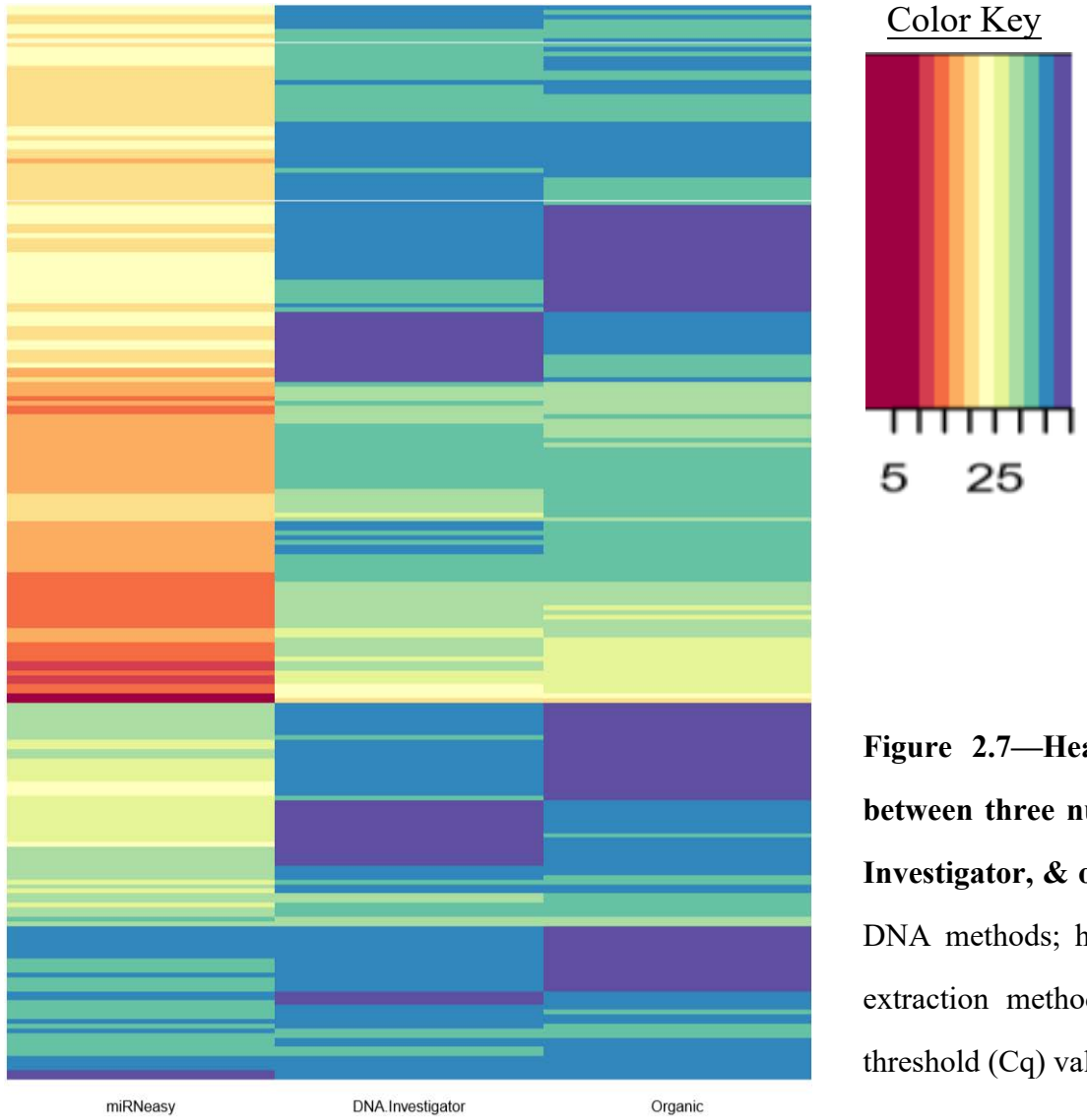

Figure 2.7—Heatmap comparing global detection of 228 human microRNAs between three nucleic acid isolation methods (miRNeasy® Mini, QIAamp® DNA Investigator, & organic). A significant difference was observed between the RNA and DNA methods; however, there was no significant difference between the two DNA extraction methods (unpaired Student's *t*-test, $p<0.05$). Color key represents cycle threshold (Cq) values, and each row represents Cq values for individual miRNAs.

various types of DNA extraction methods, but this result is limited by a single body fluid from a single donor; therefore, any miRNAs identified using RNA extracts will need to be further validated in DNA samples of a larger population size prior to use in casework.

Body Fluid Identification using miRNA Panel

Previous work in our laboratory identified a miRNA panel for distinguishing between several forensically relevant biological fluids: blood, menstrual secretions, feces, urine, saliva, and semen. These body fluids are identified through a decision matrix based on differential expression patterns between target miRNAs (miR-200b-3p, miR-1246, miR-320c, miR-10b, miR-26b, miR-891a, respectively and in Table 2.1) and endogenous reference miRNAs (let-7g and let-7i). Once we observed that miRNAs let-7g and let-7i were co-extracting with DNA in all methods except for the FTA method, we wanted to address if a body fluid can be accurately identified from this miRNA panel using QIAamp® DNA Investigator extracts. We found that out of the 12 samples tested (three donors each of four body fluids), seven samples were accurately identified using the miRNA panel (Table 2.4). All semen samples were correctly identified, two out of three were correctly identified for saliva and blood samples, but none of the menstrual secretions were accurately identified. The inaccurate classification of menstrual secretions could be due to the low detection of miR-200b in the DNA extracts, which affected the predicted differential expression pattern. Shown in Table 2.4, the differential expression pattern for miR-200b was the reason for the failed classification of menstrual blood, whereas the differential expression pattern for miR-1246 was within the optimal ΔCq range for menstrual secretions ($\Delta Cq < -2$). This same phenomenon was observed in the blood samples, where the detection of miR-200b was low in DNA extracts compared to the differential expression values for blood samples observed by Seashols-Williams et al.[76] Although more work would be required to

Table 2.4—microRNA body fluid specificity in DNA extracts. Data reflect optimal and observed differential expression values (ΔCq) in DNA extracts of forensically relevant body fluids using a miRNA body fluid identification panel.

	Menstrual Secretions		Seminal Fluid		Blood		Saliva
Optimal ΔCq for differentiation	miR-200b > 6	miR-1246 < -2	miR-200b < 6	miR-891a < 0	miR-200b > 6	miR-1246 > -2	miR-26b < -2
Donor A	0.90*	-6.87	-3.08	-2.96	2.62*	-0.35	-0.94*
Donor B	-0.63*	-6.25	-1.93	-2.85	7.65	1.00	-2.52
Donor C	2.13*	-3.87	-3.90	-3.28	7.58	0.11	-2.54

* indicates value that failed the optimal ΔCq range (red)

validate the miRNA panel for detection in DNA extracts, these initial results show promise in the detection of miRNAs from DNA extractions for forensic body fluid identification purposes. Two reports specifically address the potential use of miR-451 and miR-205 for the identification of blood and saliva from DNA extracts (respectively), but neither addresses normalization or semen identification.[206,207] In this study, we included semen in our evaluation of DNA extraction methods and tested a more comprehensive miRNA analysis method for body fluid identification from DNA extracts. Consequently, this work provides additional support to the literature that forensic body fluid identification using miRNAs from DNA extracts is achievable and could alleviate many of the challenges in terms of sample usage and serological analysis faced by forensic biologists.

CONCLUSIONS

The utilization of miRNAs for forensic applications is well documented in the literature as a promising method for the identification of forensically relevant body fluids.[76,144,177,191,202–204,206,219] Although the use of miRNAs for forensic body fluid identification is an improvement to the sensitivity and specificity of the current serological tests, it often involves a separate RNA extraction, consuming evidentiary sample and analyst time. RNA and DNA co-extraction kits are commercially available but can reduce DNA yield as the same sample is being used for one extraction. We first evaluated miRNA detection levels in DNA extracts using several of the most commonly used forensic DNA extraction methods, and then we tested if we were able to accurately classify the source of the biological fluid. The benefit of this would be to obtain biological source information as well as STR profiles for individualization.

Our data suggest that miRNAs are detectable in DNA extracts without any modifications to the manufacturer protocol, allowing for automation capability and use of excess DNA in a

sample for body fluid identification purposes. miRNAs were also detected in lysates or wash buffer at similar detection levels, indicating one could potentially use a sample lysate for body fluid identification to conserve the amount DNA extract consumed.

In conclusion, separate RNA extractions and DNase-treatment are not strictly necessary for miRNA detection. Given that these two steps are significant barriers to implementation, miRNA analysis could provide forensic laboratories with the ability to identify the body fluid(s) present in a timely manner with stronger confidence in the results obtained.

CHAPTER 3:

Design and optimization of a 16S microbial qPCR multiplex for the identification of feces, saliva, vaginal and menstrual secretions

INTRODUCTION

The identification of biological fluids on crime scene evidence can play a crucial role in determining the outcome of a criminal case. Most methods currently used in forensic serology rely on enzymatic-based tests that result in a color change that is interpreted and recorded by an analyst. Although these serological methods have been utilized for decades, there are well-documented flaws associated with each.[4,11,16,220] Therefore, there has been extensive research among the forensic community that addresses molecular-based methods for body fluid identification (BFID), such as mRNA or miRNA analysis, DNA methylation, and microbial DNA analysis.[66,67,126,138,169]

There are numerous studies that focus on microbiome based BFID, but most rely on 16S rRNA gene sequencing, as this is a gold standard in microbiome analysis.[90,92] The 16S ribosomal subunit is specific to prokaryotes and is highly conserved within the same genus and species but has several hypervariable regions, all of which allow for taxonomical microbial classification.[221] One limitation that has been reported is the inability to differentiate menstrual blood from vaginal secretions based on taxonomical classification.[81] Another consideration is the high cost and complex workflow of high-throughput sequencing (HTS) methods. As of now, HTS is not standard in forensic DNA analysis due to cost, sample preparation time, hands-on training requirements, and complicated back end bioinformatic analyses.

To better align with the current DNA analysis workflow, real-time PCR methods for bacterial BFID have been proposed; however, most of these amplify multiple bacterial species to classify a single body fluid,[107,118,120] which could be problematic when developing a qPCR multiplex for multiple body fluids due to the limitations of dye channels in real-time PCR instruments. Furthermore, the most successful microbiome qPCR studies for BFID involve body

fluids with high bacterial content, such as vaginal fluid, feces, and saliva.[75,123,222–229] Body fluids considered to be sterile (i.e., venous blood, semen, and urine) are harder to identify using microbial characterization because low bacterial cell counts often result in poor DNA yields, a problem when considering the often-compromised nature of forensic evidence.[226]

The purpose of this research was to design and validate a qPCR multiplex that targets the 16S gene of three microbial species that are highly abundant in the respective body fluids—*B. uniformis* for fecal samples, *S. salivarius* for saliva samples, and *L. crispatus* for female intimate samples (vaginal fluid/menstrual blood). Additionally, these three species have been reportedly successful for BFID in multiple forensic studies (Table 1.1). As previously mentioned, it is reportedly difficult to distinguish menstrual blood from vaginal fluid;[81] therefore, differentiating between the two fluids was not a primary goal of this study.

MATERIALS AND METHODS

Primer and Probe Design

Primers and probes were designed using default parameters in Beacon Designer 8 (Premier Biosoft, Palo Alto, CA). The same primer sequences from the SYBR Green mode were used to design the probes in TaqMan mode, which allowed for primer specificity testing before ordering/testing the hydrolysis probe. Primers and probes were ordered from Integrated DNA Technologies (IDT, Coralville, IA); dual-labeled hydrolysis probes were labeled with internal quenchers, HPLC-purified and normalized to 100 µM in TE buffer. Sequence information for qPCR primers, probes, and respective targets are listed in Table 3.1.

Table 3.1—Microbial target and sequence information utilized in the 16S qPCR triplex. HPLC-purified hydrolysis probes include 5' reporters, internal and 3' quenchers.

Microbial Target & [Accession No.]	**Forward Primer** 5' – 3'	**Reverse Primer** 5' – 3'	**Hydrolysis Probe** 5' – 3'	**Amplicon Length** (bp)
Lactobacillus crispatus [MN744551]	CAGCAGTAGGGAATCTTC	CTGGTTGATTACCGTCAA	/ATTO550N/ACCTCTATC/TAO/CTTCTTCACCAACAACA/IAbRQSp/	145
Bacteroides uniformis [AP019724.1]	TAGCGGTGAAATGCTTAG	CATCGTTTACTGTGTGGA	/6FAM/CACGAAGAA/ZEN/CTCCGATTGCGAAG/IABkFQ/	136
Streptococcus salivarius [CP015282.1]	ATGCGTAGATATATGGAGG	CTACCAGGGTATCTAATCC	/SUN/CGAGCCTCA/ZEN/GCGTCAGTTACA/IABkFQ/	108

Sample Collection and DNA Extraction

Venous blood, menstrual blood, semen, saliva, feces, and vaginal samples were collected from 30 volunteers according to VCU's IRB approved protocol for research with human subjects (HM20002931). Menstrual blood, feces, saliva, and vaginal secretions were collected by the donor using polyester swabs, while semen was collected in sterile plastic containers and aliquoted onto polyester swabs (50 µL). Venous blood was deposited onto polyester swabs by pricking the donor's sterilized finger with a finger lancet. Swabs were placed in swab boxes upon collection and dried at room temperature for at least 24 hours prior to DNA extraction. The donations were collected between 2017 and 2019 for a biological sample registry, so they had been stored at room temperature for a comparable amount of time as they might in a forensic evidence room. Other considerations when selecting samples from the registry were antibiotic usage, equal number of male/female donors (when applicable), various ethnicities and ages, different days of the menstrual cycle, and at least seven days post-coital for intimate fluids (semen and vaginal/menstrual secretions).

Whole swabs were extracted using the QIAamp® DNA Investigator Kit following the "Isolation of Total DNA from Body Fluid Stains" protocol from the manufacturer. Reagent blanks were included in each batch of extractions to assess potential contamination. During cell lysis, no carrier RNA was added, and 20 µL of 1 M DTT was added to semen samples. After the 10-min incubation at 70°C, residual liquid was collected by placing the swab in a DNA IQ™ spin basket (Promega, Madison, WI), returning it to the lysate tube, and centrifuging at 5,000 rpm for 5 min. Once the spin basket and swab were discarded, DNA purification was performed according to protocol without modification. All samples and reagent blanks were eluted in 50 µL of ATE buffer (Qiagen) and stored at -80°C.

qPCR

The multiplex is technically a qualitative-PCR assay since the goal is not to quantify any microbial DNA in the sample; however, since the multiplex was validated using quantities of synthetic DNA standards, the MIQE guidelines for quantitative-PCR reports were followed throughout the project.[230]

Initially, each microbial target was evaluated as a single-plex using gBlocks® Gene Fragments (IDT) as the qPCR standards. The gBlocks® were chosen as standards because there were no commercially available mock microbial community standards that included all three bacterial species of interest and because gBlocks® are manufactured (i.e., undergo extra quality control measures) for the purpose of validating qPCR assays. Sequences for each gBlock® standard are listed in Table 3.2**.** Each gBlock® was resuspended in TE buffer at 10 ng/µL and incubated at 50°C for 20 min, per manufacturer's instructions. Ten-fold serial dilutions were prepared at the concentration range of 5 pg/µL – 0.05 fg/µL, aliquoted and stored at -20°C. To validate the multiplex, the three gBlocks® sequences were pooled together at a concentration of 1 ng/µL before making ten-fold serial dilutions to the optimized concentration range (5 pg/µL – 0.05 fg/µL).

SYBR Green Assay

To ensure amplification of a single product, each primer set was evaluated first via a SYBR Green assay with melt curve analysis to ensure a single amplified product. Standards and no-template controls were analyzed in triplicate using 6.25 µL of PerfeCTa SYBR Green SuperMix (2X) (VWR, Radnor, PA), 3.75 µL of nuclease-free water, 0.25 µL (10 µM) of forward and reverse primers (Table 3.1), and 2 µL of standard for a 12.5 µL total reaction volume. Thermal cycling was conducted on the Applied Biosystems™ QuantStudio™ 6 Flex

Table 3.2—gBlock® Gene Fragment sequences for each microbial species. Sequences were used as qPCR standards during the microbial 16S triplex validation.

Microbial Target & [Accession No.]	**gBlock® Gene Fragment Sequence** 5' – 3'
Lactobacillus crispatus [MN744551]	CCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGAC GCAAGTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATC GTAAAGCTCTGTTGTTGGTGAAGAAGGATAGAGGTAGTAACTGGCCTT TATTTGACGGTAATCAACCAGAAAGTCACGGCTAACTACGTGCCAGC
Bacteroides uniformis [AP019724.1]	AGGCAGGCGGAATTCGTGGTGTAGCGGTGAAATGCTTAGATATCACGA AGAACTCCGATTGCGAAGGCAGCTTGCTGGACTGTAACTGACGCTGAT GCTCGAAAGTGTGGGTATCAAACAGGATTAGATACCCTGGTAGTCCAC ACAGTAAACGATGAATACTCGCTGTTTGCGATATAC
Streptococcus salivarius [CP015282.1]	GAATTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAGGAACACCG GTGGCGAAAGCGGCTCTCTGGTCTGTAACTGACGCTGAGGCTCGAAAG CGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAAC

Real-Time PCR System (Thermo Fisher Scientific) with PCR parameters set to 95°C for 3 min followed by 35 cycles of 95°C for 15 sec, 58°C for 45 sec, 72°C for 30 sec with instrument default melt curve parameters. Raw data were analyzed in QuantStudio™ Software v1.3 (Thermo Fisher Scientific) with the baseline set at 1-6 cycles and threshold set to 0.02 for all targets.

Hydrolysis Probe-Based Assay

Once a single amplification product was confirmed in the SYBR Green assay, each target was individually evaluated in a probe-based assay before multiplexing. After multiplex validation using gBlock® standards, the assay was tested using body fluid samples.

Each single-plex qPCR reaction consisted of 5 µL of PrimeTime® Gene Expression Master Mix (2X) (IDT), 2.5 µL of nuclease-free water, 0.5 µL of 20X assay mix, and 2 µL of standard for a total reaction volume of 10 µL. The 20X assay mixes for all targets were optimized to final determined concentrations of 400 nM for each primer and 200 nM for the probe. For the triplex reactions, 5 µL of PrimeTime® Gene Expression Master Mix (2X), 1.5 µL of nuclease-free water, 0.5 µL of *L. crispatus* 20X assay mix, 0.5 µL of *B. uniformis* 20X assay mix, 0.5 µL of *S. salivarius* 20X assay mix, and 2 µL of sample or standard were combined for a 10 µL total reaction volume. Thermal cycling parameters on the QuantStudio™ 6 Flex Real-Time PCR instrument were set to 95°C for 3 min followed by 40 cycles of 95°C for 5 sec and 60°C for 30 sec in fast cycling mode. All six standards were analyzed in duplicate with NTCs on each plate. To conserve DNA extract, single technical replicates were run when testing body fluids; however, various extracts were tested multiple times throughout the validation to ensure repeatable results from body fluid samples. In QuantStudio™ Software v1.3, auto-baseline and

cycle threshold of 0.06 were applied initially during assay optimization, but the threshold was lowered to 0.04 for all targets during final validation and when evaluating body fluid specificity.

Data Analysis

During assay optimization, standard curve metrics such as slope, Y-intercept, and PCR efficiency (>90%) were analyzed for all targets, and technical validation was complete when the single-plex and multiplex quantification cycle (Cq) values for each standard were within one cycle of one another. Raw data was exported from QuantStudio™ Software v1.3 and input into Microsoft Excel to calculate averages between technical replicates, replace a value of 40 for any "undetermined" result, and omit eight outliers that were greater than three standard deviations from the mean (yellow highlight in Appendix 1: Table 1). All data are reported as averages with standard deviations, where applicable.

Subsequent data analysis was performed in R version 4.1.1 (R foundation, Vienna, Austria). The data were randomly split into training and validation sets (70% and 30%, respectively) using the sample() function, and a classification regression tree (CART) model was created using the rpart package. Confusion matrices and classification accuracy percentages are reported for only one iteration of the validation dataset.

Linear Range of Classification

The goal of the linear range of classification study was to determine the lowest DNA concentration at which the qPCR assay will accurately classify as the correct body fluid. Total DNA was quantified using the NanoDrop™2000 UV Spectrophotometer (Thermo Fisher Scientific), and averaged among the 30 donors of each body fluid in Microsoft Excel. Five donors of each body fluid were selected based on having DNA concentrations close to the calculated averages (blood=4.13 ng/µL, menstrual blood=65.19 ng/µL, semen=22.04 ng/µL,

feces=117.16 ng/µL, vaginal fluid=57.34 ng/µL, and saliva=19.04 ng/µL). Each DNA extract was serially diluted ten-fold until the dilution concentration was less than the lower limit of the linear dynamic range (0.05 fg/µL), and then qPCR using the validated 16S triplex was performed as mentioned above. Data were analyzed using the trained CART model; however, since the confusion matrix output does not show which samples are misclassifying, the analysis was performed manually in Microsoft Excel for each sample.

RESULTS AND DISCUSSION

Assay Validation

In both SYBR Green and probe-based assays, there was no amplification detected in any of the negative controls, including extraction reagent blanks. Single peaks were observed in the SYBR Green melt curve analysis, which verified that there was only one PCR product for each primer pair (data not shown). The same primer sequences were then used for the probe-based assay, in which various primer/probe concentrations and 5' reporter dyes were evaluated during optimization. The final 16S triplex primer and probe concentrations for all three microbial targets were 400 nM (forward and reverse) and 200 nM, respectively. All reported data are representative of the 16S triplex at these concentrations. The 5' reporter dyes were chosen based on having similar excitation and emission wavelengths as dyes that are in use in commercial STR multiplex kits. For example, the ATTO550 and SUN dyes (IDT) emit in the same filters as ABY and VIC (Thermo Fisher Scientific), respectively. These choices were designed to ensure that qPCR instruments would already be calibrated for the requisite probe emission spectra, which would ease implementation in forensic laboratories.

The slopes, amplification efficiencies, and R^2 values of the standard curves (Table 3.3) were all within the recommended ranges for qPCR assays.[231] The reported standard curve data in

Table 3.3—Standard curve metrics for the microbial 16S triplex assay validation. Values are representative of averages and standard deviations across six experiments.

Microbial Target	Slope	Y-intercept	R^2	% Efficiency
Lactobacillus crispatus	-3.525 ± 0.05	2.719 ± 0.58	0.997 ± 0.002	92.2 ± 1.78
Bacteroides uniformis	-3.491 ± 0.03	2.433 ± 0.48	0.998 ± 0.002	93.4 ± 1.00
Streptococcus salivarius	-3.524 ± 0.03	2.549 ± 0.44	0.998 ± 0.002	92.2 ± 0.85

Table 3.3 are averaged across six experiments, demonstrating repeatability and reproducibility of the assay. The linear dynamic range was determined to be 5 pg/µL – 0.05 fg/µL. We acknowledge that the lower limit of this range is not the lowest limit of detection since each microbial target amplifies at approximately 28 cycles (Table 3.4); however, lower concentrations were not tested to minimize reagent consumption. Additionally, we felt that six standards were sufficient to validate assay performance because the overall purpose is to obtain a raw Cq value (qualitative-PCR), rather than to quantify any microbial DNA from a sample (quantitative-PCR). Although it is possible to quantify DNA of each microbial species using this assay, further research expanding the lower limit of detection would be required to quantify microbial DNA for forensic applications.

An important consideration during assay validation was equivalent amplification of the microbial DNA from forensic body fluid samples in a single-plex assay when compared to the multiplex. To address this concern, multiple DNA extracts were tested for each target species both in single-plex and multiplex reactions and verified that Cq values were within one cycle of each other (data not shown).

Body Fluid Specificity of the 16S Triplex

When evaluating raw data (Appendix 1: Table 1), *S. salivarius* was detected in all body fluid samples at higher/similar abundances (lower Cq values) in saliva and menstrual blood compared to blood, semen, vaginal fluid, and feces. As expected, *B. uniformis* and *L. crispatus* were most abundant in feces and vaginal/menstrual secretions, respectively. Of the three microbial species, detection of *L. crispatus* was the most variable, especially in vaginal fluid. One possible explanation for such high variability in vaginal fluid is PCR inhibition due to over-input of template DNA (*L. crispatus* was undetected in nine vaginal samples), which is further

Table 3.4—Example of microbial 16S triplex standard curve data. Raw data is reflective of one run of the 16S microbial triplex demonstrating equal amplification of all three targets in a single well (Conc.=DNA concentration of pooled standard of IDT gBlocks® Gene Fragments).

A) *Lactobacillus crispatus*

Target	Reporter	Conc. (pg/µL)	Cq Mean	SD	Slope	Y-int	R^2	Efficiency %
L.crispatus_16S_ ATTO550	ABY	5	10.181	0.47	-3.534	2.062	0.999	91.86%
		0.5	13.947	0.12				
		0.05	17.166	0.02				
		0.005	20.559	0.06				
		0.0005	24.265	0.01				
		0.00005	28.049	0.08				

B) *Bacteroides uniformis*

Target	Reporter	Conc. (pg/µL)	Cq Mean	SD	Slope	Y-int	R^2	Efficiency %
B.uniformis_16S_ FAM	FAM	5	9.876	0.08	-3.483	1.821	0.999	93.70%
		0.5	13.413	0.07				
		0.05	16.760	0.13				
		0.005	20.072	0.01				
		0.0005	23.717	0.09				
		0.00005	27.410	0.11				

C) *Streptococcus salivarius*

Target	Reporter	Conc. (pg/µL)	Cq Mean	SD	Slope	Y-int	R^2	Efficiency %
S.salivarius_16S_ SUN	VIC	5	10.079	0.14	-3.518	1.975	0.999	92.43%
		0.5	13.736	0.13				
		0.05	17.090	0.11				
		0.005	20.375	0.03				
		0.0005	24.043	0.05				
		0.00005	27.861	0.01				

discussed in a later section. Another possible explanation is that only one *Lactobacillus* species was included in the assay, compared to other microbial qPCR studies that have targeted more than one species for accurate vaginal fluid identification.[78,107,119,120] Incorporating more markers into the assay would account for the diversity of the vaginal microbiome across females, but it also increases cost and consumption of DNA extract, which is something we wanted to avoid when designing the assay. An alternative solution could be to utilize the same approach as Doi et al.,[120] in which qPCR primers were designed flanking a conserved region of 16S at the genus level of four *Lactobacillus* species.

Classification Regression Tree Analysis

It should first be noted that although blood and semen samples were included in the CART model, the goal was not to differentiate between them using this assay, as they have low bacterial DNA yields.[90] For this reason, blood and semen were grouped together as "Bld/SF" in the dataset and were used to eliminate either fluid as the biological source in an unknown sample. Two regression trees were created—one with vaginal fluid (VF) and menstrual blood (MB) as separate fluids (Figure 3.1) and one with them as a combined group (VF/MB) (Figure 3.2). While the original intent of this study was not to differentiate between vaginal/menstrual secretions, both were evaluated out of curiosity to see how each model would perform. The individual model resulted in an 84% overall classification accuracy with 54.5% of MB samples misclassified. Four of the MB samples were classified as VF while two were classified as saliva (Table 3.5).

When female intimate sample data were combined (VF/MB), the tree plot (Figure 3.2) looked similar to that in Figure 3.1; however, the overall classification accuracy increased to 96.5% with only two samples (one saliva and one VF/MB) misclassified (Table 3.6). This

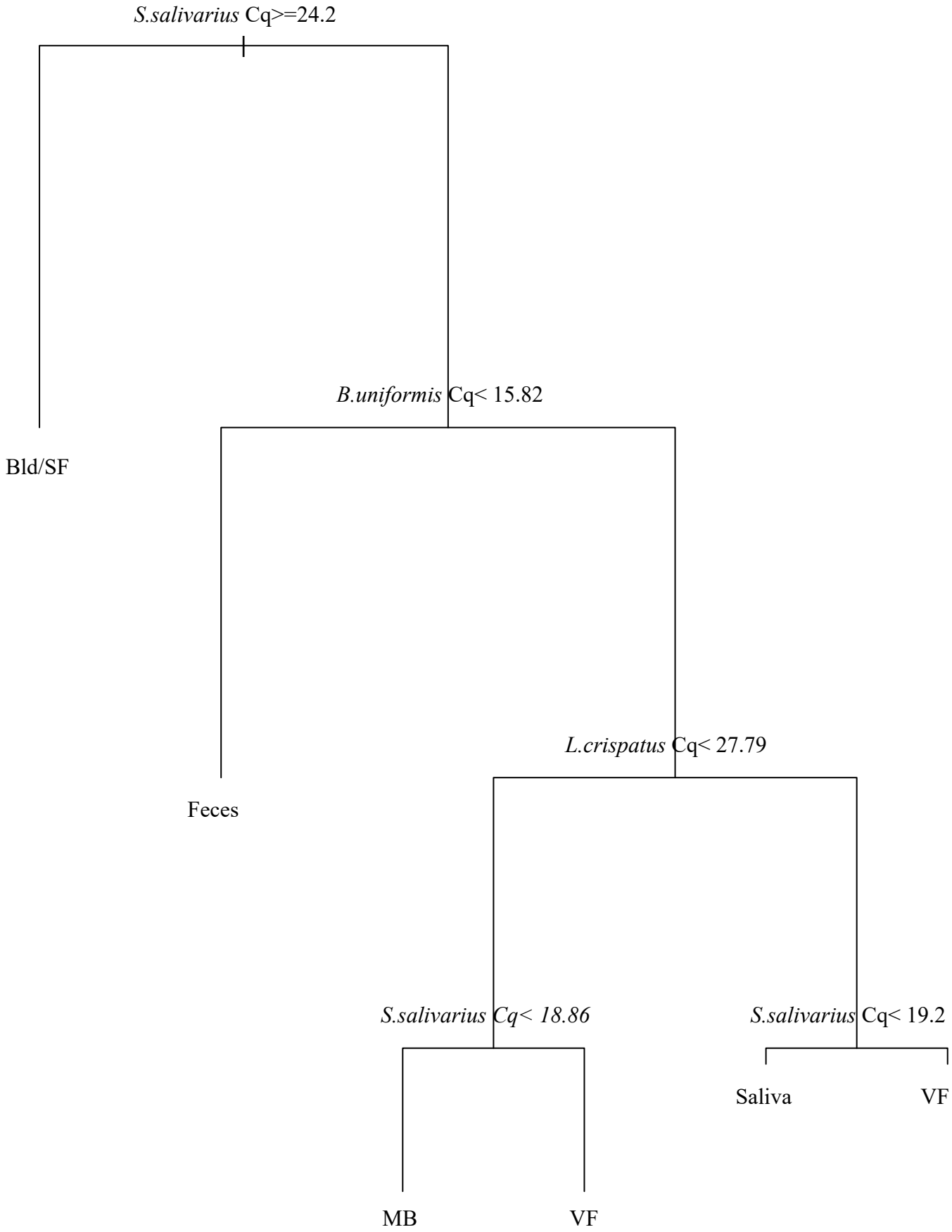

Figure 3.1—Classification Regression Tree (CART) model for the microbial 16S triplex data when vaginal fluid (VF) and menstrual blood (MB) are analyzed as separate fluids (n=30 donors of each body fluid, Bld/SF=blood/semen).

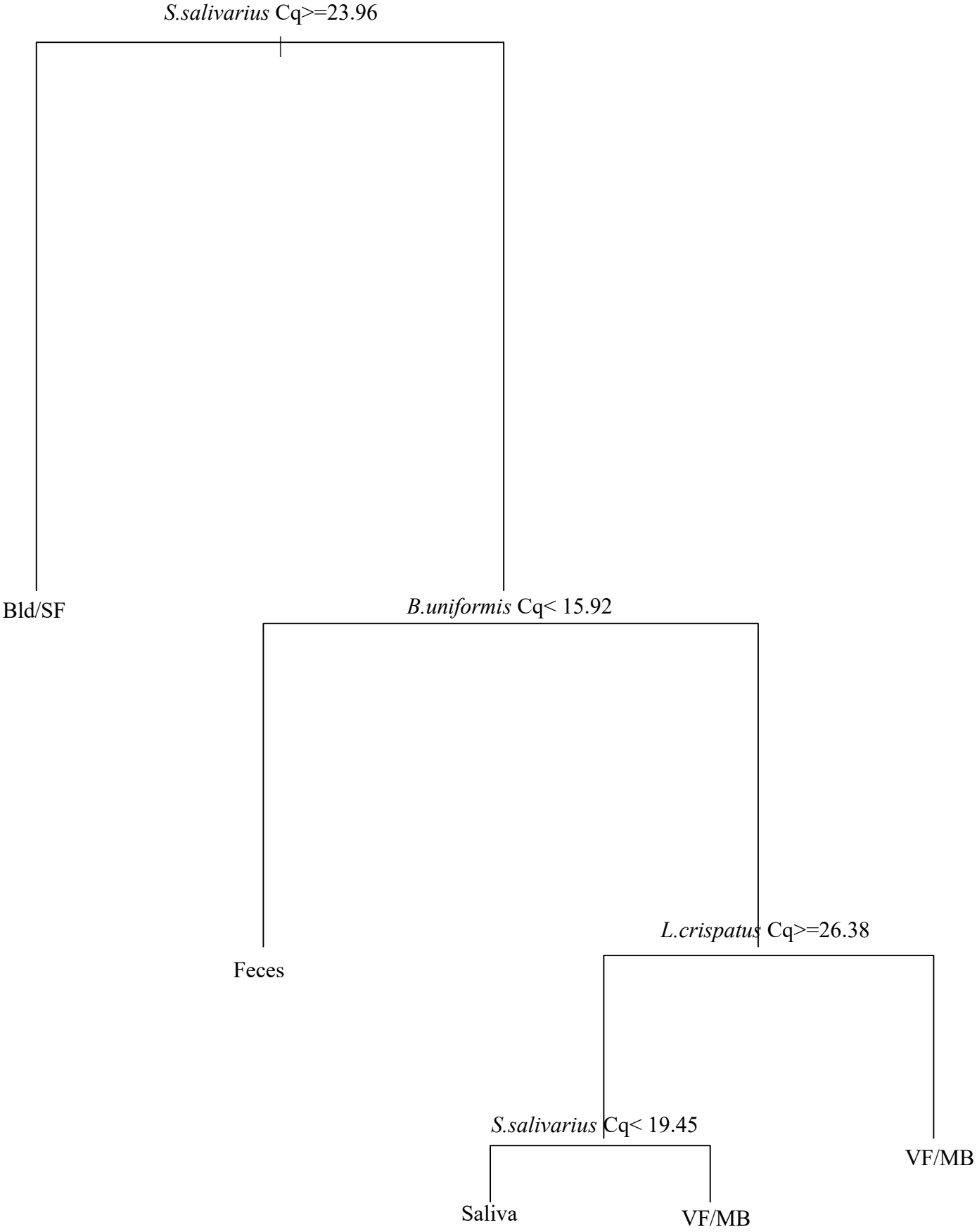

Figure 3.2—Classification Regression Tree (CART) model for a microbial 16S qPCR triplex when vaginal and menstrual secretions are grouped as female intimate samples (n=30 donors of each body fluid, Bld/SF=blood/seminal fluid, VF/MB=vaginal fluid/menstrual blood).

Table 3.5—Confusion matrix classifying body fluids using the 16S triplex in a trained Classification Regression Tree (CART) model with VF and MB as separate fluids. An 84% overall classification rate was achieved when analyzing vaginal and menstrual secretions as individual body fluids. Bold numbers indicate correct classifications (Bld/SF=blood/seminal fluid, VF=vaginal fluid, MB=menstrual blood).

		Predicted				
		Bld/SF	Feces	MB	Saliva	VF
Actual	Bld/SF	**19**	0	0	0	0
	Feces	0	**9**	0	0	0
	MB	0	0	**5**	2	4
	Saliva	0	0	0	**6**	1
	VF	0	0	1	1	**8**

Table 3.6—Confusion matrix classifying body fluids using the 16S triplex in a trained Classification Regression Tree (CART) model with VF/MB combined. A 96.5% overall classification rate was achieved when grouping female intimate samples together (VF/MB). Bold numbers indicate correct classifications (Bld/SF=blood/seminal fluid, VF/MB=vaginal fluid/ menstrual blood).

		Predicted			
		Bld/SF	Feces	Saliva	VF/MB
Actual	Bld/SF	**23**	0	0	0
	Feces	0	**8**	0	0
	Saliva	0	0	**6**	1
	VF/MB	0	0	1	**17**

demonstrated that combining female intimate samples in a dataset can increase classification accuracy, particularly for MB samples. Furthermore, it supports the reported claim that it is difficult to differentiate vaginal fluid from menstrual blood using microbial signatures since both fluids originate from the same body cavity and can contain similar bacterial compositions at any point during the menstrual cycle.[121,123,222].

It should be kept in mind that data are reflective of 30% of the dataset and that the true sample size is larger than depicted (n=30 donors of each body fluid). Also of note is that the training and validation data were randomly split for each of the two microbial CART models; therefore, sample sizes of each body fluid group slightly differ, and reported classifications (Tables 3.5 and 3.6) are not necessarily reflective of the same body fluid samples.

Importantly, 100% of fecal samples were correctly classified regardless of VF/MB grouping., and there were no misclassifications involving blood/semen samples. Saliva misclassifications were observed in both CART models, which could be due to higher-than-expected *S. salivarius* detection in other body fluids thus negatively impacting its anticipated saliva specificity. Another possible explanation is that *S. salivarius* primers and probe were designed at the species level, and differentiation among *Streptococcus* species in saliva has been reportedly more difficult using 16S compared to other genes.[75,232] If this is true, there could be other *Streptococcus* species amplifying, which would result in lower Cq values. This could possibly explain why *S. salivarius* was detected in all donors in every body fluid sample, whereas *L. crispatus* and *B. uniformis* were undetected in at least one donor of all body fluids (Appendix 1: Table 1). These results are contrary to what has been reported in the literature, which state that *S. salivarius* was not detected in other body fluids; however, one group did not examine feces,[102] and different methodologies were used, such as LAMP, RT-LAMP, or direct

PCR combined with immunochromatographic strip.[102,106,233] Importantly, none of these studies amplified 16S, which supports the previous statement that the 16S rRNA gene may not be the best target for saliva identification, especially at the species taxonomic level. Since saliva is commonly present on crime scene evidence, replacing and/or incorporating additional saliva markers into the proposed qPCR multiplex may be useful for forensic casework implementation.

Linear Range of Classification

The goal of this study was to determine at which ten-fold dilution of DNA extract the body fluid will classify correctly using the grouped female intimate CART model. The three microbial targets were undetectable in all blood and semen dilutions and only sometimes were detected in DNA extracts. All saliva samples could only be accurately classified when the DNA extract was input into the qPCR reaction (Table 3.7). The lowest DNA concentrations quantified via UV-spectrophotometry were observed in blood, semen, and saliva compared to vaginal fluid, menstrual blood, and feces (Figure 3.3); therefore, it was expected that the ten-fold dilutions of DNA extracts from these fluids would result be undetectable and thus yield inaccurate classifications. It should be noted that, unless otherwise stated, any dilution that was correctly classified was also correctly classified in the DNA extract; for example, vaginal fluid and feces samples were correctly classified in DNA extracts of all five donors but only in the first dilution (D1) of three donors (Table 3.7).

There were no fluids that classified correctly beyond the second dilution (D2), except for MB, which was still accurately classified in the second dilution for four out of five donors (Table 3.7). There was one MB sample that could only be classified in the DNA extract; however, *L. crispatus* was never detected in that individual throughout the project. This could be due to a lower abundance (or absence) of that *Lactobacillus* species in the vaginal microbiome of that

Table 3.7—Linear range of classification for the 16S triplex demonstrating at which DNA dilution the body fluid remained correctly classified. Five donors of each were serially diluted ten-fold based on total DNA concentration obtained via UV-spectrophotometry and evaluated using the CART model with female intimate samples grouped together (VF=vaginal fluid, MB=menstrual blood, D=dilution).

Body Fluid (n=5 donors)

Dilution	Blood	Semen	Saliva	Feces	VF	MB
Extract	5/5	5/5	5/5	5/5	4/5	5/5
D1	0	0	0	3/5	3/5	4/5
D2	0	0	0	0	0	4/5

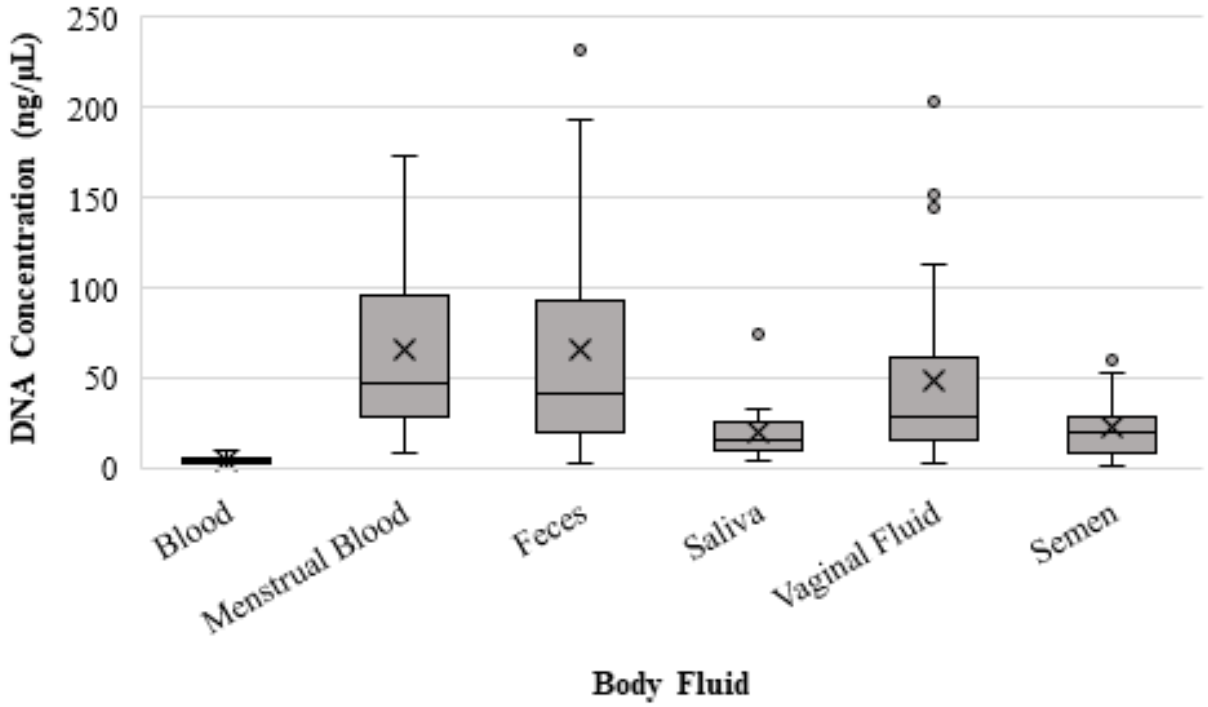

Figure 3.3—Quantification of total DNA from blood, menstrual blood, feces, saliva, vaginal fluid, and semen. DNA concentrations obtained via UV-spectrophotometry were higher and most variable in fluids with an abundance of bacterial DNA, while blood, semen and saliva yielded relatively low DNA concentrations (n=30 donors for blood, menstrual blood, saliva, semen; n=29 for vaginal fluid and n=26 for feces).

particular donor, which could be a result from a clinical infection, such as BV. Alternatively, that person may naturally have decreased *L. crispatus* abundance during their menstrual cycle. These are reasons why large population sizes of various demographics should be evaluated for any microbial assay so that inter- and intra- person variability can be assessed and so that false positive/negative rates can be described.

An interesting observation in this study was that in two vaginal samples, *L. crispatus* was undetected in the DNA extract yet was detected in subsequent dilutions, which supports the previous hypothesis that inputting too much DNA template into the reaction may lead to PCR inhibition. This only affected classification accuracy in one of the VF samples, where classification was only achieved in the first dilution but not in the DNA extract. Of note, the proposed CART analysis method evaluates raw Cq values, so it was expected that dilution of a DNA extract will equally dilute all DNA in the sample; therefore, a different analysis method or data normalization may be useful for compromised evidence samples.

CONCLUSIONS

In this study, a microbial 16S qPCR multiplex was proposed, which can classify vaginal fluid, menstrual blood, feces, and saliva with 84% overall accuracy through classification regression tree analysis. When female intimate samples were grouped together in the CART model, overall classification accuracy increased to 96.5%. All observed misclassifications involved saliva samples, which indicates that different saliva-specific markers or redesign of *Streptococcus* primers may increase the assay's ability to correctly classify saliva. Importantly, high accuracy was achieved using forensically relevant dried samples of appropriate volumes that had been stored at room temperature for an extended period of time, which suggest that bacterial signatures remain stable enough to characterize dried body fluids. The method does not

require additional steps in the current DNA workflow and consumes minimal volume of DNA extract, which would allow for easy implementation to forensic laboratories.

The incorporation of additional molecular markers into the assay or analysis method could allow for differentiation among blood and semen, which is essential for forensic applications since they are two of the most common fluids found on crime scene evidence. Biomarkers for other forensically relevant fluids, such as skin or sweat, could also be incorporated. Future research should address body fluid mixtures, compromised or degraded samples, differences in microbial signatures across and within populations and individuals, and other forensic considerations, such as antibiotic usage.

CHAPTER 4:

A combined molecular approach utilizing microbial DNA and microRNAs in a qPCR multiplex for the classification of five forensically relevant body fluids

INTRODUCTION

Forensic body fluid identification (BFID) is an essential step of the DNA analysis workflow that can assist in criminal investigations or story corroborations. Most of the current serological tests lack the sensitivity and specificity that newer techniques could provide; therefore, many researchers have proposed molecular methods for identifying body fluids, including RNA-based approaches, proteomic analyses, DNA methylation patterns, and the use of body fluid-specific microbial signatures.[4,16]

Microbial forensics has been applied to bioterrorism and infectious agent detection for years;[84] however, the advancement of sequencing technologies in the last 15 years has expanded its use to human identification,[89] body fluid identification,[70,90] and postmortem interval estimation.[91] Numerous studies address microbiome based BFID, but the most successful microbiome qPCR studies for BFID address fluids with high abundances of microbial DNA, such as vaginal fluid, feces, and saliva,[75,81,90,103,107,118,120,123,223,224] while venous blood, semen, and urine are harder to identify because low bacterial cell counts often result in poor DNA yields.[90] In Chapter 3, a 16S qPCR triplex that amplifies the 16S rRNA gene of *L. crispatus*, *B. uniformis*, and *S. salivarius* to classify vaginal/menstrual secretions, feces, and saliva, respectively, was developed and evaluated. In this study, the goal was to incorporate additional molecular markers into the analysis that could differentiate between blood and semen since they are two of the most common fluids present on forensic evidence.

microRNAs (miRNAs) are short sequences that assist in cell regulatory processes by binding the 3' untranslated region of target messenger RNA to either signal for messenger RNA degradation or to repress translation.[145,152,169] This biological function allows for consistent expression in all human tissues and for tissue-specificity, as regulatory processes differ between

tissues.[146,152,153] Their short length and encapsulation in the Argonaute complex or lipid bilayer vesicles contribute to their stability under compromising conditions,[234] which has been confirmed in multiple forensic reports.[190,192,193,235] Additionally, it has been demonstrated that miRNAs co-extract with DNA using a wide variety of nucleic acid isolation methods, including commercially available forensic DNA extraction kits.[204–207,211,214,236] For these reasons, there have been many reports addressing the use of miRNAs for forensic BFID; however, lack of consensus on markers and the use of different platforms, data analyses, and chemistries have hindered implementation of any miRNA assay into casework in the United States.[73,74,76,161,170,177,202,219,237]

With advantages and disadvantages well-described for each methodology, it has been proposed that an integrated molecular approach would be best suited for a comprehensive BFID assay. To our knowledge, there are only three reported integrated molecular approaches for forensic BFID; Choi et al. uses DNA methylation patterns and microbial DNA to identify four types of body fluids,[123] while Fleming et al. and Jakubowska et al. used mRNA and microbial markers for vaginal fluid identification.[103,124] There has never been a combined molecular approach utilizing microRNA and microbial DNA sequences in a qPCR assay to identify forensically relevant body fluids. Therefore, we propose in this study the expansion of the previously reported microbial 16S triplex to incorporate a miRNA reverse transcription-qPCR (RT-qPCR) duplex that can now differentiate between vaginal/menstrual secretions, feces, saliva, blood, and semen at the quantification step of the forensic DNA workflow.

MATERIALS AND METHODS

Primer and Probe Design

Since commercially available reverse transcription (RT) kits contain proprietary primer sequences, a stem-loop RT primer needed to be designed for reverse transcription to occur. Primers were designed for let-7g and miR-891a according to the method reported by Kramer 2011.[186] Hydrolysis probes were designed following the same method; however, locked nucleic acid (LNA®) probes were used in place of minor groove binder (MGB) probes to increase the melting temperature (T_m) near the recommended 70°C. This decision was based on the limitation of MGB probes to multiplex more than two molecules in qPCR reaction (Thermo Fisher Scientific, personal communication, November 20, 2018), and LNA bases allow for more flexibility for short probe sequences due to their ability to increase T_m of the probe by >15°C.[238]

Stem-loop RT and qPCR primers were purchased from IDT as HPLC-purified DNA oligos normalized to 100 µM in TE buffer. HPLC-purified Affinity Plus™ qPCR probes (IDT) were normalized to 100 µM in TE and aliquoted to prevent freeze/thaw degradation. All qPCR primers and probes were stored at -20°C. In accordance with MIQE guidelines for qPCR,[231] sequence information for all primers, probes and miRNA targets are shown in Table 4.1.

The RT primers require a one-time folding step upon arrival to form the stem-loop structure. The folding protocol reported by Kramer 2011[186] was performed on the Applied Biosystems™ Proflex™ PCR System (Thermo Fisher Scientific) by transferring the entire volume to a PCR tube and incubating at 95°C for 10 min. The temperature was slowly reduced to 75°C (ramp rate = 0.1°C/sec) and held at 75°C, 68°C, 65°C, and 62°C for 1 hour each with a final hold at 60°C for 6 hours (instrument default ramp rates). The folded RT primer was

Table 4.1—Reverse transcription (RT) and qPCR primer sequence information for the microRNA targets included in the RT-qPCR duplex. HPLC-purified hydrolysis probes include 5' reporters and 3' quenchers ('+' = LNA® base).

miRNA Target & [miRBase Accession ID]	**Stem-loop RT primer** 5' – 3'	**Forward Primer** 5' – 3'	**Reverse Primer** 5' – 3'	**Hydrolysis Probe** 5' – 3'
hsa-let-7g-5p [MIMAT0000414]	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAACTGT	CACGCATGAGGTAGTAGTTT	CCAGTGCAGGGTCCGAGGTA	6FAM/TGGATA+C+GA+CTGTA/3IABkFQ
hsa-miR-891a-5p [MIMAT0004902]	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCAGTG	AGTTAATGCAACGAACCT	CCAGTGCAGGGTCCGAGGTA	HEX/ATACGA+CT+CA+GT+GGCT/3IABkFQ

aliquoted, diluted to working stock concentrations of 1 µM, 500 nM, and 250 nM, and stored at -20°C until reverse transcription.

Sample Preparation and Nucleic Acid Isolation

Synthetic miRNA sequences were used as positive controls or copy number standards during assay optimization. RNase-free HPLC-purified RNA oligos for let-7g (5'-/5Phos/rUGAGGrUAGrUAGrUrUrUGrUACAGrU-3') and miR-891a (5'-/5Phos/rUGCAACGAACCrUGAGCCACrUGA-3') were purchased from IDT and resuspended at 100 µM in nuclease-free water. The number of molecules per microliter (copies/µL) in the 100 µM stock were calculated using the copy number calculator tool (www.scienceprimer.com) based on delivered amount and molecular weight of the RNA oligo. Ten-fold serial dilutions (10^9 – 10^4 copies/µL) were prepared in nuclease-free water, aliquoted, and stored at -80°C.

The same DNA extracts from the previous 16S triplex validation from Chapter 3 were evaluated using the miRNA duplex. Briefly, samples were collected from 30 volunteers according to VCU's IRB approved protocol for human subject research (HM20002931). Menstrual blood, feces, saliva, and vaginal fluid were collected by the donor onto polyester swabs, while semen was collected in sterile plastic containers and aliquoted onto polyester swabs (50 µL). Peripheral blood was deposited onto polyester swabs by pricking the donor's sterilized finger with a finger lancet. Swabs were dried and placed in swab boxes, which were stored at room temperature for at least one year until DNA extraction was performed using the QIAamp® DNA Investigator Kit (refer to Chapter 3 for detailed sample selection and DNA extraction methods).

Body Fluid Mixture Sample Collection and Preparation

Forensically relevant two-source body fluid mixtures were prepared by depositing 50 µL of liquid blood, saliva and/or semen onto dried swabs of feces, menstrual blood, or vaginal fluid. Feces, menstrual blood, and vaginal fluid of the same donor were collected on polyester swabs and allowed to dry at room temperature for at least 24 hours. Liquid blood, semen, and saliva were collected according to the reported methods in Chapter 2. Each was aliquoted onto one or more of the swabs containing one of the dried fluids, depending on the mixture type. Single-source samples from the mixture sample donors were also collected and evaluated for comparison purposes. Whole swabs were used for DNA extraction as described above, and the same RT-qPCR methods mentioned below were performed. Notably, the mixture samples were anonymized prior to DNA extraction and RT-qPCR to avoid any potential bias during classification analysis.

Reverse Transcription

Reverse transcription was performed using the TaqMan™ MicroRNA Reverse Transcription Kit (Thermo Fisher Scientific). Each RT reaction included 0.15 µL of 100 mM dNTPs, 1 µL of MultiScribe™ Reverse Transcriptase (50 U/µL), 1.5 µL of 10X Reverse Transcription Buffer, and 0.19 µL of RNase Inhibitor (20 U/µL). For copy number standards, 8.16 µL of nuclease-free water and 1 µL synthetic RNA were added. For body fluid samples, 4.16 µL of nuclease-free water and 5 µL of DNA extract were added. For single-plex RT reactions, 3 µL of 250 nM RT primer was added for a final RT primer concentration of 50 nM and total reaction volume of 15 µL. For duplex RT reactions with synthetic RNA, the volume of nuclease-free water was adjusted to 7.16 µL to account for 1 µL input of each synthetic sequence, and 1.5 µL of each RT primer (500 nM) was added to bring the final reaction volume

to 15 µL. The manufacturer's protocol—16°C for 30 min, 42°C for 30 min and 85°C for 5 min—was performed on the Proflex™ PCR System, and cDNA was stored at -20°C until qPCR. To avoid freeze/thaw degradation, qPCR was performed immediately after reverse transcription, when possible. Two negative controls were included in each RT batch—one with RT primer but no RNA and one without RT primer or RNA added. The commercially available let-7g assay for the RT kit (Thermo Fisher Scientific, Cat. No. 4427975, Assay ID No. 002282) was initially tested as a positive control and to compare standard curve data.

qPCR

The qPCR reaction was first optimized using gBlocks® Gene Fragments (IDT). The gBlocks® were designed to serve as positive controls for the qPCR rection, so the sequences include the second strand cDNA product from the RT reaction with extra nucleotides on the 5' and 3' ends (Table 4.2). Each gBlock® was resuspended in 25 µL at 10 ng/µL in TE, incubated at 50°C for 20 min, and diluted to the optimized copy number range (10^9 – 10^4 copies/µL) in nuclease-free water. Aliquots were prepared and stored at -20°C.

The optimized qPCR primer and probe concentrations for let-7g and miR-891a were 4/4/0.8 µM and 1.25/1.25/0.25 µM, respectively (forward/reverse/probe). Primer and probe assay mixes for each miRNA target were prepared at the optimized concentrations immediately prior to setting up qPCR. Single-plex reactions for let-7g included 5 µL of PrimeTime® Gene Expression Master Mix (2X) (IDT), 1 µL of 10X assay mix, 2 µL of nuclease-free water and 2 µL of sample for a 10 µL total reaction volume. For miR-891a single-plex reactions, 5 µL of PrimeTime® Gene Expression Master Mix, 0.5 µL of 20X assay mix, 2.5 µL of nuclease-free water and 2 µL of sample were added for a 10 µL reaction volume. Duplex reactions consisted of 5 µL of PrimeTime® Gene Expression Master Mix, 1 µL of let-7g 10X assay mix, 0.5 µL of

Table 4.2—gBlock® Gene Fragment sequences for the miRNA assay validation. Each qPCR standard contains the second strand cDNA sequence of the corresponding miRNA for qPCR optimization.

miRNA Target	**gBlock® Gene Fragment Sequence** 5' – 3'
let-7g	ATCGGATCGAATTCGATATGCGTATAGCCACGCATGAGGTAGTAGTTTGT ACAGTTGTCGTATCCAGTGCGAATACCTCGGACCCTGCACTGGATGGATT CCAGCTACGTAAGTTCCATATACGGAACT
miR-891a	CTGAACTGTCAGATAGCCAGGATGTCATTGCTATGTGCAACGAACCTGA GCCACTGAGTCGTATCCAGTGCGAATACCTCGGACCCTGCACTGGCCTCA GTAGACTGTGACCGACTGTTAGAATA

miR-891a 20X assay mix, 1.5 µL of nuclease-free water and 2 µL of cDNA for a 10 µL total reaction volume.

Thermal cycling was performed on the Applied Biosystems™ QuantStudio™ 6 Flex Real-Time PCR System (Thermo Fisher Scientific) in fast cycling mode with the following parameters—95°C for 3 min followed by 40 cycles of 95°C for 5 sec and 60°C for 30 sec. All standards (synthetic cDNA or gBlocks®) were run in duplicate with NTCs on each plate, while body fluid samples were run in single technical replicates to conserve cDNA and qPCR reagents.

Data Analysis

Raw qPCR data were analyzed in QuantStudio™ Software v1.3 (Thermo Fisher Scientific) with auto-baseline and cycle threshold of 0.04 set for both miRNA targets. Standard curve metrics of the synthetic cDNA standards, such as slope, Y-intercept, and PCR efficiency were analyzed during assay validation, which was complete when the single-plex and duplex quantification cycle (Cq) values for all standards were within one cycle of one another. Data were exported into Microsoft Excel to calculate averages of Cq values between technical replicates and to calculate miR-891a differential expression ($\Delta Cq = Cq_{miR\text{-}891a} - Cq_{let\text{-}7g}$). A value of 40 was used in place of any "undetermined" value when calculating differential expression, and any outliers greater than three standard deviations from the mean were omitted prior to further analysis. JMP® v14.2.0 was used to analyze data distributions and subsequently perform a non-parametric Steel-Dwass test on the ΔCq data (α=0.05). The miR-891a ΔCq values were added to the previously reported 16S triplex dataset (Appendix 1: Table 1), and all data were randomly split into training and validation sets (70% and 30%, respectively) using the sample() function. A new classification regression tree (CART) model was generated using the rpart package in R version 4.1.1. The tree plot, confusion matrix, and accuracy percentage are reported

for one iteration of the validation dataset, and all data are reported as averages with standard deviations, when applicable.

Limit of Detection and Classification

The goal of this portion of the study was to evaluate the lower limit at which blood and semen samples still exhibit the same body fluid-specific differential expression patterns. Since specific miRNA concentration in a DNA extract would be unknown in a forensic sample, we evaluated the limit of detection by decreasing the volume of extract input into the RT reaction. Five donors of each body fluid were evaluated, in which RT reactions were set up as described above but with four input volumes: 5 µL, 2.5 µL, 1.25 µL, and 0.625 µL. All reactions were brought to final volume of 15 µL with nuclease-free water, and RT-qPCR was performed as described above.

RESULTS AND DISCUSSION

RT-qPCR Assay Validation

The selection of miR-891a and let-7g for the combinatorial assay was based on previously reported data from our laboratory that showed significant differences in miR-891a ΔCq values between blood and semen.[76] Additionally, miR-891a has been confirmed in the literature as a semen-specific miRNA marker;[74,163,166,171,201] therefore, we were confident that we could achieve differentiation without an additional blood-specific marker. If accurate identification of blood and semen could still be achieved without a blood-specific marker, it would greatly reduce the overall cost of the assay—an important consideration in terms of forensic implementation.

qPCR Primer and Probe Optimization

Initially, let-7g was evaluated as a single-plex assay to ensure that amplification of the normalization marker was possible using the chosen primer design method. Initial amplification attempts were unsuccessful (data not shown); however, results were obtained results increasing the let-7g final primer/probe concentrations to 4/4/0.8 µM from those reported by Kramer 2011.[186] Additionally, it was determined that increasing the final RT primer concentration to 50 nM, originally reported by Chen et al.,[184] was the most effective and was used throughout the remainder of the study. Once the let-7g qPCR assay was optimized, the same reaction conditions were used to evaluate miR-891a as a single-plex and found that 1.25/1.25/0.25 µM were optimal qPCR concentrations for miR-891a. Both miRNA primer sets were evaluated with these reduced concentrations for multiple reasons: the qPCR master mix manufacturer's recommended upper limit of final primer and probe concentrations (1000 nM and 250 nM, respectively),[239] the recommendation to use a 5:1 primer:probe ratio (Personal communication, IDT, March 16, 2021), and most importantly, to significantly reduce reagent cost/consumption. While miR-891a Cq values were not impacted, lowering concentrations for let-7g delayed amplification around three cycles for all standards (data not shown); therefore, only miR-891a qPCR concentrations were reduced in the final assay. All negative controls (extraction reagent blanks, RT-RBs, NTCs) performed as expected using the optimized RT-qPCR assay (Cq > 38).

Optimization of the Duplex RT Reaction

The average slopes and Y-intercepts of let-7g standard curves were greater than miR-891a, which resulted in lower amplification efficiencies for let-7g (Table 4.3). Although the PCR efficiency of let-7g is lower than the 90-105% recommended range for qPCR assay validation,[231] similar and/or lower percent efficiencies were observed when testing a commercially available

Table 4.3—Standard curve metrics of synthetic let-7g and miR-891a in a microRNA RT-qPCR duplex. Averages and standard deviations are representative of six standards across eight experiments. Two duplex RT batches were each run four to five separate qPCR plates.

microRNA	Slope	Y-intercept	R^2	% Efficiency
let-7g	-3.88 ± 0.07	51.92 ± 0.54	0.996 ± 0.004	81.03 ± 0.02
miR-891a	-3.48 ± 0.04	42.78 ± 0.71	0.999 ± 0.000	93.84 ± 0.02

let-7g assay. Furthermore, previous work in our laboratory involving synthetic miRNA standard curves resulted in PCR efficiencies between 70-85% using commercially validated RT kits and miRNA primer sets (data not shown).

When developing a qPCR multiplex, it is important to ensure that there is no preferential amplification of a particular target. In practice, this means that Cq values for all targets would be similar for each standard. Throughout our assay validation, we consistently observed lower Cq values with synthetic miR-891a standards compared to let-7g (Table 4.4); however, similar Cq values between let-7g and miR-891a were observed when using gBlocks® as standards (data not shown), which indicated that the let-7g RT reaction is less efficient than miR-891a. Since RT efficiency is difficult to measure and varies for each target miRNA using the stem-loop primer method,[162,240–242] we evaluated various RT primer concentrations but found no significant difference in resulting Cq values (data not shown). Nonetheless, RT and qPCR reproducibility and repeatability were demonstrated across eight experiments—two RT batches each run on four or five separate plates (Table 4.4); therefore, we felt confident in moving forward with body fluid testing.

Body Fluid Specificity of the Combinatorial RT-qPCR Assay

When comparing raw Cq values in 30 donors of each body fluid, vaginal fluid and menstrual blood were the most variable for both miRNA targets (Appendix 1: Table 1), which resulted in large variation of miR-891a ΔCq values. Subsequently, a Steel-Dwass non-parametric test on the differential expression data was performed and determined that all body fluids were significantly different from one another except for vaginal fluid and menstrual blood (Figure 4.1). This was not surprising based on the findings from Chapter 3 as well as other reports that claim difficulty differentiating between menstrual and vaginal secretions using microbial

Table 4.4—Average cycle threshold (Cq) values and standard curve metrics of synthetic miRNA copy number standards when evaluated using the developed RT-qPCR duplex for let-7g and miR-891a (shaded colors coordinate the two RT batches, PX=plate number, Conc.=copy number concentration of the synthetic sequence).

	let-7g								miR-891a								
	50 nM RT-1				50 nM RT-2				50 nM RT-1					50 nM RT-2			
Conc.	4/4/0.8 µM				4/4/0.8 µM				1.25/1.25/0.25 µM					1.25/1.25/0.25 µM			
(copies/uL)	P1	P2	P3	P4	P1	P2	P3	P4	P1	P2	P3	P4	P5	P1	P2	P3	P4
10^9	17.846	17.26	16.829	16.83	16.583	16.453	16.469	16.544	12.803	13.397	11.480	11.737	11.457	10.827	10.550	10.648	10.460
10^8	21.672	20.992	20.524	20.523	20.830	20.576	20.641	20.871	16.293	16.703	14.900	15.080	14.768	14.723	14.348	14.554	14.292
10^7	25.506	24.786	24.398	24.425	24.763	24.577	24.429	24.762	19.624	20.012	18.244	18.388	18.067	18.327	17.967	18.102	17.911
10^6	29.396	28.786	28.508	28.514	29.180	29.113	29.038	29.164	23.058	23.535	21.788	21.886	21.556	21.399	21.130	21.195	20.985
10^5	33.378	32.99	32.534	32.721	32.829	32.930	32.829	33.091	26.931	27.351	25.525	25.719	25.347	25.161	24.828	24.929	24.727
10^4	36.8	37.099	36.2	36.261	34.874	35.275	35.272	35.547	29.889	30.288	28.612	28.882	28.321	28.530	28.320	28.403	28.182
Slope	-3.822	-3.977	-3.914	-3.938	-3.768	-3.877	-3.862	-3.888	-3.451	-3.426	-3.459	-3.461	-3.416	-3.511	-3.527	-3.514	-3.514
Y-intercept	52.278	52.835	51.94	52.144	51.000	51.690	51.553	51.935	43.864	44.153	42.578	42.780	42.120	42.652	42.450	42.479	42.268
R^2	0.999	0.999	1	0.999	0.990	0.993	0.994	0.994	0.999	0.999	1.000	0.999	0.999	0.999	0.999	0.999	0.999
Efficiency	82.65%	78.42%	80.09%	79.44%	84.25%	81.10%	81.51%	80.80%	94.89%	95.82%	94.57%	94.50%	96.24%	92.66%	92.10%	92.56%	92.56%

RT batch 1
RT batch 2
P=plate #

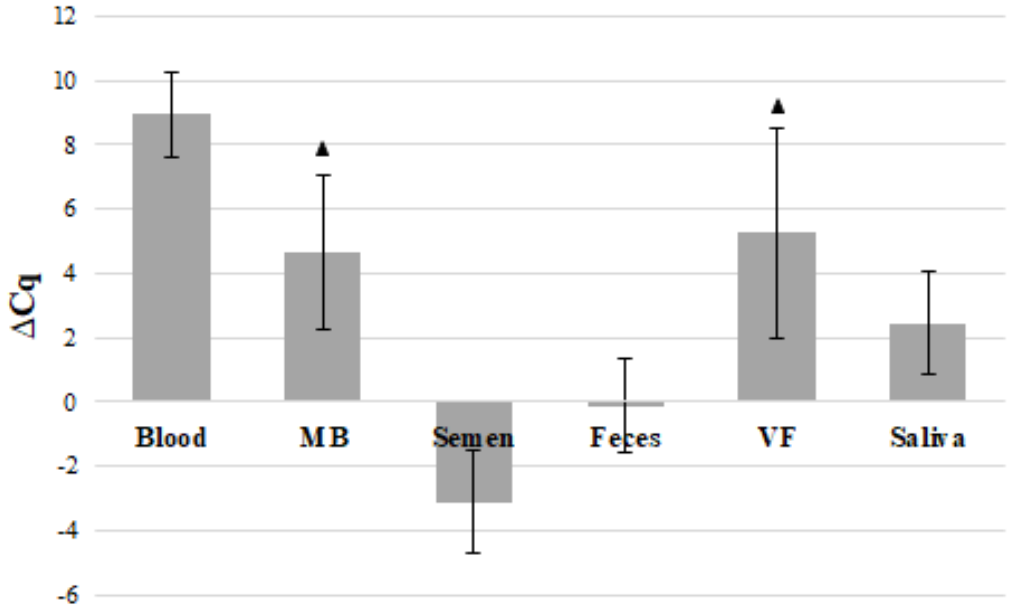

Figure 4.1—Differential expression (ΔCq) of miR-891a in forensically relevant body fluids using the proposed miRNA RT-qPCR duplex (ΔCq = $Cq_{miR\text{-}891a} - Cq_{let\text{-}7g}$). Blood, semen, feces, and saliva all had statistically different ΔCq values from one another using a non-parametric Steel-Dwass method ($p<0.05$). Conversely, there was no statistical difference between vaginal fluid (VF) and menstrual blood (MB), indicated by the shared symbol "▲" (n=30 donors of each body fluid, error bars represent 1 SD from the mean).

DNA.[81,103,121,123] Figure 4.1 visually demonstrates the significant difference of miR-891a ΔCq values between 30 donors each of blood and semen. The negative miR-891a ΔCq values in semen and large positive ΔCq values in blood are similar to what we had previously reported, even when using different RT-qPCR chemistries.[76]

It should be remembered that the main goal of this study was to incorporate miRNA markers the previous microbial 16S triplex CART model (Figure 3.2) that could differentiate between blood and semen. The new CART model that included miR-891a ΔCq values (Figure 4.2) correctly classified all VF/MB samples and 100% of blood and semen samples as individual fluids (Table. 4.5). Although the overall classification accuracy rate for the combinatorial CART model was slightly reduced from that of the 16S triplex model (96.5% to 94.6%, respectively), the combinatorial analysis method has a major advantage of discriminating blood from semen—two of the most common fluids observed in forensic casework. Importantly, the misclassification rate was low (5.4%) and only due to three saliva samples inaccurately classifying as VF/MB (Table 4.5). Misclassifications of saliva and VF/MB as one another were also observed when the previous 16S triplex model was evaluated (Table 3.6), which further supports the claim that an additional saliva marker should be considered to improve classification accuracy of saliva samples. An important note in terms of forensic potential is that significantly different miR-891a ΔCq values between semen and VF/MB were observed, which could be valuable information in sexual assault cases.

Evaluation of Body Fluid Mixtures

Ten forensically relevant two-source body fluid mixtures were prepared, extracted, and evaluated following the methods described above. Unfortunately, the statistical CART model can only result in a single categorical outcome and thus would not be capable of predicting both

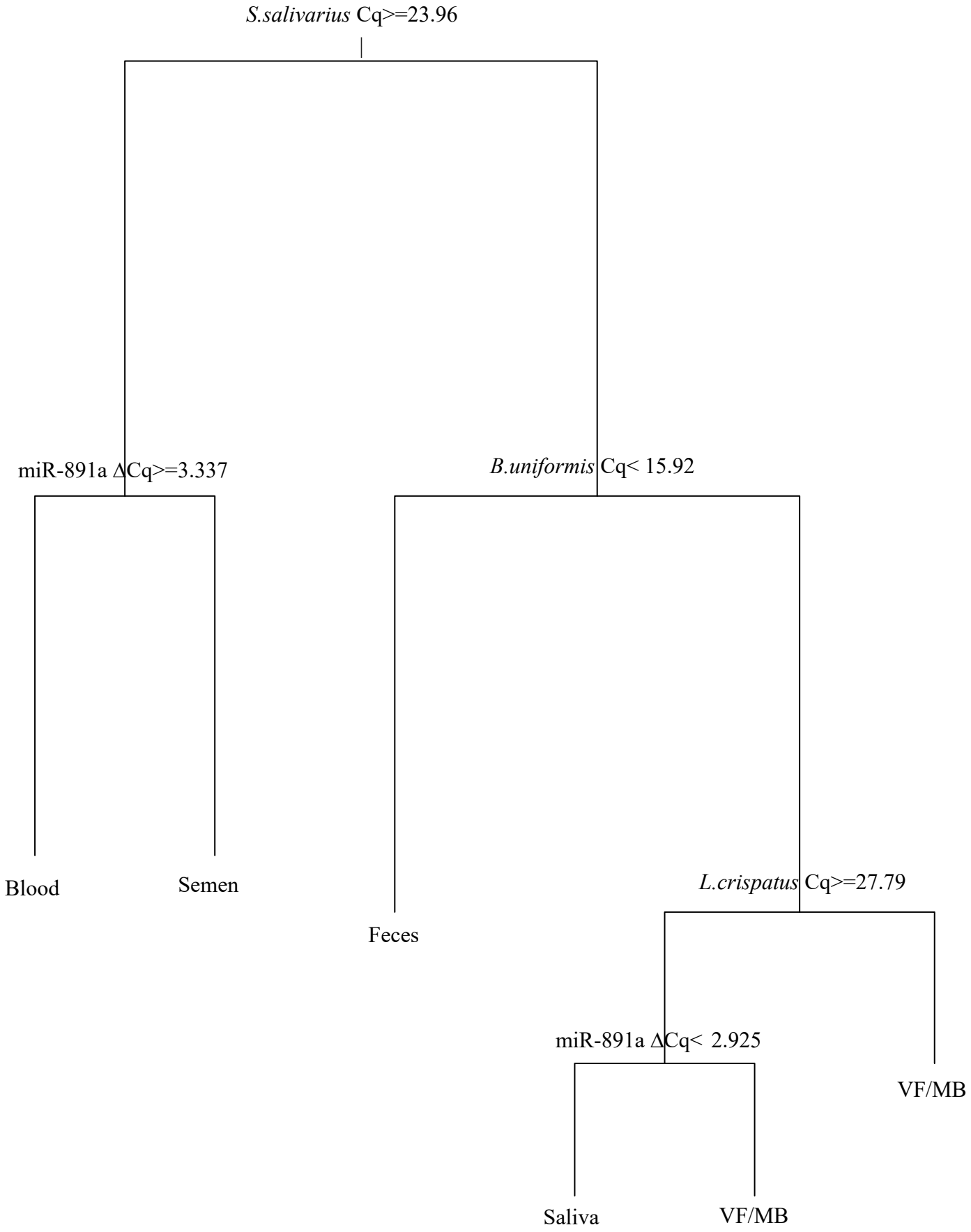

Figure 4.2—Classification Regression Tree (CART) model for a combinatorial RT-qPCR assay utilizing microbial DNA and microRNAs (n=30 donors of each body fluid, VF/MB=vaginal fluid/menstrual blood, $\Delta Cq = Cq_{miR\text{-}891a} - Cq_{let\text{-}7g}$).

Table 4.5—Confusion matrix for classifying body fluids using a combinatorial RT-qPCR assay. A trained Classification Regression Tree (CART) model resulted in 94.6% overall classification accuracy for blood, semen, saliva, and vaginal fluid/menstrual blood (VF/MB). Bold numbers indicate correct classifications.

		Predicted				
		Blood	Feces	Saliva	Semen	VF/MB
Actual	Blood	**9**	0	0	0	0
	Feces	0	**9**	0	0	0
	Saliva	0	0	**8**	0	3
	Semen	0	0	0	**10**	0
	VF/MB	0	0	0	0	**17**

components of a mixture sample, as that would require multiple categorical outcomes. Even with this knowledge, body fluid mixtures were analyzed manually (not in R) to determine if one (if any) of the two fluids could be predicted using the combinatorial assay CART model.

One of the fluids was accurately predicted in eight out of ten (80%) of the mixture samples (Table 4.6). It was observed that any mixture containing saliva was classified as feces, including saliva mixed with blood, VF, semen, and feces. This supports the previous claim that an additional saliva-specific marker may be useful. Slightly lower *B. uniformis* Cq values in the single-source samples were observed compared to the original population data, which would have consequently affected the mixture results. However, it should be noted that these mixtures were freshly collected and prepared rather than being stored for the same amount of time as the population samples that the CART model was trained with. It should be additionally noted that most of the mixtures were prepared using multiple fluids of the same donor; therefore, future work should include a much larger sample size containing multiple donors of each fluid mixed at various ratios that have been stored for different amounts of time.

An interesting trend that we observed was that any negative ΔCq resulted from a mixture containing semen or feces, which is concordant with the single source population data from Figure 4.1. Furthermore, two mixtures with neat semen (VF/semen and saliva/semen) had the lowest ΔCq values around -4.5, while the 1:20 diluted semen/VF mixture ΔCq was slightly higher at -1.172 (Table 4.6). Conversely, all blood mixtures had positive ΔCq values, even when mixed with neat semen, which indicates that the differential expression is reflective of each mixture component. It should be noted that the body fluid predictions did not change when evaluating mixtures with and without ΔCq incorporated into the classification model; therefore, analyzing the differential expression of miR-891a alongside the 16S triplex model, rather than

Table 4.6—Analysis of forensically relevant body fluid mixtures using the CART model developed for the combinatorial RT-qPCR assay. Cycle threshold (Cq) and miR-891a ΔCq values obtained from each mixture are reported with the respective body fluid prediction. Any undetermined value was manually replaced with a value of 40. The bold* indicates which of the two body fluids was classified correctly using the CART model ($\Delta Cq = Cq_{miR\text{-}891a} - Cq_{let\text{-}7g}$, VF=vaginal fluid, MB=menstrual blood).

Body Fluid Mixture	*S. salivarius* Cq	*L. crispatus* Cq	*B. uniformis* Cq	miR-891a ΔCq	Body Fluid Prediction
Blood/Saliva	14.641	40	13.772	4.109	Feces
VF*/Blood	13.010	16.160	28.681	5.516	VF/MB
VF*/Saliva	11.321	13.866	13.279	1.712	Feces
Feces*/Blood	16.451	28.442	10.989	3.315	Feces
Feces*/Saliva	14.500	40	10.548	-2.910	Feces
Blood/**Semen***	27.869	35.535	30.724	1.489	Semen
VF*/Neat Semen	12.166	14.939	32.561	-4.845	VF/MB
Semen/Saliva	15.538	40	13.917	-4.363	Feces
VF*/1:20 Semen	12.196	15.269	26.129	-1.172	VF/MB
MB*/Semen	14.225	14.851	37.550	-0.495	VF/MB

incorporated into the model, may provide more useful information for predicting mixtures. As previously mentioned, CART analysis only results in a single categorical outcome; therefore, other data analysis methods could be evaluated, including, quadratic discriminant analysis or machine learning cluster models. Additionally, a different data analysis approach that does not rely on raw qPCR data may improve classification accuracy of forensic samples with low template DNA, especially in the microbial multiplex, as miRNA data is normalized and not solely dependent on raw Cq values.

Limit of Detection and Classification

The linear dynamic range for the miRNA RT-qPCR duplex was determined to be 10^9 – 10^4 copies/µL. The lower limit of this range is comparable to what we have previously observed in commercially available RT-qPCR kits (Table 1.2). It is also similar the findings of Androvic et al.,[243] in which they report 10^3 or 10^2 copies/µL as the lower limit of detection across various RT-qPCR chemistries. It should be noted that the limit of detection refers to both miRNAs together and is limited by RT efficiency of let-7g. The lower limit of detection for miR-891a is likely a few magnitudes lower than 10^4 copies/µL based on the observed raw Cq values of the synthetic standards (Table 4.4); however, we did not test this theory as the results are dependent on the detection of let-7g as an endogenous reference gene to distinguish blood and semen from one another.

Since miRNA concentration in DNA extracts of an evidence sample would be unknown, the limit of detection (or limit of classification) was evaluated in terms of the lowest input volume of DNA extract into the RT reaction that would result in correct classification of blood and semen samples. Blood and semen are differentiated by a miR-891a ΔCq threshold based on the CART model (Figure 4.2); therefore, $\Delta Cq > 3.34$ indicates blood, while $\Delta Cq < 3.34$ indicates

semen. We found that all blood and semen samples from all five donors classified correctly using this threshold even at the lowest input volume (0.625 µL). This is beneficial in terms of sample consumption when considering forensic implementation, as discrimination between blood and semen could be achieved with as little as 0.625 µL of DNA extract.

CONCLUSIONS

In this report, the first known combined molecular approach that utilizes both microbial DNA and miRNAs for forensic BFID was proposed. A miRNA RT-qPCR duplex was developed, and significant differences in differential expression between blood and semen were observed when miR-891a is normalized to let-7g. These markers were incorporated into our previously described qPCR multiplex that amplifies the 16S rRNA gene of *L. crispatus*, *S. salivarius*, and *B. uniformis*. The new CART model generated from the combinatorial RT-qPCR data could classify blood, semen, saliva, feces, and vaginal/menstrual secretions with 94.6% overall accuracy, which was achieved using a small input volume of DNA extract from forensically relevant samples that had been stored for extended time periods of up to two years.

Future research should further investigate other forensically relevant body fluid mixtures and other forensic considerations. Efforts should be made to distinguish between vaginal and menstrual secretions, as this could be important in sexual assault cases. Different statistical approaches, such as one with multiple categorical outcomes, may be more useful for forensic body fluid mixtures. Additional saliva miRNA or microbial markers may reduce saliva misclassifications, and markers for other body fluids, such as urine, sweat, or skin, could be incorporated for a full comprehensive molecular assay for forensic BFID.

CHAPTER 5:

CONCLUSIONS AND FUTURE DIRECTIONS

CONCLUSIONS

The ability to locate and identify biological fluids on evidentiary samples is an important step in forensic casework. Although traditional serological tests have been used for many years, they lack the desired specificity of a serological assay, and there is no comprehensive test capable of identifying multiple body fluids simultaneously. With flaws of current serological tests well-described in the literature, advancements in genomic technologies have allowed researchers to consider molecular-based methods for forensic BFID. Microbial DNA and microRNAs have both been individually explored in forensic research, and the benefits and limitations of each have led to the idea that combining molecular methods will increase discrimination among forensically relevant body fluids.

This dissertation is the first approach at forensic BFID that analyzes both microRNAs and microbial DNA simultaneously utilizing a dual-amplification qPCR assay. Before developing the combinatorial RT-qPCR assay, it was important to ensure that DNA extracts could be used in subsequent microRNA analysis. Detection of let-7g and let-7i were similar in all DNA isolation methods, and similar body fluid-specific differential expression patterns were observed in DNA extracts compared to those previously observed using RNA extracts. Extensive former research on miRNAs for BFID within our laboratory provided confidence that differential expression of miR-891a and miR-200b-3p were effective markers for the identification of semen and blood in the combinatorial assay, respectively. Forensic implementation was a huge consideration during the design of the combinatorial assay; therefore, we wanted highly accurate body fluid discrimination with as few markers as possible. Because let-7g and let-7i were detected at similar levels in DNA extracts in our early work, we chose to normalize miR-891a to let-7g to differentiate semen from blood. It was necessary to include an additional blood marker

(miR-200b-3p) as originally planned since the differential expression patterns of miR-891a alone were sufficient in distinguishing semen from blood.

The combinatorial assay design was ultimately split into two objectives—development of a microbial qPCR multiplex and development of miRNA RT-qPCR duplex. This was necessary since the microbial markers selected were entirely based a review of clinical microbiology and forensic literature, thus, preliminary confirmation of body fluid specificity for these markers needed to be internally evaluated. Further, the microbial primer design would be more accurate using a software tool rather than designing miRNA primers independently. Lastly, the microbial assay would not require an RT step and therefore would allow for easier optimization of the qPCR reaction before applying the same reaction conditions to the miRNA assay.

A microbial qPCR triplex first was developed to amplify the 16S rRNA genes in *L. crispatus*, *B. uniformis*, and *S. salivarius* that classified female intimate secretions, feces, saliva, and blood/semen with around 96.5% overall accuracy using a trained CART model. It is important to note that, this model was unable to appropriately distinguish between blood and samples but could accurately group them together without misclassifications as saliva, feces, or female intimate samples. When the data were modeled using separate classification categories for vaginal fluid and menstrual blood, prediction accuracies dropped to 84%, This supports the claim that distinguishing vaginal fluid and menstrual blood using microbial signatures is difficult since they originate from the same body cavity. To differentiate between blood and semen samples, a miRNA RT-qPCR duplex was validated, and the CART model was retrained with miR-891a ΔCq values incorporated. This slightly reduced the overall classification accuracy rate to 94.6%; however, the addition of miR-891a and let-7g allowed for blood and semen samples to

be individually classified. This was an important step given that they are two of the most common fluids found on evidentiary samples.

Overall, these findings demonstrate proof of concept that microbial DNA and microRNAs can be used in conjunction to differentiate between forensically relevant body fluids, and the utilization of both molecule types in one analysis method allows for a highly accurate comprehensive BFID method with higher sensitivity and specificity than current serological tests. Most importantly, the combinatorial assay was designed with the typical forensic DNA workflow in mind, using universal instrumentation for techniques that would require minimal training, to allow for easy implementation into forensic casework. Also of importance is that RNA extraction and DNase-treatment were not necessary, which would minimize sample consumption. If human autosomal and Y chromosome DNA quantification was performed on the same qPCR plate, minimal steps, such as reverse transcription and CART analysis, would be additional to the current DNA workflow. However, the CART body fluid predictions could be generated quickly if a bioinformatic web interface tool was automatically coupled to the raw and/or normalized qPCR data. In this case, the RT reaction set up, 1-hour RT protocol, and subsequent qPCR set up would be the only factors could add significant time depending on how many samples are batched; however, feces, semen, blood, saliva, and/or female intimate samples could all be identified using DNA extracts, further minimizing evidentiary sample consumption.

FUTURE DIRECTIONS

Future research should first focus on incorporating an additional (or improved) saliva marker, as each of the few observed sample misclassifications involved saliva samples. Distinguishing between menstrual and vaginal secretions—whether it is statistical differentiation or through use of another marker—should also be explored further as this distinction could

provide (single-handedly) important probative information in some sexual assault cases. Other statistical methods could be explored, such as those with multiple categorical outcomes to potentially identify more than one fluid in body fluid mixture samples. Importantly, the 16S triplex assay should be tested on the reverse transcribed body fluids to ensure the RT step would not affect the results. A small preliminary study was performed, but it should be further evaluated in an expanded formal study. With that, the limit of detection would need to be identified as it would be beneficial to obtain accurate identification of five body fluids using 0.625 µL or less of DNA extract.

Developmental validation studies outlined by SWGDAM should be performed on any method that is intended for forensic casework. Several of these have been preliminarily evaluated in this project, such as characterization of molecular targets, accuracy and precision, limit of detection, and human specificity of a medium/large sized population. A small-scale case-type mixture study was conducted; however, more in-depth research should be performed regarding body fluid mixtures, population differences, and inter- and intra- person variability, to fully validate the assay before implementation into forensic casework. The findings and conclusions of this research provide sufficient preliminary data to confirm and/or further evaluate forensic utility of the proposed combinatorial RT-qPCR for body fluid identification.

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APPENDIX 1: Raw qPCR data obtained from the combinatorial RT-qPCR assay

Table 1—Cycle threshold (Cq) and ΔCq values for each body fluid sample tested with the combinatorial RT-qPCR assay. A value of 40 indicates the target was undetermined, and the yellow highlight indicates the value was an outlier (3 standard deviations from the mean) and not included in downstream analyses (VF=vaginal fluid, MB=menstrual blood).

		Cq Values					
Body Fluid	**Sample ID**	*S.salivarius*	*L.crispatus*	*B.uniformis*	miR-891a	let-7g	**ΔCq**
Blood	B-1093	33.58	40	34.71	38.24	31.09	7.14
Blood	B-7041	29.90	40	32.65	40	30.37	9.63
Blood	B-7034	31.02	33.68	35.00	40	28.56	11.445
Blood	B-7511	34.09	36.48	40	40	30.44	9.565
Blood	B-1092	32.73	30.59	33.61	40	31.38	8.625
Blood	B-1219	30.21	26.55	38.78	39.92	28.79	11.133
Blood	B-1084	34.22	36.18	34.64	40	32.98	7.025
Blood	B-1214	40	40	40	40	36.60	3.401
Blood	B-1217	36.73	38.06	35.52	40	33.39	6.606
Blood	B-1218	40	40	40	40	31.81	8.190739
Blood	B-1221	38.57	40	40	40	31.52	8.477762
Blood	B-1026	29.33	36.35	34.52	38.89	32.24	6.641251
Blood	B-3507	24.93	34.69	29.87	40	30.13	9.867558
Blood	B-3548	29.01	40	35.91	38.52	30.92	7.594457
Blood	B-5008	29.23	36.03	32.45	40	31.88	8.124456
Blood	B-5018	33.42	40	39.41	40	30.39	9.608332
Blood	B-5056	29.84	33.60	37.59	40	30.60	9.399527
Blood	B-5122	27.53	34.84	31.92	40	30.22	9.777845
Blood	B-6036	30.58	36.07	32.92	38.30	30.91	7.389233
Blood	B-5016	33.03	40	38.10	40	31.32	8.684254
Blood	B-5019	35.43	34.79	39.95	40	31.39	8.606457
Blood	B-6046	33.74	36.66	35.22	40	30.89	9.108818
Blood	B-1079	33.81	40	37.70	40	31.49	8.515
Blood	B-1086	35.18	40	40	39.60	29.27	10.331
Blood	B-1215	35.46	40	34.41	40	30.14	9.861635
Blood	B-5521	30.98	37.12	33.97	40	31.34	8.664278
Blood	B-7043	37.43	36.36	40	40	33.27	6.72681
Blood	B-7033	36.01	32.81	36.97	40	30.11	9.89263
Blood	B-7503	30.40	40	34.07	40	30.36	9.642946
Blood	B-5522	29.99	40	36.22	39.52	29.54	9.984411
Blood	B-1076	36.48	40	36.80	40	29.41	10.595
Blood	B-1227	27.53	40	31.37	40	29.91	10.094
MB	MB-7502	14.77	15.40	22.27	34.01	30.40	3.610876
MB	MB-7503	15.46	12.00	35.67	34.18	31.56	2.624614
MB	MB-1084	16.00	15.00	20.80	35.55	31.04	4.511
MB	MB-1090	14.85	15.44	16.84	35.60	27.90	7.693
MB	MB-1206	17.37	40	17.37	34.99	28.22	6.772
MB	MB-1211	16.54	40	24.74	38.30	35.08	3.212598

MB	MB-5520	15.10	18.24	31.99	36.21	30.32	5.892834
MB	MB-7033	16.80	17.02	21.97	35.95	28.78	7.170988
MB	MB-7504	14.84	12.39	20.83	35.98	29.65	6.335888
MB	MB-7507	16.77	13.26	27.95	37.58	28.86	8.728273
MB	MB-7508	17.04	15.56	26.04	35.57	31.54	4.024341
MB	MB-7509	15.95	16.41	40	35.25	31.61	3.644566
MB	MB-1219	15.09	13.83	29.70	35.51	30.26	5.256
MB	MB-1221	16.07	14.33	24.35	36.71	35.42	1.286972
MB	MB-1222	24.90	22.77	25.25	34.54	30.12	4.415567
MB	MB-5075	17.74	14.46	18.01	35.02	28.84	6.177838
MB	MB-5093	18.80	15.14	40	36.44	30.72	5.71966
MB	MB-5098	15.76	16.29	34.08	36.22	34.76	1.463413
MB	MB-6032	19.34	22.93	18.66	35.32	29.94	5.38437
MB	MB-7043	19.70	40	21.00	34.83	26.73	8.103753
MB	MB-1081	16.89	40	16.07	34.26	31.23	3.030611
MB	MB-1086	18.93	17.58	27.00	35.61	32.52	3.088724
MB	MB-1089	19.85	17.47	32.11	34.98	32.63	2.348363
MB	MB-1091	21.31	18.35	40	35.13	28.73	6.397213
MB	MB-1092	16.10	15.06	35.55	34.15	35.98	-1.832386
MB	MB-1207	20.57	18.89	40	35.54	29.72	5.818771
MB	MB-1212	17.29	18.97	20.75	35.57	32.76	2.803604
MB	MB-1214	17.93	40	29.21	35.71	27.84	7.874735
MB	MB-1218	21.96	40	24.18	35.33	31.99	3.336454
MB	MB-7034	17.96	15.98	40	39.17	25.14	14.029871
Semen	SF-1025	26.18	40	40	32.67	34.91	-2.238
Semen	SF-1026	24.43	40	40	32.16	36.92	-4.764
Semen	SF-1030	33.22	40	35.21	32.69	38.60	-5.904
Semen	SF-1032	27.94	40	36.88	30.22	34.52	-4.298343
Semen	SF-1043	36.14	40	37.61	30.82	35.48	-4.659181
Semen	SF-1045	30.55	40	40	31.44	35.26	-3.817056
Semen	SF-1054	28.63	40	35.61	32.91	37.55	-4.641525
Semen	SF-3507	35.41	40	35.48	31.10	38.10	-7.008926
Semen	SF-3509	32.05	40	37.25	34.05	36.87	-2.823687
Semen	SF-3514	29.53	40	32.88	36.73	37.39	-0.665143
Semen	SF-3521	28.72	40	30.65	30.61	34.62	-4.011783
Semen	SF-4015	27.62	40	29.80	32.84	34.80	-1.958549
Semen	SF-4525	31.48	40	34.30	33.82	36.26	-2.43739
Semen	SF-5001	30.18	38.67	31.72	32.15	35.96	-3.816243
Semen	SF-5002	32.05	40	37.71	33.08	35.84	-2.763783
Semen	SF-5007	29.24	40	38.55	29.91	33.79	-3.878867
Semen	SF-5010	30.46	29.14	32.87	31.15	35.06	-3.903415
Semen	SF-5012	27.71	40	36.44	30.61	32.53	-1.916418
Semen	SF-5152	32.54	40	40	30.75	34.47	-3.717697
Semen	SF-8027	33.59	40	40	36.51	36.44	0.067992
Semen	SF-1093	32.76	40	36.74	32.55	34.95	-2.402452

Semen	SF-1210	28.95	40	36.88	32.23	34.00	-1.778008
Semen	SF-1213	25.11	40	29.63	32.17	35.40	-3.233257
Semen	SF-1216	26.85	36.82	38.32	32.58	33.77	-1.191898
Semen	SF-1224	29.79	35.50	30.80	34.22	35.00	-0.784307
Semen	SF-5142	32.33	40	34.30	31.06	33.45	-2.395531
Semen	SF-5522	33.78	38.59	40	34.54	36.98	-2.441475
Semen	SF-7046	33.27	37.10	36.67	31.41	36.09	-4.678972
Semen	SF-7042	33.34	40	37.44	33.42	35.37	-1.94825
Semen	SF-7505	29.95	40	31.74	31.43	34.95	-3.515968
Feces	Fe-1073	19.60	40	10.06	39.24	40	-0.765
Feces	Fe-1075	16.88	40	11.32	36.64	37.57	-0.93
Feces	Fe-1092	21.90	40	11.84	40	40	0
Feces	Fe-1205	21.06	40	7.90	40	40	0
Feces	Fe-1226	16.55	40	10.60	36.32	37.52	-1.207641
Feces	Fe-5119	14.26	30.97	9.93	34.43	38.09	-3.658333
Feces	Fe-7034	16.72	40	10.26	33.17	34.49	-1.323018
Feces	Fe-7507	14.86	40	9.43	33.46	36.36	-2.898285
Feces	Fe-7511	15.78	31.16	10.25	31.53	31.14	0.393967
Feces	Fe-1013	19.19	40	14.96	36.00	35.55	0.442144
Feces	Fe-1076	16.74	40	12.11	40	40	0
Feces	Fe-1210	23.15	40	14.95	36.07	37.41	-1.338318
Feces	Fe-1213	40	37.13	9.53	40	40	-0.003475
Feces	Fe-1220	18.37	40	12.91	35.34	35.54	-0.196607
Feces	Fe-1224	18.28	40	12.41	33.58	34.74	-1.164585
Feces	Fe-5141	20.34	40	15.77	35.45	29.58	5.86528
Feces	Fe-5142	16.26	40	11.85	33.29	33.80	-0.513797
Feces	Fe-5048	40	40	15.10	35.05	35.41	-0.360687
Feces	Fe-7505	18.90	40	15.46	37.15	36.18	0.967602
Feces	Fe-7506	20.52	35.84	11.91	33.60	32.17	1.428444
Feces	Fe-1211	21.28	40	13.34	35.17	36.74	-1.567322
Feces	Fe-7509	18.10	40	12.44	36.24	36.65	-0.415237
Feces	Fe-1219	16.56	40	11.89	33.90	33.26	0.642411
Feces	Fe-7508	19.30	36.32	15.26	34.58	33.80	0.77337
Feces	Fe-7503	17.47	40	13.15	32.12	32.76	-0.644205
Feces	Fe-1084	19.31	24.40	13.96	40	40	0
Feces	Fe-7042	21.04	40	13.19	33.17	30.70	2.471134
Feces	Fe-5520	17.23	40	14.12	34.81	33.75	1.062144
Feces	Fe-1225	16.60	32.41	11.98	35.94	31.99	3.94459
Feces	Fe-7046	18.27	40	13.41	34.90	34.41	0.48856
Feces	Fe-6010.3	18.63	40	15.56	not tested	not tested	not tested
VF	VF-3550	20.50	15.99	33.19	39.13	28.94	10.187
VF	VF-5083	18.92	15.61	19.37	37.46	35.70	1.758
VF	VF-5086	23.46	20.02	28.96	37.38	40	-2.623
VF	VF-5088	20.09	18.18	40	40	34.68	5.324112
VF	VF-5090	19.07	16.85	40	38.38	33.32	5.059827

VF	VF-6028	21.55	16.50	40	38.50	35.33	3.17223
VF	VF-6040	14.74	40	40	36.75	28.90	7.85225
VF	VF-5042	23.13	20.11	36.47	40	30.58	9.421999
VF	VF-6021	20.97	17.38	30.10	38.37	33.06	5.313461
VF	VF-6025	16.93	40	20.50	35.75	31.88	3.873989
VF	VF-6047	22.57	40	28.93	40	33.73	6.26999
VF	VF-7001	19.48	40	28.55	37.75	31.73	6.022555
VF	VF-7014	21.42	40	33.68	37.97	30.54	7.432797
VF	VF-8001	21.30	16.35	18.75	38.52	37.71	0.807873
VF	VF-1046	21.06	16.25	39.02	37.77	35.38	2.39339
VF	VF-1055	21.51	35.23	40	40	27.42	12.584402
VF	VF-3500	19.46	40	22.24	37.87	30.16	7.711405
VF	VF-3541	16.59	16.77	23.98	36.63	31.84	4.788799
VF	VF-5102	16.72	13.02	35.63	36.97	29.79	7.176329
VF	VF-6500	15.39	11.23	27.99	35.34	35.81	-0.4654
VF	VF-5026	21.12	16.32	35.93	38.07	35.63	2.431248
VF	VF-1044	21.27	40	33.19	37.00	30.16	6.846455
VF	VF-4012	21.44	14.12	40	36.00	32.57	3.42723
VF	VF-1037	23.50	17.79	33.05	36.48	31.71	4.770924
VF	VF-5036	22.42	17.31	32.15	35.84	30.85	4.988843
VF	VF-3508	19.23	19.94	20.73	36.10	34.09	2.013477
VF	VF-5016	22.72	40	40	37.89	32.42	5.462075
VF	VF-3534	19.25	14.95	19.72	36.07	30.78	5.285513
VF	VF-6039	20.26	17.30	21.72	37.75	28.38	9.371921
VF	VF-1027	22.15	38.76	30.02	37.15	28.28	8.86834
VF	VF-1010	15.19	12.80	32.88	not tested	not tested	not tested
Saliva	SA-1029	17.85	40	24.50	37.39	35.20	2.196
Saliva	SA-1037	19.54	40	23.07	39.34	35.62	3.724
Saliva	SA-3519	21.00	40	26.98	38.05	35.81	2.239
Saliva	SA-3520	17.27	40	23.76	39.12	35.45	3.67326
Saliva	SA-5019	22.01	40	26.89	38.67	35.51	3.161368
Saliva	SA-5024	15.24	40	17.47	38.27	35.48	2.792385
Saliva	SA-5026	15.47	40	23.77	37.17	34.78	2.388951
Saliva	SA-3508	18.94	40	22.38	38.73	36.64	2.092408
Saliva	SA-3512	17.58	32.64	28.65	38.54	35.93	2.607906
Saliva	SA-4012	17.18	40	24.43	36.14	36.74	-0.5984
Saliva	SA-1026	14.11	40	23.75	36.09	35.07	1.018788
Saliva	SA-1030	17.24	40	20.34	37.80	34.99	2.818494
Saliva	SA-1045	16.35	40	19.25	36.11	32.01	4.101231
Saliva	SA-3509	18.51	40	27.81	36.74	34.14	2.602348
Saliva	SA-3514	17.62	40	28.80	36.24	33.20	3.032971
Saliva	SA-5003	16.21	40	16.35	36.87	35.15	1.725835
Saliva	SA-5008	19.44	40	23.74	37.01	34.21	2.790588
Saliva	SA-5010	14.90	40	19.16	35.74	31.08	4.662554
Saliva	SA-5012	15.77	40	23.28	36.02	33.42	2.597424

Saliva	SA-5018	14.35	23.77	23.50	35.98	33.85	2.120786
Saliva	SA-1062	16.59	40	16.63	35.08	35.32	-0.245155
Saliva	SA-1069	13.74	40	18.07	35.10	32.59	2.515783
Saliva	SA-5061	16.01	40	19.40	37.01	30.79	6.219633
Saliva	SA-5077	17.88	40	26.39	35.75	34.80	0.957752
Saliva	SA-7009	18.64	40	24.01	36.95	34.33	2.62226
Saliva	SA-1057	16.98	40	25.03	36.52	36.26	0.263
Saliva	SA-5092	15.82	40	17.98	35.62	29.59	6.022
Saliva	SA-1072	16.31	40	19.24	34.30	34.12	0.179617
Saliva	SA-1075	15.96	37.89	24.52	36.17	35.77	0.395246
Saliva	SA-6008	18.11	21.45	21.44	35.89	33.12	2.770344
Saliva	SA-3500	15.45	40	21.26	not tested	not tested	not tested

VITA

Carolyn A. Lewis

Email: lewisca4@vcu.edu
Phone: (757) 879-2491
LinkedIn: https://www.linkedin.com/in/carolyn-lewis-05896912a/
ResearchGate: https://www.researchgate.net/profile/Carolyn-Lewis-2

Education

Virginia Commonwealth University, Richmond, VA — *Aug 2016-Dec 2021*

Ph.D. in Integrative Life Sciences
ILS Student Organization Board Member:
-President (2019-2020)
-Secretary (2018-2019)
-Member at large (2017-2018)

Virginia Commonwealth University, Richmond, VA — *Aug 2011-May 2015*

B.S. in Forensic Science, Biology concentration
Chemistry and Biology minors
GPA: 3.81

Research Grants

1. Lewis, CA, Seashols-Williams, SJ. Developmental evaluation of a combinatorial qPCR multiplex for forensic body fluid identification. $49,994. Graduate Research Fellowship, Fiscal Year 2020. Submitted in April 2019 to the National Institute of Justice for Jan 2021-Dec 2021.

Patents

1. miRNA panel for forensic body fluid identification. Provisional US Patent 62/288,788, filed January 29, 2016.

Publications

1. Lewis C, Seashols-Williams S. Design and optimization of a 16S microbial qPCR multiplex for the identification of feces, saliva, vaginal and menstrual secretions. Submitted to Journal of Forensic Sciences on October 26, 2021.
2. Rhodes C, Lewis C, Szekely J, Campbell A, Boone E, Seashols-Williams S. Developmental validation of a microRNA panel using quadratic discriminant analysis for the identification of seven forensically relevant body fluids. Manuscript in final preparation to be submitted to FSIG.
3. Smith C, Cox J, Rhodes C, Lewis C, Koroma M, Hudson B, Dawson Cruz T, Seashols-Williams S. Comparison of DNA typing success in compromised blood and touch samples based on sampling swab composition. Journal of Forensic Sciences 2021;00:1-8. doi: 10.1111/1556-4029.14694
4. Layne T, Green R, Lewis C, Nogales F, Dawson Cruz T, Zehner Z, Seashols-Williams S. microRNA detection in blood, urine, semen, and saliva stains after compromising treatments. Journal of Forensic Sciences 2019;64(6):1831-37. doi: 10.1111/1556-4029.14113
5. Lewis C, Layne T, Seashols-Williams S. Detection of microRNAs in DNA extractions for forensic biological source identification. Journal of Forensic Sciences 2019;64(6):1823-30. doi: 10.1111/1556-4029.14070

6. Seashols-Williams S, Lewis C, Calloway C, Peace N, Harrison A, Hayes-Nash C, et al. High-throughput miRNA sequencing and identification of biomarkers for forensically relevant biological fluids. Electrophoresis 2016;37(21):2780–8. doi:10.1002/elps.201600258

Honors/Awards

2018, Mid-Atlantic Association of Forensic Scientists, Annual Scholarship Award
2015, Virginia Commonwealth University, Outstanding Academic Achievement Award

Oral Presentations

1. C Lewis*, S Seashols-Williams. "Forensic Science Education & Research Through an Interdisciplinary Program: Utilizing MicroRNAs from DNA Extracts for Biological Source Identification." Invited talk, presented at the National Institute of Standards and Technology, October 2018.
2. C Lewis*, T Layne, S Seashols-Williams. "Detection of MicroRNAs in DNA Extractions for Biological Source Identification." Talk, presented at the Mid-Atlantic Association of Forensic Scientists Annual Meeting, May 2018.
3. C Lewis*, C Calloway, N Peace, A Albornoz, S Fleming, C Hayes, Z Zehner, S Seashols-Williams. "Developmental Validation of a miRNA Panel for the Identification of Six Forensically Relevant Body Fluids." Talk, presented at the Mid-Atlantic Association of Forensic Scientists Annual Meeting, May 2016.
4. C Lewis*, J Gentry, C Calloway, N Peace, A Albornoz, S Fleming, C Hayes, Z Zehner, S Seashols-Williams. "Developmental Validation of microRNAs for Body Fluid Identification." Talk, presented at the American Academy of Forensic Sciences Annual Meeting, February 2016.

Poster Presentations

1. C Lewis*, S Seashols-Williams. "Developmental evaluation of a combinatorial reverse transcription-qPCR multiplex for forensic body fluid identification." Poster, presented at the International Symposium on Human Identification Annual Meeting, September 2021.
2. C Lewis*, T Layne, S Seashols-Williams. "Detection of MicroRNAs in DNA Extractions for Biological Source Identification." Poster, presented at the Gordon Research Conference on Forensic Analysis of Human DNA, June 2018.
3. C Lewis*, T Layne, S Seashols-Williams. "MicroRNA Detection in DNA Extraction Methods Commonly Used in Forensic Casework." Poster, presented at the International Symposium on Human Identification Annual Meeting, October 2017.
4. C Lewis*, T Layne, S Seashols-Williams. "Detection of microRNAs in DNA Extraction Methods Commonly Used for Forensic Casework." Poster, presented at the American Academy of Forensic Sciences Annual Meeting, February 2017.

Professional Activities & Continuing Education

- Virtual Summer & Winter Seminars – Students and Young Researchers in Forensic DNA (Qiagen): 2020, 2021
- Gordon Research Seminar on Forensic Analysis of Human DNA: 2018 (co-chair)
- International Symposium on Human Identification Annual Meeting: 2017, 2021
- Gordon Research Conference on Forensic Analysis of Human DNA: 2016, 2018
- American Academy of Forensic Sciences Annual Meeting: 2016, 2017

- Potomac Regional Symposium on Forensic DNA Analysis: 2015, 2019
- Future Trends in Forensic DNA Technologies (Thermo Fisher Scientific): 2015, 2018, 2020
- Mid-Atlantic Association of Forensic Scientists Annual Meeting: 2015, 2016, 2018

Service & Professional Involvement

- Gordon Research Seminar: Forensic Analysis of Human DNA
  -Co-chair, 2018 Executive Committee
- American Academy of Forensic Sciences
  -Student Affiliate Member, 2017-present
- Mid-Atlantic Association of Forensic Scientists
  -Member, 2016-present
- Community Engagement & Outreach
  -New Community High School forensic demonstration—February 2020
  -Silent No More Overdose Symposium—January 2020
  -Grace Christian High School forensic demonstration—October 2019
  -VCU Honors Summer Undergraduate Research Program Workshop
  Graduate Student Panelist—June 2019
  -William Monroe High School forensic demonstration—September 2018
  -Richmond Minorities in Engineering Partnership—July 2018
  -Girl Scout activity & panel discussion—October 2017
  -STEM field exploration for local Richmond middle schools—December 2016
  -Richmond Public Schools Review Day—October 2016