DEVELOPMENT AND EVALUATION OF MIRNA AND MRNA PANELS FOR BODY FLUID IDENTIFICATION

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Carrie Ann Mayes

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Carrie Ann Mayes

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APPROVED:

Sheree Hughes-Stamm, PhD
Committee Director

Bobby LaRue, PhD
Committee Co-Director

Sarah Seashols-Williams, PhD
Committee Member

Rachel Houston, PhD
Committee Member

Phillip Lyons, PhD
Dean, College of Criminal Justice

DEDICATION

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Finally, to the eccentric lady at my bar all those years ago that told me that she saw a lot of writing in my future – touché.

ABSTRACT

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The attribution of biological material to a tissue source, also known as body fluid identification (BFID), can aid investigators in corroborating statements and in the reconstruction of events. Chemical tests, microscopy, enzymatic activity, and immunochromatographic tests are widely employed in crime laboratories at present but have a high false-positive rate and interpretations are often subject to conjecture. Forensic testing requires a methodology that can analyze low level and challenging samples with high specificity. While several molecular targets have been considered for BFID, this project explores the use of microRNA (miRNA) and messenger RNA (mRNA) for BFID. The results of this study provide the forensic community with information on the performance of miRNA and mRNA for BFID using capillary electrophoresis (CE), quantitative reverse transcription PCR (RT-qPCR), and massively parallel sequencing (MPS) technologies, particularly for challenging samples.

In the first study, an eight-marker miRNA multiplex was developed for capillary electrophoresis using a linear primer system. Markers were chosen to identify venous blood, menstrual blood, semen, saliva, as well as an endogenous reference gene. Reverse transcription and PCR primers were developed for each marker, evaluated in singleplex, and then multiplexed. Each fluid were co-extracted (DNA/RNA) and amplified with STR and miRNA multiplexes, respectively. All DNA profiles were complete and miRNA profiles correctly identified the body fluid using a decision tree-based interpretation strategy.

In the second study, the stability and persistence of miRNA and mRNA was evaluated in challenging samples. Custom reverse transcription quantitative PCR (RT-qPCR) assays were performed to detect the presence of miRNA and mRNA in samples. mRNA targets and miRNA targets were chosen for blood, semen, and an endogenous reference gene respectively. For the evaluation of these markers over time, blood and semen samples were placed in a glass enclosed area exposed to natural heat, humidity, and UV light as well as controlled conditions in a lab cabinet (room temperature, low humidity, and darkness) for up to six months. mRNA was undetected in experimental samples after 30 days, while control mRNA and all miRNA transcripts were detected for the duration of the experiment. A persistence study was also performed by laundering blood and semen stained swatches and either machine drying or allowing the samples to air dry. Blood specific markers were detected in all bloodstained samples, while semen specific markers were observed in all but one semen stained sample. Transfer of both miRNA and mRNA was observed by taking an unstained portion of the swatch.

In the final study, an early access mRNA panel for BFID was evaluated. Samples of venous blood, menstrual blood, semen, saliva, and vaginal secretions were placed in a variety of challenging conditions including outside with and without exposure to direct precipitation, buried, on a decomposing cadaver, laundered, aged, and post-coital samples. Co-extracted DNA profiles were evaluated, and RNA was successfully extracted and typed in most samples. Reverse transcription negatives, total reads, and composition of reads attributed to body fluid specific markers were used to evaluate the performance of the panel. Based on the results of the study, potential areas for improvement were highlighted; however, the performance of the panel overall is encouraging.

KEY WORDS: Body fluid identification, mRNA, miRNA, Persistence, Forensic science, Serology

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ABBREVIATIONS

BFID	Body fluid identification
CE	Capillary electrophoresis
CpG	Cytosine-phosphate-guanine
cDNA	Complementary DNA
DNA	Deoxyribonucleic acid
EDNAP	European DNA Profiling Group
ESR	Environmental Science and Research Ltd
EUROFORGEN	European Forensic Genetics Network
FBI	Federal Bureau of Investigation
FITC	Fluorescein isothiocyanate
KM	Kastle-Meyer
KPIC	Kernechtrot–Picroindigocarmine
LMG	Leucomalachite green
LNA	Locked nucleic acid
miRNA	Micro RNA
mRNA	Messenger RNA
MPS	Massively parallel sequencing
NFI	Netherlands Forensic Institute
NGS	Next generation sequencing
PIC	Preinitiation complex
PCR	Polymerase chain reaction
PSA	Prostate-specific antigen
qPCR	Quantitative PCR
RT-qPCR	Reverse transcription quantitative PCR
RNA	Ribonucleic acid
SAAP	Single amino acid polymorphism
SNP	Single nucleotide polymorphism

STR	Short tandem repeat
TPPR	Transfer, persistence, prevalence, and recovery
WGA	Whole genome amplification

GLOSSARY

Allele	Alternate versions of a gene or locus.
DNase	Deoxyribonuclease commonly used to remove DNA from samples through the hydrolysis of phosphodiester bonds in the sugar-phosphate backbone.
Gene expression	The process of using genetic information to generate products used by the organism.
Gene expression analysis	Pattern of expressed genes at the transcriptional level.
Polymerase chain reaction	A method used to amplify DNA. Primers are used to anneal to DNA, allowing polymerase to attach and extend. Several temperature cycles are performed to generate millions of copies of the designated regions.
Reverse transcription	The conversion of RNA molecules to cDNA by using reverse transcriptase
Transcript	RNA product of DNA transcription
Transcription	Process by which DNA is converted to RNA

CHAPTER I

Introduction

Current methods for body fluid identification (BFID)

Purpose of BFID tests

In crime laboratories, BFID, also referred to as serology, is testing of evidence with the intent of classifying biological material to its body fluid type. The testing also functions to localize possible biological material that may not be visible to the naked eye or large items of evidence. Several current tests allow investigators to preliminarily screen items at the crime scene and in the laboratory to identify potentially probative evidence.

Tests are designed on the basis that body fluids are composed of a combination of liquid and terminally differentiated cells, each having a separate function. Each cell type requires a different set of cellular machinery, such as proteins, mRNA, and miRNA, to perform its required function in the body. These differences are the targets for BFID tests. The ability to determine the body fluid of origin may be highly relevant in the course of forensic investigations, particularly in sexual assault cases. Although DNA typing can identify the individual that deposited a stain, current methods cannot conclusively determine the biological origin of the sample (epithelial cells due to touch, saliva, vaginal material, semen from an oligo- or azoospermic individual, or distinguish between venous and menstrual blood). This determination may be critical in the reconstruction of events.

Chemical and catalytic tests

Current BFID methods employ a variety of chemical tests for presumptive testing. The tests are generally fast, inexpensive, and allow examiners to locate latent stains on large or dark items of evidence. In general, chemical and catalytic tests are coupled with a

color change and target a protein or enzyme inherent to the body fluid. The Kastle-Meyer (KM) and leucomalachite green (LMG) tests both rely on the peroxidase activity of heme in blood (human and non-human) to reduce the indicator molecule which elicits a visible color change [1]. While these tests have shown high sensitivity, (e.g. luminol tests have detected blood in dilutions down to one in five million [2] and after cleaning attempts [3]), they lack in specificity. False positives have been observed in commonly encountered household items such as tomatoes, potatoes, kidney beans, onions, horseradish, and bleach [1,4].

Enzymes endogenously present in the body have also been targeted for forensic use. Acid phosphatase and α-amylase have been found to be moderately body fluid specific in semen and saliva, respectively, and are helpful in localizing latent stains [5,6]. However, enzymes have conserved functions across body fluids and species, such as α-amylase in semen, vaginal fluid, rat saliva, and other body fluids [7-9]. False negatives for the presence of a body fluid can also occur in samples exposed to harsh conditions, for example heat or mold, due to degradation of target enzymes [8,10].

Due to their convenience and low cost, chemical and catalytic presumptive testing are unlikely to be usurped by molecular-based techniques at this time. However, the high level of cross-reactivity with other body fluids and even non-human common household materials provides only presumptive information to examiners. The results of these tests cannot be used with any confidence to definitively identity a body fluid in a court of law.

Immunochromatographic tests

Proteomics was first advocated for BFID in immunochromatographic assays (such as the PSA test) and has shown success for both human specificity and BFID [11-15]. This

testing is based on anti-human antibodies of a body fluid specific protein conjugated to a dye. In the presence of the suspected body fluid, the antibodies will attach to the target molecules. The solution is placed on a membrane and migrates to a reaction zone containing immobile antibodies also specific to the targeted protein. The accumulation of antibody-antigen-antibody complexes allows the dye to become visible to the user. An immobilized line of anti-Ig antibodies allows unbound antibodies to attach and which are also visualized and used as a control [16].

Commercial test kits are available for many of the forensically relevant body fluids including blood [12,17,18], semen [13,19,20], saliva [21,22], menstrual blood [23], and urine [24], though a test for vaginal secretions is not available to date. These tests are commonly used due to their ease of use, relatively low cost, and portability which allows them to be used both on-site at crime scenes and in the laboratory. Conversely, the tests have many similar drawbacks as chemical testing such as sample destruction, mixture detection, cross-reactivity across species [17,24,25,22] and body fluids [12,13,20,22,26]. Disadvantages specific to this technique are vulnerability to the high-dose hook effect and environmental factors [11] and the requirement that an analyst much make assumptions about the evidence to choose the most appropriate test(s). Holtkötter et al. (2018) constructed a multiplexed immunochromatographic test by combining commercially available tests which was able to detect mixtures [27]. This type of testing may become more popular as a cost-effective way to detect body fluids without prior assumptions about the evidence.

Microscopic testing

To date, the visualization of spermatozoa using histological staining is still the most commonly employed confirmatory test for the presence of semen [9,10]. The most prominently used stain, the Kernechtrot–Picroindigocarmine stain (also known as the Christmas Tree stain or KPIC), differentially stains nuclear material red and cytoplasm green. Despite the popularity and strength of this method, it cannot indicate the presence of semen in azoospermic individuals, spermatozoa can be easily obscured by the presence of other cells and searching microscopically can be extremely time-consuming [28-30]. Differential staining has also been investigated more broadly to distinguish tissue types. Paterson et al. (2006) used immunohistochemical staining targeting antigens present in different cell types in combination with cellular morphology to attempt to identify vaginal epithelial cells [31]. While the group did have some success, they were not able to identify an antigen specific for vaginal epithelial cells and it is unknown whether this method would be successful with mixed or environmentally challenged samples. Miller et al. (2011) augmented the immunohistochemical staining method by attaching a fluorescent dye to a human spermatozoa-specific antibody [30]. Using a fluorescein isothiocyanate (FITC) filter on a microscope, spermatozoa can be visualized using this commercial product. Compared to KPIC staining, analysts would be able to visualize spermatozoa more quickly and more accurately in cell abundant samples where spermatozoa may be otherwise obscured [28-30].

Crystal tests, also visualized microscopically, have also been used in forensics. In these tests, blood samples are exposed to chemicals; pyridine and ammonium sulfide for the Takayama test, and acetic acid for the Teichmann test, to form distinctive crystals that

could be viewed under polarized light [32]. The Takayama and Teichmann tests were considered a confirmatory test for blood, but not specifically human blood. The tests were also time consuming, had a high rate of false negatives, and generally require a larger amount of sample [11,32]. As a result, they are not widely employed by forensic laboratories.

Proposed Methods for BFID

The development of a successful BFID system must overcome the deficiencies inherent with current methods. First, the method must be confirmatory and not be prone to false positives from other fluids or substances. Distinguishing between similar body fluids, such as venous and menstrual blood, or the identification of semen without the presence of spermatozoa may be critical elements in the investigation of sexual assault cases. Secondly, BFID testing should not totally consume or dilute the evidentiary sample. In casework, it is not uncommon that stains are too small for separate and consecutive DNA and BFID analyses to be performed. The following techniques have been proposed as potential supplements to current BFID methods.

Spectroscopic testing

Interest in Raman spectroscopy has recently increased within the forensic community due to the development of a portable Raman spectrometer. Raman spectroscopy, a type of vibrational spectroscopy, measures the inelastic scattering of light applied to a sample. The method is non-destructive and is currently being evaluated as a body fluid identification technique in addition to other forensic disciplines such as forensic trace, chemistry, and document examination [9,33-36].

Preliminary research was able to identify forensically relevant body fluids and discriminate between species in samples of the same fluid [37,38]. These initial studies required samples to be placed on aluminum foil covered slides to reduce background interference. Challenges for using Raman spectroscopy for forensic samples in general include the heterogeneous nature of body fluids and both substrate and cellular luminescence which can obfuscate emissions from the sample itself [39]. Virkler et al. (2009) employed spectral mapping of an area to accommodate heterogeneity in samples and McLaughlin et al. (2013) were able to correct for substrate interference for samples on homogenous substrates using different excitation wavelengths, baseline treatments, and the subtraction of a reference spectra, however neither of these techniques has been used with a portable device [40,41].

Given the variety of substrates and conditions body fluid stains are found, substantial research is required to investigate the robustness of this technique. Studies have been performed examining stains on different types of substrates and the effect of various environmental contaminants, such as soil [41-43]. While these studies have yielded promising results, the majority of the research regarding Raman spectroscopy for body fluid identification has been performed at the same institution, and investigation of this method by other groups would be beneficial to demonstrate widespread applicability, robustness, reliability, and reproducibility of the technique. Overall, the use of a single instrument to test across several disciplines on-site would undoubtedly be highly desired by forensic examiners.

Proteomics

Recently, advances in spectroscopic technologies have allowed for a more comprehensive study of proteomics [44]. In proteomic analysis, polypeptide chains are digested into small peptides, desalted, separated by liquid chromatography, and detected by mass spectroscopy. DNA and proteins can be co-extracted, allowing for traditional human identification techniques using DNA and BFID through protein analysis. Sample preparation techniques used by Dammeier et al. [45] and Van Steendam et al. [46] used centrifugal methods to pellet DNA-containing cells and used the supernatant for protein analysis; however, the supernatant also contains cell-free DNA and its absence may affect traditional downstream DNA typing results [47]. Efforts have been made to identify body fluids using a targeted set of marker proteins [46,48-50] as well as machine learning assisted untargeted protein analysis [45]. The discovery of single amino acid polymorphisms (SAAPs) has bolstered the utility of proteomics in forensics. SAAPs in hair samples have shown a power of discrimination similar to mitochondrial DNA [51] and bone samples have been matched to ancestry [52], but body fluids have not yet been investigated for these markers. Additionally, proteomic analysis requires mass spectrometry techniques and instrumentation not typically used in forensic biology laboratories [45,53].

DNA methylation patterns

DNA methylation-based assays have also been suggested as a possible method for BFID. Although DNA methylation-based tests require the consumption of DNA, they can be easily applied retrospectively to archived DNA extracts. Gene expression at the DNA level is achieved through differences in the chromatin structure of the DNA molecule

through a methyl group at the 5’ position of the pyrimidine ring in CpG dinucleotides [54,55]. Differentially methylated regions can be used to distinguish different cell types to provide BFID [56,57]. Other applications of differential methylation are being investigated for forensic intelligence purposes including biological age determination, monozygotic twin differentiation, and lifestyle choices [58-60]. The detection of DNA methylation patterns can be performed using two different methods. First, methylation-based restriction enzymes can be used to digest unmethylated regions of DNA, leaving only the areas of interest, although a limited number of these enzymes have been identified [61]. Additionally, restriction enzymes are not compatible with extraction techniques that yield single stranded DNA. Second, DNA may be treated with sodium bisulphite to change unmethylated cytosines to uracil, which can then be detected by quantitative PCR (qPCR), pyrosequencing, or single base extension reactions [62,63]. Sodium bisulphite treatment has traditionally required more DNA (>40 ng) than is typically present in a forensic sample. However, bisulphite conversion followed by whole genome amplification (WGA) of 100 pg DNA has been successfully analyzed [64,65] via pyrosequencing and may provide a means to analyze forensically relevant amounts of DNA. WGA has been shown to exhibit amplification bias [66] but no significant bias in quantitative methylation statuses have yet to be observed [64,65,67]. A method to assess DNA methylation status using MPS has recently been utilized in studies examining chronological age prediction [68,69]. MPS would allow for an increase in through-put compared to the traditional pyrosequencer, but these preliminary studies required high minimum read counts (800 and 1000 reads) to accurately determine methylation status [68,69].

Emphasis has been placed on identifying CpG sites that are completely methylated in the target body fluid and not methylated in others, or 'on/off' methylation [70], as described in recent studies [63,71,72]. Another important concern regarding the use of DNA methylation-based assays is that methylation patterns have been shown to change with a person's age and exposure to different environmental conditions [61,73], and some of these changes may also influence the reliability/accuracy of methylation-based BFID tests. Independent validation and rigorous testing of any markers is imperative [60,74].

mRNA

mRNA biogenesis and function

The beginning of the mRNA transcription process occurs in the nucleus with the recruitment of general transcription factors along with Pol II to form the preinitiation complex (PIC) [75]. The Mediator complex then binds to Pol II, assists with gene and general transcription factor recruitment, and is responsible for the initiation of Pol II during transcription [76]. After initiation, elongation occurs until termination is signaled. The pre-mRNA is processed through a series of modifications before being exported to the cytoplasm, which generally occurs as transcription is taking place [75]. The 5' end is capped through the addition of an m7GpppN structure by human capping enzyme and RNA 7-methyltransferase to protect the molecule from nucleases [77]. Spliceosomes remove any introns through the recognition of various elements, such as the 5' and 3' splice sites, excise intronic regions, and ligate the exons [75]. Most protein coding mRNAs are also polyadenylated. For this modification, the pre-mRNA is cleaved and poly(A) polymerase adds a poly(A) tail [78]. Transcription is terminated after Pol II is slowed down through various mechanisms, such as encountering heterochromatin patches, R-loop formation, or

proteins associated cleavage, and eventually dissociated from the pre-mRNA strand [79]. Many of the protein factors required for processing remain bound to the mRNA and take part in a quality control check before the molecule is exported to the cytoplasm through the nuclear pore complex [75,80,81].

Once exported to the cytoplasm, mRNA functions as the template for protein formation. Cellular signaling recruits initiation factors such as RNA helicase and 5’ cap binding protein to circularize the molecule for stability during translation and ribosomal subunits then scan the molecule for the start codon to begin translation [81,82]. However, post-transcriptional regulation such as miRNA-mediated repression may halt or delay translation (Figure 1.1).

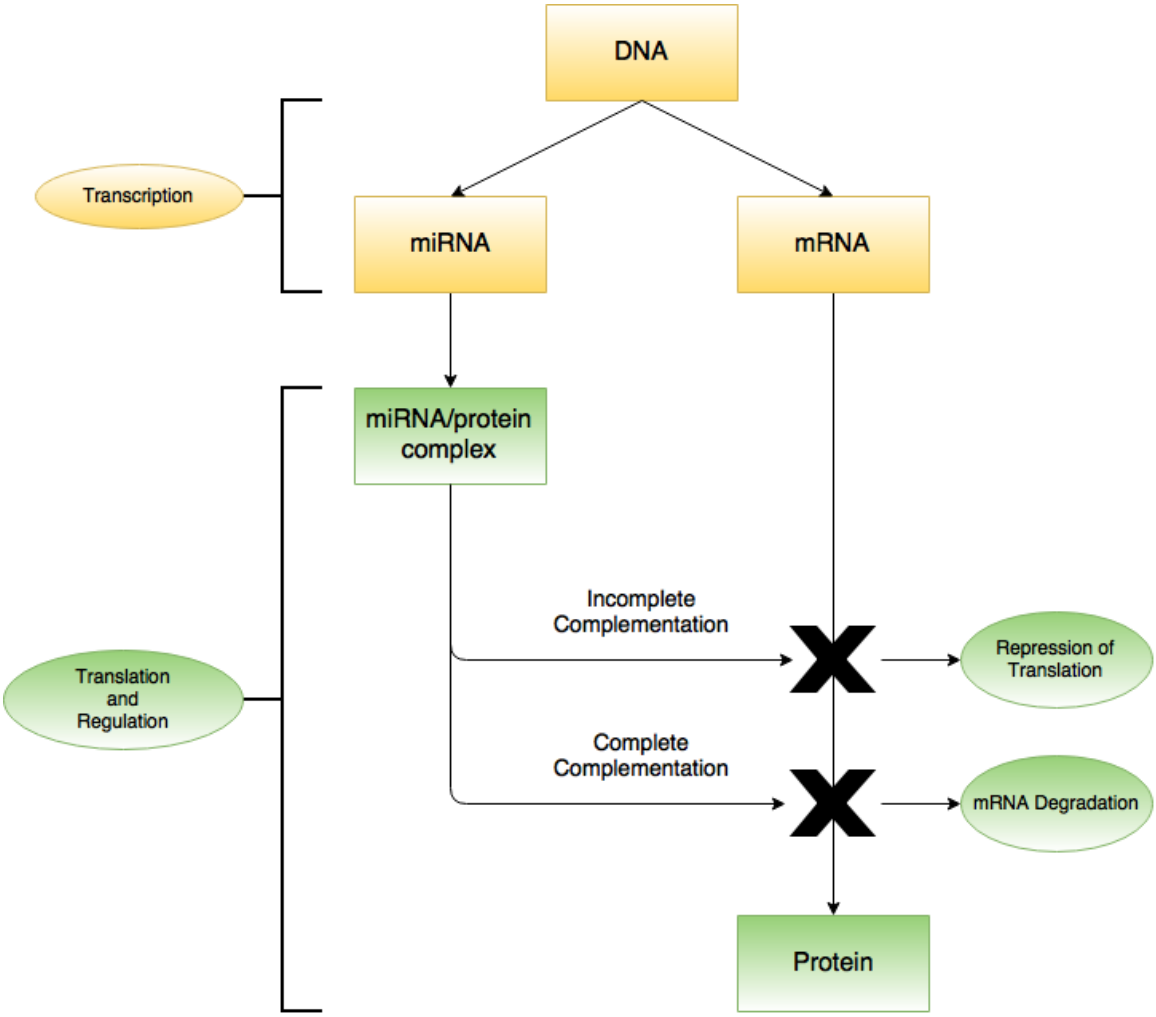

Fig. 1.1. Schematic of how miRNA may regulate gene expression (and the eventual translation of proteins) in the cell.

Initial experiments and marker candidacy

Bauer et al. (1999) performed some of the initial experiments examining mRNA for forensic use after it was found that RNA recovered from blood spotted filter paper used in a medical context was still viable for analysis [83,84]. Cytokeratin and progesterone receptor transcripts were used as the basis for distinguishing between menstrual blood, which contains epithelial cells in the shedding endometrium, and venous blood. Overall,

the group was successful in detecting transcripts and continued to perform further research into the field [83].

The completion and ongoing efforts of such projects as the Human Genome Project and the Cancer Genome Anatomy Project as well as a plethora of medical literature have allowed researchers to search for genes differentially expressed in forensically relevant body fluids in silico and through literature reviews [85,86]. Several groups used these resources to evaluate transcripts for BFID. Bauer and Patzelt (2002) evaluated a battery of transcripts associated with the endometrium and menstrual cycle and were able to find transcripts present in menstrual blood that were not present in vaginal fluid nor saliva samples [87]. Juusola and Ballatyne (2003) evaluated saliva specific transcripts, and Visser et al. (2011) explored skin specific transcripts [88,89]. Further studies also found spermatozoa specific transcripts and applied a more sensitive detection method of capillary electrophoresis rather than agarose gel electrophoresis [90].

Upon the observation that all work investigating mRNA for BFID had been targeting transcripts through database searches or literature reviews, Zubakov et al. (2008) performed a microarray analysis of blood, semen, saliva, and vaginal fluid deliberately screening degrading samples [91]. A time course study of blood and saliva samples over a period of 180 days was included in the analysis in an effort to identify stable transcripts and increase the likelihood of BFID in degraded samples. These markers did not overlap with any previously used markers and were used to successfully detect blood and saliva specific transcripts in samples up to 16 and 6 years, respectively [92].

It should be noted that considerable discordance has been observed in the performance of markers reported to uniquely identify saliva and vaginal samples. It is not

surprising that overlap was found as mucosal cells in both anatomical regions perform similar physiological functions. Histatin 3 (HTN3) and statherin (STATH) were both first reported for forensic use as saliva specific markers, though the initial specificity testing did not include vaginal samples [88]. These markers were found to be saliva specific in several studies [93-96] and later found in nasal secretions (only STATH) [97,98], nose bleeds [97], and tears [97] which also share some physiological pathway overlap. However, STATH was observed in menstrual blood [99-101], vaginal fluid [102-104], and semen [100] and HTN3 was detected in vaginal fluid [105]. A possible isoform of STATH was detected in menstrual blood, which may account for some discrepancies [93].

Mucin 4 (MUC4) was originally considered to be specific to vaginal fluids (though also expected in menstrual samples) as seen in [93,95,106] and urine [98], which, again, makes physiological sense as the urethra is in close proximity to the vagina. As MUC4 takes part in mucus production, it was also observed in fluids in proximity to mucus membranes such as saliva [98-100,102,107-109], nasal mucosa [97,102], urine [97,109], and tears and nosebleeds [97], as well as semen [100,107]. HBD1, also intended as vaginal fluid specific, was also observed in non-target fluids whilst in a multiplex which was resolved when run independently in one study [95], but not another [109]. Both markers were also observed in samples from various orifices (such as the ear canal and anus) in singleplex reactions [108]. These discordances may be the result of primer design, PCR parameters such as annealing temperature, or cDNA input in PCR. The cross-reactivity of markers with nasal mucosa and urine were likely only discovered due to the intentional sampling of these body fluids and were not originally considered during marker screening.

Overall, the incongruous results demonstrate the importance of comprehensive validation testing.

Multiplex development and implementation

In 2005, the first of several BFID multiplexes targeting mRNA was reported using capillary electrophoresis (CE) [93]. Although RT-qPCR multiplexes were also developed, its use is hampered by the number of targets that can be amplified simultaneously [93,106,107]. While Juusola et al. (2007) found the RT-qPCR method to be more sensitive, Haas et al. (2009) found it to be comparable with CE and enzymatic tests [106,110]. The ability to multiplex reduces reagent cost, sample consumption, and preparation time, and led CE to become the chosen platform for forensic casework to date.

The Institute of Environmental Science and Research Ltd (ESR) developed and implemented mRNA multiplexing for BFID into casework in 2010. This test is able to identify venous blood, menstrual blood, semen, vaginal secretions, and saliva [94,111,112]. Interestingly, the group implemented markers targeting bacterial RNA in naturally occurring vaginal microbial flora [112], but recent concerns have been raised regarding their use due to the detection of these microbes on hands, groin area, and penile swabs [97,113]. A group at the Federal Bureau of Investigation (FBI) also created a multiplex for BFID but was not implemented likely due to lack of consensus markers and interpretation schema for cross-reactive markers [100]. Xu et al. (2014) developed the mRNA multiplex for BFID, termed XCYR1, to identify urine, sweat, nasal secretions, and oral mucosa in addition to the commonly typed venous blood, menstrual blood, semen, saliva, and vaginal fluid. However, it is unknown whether it was ever implemented into casework [98]. An assay was created [114] and developmentally validated [115] to be used

in a portable device with the intent for use at crime scenes, but the manufacturer halted production and support of the instrument soon after its design.

Not only is the choice of markers important, but an effective interpretation plan is also required. An interpretation strategy was developed by Roeder and Haas (2013) for use with four BFID multiplexes [99]. A score was assigned to each marker based on the number of times the marker was observed in known samples. The score was lowered in instances of false positives, effectively giving the marker less weight during interpretation. For each sample, marker scores were summed and must reach a pre-defined minimum score threshold for a positive result. Minimum threshold scores were also determined using known samples and set to minimize false positives. Using the scoring system, saliva, semen, and blood samples were correctly identified in over 90% of samples. Menstrual and vaginal samples, however, were only correctly identified in 52% and 70% of samples, respectively. The authors attributed the low identification rates of these samples to the higher minimum threshold scores required for these samples. Although artifacts in the electropherograms provided in the manuscript suggest that the multiplexes would benefit from further optimization due to numerous artifacts, the authors did propose a novel scoring system as reporting guidelines [99].

The Netherlands Forensic Institute (NFI) created several BFID multiplexes and has published extensively on their use and interpretation strategies [116,97,95,117,118]. The original multiplex contained at least two markers per target fluid and three housekeeping markers. Multiple PCR reactions containing a range of cDNA input amounts were used due to the lack of human specific RNA quantification and unknown composition of the extract. Consensus markers were used to account for inter-person variation of expression

and drop out from stochastic effects. As substantial cross-reactivity of markers was observed in previous studies [98-100,102,107-109,119], fluid specific and general mucosal markers were included to differentiate between vaginal, menstrual, and saliva samples. Sensitivity and specificity testing of the multiplex yielded sufficient results for implementation [95]. The NFI reported that routine casework applying the mRNA BFID multiplex was completed by specially trained analysts [116]. Interpretation guidelines were developed based on the number of times relevant markers were observed (x) relative to the number of times the markers could have been observed in the profiles (n) generated from serial cDNA inputs. The presence of each body fluid is assessed in each profile and are designated as "observed" if $x \geq n/2$ and potentially mixed samples with overlapping cell types (such as sharing of mucosal markers in saliva and vaginal samples) reaching the threshold are designated as "observed and fits with." General housekeeping marker peak height and peak heights of body fluid specific markers relative to one another are also considered. Profiles not reaching the $x \geq n/2$ threshold are scored based on whether co-expressed markers (e.g. mucosal) are present and generally scored as "sporadically observed, no reliable statement possible" or "sporadically observed and fits with." Body fluids are labelled as "not observed" if no amplicons were detected in any of the profiles and "non-specific due to high input" is termed for overloaded samples in which no definitive interpretations can be made [116]. Although the interpretation strategy can be confusing to those unfamiliar with RNA profiling, it has been thoroughly tested and can accommodate mixtures and some degradation.

Based on the reported cross-reactivity of mucosal markers in nasal secretions [98,103,119] and various cross-reactivities in urine [98], the NFI updated their multiplex

and interpretation protocols [97]. During this time, the analysis of profiles generated from differentially extracted samples was incorporated and vaginal markers were updated based on the cross-reactivity discussed above. Notably, the inclusion of technical replicates was also recommended for RNA profiling due to high variation of replicate amplifications, including technical replicates [97].

The relationship between contributor proportions in electropherograms has also been investigated to determine if RNA profiling results mimicked cell type proportion and/or DNA contribution [94,98,120]. Those studies reveal that while some inference could be made regarding proportions of cell-type in the RNA profiles, further attribution to a donor based on contributor proportions through DNA profiling was not advisable. These results were not unexpected because DNA and RNA are not found in consistent proportions across cell types and can show both inter- and intra-person variation within cell types [121].

A marked improvement was seen in sensitivity and specificity of mRNA markers after primers were redesigned by Lin et al. (2016) [122]. Degraded samples were sequenced using MPS and specific, partial regions of mRNA molecules were consistently seen compared to the rest of the transcript. Primers were then designed around the transcript stable region RNAs, or "StaRs," using conventional CE-based methods. In comparison to previous primers, the "StaR" primers improved peak height and detection in degraded samples. These primers were incorporated into a multiplex by Albani and Fleming (2018) along with markers for seminal fluid (azoospermic) and semen [123]. Any cross-reactivity of markers in non-target body fluids was not reproducible, including between mucosal saliva and vaginal fluid samples. The performance of the new multiplex [123] generated

significantly higher signals in blood, semen, and menstrual blood samples when directly compared to the previous version [94].

The adoption of Bayesian statistics has been encouraged in the forensic community to help answer questions about the nature of the deposition of cellular material [124-128]. Akin to probabilistic genotyping for identification [129], Dørum et al. (2018) developed a method for probabilistic BFID based on MPS data [130]. The probabilistic method may be an appropriate alternative to the somewhat complicated interpretation strategies mentioned above.

Transfer, persistence, prevalence, and recoverability (TPPR) of mRNA

In general, mRNA is considered relatively unstable compared to DNA [131,132] with some transcripts having a half-life of only 5-10 minutes [133]. Despite a higher resistance to depurination and depyrimidation than DNA, mRNA undergoes hydrolysis, particularly in basic conditions [132,134]. Additionally, mRNAs are subject to degradation by ubiquitous endogenous and exogenous ribonucleases, particularly in post-mortem samples [135]. However, the evaporation of water from stains may decrease microbial and RNase activity, effectively preserving RNA [132].

Researchers have endeavored to provide evidence that despite its volatile reputation, mRNA can be used in a forensic setting. Setzer et al. (2008) performed a time course study evaluating mRNA markers in various environmental conditions up to 547 days using a less sensitive gel electrophoresis technique [136]. Predictably, samples exposed to precipitation were only detected up to 90 days, but some transcripts in each of blood, semen, saliva, and vaginal fluid were detected throughout the 547-day period,

establishing that mRNA of adequate quality and quantity could be recovered from forensically relevant types of samples.

Additional studies were also performed to bolster confidence in mRNA profiling in aged samples. Blood stain samples aged 23 years [131] up to 50 years [137] have been successfully body fluid typed. Blood was even detected in samples recovered from backspatter on the barrel of a rifle collected 40-years previously [138], adhesive tapes taken from the hands for gunshot residue up to 18 years after collection [139], and polyvinyl-alcohol gloves up to 19 years after collection [140]. Nakanishi et al. (2014) was able to detect semen specific transcripts in samples up to 56 years old [141]. Sirker et al. (2016) examined small stains (0.05 – 5 μL) in high humidity (approaching 100%) over 17 months [142]. Although humidity did have a deleterious effect on mRNA, transcripts were detected over the entire 17-month period in 5 μL semen and saliva samples.

The increase in sensitivity of DNA testing has raised important questions regarding the circumstances leading to the TPPR of cellular material along with the donor of the material [124]. Kulstein et al. (2018, 2018) examined laundered blood, saliva, and semen stains [143,144]. Intriguingly, mRNA was most frequently detected in blood and semen samples that had been stored for 30 days before laundering. It was theorized that this time allowed cellular material to become embedded into the fabric and less likely to be removed during the washing process [143,144]. A survey of a variety of public and private items specifically assessing the TPPR of DNA and RNA found that 13% of samples contained traces of residual non-skin body fluids [118]. Though more work is necessary, these studies are important for the most accurate interpretation of evidence [124-128].

European DNA Profiling Group (EDNAP) Exercises

The EDNAP group recognized the need for a collaborative effort to compare mRNA data across laboratories. The group conducted a series of experiments evaluating body fluid specific markers across several different laboratories internationally, specifically including crime laboratories with no prior experience with RNA profiling [101,109,145-148]. This range of analyst experience was included to both evaluate the robustness of methods and protocols as well as identify potential problem areas. In these exercises, participating laboratories were provided with an example protocol, amplification reagents, and pre-prepared samples but were allowed to make modifications based on the laboratories' own resources and include additional in-house samples. Common modifications included the use of different extraction kits, reverse transcription kits, and PCR parameters. The makeup of the collaboration was not static and not all laboratories were involved in all experiments. Studies were generally conducted in a body fluid specific manner, evaluating small multiplexes targeting single body fluids to generate consensus data and help refine marker selection. Variation in results were expected due to the inability to generate truly uniform samples (eg. vaginal and menstrual samples), various experimental modifications, and possible complications from shipping samples and reagents. Experiments included the evaluation of sensitivity, body fluid specificity, human specificity, and casework type samples.

The first study was performed in 2011 and evaluated two blood multiplexes where results were generally concordant [145]. The second study used the same blood multiplexes but stipulated that laboratories perform a DNA/RNA co-extraction and, based on results from the first collaboration, encouraged groups to perform a post-PCR purification step to

increase peak heights and generate a smoother baseline [146]. Although PCR optimization was recommended, results were again generally concordant. Semen and saliva were tested in the third collaboration that also generally reported similar results between laboratories, although instances of cross-reactive saliva markers were noted in vaginal samples [147]. Other reports of non-target fluid amplification of saliva markers have been observed [107,108], although a majority of laboratories in the collaboration and other studies did not observe the cross-reactivity [95]. Samples from a vasectomized male were intentionally included to ensure identification of azoospermic samples could be correctly identified.

The fourth and fifth studies focusing on menstrual blood and vaginal fluid were performed in parallel and all pre-prepared stains were correctly identified by all participants [109]. Special care was taken to prepare stains from different times throughout the menstrual cycle to accommodate potential variation in expression during menstruation. Interestingly, in this iteration of the collaboration, two groups were able to use their in-house developed multiplexes that had been implemented for casework and correctly identified each of the stains provided.

Skin samples, which are viewed as challenging because of their naturally low levels in samples and ubiquity in our surroundings [118], were also investigated in an EDNAP exercise [148]. Compared to other EDNAP studies, these results showed less concordance in detected markers, and the most frequently detected skin markers were also observed in saliva, vaginal, and menstrual samples. Regardless of whether the cross-reactivity is genuinely expressed in saliva or from accidental substrate interaction with skin, this would likely be commonly observed in forensic samples. Overall, caution during interpretation of these markers was recommended if implemented.

The most recent EDNAP exercise, in conjunction with European Forensic Genetics Network (EUROFORGEN), evaluated the use of MPS for BFID [101]. Based on the results of previous EDNAP studies and some preliminary evaluations by the organizing laboratory, markers were combined into panels for use on the Ion Torrent (PGM/S5) and MiSeq MPS platforms. Some variation was noted in the results that was particularly attributed to differences in input RNA due to different quantification methods. Although number of reads varied across platforms and laboratories, stains were correctly identified by the majority of participants. As seen in previous studies, some cross-reactivity of markers between saliva and vaginal fluid were observed. A preliminary interpretation schema using Partial Least Squares analysis was applied to group samples according to similarities and some grouping was observed [101]. Samples with mixed cell types were also analyzed and was found that fluid types with typically lower RNA yields (venous blood, semen, and saliva) may be overshadowed by other fluids in the mixture. The authors state that the performance of the prototype panels will be used to drive panel and library preparation optimization in the future including single nucleotide polymorphisms (SNPs) which may assist in associating a body fluid to a donor [101].

Overall, the EDNAP exercises have given the forensic community evidence that mRNA profiling is a practicable option for BFID. While the forensic community is undoubtedly moving towards MPS, only a few labs have started using MPS for DNA analysis likely because the cost is still prohibitive. Therefore, it would still be beneficial to conduct a collaborative study using a comprehensive multiplex for capillary electrophoresis to encourage additional forensic crime laboratories to adopt this methodology using their existing equipment. A study in this manner may also prompt the

development of more effective interpretation strategies. It was repeatedly stated throughout the collaborations that the scope of the studies did not include interpretation strategies and the role of housekeeping markers were included for qualitative purposes and did not contribute information to body fluid determination [109,145-148]. The exclusion of this aspect is not an inherent flaw in the studies individually; however, the discussion of interpretation strategies across a variety of laboratories may improve the robustness of the technique and the community's confidence in RNA-based BFID methods.

miRNA

miRNA biogenesis and function

MicroRNAs are short non-coding RNAs approximately 18-22 nucleotides long first discovered by Lee et al. in 1993 [149]. DNA sequences that code for miRNAs are located in the genome from either independent genes or from within introns of protein coding genes [150]. Transcription of miRNAs is performed by RNA Polymerase II (Pol II) in the nucleus and processed through two different pathways; canonical or non-canonical [151]. In canonical transcription, the transcript forms a primary pre-cursor (pri-miRNA) hairpin molecule of 70-100 nucleotides with tails at the end. The tails of the pri-miRNA molecule are cleaved by the Droshsa-DGCR8 protein complex to form a precursor hairpin (pre-miRNA) [151]. In non-canonical transcription, the precursor miRNA hairpin (pre-miRNA) is produced through the removal of introns by spliceosomes [151,152]. The pre-miRNA is then exported into the cytoplasm by Exportin 5 where the folded hairpin is cleaved off by the Dicer protein, leaving the miRNA and its complement (miRNA*) duplex approximately 20 nucleotides in length. An Argonaute (Ago) protein then displaces the miRNA* and forms a complex with the mature miRNA. This complex is commonly termed

a miRNA-induced silencing complex (miRISC) [153]. The miRISC complex anneals to mRNA by exposed portions of the mature miRNA termed the seed region [154]. The long-term association with the Ago protein protects the mature miRNA from hydrolysis and RNase activity [155].

Within the cell, miRNAs facilitate gene expression as a post-transcriptional factor through degradation of mRNA and repression of translation (Figure 1.1) [150,151,154]. Complete complementation between the seed region and the mRNA elicits endonucleolytic cleavage and the mRNA is degraded, preventing translation of the mRNA [150,151,154]. However, if the seed region anneals to mRNA and there is incomplete complementation, the Ago protein recruits additional proteins and represses translation by deadenylating the mRNA, providing steric hindrance, or through other mechanisms that remain unclear at this time [151,153,156].

Screening for candidate markers

miRNAs were first proposed as a potential biomarker for BFID by Hanson et al. in 2009 [157]. The group theorized that the expression profiles in terminally differentiated cells would allow analysts to distinguish between body fluids. Using a SYBR RT-qPCR method targeting 452 miRNAs, samples of forensically relevant body fluids including venous blood, menstrual blood, semen, saliva, and vaginal fluid as well as RNA from various human tissues were compared to find markers that may be targeted for forensic BFID use. In this study and the subsequent experiments to identify candidate miRNAs, no miRNAs were found that solely identify the body fluid of interest but suggested a panel of consensus miRNAs in tandem with data analysis metrics for BFID (Table 1.1).

Table 1.1. Candidate body fluid specific markers identified through forensic screening studies. Asterisks (*) indicate markers used to identify blood from non-blood samples. (†) were used as probabilistic markers and not body fluid specific. NS signifies that the fluid was not screened in the study. Markers are human unless designated otherwise.

Body Fluid	Marker	Hanson et al. (2009)	Zubakov et al. (2010)	Courts and Madea (2010)	Wang et al. (2013)	Park et al. (2014)	Sauer et al. (2016)	Seashols-Williams et al. (2016)	Wang et al. (2016)	Li et al. (2017)	Dørum et al. (2019)	Fang et al. (2019)
Venous Blood	miR-16	X			X				X			X
	miR-144		X						X			
	miR-185		X			X						
	miR-126					X						
	miR-150			X								
	miR-126			X					X		X†	
	miR-486				X	X			X		X†	X
	miR-484					X						

(continued)

Body Fluid	Marker	Hanson et al. (2009)	Zubakov et al. (2010)	Courts and Madea (2010)	Wang et al. (2013)	Park et al. (2014)	Sauer et al. (2016)	Seashols-Williams et al. (2016)	Wang et al. (2016)	Li et al. (2017)	Dørum et al. (2019)	Fang et al. (2019)
	miR-182					X						
	miR-200b							X				
	miR-144-3p						X*		X			
	miR-144-5p						X*					
	miR-20a-5p											X
	miR-148a-3p											X
	miR-151a-3p											X
Menstrual	miR-412	X		NS		NS						NS
Blood	miR-214				X							
	miR-1246							X			X†	
	miR-141-3p									X		

(continued)

Body Fluid	Marker	Hanson et al. (2009)	Zubakov et al. (2010)	Courts and Madea (2010)	Wang et al. (2013)	Park et al. (2014)	Sauer et al. (2016)	Seashols-Williams et al. (2016)	Wang et al. (2016)	Li et al. (2017)	Dørum et al. (2019)	Fang et al. (2019)
	miR-497-5p									X		
	miR143-5p									X		
	miR-144-3p						X*					
	miR-144-5p						X*					
Semen	miR-135b	X		NS								
	miR-10b	X					X					NS
	miR-891a		X		X	X	X	X				
	miR-135a		X				X					
	miR-888				X							
	miR-2392					X						
	miR-3197					X						

(continued)

Body Fluid	Marker	Hanson et al. (2009)	Zubakov et al. (2010)	Courts and Madea (2010)	Wang et al. (2013)	Park et al. (2014)	Sauer et al. (2016)	Seashols-Williams et al. (2016)	Wang et al. (2016)	Li et al. (2017)	Dørum et al. (2019)	Fang et al. (2019)
Saliva	miR-205	X		X		X			X			NS
	miR-658	X										
	miR-200c			X					X			
	miR-203			X		X	X		X			
	miR-223					X			X			
	miR-445					X						
	miR-26b							X				
	miR-141-3p								X			
	miR-375								X		X†	
	miR-34a-5p								X			
	let-7c								X			

(continued)

Body Fluid	Marker	Hanson et al. (2009)	Zubakov et al. (2010)	Courts and Madea (2010)	Wang et al. (2013)	Park et al. (2014)	Sauer et al. (2016)	Seashols-Williams et al. (2016)	Wang et al. (2016)	Li et al. (2017)	Dørum et al. (2019)	Fang et al. (2019)
	miR-27b-3p								X			
	miR-125b-5p								X			
	miR-23b-3p								X			
	miR-99a-5p								X			
	miR-29a-3p								X			
	miR-23a-3p								X			
	miR-27a-3p								X			
	miR-210-3p								X			
	miR-24-3p								X			
	miR-29b-3p								X			
	miR-22-3p								X			

(continued)

Body Fluid	Marker	Hanson et al. (2009)	Zubakov et al. (2010)	Courts and Madea (2010)	Wang et al. (2013)	Park et al. (2014)	Sauer et al. (2016)	Seashols-Williams et al. (2016)	Wang et al. (2016)	Li et al. (2017)	Dørum et al. (2019)	Fang et al. (2019)
	miR-203b-5p										X†	
Vaginal	miR-124	X		NS			X					
Material	miR-372	X										NS
	miR-1260b					X						
	miR-645-5p					X						
	miR-200a-3p										X†	
Reference	RNU6b	X		X								
	RNU44	X	X									
	RNU24		X									
	RNU48		X									
	RNU6				X	X				X		

(continued)

Body Fluid	Marker	Hanson et al. (2009)	Zubakov et al. (2010)	Courts and Madea (2010)	Wang et al. (2013)	Park et al. (2014)	Sauer et al. (2016)	Seashols-Williams et al. (2016)	Wang et al. (2016)	Li et al. (2017)	Dørum et al. (2019)	Fang et al. (2019)
	let-7g							X			X	
	let-7i							X			X	
	cer-miR-39											X

After the initial study, several other groups also proposed candidate markers using various platforms and strategies. Zubakov et al. (2010) screened for candidate markers using a custom microarray targeting 718 LNA™-modified oligonucleotides and evaluated the potential markers using RT-qPCR with TaqMan probes [158]. While results between platforms for blood and semen were largely concordant, discrepancies were noted in menstrual blood, vaginal fluid, and saliva results. Upon further investigation, it was suggested that small bacterial/fungal RNAs or pre-cursor miRNAs may have cross-hybridized with specific oligonucleotide probes on the microarray, causing the platform to artificially recognize the miRNAs in question [158]. After also performing sensitivity and a one-year stability study, the group proposed markers for blood and semen (Table 1.1) but did not identify any markers that could be verified by RT-qPCR for the remaining fluids.

In 2011, Courts and Madea performed a similar experiment screening for markers in blood and saliva using a microarray platform targeting approximately 800 miRNAs and then further evaluating those markers with RT-qPCR [159]. Three markers for blood and saliva each were proposed (Table 1.1). Although this study provided beneficial information regarding potential candidate markers, the markers were not evaluated in other forensic body fluids of interest. While these markers may be able to distinguish between blood and saliva, they may have similar expression levels in other fluids, precluding them as a means to distinguish between fluids.

Using a qPCR-array with 754 targets, Wang et al. (2013) screened for markers in venous blood, menstrual blood, semen, saliva, and vaginal fluid [160]. After analyzing the qPCR-array data, giving priority to more highly abundant markers, seven miRNAs were further evaluated using RT-qPCR assays. Candidate markers for venous blood, menstrual

blood, and semen were validated (Table 1.1), including three markers that were also proposed in previous studies. Due to discrepancies between the qPCR-array, RT-qPCR data, and the microarray screening studies, miRNA markers for saliva and vaginal fluid could not be validated. The group also advocated that multiple reference genes are necessary for the application of this technique. However, after extensive analyses of reference genes, no markers tested demonstrated stable expression across all the body fluids tested, but U6 was deemed as a reference gene appropriate for the study [160].

Park et al. (2014) also employed microarray screening and RT-qPCR confirmation techniques [161]. Blood, semen, saliva, and vaginal fluid samples were processed using a microarray targeting 1733 miRNAs. Interestingly, to increase the overall number of candidate markers in the literature, marker proposal criteria prioritized miRNAs that had not been reported in previous studies. Microarray candidates from the study as well as miRNAs previously reported were evaluated by RT-qPCR for sensitivity and accuracy. The study proposed eight novel miRNAs and validated nine previously reported miRNAs (Table 1.1).

Another study using LNA™ modified oligonucleotides in a microarray was performed by Sauer et al. (2016), examining venous blood, menstrual blood, semen, saliva, and vaginal secretions [162]. In addition to prioritizing markers high in abundance or expressed solely in one body fluid, markers were evaluated based on their ability to distinguish between similar markers, particularly between fluids with similar biological components (e.g. epithelial cells in vaginal and saliva samples) or overlapping physical locations (e.g. vaginal and menstrual samples). Relevant literature was also reviewed for marker selection and subsequent RT-qPCR evaluations. Contrary to the previous

recommendation of consensus markers for BFID [157], the authors suggested fewer markers with high discriminatory power because of the complexity of overlapping expression data between fluids (Table 1.1) [162]. A preliminary decision algorithm designed and applied to blinded and casework type samples (ages range up to 37 years) was able to correctly identify all tested single source samples of venous blood, menstrual blood, and semen. Despite the inability of the algorithm to consistently correctly distinguish saliva or vaginal samples or accommodate mixtures, the application of the technique to blinded and aged samples is promising.

More recently, MPS has emerged as a platform available for small RNA sequencing. Unlike microarray and RT-qPCR analysis, MPS does not require any pre-established sequence information necessary for oligonucleotides or primers which may allow for the discovery of novel miRNAs with greater body fluid specificity. Seashols-Williams et al. (2016) used MPS to screen for candidate markers in venous blood, menstrual blood, semen, saliva, vaginal fluid, feces, urine, and sweat [163]. Forensically relevant volumes (200 μL for all fluids except urine) were used in the study to ensure possible targets would be applicable in the small volumes of sample generally seen in evidence submissions. As seen in other studies, the data suggests that a combinatorial approach for markers is necessary for distinguishing between body fluids. With RT-qPCR confirmation, two reference markers and six body fluid specific markers were proposed for venous blood, menstrual blood, semen, saliva, urine, and feces (Table 1.1).

Wang et al. (2016) used the Ion Personal Genome Machine (PGM) for massive parallel sequencing of blood and saliva samples [164]. Targets with the highest abundance with a differential fold change of at least ten were proposed. Out of 2588 unique miRNAs

identified, 25 miRNAs were proposed to distinguish between blood and saliva samples (Table 1.1). However, the study did not perform subsequent RT-qPCR experiments to validate the markers. Additionally, it is unknown whether the markers would maintain specificity compared to other forensically relevant body fluids, as seen in the Courts and Madea (2011) study; nevertheless, the need for similar studies in other fluids and validation was noted [159,164].

Li et al. (2017) performed a microarray screen containing 3100 capture probes focusing on the differences in expression between venous blood and menstrual blood, though semen, vaginal fluid, and saliva samples were also screened for comparative purposes [165]. Five of the candidate markers were then evaluated using both TaqMan probes and SYBR Green RT-qPCR methods to investigate the differences observed between the two chemistries in previous studies. Two of the markers were concordant and three showed significant differences, one of which demonstrated a negative correlation. Further studies included the analysis of menstrual samples collected during various timepoints during the menstrual cycle to ensure that differential expression was still able to distinguish venous blood and menstrual blood. Overall, three markers were proposed to differentiate venous and menstrual blood (designated as menstrual blood markers in Table 1.1) [165].

Using a probabilistic approach, Dørum et al. (2019) screened casework type samples of venous blood, semen, saliva, menstrual blood, and skin [166]. Samples were separated into model training sets and a test set. Partial Least Squares (PLS) and Least Discriminant Analysis (LDA) were applied to determine the markers that were able to provide maximum separability between the body fluid types. In the training set,

components one and two were able to account for 72% of the variation between samples. A series of models were generated with emphasis on either overall accuracy or to identify the fewest number of miRNAs needed that were able to predict stains most accurately. One of the models was able to predict stains with 93% accuracy using nine markers in the training set but dropped to 65% in the test set. Using 100 markers, the model's accuracy was 98% in the training set and 90% in the test set. Current technology may limit the number of markers a laboratory may practically analyze, meaning the nine markers would require three different assays, but MPS would be able to analyze all 100 markers simultaneously. As seen in mRNA studies [98,102], the misidentified samples were saliva and vaginal in origin. Interestingly, seven of the nine markers used in the model (Table 1.1) had been previously identified as candidate markers in other screening studies. The authors stated that the poor results from the test set may be from model overfitting due to a somewhat small sample size. Model overfitting may also be due to the inclusion of both miR-486-5p and miR-486-3p which are the result of two mature miRNAs from a single pre-miRNA. These markers appear to have similar regression values and may be co-expressed [167]. Fecal and urine samples were also included in the test set but not the training set. As the model is only able to predict body fluid origin based on the categories supplied in the training set, the authors hope to include an "unknown" category in future iterations to reduce the likelihood of a false positive and mixed body fluid samples.

Fang et al. (2019) screened blood and bloodstains for candidate markers in compromised samples including freeze/thaw cycles, storage in elevated temperatures, and treated with an oxidant [168]. Candidates were chosen based on the highest abundance present in the samples and confirmed with RT-qPCR (Table 1.1). Samples were normalized

to a spiked-in non-human miRNA that was observed to be stable in all conditions tested. Expression levels were not significantly different between whole blood and blood-stained samples, confirming that results using either of the sample types are comparable. While stable at room and elevated temperatures, repeated freeze/thaw events and the presence of an oxidant significantly reduced expression levels of several candidate markers. The screen identified four previously identified markers and two novel markers; however, as the novel markers were not evaluated in other forensically relevant body fluids, they may not be appropriate for BFID purposes [168].

Based on Table 1.1, it is clearly seen that there is little consensus on candidate markers for BFID using miRNAs. The lack of concordance is not unexpected due to the relatively recent discovery of miRNAs and accompanying technological advancements as well as the physical and biological similarities of the matrices. Vaginal fluid and saliva are both mucosal cells, blood is a component of menstrual blood, and the endometrial lining is shed through the vagina; the overlap in function or physical location is reflected in the lack of definitive markers for BFID. Vaginal and menstrual candidates are further complicated due to natural expression changes throughout the menstrual cycle [169]. In males, fertility status has been associated with differences in semen marker expression [170]. The differences in candidacy are also likely due to many other factors including platform and chemistry differences, the number of donors, and the number of fluids screened.

The most commonly cited theory for candidacy discordance has been the use of different RT-qPCR techniques [158,159,161,163,171,165,172]. Typically, RT-qPCR reactions amplifying miRNAs employ one of two general strategies (Fig. 1.2). The first strategy involves stem-loop reverse transcription primers, followed by PCR using miRNA

specific forward and reverse primers, and is detected via fluorescently labelled hydrolysis probe [173]. The second strategy polyadenylates all small RNAs, oligo-T primers containing a universal tag are used for reverse transcription, PCR primers consist of the complement to the universal tag and a miRNA sequence specific primer, and amplification is detected with an intercalating dye [174]. Dunnett et al. (2014) compared the two strategies using forensically relevant sample sizes and workflows [175]. It was suggested that due to lower abundances detected compared to polyadenylated samples, stem-loop primer-based assays may not be the most appropriate approach for forensic applications [175]. However, as the amplicons were not sequenced, it is unknown whether the higher abundances noted in polyadenylated samples were truly higher or if non-specific amplification was artificially inflating reported abundance. A study examining different RT-qPCR approaches indicated that non-specific amplification was frequently observed after polyadenylation and subsequent amplification, while stem-loop primers demonstrate relatively low reverse transcription efficiency which was then reflected in downstream amplification [176]. Non-specific amplification may be a possible reason miR-658 in saliva was detected by Hanson et al. (2009) but has not been replicated by any other groups [157].

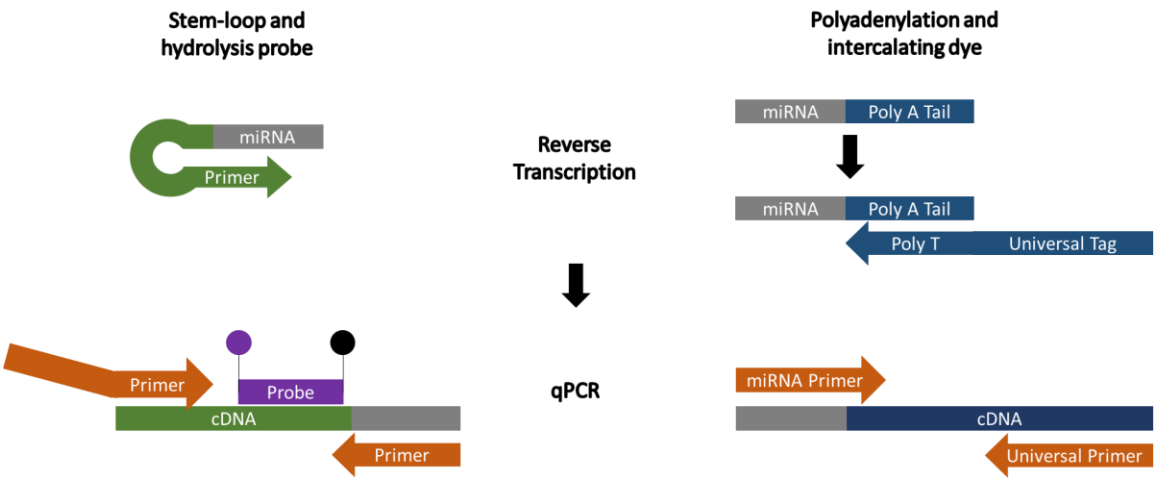

Fig. 1.2. General strategies for RT-qPCR analysis for miRNAs are depicted: stem-loop primers (left) and polyadenylation (right). Colored arrows indicate direction of polymerase travel.

Several studies have examined differences in miRNA expression profiles via different platforms and/or chemistries and some have yielded conflicting results. In one study, RT-qPCR assays were highly correlated with qPCR-assays but had little correlation with microarray results [177], while a different study found that RT-qPCR arrays were generally concordant with each of the four microarrays tested [178]. Similar relative expression ratios, but different absolute quantifications were observed in a study using known amounts of synthetic RNA between microarray and MPS results [179]. Mestagh et al. (2014) performed a comprehensive study examining performance variation in 12 different platforms across RT-qPCR, microarrays, and MPS technologies and found that while differential expression concordance between any two platforms was close to 86%, validation rates averaged only approximately 56%, highlighting the need for cross-platform validation [180]. A majority of the miRNA screening studies for BFID described above confirmed screening results with another platform to verify differential expression.

General challenges to miRNA analysis are also observed due to their small size. In microarray platforms, vastly different melting temperatures and similarity between sequences can complicate probe design [181,182]. The type of probe employed, such as

LNA™, may also factor in to microarray screening, as previously mentioned [158]. Additionally, downstream analysis may be affected by primer efficiencies and inhibitors present in tissues during both reverse transcription, hybridization, and PCR [160,162,180,183,184]. However, as described in Seashols-Williams et al. (2016), the results of each screening are not completely incongruous with each other [163]. For instance, miR-891a, proposed by Zubakov et al. (2011), was not present in other screens as a potential candidate but has shown to be semen specific by several other groups [158,160-163].

The types of body fluids screened simultaneously or the confirmation of marker specificity across fluid types, described several times above, is important for candidate evaluation. Some studies initially evaluated markers based on their abundance [168,171]. Abundance is an important factor because they would be more likely to be detected in low-level or degraded stains, but abundant markers may not necessarily be body fluid specific. Expression profiles are generated based off the expression of markers relative to normalization markers, which varied in the screening studies (Table 1.1). It may be of use to reanalyze available data using different normalization markers to either resolve discordances or strengthen candidate markers.

Forensic studies examining miRNAs

Marker selection is paramount to the implementation of miRNA for BFID, however other studies must also be performed prior to incorporation into a crime laboratory workflow. Several m(i)RNA specific extraction kits are commercially available; however, identity determination is generally a priority in casework and any extraction technique must also recover the most DNA possible in a sample. Any extraction technique must effectively

recover nucleic acids and remove possible inhibitors across different body fluids as well as a variety of substrates that may be submitted as evidence. Omelia et al. (2013) compared DNA and RNA specific spin column based extraction kits and found that a higher abundance of miRNAs were recovered in the DNA extract rather than any washes or from the RNA specific extraction kit, although it is unknown whether the Small RNA protocol was followed for the particular RNA kit [185]. The ability to analyze miRNAs in DNA extracts has interesting implications due to the potential to retest archived DNA extracts and laboratories would not have to purchase, validate, and implement additional extraction kits. Four extraction kits specific to m(i)RNAs and one DNA/RNA co-extraction kit were evaluated by Grabmüller et al. (2013) which were not able to recover sufficient DNA via washes or lysate for casework applications except for the co-extraction kit [186].

A common strategy for interpretation of miRNA profiling systems is based on the relative differences in expression of various miRNAs of samples using real-time PCR systems which are frequently used in forensic laboratories. These differences are calculated via Cq values determined during RT-qPCR and normalized to endogenous reference genes to produce ΔCq values. As with DNA profiling, interpretation problems arise when evidence is degraded, or very small amounts of biological material are available for analysis. Differential expression relies on the amplification of the targets of interest and the normalization marker; however, degradation rates of different cell types may alter differential expression values, and small volume samples may exhibit stochastic effects. The former situation was observed by Sauer et al. (2016) in an aged semen sample in which the semen specific marker was amplified, but the reference marker was not detected [162]. Stochastic effects were theorized as a possible reason that reference markers were not

detected in blood backspatter samples [138]. The potential loss of detection of reference markers reinforces the need for either markers expressed solely in the body fluid of interest, or the incorporation of a data interpretation scheme to accommodate degradation and stochastic effects.

A limitation of quantitative analysis via RT-qPCR as the primary analysis method of BFID using miRNAs is the ability to multiplex [187]. Currently, most instrumentation for qPCR can detect up to five different fluorescent dyes, limiting the number of markers that can be amplified simultaneously. Normalization strategies can include the use of more than one normalization gene thereby further reducing the number of tissue-specific markers analyzed at once [188]. Additional reactions would be required to analyze multiple markers, which increases sample consumption, the risk of contamination, cost of reagents, and time of analysis [189].

Van der Meer et al. (2013) developed a method to co-analyze DNA (identification via STRs) and miRNA with stem-loop primers using capillary electrophoresis, thereby avoiding separate extractions of RNA and DNA [190]. While this method would eliminate a separate analysis, the amplicon sizes in this study (65 – 67 base pairs) were close to both unincorporated primers (typically 20 bp), and the lower range mini-STR amplicons (typically 80 bp) which may limit the number of miRNA markers that can be included in the assay [191]. It is also not well established whether the use of stem-loop primers would result in PCR products that would form hair-pins or anneal to each other and cause PCR artifacts. Li et al. (2014) addressed the potential problems of using stem-loop reverse transcription primers by designing a linear primer set for amplification [187]. While the linear primer system does address the technical challenges observed from the use of stem-

loop primers, only one universal primer for use on one dye channel was designed and tested. As novel markers are continually being discovered for BFID, it would be advantageous to expand the linear primer system to additional dye channels to be able to incorporate more markers.

The use of miRNA for BFID is relatively new and would benefit from additional research in many aspects. As seen in mRNA profiling, development of assays is an iterative process [97,105,123]. The performance of miRNAs in forensic samples is promising, but additional research is necessary including marker validation and TPPR studies.

Statement of the problem

BFID can be of great importance during the course of an investigation and in the courtroom. Determining the body fluid of a stain may provide probative information about the events that took place during the commission of a crime. Current methods for BFID, such as chemical tests, microscopy, enzymatic activity, and immunological tests do not offer the required specificity and results are often open to conjecture [1,2,8,9,11]. Furthermore, current methods are not widely employed to differentiate between menstrual and venous blood (and other forensically relevant fluids). To alleviate these concerns, alternate methods must be considered.

The most efficient use of a sample would be co-extraction and co-analysis methods for both DNA and BFID. DNA offers more probative information (individualization), and is the priority in most cases, but RNA co-analysis from a single sample could provide more information for investigation. Recently, miRNAs have been suggested as a potential biomarker for conclusive BFID and research to date has suggested that these markers meet the required criteria (specificity and lack of sample consumption) [157,190].

The robustness of any new methodology for BFID will also require testing with environmentally challenged samples typically encountered in forensic casework. Any potential biomarker must be able to endure and be detected after exposure to ultraviolet light, heat, pH changes, and humidity for lengths of time to be effective for forensic analysis. These markers must persist in tissue fluids until the evidence is collected and processed. Although previous research has been conducted investigating various miRNA markers for BFID, few studies have directly assessed miRNA stability, persistence, and recoverability in challenging conditions [192]. Additionally, while it has been theorized that miRNAs are more stable than mRNA due to their small size [193], no literature is currently available which directly compares the stability of these molecules.

In order to evaluate the utility and stability of miRNAs in forensically relevant samples, this project will employ several different approaches. The first stage will be the development of a miRNA analysis methodology designed to identify venous blood, menstrual blood, semen, and saliva via capillary electrophoresis (CE) techniques and instrumentation commonly used in forensic genetics laboratories [187]. The second stage will focus on assessing the stability of miRNAs compared to mRNAs using conventional techniques currently observed in the literature. The final approach will incorporate emerging techniques in forensic science, specifically massively parallel sequencing (MPS), using novel markers and specially designed primers to analyze mock samples placed in various environmentally challenging conditions.

References

1. Cox M (1991) A study of the sensitivity and specificity of four presumptive tests for blood. J Forensic Sci 36 (5):1503-1511
2. Webb JL, Creamer JI, Quickenden TI (2006) A comparison of the presumptive luminol test for blood with four non-chemiluminescent forensic techniques. Luminescence 21 (4):214-220. doi:10.1002/bio.908
3. Creamer JI, Quickenden TI, Crichton LB, Robertson P, Ruhayel RA (2005) Attempted cleaning of bloodstains and its effect on the forensic luminol test. Luminescence 20 (6):411-413. doi:10.1002/bio.865
4. Tobe SS, Watson N, Daeid NN (2007) Evaluation of six presumptive tests for blood, their specificity, sensitivity, and effect on high molecular-weight DNA. Journal of forensic sciences 52 (1):102-109
5. Willott GM (1974) An Improved Test for the Detection of Salivary Amylase in Stains. Journal of the Forensic Science Society 14 (4):341-344. doi:10.1016/S0015-7368(74)70923-9
6. Kaye S (1949) Acid phosphatase test for identification of seminal stains. J Lab Clin Med 34 (5):728-732
7. Auvdel MJ (1986) Amylase levels in semen and saliva stains. J Forensic Sci 31 (2):426-431
8. Keating SM, Higgs DF (1994) The detection of amylase on swabs from sexual assault cases. J Forensic Sci Soc 34 (2):89-93

9. Virkler K, Lednev IK (2009) Analysis of body fluids for forensic purposes: from laboratory testing to non-destructive rapid confirmatory identification at a crime scene. Forensic Sci Int 188 (1-3):1-17. doi:10.1016/j.forsciint.2009.02.013

10. Jones Jr E (2005) The identification of semen and other body fluids. Forensic Science Handbook, Prentice Hall, Upper Saddle River, NJ:329-382

11. Harbison S, Fleming R (2016) Forensic body fluid identification: state of the art. Research and Reports in Forensic Medical Science 6:11-23

12. Horjan I, Barbaric L, Mrsic G (2016) Applicability of three commercially available kits for forensic identification of blood stains. J Forensic Leg Med 38:101-105. doi:10.1016/j.jflm.2015.11.021

13. Boward ES, Wilson SL (2013) A comparison of ABAcard® p30 and RSID™-Semen test kits for forensic semen identification. Journal of forensic and legal medicine 20 (8):1126-1130

14. Holtkötter H, Dierig L, Schürenkamp M, Sibbing U, Pfeiffer H, Vennemann M (2015) Validation of an immunochromatographic D-dimer test to presumptively identify menstrual fluid in forensic exhibits. International journal of legal medicine 129 (1):37-41

15. Ohta J, Noda N, Sakurada K (2018) Comparison of Catalytic and Immunological Amylase Tests for Identifying of Saliva from Degraded Samples. J Forensic Sci. doi:10.1111/1556-4029.13936

16. Hochmeister MN, Budowle B, Sparkes R, Rudin O, Gehrig C, Thali M et al. (1999) Validation studies of an immunochromatographic 1-step test for the forensic identification of human blood. J Forensic Sci 44 (3):597-602

17. Johnston S, Newman J, Frappier R (2003) Validation study of the Abacus Diagnostics ABAcard® HemaTrace® membrane test for the forensic identification of human blood. Canadian Society of Forensic Science Journal 36 (3):173-183

18. Schweers BA, Old J, Boonlayangoor P, Reich KA (2008) Developmental validation of a novel lateral flow strip test for rapid identification of human blood (Rapid Stain Identification™-Blood). Forensic Science International: Genetics 2 (3):243-247

19. Old J, Schweers BA, Boonlayangoor PW, Fischer B, Miller KW, Reich K (2012) Developmental Validation of RSID™-Semen: A Lateral Flow Immunochromatographic Strip Test for the Forensic Detection of Human Semen. Journal of forensic sciences 57 (2):489-499

20. Bitner SE (2012) False positives observed on the Seratec® PSA SemiQuant cassette test with condom lubricants. Journal of forensic sciences 57 (6):1545-1548

21. Myers JR, Adkins WK (2008) Comparison of modern techniques for saliva screening. J Forensic Sci 53 (4):862-867. doi:10.1111/j.1556-4029.2008.00755.x

22. Old JB, Schweers BA, Boonlayangoor PW, Reich KA (2009) Developmental Validation of RSID™-Saliva: A Lateral Flow Immunochromatographic Strip Test for the Forensic Detection of Saliva. Journal of forensic sciences 54 (4):866-873

23. Holtkötter H, Dias Filho CR, Schwender K, Stadler C, Vennemann M, Pacheco AC et al. (2018) Forensic differentiation between peripheral and menstrual blood in cases of alleged sexual assault—validating an immunochromatographic multiplex assay for simultaneous detection of human hemoglobin and D-dimer. International journal of legal medicine 132 (3):683-690

24. Akutsu T, Watanabe K, Sakurada K (2012) Specificity, Sensitivity, and Operability of RSID™-Urine for Forensic Identification of Urine: Comparison with ELISA for Tamm-Horsfall Protein. Journal of forensic sciences 57 (6):1570-1573

25. Misencik A, Laux DL (2007) Validation study of the seratec hemdirect hemoglobin assay for the forensic identification of human blood. MAFS Newslett 36 (2):18-26

26. Lunetta P, Sippel H (2009) Positive prostate-specific antigen (PSA) reaction in post-mortem rectal swabs: a cautionary note. J Forensic Leg Med 16 (7):397-399. doi:10.1016/j.jflm.2009.04.002

27. Holtkötter H, Schwender K, Wiegand P, Peiffer H, Vennemann M (2018) Improving body fluid identification in forensic trace evidence—construction of an immunochromatographic test array to rapidly detect up to five body fluids simultaneously. International journal of legal medicine 132 (1):83-90

28. Christoffersen S (2011) Immunohistochemical staining of human sperm cells in smears from sexual assault cases. Int J Legal Med 125 (6):887-890. doi:10.1007/s00414-011-0612-3

29. Herr J (2007) SpermPaint optimization and validation. Report submitted to the US Department of Justice, Washington, DC

30. Miller KW, Old J, Fischer BR, Schweers B, Stipinaite S, Reich K (2011) Developmental Validation of the SPERM HY-LITERTM Kit for the Identification of Human Spermatozoa in Forensic Samples. Journal of forensic sciences 56 (4):853-865

31. Paterson SK, Jensen CG, Vintiner SK, McGlashan SR (2006) Immunohistochemical staining as a potential method for the identification of

vaginal epithelial cells in forensic casework. Journal of forensic sciences 51 (5):1138-1143

32. Sheehan FX, Kobilinsky L (1984) Human blood identification: A forensic science approach. Journal of Chemical Education 61 (6):542

33. Thomas J, Buzzini P, Massonnet G, Reedy B, Roux C (2005) Raman spectroscopy and the forensic analysis of black/grey and blue cotton fibres Part 1: investigation of the effects of varying laser wavelength. Forensic Sci Int 152 (2-3):189-197. doi:10.1016/j.forsciint.2004.08.009

34. Braz A, Lopez-Lopez M, Garcia-Ruiz C (2013) Raman spectroscopy for forensic analysis of inks in questioned documents. Forensic Sci Int 232 (1-3):206-212. doi:10.1016/j.forsciint.2013.07.017

35. Izake EL (2010) Forensic and homeland security applications of modern portable Raman spectroscopy. Forensic Sci Int 202 (1-3):1-8. doi:10.1016/j.forsciint.2010.03.020

36. Hargreaves MD, Page K, Munshi T, Tomsett R, Lynch G, Edwards HG (2008) Analysis of seized drugs using portable Raman spectroscopy in an airport environment—a proof of principle study. Journal of Raman Spectroscopy 39 (7):873-880

37. Virkler K, Lednev IK (2008) Raman spectroscopy offers great potential for the nondestructive confirmatory identification of body fluids. Forensic Sci Int 181 (1-3):e1-5. doi:10.1016/j.forsciint.2008.08.004

38. Virkler K, Lednev IK (2009) Blood species identification for forensic purposes using Raman spectroscopy combined with advanced statistical analysis. Analytical chemistry 81 (18):7773-7777

39. Boyd S, Bertino MF, Seashols SJ (2011) Raman spectroscopy of blood samples for forensic applications. Forensic Sci Int 208 (1-3):124-128. doi:10.1016/j.forsciint.2010.11.012

40. Virkler K, Lednev IK (2009) Raman spectroscopic signature of semen and its potential application to forensic body fluid identification. Forensic Sci Int 193 (1-3):56-62. doi:10.1016/j.forsciint.2009.09.005

41. McLaughlin G, Sikirzhytski V, Lednev IK (2013) Circumventing substrate interference in the Raman spectroscopic identification of blood stains. Forensic Sci Int 231 (1-3):157-166. doi:10.1016/j.forsciint.2013.04.033

42. Sikirzhytskaya A, Sikirzhytski V, McLaughlin G, Lednev IK (2013) Forensic identification of blood in the presence of contaminations using Raman microspectroscopy coupled with advanced statistics: effect of sand, dust, and soil. Journal of forensic sciences 58 (5):1141-1148

43. McLaughlin G, Lednev IK (2015) In Situ Identification of Semen Stains on Common Substrates via Raman Spectroscopy. J Forensic Sci 60 (3):595-604. doi:10.1111/1556-4029.12708

44. Merkley ED, Wunschel DS, Wahl KL, Jarman KH (2019) Applications and challenges of forensic proteomics. Forensic Sci Int 297:350-363. doi:10.1016/j.forsciint.2019.01.022

45. Dammeier S, Nahnsen S, Veit J, Wehner F, Ueffing M, Kohlbacher O (2015) Mass-spectrometry-based proteomics reveals organ-specific expression patterns to be used as forensic evidence. J Proteome Res 15 (1):182-192

46. Van Steendam K, De Ceuleneer M, Dhaenens M, Van Hoofstat D, Deforce D (2013) Mass spectrometry-based proteomics as a tool to identify biological matrices in forensic science. Int J Legal Med 127 (2):287-298

47. Quinones I, Daniel B (2012) Cell free DNA as a component of forensic evidence recovered from touched surfaces. Forensic science international: Genetics 6 (1):26-30

48. Danielson PB (2011) Isolation of Highly Specific Protein Markers for the Identification of Biological Stains: Adapting Comparative Proteomics to Forensics. National Institute of Justice,

49. Legg KM, Powell R, Reisdorph N, Reisdorph R, Danielson PB (2017) Verification of protein biomarker specificity for the identification of biological stains by quadrupole time-of-flight mass spectrometry. Electrophoresis 38 (6):833-845. doi:10.1002/elps.201600352

50. Yang H, Zhou B, Deng H, Prinz M, Siegel D (2013) Body fluid identification by mass spectrometry. Int J Legal Med 127 (6):1065-1077. doi:10.1007/s00414-013-0848-1

51. Parker GJ, Leppert T, Anex DS, Hilmer JK, Matsunami N, Baird L et al. (2016) Demonstration of protein-based human identification using the hair shaft proteome. PloS one 11 (9):e0160653

52. Mason KE, Anex D, Grey T, Hart B, Parker G (2018) Protein-based forensic identification using genetically variant peptides in human bone. Forensic science international 288:89-96

53. Seraglia R, Teatino A, Traldi P (2004) MALDI mass spectrometry in the solution of some forensic problems. Forensic Sci Int 146 Suppl:S83-85. doi:10.1016/j.forsciint.2004.09.031

54. Miranda TB, Jones PA (2007) DNA methylation: the nuts and bolts of repression. J Cell Physiol 213 (2):384-390. doi:10.1002/jcp.21224

55. Hashimshony T, Zhang J, Keshet I, Bustin M, Cedar H (2003) The role of DNA methylation in setting up chromatin structure during development. Nat Genet 34 (2):187-192. doi:10.1038/ng1158

56. Frumkin D, Wasserstrom A, Budowle B, Davidson A (2011) DNA methylation-based forensic tissue identification. Forensic Sci Int Genet 5 (5):517-524. doi:10.1016/j.fsigen.2010.12.001

57. Wasserstrom A, Frumkin D, Davidson A, Shpitzen M, Herman Y, Gafny R (2013) Demonstration of DSI-semen--A novel DNA methylation-based forensic semen identification assay. Forensic Sci Int Genet 7 (1):136-142. doi:10.1016/j.fsigen.2012.08.009

58. Horvath S (2013) DNA methylation age of human tissues and cell types. Genome biology 14 (10):3156

59. Vidaki A, López CD, Carnero-Montoro E, Ralf A, Ward K, Spector T et al. (2017) Epigenetic discrimination of identical twins from blood under the forensic scenario. Forensic Science International: Genetics 31:67-80

60. Vidaki A, Kayser M (2018) Recent progress, methods and perspectives in forensic epigenetics. Forensic Sci Int Genet 37:180-195. doi:https://doi.org/10.1016/j.fsigen.2018.08.008

61. Sijen T (2015) Molecular approaches for forensic cell type identification: On mRNA, miRNA, DNA methylation and microbial markers. Forensic Sci Int Genet 18:21-32. doi:10.1016/j.fsigen.2014.11.015

62. An JH, Choi A, Shin KJ, Yang WI, Lee HY (2013) DNA methylation-specific multiplex assays for body fluid identification. Int J Legal Med 127 (1):35-43. doi:10.1007/s00414-012-0719-1

63. Park JL, Kwon OH, Kim JH, Yoo HS, Lee HC, Woo KM et al. (2014) Identification of body fluid-specific DNA methylation markers for use in forensic science. Forensic Sci Int Genet 13:147-153. doi:10.1016/j.fsigen.2014.07.011

64. Paliwal A, Vaissière T, Herceg Z (2010) Quantitative detection of DNA methylation states in minute amounts of DNA from body fluids. Methods 52 (3):242-247. doi:https://doi.org/10.1016/j.ymeth.2010.03.008

65. Vaissière T, Cuenin C, Paliwal A, Vineis P, Hainaut P, Herceg Z (2009) Quantitative analysis of DNA methylation after whole bisulfitome amplification of a minute amount of DNA from body fluids. Epigenetics 4 (4):221-230

66. Pinard R, de Winter A, Sarkis GJ, Gerstein MB, Tartaro KR, Plant RN et al. (2006) Assessment of whole genome amplification-induced bias through high-throughput, massively parallel whole genome sequencing. BMC genomics 7 (1):216

67. Mill J, Yazdanpanah S, Gückel E, Ziegler S, Kaminsky Z, Petronis A (2006) Whole genome amplification of sodium bisulfite-treated DNA allows the accurate estimate

of methylated cytosine density in limited DNA resources. Biotechniques 41 (5):603-607

68. Naue J, Hoefsloot HCJ, Mook ORF, Rijlaarsdam-Hoekstra L, van der Zwalm MCH, Henneman P et al. (2017) Chronological age prediction based on DNA methylation: Massive parallel sequencing and random forest regression. Forensic Science International: Genetics 31:19-28. doi:https://doi.org/10.1016/j.fsigen.2017.07.015

69. Vidaki A, Ballard D, Aliferi A, Miller TH, Barron LP, Syndercombe Court D (2017) DNA methylation-based forensic age prediction using artificial neural networks and next generation sequencing. Forensic Science International: Genetics 28:225-236. doi:https://doi.org/10.1016/j.fsigen.2017.02.009

70. Vidaki A, Daniel B, Court DS (2013) Forensic DNA methylation profiling—Potential opportunities and challenges. Forensic Science International: Genetics 7 (5):499-507. doi:https://doi.org/10.1016/j.fsigen.2013.05.004

71. Forat S, Huettel B, Reinhardt R, Fimmers R, Haidl G, Denschlag D et al. (2016) Methylation Markers for the Identification of Body Fluids and Tissues from Forensic Trace Evidence. PLOS ONE 11 (2):e0147973. doi:10.1371/journal.pone.0147973

72. Lee HY, An JH, Jung S-E, Oh YN, Lee EY, Choi A et al. (2015) Genome-wide methylation profiling and a multiplex construction for the identification of body fluids using epigenetic markers. Forensic Science International: Genetics 17:17-24

73. Koch CM, Wagner W (2011) Epigenetic-aging-signature to determine age in different tissues. Aging (Albany NY) 3 (10):1018-1027. doi:10.18632/aging.100395

74. Holtkotter H, Beyer V, Schwender K, Glaub A, Johann KS, Schurenkamp M et al. (2017) Independent validation of body fluid-specific CpG markers and construction of a robust multiplex assay. Forensic Sci Int Genet 29:261-268. doi:10.1016/j.fsigen.2017.05.002

75. Fujiwara N, Shiki T, Masuda S (2012) mRNA Biogenesis in the Nucleus and Its Export to the Cytoplasm. In: Current Frontiers and Perspectives in Cell Biology. InTech,

76. Conaway RC, Conaway JW (2011) Function and regulation of the Mediator complex. Curr Opin Genet Dev 21 (2):225-230. doi:10.1016/j.gde.2011.01.013

77. Hirose Y, Manley JL (2000) RNA polymerase II and the integration of nuclear events. Genes Dev 14 (12):1415-1429

78. Bienroth S, Keller W, Wahle E (1993) Assembly of a processive messenger RNA polyadenylation complex. EMBO J 12 (2):585-594. doi:10.1002/j.1460-2075.1993.tb05690.x

79. Proudfoot NJ (2016) Transcriptional termination in mammals: Stopping the RNA polymerase II juggernaut. Science 352 (6291):aad9926. doi:10.1126/science.aad9926

80. Köhler A, Hurt E (2007) Exporting RNA from the nucleus to the cytoplasm. Nature reviews Molecular cell biology 8 (10):761

81. Fasken MB, Corbett AH (2005) Process or perish: quality control in mRNA biogenesis. Nat Struct Mol Biol 12 (6):482-488. doi:10.1038/nsmb945

82. Livingstone M, Atas E, Meller A, Sonenberg N (2010) Mechanisms governing the control of mRNA translation. Phys Biol 7 (2):021001. doi:10.1088/1478-3975/7/2/021001

83. Bauer M, Kraus A, Patzelt D (1999) Detection of epithelial cells in dried blood stains by reverse transcriptase-polymerase chain reaction. J Forensic Sci 44 (6):1232-1236

84. Matsubara Y, Ikeda H, Endo H, Narisawa K (1992) Dried blood spot on filter paper as a source of mRNA. Nucleic acids research 20 (8):1998

85. Venter JC, Adams MD, Myers EW, Li PW, Mural RJ, Sutton GG et al. (2001) The sequence of the human genome. Science 291 (5507):1304-1351. doi:10.1126/science.1058040

86. Riggins GJ, Strausberg RL (2001) Genome and genetic resources from the Cancer Genome Anatomy Project. Hum Mol Genet 10 (7):663-667

87. Bauer M, Patzelt D (2002) Evaluation of mRNA markers for the identification of menstrual blood. J Forensic Sci 47 (6):1278-1282

88. Juusola J, Ballantyne J (2003) Messenger RNA profiling: a prototype method to supplant conventional methods for body fluid identification. Forensic Sci Int 135 (2):85-96. doi:10.1016/S0379-0738(03)00197-X

89. Visser M, Zubakov D, Ballantyne KN, Kayser M (2011) mRNA-based skin identification for forensic applications. Int J Legal Med 125 (2):253-263. doi:10.1007/s00414-010-0545-2

90. Bauer M, Patzelt D (2003) Protamine mRNA as molecular marker for spermatozoa in semen stains. Int J Legal Med 117 (3):175-179. doi:10.1007/s00414-002-0347-2

91. Zubakov D, Hanekamp E, Kokshoorn M, van Ijcken W, Kayser M (2008) Stable RNA markers for identification of blood and saliva stains revealed from whole genome expression analysis of time-wise degraded samples. Int J Legal Med 122 (2):135-142. doi:10.1007/s00414-007-0182-6

92. Zubakov D, Kokshoorn M, Kloosterman A, Kayser M (2009) New markers for old stains: stable mRNA markers for blood and saliva identification from up to 16-year-old stains. Int J Legal Med 123 (1):71-74. doi:10.1007/s00414-008-0249-z

93. Juusola J, Ballantyne J (2005) Multiplex mRNA profiling for the identification of body fluids. Forensic Sci Int 152 (1):1-12. doi:10.1016/j.forsciint.2005.02.020

94. Fleming RI, Harbison S (2010) The development of a mRNA multiplex RT-PCR assay for the definitive identification of body fluids. Forensic Sci Int Genet 4 (4):244-256. doi:10.1016/j.fsigen.2009.10.006

95. Lindenbergh A, de Pagter M, Ramdayal G, Visser M, Zubakov D, Kayser M et al. (2012) A multiplex (m)RNA-profiling system for the forensic identification of body fluids and contact traces. Forensic Sci Int Genet 6 (5):565-577. doi:10.1016/j.fsigen.2012.01.009

96. Sakurada K, Ikegaya H, Fukushima H, Akutsu T, Watanabe K, Yoshino M (2009) Evaluation of mRNA-based approach for identification of saliva and semen. Leg Med (Tokyo) 11 (3):125-128. doi:10.1016/j.legalmed.2008.10.002

97. van den Berge M, Bhoelai B, Harteveld J, Matai A, Sijen T (2016) Advancing forensic RNA typing: On non-target secretions, a nasal mucosa marker, a differential co-extraction protocol and the sensitivity of DNA and RNA profiling. Forensic Sci Int Genet 20:119-129. doi:10.1016/j.fsigen.2015.10.011

98. Xu Y, Xie J, Cao Y, Zhou H, Ping Y, Chen L et al. (2014) Development of highly sensitive and specific mRNA multiplex system (XCYR1) for forensic human body fluids and tissues identification. PLoS One 9 (7):e100123. doi:10.1371/journal.pone.0100123

99. Roeder AD, Haas C (2013) mRNA profiling using a minimum of five mRNA markers per body fluid and a novel scoring method for body fluid identification. Int J Legal Med 127 (4):707-721. doi:10.1007/s00414-012-0794-3

100. Richard ML, Harper KA, Craig RL, Onorato AJ, Robertson JM, Donfack J (2012) Evaluation of mRNA marker specificity for the identification of five human body fluids by capillary electrophoresis. Forensic Sci Int Genet 6 (4):452-460. doi:10.1016/j.fsigen.2011.09.007

101. Ingold S, Dørum G, Hanson E, Berti A, Branicki W, Brito P et al. (2018) Body fluid identification using a targeted mRNA massively parallel sequencing approach–results of a EUROFORGEN/EDNAP collaborative exercise. Forensic Sci Int Genet 34:105-115

102. Sakurada K, Akutsu T, Watanabe K, Miyasaka S, Kasai K (2013) Identification of Body Fluid Stains Using Real-time RT-PCR: Discrimination Between Salivary, Nasal, and Vaginal Secretions. Jpn J Forensic Sci Tech 18 (1):1-11. doi:10.3408/jafst.18.1

103. Sakurada K, Akutsu T, Watanabe K, Fujinami Y, Yoshino M (2011) Expression of statherin mRNA and protein in nasal and vaginal secretions. Leg Med (Tokyo) 13 (6):309-313. doi:10.1016/j.legalmed.2011.07.002

104. Lin MH, Jones DF, Fleming R (2015) Transcriptomic analysis of degraded forensic body fluids. Forensic Sci Int Genet 17:35-42. doi:10.1016/j.fsigen.2015.03.005

105. Hanson E, Ingold S, Haas C, Ballantyne J (2015) Targeted multiplexed next generation RNA sequencing assay for tissue source determination of forensic samples. Forensic Sci Int Genet Suppl Ser 5:E441-E443. doi:10.1016/j.fsigss.2015.09.175

106. Haas C, Klesser B, Maake C, Bar W, Kratzer A (2009) mRNA profiling for body fluid identification by reverse transcription endpoint PCR and realtime PCR. Forensic Sci Int Genet 3 (2):80-88. doi:10.1016/j.fsigen.2008.11.003

107. Nussbaumer C, Gharehbaghi-Schnell E, Korschineck I (2006) Messenger RNA profiling: a novel method for body fluid identification by real-time PCR. Forensic Sci Int 157 (2-3):181-186. doi:10.1016/j.forsciint.2005.10.009

108. Cossu C, Germann U, Kratzer A, Bär W, Haas C (2009) How specific are the vaginal secretion mRNA-markers HBD1 and MUC4? Forensic Sci Int Genet Suppl Ser 2 (1):536-537. doi:10.1016/j.fsigss.2009.08.063

109. Haas C, Hanson E, Anjos MJ, Ballantyne KN, Banemann R, Bhoelai B et al. (2014) RNA/DNA co-analysis from human menstrual blood and vaginal secretion stains: results of a fourth and fifth collaborative EDNAP exercise. Forensic Sci Int Genet 8 (1):203-212. doi:10.1016/j.fsigen.2013.09.009

110. Juusola J, Ballantyne J (2007) mRNA profiling for body fluid identification by multiplex quantitative RT-PCR. J Forensic Sci 52 (6):1252-1262. doi:10.1111/j.1556-4029.2007.00550.x

111. Fleming RF BH, Fallow MV, Simon PM, Stacey JK, Wivell RJ and Harbison, SA Implementation of Messenger RNA Body Fluid Testing in Forensic Case Work. In: International Symposium on Human Identification, 2011.

112. Fleming RI, Harbison S (2010) The use of bacteria for the identification of vaginal secretions. Forensic Sci Int Genet 4 (5):311-315. doi:10.1016/j.fsigen.2009.11.008

113. Benschop CC, Quaak FC, Boon ME, Sijen T, Kuiper I (2012) Vaginal microbial flora analysis by next generation sequencing and microarrays; can microbes indicate vaginal origin in a forensic context? Int J Legal Med 126 (2):303-310. doi:10.1007/s00414-011-0660-8

114. Stafford-Allen B, Dawnay N, Hanson EK, Ball G, Gupta A, Blackman S et al. (2018) Development of HyBeacon® probes for specific mRNA detection using body fluids as a model system. Molecular and Cellular Probes 38:51-59. doi:https://doi.org/10.1016/j.mcp.2017.11.007

115. Blackman S, Stafford-Allen B, Hanson EK, Panasiuk M, Brooker A-L, Rendell P et al. (2018) Developmental validation of the ParaDNA® Body Fluid ID System—A rapid multiplex mRNA-profiling system for the forensic identification of body fluids. Forensic Science International: Genetics 37:151-161. doi:https://doi.org/10.1016/j.fsigen.2018.08.012

116. Lindenbergh A, Maaskant P, Sijen T (2013) Implementation of RNA profiling in forensic casework. Forensic Sci Int Genet 7 (1):159-166. doi:10.1016/j.fsigen.2012.09.003

117. van den Berge M, Wiskerke D, Gerretsen RR, Tabak J, Sijen T (2016) DNA and RNA profiling of excavated human remains with varying postmortem intervals. Int J Legal Med 130 (6):1471-1480. doi:10.1007/s00414-016-1438-9

118. van den Berge M, Ozcanhan G, Zijlstra S, Lindenbergh A, Sijen T (2016) Prevalence of human cell material: DNA and RNA profiling of public and private objects and after activity scenarios. Forensic Sci Int Genet 21:81-89. doi:10.1016/j.fsigen.2015.12.012

119. Sakurada K, Akutsu T, Watanabe K, Yoshino M (2012) Identification of nasal blood by real-time RT-PCR. Leg Med (Tokyo) 14 (4):201-204. doi:10.1016/j.legalmed.2012.01.014

120. Harteveld J, Lindenbergh A, Sijen T (2013) RNA cell typing and DNA profiling of mixed samples: can cell types and donors be associated? Sci Justice 53 (3):261-269. doi:10.1016/j.scijus.2013.02.001

121. Whitehead A, Crawford DL (2005) Variation in tissue-specific gene expression among natural populations. Genome Biol 6 (2):R13. doi:10.1186/gb-2005-6-2-r13

122. Lin MH, Albani PP, Fleming R (2016) Degraded RNA transcript stable regions (StaRs) as targets for enhanced forensic RNA body fluid identification. Forensic Sci Int Genet 20:61-70. doi:10.1016/j.fsigen.2015.09.012

123. Albani PP, Fleming R (2018) Novel messenger RNAs for body fluid identification. Sci Justice 58 (2):145-152. doi:10.1016/j.scijus.2017.09.002

124. Biedermann A, Champod C, Jackson G, Gill P, Taylor D, Butler J et al. (2016) Evaluation of Forensic DNA Traces When Propositions of Interest Relate to Activities: Analysis and Discussion of Recurrent Concerns. Front Genet 7 (215):215. doi:10.3389/fgene.2016.00215

125. Taroni F, Biedermann A, Garbolino P, Aitken CG (2004) A general approach to Bayesian networks for the interpretation of evidence. Forensic Sci Int 139 (1):5-16. doi:https://doi.org/10.1016/j.forsciint.2003.08.004

126. Taylor D, Biedermann A, Hicks T, Champod C (2018) A template for constructing Bayesian networks in forensic biology cases when considering activity level propositions. Forensic Sci Int Genet 33:136-146. doi:10.1016/j.fsigen.2017.12.006

127. Curran JM, Triggs CM, Buckleton JS, Walsh K, Hicks T (1998) Assessing transfer probabilities in a Bayesian interpretation of forensic glass evidence. Sci Justice 38 (1):15-21. doi:https://doi.org/10.1016/S1355-0306(98)72068-4

128. Cook R, Evett IW, Jackson G, Jones PJ, Lambert JA (1998) A hierarchy of propositions: deciding which level to address in casework. Sci Justice 38 (4):231-239. doi:Doi 10.1016/S1355-0306(98)72117-3

129. Coble MD, Bright J-A (2019) Probabilistic genotyping software: An overview. Forensic Sci Int Genet 38:219-224. doi:https://doi.org/10.1016/j.fsigen.2018.11.009

130. Dørum G, Ingold S, Hanson E, Ballantyne J, Snipen L, Haas C (2018) Predicting the origin of stains from next generation sequencing mRNA data. Forensic Sci Int Genet 34:37-48. doi:10.1016/j.fsigen.2018.01.001

131. Kohlmeier F, Schneider PM (2012) Successful mRNA profiling of 23 years old blood stains. Forensic Sci Int Genet 6 (2):274-276. doi:10.1016/j.fsigen.2011.04.007

132. Fordyce SL, Kampmann M-L, Van Doorn NL, Gilbert MTP (2013) Long-term RNA persistence in post mortem contexts. Investig Genet 4 (7)

133. Guhaniyogi J, Brewer G (2001) Regulation of mRNA stability in mammalian cells. Gene 265 (1-2):11-23. doi:https://doi.org/10.1016/S0378-1119(01)00350-X

134. Brown D, Todd A (1952) 13. Nucleotides. Part X. Some observations on the structure and chemical behaviour of the nucleic acids. Journal of the Chemical Society (Resumed):52-58

135. Bauer M, Gramlich I, Polzin S, Patzelt D (2003) Quantification of mRNA degradation as possible indicator of postmortem interval—a pilot study. Legal Medicine 5 (4):220-227. doi:10.1016/j.legalmed.2003.08.001

136. Setzer M, Juusola J, Ballantyne J (2008) Recovery and stability of RNA in vaginal swabs and blood, semen, and saliva stains. J Forensic Sci 53 (2):296-305. doi:10.1111/j.1556-4029.2007.00652.x

137. Zhao H, Wang C, Yao L, Lin Q, Xu X, Hu L et al. (2017) Identification of aged bloodstains through mRNA profiling: Experiments results on selected markers of 30- and 50-year-old samples. Forensic Sci Int 272:e1-e6. doi:10.1016/j.forsciint.2017.01.006

138. Grabmuller M, Schyma C, Euteneuer J, Madea B, Courts C (2015) Simultaneous analysis of nuclear and mitochondrial DNA, mRNA and miRNA from backspatter

from inside parts of firearms generated by shots at "triple contrast" doped ballistic models. Forensic Sci Med Pathol 11 (3):365-375. doi:10.1007/s12024-015-9695-3

139. Grabmuller M, Schyma C, Madea B, Eichhorst T, Courts C (2017) RNA/DNA co-analysis on aged bloodstains from adhesive tapes used for gunshot residue collection from hands. Forensic Sci Med Pathol 13 (2):161-169. doi:10.1007/s12024-017-9864-7

140. Grabmuller M, Courts C, Madea B, Eichhorst T, Schyma C (2018) RNA/DNA co-analysis from bloodstains on aged polyvinyl-alcohol gloves prepared for securing evidence from the hands of victims of fatal gunshot injuries. Int J Legal Med 132 (1):53-66. doi:10.1007/s00414-017-1687-2

141. Nakanishi H, Hara M, Takahashi S, Takada A, Saito K (2014) Evaluation of forensic examination of extremely aged seminal stains. Leg Med (Tokyo) 16 (5):303-307. doi:10.1016/j.legalmed.2014.04.002

142. Sirker M, Schneider PM, Gomes I (2016) A 17-month time course study of human RNA and DNA degradation in body fluids under dry and humid environmental conditions. Int J Legal Med 130 (6):1431-1438. doi:10.1007/s00414-016-1373-9

143. Kulstein G, Wiegand P (2018) Comprehensive examination of conventional and innovative body fluid identification approaches and DNA profiling of laundered blood- and saliva-stained pieces of cloths. Int J Legal Med 132 (1):67-81. doi:10.1007/s00414-017-1691-6

144. Kulstein G, Schacker U, Wiegand P (2018) Old meets new: Comparative examination of conventional and innovative RNA-based methods for body fluid

identification of laundered seminal fluid stains after modular extraction of DNA and RNA. Forensic Sci Int Genet 36:130-140. doi:10.1016/j.fsigen.2018.06.017

145. Haas C, Hanson E, Bär W, Banemann R, Bento A, Berti A et al. (2011) mRNA profiling for the identification of blood—results of a collaborative EDNAP exercise. Forensic Sci Int Genet 5 (1):21-26

146. Haas C, Hanson E, Anjos M, Bär W, Banemann R, Berti A et al. (2012) RNA/DNA co-analysis from blood stains—results of a second collaborative EDNAP exercise. Forensic Sci Int Genet 6 (1):70-80

147. Haas C, Hanson E, Anjos M, Banemann R, Berti A, Borges E et al. (2013) RNA/DNA co-analysis from human saliva and semen stains–results of a third collaborative EDNAP exercise. Forensic Sci Int Genet 7 (2):230-239

148. Haas C, Hanson E, Banemann R, Bento A, Berti A, Carracedo Á et al. (2015) RNA/DNA co-analysis from human skin and contact traces–results of a sixth collaborative EDNAP exercise. Forensic Sci Int Genet 16:139-147

149. Lee RC, Feinbaum RL, Ambros V (1993) The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. Cell 75 (5):843-854

150. Carthew RW, Sontheimer EJ (2009) Origins and Mechanisms of miRNAs and siRNAs. Cell 136 (4):642-655. doi:10.1016/j.cell.2009.01.035

151. Krol J, Loedige I, Filipowicz W (2010) The widespread regulation of microRNA biogenesis, function and decay. Nat Rev Genet 11 (9):597-610. doi:10.1038/nrg2843

152. Westholm JO, Lai EC (2011) Mirtrons: microRNA biogenesis via splicing. Biochimie 93 (11):1897-1904. doi:10.1016/j.biochi.2011.06.017

153. Pratt AJ, MacRae IJ (2009) The RNA-induced silencing complex: a versatile gene-silencing machine. J Biol Chem 284 (27):17897-17901. doi:10.1074/jbc.R900012200

154. Du T, Zamore PD (2005) microPrimer: the biogenesis and function of microRNA. Development 132 (21):4645-4652. doi:10.1242/dev.02070

155. Arroyo JD, Chevillet JR, Kroh EM, Ruf IK, Pritchard CC, Gibson DF et al. (2011) Argonaute2 complexes carry a population of circulating microRNAs independent of vesicles in human plasma. Proceedings of the National Academy of Sciences 108 (12):5003-5008

156. Filipowicz W, Bhattacharyya SN, Sonenberg N (2008) Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? Nat Rev Genet 9 (2):102-114. doi:10.1038/nrg2290

157. Hanson EK, Lubenow H, Ballantyne J (2009) Identification of forensically relevant body fluids using a panel of differentially expressed microRNAs. Anal Biochem 387 (2):303-314. doi:10.1016/j.ab.2009.01.037

158. Zubakov D, Boersma AW, Choi Y, van Kuijk PF, Wiemer EA, Kayser M (2010) MicroRNA markers for forensic body fluid identification obtained from microarray screening and quantitative RT-PCR confirmation. Int J Legal Med 124 (3):217-226. doi:10.1007/s00414-009-0402-3

159. Courts C, Madea B (2011) Specific micro-RNA signatures for the detection of saliva and blood in forensic body-fluid identification. J Forensic Sci 56 (6):1464-1470. doi:10.1111/j.1556-4029.2011.01894.x

160. Wang Z, Zhang J, Luo H, Ye Y, Yan J, Hou Y (2013) Screening and confirmation of microRNA markers for forensic body fluid identification. Forensic Sci Int Genet 7 (1):116-123. doi:10.1016/j.fsigen.2012.07.006

161. Park JL, Park SM, Kwon OH, Lee HC, Kim JY, Seok HH et al. (2014) Microarray screening and qRT-PCR evaluation of microRNA markers for forensic body fluid identification. Electrophoresis 35 (21-22):3062-3068. doi:10.1002/elps.201400075

162. Sauer E, Reinke AK, Courts C (2016) Differentiation of five body fluids from forensic samples by expression analysis of four microRNAs using quantitative PCR. Forensic Sci Int Genet 22:89-99. doi:10.1016/j.fsigen.2016.01.018

163. Seashols-Williams S, Lewis C, Calloway C, Peace N, Harrison A, Hayes-Nash C et al. (2016) High-throughput miRNA sequencing and identification of biomarkers for forensically relevant biological fluids. Electrophoresis 37 (21):2780-2788. doi:10.1002/elps.201600258

164. Wang Z, Zhou D, Cao Y, Hu Z, Zhang S, Bian Y et al. (2016) Characterization of microRNA expression profiles in blood and saliva using the Ion Personal Genome Machine® System (Ion PGM™ System). Forensic Sci Int Genet 20:140-146

165. Li Z, Bai P, Peng D, Wang H, Guo Y, Jiang Y et al. (2017) Screening and confirmation of microRNA markers for distinguishing between menstrual and peripheral blood. Forensic Sci Int Genet 30:24-33. doi:10.1016/j.fsigen.2017.05.012

166. Dorum G, Ingold S, Hanson E, Ballantyne J, Russo G, Aluri S et al. (2019) Predicting the origin of stains from whole miRNome massively parallel sequencing data. Forensic Sci Int Genet 40:131-139. doi:10.1016/j.fsigen.2019.02.015

167. Huang CJ, Nguyen PN, Choo KB, Sugii S, Wee K, Cheong SK et al. (2014) Frequent co-expression of miRNA-5p and -3p species and cross-targeting in induced pluripotent stem cells. Int J Med Sci 11 (8):824-833. doi:10.7150/ijms.8358

168. Fang C, Zhao J, Li JB, Qian JL, Liu X, Sun QF et al. (2019) Massively parallel sequencing of microRNA in bloodstains and evaluation of environmental influences on miRNA candidates using realtime polymerase chain reaction. Forensic Sci Int Genet 38:32-38. doi:10.1016/j.fsigen.2018.10.001

169. Bexon K, Williams G (2015) Characterising the fluctuation of microRNA expression throughout a full menstrual cycle. Forensic Sci Int Genet Suppl Ser 5:E264-E266. doi:10.1016/j.fsigss.2015.09.105

170. Tian H, Lv M, Li Z, Peng D, Tan Y, Wang H et al. (2018) Semen-specific miRNAs: Suitable for the distinction of infertile semen in the body fluid identification? Forensic Sci Int Genet 33:161-167. doi:10.1016/j.fsigen.2017.12.010

171. Wang Z, Zhou D, Cao Y, Hu Z, Zhang S, Bian Y et al. (2016) Characterization of microRNA expression profiles in blood and saliva using the Ion Personal Genome Machine((R)) System (Ion PGM System). Forensic Sci Int Genet 20:140-146. doi:10.1016/j.fsigen.2015.10.008

172. Li ZL, Bai P, Peng D, Long B, Zhang L, Liang WB (2015) Influences of different RT-qPCR methods on forensic body fluid identification by microRNA. Forensic Sci Int Genet Suppl Ser 5:E295-E297. doi:10.1016/j.fsigss.2015.09.117

173. Chen C, Ridzon DA, Broomer AJ, Zhou Z, Lee DH, Nguyen JT et al. (2005) Real-time quantification of microRNAs by stem-loop RT-PCR. Nucleic Acids Res 33 (20):e179. doi:10.1093/nar/gni178

174. Fu HJ, Zhu J, Yang M, Zhang ZY, Tie Y, Jiang H et al. (2006) A novel method to monitor the expression of microRNAs. Mol Biotechnol 32 (3):197-204. doi:10.1385/MB:32:3:197

175. Dunnett H, van der Meer D, Williams GA (2014) Evaluation of stem-loop reverse transcription and poly-A tail extension in microRNA analysis of body fluids. MicroRNA 3 (3):150-154

176. Kang K, Zhang XY, Liu HT, Wang ZW, Zhong JS, Huang ZT et al. (2012) A Novel Real-Time PCR Assay of microRNAs Using S-Poly(T), a Specific Oligo(dT) Reverse Transcription Primer with Excellent Sensitivity and Specificity. Plos One 7 (11):e48536. doi:10.1371/journal.pone.0048536

177. Chen Y, Gelfond JA, McManus LM, Shireman PK (2009) Reproducibility of quantitative RT-PCR array in miRNA expression profiling and comparison with microarray analysis. BMC Genomics 10 (1):407. doi:10.1186/1471-2164-10-407

178. Yauk CL, Rowan-Carroll A, Stead JD, Williams A (2010) Cross-platform analysis of global microRNA expression technologies. BMC Genomics 11 (1):330. doi:10.1186/1471-2164-11-330

179. Willenbrock H, Salomon J, Sokilde R, Barken KB, Hansen TN, Nielsen FC et al. (2009) Quantitative miRNA expression analysis: comparing microarrays with next-generation sequencing. RNA 15 (11):2028-2034. doi:10.1261/rna.1699809

180. Mestdagh P, Hartmann N, Baeriswyl L, Andreasen D, Bernard N, Chen C et al. (2014) Evaluation of quantitative miRNA expression platforms in the microRNA quality control (miRQC) study. Nat Methods 11 (8):809-815. doi:10.1038/nmeth.3014

181. Saba R, Booth SA (2006) Target labelling for the detection and profiling of microRNAs expressed in CNS tissue using microarrays. BMC Biotechnol 6 (1):47

182. Git A, Dvinge H, Salmon-Divon M, Osborne M, Kutter C, Hadfield J et al. (2010) Systematic comparison of microarray profiling, real-time PCR, and next-generation sequencing technologies for measuring differential microRNA expression. RNA 16 (5):991-1006. doi:10.1261/rna.1947110

183. Wang Z, Luo H, Pan X, Liao M, Hou Y (2012) A model for data analysis of microRNA expression in forensic body fluid identification. Forensic Sci Int Genet 6 (3):419-423. doi:10.1016/j.fsigen.2011.08.008

184. Sauer E, Madea B, Courts C (2014) An evidence based strategy for normalization of quantitative PCR data from miRNA expression analysis in forensically relevant body fluids. Forensic Sci Int Genet 11:174-181. doi:10.1016/j.fsigen.2014.03.011

185. Omelia EJ, Uchimoto ML, Williams G (2013) Quantitative PCR analysis of blood- and saliva-specific microRNA markers following solid-phase DNA extraction. Anal Biochem 435 (2):120-122. doi:10.1016/j.ab.2012.12.024

186. Grabmuller M, Madea B, Courts C (2015) Comparative evaluation of different extraction and quantification methods for forensic RNA analysis. Forensic Sci Int Genet 16:195-202. doi:10.1016/j.fsigen.2015.01.006

187. Li Y, Zhang J, Wei W, Wang Z, Prinz M, Hou Y (2014) A strategy for co-analysis of microRNAs and DNA. Forensic Sci Int Genet 12:24-29. doi:10.1016/j.fsigen.2014.04.011

188. Sauer E, Reinke AK, Courts C (2015) Validation of forensic body fluid identification based on empirically normalized miRNA expression data. Forensic Sci Int Genet Suppl Ser 5:E462-E464. doi:10.1016/j.fsigss.2015.09.183

189. van der Meer DJ, Williams GA (2015) Performing body fluid identification with microRNAs using capillary electrophoresis. Forensic Sci Int Genet Suppl Ser

190. van der Meer D, Uchimoto ML, Williams G (2013) Simultaneous analysis of micro-RNA and DNA for determining the body fluid origin of DNA profiles. J Forensic Sci 58 (4):967-971. doi:10.1111/1556-4029.12160

191. Williams G, Uchimoto ML, Coult N, World D, Beasley E, Avenell P (2013) Characterisation of body fluid specific microRNA markers by capillary electrophoresis. Forensic Sci Int Genet Suppl Ser 4 (1):e274-e275

192. Silva SS, Lopes C, Teixeira AL, Carneiro de Sousa MJ, Medeiros R (2015) Forensic miRNA: potential biomarker for body fluids? Forensic Sci Int Genet 14:1-10. doi:10.1016/j.fsigen.2014.09.002

193. Arroyo JD, Chevillet JR, Kroh EM, Ruf IK, Pritchard CC, Gibson DF et al. (2011) Argonaute2 complexes carry a population of circulating microRNAs independent of vesicles in human plasma. Proc Natl Acad Sci U S A 108 (12):5003-5008. doi:10.1073/pnas.1019055108

CHAPTER II

A capillary electrophoresis method for identifying forensically relevant body fluids using miRNAs[1]

This dissertation follows the style and format of *International Journal of Legal Medicine*.

[1] Mayes C, Seashols-Williams S, Hughes-Stamm S. (2018) A capillary electrophoresis method for identifying forensically relevant body fluids using miRNAs. Legal Med (Tokyo, Japan) 30:1-4. https://doi.org/10.1016/j.legalmed.2017.10.013

Abstract

Body fluid identification (BFID) can provide crucial information during the course of an investigation. In recent years, microRNAs (miRNAs) have shown considerable body fluid specificity, are able to be co-extracted with DNA, and their small size (18-25 nucleotides) make them ideal for analyzing highly degraded forensic samples. In this study, we designed a preliminary 8-marker system for BFID including an endogenous reference gene (let-7g) to differentiate between venous blood (miR-451a and miR-142-3p), menstrual blood (miR-141-3p and miR-412-3p), semen (miR-891a and miR-10b), and saliva (miR-205) using a capillary electrophoresis approach. This panel uses a linear primer system in order to incorporate additional miRNA markers by forming a multiplex system. The miRNA system was able to distinguish between venous blood, menstrual blood, semen, and saliva using a rudimentary data interpretation strategy. All STR amplifications from co-extracted DNA yielded complete profiles from human identification purposes.

Keywords: Forensic genetics; microRNAs; capillary electrophoresis; body fluid identification

Introduction

Body fluid identification (BFID) can be of importance during the course of an investigation and in the courtroom. Determining the origin of a stain may provide probative information about the events that took place during the commission of a crime. Current methods for BFID such as chemical tests, microscopy, enzymatic activity, and immunological tests do not offer the level of specificity or sensitivity required by the forensic field, and results from these methods are often subject to doubt [1-5].

Recently, microRNAs (miRNAs) have been suggested as a potential biomarker for conclusive BFID, and research to date indicates that these markers may meet the desired specificity and sensitivity for forensic use [6-10]. miRNAs are short, non-coding RNA sequences (18 – 25 nucleotides) that participate in post-transcriptional gene regulation [11]. These molecules are thought to be stable and resistant to degradation due to their association with proteins that provide protection from hydrolysis and RNase activity [12]. Though there has been little research on the stability of miRNAs in challenging conditions, extracted miRNAs have shown considerable stability at room temperature for one year and remained detectable in storage at -20°C for up to ten years [8, 13].

A common strategy for miRNA profiling systems is to use reverse transcription quantitative PCR (RT-qPCR) to determine the relative differences in expression of various miRNAs. Although this method has generated successful interpretation strategies [14, 15], it requires multiple reactions, which increases sample consumption, the risk of contamination, cost of reagents, and time of analysis [16]. To address these issues, Li et al. developed a four marker miRNA BFID profiling system using capillary electrophoresis

based on a set of linear primers [17]. The system was originally developed using one dye channel with the intent of expansion in order to incorporate additional markers.

In this study, we designed additional primers for the linear primer set to generate an eight marker miRNA multiplex to distinguish between venous blood, menstrual blood, semen, and saliva. A co-extraction strategy was utilized to generate DNA for STR analysis and miRNA for BFID profiles from a single sample.

Materials and methods

Sample collection and extraction

Five samples each of venous blood, menstrual blood, semen, and saliva were collected from volunteers in accordance with Sam Houston State University Institutional Review Board approval (#2015-09-26124). Menstrual blood was collected with cotton swabs. Venous blood was collected via venipuncture and semen provided by donors in specimen containers. Saliva was collected in plastic tubes. Aliquots (50 µL) of venous blood, semen, and saliva were used for extraction, and one swab was used for menstrual blood samples. Samples were extracted using the AllPrep® DNA/RNA Micro Kit (Qiagen®, Valencia, CA) following the Small RNA Purification Protocol [18] with the addition of an initial incubation of 2 hr at 56 °C with 900 rpm shaking to improve lysis. The elution volumes for DNA and RNA were 50 µL and 18 µL, respectively.

DNA quantification and amplification

DNA was quantified with the Quantifiler® Trio DNA Quantification Kit (ThermoFisher Scientific) on a 7500 Real Time PCR System (ThermoFisher Scientific), and amplified using the GlobalFiler™ PCR Amplification Kit (ThermoFisher Scientific) on a Veriti™ Thermal Cycler (ThermoFisher Scientific) as per manufacturer's instructions.

Marker selection and primer design

Markers were selected based on a consensus of candidate markers in various published studies [6, 8-10]. The endogenous reference gene let-7g was included as an internal control, miR-451a and miR-142-3p were chosen for venous blood, miR-141-3p and miR-412-3p for menstrual blood, miR-891a and miR-10b for semen, and miR-205 for saliva. The reverse transcription and specific primers for miR-451a as well as the universal primer designed by Li et al. [17] were also used in this study. Additional primers (reverse transcription and specific primers for all markers except miR-451a and three universal primers) were adapted from this linear primer system. To minimize non-specific binding, M13 sequences and a sequence designed by Lindblad-Toh et al. [19] were used for the universal primer sequences. All primer sequences are listed in Table 2.1.

Table 2.1. Primer sequences and concentrations.

Body Fluid	Marker	Accession Number	Sequence	Reverse Transcription Primer	Reverse Transcription Primer Conc.	Specific Primer	Specific Primer Final Conc.	Universal Primer	Universal Primer Conc.
End. Ref. Gene	let-7g	MIMAT0000414	UGAGGUAGUAGUUGUACAGUU	GTTCTTGCTGTCAACGATACGCTACGTTTTCTTTTCTTTTAACTGTAC	0.2 µM	GTTCTTCTTTTTCTTTTTCTTTTCTTTCTTTCTTTCTTTTGAGGTAGTAGTTT	1.54 µM	FAM′GTTCTTCTTTTTCTTTTCTGCTGTCAACGATACGCTACG	0.5 µM
Venous Blood	miR-451a	MIMAT0001631	AAACCGUUACCAUUACUGAGUU	GTTCTTGCTGTCAACGATACGCTACGTTTTCTTTTCTTTTAACTCAGT	0.16 µM	GTTCTTCTTTTTCTTTTTCTTTTTCAAACCGTTACCATT	0.08 µM		
	miR-142-3p	MIMAT0000434	UGUAGUGUUUCCUACUUUAUGGA	GTTCTTGCTGTCAACGATACGCTACGTTTTCTTTTCTTTTTCCATAAA	0.16 µM	GTTCTTCTTTTTCTTTTTCTTTTTCTTGTAGTGTTTCCTAC	0.12 µM		
Menstrual Blood	miR-141-3p	MIMAT0000432	UAACACUGUCUGGUAAAGAUGG	GTTCTTAACTGACTAAACTAGGTGCCTTTTCTTTTCTTTTCCATCTTT	0.2 µM	GTTCTTCTTTTTCTTTTTCTTAACACTGTCTGGT	0.04 µM	VIC'GTTCTTCTTTTTCTTTTCTAACTGACTAAACTAGGTGCC	0.5 µM
	miR-412-3p	MIMAT0002170	ACUUCACCUGGUCCACUAGCCGU	GTTCTTAACTGACTAAACTAGGTGCCTTTTCTTTTCTTTTACGGCTAG	0.3 µM	GTTCTTCTTTTTCTTTTTCTTTTTCTACTTCACCTGGTCCA	6.9 µM		

(continued)

Body Fluid	Marker	Accession Number	Sequence	Reverse Transcription Primer	Reverse Transcription Primer Conc.	Specific Primer	Specific Primer Final Conc.	Universal Primer	Universal Primer Conc.
Semen	miR-891a	MIMAT0004902	UGCAACGAACCUGAGCCACUGA	GTTCTTACGTCGTGAAAGTCTGACAATTTTCTTTTCTTTTCAGTGGC	0.2 µM	GTTCTTCTTTTTCTTTTTCTTTTTTCTTTTTCTTTTGCAACGAACCTGA	0.04 µM	NED'GTTCTTCTTTTCTTTCTACGTCGTGAAAGTCTGACAA	0.5 µM
	miR-10b	MIMAT0000254	UACCCUGUAGAACCGAAUUUGUG	GTTCTTACGTCGTGAAAGTCTGACAATTTTCTTTTCTTTCACAAATT	0.3 µM	GTTCTTCTTTTTCTTTTTCTTTTTTCTTACCCTGTAGAACCG	3.8 µM		
Saliva	miR-205	MIMAT0000266	UCCUUCAUUCCACCGGAGUCUG	GTTCTTCCCTTGAACCTCCTCGTTCGACCTTTTCTTTTCTTTTCAGACTCC	0.2 µM	GTTCTTCTTTTTCTTTTTCTTTTTTCTTTTCCTTCATTCCACC	0.2 µM	PET'GTTCTTCTTTTCTCCCTTGAACCTCCTCGTTCGACC	0.5 µM

miRNA reverse transcription and amplification

Reverse Transcription was performed using the SuperScript® III First Strand Synthesis System (ThermoFisher Scientific) as per manufacturer's instructions (20) using 7 µL of RNA and 2 µL mix of pooled PAGE purified custom reverse transcription primers (Integrated DNA Technologies, Coralville, IA) (final concentrations listed in Table 2.1) in the reaction. A reverse transcription negative control was performed for each body fluid. Multiplex amplification reactions were performed using the Multiplex PCR Kit (Qiagen) on a T100™ Thermal Cycler (Bio-Rad, Hercules, CA). Primers were evaluated in singlepex reactions before being combined. PCR amplification was performed in a reaction volume of 26 µL composed of 13 µL 2X PCR Mix, 3 µL cDNA, and 10 µL of specific primers (Integrated DNA Technologies) and fluorescently tagged universal primers (ThermoFisher Scientific) (final concentrations listed in Table 2.1). Cycling conditions for the reactions were as follows: 95 °C for 15 min, 35 cycles of 94 °C for 30 s, 57 °C for 1 min, and 72 °C for 1 min, with a final extension at 72 °C for 10 min.

Detection

Amplified DNA products were detected using a 3500 Genetic Analyzer equipped with POP-4™ and a 36 cm capillary array (ThermoFisher Scientific). Amplified cDNA products were detected using a 3500 Genetic Analyzer equipped with POP-7™ and a 50 cm capillary array (ThermoFisher Scientific). Data was analyzed using Genemapper® Software ID-X v. 4.1 (ThermoFisher Scientific). An analytical threshold of 150 RFU was applied to both DNA and miRNA profiles.

Results and discussion

All samples tested yielded full STR profiles from the DNA fraction (data not shown) as also observed in previous studies [17, 21, 22]. No amplification was observed in the reverse transcription negatives, indicating that there was no observable interaction with genomic DNA. The results of the miRNA profiling are presented in Table 2.2 and representative profiles can be seen in Fig. 2.1a-d. Average peak heights for each of the body fluids can be seen in Fig. 2.2. Although the intent of this method was to choose markers that were both specific and high in abundance to reduce amplification of background transcription, some marker cross-reactivity was observed. However, we have adopted an interpretation strategy based on patterns of marker amplification to distinguish between the fluid types. A decision tree for the identification of single source samples can be seen in Fig. 2.3.

Table 2.2. Proportion of markers observed in the miRNA profiles generated in this study (N = 5).

Body Fluid	Reference	Venous Blood		Menstrual Blood		Semen		Saliva
	let-7g	miR-451a	miR-142-3p	miR-141-3p	miR-412-3p	miR-10b	miR-891a	miR-205
Venous Blood	1	1	1	0	0	0	0	0
Menstrual Blood	1	1	0	1	0.6	0	0	1
Semen	1	0	1	0.8	0.8	1	1	1
Saliva	1	0	1	0.2	0.2	0	0	1

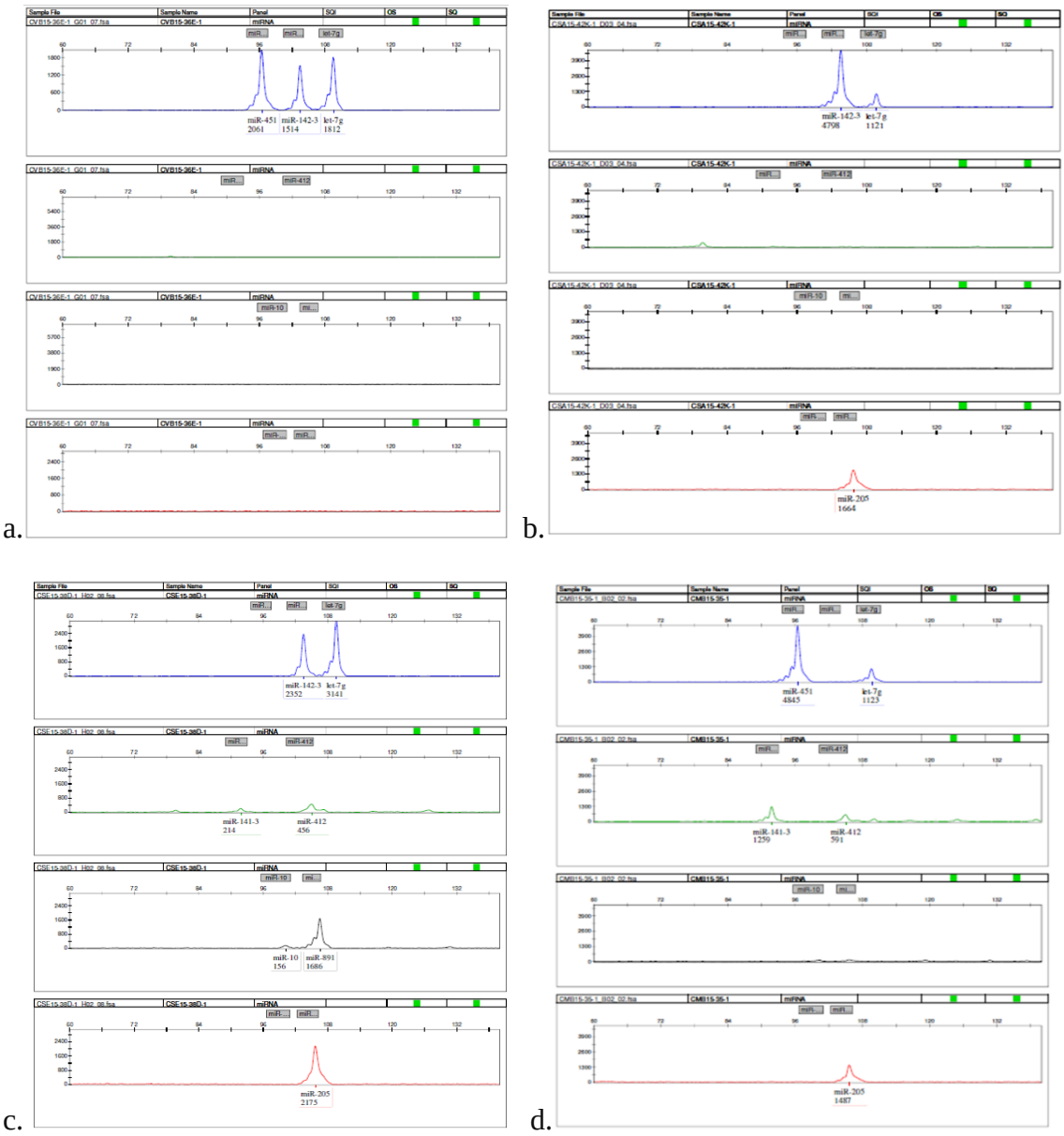

Fig. 2.1. Representative electropherograms from miRNA multiplex analysis allow for body fluid discrimination. a. Venous blood sample b. Saliva sample c. Semen sample d. Menstrual blood sample

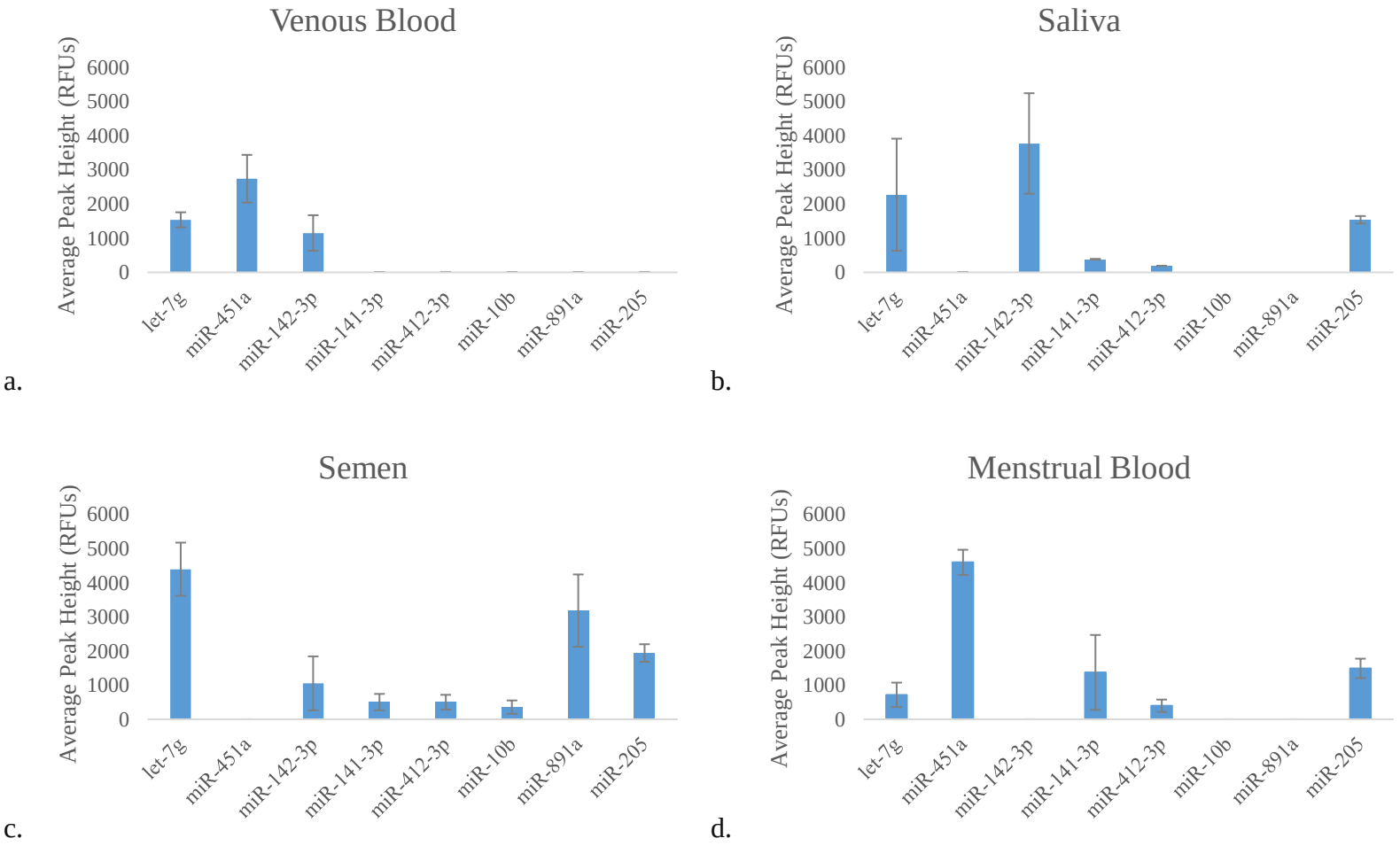

Fig. 2.2. Average peak heights for each body fluid. Error bars indicate standard deviation. a. Venous blood sample b. Saliva sample c. Semen sample d. Menstrual blood sample.

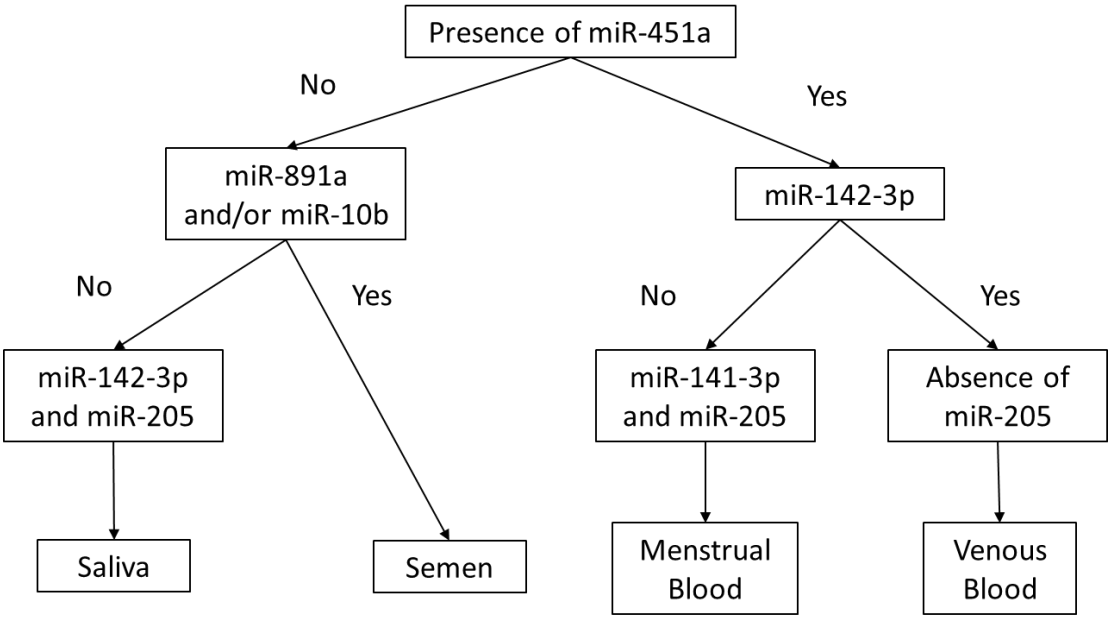

Fig. 2.3. Decision tree for body fluid identification of single source samples.

The endogenous reference gene let-7g was present in all samples. In the venous profiles, the markers miR-451a and miR-142-3p were observed in all profiles and no other markers were observed. miR-205 was seen in all fluids except venous blood. This is not unexpected, as the marker has been associated with epithelial cells [23]. For unknown samples, venous blood profiles would include the presence of miR-451a and miR-142-3p and the absence of other markers (including miR-205).

In menstrual blood samples, miR-451a, miR-141-3p, and miR-205 were observed in all samples and miR-412-3p was observed in three out of the five samples. While marker miR-142-3p was expected to be present solely in blood samples as seen by van der Meer et al. [16], it was observed in all fluids except menstrual blood. Sirker et al. [24] observed elevated levels of miR-142-3p in venous blood, menstrual blood, skin, and vaginal secretions, but differences in expression patterns in laboratories using different platforms and amplification strategies have been documented [25, 26]. Reported expression patterns

can also differ due to the choice of normalization strategies and/or markers used in RT-qPCR studies [27, 28]. During interpretation, the presence of miR-451a and miR-141-3p and the absence of miR-142-3p would indicate the presence of menstrual blood.

The markers miR-891a, miR-10b, miR-142-3p, and miR-205 were observed in all semen samples. In cases of vasectomized males, miR-891a would likely not be present in seminal fluid as it originates in epididymis tissue, but miR-10b would still be present [6]. Although miR-141-3p and miR-412-3p were observed in four of the five semen samples, they are essentially irrelevant for semen identification, as they were chosen based on their ability to distinguish between venous and menstrual blood and are known to be expressed in other fluids [6, 25, 29, 30]. For this reason, we would suggest using only markers miR-891a and miR-10b as indicators for the presence of semen.

In the saliva profiles, miR-142-3p and miR-205 were observed in all samples. Although markers miR-141-3p and miR-412-3p were both observed once, several peaks outside of the expected marker range were also seen in the green dye channel, likely due to amplification of bacterial RNAs in saliva or miRNAs with a similar motif. However, as there were no other peaks to indicate menstrual blood or semen were present, saliva samples can be distinguished from the other body fluids. The presence of miR-142-3p and miR-205 and absence of all other markers suggest saliva. Peaks under the analytical threshold were also present in the green dye channel outside of expected marker ranges in menstrual blood profiles, which also contains bacterial flora.

Although this miRNA multiplex was able to discriminate between single source fluids using the data interpretation strategy described in this paper, refinement of markers may be necessary. For instance, the marker miR-412-3p does not provide additional

information to the multiplex. Body fluid mixtures would be problematic due to some cross reactivity and differential expression patterns. Due to the cross-reactivity of markers miR-141-3p and miR-412-3p, a venous blood/semen mixture would not be distinguishable from a menstrual blood/semen mixture. Furthermore, the presence of saliva would be masked if semen were also present. Novel markers are continually being reported which may improve the specificity of BFID and mixture resolution as well as identify organ tissues [10, 31, 32]. The identification and inclusion of these markers would warrant additional validation studies including an increased sample size, sensitivity studies, and testing of additional relevant body fluids (such as vaginal fluid).

In conclusion, the additional universal primer sequences allowed for a greater number of markers to be incorporated into the miRNA BFID multiplex. Using this system, we were able to discriminate between venous blood, menstrual blood, semen, and saliva using the miRNA BFID multiplex and development of a crude data interpretation strategy. STR and BFID profiles were generated from single samples using techniques and instrumentation currently employed in crime laboratories.

Acknowledgements

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References

1. Vennemann M, Koppelkamm A (2010) mRNA profiling in forensic genetics I: Possibilities and limitations. Forensic Sci Int 203 (1-3):71-75. doi:10.1016/j.forsciint.2010.07.006
2. An JH, Shin KJ, Yang WI, Lee HY (2012) Body fluid identification in forensics. BMB Rep 45 (10):545-553
3. Yu H, Berkel H (1999) Prostate-specific antigen (PSA) in women. J La State Med Soc 151 (4):209-213
4. Romero-Montoya L, Martinez-Rodriguez H, Perez MA, Arguello-Garcia R (2011) Relationship of spermatoscopy, prostatic acid phosphatase activity and prostate-specific antigen (p30) assays with further DNA typing in forensic samples from rape cases. Forensic Sci Int 206 (1-3):111-118. doi:10.1016/j.forsciint.2010.07.012
5. Virkler K, Lednev IK (2009) Analysis of body fluids for forensic purposes: from laboratory testing to non-destructive rapid confirmatory identification at a crime scene. Forensic Sci Int 188 (1-3):1-17. doi:10.1016/j.forsciint.2009.02.013
6. Hanson EK, Lubenow H, Ballantyne J (2009) Identification of forensically relevant body fluids using a panel of differentially expressed microRNAs. Anal Biochem 387 (2):303-314. doi:10.1016/j.ab.2009.01.037
7. van der Meer D, Uchimoto ML, Williams G (2013) Simultaneous analysis of micro-RNA and DNA for determining the body fluid origin of DNA profiles. J Forensic Sci 58 (4):967-971. doi:10.1111/1556-4029.12160
8. Zubakov D, Boersma AW, Choi Y, van Kuijk PF, Wiemer EA, Kayser M (2010) MicroRNA markers for forensic body fluid identification obtained from microarray

screening and quantitative RT-PCR confirmation. Int J Legal Med 124 (3):217-226. doi:10.1007/s00414-009-0402-3

9. Wang Z, Zhang J, Luo H, Ye Y, Yan J, Hou Y (2013) Screening and confirmation of microRNA markers for forensic body fluid identification. Forensic Sci Int Genet 7 (1):116-123. doi:10.1016/j.fsigen.2012.07.006

10. Seashols-Williams S, Lewis C, Calloway C, Peace N, Harrison A, Hayes-Nash C, Fleming S, Wu Q, Zehner ZE (2016) High-throughput miRNA sequencing and identification of biomarkers for forensically relevant biological fluids. Electrophoresis 37 (21):2780-2788. doi:10.1002/elps.201600258

11. Bartel DP (2004) MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 116 (2):281-297

12. Arroyo JD, Chevillet JR, Kroh EM, Ruf IK, Pritchard CC, Gibson DF, Mitchell PS, Bennett CF, Pogosova-Agadjanyan EL, Stirewalt DL, Tait JF, Tewari M (2011) Argonaute2 complexes carry a population of circulating microRNAs independent of vesicles in human plasma. Proc Natl Acad Sci U S A 108 (12):5003-5008. doi:10.1073/pnas.1019055108

13. Grasedieck S, Scholer N, Bommer M, Niess JH, Tumani H, Rouhi A, Bloehdorn J, Liebisch P, Mertens D, Dohner H, Buske C, Langer C, Kuchenbauer F (2012) Impact of serum storage conditions on microRNA stability. Leukemia 26 (11):2414-2416. doi:10.1038/leu.2012.106

14. Hanson EK, Rekab K, Ballantyne J (2013) Binary logistic regression models enable miRNA profiling to provide accurate identification of forensically relevant body

fluids and tissues. Forensic Science International: Genetics Supplement Series 4 (1):e127-e128

15. Sauer E, Reinke AK, Courts C (2015) Validation of forensic body fluid identification based on empirically normalized miRNA expression data. Forensic Science International Genetics Supplement Series 5:E462-E464. doi:10.1016/j.fsigss.2015.09.183

16. van der Meer DJ, Williams GA (2015) Performing body fluid identification with microRNAs using capillary electrophoresis. Forensic Science International: Genetics Supplement Series

17. Li Y, Zhang J, Wei W, Wang Z, Prinz M, Hou Y (2014) A strategy for co-analysis of microRNAs and DNA. Forensic Sci Int Genet 12:24-29. doi:10.1016/j.fsigen.2014.04.011

18. Qiagen (2014) AllPrep DNA/RNA Micro Handbook.

19. Lindblad-Toh K, Winchester E, Daly MJ, Wang DG, Hirschhorn JN, Laviolette JP, Ardlie K, Reich DE, Robinson E, Sklar P, Shah N, Thomas D, Fan JB, Gingeras T, Warrington J, Patil N, Hudson TJ, Lander ES (2000) Large-scale discovery and genotyping of single-nucleotide polymorphisms in the mouse. Nat Genet 24 (4):381-386. doi:10.1038/74215

20. Life Technologies (2013) SuperScript III First-Strand Synthesis System for RT-PCR.

21. Alvarez M, Juusola J, Ballantyne J (2004) An mRNA and DNA co-isolation method for forensic casework samples. Anal Biochem 335 (2):289-298. doi:10.1016/j.ab.2004.09.002

22. Schweighardt AJ, Tate CM, Scott KA, Harper KA, Robertson JM (2015) Evaluation of commercial kits for dual extraction of DNA and RNA from human body fluids. J Forensic Sci 60 (1):157-165. doi:10.1111/1556-4029.12586

23. Wang Z, Luo H, Pan X, Liao M, Hou Y (2012) A model for data analysis of microRNA expression in forensic body fluid identification. Forensic Sci Int Genet 6 (3):419-423. doi:10.1016/j.fsigen.2011.08.008

24. Sirker M, Fimmers R, Schneider PM, Gomes I (2017) Evaluating the forensic application of 19 target microRNAs as biomarkers in body fluid and tissue identification. Forensic Science International-Genetics 27:41-49. doi:10.1016/j.fsigen.2016.11.012

25. Li ZL, Bai P, Peng D, Wang H, Guo YD, Jiang YJ, He W, Tian H, Yang Y, Huang Y, Long B, Liang WB, Zhang L (2017) Screening and confirmation of microRNA markers for distinguishing between menstrual and peripheral blood. Forensic Science International-Genetics 30:24-33. doi:10.1016/j.fsigen.2017.05.012

26. Li ZL, Bai P, Peng D, Long B, Zhang L, Liang WB (2015) Influences of different RT-qPCR methods on forensic body fluid identification by microRNA. Forensic Science International Genetics Supplement Series 5:E295-E297. doi:10.1016/j.fsigss.2015.09.117

27. Sauer E, Babion I, Madea B, Courts C (2014) An evidence based strategy for normalization of quantitative PCR data from miRNA expression analysis in forensic organ tissue identification. Forensic Sci Int Genet 13:217-223. doi:10.1016/j.fsigen.2014.08.005

28. Sirker M, Liang W, Zhang L, Fimmers R, Rothschild A, Gomes I, Schneider M, Consortium E-N (2015) Impact of using validated or standard reference genes for miRNA qPCR data normalization in cell type identification. Forensic Science International Genetics Supplement Series 5:E199-E201. doi:10.1016/j.fsigss.2015.09.080

29. Bexon K, Williams G (2015) Characterising the fluctuation of microRNA expression throughout a full menstrual cycle. Forensic Science International Genetics Supplement Series 5:E264-E266. doi:10.1016/j.fsigss.2015.09.105

30. Li ZL, Bai P, Peng D, Long B, Zhang L, Liang WB (2015) Screening and confirmation of microRNA markers for distinguishing between menstrual and peripheral blood. Forensic Science International Genetics Supplement Series 5:E353-E355. doi:10.1016/j.fsigss.2015.09.140

31. Wang Z, Zhou D, Cao Y, Hu Z, Zhang S, Bian Y, Hou Y, Li C (2016) Characterization of microRNA expression profiles in blood and saliva using the Ion Personal Genome Machine® System (Ion PGM™ System). Forensic Science International: Genetics 20:140-146

32. Sauer E, Extra A, Cachee P, Courts C (2017) Identification of organ tissue types and skin from forensic samples by microRNA expression analysis. Forensic Sci Int Genet 28:99-110. doi:10.1016/j.fsigen.2017.02.002

CHAPTER III

The stability and persistence of blood and semen mRNA and miRNA targets for body fluid identification in environmentally challenged and laundered samples[1]

This dissertation follows the style and format of *International Journal of Legal Medicine*.

[1] Mayes C, Houston R, Seashols-Williams S, LaRue B, Hughes-Stamm S. (2019) The stability and persistence of blood and semen mRNA and miRNA targets for body fluid identification in environmentally challenged and laundered samples. Leg Med (Tokyo, Japan) 38:45-50. https://doi.org/10.1016/j.legalmed.2019.03.007

Abstract

The identification of body fluids in evidentiary stains may provide investigators with probative information during an investigation. In this study, quantitative reverse transcription polymerase chain reaction (RT-qPCR) assays were performed to detect the presence of mRNA and miRNA in fresh and environmentally challenged samples. Blood, semen, and reference markers were chosen for both mRNA/miRNA testing. Samples of blood and semen were exposed to heat, humidity, and sunlight, and controlled conditions (room temperature, low humidity, and darkness) for 6 months. All mRNA targets were observed through six months under controlled conditions, but were undetected after 30 days in experimental conditions. However, miRNA targets persisted under all test conditions for the duration of the study.

Additionally, cotton stained with blood or semen was laundered using a liquid detergent in various washing and drying conditions. An unstained cutting was evaluated for potential transfer. Both miRNA targets were observed in all stained samples regardless of the wash protocol used. Of the mRNA markers, HBB was detected in all bloodstained samples and PRM1 persisted in all but one semen stained sample. The unstained samples showed transfer of at least one body fluid specific miRNA marker in all samples and at least one body fluid specific mRNA in approximately half of the samples. These results support that RNA markers can be used for body fluid identification in challenging samples, and that miRNA markers may be more persistent than mRNA for blood and semen stains. However, some caution is warranted with laundered items due to possible transfer.

Keywords: Body fluid identification, mRNA, miRNA, persistence, forensic science, serology

Introduction

The identification of body fluids in evidentiary stains may provide investigators with probative information during the course of an investigation. Traditional serological tests currently used in forensic labs are presumptive in nature and have varying levels of specificity and sensitivity [1-3]. The interrogation of nucleic acids, such as mRNA and miRNA, has been introduced to assist analysts in determining the body fluid of origin in forensic samples [4-9]. These methods attempt to improve upon existing techniques and allow for the co-extraction with DNA, the consumption of less sample, and the potential to identify a greater number of body fluids [10,11].

As STR typing kits are becoming more sensitive, we are beginning to probe and understand the mechanisms behind the transfer, persistence, prevalence, and recovery (TPPR) of cellular material [12,13]. The vast majority of this research is geared toward DNA analysis, but assessing the TPPR of molecular based methods for body fluid identification should also be investigated. This information can ascertain scenarios in which these methods would be successful, identify possible limitations, and provide context for profile interpretation or activity level. While several experiments have been performed investigating the persistence of mRNA in environmentally challenged samples [14,15] and aged stains [16,17], concerns remain regarding the use of RNA in forensic casework type samples. Due to their small size (19 – 25 nucleotides) and encapsulation in protein, miRNAs are thought to be more resistant to degradation. Grabmüller et al. [18] detected miRNAs in evidentiary items aged up to 20 years; however, to our knowledge, a time course evaluation of miRNAs has not yet been performed. Additionally, only one

study has examined mRNA and miRNA in parallel outside of evidentiary items [19], but did not yield any conclusive results regarding miRNA.

A sample may also be compromised through means other than time and environmental exposure. It is not uncommon for items of evidence to be washed by the victim or perpetrator in an attempt to remove biological material prior to seizure by police. While it has been reported that laundered items may not be submitted for analysis based on the belief that these items are unsuitable for forensic testing [19], both serological testing [20] and STR profiling has been successful [20-23].

In this study, the persistence of both mRNA and miRNA was evaluated in the same set of environmentally challenged and laundered samples. The following mRNA and miRNA targets were chosen based on previous literature: hemoglobin beta (HBB) [24] and miR-451a [4] for blood, protamine 1 (PRM1) [25] and miR-891a [4] for semen, and beta-2-microglobulin (B2M) [26] and let-7g [27] for reference genes. A real-time quantitative PCR (RT-qPCR) method was used to compare mRNA and miRNA targets. The persistence of mRNA and miRNA was monitored over a period of six months in blood and semen stains on cotton swatches exposed to two conditions: 1) in a glass enclosure with natural heat, humidity, and UV light, and 2) controlled conditions in a cabinet (room temperature, low humidity, and darkness). Additionally, a preliminary investigation of laundered items was performed examining cotton swatches stained with blood and semen laundered with a liquid detergent in cold or hot wash cycles, washed once or twice, and either air-dried or machine dried with or without the presence of additional clothes to test for persistence and any transfer.

Materials and methods

Sample collection

Samples of venous blood (n=3) and semen (n=3) were collected with informed consent according to a University approved protocol. Venous blood was collected by venipuncture into BD Vacutainer™ tubes treated with an anti-coagulant (EDTA). Semen was provided in specimen containers. Cotton swatches were UV treated to eliminate possible residual nucleic acids.

Sample treatment

Time course study

Time course study samples were prepared by staining each swatch with 50 µL of blood (n=3) or semen (n=3) on cotton swatches approximately 2 cm by 1 cm and allowed to dry overnight. The stained swatches were placed in a glass enclosure with exposure to natural heat, humidity, and sunlight. The control samples were placed in a laboratory cabinet (room temperature, low humidity, and darkness). Temperature and humidity were monitored in both environments. Samples were taken at days 0, 1, 3, 5, 10, 14, 60, 120, and 180.

Laundry study

Aliquots of 50 µL of blood (n=3) and semen (n=3) were applied to sections of cotton bedsheets approximately 24 cm by 16 cm in size. Each section, one per treatment and one unwashed control, was spotted with each blood and semen on different areas roughly 8 cm apart. Samples were allowed to dry overnight before being washed. The washer used in the laundry experiment was a top-loading Whirlpool® Commercial Washer Heavy Duty Series. The experimental design can be seen in Fig 3.1. Items washed at the

same temperature were washed together. The preprogrammed settings for cold (15°C) and hot (42°C) were selected and run for 30 minutes with a drum speed of 700 rpm. One Tide PODS® packet (Proctor & Gamble, Cincinnati, OH) was used per wash as the detergent. Individual stains were marked with permanent marker prior to washing. The stains were laundered with and without additional clothes and were allowed to air dry overnight or were machine dried in a front-loading Whirlpool® Commercial Single Load, Super Capacity Stack Dryer on the permanent-press setting (57°C) for 45 minutes. Cuttings (approximately 2 cm by 1 cm) were taken of unstained areas to evaluate potential transfer. Neither the washer nor dryer were cleaned before the experiment. The swatches were examined visually for bloodstains and with a Crime-Lite™ (Foster & Freeman, Sterling, VA) with orange goggles before and after laundering to visualize semen stains.

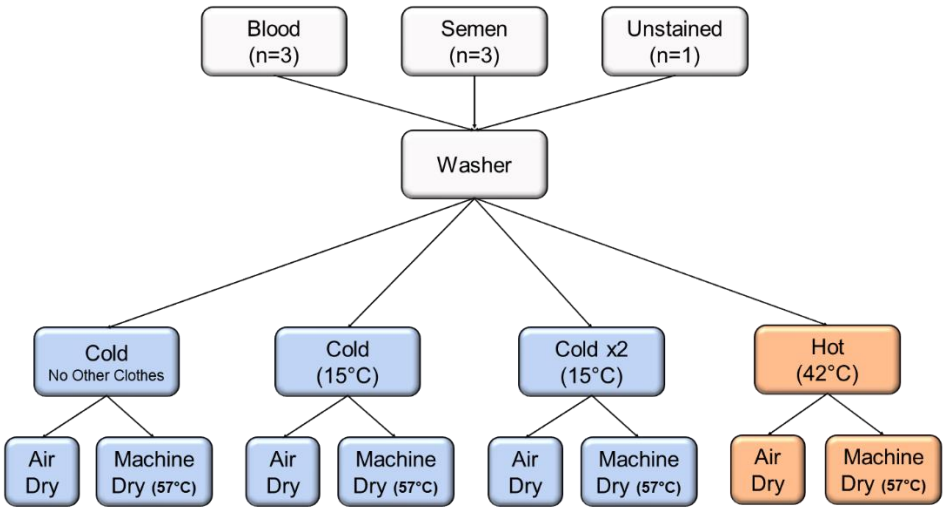


Fig. 3.1. Experimental conditions of laundered items. Blood, semen, and an unstained control were washed with liquid detergent and dried according to the conditions listed.

Sample extraction

Samples were extracted with the miRNeasy RNA Mini Kit (QIAGEN, Germantown, MD) according to manufacturer's recommendations with the addition of a centrifuge step with spin basket (5 min at 2000 g) to remove liquid from fabric and an elution volume of 50 µL.

Reverse transcription

Reverse transcription was performed using the QuantiTect® Reverse Transcription Kit (QIAGEN) on the Veriti™ 96-Well Thermal Cycler (ThermoFisher Scientific, Waltham, MA) for mRNA targets. cDNA for miRNA targets was generated using the miScript II Reverse Transcription Kit (QIAGEN) on the ProFlex™ 96-Well PCR System (ThermoFisher Scientific). All reactions were performed according to manufacturer's protocols. A 10 µL volume of neat RNA extract was used for each reaction. Reverse transcription negatives were performed to ensure no genomic material was amplified.

Quantitative PCR and data analysis

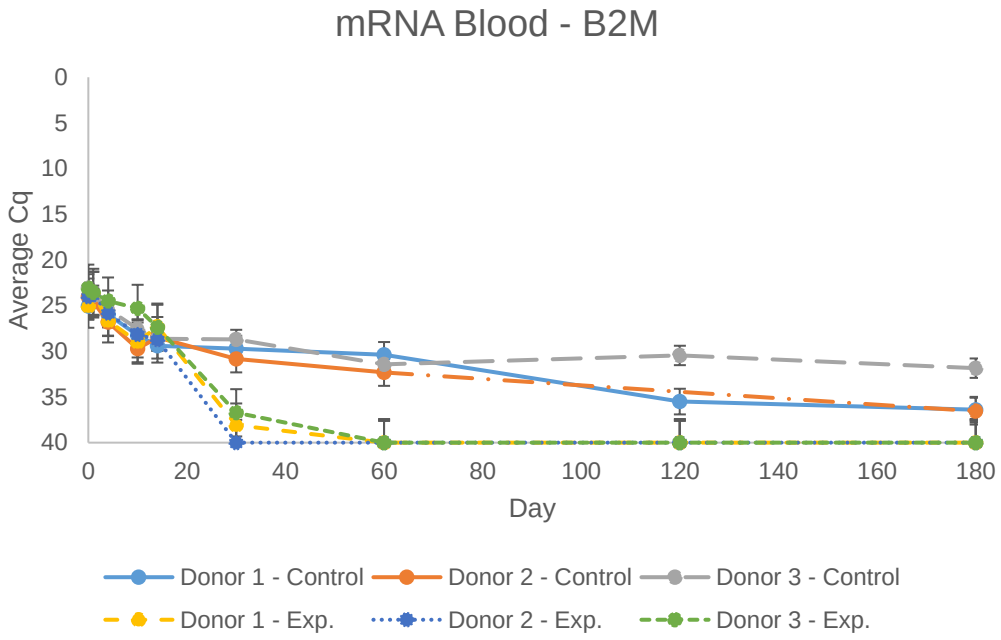
Real-time PCR amplification was performed in duplicate using the miScript SYBR® Green PCR kit with miScript Primer Assays (let-7g, miR-451a, and miR-891a) and QuantiTect® Primer Assays (B2M, HBB, and PRM1) (QIAGEN). Experiments were performed and analyzed according to MIQE guidelines [28]. Primer validation experiments were performed to ensure acceptable amplification efficiency (87.7-98.0%) and specificity. A 1 in 10 dilution of cDNA was used for amplification. Amplification was detected using a 7500 Real-Time PCR System (Applied Biosystems) with HID Real-Time Analysis Software v1.2. Cycling conditions were as follows: 95°C for 15 min, 40 cycles of 94°C for 15 s, 55°C for 30 s, and 70°C for 30 s. A threshold of 0.08 was applied for all markers and

C_q values were calculated by averaging technical duplicates. Melt curve analysis was used to ensure specific target amplification in samples with a $C_q \geq 35$. Samples with replicates deviating more than ±1˚C in Tm were removed from analysis. Although ΔC_q or $2^{-\Delta\Delta Cq}$ values (C_q of the fluid specific marker subtracted from the C_q of the reference marker) are traditionally used for RT-qPCR studies, individual variation has been known to produce false negatives [29] and environmentally challenging conditions may alter the proportional abundance of transcripts within samples. In this study, ΔC_q values were calculated and compared to control samples. C_q values and ΔC_q values were evaluated in the time course study and ΔC_q values were determined for the laundry study.

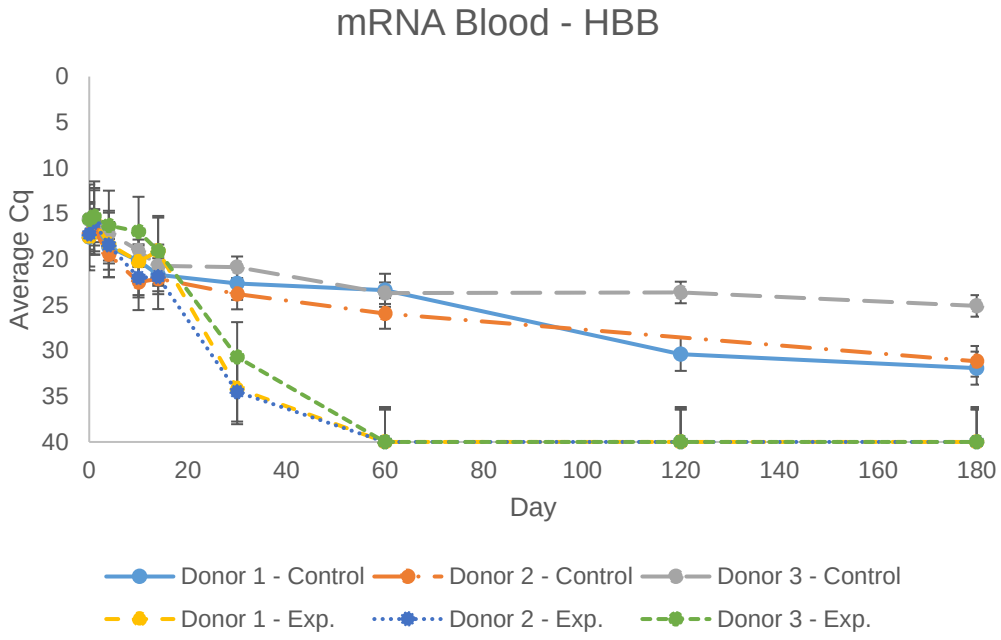
Results and discussion

Time course study

The results of the time course study can be seen in Fig. 3.2a-d and Fig 3.3a-d. Temperatures ranged from 19.0-23.7˚C in controlled conditions and 8.9-61.0˚C in experimental conditions over the 6-month period. Humidity ranged from 38-52% and 10-99% in controlled and experimental conditions, respectively. A more detailed record of daily temperatures is provided in Fig. 3.4.


mRNA Blood - B2M
Average Cq
0
5
10
15
20
25
30
35
40
0
20
40
60
80
100
120
140
160
180
Day
Donor 1 - Control
Donor 2 - Control
Donor 3 - Control
Donor 1 - Exp.
Donor 2 - Exp.
Donor 3 - Exp.

a.


mRNA Blood - HBB
Average Cq
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5
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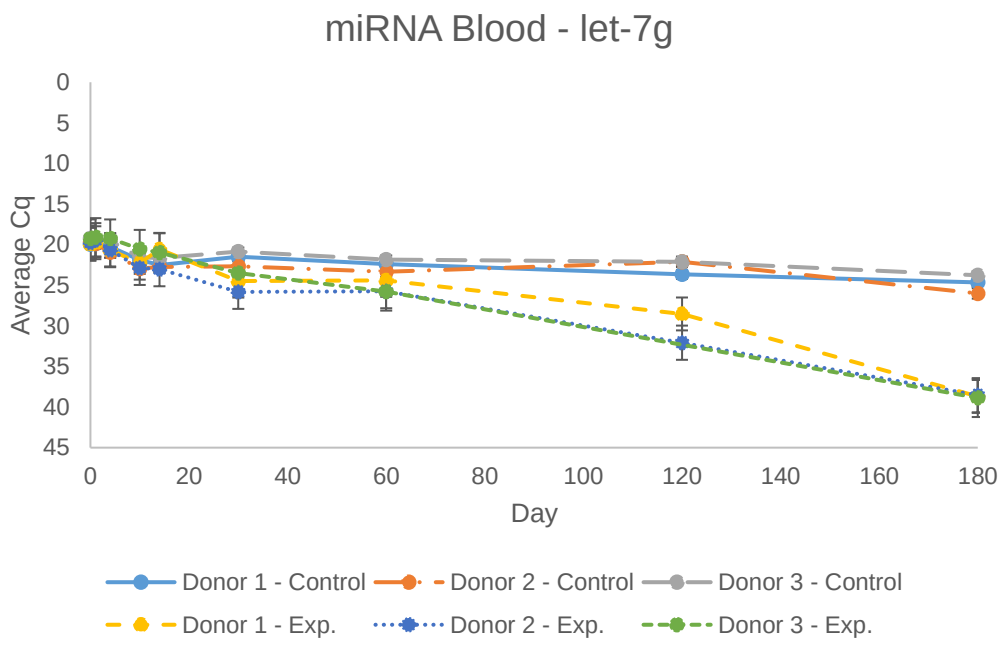
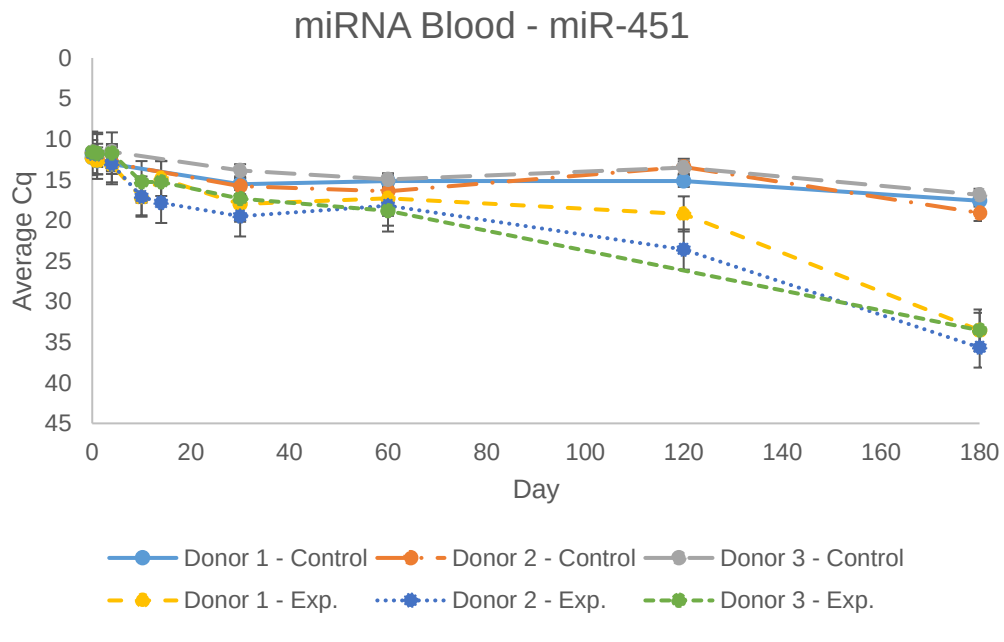
Donor 1 - Control
Donor 2 - Control
Donor 3 - Control
Donor 1 - Exp.
Donor 2 - Exp.
Donor 3 - Exp.

b.

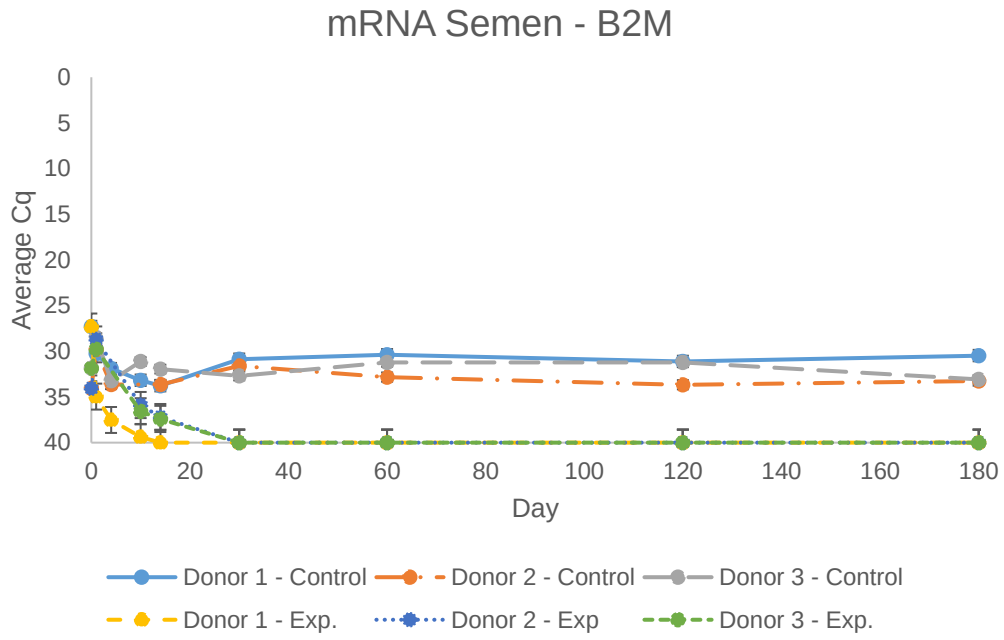


c.



d.

Fig. 3.2.a-d. Results of the time course study examining C_q values of reference markers (B2M and let-7g) and blood specific targets. Data presented as average (n=3) ± SE **a** B2M **b** HBB **c** let-7g **d** miR-451a


mRNA Semen - B2M
Average Cq
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15
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140
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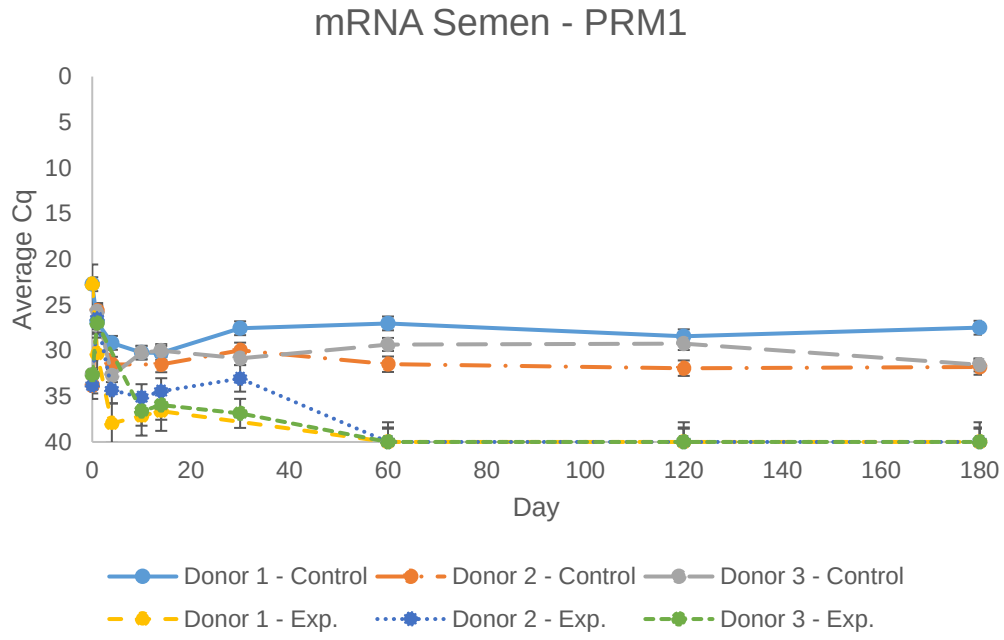
Day
Donor 1 - Control
Donor 2 - Control
Donor 3 - Control
Donor 1 - Exp.
Donor 2 - Exp
Donor 3 - Exp.

a.


mRNA Semen - PRM1
Average Cq
0
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30
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40
0
20
40
60
80
100
120
140
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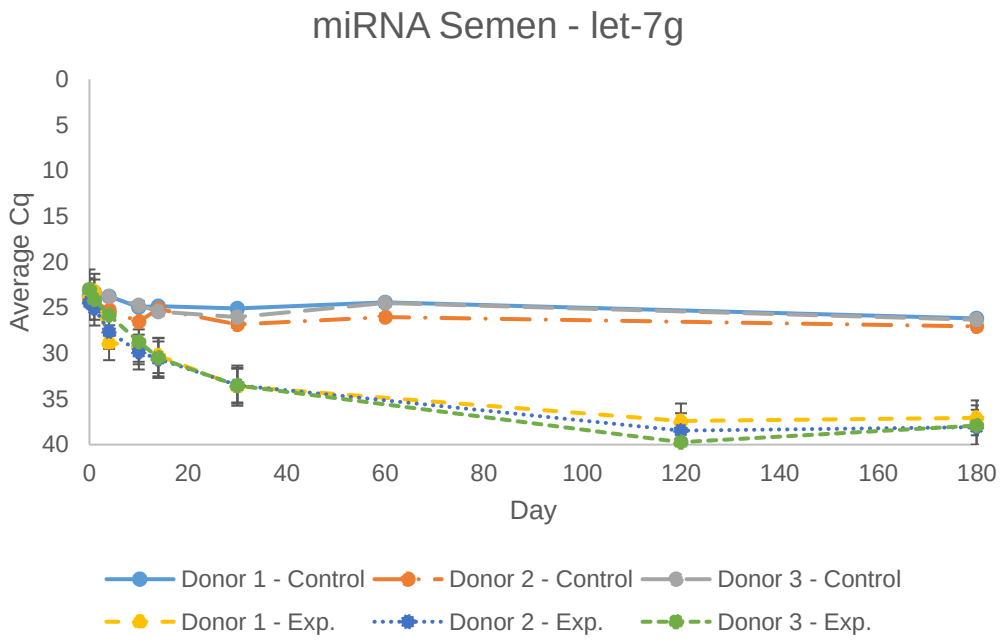
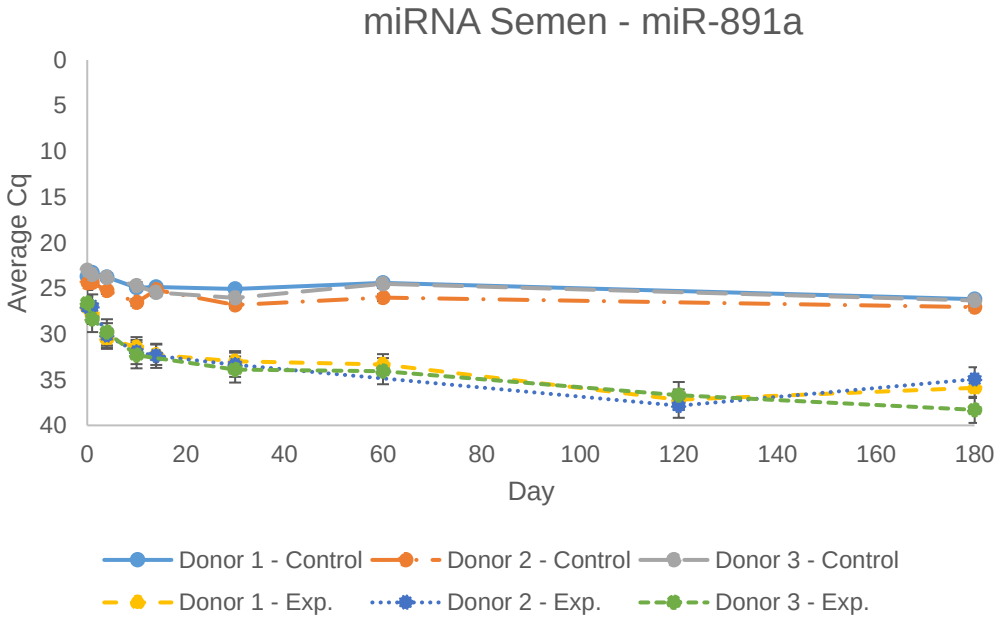
Day
Donor 1 - Control
Donor 2 - Control
Donor 3 - Control
Donor 1 - Exp.
Donor 2 - Exp.
Donor 3 - Exp.

b.



c.



d.

Fig. 3.3.a-d. Results of the time course study examining C_q values of reference markers (B2M and let-7g) and semen specific targets. Data presented as average (n=3) ± SE **a** B2M **b** PRM1 **c** let-7g **d** miR-891a

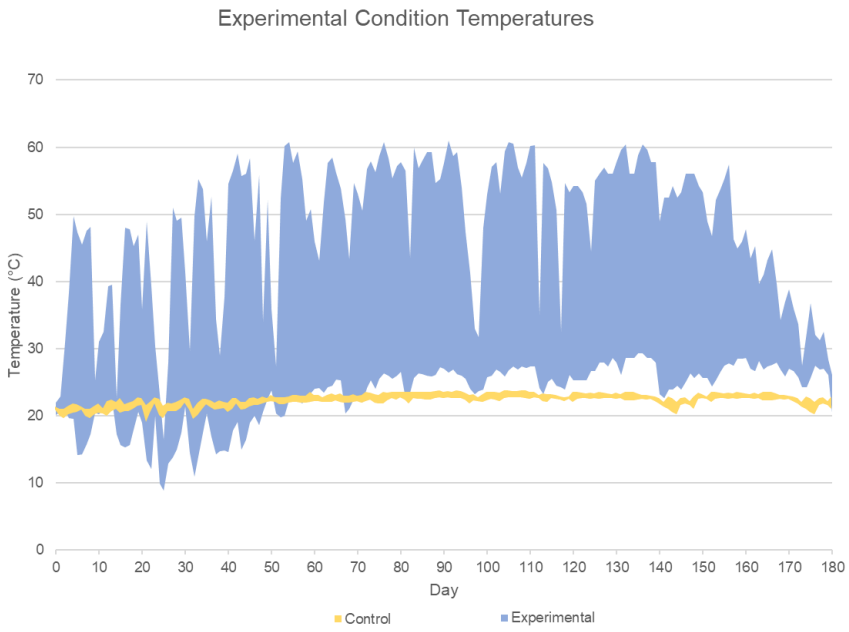


Fig. 3.4. High and low temperatures recorded within the control and experimental environments.

For the blood samples, the mRNA markers (B2M and HBB) in samples left at room temperature were amplifiable at every time point while samples exposed to variable environmental conditions steadily declined and were not amplified beyond day 30 (Fig. 3.2a-b). As the same volume of extract was used for reverse transcription, variation in C_q values was observed between biological sources. Relative expression remained roughly proportional (ΔC_q values remained within ±2 cycles compared to controls) across the 180 days stored in room temperature conditions, and for 30 days under the test conditions. These results are similar to Setzer et al. [14], while the inclusion of variable temperature appears to drastically reduce the period of detection of mRNA, as compared to Sirker et al. [15]. In contrast to mRNA, miRNA targets (let-7g and miR-451a) were more robust and were observed for the 180-day length of the experiment in both conditions. Similar to blood mRNA expression, ΔC_q values for the miRNA targets were proportional (±3.5 cycles

compared to controls) throughout the experiment (Fig. 3.2c-d). Our results support those of Fang et al. [30] who also observed minimal degradation of miRNAs in bloodstains aged 5 months in elevated temperatures (37°C).

Similar results were observed in the semen samples with both mRNA markers persisting for the duration of the study under controlled conditions. However, in the experimental samples PRM1 was detected up to day 30, while the reference B2M was not detected past day 14 (Fig. 3.3a-b). The persistence of semen-specific transcripts has been previously observed in challenged samples [14,15,17] and has been attributed to the robust cell membrane of the spermatozoa protecting these transcripts. This is further supported by the difference in degradation rates between the miRNA targets in the environmentally challenged samples (Fig. 3.3c-d). In one time point (day 120), reverse transcription for the experimental samples failed. The reference marker, let-7g, was observed in higher abundance during the first two weeks, but dropped below the abundance of semen specific marker, miR-891a, for the remainder of the study. The miRNA transcript miR-891a originates in epididymis tissue and has been reported in higher abundance in normozoospermic individuals [31,32], suggesting transcripts may be protected by the spermatozoa cell. Examination of the C_q values in samples stored at room temperature conditions suggests minimal miRNA degradation, as also observed by Zubakov et al. [33] in items stored for one year.

Laundry study

Upon visual inspection, blood stains were visible in all samples after washing, except those washed twice and machine dried, and samples washed in hot water and machine dried (Fig. 3.5). Previous studies [20,34] have also reported diminished visibility of bloodstains on materials washed at higher temperatures (60-95°C). The mRNA and miRNA results of the laundry study can be seen in Fig. 3.6. Both blood miRNA markers were observed in all laundered samples. In all instances where one mRNA transcript was not detected, the reference marker (B2M) was not observed and the fluid specific marker (HBB or PRM1) remained. In these stains, it appears that samples receiving more substantial physical agitation (ie. elevated wash temperature or washed twice) had a lower occurrence of mRNA transcript detection, though a more thorough investigation is needed for any definitive conclusions. Kulstein et al. [34] also observed HBB in stains washed once at 40°C, but did not observe any body fluid specific markers in warmer washes or in items washed twice. This discrepancy may be due to differences in length of wash cycles (2 hours compared to 30 minutes in this study), leading to more thorough stain removal, or differences in analysis methods (capillary electrophoresis-based assay and RT-qPCR). In another study [35], tape lifts of laundered items did not yield reportable results in blood-stained clothes, but the authors also predicted that increased recovery would be more likely with extracted cuttings such as we used in our study.

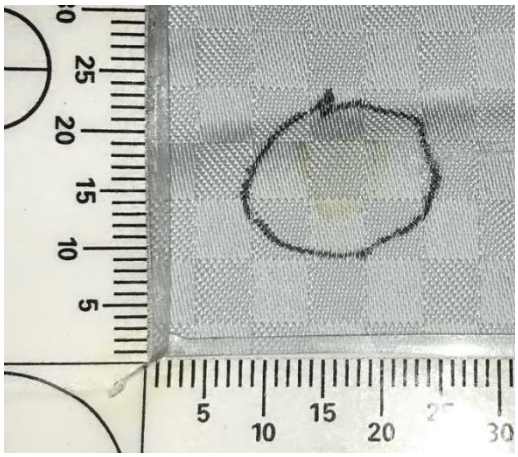


Fig. 3.5. Example of a stain visible after washing in cold water (4°C) and air dried.

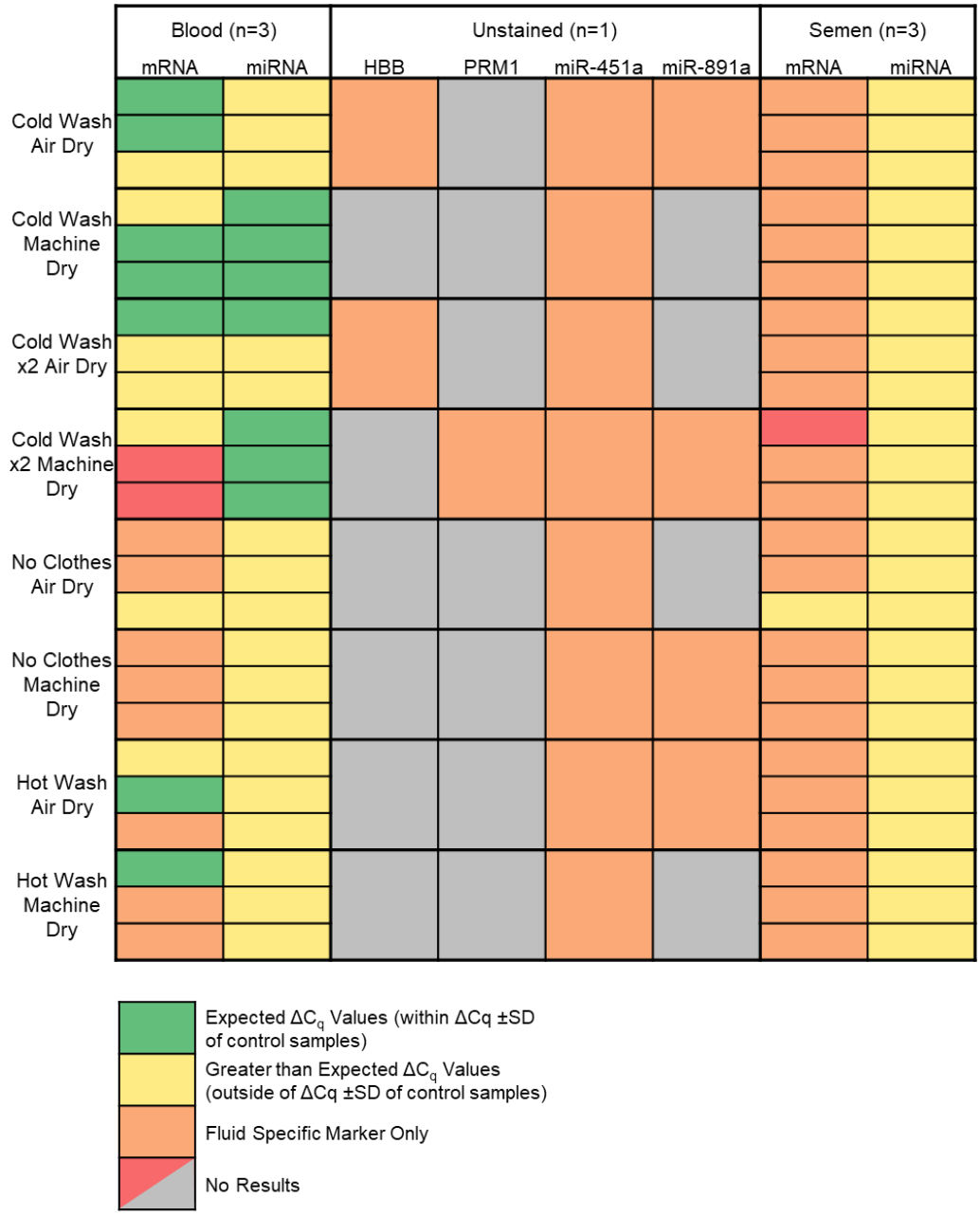


Fig. 3.6. Heat map denoting the presence or absence of mRNA or miRNA targets after being laundered. Cotton swatches stained with blood or semen (n=3), and unstained (n=1) swatches. Green – Both reference and body fluid specific markers present and ΔC_q values within ΔC_q ±SD of control samples. Yellow – Both reference and body fluid specific markers are present but deviate from ΔC_q ±SD of control samples. Orange – Only body fluid specific marker (HBB, miR-451a, PRM1, or miR-891a) detected. Reference marker not detected. Red – No transcripts detected in stained areas. Grey – No transcripts detected in unstained areas.

All semen samples prior to washing fluoresced when examined with a Crime-Lite™ and accompanying orange goggles. However, in contrast to a previous study [19], fluorescence was not detected after the samples were washed and dried. Consistent with the blood-stained items, both miRNA targets (let-7g and miR-891a) were observed in all samples, though ΔC_q values were out of normal range (outside of of ΔC_q ±SD of control samples) when compared to control values. A previous study [19] examining miRNAs in laundered items did not yield conclusive results which again may be attributed to differences in extraction techniques, amplification chemistries (probe based vs. intercalating dye), or primer design. For mRNA, PRM1 was observed in all samples except one, in which neither transcript was amplified. PRM1 was also detected more frequently than reference markers by Kulstein et al. [19] who theorized that spermatozoa cells were able to become embedded in the fabric, particularly in stains stored for a period of time before washing. Although a microscopic analysis was not performed in our study, spermatozoa have been positively identified in several other studies investigating spermatozoa persistence in laundered items [19,21,22,36-38].

The transfer of mRNA and miRNA during laundering was also evaluated in this study (Fig. 3.6). Body fluid specific markers for both blood and semen were evaluated for each of the unstained cuttings as items washed at the same temperatures were washed together. Reference markers were not included in the data analysis for the swatches evaluating transfer as they would be present in normal washing conditions through general skin contact [35] and do not specifically contribute to body fluid identification. Transfer of mRNA was observed in samples when washed in cold water and air dried, and surprisingly when washed twice in cold water air and machine dried. The transfer of blood specific

mRNA has also been previously reported by van den Berge et al. [35]. Samples washed without the addition of other clothes did not exhibit any transfer of mRNA, which is also consistent with several previous studies [19,34,35]. The blood miRNA target (miR-451a) was detected in all unstained cuttings, and transfer of the semen specific target (miR-891a) was observed in half of the unstained samples. A higher number of transfer events were noted in samples washed in colder water than hot, but more experimentation is necessary to determine any significant trends. Additionally, as no human identification techniques were performed in this study, the sources of the transfer events were not limited to experimental samples included in the wash, but may also be due to residual biological material in the washing machine itself. The transfer of cellular material through laundry can have significant implications, especially in cases regarding abuse and trafficking [21,37]. The authors agree with statements made by other groups investigating the transfer of genetic material via laundry that transfer is highly contingent on a number of variables including, but not limited to, fabric type, temperature, detergent type, storage time, collection method, and analysis methods of the laboratory and that results should be interpreted with caution [35,37].

Concluding remarks

Although this work used only one body fluid-specific mRNA and miRNA marker each for blood and semen, miRNA appears more stable and persistent than mRNA under the environmental conditions tested. All miRNA targets were observed in all samples for the duration of the 180-day experiment, while mRNA was not detected in environmentally challenged samples after 30 days. Both body fluid specific and reference miRNA targets were observed in all laundered samples, though only a cursory investigation of laundry

scenarios were explored. While the stability of these markers is promising and would likely persist beyond the 180 day limit of this experiment, any alteration in expression ratios, as seen in the degraded semen samples, may yield inconclusive results using already complicated interpretation strategies via RT-qPCR methods [1,2]. CE-based miRNA assays have been developed [3-5]; however, issues regarding mixtures, standardization of markers, and background transcription remain.

The limited stability and persistence of mRNA in forensically relevant samples has been demonstrated in this study as well as numerous others [6-11]. The body fluid specific mRNA markers, HBB and PRM1, were detected for over 30 days in challenging conditions including elevated temperature, high humidity and exposure to direct sunlight. The transcripts also persisted through wash cycles and machine drying. Implementation of stable region transcripts (STaRs) [12] or the use of massively parallel sequencing [13] may further improve body fluid identification analyses for challenging forensic samples.

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References

1. Horjan I, Barbaric L, Mrsic G (2016) Applicability of three commercially available kits for forensic identification of blood stains. J Forensic Leg Med 38:101-105. doi:10.1016/j.jflm.2015.11.021
2. Myers JR, Adkins WK (2008) Comparison of modern techniques for saliva screening. J Forensic Sci 53 (4):862-867. doi:10.1111/j.1556-4029.2008.00755.x
3. Vennemann M, Koppelkamm A (2010) mRNA profiling in forensic genetics I: Possibilities and limitations. Forensic Sci Int 203 (1-3):71-75. doi:10.1016/j.forsciint.2010.07.006
4. Hanson EK, Lubenow H, Ballantyne J (2009) Identification of forensically relevant body fluids using a panel of differentially expressed microRNAs. Anal Biochem 387 (2):303-314. doi:10.1016/j.ab.2009.01.037
5. Juusola J, Ballantyne J (2003) Messenger RNA profiling: a prototype method to supplant conventional methods for body fluid identification. Forensic Sci Int 135 (2):85-96. doi:10.1016/S0379-0738(03)00197-X
6. Sijen T (2015) Molecular approaches for forensic cell type identification: On mRNA, miRNA, DNA methylation and microbial markers. Forensic Sci Int Genet 18:21-32. doi:10.1016/j.fsigen.2014.11.015
7. Fleming RI, Harbison S (2010) The development of a mRNA multiplex RT-PCR assay for the definitive identification of body fluids. Forensic Sci Int Genet 4 (4):244-256. doi:10.1016/j.fsigen.2009.10.006

8. Silva SS, Lopes C, Teixeira AL, Carneiro de Sousa MJ, Medeiros R (2015) Forensic miRNA: potential biomarker for body fluids? Forensic Sci Int Genet 14:1-10. doi:10.1016/j.fsigen.2014.09.002

9. Bauer M (2007) RNA in forensic science. Forensic Sci Int Genet 1 (1):69-74. doi:10.1016/j.fsigen.2006.11.002

10. Alvarez M, Juusola J, Ballantyne J (2004) An mRNA and DNA co-isolation method for forensic casework samples. Anal Biochem 335 (2):289-298. doi:10.1016/j.ab.2004.09.002

11. Grabmuller M, Madea B, Courts C (2015) Comparative evaluation of different extraction and quantification methods for forensic RNA analysis. Forensic Sci Int Genet 16:195-202. doi:10.1016/j.fsigen.2015.01.006

12. Meakin G, Jamieson A (2013) DNA transfer: review and implications for casework. Forensic Sci Int Genet 7 (4):434-443. doi:10.1016/j.fsigen.2013.03.013

13. van Oorschot RA, Ballantyne KN, Mitchell RJ (2010) Forensic trace DNA: a review. Investig Genet 1 (1):14. doi:10.1186/2041-2223-1-14

14. Setzer M, Juusola J, Ballantyne J (2008) Recovery and stability of RNA in vaginal swabs and blood, semen, and saliva stains. J Forensic Sci 53 (2):296-305. doi:10.1111/j.1556-4029.2007.00652.x

15. Sirker M, Schneider PM, Gomes I (2016) A 17-month time course study of human RNA and DNA degradation in body fluids under dry and humid environmental conditions. Int J Legal Med 130 (6):1431-1438. doi:10.1007/s00414-016-1373-9

16. Zhao H, Wang C, Yao L, Lin Q, Xu X, Hu L, Li W (2017) Identification of aged bloodstains through mRNA profiling: Experiments results on selected markers of

30- and 50-year-old samples. Forensic Sci Int 272:e1-e6. doi:10.1016/j.forsciint.2017.01.006

17. Nakanishi H, Hara M, Takahashi S, Takada A, Saito K (2014) Evaluation of forensic examination of extremely aged seminal stains. Legal Med-Tokyo 16 (5):303-307. doi:10.1016/j.legalmed.2014.04.002

18. Grabmuller M, Courts C, Madea B, Eichhorst T, Schyma C (2018) RNA/DNA co-analysis from bloodstains on aged polyvinyl-alcohol gloves prepared for securing evidence from the hands of victims of fatal gunshot injuries. Int J Legal Med 132 (1):53-66. doi:10.1007/s00414-017-1687-2

19. Kulstein G, Schacker U, Wiegand P (2018) Old meets new: Comparative examination of conventional and innovative RNA-based methods for body fluid identification of laundered seminal fluid stains after modular extraction of DNA and RNA. Forensic Sci Int Genet 36:130-140. doi:10.1016/j.fsigen.2018.06.017

20. Edler C, Gehl A, Kohwagner J, Walther M, Krebs O, Augustin C, Klein A (2017) Blood Trace Evidence on Washed Textiles-a systematic approach. Int J Legal Med 131 (4):1179-1189

21. Brayley-Morris H, Sorrell A, Revoir AP, Meakin GE, Court DS, Morgan RM (2015) Persistence of DNA from laundered semen stains: Implications for child sex trafficking cases. Forensic Sci Int Genet 19:165-171. doi:10.1016/j.fsigen.2015.07.016

22. Crowe G, Moss D, Elliot D (2000) The effect of laundering on the detection of acid phosphatase and spermatozoa on cotton t-shirts. Can Soc Forensic Sci J 33 (1):1-5

23. Voskoboinik L, Amiel M, Reshef A, Gafny R, Barash M (2018) Laundry in a washing machine as a mediator of secondary and tertiary DNA transfer. Int J Legal Med 132 (2):373-378. doi:10.1007/s00414-017-1617-3

24. Levings PP, Bungert J (2002) The human β-globin locus control region. Eur J Biochem 269 (6):1589-1599. doi:10.1046/j.1432-1327.2002.02797.x

25. Bauer M, Patzelt D (2003) Protamine mRNA as molecular marker for spermatozoa in semen stains. Int J Legal Med 117 (3):175-179. doi:10.1007/s00414-002-0347-2

26. Moreno LI, Tate CM, Knott EL, McDaniel JE, Rogers SS, Koons BW, Kavlick MF, Craig RL, Robertson JM (2012) Determination of an effective housekeeping gene for the quantification of mRNA for forensic applications. J Forensic Sci 57 (4):1051-1058. doi:10.1111/j.1556-4029.2012.02086.x

27. Seashols-Williams S, Lewis C, Calloway C, Peace N, Harrison A, Hayes-Nash C, Fleming S, Wu Q, Zehner ZE (2016) High-throughput miRNA sequencing and identification of biomarkers for forensically relevant biological fluids. Electrophoresis 37 (21):2780-2788. doi:10.1002/elps.201600258

28. Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL, Vandesompele J, Wittwer CT (2009) The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem 55 (4):611-622. doi:10.1373/clinchem.2008.112797

29. Juusola J, Ballantyne J (2007) mRNA profiling for body fluid identification by multiplex quantitative RT-PCR. J Forensic Sci 52 (6):1252-1262. doi:10.1111/j.1556-4029.2007.00550.x

30. Fang C, Zhao J, Li J, Qian J, Liu X, Sun Q, Liu W, Tian Y, Ji A, Wu H, Yan J Massively parallel sequencing of microRNA in bloodstains and evaluation of environmental influences on miRNA candidates using realtime polymerase chain reaction. Forensic Sci Int Genet

31. Tian H, Lv M, Li Z, Peng D, Tan Y, Wang H, Li Q, Li F, Liang W (2018) Semen-specific miRNAs: Suitable for the distinction of infertile semen in the body fluid identification? Forensic Sci Int Genet 33:161-167. doi:10.1016/j.fsigen.2017.12.010

32. Hu L, Wu C, Guo C, Li H, Xiong C (2014) Identification of microRNAs predominately derived from testis and epididymis in human seminal plasma. Clin Biochem 47 (10-11):967-972. doi:10.1016/j.clinbiochem.2013.11.009

33. Zubakov D, Boersma AW, Choi Y, van Kuijk PF, Wiemer EA, Kayser M (2010) MicroRNA markers for forensic body fluid identification obtained from microarray screening and quantitative RT-PCR confirmation. Int J Legal Med 124 (3):217-226. doi:10.1007/s00414-009-0402-3

34. Kulstein G, Wiegand P (2018) Comprehensive examination of conventional and innovative body fluid identification approaches and DNA profiling of laundered blood- and saliva-stained pieces of cloths. Int J Legal Med 132 (1):67-81. doi:10.1007/s00414-017-1691-6

35. van den Berge M, Ozcanhan G, Zijlstra S, Lindenbergh A, Sijen T (2016) Prevalence of human cell material: DNA and RNA profiling of public and private objects and after activity scenarios. Forensic Sci Int Genet 21:81-89. doi:10.1016/j.fsigen.2015.12.012

36. Jobin R, De Gouffe M (2003) The persistence of seminal constituents on panties after laundering. Significance to investigations of sexual assault. Can Soc Forensic Sci J 36 (1):1-10

37. Noel S, Lagace K, Rogic A, Granger D, Bourgoin S, Jolicoeur C, Seguin D (2016) DNA transfer during laundering may yield complete genetic profiles. Forensic Sci Int Genet 23:240-247. doi:10.1016/j.fsigen.2016.05.004

38. Farmen RK, Cortez P, Frøyland ES (2008) Spermatozoa recovered on laundered clothing. Forensic Science International: Genetics Supplement Series 1 (1):418-420

39. Sirker M, Fimmers R, Schneider PM, Gomes I (2017) Evaluating the forensic application of 19 target microRNAs as biomarkers in body fluid and tissue identification. Forensic Sci Int Genet 27:41-49. doi:10.1016/j.fsigen.2016.11.012

40. Mayes C, Seashols-Williams S, Hughes-Stamm S (2018) A capillary electrophoresis method for identifying forensically relevant body fluids using miRNAs. Legal Med-Tokyo 30:1-4. doi:10.1016/j.legalmed.2017.10.013

41. Li Y, Zhang J, Wei W, Wang Z, Prinz M, Hou Y (2014) A strategy for co-analysis of microRNAs and DNA. Forensic Sci Int Genet 12:24-29. doi:10.1016/j.fsigen.2014.04.011

42. Williams G, Uchimoto ML, Coult N, World D, Beasley E, Avenell P (2013) Characterisation of body fluid specific microRNA markers by capillary electrophoresis. Forensic Sci Int Genet Suppl Ser 4 (1):e274-e275

43. Zubakov D, Kokshoorn M, Kloosterman A, Kayser M (2009) New markers for old stains: stable mRNA markers for blood and saliva identification from up to 16-year-old stains. Int J Legal Med 123 (1):71-74. doi:10.1007/s00414-008-0249-z

44. van den Berge M, Wiskerke D, Gerretsen RR, Tabak J, Sijen T (2016) DNA and RNA profiling of excavated human remains with varying postmortem intervals. Int J Legal Med 130 (6):1471-1480. doi:10.1007/s00414-016-1438-9

45. Lin M-H, Albani PP, Fleming R (2016) Degraded RNA transcript stable regions (StaRs) as targets for enhanced forensic RNA body fluid identification. Forensic Sci Int Genet 20:61-70

46. Hanson E, Ingold S, Haas C, Ballantyne J (2018) Messenger RNA biomarker signatures for forensic body fluid identification revealed by targeted RNA sequencing. Forensic Sci Int Genet 34:206-221. doi:10.1016/j.fsigen.2018.02.020

CHAPTER IV

Evaluation of an early-access targeted mRNA massively parallel sequencing for body fluid identification on challenging and mock casework-type samples.

This dissertation follows the style and format of *International Journal of Legal Medicine*.

Abstract

Messenger RNA (mRNA) profiling for body fluid identification has been proven as a useful tool for forensic investigations. The technique has been deemed highly sensitive, confirmatory for several different forensically relevant body fluids, and can be performed in parallel analysis to DNA. The development of massively parallel sequencing technologies has allowed for simultaneous detection of numerous targeted transcripts. The inclusion of consensus markers can increase confidence in the determination of a sample's body fluid(s) of origin. Few studies, however, have applied this technology to challenging samples.

In this study, an initial evaluation of a customized Verogen mRNA profiling panel was performed using samples of venous blood, menstrual blood, semen, saliva, and vaginal secretions exposed to various compromised conditions. Stained swatches placed on a decomposing cadaver as well as outdoors without protection from precipitation were collected over a two-week period. Mock casework samples such as post-coital underwear, laundered stains with and without machine drying, swatches placed outdoors with protection from precipitation, buried swatches, and aged samples were also collected and analyzed. While extensive validation studies will be required, the panel was able to amplify transcripts in these challenging samples, suggesting the panel could be a powerful tool for forensic analysts.

Keywords: Massive parallel sequencing, messenger RNA, body fluid identification, persistence

Introduction

Massively parallel sequencing (MPS) offers capabilities that have greatly interested the forensic community. In addition to highly valuable sequence-based information, sequencing can now be performed in a high-throughput manner and simultaneously analyze a multitude of targets. MPS panels have been commercially developed for the forensic community for the analysis of STRs [1], mitochondrial DNA [2], ancestry informative SNPS [3], identity informative SNPs [4,5], and phenotypic SNPs [6,7]. As MPS platforms are being adopted for casework use, panels are also being developed for other forensic genomic capacities including targeted messenger RNA (mRNA) sequencing for body fluid identification (BFID) [8-10].

mRNA profiling was developed as a molecular means to identify the biological material of evidentiary samples and increase the confidence of this determination both scientifically and in the courtroom. Gene expression patterns in terminally differentiated cells based on both presence and abundance of certain gene transcripts can positively associate a sample to a fluid or tissue type [11]. The profiling system also addresses many of the limitations of current serological tests, particularly human and body fluid specificity, while also increasing the number of fluid or tissue types that can be tested simultaneously. To this end, capillary electrophoresis-based mRNA assays have been developmentally validated and incorporated into casework [12,13].

In addition, the increased sensitivity of DNA analysis has highlighted the necessity of answering questions about the nature of the deposition of cellular material [14-18]. While a DNA profile may yield the identity of an individual, the context of deposition is

unknown. Information regarding the body fluid or cell type of the sample may be able to answer questions regarding the activity level source of a sample.

While several studies have demonstrated the utility of mRNA for forensic use [19-24], the reputation of RNA as being unstable endures. To further address this misconception, researchers have continued to improve the detection window of these assays. Recently, RNA sequencing on time-course degraded samples yielded specific areas of mRNA that were more likely to persist over time, termed stable region RNAs or "STaRs" [25]. The development of primers targeting these regions greatly increased the likelihood of transcript detection in degraded samples [26-28]. RNA sequencing also affords the opportunity to identify SNPs within each transcript which may be used to associate a body fluid with an individual [8]. In this study, an early access MPS panel for BFID based on STaR data was evaluated using a variety of challenging samples.

Materials and Methods

Sample Collection

Samples of venous blood, menstrual blood, semen, saliva, and vaginal secretions were collected with informed consent using an IRB approved protocol. Venous blood was collected via venipuncture into EDTA treated BD Vacutainer™ tubes, semen was self-collected, whole saliva was collected without instruction to abstain from food or beverage, and menstrual blood and vaginal secretions were self-collected via cotton swab.

Samples were dried overnight and placed in various conditions as described in Table 1. Poly-cotton fabric (approximately 13 in x 9 in) was stained with 50 µL aliquots of the designated fluid or a whole cotton swab was attached with tape to the fabric. Fabric was stained with multiple fluids (approximately 2 in apart) to prevent sample loss during

treatment. Buried samples were placed in a hole approximately six inches deep. Samples placed outside with exposure to rain were placed underneath metal mesh (0.5 cm x 0.5 cm diameter) to reduce interference from birds and small animals. Samples positioned outside without exposure to train were placed under tented polyvinyl sheeting (approximately 24 in height). Blood and semen (50 μL) were pre-stained onto swatches (100% cotton muscle shirt and cotton flannel pants) and attached to clothing on the cadaver via safety pins to reduce possible contamination during clothing (Fig 1a-b). The cadaver was placed at the Applied Anatomical Research Center (AARC) in November 2018. A covered cage was placed over the cadaver to reducing scavenging and exposure to precipitation (Fig 1c). Laundered samples were washed in a top-loading Whirlpool® Commercial Washer Heavy Duty Series at a preprogrammed cold setting of 15°C with one Tide PODS® packet (Proctor & Gamble, Cincinnati, OH) for 30 min and either air dried or machine dried for 45 minutes using a Whirlpool® Commercial Single Load, Super Capacity Stack Dryer on the permanent press setting at a reported 57°C. Aged samples were placed in an oven at 37°C for 16 weeks at high humidity. Post-coital underwear was provided by the female wearer and varied in material and color and stored at room temperature until extraction.

Table 4.1. Sample treatments for challenging samples. VB – venous blood, SE – semen, SA – saliva, MB – menstrual blood, VS – vaginal secretions.

Treatment	Biological Sources/ Fluid	Fluids	Time in Treatment/ Collection Timepoints
Control	3	VB,SE,SA,MB,VS	n/a
Buried	3	VB,SE,SA,MB,VS	3 days
Outside – No Rain	3	VB,SE,SA,MB,VS	1 month
Outside	3	VB,SE,SA,VS	Days 1,3,7,10,14
Decomposing Cadaver	3	VB,SE	Days 1,3,7,10,14
Wash (Air Dry)	3	VB,SE,SA	30 min wash
Wash (Machine Dry)	3	VB,SE,SA	30 min wash/45 min dry
Aged	3	VB,SE	16 weeks – 37°C oven
Post-Coital Underwear (Female)	3	SE,VS	As provided

a.

b.

c.

Fig. 4.1. Samples placed on a decomposing cadaver. **a** Venous blood samples placed on the trunk of the cadaver. **b** Semen samples placed on the groin/upper leg region of the cadaver. **c** Cage and tarp placed over the cadaver and samples.

Extraction and quantification

All samples were extracted with the AllPrep DNA/RNA Mini Kit (QIAGEN, Hilden, Germany) according to manufacturer's instructions with the addition of a centrifugation step of 5 min at 16,000 g to remove lysate from the substrate. DNA and RNA elution volumes were 50 µL. DNA was quantified using QuantiPlex Pro (QIAGEN) on a 7500 Real Time PCR System (Applied Biosystems, Foster City, CA). DNA was removed from RNA extracts using the TURBO-free DNase Kit (Invitrogen, Carlsbad, CA). RNA was quantified using the High Sensitivity RNA Assay (Invitrogen) on a Qubit 2.0 (Applied Biosystems) and the RNA 6000 Pico Kit (Agilent, Santa Clara, CA) on an Agilent 2100 Bioanalyzer.

DNA amplification and detection

DNA extracts were amplified using the Investigator QS 24plex Kit (QIAGEN) on a ProFlex Thermal Cycler (Applied Biosystems) with a target of 0.8 ng using recommended manufacturer protocols. The full volume (15 µL) was used for samples below the target. Fragments were detected using a 3500 Genetic Analyzer with POP-4™ and a 36 cm capillary array (30 s injection time at 13 kV) and analyzed with Genemapper ID-X v. 1.4 (all Applied Biosystems). Stochastic and analytical thresholds were 100 RFU and 200 RFU, respectively.

RNA sequencing

Reverse transcription and library amplification

Samples were sequenced using a custom library preparation kit and panel. Briefly, 10 µL of neat sample was added to 4 µL of cDNA Primer Mix and the mixture was incubated at 65 °C for 5 minutes followed by chilling to 4 °C to hybridize primers to RNA.

cDNA Reaction Mix (5 µL) and Reverse Transcriptase (1 µL) were added to each reaction and reverse transcription negatives were processed in parallel for each sample using 1 µL Nuclease Free water in lieu of enzyme. Reverse transcription was performed using the following parameters: 25 °C for 5 min, 42 °C for 50 min, 95 °C for 5 min, and at hold at 4 °C. Samples were barcoded and amplified in reactions consisting of 2 µL each of Index 1 and 2, 19.4 µL PCR1 Reaction Mix, 0.6 µL Enzyme Mix, 20 µL Primer mix, and 16 µL cDNA using the following PCR parameters: 98 °C for 3 min; 8 cycles of 96 °C for 45 sec, 80 °C for 30 sec, 54 °C for 2 min, 68 ° for 2 min; 10 cycles of 96 °C for 30 sec, 68 °C for 3 min; 15 cycles of 98 °C for 20 sec, 66 °C for 30 sec, 68 °C for 90 sec; 68 °C for 10 min, and a hold at 10 °C. Targets and amplicons sizes are listed in Table 2.

Table 4.2. Loci targeted in the BFID panel. Numbers in the parentheses are for notation purposes to distinguish between amplicons from the same gene.

Body Fluid	Gene Name	Accession Number	Exons	Amplicon Size
Circulatory Blood	ALAS2	XM_005261995.2	10,10	73
	GYPA	NM_002099.6	6,5	85
	HBD(_1)	NM_000519	1,1	69
	HBD(_2)	NM_000519	3,2	62
	SLC4A1(_1)	NM_000342.3	4,3	77
	SLC4A1(_2)	NM_000342.3	3,2	80
Menstrual Blood	MMP10(_1)	NM_002425.2	5,4	98
	MMP10(_2)	NM_002425.2	4,3	101
	MMP11	NM_005940.3	5,5	64
	MMP3	NM_002422.3	4,4	63
	PLAUR_1	NM_002659.3	6,6	69
	PLAUR_2	NM_002659.3	7,6	77
	SNORA35	NR_002993.1	1,1	62
	STC1	NM_003155.2	4,4	73
Semen	KLK2	NM_005551.4	2,2	85
	MSMB	NM_002443.3	2,2	68
	PRM1	NM_002761.2	1,1	60
	PRM2	NM_002762	2,1	60
	SEMG1	NM_003007.3	2,2	77
	SEMG2	NM_003008.2	1,2	63
	SPATA42	NR_049777.1	1,1	63
	TNP1	NM_003284.3	1,1	60
Saliva	CST1	NM_001898.2	1,1	62
	FDCSP	NM_152997.3	4,4	61
	HTN3	NM_000200.2	5,5	66

Body Fluid	Gene Name	Accession Number	Exons	Amplicon Size
	PRB4(_1)	NM_002723.5	3,3	45
	PRB4(_2)	NM_002723.5	4,4	48
	STATH	NM_003154.2	4,4	61
				(continued)
Vaginal Material	HOXA11(_1)	NM_005523.5	2,2	84
	HOXA11(_2)	NM_005523.5	2,2	95
	HOXA13(_3)	NM_000522.4	2,2	79
	HOXA13(_1)	NM_000522.4	2,2	79
	HOXA13(_2_	NM_000522.4	2,2	60
	MYOZ1	NM_021245.3	1,1	68
	PRSS21	NM_006799.3	5,5	86
	SPRR2G	NM_001014291.3	2,2	99
Skin	CCL27	NM_006664.2	3,3	63
	CST6	NM_001323.3	3,3	77
	LOR	NM_000427.2	2,2	81
Urine	AQP6(_1)	NM_001652.3	4,4	61
	AQP6(_2)	NM_001652.3	4,4	81
House-keeping Genes	TCEA(_1)	NM_006756.3	7,6	70
	TCEA(_2)	NM_006756.3	12,12	67
	G6PD(_1)	NM_000402.4	10,10	97
	G6PD(_2)	NM_000402.4	13,13	61
	UBE2D2	NM_181838.1	1,1	79

Library purification, quantification, and normalization

Sample Purification Beads (SPB2) (49.7 µL) and Proteinase K (0.3 µL) were added to 50 µL of each amplified library, vortexed for 2 min at 1800 rpm, and incubated at room temperature for 10 min. The samples were placed on a magnetic stand and the supernatant was discarded. Libraries were then washed twice as follows: 200 µL of 80% ethanol was added to each library, placed on a magnetic stand for 30 sec, and the supernatant discarded. Libraries were eluted by adding 52.5 µL Resuspension Buffer (RSB), vortexing for 2 min at 1800 rpm, and incubating at room temperature for 2 min. Purified libraries (50 µL) were collected for quantification and normalization. Libraries were quantified with the Qubit HS DNA Assay on a Qubit 2.0 according to the manufacturer's protocol. Libraries were normalized to 0.75 ng/µL and pooled (5 µL from each library).

MiSeq sequencing

Libraries were denatured and diluted immediately before sequencing. Pooled libraries (5 μL) were combined with 5 μL 0.2 N NaOH, vortexed, centrifuged for 1 min at 280 g, and incubated for 5 min at room temperature for denaturation. The denatured libraries were diluted to 20 pM by adding 990 μL of prechilled HT1. A 180 μL volume of the 20 pM libraries was combined with 418 μL prechilled HT1 and 2 μL or 4 μL pM denatured PhiX library, resulting in a final library concentration of 6 pM. The 6 pM denatured libraries (600 μL) were then deposited in the reagent cartridge for sequencing. A MiSeq Reagent Kit v3 (150 or 300 cycle) was used for sequencing.

Data Analysis

Samples were demultiplexed, parsed, and aligned using the Local Run Manager with the RNA Amplicon Analysis Module v. 1.0.2. Reads from reverse transcription negatives were subtracted from sample read values at each amplicon to reduce incorporation of reads from genomic amplification. A 100 read minimum was applied to all targets along with a 5% of total reads for each sample. Control RNA positives were evaluated using a 0.5% threshold. SNPs were identified using IGV aligned to a custom amplicon genome file.

Results and discussion

DNA

Co-extracted DNA profiles ranged from 31.1 – 100% profile completeness (Fig. 4.2). Allelic dropout was observed most frequently, nine of the fifteen profiles, in the buried samples. Buried samples can be challenging due to microbial degradation of DNA and addition of inhibitors, such as humic acid, both of which are naturally present in soil

[29,30]. DNA extracts from the buried samples had a dark appearance, though no inhibition was detected by the QS sensors. Additionally, contamination between samples was detected in four samples, likely occurring during the burial or recovery process.

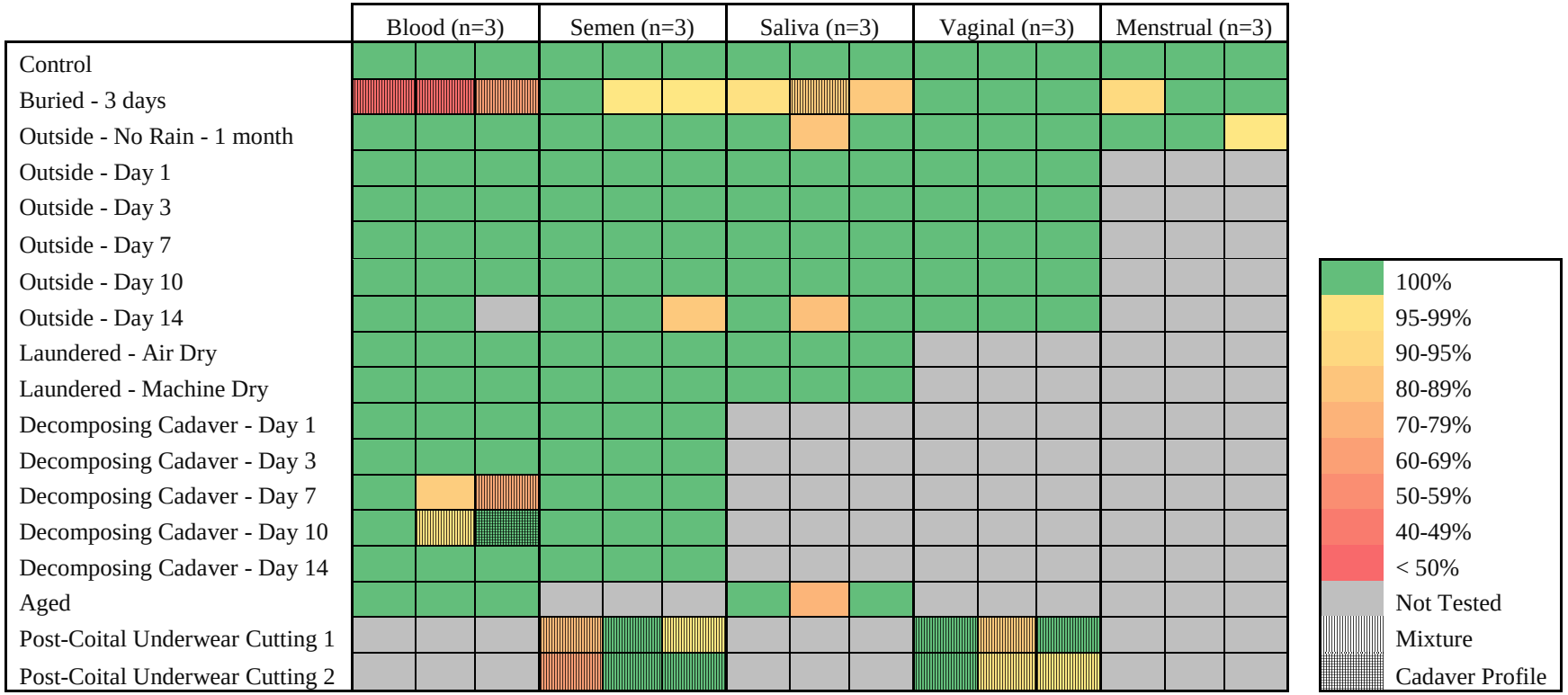


Fig. 4.2. Heat map of STR profile completeness. Each cell represents one sample.

As expected, mixtures were also observed in the post-coital samples consisting of profiles of the respective partner. Male profile completeness ranged from 59 – 100% complete and female profiles ranged from 82 – 100% complete. Similar to the buried samples, the extracts from the pair of cuttings from Pair 1 were dark in appearance due to fabric dye co-extracting with DNA, but no inhibition was detected. Interestingly, only two samples placed on the cadaver showed alleles from the cadaver. A blood sample from day 7 contained a partial profile from the cadaver and the blood donor, while a blood sample from day 10 exhibited a full, single-source profile belonging to the cadaver. As seen in previous research, full profiles were observed in both the air dried and machine dried laundered samples [24,31,32].

RNA

RNA quantification results and total read counts can be seen in Tables 3 and 4, respectively. Elevated values were noted in the buried samples as well as the samples placed on the decomposing cadaver, likely due to the presence of microbial flora as read counts were generally lower for these samples. Sequencing was performed over eight runs with % Q30 score averages ranging from 94.4 – 96.6% and 93.8% of clusters passing filter. After a run failure due to low cluster density, the 6 pM PhiX control library spike-in volume was increased from 2 μL to 4 μL in run 5 to increase cluster generation beyond the threshold and continue the run.

Table 4.3. RNA quantification values for each sample taken from Bioanalyzer results. Results are presented as ng/µL.

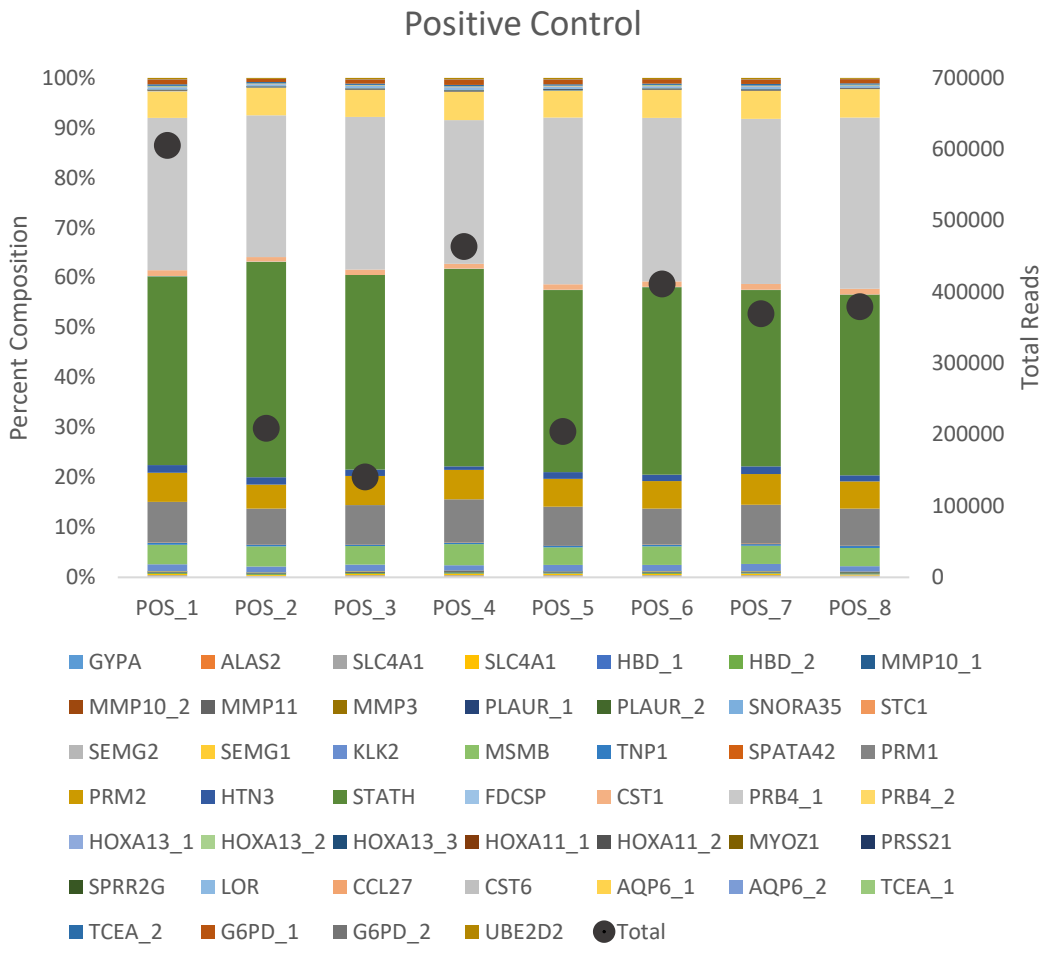
	Venous Blood			Semen			Saliva			Vaginal Secretion			Menstrual Blood		
Control	22.4	16.4	45.6	98.1	20.5	156.3	11.4	24.1	254.2	24957.0	38.9	887.1	603.3	17.0	45.5
Buried - 3 days	2481.8	7525.9	3725.8	1375.9	1622.1	1763.5	5.5	702.1	442.4	22187.2	6028.0	7469.4	29604.0	2884.8	3840.0
Outside - No Rain - 1 moth	45.5	14.4	8.3	7.37	20.0	16.1	7.6	47.8	66.0	17142.0	35.6	7584.4	1214.6	2411.8	3035.7
Outside - Day 1	53.4	3.7	19.4	408.3	78.9	64.1	66.2	29.5	62.0	2412.3	29.9	10335.4			
Outside - Day 3	9.0	13.8	9.7	144.1	174.9	845.1	32.4	20.2	33.0	596.4	290.3	567.7			
Outside - Day 7	12.5	8.1	24.6	495.4	29.3	105.2	13.1	40.2	61.7	38442.9	2762.7	6220.3			
Outside - Day 10	63.4	14.3	25.6	261.4	167.2	6.2	59.6	83.3	55.7	49979.1	387.3	4412.7			
Outside - Day 14	1.8	31.2	23.0	320.1	119.7	1166.0	31.4	42.0	41.7	10991.3	41.5	3501.0			
Laundered - Air Dry	50.3	137.3	53.6	35.5	28.7	56.6	16.1	40.9	44.8						
Laundered - Machine Dry	93.9	127.0	74.5	23.8	95.7	209.2	85.8	28.8	39.0						
Decomposing Cadaver - Day 1	284.0	97.4	188.7	522.9	110.1	233.3									
Decomposing Cadaver - Day 3	621.1	11421.7	37947.5	630.2	1858.7	468.7									
Decomposing Cadaver - Day 7	157.4	17954.8	27404.3	232.5	173.3	82.8									
Decomposing Cadaver - Day 10	459.2	1670.3	8227.3	15375.8	1172.6	5616.6									
Decomposing Cadaver - Day 14	2345.0	8456.5	469.7	246.3	84.6	75.0									
Aged	18.4	23.7	13.0				44.7	4.8	19.2						
Post-Coital Underwear Cutting 1				32.1	33.2	31.2									
Post-Coital Underwear Cutting 2				3.4	26.3	73.0									

Table 4.4. Total read counts per sample after application of thresholds.

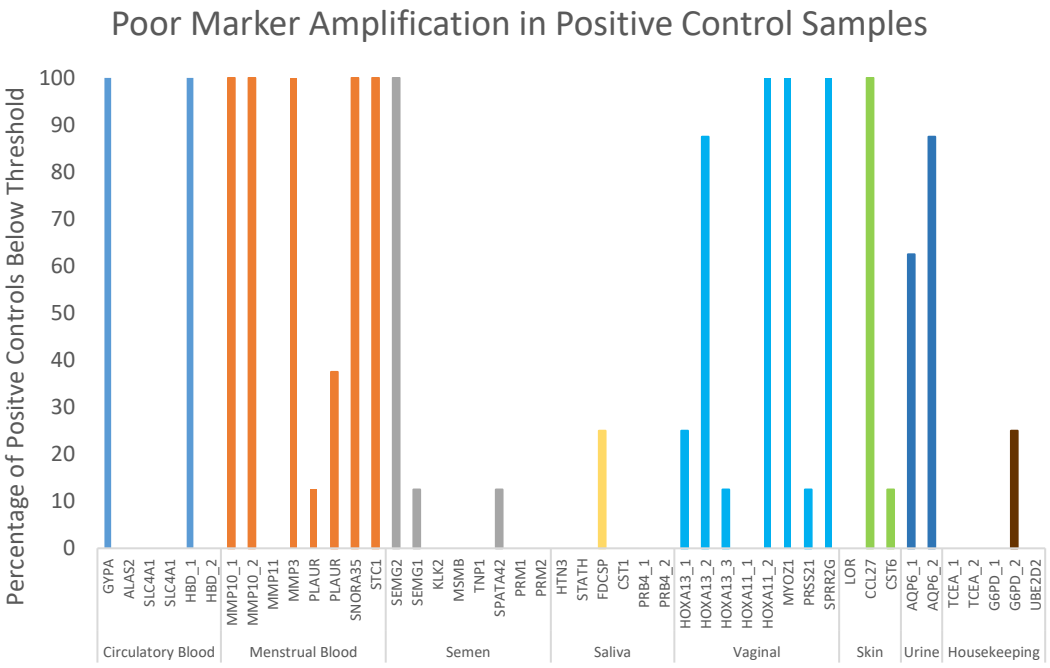
	Venous Blood			Semen			Saliva			Vaginal Secretion			Menstrual Blood		
Control	26012	14691	25979	366	27107	58847	314	109	23911	277147	289273	328032	143599	34430	113007
Buried - 3 days	0	3016	706	21039	1410	152638	0	209	0	1499	0	0	0	0	0
Outside - No Rain - 1 month	55130	45064	52951	8193	45316	210743	0	444	1705	43998	277729	141341	147811	226413	181411
Outside - Day 1	5867	1741	3504	388	1111	73220	0	0	0	115724	109651	95384			
Outside - Day 3	6046	4310	5034	27267	27470	17351	0	0	0	42886	94797	80059			
Outside - Day 7	2313	145	577	19852	2410	19208	289	0	0	36264	23828	66145			
Outside - Day 10	62884	6576	5411	9346	7582	26317	36152	626	930	97738	165791	211553			
Outside - Day 14	6408	1582	4976												
Laundered - Air Dry	415	2678	814	956	106	114	156	382	1313						
Laundered - Machine Dry	13810	7191	786	568	1955										
Decomposing Cadaver - Day 1	0	1826	580	84009	86443	86045									
Decomposing Cadaver - Day 3	137	3801	127	13377	81694	6042									
Decomposing Cadaver - Day 7	326	748	478	1235	39197	120666									
Decomposing Cadaver - Day 10	5841	519	230	89404	77287	40087									
Decomposing Cadaver - Day 14	269	1370		2445	60810	52927									
Aged	583	1126	313												
Post-Coital Underwear Cutting 1				0	148529	156466									
Post-Coital Underwear Cutting 2				0	78409	1477513									

Positive controls

Control RNA and a water negative was run with every amplification. Negative controls ranged from 8 – 32 reads. Control RNA total reads per target ranged from 140,772 – 605,444 and percentage of reads per target can be seen in Fig. 4.3a. Although total read counts were run dependent, composition remained similar between runs. In the controls as well as experimental samples, several targets exhibited less than 0.5% of total reads of the sample, and MMP10_2 spanning exon 4,3 was not detected in any positive controls or experimental samples (Fig. 4.3b). In contrast, two saliva markers, STATH and PBR4_1 represent over half of the total reads in each positive control. It is unknown whether the disproportion of targets is due to the composition of the control RNA or primer differences between targets which may affect the amplification of other targets and skew analysis.


Positive Control
Percent Composition
Total Reads
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
700000
600000
500000
400000
300000
200000
100000
0
POS_1
POS_2
POS_3
POS_4
POS_5
POS_6
POS_7
POS_8
GYPA
ALAS2
SLC4A1
SLC4A1
HBD_1
HBD_2
MMP10_1
MMP10_2
MMP11
MMP3
PLAUR_1
PLAUR_2
SNORA35
STC1
SEMG2
SEMG1
KLK2
MSMB
TNP1
SPATA42
PRM1
PRM2
HTN3
STATH
FDCSP
CST1
PRB4_1
PRB4_2
HOXA13_1
HOXA13_2
HOXA13_3
HOXA11_1
HOXA11_2
MYOZ1
PRSS21
SPRR2G
LOR
CCL27
CST6
AQP6_1
AQP6_2
TCEA_1
TCEA_2
G6PD_1
G6PD_2
UBE2D2
Total

a.



b.

Fig. 4.3 a Composition of reads (primary axis) and total reads (secondary axis) for each positive run with control RNA. **b** Percentage of occurrences in which a locus not pass thresholds in positive control samples (n=8).

Experimental Controls

Results of the experimental controls can be seen in Fig. 4.4. The total reads of the samples are substantially lower than some treated samples, particularly for the saliva controls. As the treated samples were prepared and place before the control samples were prepared, it is possible that RNA degraded during that time. Fluids were stored as whole fluids and exposure to oxygen and the presence of endogenous RNases may have decreased the overall amount of RNA in the whole fluid.

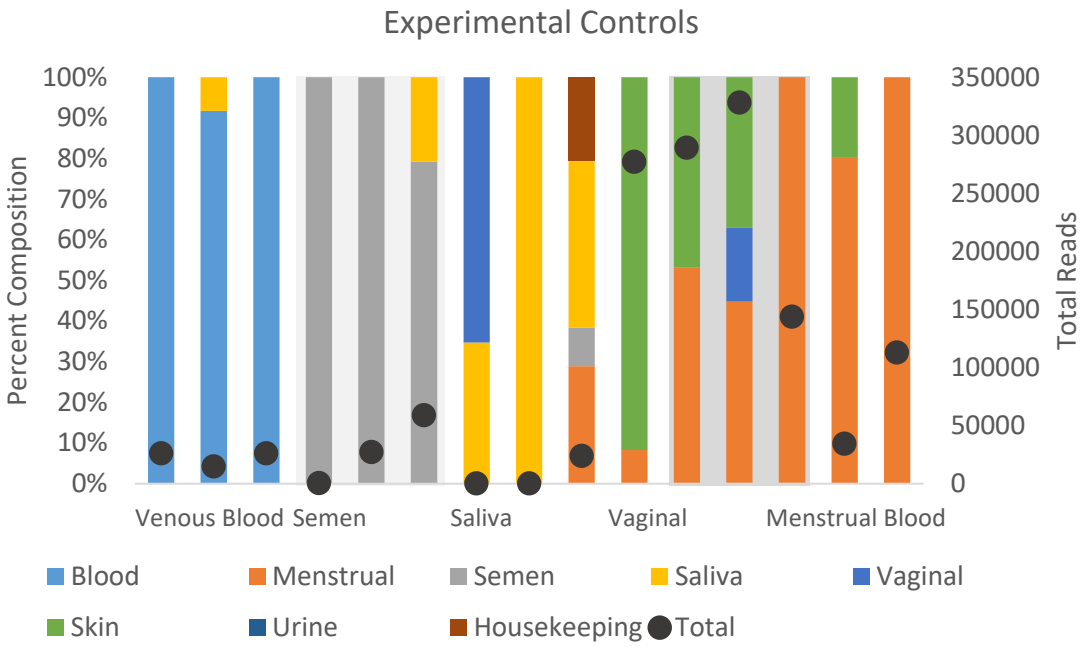


Fig. 4.4. Composition (primary axis) and total reads (secondary axis) for experimental control samples.

Venous blood, semen, and menstrual blood samples showed compositions over 50% of reads being attributed to markers of the target fluid. Saliva samples ranged from 34.7 – 100% composition of target markers. Interestingly, vaginal markers showed a high composition of skin targets (37.0 – 91.7%). Control samples for vaginal secretions were obtained from swabs self-collected from the vaginal cavity. The swab also likely collected epithelial, which likely accounts for the high proportion of reads attributed to skin. Skin targets have also been observed in vaginal samples [8,9,33], though at much lower levels. Vaginal samples also contained 8.3 – 53.3% reads attributed to menstrual targets. No specific guidelines were placed for donors for time of collection between menstrual cycles, though swabs did not appear to contain menstrual blood upon visual inspection. Low levels of menstrual markers have been observed in vaginal swabs, as well [8]. Developmental validation will certainly include numerous individuals and controls to ascertain the natural

variety of expression levels from different individuals to correctly identify body fluids in casework.

Buried samples

As seen with the corresponding DNA profiles, burying the samples had a detrimental effect on RNA profiling after only three days (Fig. 4.5). As mentioned above, extracts from the buried samples had a dark appearance. While no inhibition was noted in the STR profiles, reactions during the RNA workflow, such as reverse transcription, may have been inhibited and reduced the efficiency of the reaction. No read counts above the 100 read minimum were observed in any menstrual samples along with one venous blood sample, and two of each saliva and vaginal samples. Venous blood samples had a high composition of reads attributed to circulatory blood targets; however, the fluid of origin of the remaining samples is less obvious. Contamination, evident in the DNA profiles, may also account for non-fluid reads as seen in the remaining vaginal sample.

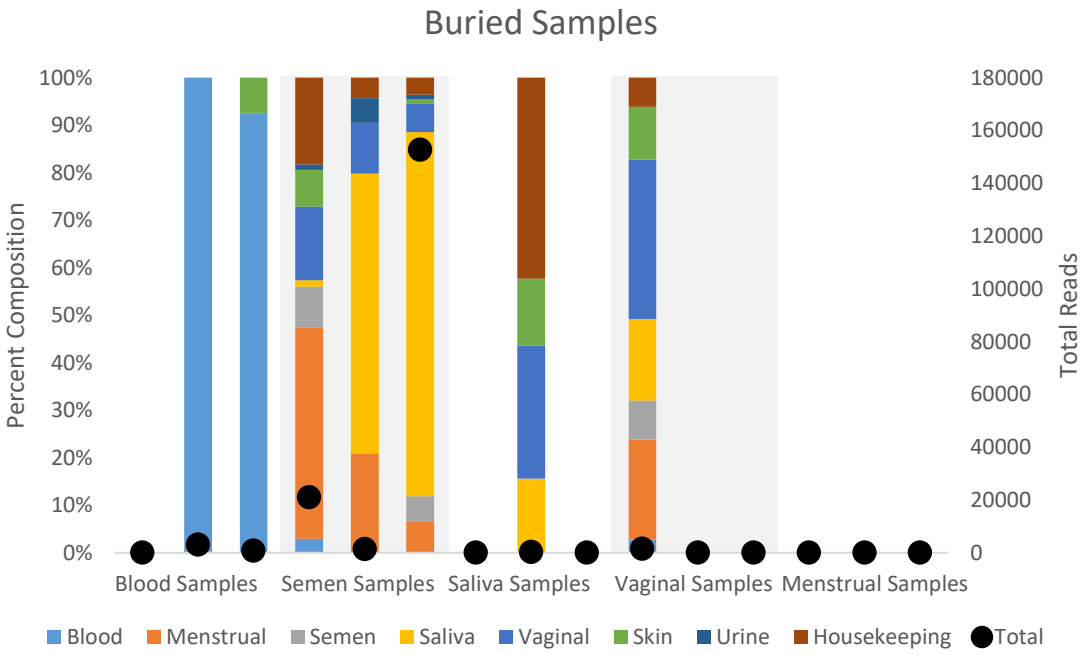


Fig. 4.5. Composition (primary axis) and total reads (secondary axis) for experimental buried samples.

Samples outside without exposure to rain

Samples placed outside for one month, with variable exposure to UV, temperature, and humidity, but protected from direct precipitation, yielded high total read counts for most samples (Fig. 4.6). With the exception of saliva samples (total reads 0 – 1705), all other samples produced read counts of above 10,000. Venous and menstrual blood targets composed of 59.8 – 100% of the reads for the respective samples. While semen target reads comprised between 0 – 23.8% of semen samples, transcripts from all body fluid categories were present in these samples. It should be noted that these samples had a large proportion of reads in the corresponding reverse transcription negatives, which will be discussed below. Similar to the control samples, vaginal and menstrual swabs included a considerable proportion of reads attribute to skin targets likely from the swab collection method. In previous studies, BFID transcripts were not able to be detected after a one month period in these conditions [19,34]. Successful typing in this study may be due to lower average temperatures experienced by the samples (average of approximately 13°C) or differences in platform.

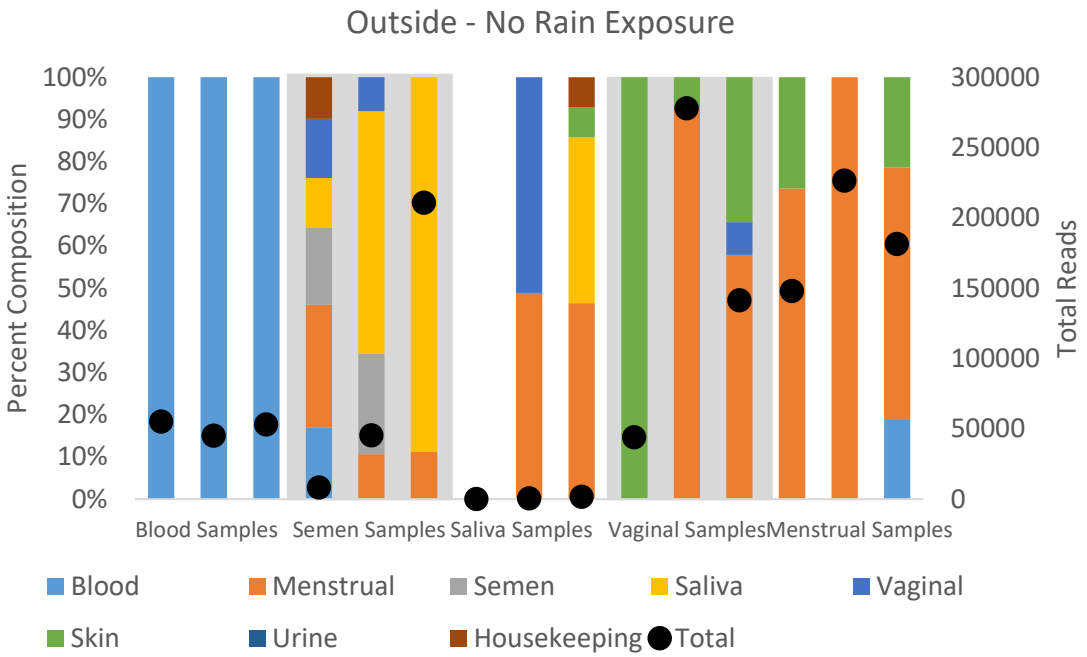


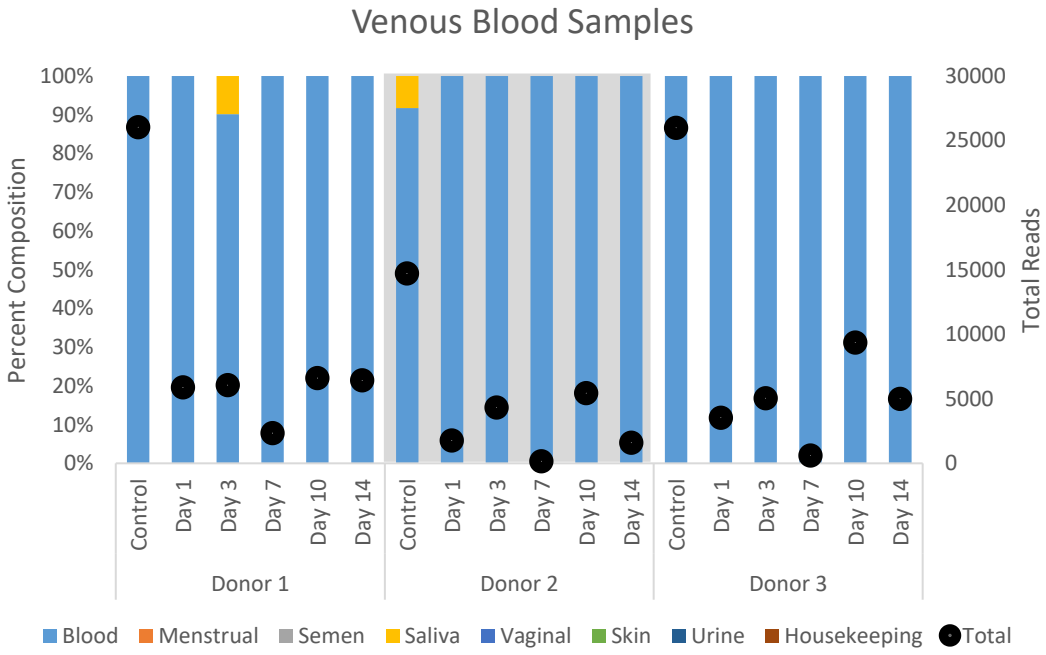
Fig. 4.6. Composition (primary axis) and total reads (secondary axis) for samples placed outside underneath polyvinyl sheeting.

Samples placed outside with exposure to rain

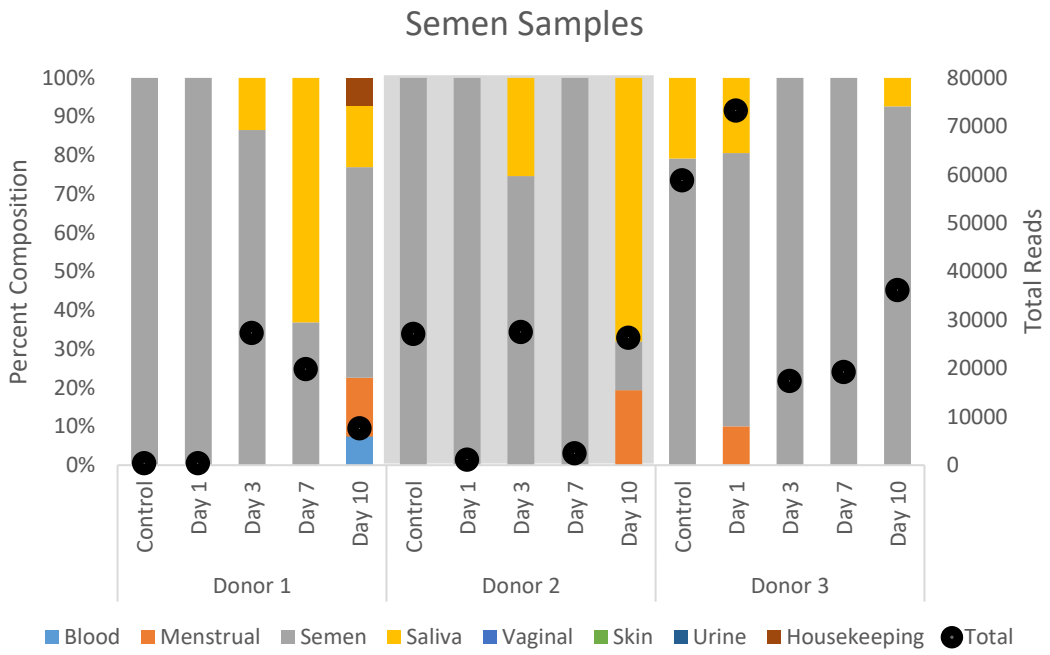
Results from the samples placed outside with exposure to precipitation can be seen in Fig. 4.7a-d, though some day 14 samples were not included in amplification due to reagent limitations. During the two-week period, it rained on three different days for a total of approximately 1.21 in. It should be noted that while samples were collected over time, it should not be considered a time-course study as samples were exposed to different amounts of direct rain due to placement. However, there is an overall trend of decreasing read counts over time in blood and vaginal samples.

Blood samples continued to exhibit high percentages of reads attributed to circulatory blood transcripts (90.2 – 100%) (Fig. 4.7a). In contrast to the samples that were buried and placed outside without rain exposure, semen samples were comprised of generally larger proportions of reads attributed to semen targets (12.5 – 100%) (Fig. 4.7b).

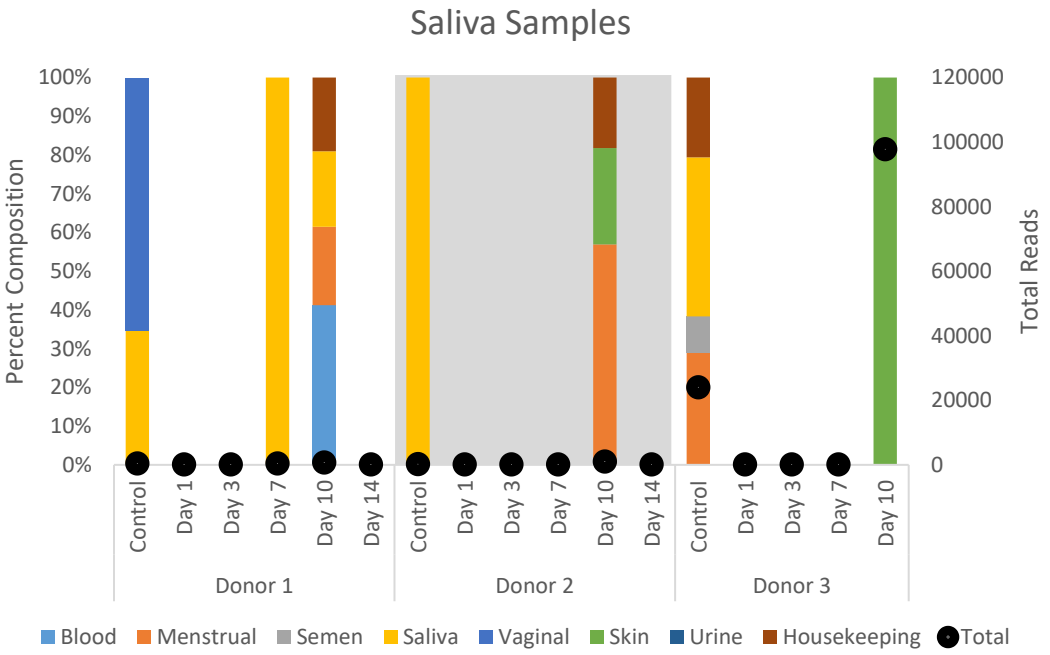
No transcripts were detected in 10 of the 15 experimental samples. One saliva sample did yield a substantially higher number of reads (97,738) with most reads attributed to skin markers (Fig. 4.7c). For this sample, it is possible that a piece of epithelial skin from another part of the body was present in the oral cavity, such as lip or from cuticle biting, then deposited into the collection container and onto the fabric substrate. Interestingly, composition of reads remained similar between donors of vaginal samples: donor 1 had consistently high levels of reads attributed to skin, donor 2 had similar ratios of menstrual to skin read and no vaginal reads, and donor 3 samples maintained similar ratios of menstrual, skin, and vaginal reads (Fig. 4.7d). In one other study profiling RNA in samples exposed to precipitation, body fluid specific transcripts could not be detected after a maximum of seven days [19]. The increase in time of detection may be due to differences in platform or elemental exposure.



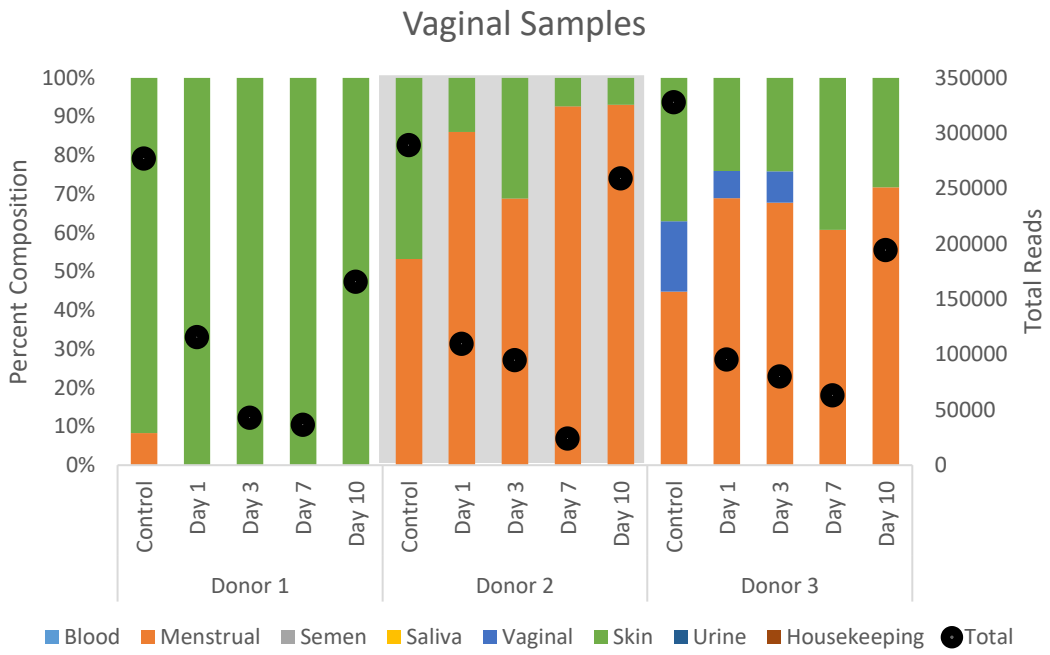
a.


Semen Samples
Percent Composition
Total Reads
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
80000
70000
60000
50000
40000
30000
20000
10000
0
Control
Day 1
Day 3
Day 7
Day 10
Donor 1
Donor 2
Donor 3
Blood
Menstrual
Semen
Saliva
Vaginal
Skin
Urine
Housekeeping
Total

b.


Saliva Samples
Percent Composition
Total Reads
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
120000
100000
80000
60000
40000
20000
0
Control
Day 1
Day 3
Day 7
Day 10
Day 14
Donor 1
Donor 2
Donor 3
Blood
Menstrual
Semen
Saliva
Vaginal
Skin
Urine
Housekeeping
Total

c.



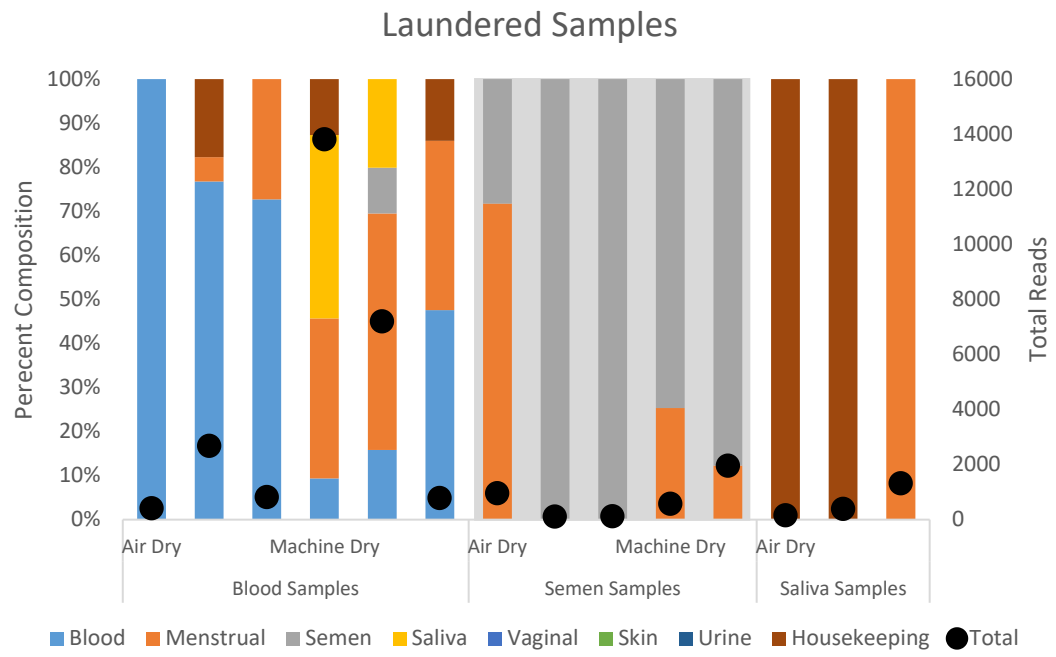
d.

Fig 4.7. Composition (primary axis) and total reads (secondary axis) for outside **a** Venous blood samples. **b** Semen samples. **c** Saliva samples. **d** Vaginal samples.

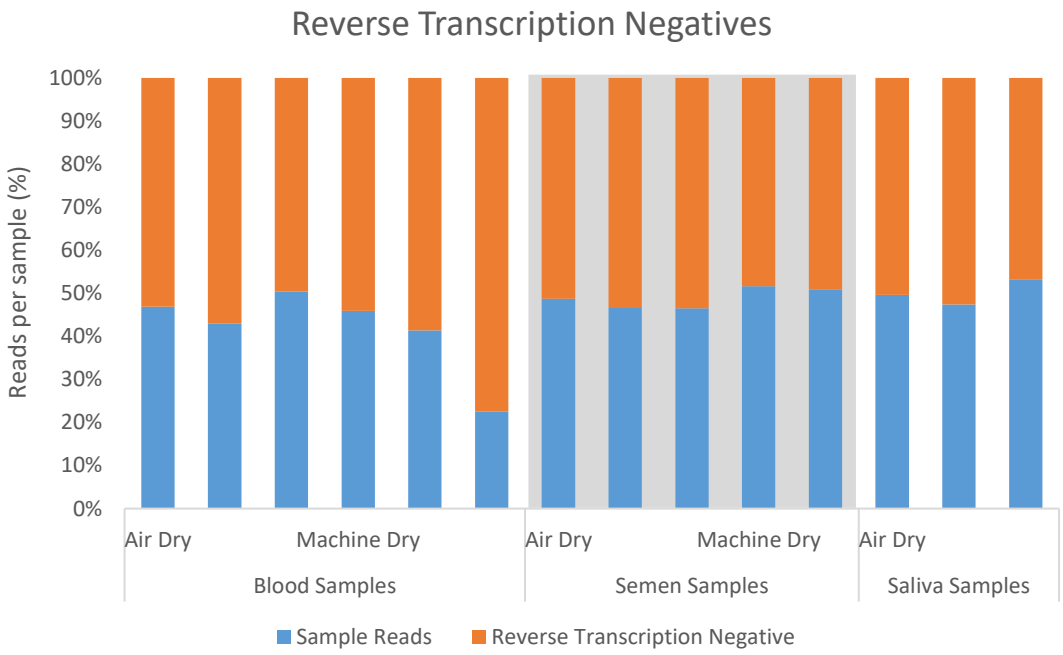
Laundered samples

Laundered samples had reads ranging from 106 – 13810 (Fig. 4.8a). Machine dried saliva samples could not be included due to technical issues. It was found that reverse transcription negatives had roughly equal amounts of reads in each sample (Fig. 8b) indicating the presence of genomic DNA in these samples. Apart from semen samples, no other sample conditions yielded consistently high reads in reverse transcription negatives, suggesting that DNase treatment was affected. DNase may have been digested by residual proteinases present in laundry detergent. While this was not observed in previous studies examining laundered items [24,31,34], different genomic DNA removal treatments were used. Also, proteinase K or specific protein denaturation steps were not performed in the

extraction process of this study. Additional experimentation is needed for any conclusive determinations.



a.

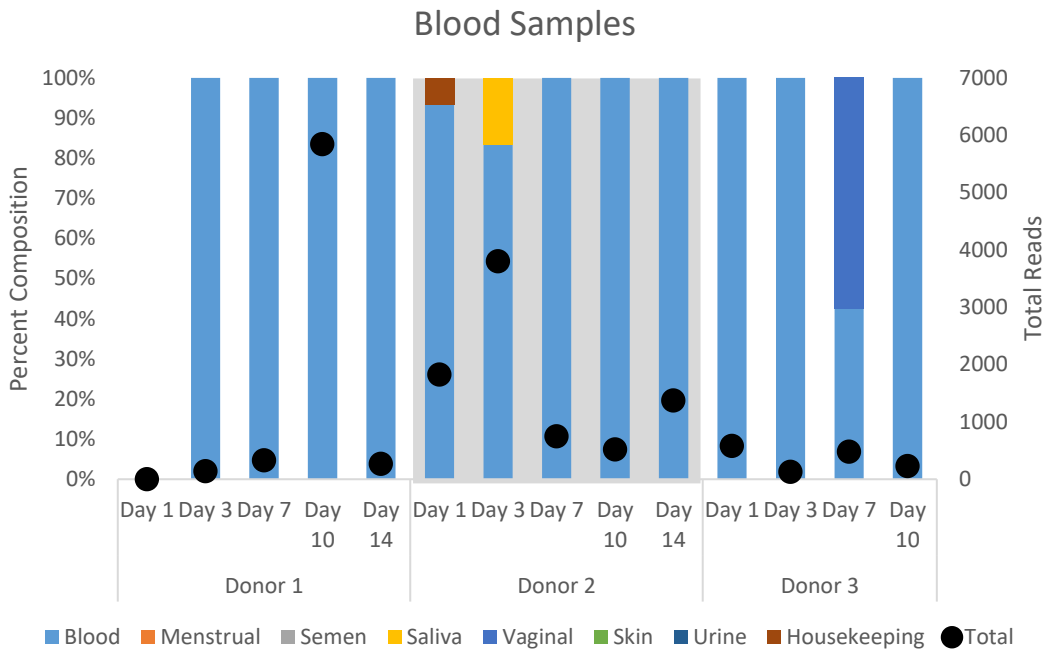


b.

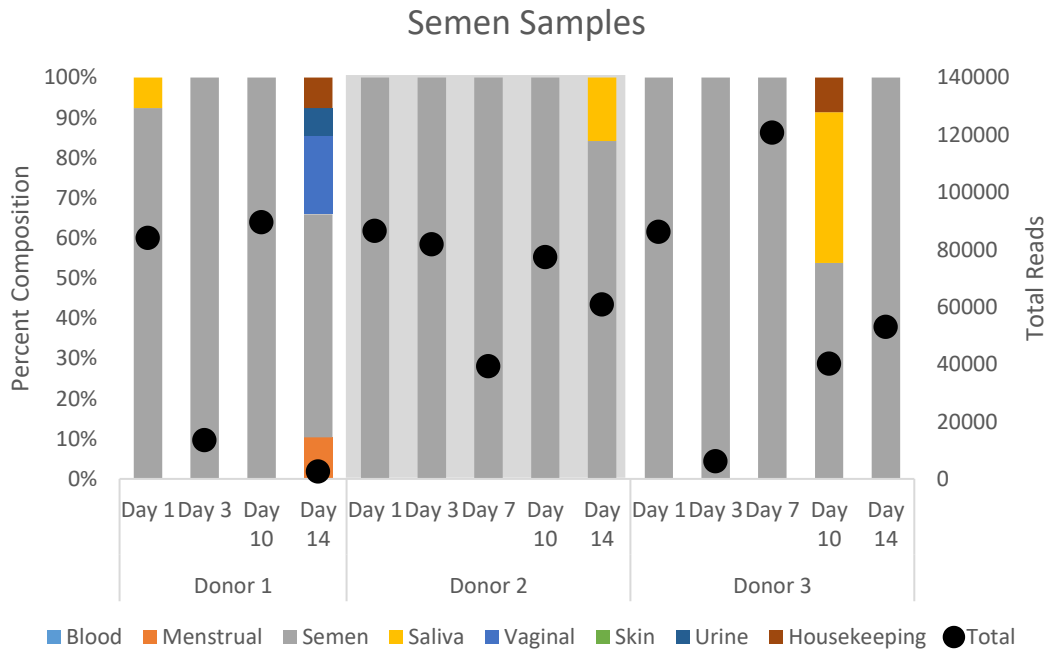
Fig 4.8. a Composition (primary axis) and total reads (secondary axis) for laundered samples. **b** RNA reads compared to reverse transcription negative reads for each sample.

Decomposing cadaver

Similar to the samples placed outdoors, transcripts were detected throughout the two-week period of collection of swatches placed on a decomposing cadaver for both venous blood and semen samples (Fig. 4.9). A blank swatch from the upper leg area taken at day 14 to evaluate possible contribution by the cadaver did not yield any reads past the thresholds (not shown). In general, total reads for blood samples were lower than the samples outside also protected from direct rain (0 – 5841 compared to 45064 - 55130). This may be due to differences in fabric used in the study. The shirt placed on the cadaver was 100% cotton and much thinner than the polycotton blend used in the other treatments. Interestingly, no non-blood targets were observed in the sample in which the corresponding DNA profile was solely that of the cadaver. One of the samples containing a mixed DNA profile, however, had reads attributed to both circulatory blood and vaginal material, suggesting possible contamination in that sample. Semen samples yielded a wide range of total reads, 2445 – 120,666 with reads attributed to semen markers ranging from 53.8 – 100%. While the total reads of these samples were lower than the other semen samples placed outside without exposure to direct rain (8193 – 210,743), a higher proportion was attributed to semen marker reads. These results may also be due to differences in fabric (poly cotton blend vs cotton flannel).



a.



b.

Fig 4.9. Composition (primary axis) and total reads (secondary axis) for samples placed on a decomposing cadaver. **a** Venous blood samples. **b** Semen samples.

Aged Samples

Results from aged samples can be seen in Fig. 4.10. While no reads from saliva samples were detected, total reads from blood samples ranged from 313 – 1126 with all reads associated with circulatory blood targets. The detection of blood transcripts, but not saliva transcripts is consistent with a previous study examining samples in hot and humid conditions at the appropriate timeframe [20].

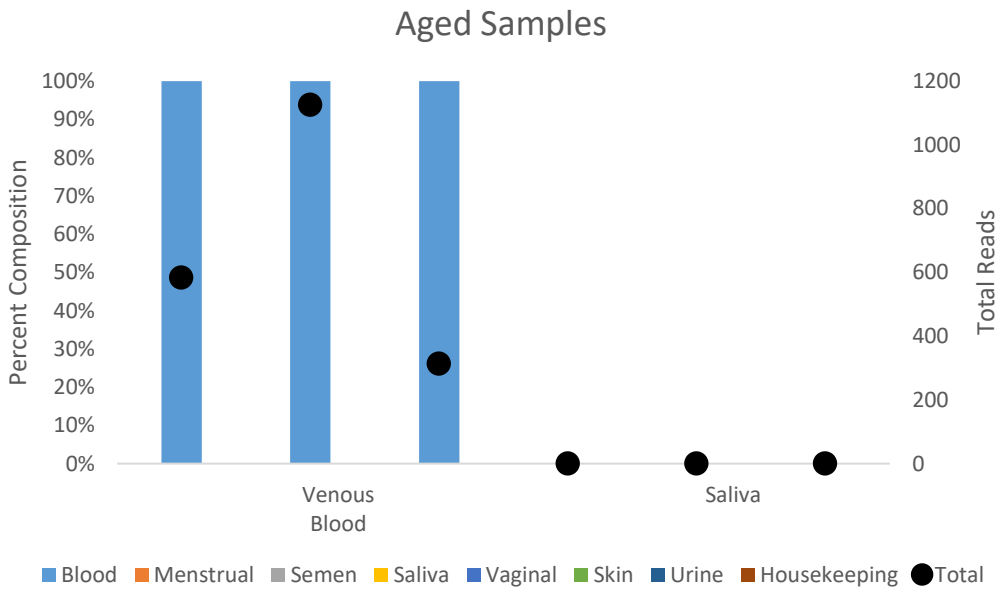


Fig 4.10. Composition (primary axis) and total reads (secondary axis) for cuttings taken from samples aged for 37 weeks in warm and humid conditions.

Post-coital samples

Unlike the corresponding DNA profiles, two samples failed to amplify any targets (Fig. 4.11). The remaining samples ranged from approximately 78k – 148k in total reads. As mentioned in the DNA section, extracts from these samples had a dark appearance which was likely dye from the underwear that had been co-extracted. While no inhibition was detected by the QS markers in the STR profiles, the dye could have negatively affected

RNA processing. Also contrary to the STR profiles that clearly indicate the presence of female cellular material, only one sample in RNA profiling contained evidence of a female contributor (0.7% of total reads). However, DNA contributor proportions are not necessarily equivalent to that of RNA [35,36] and the amplification of female targets may have been lost to stochastic effects in the presence of high amounts of semen transcripts. In a capillary electrophoresis-based assays, semen markers were also amplified at higher levels and vaginal material was only detected in profiles with higher amounts of input cDNA [12,33]. As a differential extraction is performed many cases where semen is detected [37], it is possible that RNA profiling of the non-sperm fraction will contain detectable levels of vaginal RNA.

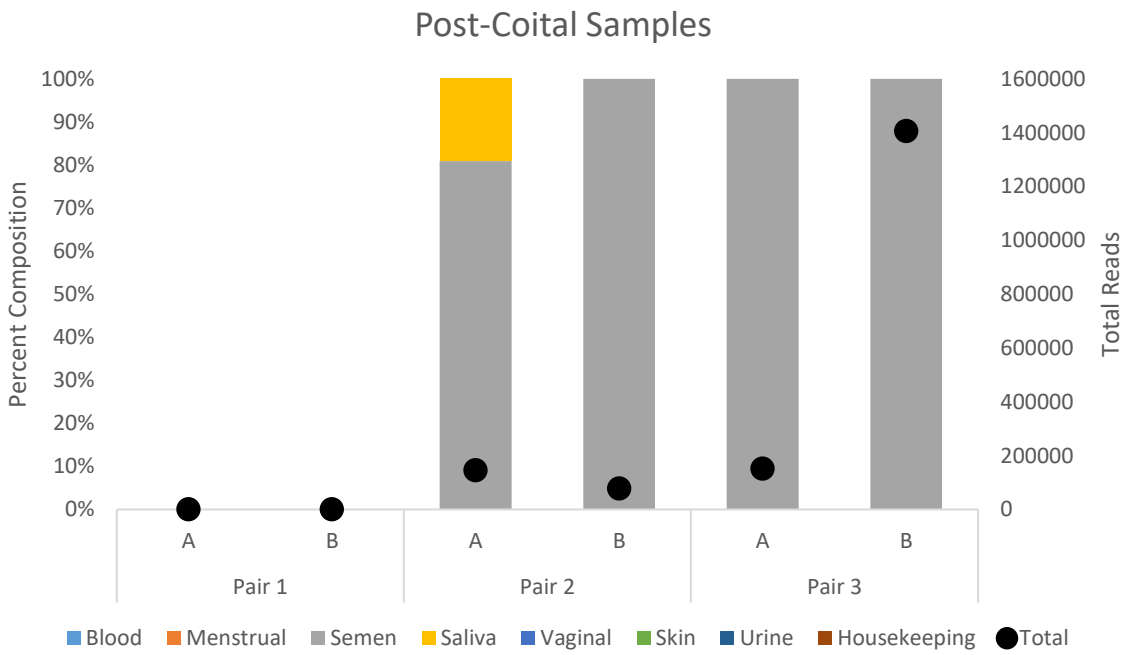


Fig 4.11. Composition (primary axis) and total reads (secondary axis) for cuttings taken from post-coital underwear.

Panel performance

As seen in the positive control samples, performance varied across markers (Table 4.5). In general, markers that were not detected in the positive controls were also not observed in experimental samples. Due to the high number of reads in reverse transcription negatives, laundered samples were not included, and markers present in semen may not be true transcripts, but amplified products from genomic DNA. Changes in the workflow may further improve results. For instance, semen samples had an average of 56.9% of reads in reverse transcription negatives, in contrast to 91.2% in venous blood samples. These results suggest that sperm persisted through extraction and likely released DNA during PCR.

Table 4.5. Percentage of samples with reads above thresholds for each marker. Markers in bold appeared with reads below thresholds in more than half of positive control samples. Shaded cells indicate markers expected in each fluid type.

	Marker	Venous Blood (n=43)	Menstrual Blood (n=9)	Semen (n=42)	Saliva (n=21)	Vaginal secretions (n=21)
Circulatory Blood	**GYPA**	0.0	0.0	0.0	0.0	0.0
	ALAS2	14.0	11.1	4.8	4.8	0.0
	SLC4A1_1	65.1	11.1	2.4	0.0	0.0
	SLC4A1_2	83.7	0.0	2.4	0.0	0.0
	HBD_1	0.0	0.0	0.0	0.0	0.0
	HBD_2	0.0	0.0	0.0	0.0	0.0
Menstrual Blood	**MMP10_1**	0.0	33.3	0.0	0.0	0.0
	MMP10_2	0.0	0.0	0.0	0.0	0.0
	MMP11	0.0	11.1	16.7	4.8	4.8
	MMP3	0.0	11.1	2.4	0.0	0.0
	PLAUR_1	0.0	66.7	7.1	23.8	66.7
	PLAUR_2	0.0	55.6	0.0	0.0	57.1
	SNORA35	0.0	0.0	0.0	0.0	0.0
	STC1	0.0	0.0	0.0	0.0	0.0
Semen	**SEMG2**	0.0	0.0	0.0	0.0	0.0
	SEMG1	0.0	0.0	2.4	0.0	0.0
	KLK2	0.0	0.0	0.0	0.0	0.0
	MSMB	0.0	0.0	11.9	0.0	0.0
	TNP1	0.0	0.0	0.0	0.0	0.0
	SPATA42	0.0	0.0	2.4	4.8	0.0
	PRM1	0.0	0.0	73.8	0.0	0.0
	PRM2	0.0	0.0	78.6	0.0	0.0
Saliva	HTN3	0.0	0.0	0.0	9.5	0.0
	STATH	0.0	0.0	26.2	19.0	4.8
	FDCSP	0.0	0.0	2.4	4.8	0.0
	CST1	0.0	0.0	7.1	0.0	0.0
	PRB4_1	4.7	0.0	19.0	9.5	0.0
	PRB4_2	0.0	0.0	7.1	0.0	0.0
Vaginal	HOXA13_1	0.0	0.0	2.4	0.0	0.0
	HOXA13_2	0.0	0.0	0.0	0.0	0.0
	HOXA13_3	0.0	0.0	4.8	0.0	0.0
	HOXA11_1	0.0	0.0	2.4	4.8	0.0
	HOXA11_2	0.0	0.0	0.0	0.0	0.0
	MYOZ1	0.0	0.0	0.0	0.0	0.0
	PRSS21	0.0	0.0	0.0	4.8	0.0
	SPRR2G	2.3	0.0	0.0	9.5	23.8
Skin	LOR	0.0	22.2	0.0	14.3	61.9

(continued)

	Marker	Venous Blood (n=43)	Menstrual Blood (n=9)	Semen (n=42)	Saliva (n=21)	Vaginal secretions (n=21)
	CCL27	0.0	0.0	0.0	0.0	0.0
	CST6	0.0	33.3	0.0	4.8	57.1
Urine	**AQP6_1**	0.0	0.0	0.0	0.0	0.0
	AQP6_2	0.0	0.0	2.4	0.0	0.0
Housekeeping	TCEA_1	0.0	0.0	7.1	0.0	0.0
	TCEA_2	2.3	0.0	2.4	4.8	4.8
	G6PD_1	0.0	0.0	2.4	14.3	0.0
	G6PD_2	0.0	0.0	0.0	0.0	0.0
	UBE2D2	0.0	0.0	0.0	4.8	0.0

SLC4A_1 and SLC4A_2 was the most persistent in venous blood samples, appearing in 83.7% and 65.1% of samples, respectively, while HBD_1 and HBD_2 were not present above thresholds in samples. GYPA was also not observed past thresholds. A SNP was identified in NM_002099.6:r.209 (A/G) in one donor sample. SNPs were also identified in SLC4A1 at rs5035 (G/T) in two donors.

In menstrual blood samples and vaginal samples, amplicons from PLAUR were observed most consistently, accounting for the majority of reads associated with menstrual blood. However, expression of PLAUR may be donor dependent, as it was not observed past thresholds for Donor 1 in several samples (Fig. 7d). Skin markers, LOR and CST6, were also present in both sample types, likely due to the collection procedure, as discussed above. Expression of vaginal marker SPRRG2G may also be donor dependent, as it was only observed in one donor consistently (data not shown) and SNPs were observed at NM_001014291.3:r.537 (A/G) in two donors. MMP10_1 was only detected in experimental menstrual blood control samples, while MMP10_2, SNORA35, and STC1 were not observed in any samples.

Although interpretation of the performance of the panel for semen samples is limited to due issues mentioned above, some general trends were observed. PRM1 and PRM2 were observed most consistently and accounted for the majority of semen attributed reads in semen samples, which is similar to sequencing results in previous studies [8,9]. Of the saliva markers, no targets were observed consistently and non-target fluid markers, such as PLAUR, were also detected in saliva. Similar results were also observed in a previous study [9] and challenges in body fluid specificity for saliva have been well documented [12,38-41].

Housekeeping markers were sporadically observed in samples. The inclusion of these markers in a multiplex is challenging due to the variable expression between both individuals and tissues [42]. Zubakov et al. (2015) used housekeeping genes as positive controls for biological material rather than as a normalizing marker, likely because of this variation [10]. Hanson et al. (2018) faced a different challenge in which high proportions of reads were attributed to housekeeping markers thus reducing efficiency of other markers [8]. While it has been suggested that these targets are necessary for the assessment of mixtures [10], a likelihood approach may be more appropriate [43].

Concluding remarks

The performance of the early access mRNA BFID panel is very promising. Overall, the panel was able to detect transcripts in samples challenged in several different manners. Based on the results of this study, it is recommended that future extraction methods ensure sperm lysis and include a proteinase to reduce possible protein contaminants. In addition to the extraction and workflow recommendations, future iterations may include primer titration adjustments to improve amplification of poor performing markers. Extensive

developmental validation studies including, but not limited to the determination of allele frequencies and expression level variation, sensitivity, and mixture studies. Additionally, robust interpretation strategies must be developed to ensure the accurate determination of the body fluid in a sample.

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References

1. Zeng X, King JL, Stoljarova M, Warshauer DH, LaRue BL, Sajantila A et al. (2015) High sensitivity multiplex short tandem repeat loci analyses with massively parallel sequencing. Forensic Sci Int Genet 16:38-47. doi:10.1016/j.fsigen.2014.11.022
2. Churchill JD, Stoljarova M, King JL, Budowle B (2018) Massively parallel sequencing-enabled mixture analysis of mitochondrial DNA samples. Int J Legal Med 132 (5):1263-1272. doi:10.1007/s00414-018-1799-3
3. Phillips C, Salas A, Sanchez J, Fondevila M, Gomez-Tato A, Alvarez-Dios J et al. (2007) Inferring ancestral origin using a single multiplex assay of ancestry-informative marker SNPs. Forensic Science International: Genetics 1 (3-4):273-280
4. Sanchez JJ, Phillips C, Børsting C, Balogh K, Bogus M, Fondevila M et al. (2006) A multiplex assay with 52 single nucleotide polymorphisms for human identification. Electrophoresis 27 (9):1713-1724
5. Kidd KK, Pakstis AJ, Speed WC, Grigorenko EL, Kajuna SL, Karoma NJ et al. (2006) Developing a SNP panel for forensic identification of individuals. Forensic science international 164 (1):20-32
6. Ruiz Y, Phillips C, Gomez-Tato A, Alvarez-Dios J, Casares de Cal M, Cruz R et al. (2013) Further development of forensic eye color predictive tests. Forensic Science International: Genetics 7 (1):28-40. doi:/10.1016/j.fsigen.2012.05.009
7. Walsh S, Liu F, Ballantyne KN, van Oven M, Lao O, Kayser M (2011) IrisPlex: A sensitive DNA tool for accurate prediction of blue and brown eye colour in the

absence of ancestry information. Forensic Science International: Genetics 5 (3):170-180. doi: 10.1016/j.fsigen.2010.02.004

8. Hanson E, Ingold S, Haas C, Ballantyne J (2018) Messenger RNA biomarker signatures for forensic body fluid identification revealed by targeted RNA sequencing. Forensic Sci Int Genet 34:206-221. doi:10.1016/j.fsigen.2018.02.020

9. Ingold S, Dørum G, Hanson E, Berti A, Branicki W, Brito P et al. (2018) Body fluid identification using a targeted mRNA massively parallel sequencing approach–results of a EUROFORGEN/EDNAP collaborative exercise. Forensic Sci Int Genet 34:105-115

10. Zubakov D, Kokmeijer I, Ralf A, Rajagopalan N, Calandro L, Wootton S et al. (2015) Towards simultaneous individual and tissue identification: A proof-of-principle study on parallel sequencing of STRs, amelogenin, and mRNAs with the Ion Torrent PGM. Forensic Sci Int Genet 17:122-128. doi:10.1016/j.fsigen.2015.04.002

11. Juusola J, Ballantyne J (2003) Messenger RNA profiling: a prototype method to supplant conventional methods for body fluid identification. Forensic Sci Int 135 (2):85-96. doi:10.1016/S0379-0738(03)00197-X

12. van den Berge M, Bhoelai B, Harteveld J, Matai A, Sijen T (2016) Advancing forensic RNA typing: On non-target secretions, a nasal mucosa marker, a differential co-extraction protocol and the sensitivity of DNA and RNA profiling. Forensic Sci Int Genet 20:119-129. doi:10.1016/j.fsigen.2015.10.011

13. Fleming RI, Harbison S (2010) The development of a mRNA multiplex RT-PCR assay for the definitive identification of body fluids. Forensic Sci Int Genet 4 (4):244-256. doi:10.1016/j.fsigen.2009.10.006

14. Biedermann A, Champod C, Jackson G, Gill P, Taylor D, Butler J et al. (2016) Evaluation of Forensic DNA Traces When Propositions of Interest Relate to Activities: Analysis and Discussion of Recurrent Concerns. Front Genet 7 (215):215. doi:10.3389/fgene.2016.00215

15. Taroni F, Biedermann A, Garbolino P, Aitken CG (2004) A general approach to Bayesian networks for the interpretation of evidence. Forensic Sci Int 139 (1):5-16. Doi:j.forsciint.2003.08.004

16. Taylor D, Biedermann A, Hicks T, Champod C (2018) A template for constructing Bayesian networks in forensic biology cases when considering activity level propositions. Forensic Sci Int Genet 33:136-146. doi:10.1016/j.fsigen.2017.12.006

17. Curran JM, Triggs CM, Buckleton JS, Walsh K, Hicks T (1998) Assessing transfer probabilities in a Bayesian interpretation of forensic glass evidence. Sci Justice 38 (1):15-21. doi: 10.1016/S1355-0306(98)72068-4

18. Cook R, Evett IW, Jackson G, Jones PJ, Lambert JA (1998) A hierarchy of propositions: deciding which level to address in casework. Sci Justice 38 (4):231-239. doi:10.1016/S1355-0306(98)72117-3

19. Setzer M, Juusola J, Ballantyne J (2008) Recovery and stability of RNA in vaginal swabs and blood, semen, and saliva stains. J Forensic Sci 53 (2):296-305. doi:10.1111/j.1556-4029.2007.00652.x

20. Sirker M, Schneider PM, Gomes I (2016) A 17-month time course study of human RNA and DNA degradation in body fluids under dry and humid environmental conditions. Int J Legal Med 130 (6):1431-1438. doi:10.1007/s00414-016-1373-9

21. van den Berge M, Ozcanhan G, Zijlstra S, Lindenbergh A, Sijen T (2016) Prevalence of human cell material: DNA and RNA profiling of public and private objects and after activity scenarios. Forensic Sci Int Genet 21:81-89. doi:10.1016/j.fsigen.2015.12.012

22. van den Berge M, Wiskerke D, Gerretsen RR, Tabak J, Sijen T (2016) DNA and RNA profiling of excavated human remains with varying postmortem intervals. Int J Legal Med 130 (6):1471-1480. doi:10.1007/s00414-016-1438-9

23. Nakanishi H, Hara M, Takahashi S, Takada A, Saito K (2014) Evaluation of forensic examination of extremely aged seminal stains. Leg Med (Tokyo) 16 (5):303-307. doi:10.1016/j.legalmed.2014.04.002

24. Kulstein G, Schacker U, Wiegand P (2018) Old meets new: Comparative examination of conventional and innovative RNA-based methods for body fluid identification of laundered seminal fluid stains after modular extraction of DNA and RNA. Forensic Sci Int Genet 36:130-140. doi:10.1016/j.fsigen.2018.06.017

25. Lin MH, Jones DF, Fleming R (2015) Transcriptomic analysis of degraded forensic body fluids. Forensic Sci Int Genet 17:35-42. doi:10.1016/j.fsigen.2015.03.005

26. Lin MH, Albani PP, Fleming R (2016) Degraded RNA transcript stable regions (StaRs) as targets for enhanced forensic RNA body fluid identification. Forensic Sci Int Genet 20:61-70. doi:10.1016/j.fsigen.2015.09.012

27. Lin M-H, Albani PP, Fleming R (2016) Degraded RNA transcript stable regions (StaRs) as targets for enhanced forensic RNA body fluid identification. Forensic Sci Int Genet 20:61-70

28. Albani PP, Fleming R (2018) Novel messenger RNAs for body fluid identification. Sci Justice 58 (2):145-152. doi:10.1016/j.scijus.2017.09.002

29. Kasu M, Shires K (2015) The validation of forensic DNA extraction systems to utilize soil contaminated biological evidence. Legal Medicine 17 (4):232-238. doi: 10.1016/j.legalmed.2015.01.004

30. Alaeddini R, Walsh SJ, Abbas A (2010) Molecular studies of time-and environment-dependent effects on bone DNA survival. Australian Journal of Forensic Sciences 42 (3):211-220

31. Kulstein G, Wiegand P (2018) Comprehensive examination of conventional and innovative body fluid identification approaches and DNA profiling of laundered blood- and saliva-stained pieces of cloths. Int J Legal Med 132 (1):67-81. doi:10.1007/s00414-017-1691-6

32. Brayley-Morris H, Sorrell A, Revoir AP, Meakin GE, Court DS, Morgan RM (2015) Persistence of DNA from laundered semen stains: Implications for child sex trafficking cases. Forensic Sci Int Genet 19:165-171. doi:10.1016/j.fsigen.2015.07.016

33. Lindenbergh A, de Pagter M, Ramdayal G, Visser M, Zubakov D, Kayser M et al. (2012) A multiplex (m)RNA-profiling system for the forensic identification of body fluids and contact traces. Forensic Sci Int Genet 6 (5):565-577. doi:10.1016/j.fsigen.2012.01.009

34. Mayes C, Houston R, Seashols-Williams S, LaRue B, Hughes-Stamm S (2019) The stability and persistence of blood and semen mRNA and miRNA targets for body fluid identification in environmentally challenged and laundered samples. Leg Med (Tokyo) 38:45-50. doi:10.1016/j.legalmed.2019.03.007

35. Harteveld J, Lindenbergh A, Sijen T (2013) RNA cell typing and DNA profiling of mixed samples: can cell types and donors be associated? Sci Justice 53 (3):261-269. doi:10.1016/j.scijus.2013.02.001

36. Whitehead A, Crawford DL (2005) Variation in tissue-specific gene expression among natural populations. Genome Biol 6 (2):R13. doi:10.1186/gb-2005-6-2-r13

37. Gill P, Jeffreys AJ, Werrett DJ (1985) Forensic application of DNA 'fingerprints'. Nature 318 (6046):577

38. Roeder AD, Haas C (2013) mRNA profiling using a minimum of five mRNA markers per body fluid and a novel scoring method for body fluid identification. Int J Legal Med 127 (4):707-721. doi:10.1007/s00414-012-0794-3

39. Richard ML, Harper KA, Craig RL, Onorato AJ, Robertson JM, Donfack J (2012) Evaluation of mRNA marker specificity for the identification of five human body fluids by capillary electrophoresis. Forensic Sci Int Genet 6 (4):452-460. doi:10.1016/j.fsigen.2011.09.007

40. Sakurada K, Akutsu T, Watanabe K, Miyasaka S, Kasai K (2013) Identification of Body Fluid Stains Using Real-time RT-PCR: Discrimination Between Salivary, Nasal, and Vaginal Secretions. Jpn J Forensic Sci Tech 18 (1):1-11. doi:10.3408/jafst.18.1

41. Sakurada K, Ikegaya H, Fukushima H, Akutsu T, Watanabe K, Yoshino M (2009) Evaluation of mRNA-based approach for identification of saliva and semen. Leg Med (Tokyo) 11 (3):125-128. doi:10.1016/j.legalmed.2008.10.002

42. 42. Moreno LI, Tate CM, Knott EL, McDaniel JE, Rogers SS, Koons BW et al. (2012) Determination of an effective housekeeping gene for the quantification of mRNA for forensic applications. J Forensic Sci 57 (4):1051-1058. doi:10.1111/j.1556-4029.2012.02086.x

43. 43. Dørum G, Ingold S, Hanson E, Ballantyne J, Snipen L, Haas C (2018) Predicting the origin of stains from next generation sequencing mRNA data. Forensic Sci Int Genet 34:37-48. doi:10.1016/j.fsigen.2018.01.001

CHAPTER V

Conclusions

BFID may assist investigators establish or verify facts during the course of an investigation and this information may also be used in the prosecution of crimes. Therefore, the adoption of a confirmatory BFID method is critical for the objective and accurate evaluation of evidence and has been identified as a Research and Development Need by the Organization of Scientific Area Committees (OSAC) for forensic science. mRNA profiling, which has been implemented in other parts of the world, affords laboratories the ability to obtain both STR and BFID information from a single sample. US laboratories, however, have been reluctant to adopt this BFID strategy due to the perception of RNA as a volatile molecule as well as the lack of availability of commercial kits. This project explores miRNA and mRNA profiling for BFID purposes to provide evidence of their utility in forensic science in both current and emerging technologies.

Traditional miRNA analysis is performed using RT-qPCR and often requires numerous reactions, thereby increasing time and cost per analysis. To avoid these issues and provide a streamlined method for miRNA analysis, an eight-marker miRNA multiplex was developed for capillary electrophoresis. A co-extraction (DNA/RNA) approach was performed to analyze both DNA and BFID profiles in parallel. For miRNA analysis, a linear primer system was modified to maximize availability for markers using both length- and dye-based separation strategies. Reverse transcription and PCR primers were developed for each marker, evaluated in singleplex, and then multiplexed. Markers were chosen to identify venous blood (miR-451a and miR-142-3p), menstrual blood (miR-412-3p and miR-141-3p), semen (miR-891a and miR-10b), saliva (miR-205), as well as an

endogenous reference gene (let-7g). All DNA profiles were complete and while several miRNAs were not found to be body fluid specific, decision tree-based interpretation strategy was developed to identify single source body fluids.

Due to the recent focus on understanding the nature of the deposition of cellular material to assist in 'activity level' assessments, the stability and persistence of miRNA and mRNA were evaluated in challenging samples. miRNA and mRNA were detected using reverse transcription quantitative PCR (RT-qPCR) assays. Targets were chosen for both mRNA (HBB, PRM1, and B2M) and miRNA (miR-451a, miR-891a, and let-7g) for blood, semen, and an endogenous reference gene, respectively. A time-course study included blood (n=3) and semen (n=3) samples placed in two different conditions. Samples were challenged through placement in an area exposed to natural heat, humidity, and UV light while controls samples were placed in an environment with controlled temperature, low humidity, and darkness for six months. In experimental samples, mRNA persisted up to 30 days and control mRNA and all miRNA transcripts were detected at all time points during the six-month experiment. Laundering and either machine or air-drying blood (n=3) and semen (n=3) stained swatches was performed as a persistence study. miR-451a, miR-891a and HBB were detected in respectively blood and semen stained samples, PRM1 was detected in all but one semen stained samples. Transfer of both miRNA and mRNA was observed by taking an unstained portion of the swatch. This study demonstrates that miRNA and mRNA can be detected in harsh and challenging conditions. Additionally, the increased stability of miRNA compared to mRNA was established which had not been directly compared in previous studies.

In the final study, an early access mRNA panel for BFID was evaluated. Samples of venous blood, menstrual blood, semen, saliva, and vaginal secretions were placed in a variety of challenging conditions including outside with and without exposure to direct precipitation, buried, on a decomposing cadaver, laundered, aged, and post-coital samples. Co-extracted DNA profiles ranged in profile completeness from 31.1 – 100%. RNA was successfully extracted and typed in most samples. Of the myriad of challenged samples (over 120), only 25 samples yielded no reads. Samples with no detected RNA included several buried samples, post-coital samples, and samples outside exposed to rain. Due to suspected inhibition and reads present in reverse transcription negatives, thorough pre-processing steps, such as extraction and DNase treatment are recommended for accurate typing. Additionally, several markers did not amplify in control or experimental samples, suggesting further panel development is necessary. However, initial results are positive.

In summary, emerging technologies and extensive research has provided the forensic community with the opportunity to advance BFID methodologies. This study contributed to the body of research by providing valuable information about the use of miRNA and mRNA for the purposes of BFID. While the widespread implementation of any new technique requires significant changes, the adoption of these techniques will likely benefit the criminal justice system.

REFERENCES

Akutsu T, Watanabe K, Sakurada K (2012) Specificity, Sensitivity, and Operability of RSID™-Urine for Forensic Identification of Urine: Comparison with ELISA for Tamm-Horsfall Protein. Journal of forensic sciences 57 (6):1570-1573

Alaeddini R, Walsh SJ, Abbas A (2010) Molecular studies of time-and environment-dependent effects on bone DNA survival. Australian Journal of Forensic Sciences 42 (3):211-220

Albani PP, Fleming R (2018) Novel messenger RNAs for body fluid identification. Sci Justice 58 (2):145-152. doi:10.1016/j.scijus.2017.09.002

Alvarez M, Juusola J, Ballantyne J (2004) An mRNA and DNA co-isolation method for forensic casework samples. Anal Biochem 335 (2):289-298. doi:10.1016/j.ab.2004.09.002

An JH, Choi A, Shin KJ, Yang WI, Lee HY (2013) DNA methylation-specific multiplex assays for body fluid identification. Int J Legal Med 127 (1):35-43. doi:10.1007/s00414-012-0719-1

An JH, Shin KJ, Yang WI, Lee HY (2012) Body fluid identification in forensics. BMB Rep 45 (10):545-553

Arroyo JD, Chevillet JR, Kroh EM, Ruf IK, Pritchard CC, Gibson DF et al. (2011) Argonaute2 complexes carry a population of circulating microRNAs independent of vesicles in human plasma. Proceedings of the National Academy of Sciences 108 (12):5003-5008

Arroyo JD, Chevillet JR, Kroh EM, Ruf IK, Pritchard CC, Gibson DF et al. (2011) Argonaute2 complexes carry a population of circulating microRNAs independent

of vesicles in human plasma. Proc Natl Acad Sci U S A 108 (12):5003-5008. doi:10.1073/pnas.1019055108

Auvdel MJ (1986) Amylase levels in semen and saliva stains. J Forensic Sci 31 (2):426-431

Bartel DP (2004) MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 116 (2):281-297

Bauer M (2007) RNA in forensic science. Forensic Sci Int Genet 1 (1):69-74. doi:10.1016/j.fsigen.2006.11.002

Bauer M, Gramlich I, Polzin S, Patzelt D (2003) Quantification of mRNA degradation as possible indicator of postmortem interval—a pilot study. Legal Medicine 5 (4):220-227. doi:10.1016/j.legalmed.2003.08.001

Bauer M, Kraus A, Patzelt D (1999) Detection of epithelial cells in dried blood stains by reverse transcriptase-polymerase chain reaction. J Forensic Sci 44 (6):1232-1236

Bauer M, Patzelt D (2002) Evaluation of mRNA markers for the identification of menstrual blood. J Forensic Sci 47 (6):1278-1282

Bauer M, Patzelt D (2003) Protamine mRNA as molecular marker for spermatozoa in semen stains. Int J Legal Med 117 (3):175-179. doi:10.1007/s00414-002-0347-2

Benschop CC, Quaak FC, Boon ME, Sijen T, Kuiper I (2012) Vaginal microbial flora analysis by next generation sequencing and microarrays; can microbes indicate vaginal origin in a forensic context? Int J Legal Med 126 (2):303-310. doi:10.1007/s00414-011-0660-8

Bexon K, Williams G (2015) Characterising the fluctuation of microRNA expression throughout a full menstrual cycle. Forensic Sci Int Genet Suppl Ser 5:E264-E266. doi:10.1016/j.fsigss.2015.09.105

Biedermann A, Champod C, Jackson G, Gill P, Taylor D, Butler J et al. (2016) Evaluation of Forensic DNA Traces When Propositions of Interest Relate to Activities: Analysis and Discussion of Recurrent Concerns. Front Genet 7 (215):215. doi:10.3389/fgene.2016.00215

Bienroth S, Keller W, Wahle E (1993) Assembly of a processive messenger RNA polyadenylation complex. EMBO J 12 (2):585-594. doi:10.1002/j.1460-2075.1993.tb05690.x

Bitner SE (2012) False positives observed on the Seratec® PSA SemiQuant cassette test with condom lubricants. Journal of forensic sciences 57 (6):1545-1548

Blackman S, Stafford-Allen B, Hanson EK, Panasiuk M, Brooker A-L, Rendell P et al. (2018) Developmental validation of the ParaDNA® Body Fluid ID System—A rapid multiplex mRNA-profiling system for the forensic identification of body fluids. Forensic Science International: Genetics 37:151-161. doi:10.1016/j.fsigen.2018.08.012

Boward ES, Wilson SL (2013) A comparison of ABAcard® p30 and RSID™-Semen test kits for forensic semen identification. Journal of forensic and legal medicine 20 (8):1126-1130

Boyd S, Bertino MF, Seashols SJ (2011) Raman spectroscopy of blood samples for forensic applications. Forensic Sci Int 208 (1-3):124-128. doi:10.1016/j.forsciint.2010.11.012

Brayley-Morris H, Sorrell A, Revoir AP, Meakin GE, Court DS, Morgan RM (2015) Persistence of DNA from laundered semen stains: Implications for child sex trafficking cases. Forensic Sci Int Genet 19:165-171. doi:10.1016/j.fsigen.2015.07.016

Braz A, Lopez-Lopez M, Garcia-Ruiz C (2013) Raman spectroscopy for forensic analysis of inks in questioned documents. Forensic Sci Int 232 (1-3):206-212. doi:10.1016/j.forsciint.2013.07.017

Brown D, Todd A (1952) 13. Nucleotides. Part X. Some observations on the structure and chemical behaviour of the nucleic acids. Journal of the Chemical Society (Resumed):52-58

Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M et al. (2009) The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem 55 (4):611-622. doi:10.1373/clinchem.2008.112797

Carthew RW, Sontheimer EJ (2009) Origins and Mechanisms of miRNAs and siRNAs. Cell 136 (4):642-655. doi:10.1016/j.cell.2009.01.035

Chen C, Ridzon DA, Broomer AJ, Zhou Z, Lee DH, Nguyen JT et al. (2005) Real-time quantification of microRNAs by stem-loop RT-PCR. Nucleic Acids Res 33 (20):e179. doi:10.1093/nar/gni178

Chen Y, Gelfond JA, McManus LM, Shireman PK (2009) Reproducibility of quantitative RT-PCR array in miRNA expression profiling and comparison with microarray analysis. BMC Genomics 10 (1):407. doi:10.1186/1471-2164-10-407

Christoffersen S (2011) Immunohistochemical staining of human sperm cells in smears from sexual assault cases. Int J Legal Med 125 (6):887-890. doi:10.1007/s00414-011-0612-3

Churchill JD, Stoljarova M, King JL, Budowle B (2018) Massively parallel sequencing-enabled mixture analysis of mitochondrial DNA samples. Int J Legal Med 132 (5):1263-1272. doi:10.1007/s00414-018-1799-3

Coble MD, Bright J-A (2019) Probabilistic genotyping software: An overview. Forensic Sci Int Genet 38:219-224. doi:https://doi.org/10.1016/j.fsigen.2018.11.009

Conaway RC, Conaway JW (2011) Function and regulation of the Mediator complex. Curr Opin Genet Dev 21 (2):225-230. doi:10.1016/j.gde.2011.01.013

Cook R, Evett IW, Jackson G, Jones PJ, Lambert JA (1998) A hierarchy of propositions: deciding which level to address in casework. Sci Justice 38 (4):231-239. doi:Doi 10.1016/S1355-0306(98)72117-3

Cossu C, Germann U, Kratzer A, Bär W, Haas C (2009) How specific are the vaginal secretion mRNA-markers HBD1 and MUC4? Forensic Sci Int Genet Suppl Ser 2 (1):536-537. doi:10.1016/j.fsigss.2009.08.063

Courts C, Madea B (2011) Specific micro-RNA signatures for the detection of saliva and blood in forensic body-fluid identification. J Forensic Sci 56 (6):1464-1470. doi:10.1111/j.1556-4029.2011.01894.x

Cox M (1991) A study of the sensitivity and specificity of four presumptive tests for blood. J Forensic Sci 36 (5):1503-1511

Creamer JI, Quickenden TI, Crichton LB, Robertson P, Ruhayel RA (2005) Attempted cleaning of bloodstains and its effect on the forensic luminol test. Luminescence 20 (6):411-413. doi:10.1002/bio.865

Crowe G, Moss D, Elliot D (2000) The effect of laundering on the detection of acid phosphatase and spermatozoa on cotton t-shirts. Can Soc Forensic Sci J 33 (1):1-5

Curran JM, Triggs CM, Buckleton JS, Walsh K, Hicks T (1998) Assessing transfer probabilities in a Bayesian interpretation of forensic glass evidence. Sci Justice 38 (1):15-21. doi:10.1016/S1355-0306(98)72068-4

Dammeier S, Nahnsen S, Veit J, Wehner F, Ueffing M, Kohlbacher O (2015) Mass-spectrometry-based proteomics reveals organ-specific expression patterns to be used as forensic evidence. J Proteome Res 15 (1):182-192

Danielson PB (2011) Isolation of Highly Specific Protein Markers for the Identification of Biological Stains: Adapting Comparative Proteomics to Forensics. National Institute of Justice,

Dorum G, Ingold S, Hanson E, Ballantyne J, Russo G, Aluri S et al. (2019) Predicting the origin of stains from whole miRNome massively parallel sequencing data. Forensic Sci Int Genet 40:131-139. doi:10.1016/j.fsigen.2019.02.015

Dørum G, Ingold S, Hanson E, Ballantyne J, Snipen L, Haas C (2018) Predicting the origin of stains from next generation sequencing mRNA data. Forensic Sci Int Genet 34:37-48. doi:10.1016/j.fsigen.2018.01.001

Du T, Zamore PD (2005) microPrimer: the biogenesis and function of microRNA. Development 132 (21):4645-4652. doi:10.1242/dev.02070

Dunnett H, van der Meer D, Williams GA (2014) Evaluation of stem-loop reverse transcription and poly-A tail extension in microRNA analysis of body fluids. MicroRNA 3 (3):150-154

Edler C, Gehl A, Kohwagner J, Walther M, Krebs O, Augustin C et al. (2017) Blood Trace Evidence on Washed Textiles-a systematic approach. Int J Legal Med 131 (4):1179-1189

Fang C, Zhao J, Li JB, Qian JL, Liu X, Sun QF et al. (2019) Massively parallel sequencing of microRNA in bloodstains and evaluation of environmental influences on miRNA candidates using realtime polymerase chain reaction. Forensic Sci Int Genet 38:32-38. doi:10.1016/j.fsigen.2018.10.001

Farmen RK, Cortez P, Frøyland ES (2008) Spermatozoa recovered on laundered clothing. Forensic Science International: Genetics Supplement Series 1 (1):418-420

Fasken MB, Corbett AH (2005) Process or perish: quality control in mRNA biogenesis. Nat Struct Mol Biol 12 (6):482-488. doi:10.1038/nsmb945

Filipowicz W, Bhattacharyya SN, Sonenberg N (2008) Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? Nat Rev Genet 9 (2):102-114. doi:10.1038/nrg2290

Fleming RF BH, Fallow MV, Simon PM, Stacey JK, Wivell RJ and Harbison, SA Implementation of Messenger RNA Body Fluid Testing in Forensic Case Work. In: International Symposium on Human Identification, 2011.

Fleming RI, Harbison S (2010) The development of a mRNA multiplex RT-PCR assay for the definitive identification of body fluids. Forensic Sci Int Genet 4 (4):244-256. doi:10.1016/j.fsigen.2009.10.006

Fleming RI, Harbison S (2010) The use of bacteria for the identification of vaginal secretions. Forensic Sci Int Genet 4 (5):311-315. doi:10.1016/j.fsigen.2009.11.008

Forat S, Huettel B, Reinhardt R, Fimmers R, Haidl G, Denschlag D et al. (2016) Methylation Markers for the Identification of Body Fluids and Tissues from Forensic Trace Evidence. PLOS ONE 11 (2):e0147973. doi:10.1371/journal.pone.0147973

Fordyce SL, Kampmann M-L, Van Doorn NL, Gilbert MTP (2013) Long-term RNA persistence in post mortem contexts. Investig Genet 4 (7)

Frumkin D, Wasserstrom A, Budowle B, Davidson A (2011) DNA methylation-based forensic tissue identification. Forensic Sci Int Genet 5 (5):517-524. doi:10.1016/j.fsigen.2010.12.001

Fu HJ, Zhu J, Yang M, Zhang ZY, Tie Y, Jiang H et al. (2006) A novel method to monitor the expression of microRNAs. Mol Biotechnol 32 (3):197-204. doi:10.1385/MB:32:3:197

Fujiwara N, Shiki T, Masuda S (2012) mRNA Biogenesis in the Nucleus and Its Export to the Cytoplasm. In: Current Frontiers and Perspectives in Cell Biology. InTech,

Gill P, Jeffreys AJ, Werrett DJ (1985) Forensic application of DNA 'fingerprints'. Nature 318 (6046):577

Git A, Dvinge H, Salmon-Divon M, Osborne M, Kutter C, Hadfield J et al. (2010) Systematic comparison of microarray profiling, real-time PCR, and next-generation sequencing technologies for measuring differential microRNA expression. RNA 16 (5):991-1006. doi:10.1261/rna.1947110

Grabmuller M, Courts C, Madea B, Eichhorst T, Schyma C (2018) RNA/DNA co-analysis from bloodstains on aged polyvinyl-alcohol gloves prepared for securing evidence from the hands of victims of fatal gunshot injuries. Int J Legal Med 132 (1):53-66. doi:10.1007/s00414-017-1687-2

Grabmuller M, Madea B, Courts C (2015) Comparative evaluation of different extraction and quantification methods for forensic RNA analysis. Forensic Sci Int Genet 16:195-202. doi:10.1016/j.fsigen.2015.01.006

Grabmuller M, Schyma C, Euteneuer J, Madea B, Courts C (2015) Simultaneous analysis of nuclear and mitochondrial DNA, mRNA and miRNA from backspatter from inside parts of firearms generated by shots at "triple contrast" doped ballistic models. Forensic Sci Med Pathol 11 (3):365-375. doi:10.1007/s12024-015-9695-3

Grabmuller M, Schyma C, Madea B, Eichhorst T, Courts C (2017) RNA/DNA co-analysis on aged bloodstains from adhesive tapes used for gunshot residue collection from hands. Forensic Sci Med Pathol 13 (2):161-169. doi:10.1007/s12024-017-9864-7

Grasedieck S, Scholer N, Bommer M, Niess JH, Tumani H, Rouhi A et al. (2012) Impact of serum storage conditions on microRNA stability. Leukemia 26 (11):2414-2416. doi:10.1038/leu.2012.106

Guhaniyogi J, Brewer G (2001) Regulation of mRNA stability in mammalian cells. Gene 265 (1-2):11-23. doi:10.1016/S0378-1119(01)00350-X

Haas C, Hanson E, Anjos M, Banemann R, Berti A, Borges E et al. (2013) RNA/DNA co-analysis from human saliva and semen stains–results of a third collaborative EDNAP exercise. Forensic Sci Int Genet 7 (2):230-239

Haas C, Hanson E, Anjos M, Bär W, Banemann R, Berti A et al. (2012) RNA/DNA co-analysis from blood stains—results of a second collaborative EDNAP exercise. Forensic Sci Int Genet 6 (1):70-80

Haas C, Hanson E, Anjos MJ, Ballantyne KN, Banemann R, Bhoelai B et al. (2014) RNA/DNA co-analysis from human menstrual blood and vaginal secretion stains: results of a fourth and fifth collaborative EDNAP exercise. Forensic Sci Int Genet 8 (1):203-212. doi:10.1016/j.fsigen.2013.09.009

Haas C, Hanson E, Banemann R, Bento A, Berti A, Carracedo Á et al. (2015) RNA/DNA co-analysis from human skin and contact traces–results of a sixth collaborative EDNAP exercise. Forensic Sci Int Genet 16:139-147

Haas C, Hanson E, Bär W, Banemann R, Bento A, Berti A et al. (2011) mRNA profiling for the identification of blood—results of a collaborative EDNAP exercise. Forensic Sci Int Genet 5 (1):21-26

Haas C, Klesser B, Maake C, Bar W, Kratzer A (2009) mRNA profiling for body fluid identification by reverse transcription endpoint PCR and realtime PCR. Forensic Sci Int Genet 3 (2):80-88. doi:10.1016/j.fsigen.2008.11.003

Hanson E, Ingold S, Haas C, Ballantyne J (2015) Targeted multiplexed next generation RNA sequencing assay for tissue source determination of forensic samples. Forensic Sci Int Genet Suppl Ser 5:E441-E443. doi:10.1016/j.fsigss.2015.09.175

Hanson E, Ingold S, Haas C, Ballantyne J (2018) Messenger RNA biomarker signatures for forensic body fluid identification revealed by targeted RNA sequencing. Forensic Sci Int Genet 34:206-221. doi:10.1016/j.fsigen.2018.02.020

Hanson EK, Lubenow H, Ballantyne J (2009) Identification of forensically relevant body fluids using a panel of differentially expressed microRNAs. Anal Biochem 387 (2):303-314. doi:10.1016/j.ab.2009.01.037

Hanson EK, Rekab K, Ballantyne J (2013) Binary logistic regression models enable miRNA profiling to provide accurate identification of forensically relevant body fluids and tissues. Forensic Science International: Genetics Supplement Series 4 (1):e127-e128

Harbison S, Fleming R (2016) Forensic body fluid identification: state of the art. Research and Reports in Forensic Medical Science 6:11-23

Hargreaves MD, Page K, Munshi T, Tomsett R, Lynch G, Edwards HG (2008) Analysis of seized drugs using portable Raman spectroscopy in an airport environment—a proof of principle study. Journal of Raman Spectroscopy 39 (7):873-880

Harteveld J, Lindenbergh A, Sijen T (2013) RNA cell typing and DNA profiling of mixed samples: can cell types and donors be associated? Sci Justice 53 (3):261-269. doi:10.1016/j.scijus.2013.02.001

Hashimshony T, Zhang J, Keshet I, Bustin M, Cedar H (2003) The role of DNA methylation in setting up chromatin structure during development. Nat Genet 34 (2):187-192. doi:10.1038/ng1158

Herr J (2007) SpermPaint optimization and validation. Report submitted to the US Department of Justice, Washington, DC

Hirose Y, Manley JL (2000) RNA polymerase II and the integration of nuclear events. Genes Dev 14 (12):1415-1429

Hochmeister MN, Budowle B, Sparkes R, Rudin O, Gehrig C, Thali M et al. (1999) Validation studies of an immunochromatographic 1-step test for the forensic identification of human blood. J Forensic Sci 44 (3):597-602

Holtkotter H, Beyer V, Schwender K, Glaub A, Johann KS, Schurenkamp M et al. (2017) Independent validation of body fluid-specific CpG markers and construction of a robust multiplex assay. Forensic Sci Int Genet 29:261-268. doi:10.1016/j.fsigen.2017.05.002

Holtkötter H, Dias Filho CR, Schwender K, Stadler C, Vennemann M, Pacheco AC et al. (2018) Forensic differentiation between peripheral and menstrual blood in cases of alleged sexual assault—validating an immunochromatographic multiplex assay for simultaneous detection of human hemoglobin and D-dimer. International journal of legal medicine 132 (3):683-690

Holtkötter H, Dierig L, Schürenkamp M, Sibbing U, Pfeiffer H, Vennemann M (2015) Validation of an immunochromatographic D-dimer test to presumptively identify menstrual fluid in forensic exhibits. International journal of legal medicine 129 (1):37-41

Holtkötter H, Schwender K, Wiegand P, Peiffer H, Vennemann M (2018) Improving body fluid identification in forensic trace evidence—construction of an immunochromatographic test array to rapidly detect up to five body fluids simultaneously. International journal of legal medicine 132 (1):83-90

Horjan I, Barbaric L, Mrsic G (2016) Applicability of three commercially available kits for forensic identification of blood stains. J Forensic Leg Med 38:101-105. doi:10.1016/j.jflm.2015.11.021

Horvath S (2013) DNA methylation age of human tissues and cell types. Genome biology 14 (10):3156

Hu L, Wu C, Guo C, Li H, Xiong C (2014) Identification of microRNAs predominately derived from testis and epididymis in human seminal plasma. Clin Biochem 47 (10-11):967-972. doi:10.1016/j.clinbiochem.2013.11.009

Huang CJ, Nguyen PN, Choo KB, Sugii S, Wee K, Cheong SK et al. (2014) Frequent co-expression of miRNA-5p and -3p species and cross-targeting in induced pluripotent stem cells. Int J Med Sci 11 (8):824-833. doi:10.7150/ijms.8358

Ingold S, Dørum G, Hanson E, Berti A, Branicki W, Brito P et al. (2018) Body fluid identification using a targeted mRNA massively parallel sequencing approach–results of a EUROFORGEN/EDNAP collaborative exercise. Forensic Sci Int Genet 34:105-115

Izake EL (2010) Forensic and homeland security applications of modern portable Raman spectroscopy. Forensic Sci Int 202 (1-3):1-8. doi:10.1016/j.forsciint.2010.03.020

Jobin R, De Gouffe M (2003) The persistence of seminal constituents on panties after laundering. Significance to investigations of sexual assault. Can Soc Forensic Sci J 36 (1):1-10

Johnston S, Newman J, Frappier R (2003) Validation study of the Abacus Diagnostics ABAcard® HemaTrace® membrane test for the forensic identification of human blood. Canadian Society of Forensic Science Journal 36 (3):173-183

Jones Jr E (2005) The identification of semen and other body fluids. Forensic Science Handbook, Prentice Hall, Upper Saddle River, NJ:329-382

Juusola J, Ballantyne J (2003) Messenger RNA profiling: a prototype method to supplant conventional methods for body fluid identification. Forensic Sci Int 135 (2):85-96. doi:10.1016/S0379-0738(03)00197-X

Juusola J, Ballantyne J (2005) Multiplex mRNA profiling for the identification of body fluids. Forensic Sci Int 152 (1):1-12. doi:10.1016/j.forsciint.2005.02.020

Juusola J, Ballantyne J (2007) mRNA profiling for body fluid identification by multiplex quantitative RT-PCR. J Forensic Sci 52 (6):1252-1262. doi:10.1111/j.1556-4029.2007.00550.x

Kang K, Zhang XY, Liu HT, Wang ZW, Zhong JS, Huang ZT et al. (2012) A Novel Real-Time PCR Assay of microRNAs Using S-Poly(T), a Specific Oligo(dT) Reverse Transcription Primer with Excellent Sensitivity and Specificity. Plos One 7 (11):e48536. doi:ARTN e48536

10.1371/journal.pone.0048536

Kasu M, Shires K (2015) The validation of forensic DNA extraction systems to utilize soil contaminated biological evidence. Legal Medicine 17 (4):232-238. doi:10.1016/j.legalmed.2015.01.004

Kaye S (1949) Acid phosphatase test for identification of seminal stains. J Lab Clin Med 34 (5):728-732

Keating SM, Higgs DF (1994) The detection of amylase on swabs from sexual assault cases. J Forensic Sci Soc 34 (2):89-93

Kidd KK, Pakstis AJ, Speed WC, Grigorenko EL, Kajuna SL, Karoma NJ et al. (2006) Developing a SNP panel for forensic identification of individuals. Forensic science international 164 (1):20-32

Koch CM, Wagner W (2011) Epigenetic-aging-signature to determine age in different tissues. Aging (Albany NY) 3 (10):1018-1027. doi:10.18632/aging.100395

Köhler A, Hurt E (2007) Exporting RNA from the nucleus to the cytoplasm. Nature reviews Molecular cell biology 8 (10):761

Kohlmeier F, Schneider PM (2012) Successful mRNA profiling of 23 years old blood stains. Forensic Sci Int Genet 6 (2):274-276. doi:10.1016/j.fsigen.2011.04.007

Krol J, Loedige I, Filipowicz W (2010) The widespread regulation of microRNA biogenesis, function and decay. Nat Rev Genet 11 (9):597-610. doi:10.1038/nrg2843

Kulstein G, Schacker U, Wiegand P (2018) Old meets new: Comparative examination of conventional and innovative RNA-based methods for body fluid identification of laundered seminal fluid stains after modular extraction of DNA and RNA. Forensic Sci Int Genet 36:130-140. doi:10.1016/j.fsigen.2018.06.017

Kulstein G, Wiegand P (2018) Comprehensive examination of conventional and innovative body fluid identification approaches and DNA profiling of laundered blood- and saliva-stained pieces of cloths. Int J Legal Med 132 (1):67-81. doi:10.1007/s00414-017-1691-6

Lee HY, An JH, Jung S-E, Oh YN, Lee EY, Choi A et al. (2015) Genome-wide methylation profiling and a multiplex construction for the identification of body fluids using epigenetic markers. Forensic Science International: Genetics 17:17-24

Lee RC, Feinbaum RL, Ambros V (1993) The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. Cell 75 (5):843-854

Legg KM, Powell R, Reisdorph N, Reisdorph R, Danielson PB (2017) Verification of protein biomarker specificity for the identification of biological stains by quadrupole time-of-flight mass spectrometry. Electrophoresis 38 (6):833-845. doi:10.1002/elps.201600352

Levings PP, Bungert J (2002) The human β-globin locus control region. Eur J Biochem 269 (6):1589-1599. doi:10.1046/j.1432-1327.2002.02797.x

Li Y, Zhang J, Wei W, Wang Z, Prinz M, Hou Y (2014) A strategy for co-analysis of microRNAs and DNA. Forensic Sci Int Genet 12:24-29. doi:10.1016/j.fsigen.2014.04.011

Li Z, Bai P, Peng D, Wang H, Guo Y, Jiang Y et al. (2017) Screening and confirmation of microRNA markers for distinguishing between menstrual and peripheral blood. Forensic Sci Int Genet 30:24-33. doi:10.1016/j.fsigen.2017.05.012

Li ZL, Bai P, Peng D, Long B, Zhang L, Liang WB (2015) Influences of different RT-qPCR methods on forensic body fluid identification by microRNA. Forensic Sci Int Genet Suppl Ser 5:E295-E297. doi:10.1016/j.fsigss.2015.09.117

Li ZL, Bai P, Peng D, Long B, Zhang L, Liang WB (2015) Screening and confirmation of microRNA markers for distinguishing between menstrual and peripheral blood. Forens Sci Int-Gen S 5:E353-E355. doi:10.1016/j.fsigss.2015.09.140

Li ZL, Bai P, Peng D, Wang H, Guo YD, Jiang YJ et al. (2017) Screening and confirmation of microRNA markers for distinguishing between menstrual and

peripheral blood. Forensic Sci Int Genet 30:24-33. doi:10.1016/j.fsigen.2017.05.012

Lin M-H, Albani PP, Fleming R (2016) Degraded RNA transcript stable regions (StaRs) as targets for enhanced forensic RNA body fluid identification. Forensic Sci Int Genet 20:61-70

Lin MH, Albani PP, Fleming R (2016) Degraded RNA transcript stable regions (StaRs) as targets for enhanced forensic RNA body fluid identification. Forensic Sci Int Genet 20:61-70. doi:10.1016/j.fsigen.2015.09.012

Lin MH, Jones DF, Fleming R (2015) Transcriptomic analysis of degraded forensic body fluids. Forensic Sci Int Genet 17:35-42. doi:10.1016/j.fsigen.2015.03.005

Lindblad-Toh K, Winchester E, Daly MJ, Wang DG, Hirschhorn JN, Laviolette JP et al. (2000) Large-scale discovery and genotyping of single-nucleotide polymorphisms in the mouse. Nat Genet 24 (4):381-386. doi:10.1038/74215

Lindenbergh A, de Pagter M, Ramdayal G, Visser M, Zubakov D, Kayser M et al. (2012) A multiplex (m)RNA-profiling system for the forensic identification of body fluids and contact traces. Forensic Sci Int Genet 6 (5):565-577. doi:10.1016/j.fsigen.2012.01.009

Lindenbergh A, Maaskant P, Sijen T (2013) Implementation of RNA profiling in forensic casework. Forensic Sci Int Genet 7 (1):159-166. doi:10.1016/j.fsigen.2012.09.003

Livingstone M, Atas E, Meller A, Sonenberg N (2010) Mechanisms governing the control of mRNA translation. Phys Biol 7 (2):021001. doi:10.1088/1478-3975/7/2/021001

Lunetta P, Sippel H (2009) Positive prostate-specific antigen (PSA) reaction in post-mortem rectal swabs: a cautionary note. J Forensic Leg Med 16 (7):397-399. doi:10.1016/j.jflm.2009.04.002

Mason KE, Anex D, Grey T, Hart B, Parker G (2018) Protein-based forensic identification using genetically variant peptides in human bone. Forensic science international 288:89-96

Matsubara Y, Ikeda H, Endo H, Narisawa K (1992) Dried blood spot on filter paper as a source of mRNA. Nucleic acids research 20 (8):1998

Mayes C, Houston R, Seashols-Williams S, LaRue B, Hughes-Stamm S (2019) The stability and persistence of blood and semen mRNA and miRNA targets for body fluid identification in environmentally challenged and laundered samples. Leg Med (Tokyo) 38:45-50. doi:10.1016/j.legalmed.2019.03.007

Mayes C, Seashols-Williams S, Hughes-Stamm S (2018) A capillary electrophoresis method for identifying forensically relevant body fluids using miRNAs. Leg Med (Tokyo) 30:1-4. doi:10.1016/j.legalmed.2017.10.013

McLaughlin G, Lednev IK (2015) In Situ Identification of Semen Stains on Common Substrates via Raman Spectroscopy. J Forensic Sci 60 (3):595-604. doi:10.1111/1556-4029.12708

McLaughlin G, Sikirzhytski V, Lednev IK (2013) Circumventing substrate interference in the Raman spectroscopic identification of blood stains. Forensic Sci Int 231 (1-3):157-166. doi:10.1016/j.forsciint.2013.04.033

Meakin G, Jamieson A (2013) DNA transfer: review and implications for casework. Forensic Sci Int Genet 7 (4):434-443. doi:10.1016/j.fsigen.2013.03.013

Merkley ED, Wunschel DS, Wahl KL, Jarman KH (2019) Applications and challenges of forensic proteomics. Forensic Sci Int 297:350-363. doi:10.1016/j.forsciint.2019.01.022

Mestdagh P, Hartmann N, Baeriswyl L, Andreasen D, Bernard N, Chen C et al. (2014) Evaluation of quantitative miRNA expression platforms in the microRNA quality control (miRQC) study. Nat Methods 11 (8):809-815. doi:10.1038/nmeth.3014

Mill J, Yazdanpanah S, Gückel E, Ziegler S, Kaminsky Z, Petronis A (2006) Whole genome amplification of sodium bisulfite-treated DNA allows the accurate estimate of methylated cytosine density in limited DNA resources. Biotechniques 41 (5):603-607

Miller KW, Old J, Fischer BR, Schweers B, Stipinaite S, Reich K (2011) Developmental Validation of the SPERM HY-LITERTM Kit for the Identification of Human Spermatozoa in Forensic Samples. Journal of forensic sciences 56 (4):853-865

Miranda TB, Jones PA (2007) DNA methylation: the nuts and bolts of repression. J Cell Physiol 213 (2):384-390. doi:10.1002/jcp.21224

Misencik A, Laux DL (2007) Validation study of the seratec hemdirect hemoglobin assay for the forensic identification of human blood. MAFS Newslett 36 (2):18-26

Moreno LI, Tate CM, Knott EL, McDaniel JE, Rogers SS, Koons BW et al. (2012) Determination of an effective housekeeping gene for the quantification of mRNA for forensic applications. J Forensic Sci 57 (4):1051-1058. doi:10.1111/j.1556-4029.2012.02086.x

Myers JR, Adkins WK (2008) Comparison of modern techniques for saliva screening. J Forensic Sci 53 (4):862-867. doi:10.1111/j.1556-4029.2008.00755.x

Nakanishi H, Hara M, Takahashi S, Takada A, Saito K (2014) Evaluation of forensic examination of extremely aged seminal stains. Leg Med (Tokyo) 16 (5):303-307. doi:10.1016/j.legalmed.2014.04.002

Naue J, Hoefsloot HCJ, Mook ORF, Rijlaarsdam-Hoekstra L, van der Zwalm MCH, Henneman P et al. (2017) Chronological age prediction based on DNA methylation: Massive parallel sequencing and random forest regression. Forensic Science International: Genetics 31:19-28. doi:https://doi.org/10.1016/j.fsigen.2017.07.015

Noel S, Lagace K, Rogic A, Granger D, Bourgoin S, Jolicoeur C et al. (2016) DNA transfer during laundering may yield complete genetic profiles. Forensic Sci Int Genet 23:240-247. doi:10.1016/j.fsigen.2016.05.004

Nussbaumer C, Gharehbaghi-Schnell E, Korschineck I (2006) Messenger RNA profiling: a novel method for body fluid identification by real-time PCR. Forensic Sci Int 157 (2-3):181-186. doi:10.1016/j.forsciint.2005.10.009

Ohta J, Noda N, Sakurada K (2018) Comparison of Catalytic and Immunological Amylase Tests for Identifying of Saliva from Degraded Samples. J Forensic Sci. doi:10.1111/1556-4029.13936

Old J, Schweers BA, Boonlayangoor PW, Fischer B, Miller KW, Reich K (2012) Developmental Validation of RSID™-Semen: A Lateral Flow Immunochromatographic Strip Test for the Forensic Detection of Human Semen. Journal of forensic sciences 57 (2):489-499

Old JB, Schweers BA, Boonlayangoor PW, Reich KA (2009) Developmental Validation of RSID™-Saliva: A Lateral Flow Immunochromatographic Strip Test for the Forensic Detection of Saliva. Journal of forensic sciences 54 (4):866-873

Omelia EJ, Uchimoto ML, Williams G (2013) Quantitative PCR analysis of blood- and saliva-specific microRNA markers following solid-phase DNA extraction. Anal Biochem 435 (2):120-122. doi:10.1016/j.ab.2012.12.024

Paliwal A, Vaissière T, Herceg Z (2010) Quantitative detection of DNA methylation states in minute amounts of DNA from body fluids. Methods 52 (3):242-247. doi:10.1016/j.ymeth.2010.03.008

Park JL, Kwon OH, Kim JH, Yoo HS, Lee HC, Woo KM et al. (2014) Identification of body fluid-specific DNA methylation markers for use in forensic science. Forensic Sci Int Genet 13:147-153. doi:10.1016/j.fsigen.2014.07.011

Park JL, Park SM, Kwon OH, Lee HC, Kim JY, Seok HH et al. (2014) Microarray screening and qRT-PCR evaluation of microRNA markers for forensic body fluid identification. Electrophoresis 35 (21-22):3062-3068. doi:10.1002/elps.201400075

Parker GJ, Leppert T, Anex DS, Hilmer JK, Matsunami N, Baird L et al. (2016) Demonstration of protein-based human identification using the hair shaft proteome. PloS one 11 (9):e0160653

Paterson SK, Jensen CG, Vintiner SK, McGlashan SR (2006) Immunohistochemical staining as a potential method for the identification of vaginal epithelial cells in forensic casework. Journal of forensic sciences 51 (5):1138-1143

Phillips C, Salas A, Sanchez J, Fondevila M, Gomez-Tato A, Alvarez-Dios J et al. (2007) Inferring ancestral origin using a single multiplex assay of ancestry-informative marker SNPs. Forensic Science International: Genetics 1 (3-4):273-280

Pinard R, de Winter A, Sarkis GJ, Gerstein MB, Tartaro KR, Plant RN et al. (2006) Assessment of whole genome amplification-induced bias through high-throughput, massively parallel whole genome sequencing. BMC genomics 7 (1):216

Pratt AJ, MacRae IJ (2009) The RNA-induced silencing complex: a versatile gene-silencing machine. J Biol Chem 284 (27):17897-17901. doi:10.1074/jbc.R900012200

Proudfoot NJ (2016) Transcriptional termination in mammals: Stopping the RNA polymerase II juggernaut. Science 352 (6291):aad9926. doi:10.1126/science.aad9926

Qiagen (2014) AllPrep DNA/RNA Micro Handbook.

Quinones I, Daniel B (2012) Cell free DNA as a component of forensic evidence recovered from touched surfaces. Forensic science international: Genetics 6 (1):26-30

Richard ML, Harper KA, Craig RL, Onorato AJ, Robertson JM, Donfack J (2012) Evaluation of mRNA marker specificity for the identification of five human body fluids by capillary electrophoresis. Forensic Sci Int Genet 6 (4):452-460. doi:10.1016/j.fsigen.2011.09.007

Riggins GJ, Strausberg RL (2001) Genome and genetic resources from the Cancer Genome Anatomy Project. Hum Mol Genet 10 (7):663-667

Roeder AD, Haas C (2013) mRNA profiling using a minimum of five mRNA markers per body fluid and a novel scoring method for body fluid identification. Int J Legal Med 127 (4):707-721. doi:10.1007/s00414-012-0794-3

Romero-Montoya L, Martinez-Rodriguez H, Perez MA, Arguello-Garcia R (2011) Relationship of spermatoscopy, prostatic acid phosphatase activity and prostate-specific antigen (p30) assays with further DNA typing in forensic samples from rape cases. Forensic Sci Int 206 (1-3):111-118. doi:10.1016/j.forsciint.2010.07.012

Ruiz Y, Phillips C, Gomez-Tato A, Alvarez-Dios J, Casares de Cal M, Cruz R et al. (2013) Further development of forensic eye color predictive tests. Forensic Science International: Genetics 7 (1):28-40. doi:10.1016/j.fsigen.2012.05.009

Saba R, Booth SA (2006) Target labelling for the detection and profiling of microRNAs expressed in CNS tissue using microarrays. BMC Biotechnol 6 (1):47

Sakurada K, Akutsu T, Watanabe K, Fujinami Y, Yoshino M (2011) Expression of statherin mRNA and protein in nasal and vaginal secretions. Leg Med (Tokyo) 13 (6):309-313. doi:10.1016/j.legalmed.2011.07.002

Sakurada K, Akutsu T, Watanabe K, Miyasaka S, Kasai K (2013) Identification of Body Fluid Stains Using Real-time RT-PCR: Discrimination Between Salivary, Nasal, and Vaginal Secretions. Jpn J Forensic Sci Tech 18 (1):1-11. doi:10.3408/jafst.18.1

Sakurada K, Akutsu T, Watanabe K, Yoshino M (2012) Identification of nasal blood by real-time RT-PCR. Leg Med (Tokyo) 14 (4):201-204. doi:10.1016/j.legalmed.2012.01.014

Sakurada K, Ikegaya H, Fukushima H, Akutsu T, Watanabe K, Yoshino M (2009) Evaluation of mRNA-based approach for identification of saliva and semen. Leg Med (Tokyo) 11 (3):125-128. doi:10.1016/j.legalmed.2008.10.002

Sanchez JJ, Phillips C, Børsting C, Balogh K, Bogus M, Fondevila M et al. (2006) A multiplex assay with 52 single nucleotide polymorphisms for human identification. Electrophoresis 27 (9):1713-1724

Sauer E, Babion I, Madea B, Courts C (2014) An evidence based strategy for normalization of quantitative PCR data from miRNA expression analysis in forensic organ tissue identification. Forensic Sci Int Genet 13:217-223. doi:10.1016/j.fsigen.2014.08.005

Sauer E, Extra A, Cachee P, Courts C (2017) Identification of organ tissue types and skin from forensic samples by microRNA expression analysis. Forensic Sci Int Genet 28:99-110. doi:10.1016/j.fsigen.2017.02.002

Sauer E, Madea B, Courts C (2014) An evidence based strategy for normalization of quantitative PCR data from miRNA expression analysis in forensically relevant body fluids. Forensic Sci Int Genet 11:174-181. doi:10.1016/j.fsigen.2014.03.011

Sauer E, Reinke AK, Courts C (2015) Validation of forensic body fluid identification based on empirically normalized miRNA expression data. Forensic Sci Int Genet Suppl Ser 5:E462-E464. doi:10.1016/j.fsigss.2015.09.183

Sauer E, Reinke AK, Courts C (2016) Differentiation of five body fluids from forensic samples by expression analysis of four microRNAs using quantitative PCR. Forensic Sci Int Genet 22:89-99. doi:10.1016/j.fsigen.2016.01.018

Schweers BA, Old J, Boonlayangoor P, Reich KA (2008) Developmental validation of a novel lateral flow strip test for rapid identification of human blood (Rapid Stain Identification™-Blood). Forensic Science International: Genetics 2 (3):243-247

Schweighardt AJ, Tate CM, Scott KA, Harper KA, Robertson JM (2015) Evaluation of commercial kits for dual extraction of DNA and RNA from human body fluids. J Forensic Sci 60 (1):157-165. doi:10.1111/1556-4029.12586

Seashols-Williams S, Lewis C, Calloway C, Peace N, Harrison A, Hayes-Nash C et al. (2016) High-throughput miRNA sequencing and identification of biomarkers for forensically relevant biological fluids. Electrophoresis 37 (21):2780-2788. doi:10.1002/elps.201600258

Seraglia R, Teatino A, Traldi P (2004) MALDI mass spectrometry in the solution of some forensic problems. Forensic Sci Int 146 Suppl:S83-85. doi:10.1016/j.forsciint.2004.09.031

Setzer M, Juusola J, Ballantyne J (2008) Recovery and stability of RNA in vaginal swabs and blood, semen, and saliva stains. J Forensic Sci 53 (2):296-305. doi:10.1111/j.1556-4029.2007.00652.x

Sheehan FX, Kobilinsky L (1984) Human blood identification: A forensic science approach. Journal of Chemical Education 61 (6):542

Sijen T (2015) Molecular approaches for forensic cell type identification: On mRNA, miRNA, DNA methylation and microbial markers. Forensic Sci Int Genet 18:21-32. doi:10.1016/j.fsigen.2014.11.015

Sikirzhytskaya A, Sikirzhytski V, McLaughlin G, Lednev IK (2013) Forensic identification of blood in the presence of contaminations using Raman

microspectroscopy coupled with advanced statistics: effect of sand, dust, and soil. Journal of forensic sciences 58 (5):1141-1148

Silva SS, Lopes C, Teixeira AL, Carneiro de Sousa MJ, Medeiros R (2015) Forensic miRNA: potential biomarker for body fluids? Forensic Sci Int Genet 14:1-10. doi:10.1016/j.fsigen.2014.09.002

Sirker M, Fimmers R, Schneider PM, Gomes I (2017) Evaluating the forensic application of 19 target microRNAs as biomarkers in body fluid and tissue identification. Forensic Sci Int Genet 27:41-49. doi:10.1016/j.fsigen.2016.11.012

Sirker M, Fimmers R, Schneider PM, Gomes I (2017) Evaluating the forensic application of 19 target microRNAs as biomarkers in body fluid and tissue identification. Forensic Science International-Genetics 27:41-49. doi:10.1016/j.fsigen.2016.11.012

Sirker M, Liang W, Zhang L, Fimmers R, Rothschild A, Gomes I et al. (2015) Impact of using validated or standard reference genes for miRNA qPCR data normalization in cell type identification. Forens Sci Int-Gen S 5:E199-E201. doi:10.1016/j.fsigss.2015.09.080

Sirker M, Schneider PM, Gomes I (2016) A 17-month time course study of human RNA and DNA degradation in body fluids under dry and humid environmental conditions. Int J Legal Med 130 (6):1431-1438. doi:10.1007/s00414-016-1373-9

Stafford-Allen B, Dawnay N, Hanson EK, Ball G, Gupta A, Blackman S et al. (2018) Development of HyBeacon® probes for specific mRNA detection using body fluids as a model system. Molecular and Cellular Probes 38:51-59. doi:/10.1016/j.mcp.2017.11.007

Taroni F, Biedermann A, Garbolino P, Aitken CG (2004) A general approach to Bayesian networks for the interpretation of evidence. Forensic Sci Int 139 (1):5-16. doi:https://doi.org/10.1016/j.forsciint.2003.08.004

Taylor D, Biedermann A, Hicks T, Champod C (2018) A template for constructing Bayesian networks in forensic biology cases when considering activity level propositions. Forensic Sci Int Genet 33:136-146. doi:10.1016/j.fsigen.2017.12.006

Technologies L (2013) SuperScript III First-Strand Synthesis System for RT-PCR.

Thomas J, Buzzini P, Massonnet G, Reedy B, Roux C (2005) Raman spectroscopy and the forensic analysis of black/grey and blue cotton fibres Part 1: investigation of the effects of varying laser wavelength. Forensic Sci Int 152 (2-3):189-197. doi:10.1016/j.forsciint.2004.08.009

Tian H, Lv M, Li Z, Peng D, Tan Y, Wang H et al. (2018) Semen-specific miRNAs: Suitable for the distinction of infertile semen in the body fluid identification? Forensic Sci Int Genet 33:161-167. doi:10.1016/j.fsigen.2017.12.010

Tobe SS, Watson N, Daeid NN (2007) Evaluation of six presumptive tests for blood, their specificity, sensitivity, and effect on high molecular-weight DNA. Journal of forensic sciences 52 (1):102-109

Vaissière T, Cuenin C, Paliwal A, Vineis P, Hainaut P, Herceg Z (2009) Quantitative analysis of DNA methylation after whole bisulfitome amplification of a minute amount of DNA from body fluids. Epigenetics 4 (4):221-230

van den Berge M, Bhoelai B, Harteveld J, Matai A, Sijen T (2016) Advancing forensic RNA typing: On non-target secretions, a nasal mucosa marker, a differential co-

extraction protocol and the sensitivity of DNA and RNA profiling. Forensic Sci Int Genet 20:119-129. doi:10.1016/j.fsigen.2015.10.011

van den Berge M, Ozcanhan G, Zijlstra S, Lindenbergh A, Sijen T (2016) Prevalence of human cell material: DNA and RNA profiling of public and private objects and after activity scenarios. Forensic Sci Int Genet 21:81-89. doi:10.1016/j.fsigen.2015.12.012

van den Berge M, Wiskerke D, Gerretsen RR, Tabak J, Sijen T (2016) DNA and RNA profiling of excavated human remains with varying postmortem intervals. Int J Legal Med 130 (6):1471-1480. doi:10.1007/s00414-016-1438-9

van der Meer D, Uchimoto ML, Williams G (2013) Simultaneous analysis of micro-RNA and DNA for determining the body fluid origin of DNA profiles. J Forensic Sci 58 (4):967-971. doi:10.1111/1556-4029.12160

van der Meer DJ, Williams GA (2015) Performing body fluid identification with microRNAs using capillary electrophoresis. Forensic Sci Int Genet Suppl Ser

van Oorschot RA, Ballantyne KN, Mitchell RJ (2010) Forensic trace DNA: a review. Investig Genet 1 (1):14. doi:10.1186/2041-2223-1-14

Van Steendam K, De Ceuleneer M, Dhaenens M, Van Hoofstat D, Deforce D (2013) Mass spectrometry-based proteomics as a tool to identify biological matrices in forensic science. Int J Legal Med 127 (2):287-298

Vennemann M, Koppelkamm A (2010) mRNA profiling in forensic genetics I: Possibilities and limitations. Forensic Sci Int 203 (1-3):71-75. doi:10.1016/j.forsciint.2010.07.006

Venter JC, Adams MD, Myers EW, Li PW, Mural RJ, Sutton GG et al. (2001) The sequence of the human genome. Science 291 (5507):1304-1351. doi:10.1126/science.1058040

Vidaki A, Ballard D, Aliferi A, Miller TH, Barron LP, Syndercombe Court D (2017) DNA methylation-based forensic age prediction using artificial neural networks and next generation sequencing. Forensic Science International: Genetics 28:225-236. doi:10.1016/j.fsigen.2017.02.009

Vidaki A, Daniel B, Court DS (2013) Forensic DNA methylation profiling—Potential opportunities and challenges. Forensic Science International: Genetics 7 (5):499-507. doi:10.1016/j.fsigen.2013.05.004

Vidaki A, Kayser M (2018) Recent progress, methods and perspectives in forensic epigenetics. Forensic Sci Int Genet 37:180-195. doi: 10.1016/j.fsigen.2018.08.008

Vidaki A, López CD, Carnero-Montoro E, Ralf A, Ward K, Spector T et al. (2017) Epigenetic discrimination of identical twins from blood under the forensic scenario. Forensic Science International: Genetics 31:67-80

Virkler K, Lednev IK (2008) Raman spectroscopy offers great potential for the nondestructive confirmatory identification of body fluids. Forensic Sci Int 181 (1-3):e1-5. doi:10.1016/j.forsciint.2008.08.004

Virkler K, Lednev IK (2009) Analysis of body fluids for forensic purposes: from laboratory testing to non-destructive rapid confirmatory identification at a crime scene. Forensic Sci Int 188 (1-3):1-17. doi:10.1016/j.forsciint.2009.02.013

Virkler K, Lednev IK (2009) Blood species identification for forensic purposes using Raman spectroscopy combined with advanced statistical analysis. Analytical chemistry 81 (18):7773-7777

Virkler K, Lednev IK (2009) Raman spectroscopic signature of semen and its potential application to forensic body fluid identification. Forensic Sci Int 193 (1-3):56-62. doi:10.1016/j.forsciint.2009.09.005

Visser M, Zubakov D, Ballantyne KN, Kayser M (2011) mRNA-based skin identification for forensic applications. Int J Legal Med 125 (2):253-263. doi:10.1007/s00414-010-0545-2

Voskoboinik L, Amiel M, Reshef A, Gafny R, Barash M (2018) Laundry in a washing machine as a mediator of secondary and tertiary DNA transfer. Int J Legal Med 132 (2):373-378. doi:10.1007/s00414-017-1617-3

Walsh S, Liu F, Ballantyne KN, van Oven M, Lao O, Kayser M (2011) IrisPlex: A sensitive DNA tool for accurate prediction of blue and brown eye colour in the absence of ancestry information. Forensic Science International: Genetics 5 (3):170-180. doi:10.1016/j.fsigen.2010.02.004

Wang Z, Luo H, Pan X, Liao M, Hou Y (2012) A model for data analysis of microRNA expression in forensic body fluid identification. Forensic Sci Int Genet 6 (3):419-423. doi:10.1016/j.fsigen.2011.08.008

Wang Z, Zhang J, Luo H, Ye Y, Yan J, Hou Y (2013) Screening and confirmation of microRNA markers for forensic body fluid identification. Forensic Sci Int Genet 7 (1):116-123. doi:10.1016/j.fsigen.2012.07.006

Wang Z, Zhou D, Cao Y, Hu Z, Zhang S, Bian Y et al. (2016) Characterization of microRNA expression profiles in blood and saliva using the Ion Personal Genome Machine((R)) System (Ion PGM System). Forensic Sci Int Genet 20:140-146. doi:10.1016/j.fsigen.2015.10.008

Wang Z, Zhou D, Cao Y, Hu Z, Zhang S, Bian Y et al. (2016) Characterization of microRNA expression profiles in blood and saliva using the Ion Personal Genome Machine® System (Ion PGM™ System). Forensic Sci Int Genet 20:140-146

Wasserstrom A, Frumkin D, Davidson A, Shpitzen M, Herman Y, Gafny R (2013) Demonstration of DSI-semen--A novel DNA methylation-based forensic semen identification assay. Forensic Sci Int Genet 7 (1):136-142. doi:10.1016/j.fsigen.2012.08.009

Webb JL, Creamer JI, Quickenden TI (2006) A comparison of the presumptive luminol test for blood with four non-chemiluminescent forensic techniques. Luminescence 21 (4):214-220. doi:10.1002/bio.908

Westholm JO, Lai EC (2011) Mirtrons: microRNA biogenesis via splicing. Biochimie 93 (11):1897-1904. doi:10.1016/j.biochi.2011.06.017

Whitehead A, Crawford DL (2005) Variation in tissue-specific gene expression among natural populations. Genome Biol 6 (2):R13. doi:10.1186/gb-2005-6-2-r13

Willenbrock H, Salomon J, Sokilde R, Barken KB, Hansen TN, Nielsen FC et al. (2009) Quantitative miRNA expression analysis: comparing microarrays with next-generation sequencing. RNA 15 (11):2028-2034. doi:10.1261/rna.1699809

Williams G, Uchimoto ML, Coult N, World D, Beasley E, Avenell P (2013) Characterisation of body fluid specific microRNA markers by capillary electrophoresis. Forensic Sci Int Genet Suppl Ser 4 (1):e274-e275

Willott GM (1974) An Improved Test for the Detection of Salivary Amylase in Stains. Journal of the Forensic Science Society 14 (4):341-344. doi:10.1016/S0015-7368(74)70923-9

Xu Y, Xie J, Cao Y, Zhou H, Ping Y, Chen L et al. (2014) Development of highly sensitive and specific mRNA multiplex system (XCYR1) for forensic human body fluids and tissues identification. PLoS One 9 (7):e100123. doi:10.1371/journal.pone.0100123

Yang H, Zhou B, Deng H, Prinz M, Siegel D (2013) Body fluid identification by mass spectrometry. Int J Legal Med 127 (6):1065-1077. doi:10.1007/s00414-013-0848-1

Yauk CL, Rowan-Carroll A, Stead JD, Williams A (2010) Cross-platform analysis of global microRNA expression technologies. BMC Genomics 11 (1):330. doi:10.1186/1471-2164-11-330

Yu H, Berkel H (1999) Prostate-specific antigen (PSA) in women. J La State Med Soc 151 (4):209-213

Zeng X, King JL, Stoljarova M, Warshauer DH, LaRue BL, Sajantila A et al. (2015) High sensitivity multiplex short tandem repeat loci analyses with massively parallel sequencing. Forensic Sci Int Genet 16:38-47. doi:10.1016/j.fsigen.2014.11.022

Zhao H, Wang C, Yao L, Lin Q, Xu X, Hu L et al. (2017) Identification of aged bloodstains through mRNA profiling: Experiments results on selected markers of 30- and 50-year-old samples. Forensic Sci Int 272:e1-e6. doi:10.1016/j.forsciint.2017.01.006

Zubakov D, Boersma AW, Choi Y, van Kuijk PF, Wiemer EA, Kayser M (2010) MicroRNA markers for forensic body fluid identification obtained from microarray screening and quantitative RT-PCR confirmation. Int J Legal Med 124 (3):217-226. doi:10.1007/s00414-009-0402-3

Zubakov D, Hanekamp E, Kokshoorn M, van Ijcken W, Kayser M (2008) Stable RNA markers for identification of blood and saliva stains revealed from whole genome expression analysis of time-wise degraded samples. Int J Legal Med 122 (2):135-142. doi:10.1007/s00414-007-0182-6

Zubakov D, Kokmeijer I, Ralf A, Rajagopalan N, Calandro L, Wootton S et al. (2015) Towards simultaneous individual and tissue identification: A proof-of-principle study on parallel sequencing of STRs, amelogenin, and mRNAs with the Ion Torrent PGM. Forensic Sci Int Genet 17:122-128. doi:10.1016/j.fsigen.2015.04.002

Zubakov D, Kokshoorn M, Kloosterman A, Kayser M (2009) New markers for old stains: stable mRNA markers for blood and saliva identification from up to 16-year-old stains. Int J Legal Med 123 (1):71-74. doi:10.1007/s00414-008-0249-z

APPENDIX

Consent for Participation in Research

Development and evaluation of a combined DNA and miRNA system for forensically relevant body fluids using capillary electrophoresis.

Why am I being asked?

You are being asked to be a participant in a research study about the forensic identification of human bodily fluids using miRNA markers conducted by Dr. Sheree Hughes-Stamm, the Department of Forensic at Sam Houston State University. This study is funded by the National Institute of Justice. You have been asked to participate in the research because you are eligible to participate. We ask that you read this form and ask any questions you may have before agreeing to be in the research.

Your participation in this research is voluntary. Your decision whether or not to participate will not affect your current or future relations with Sam Houston State University or the Forensic Science program. If you decide to participate, you are free to withdraw at any time without affecting that relationship.

Why is this research being done?The biological samples provided in this study will serve as mock crime scene evidence. These materials will be used for research, and will provide researchers with the appropriate human samples for forensic analysis investigating the utility of miRNA markers for the identification of body fluids.

Biological samples from living humans are required in order to simulate mock crime scene samples such as small amounts of blood, semen and saliva on items of evidence. The ability to recover and get a "match" from the minute amounts of human DNA and RNA from these types of samples is vital for forensic analysis.

What is the purpose of this research?

The purpose of this project is to evaluate whether miRNA markers can be used to identify the biological source of evidence (saliva, semen, vaginal material, and venous or menstrual blood) in conjunction with traditional DNA profiling methods used for human identification.

What procedures are involved?

If you agree to be in this research, we may ask you to do one or more of the following things:

- Wipe the inside of your cheek with two swabs for approximately 30 seconds.
- Collect saliva in a tube (approximately 1 mL).
- Have your venous blood collected by a qualified phlebotomist.
- Collect a sample of menstrual blood using a cotton swab in the privacy in your own home.
- Collect a sample of vaginal material using a cotton swab in the privacy in your own home.
- Collect one emission of semen in a container in the privacy in your own

home.

Samples (and consent form with name) will be destroyed after a period of 2 years after the completion of the project. Samples will be disposed of via the standard pathological waste collection service.

Approximately 10-20 subjects may be involved in this research at Sam Houston State University.

What are the potential risks and discomforts?

There is minimal risk for the participants. These may include:

- Mild irritation and bruising whilst getting blood drawn (arm).

There are no significant physical or psychological, legal or reputational risks to participation.

If you feel uncomfortable at any time during the study, please notify Dr. Sheree Hughes-Stamm on 936-294-4359.

All collection procedures will be done at the same time and no future participation is required.

What other options are there?

There are no other options. Animal samples cannot be used for forensic human identification work.

What about privacy and confidentiality?

The only person who will know that you are a research participant is the principle investigator (PI) of this research project. No information about you, or provided by you during the research will be disclosed to others without your written permission, except:

- -if necessary to protect your rights or welfare (for example, if you are injured and need emergency care or when the SHSU Protection of Human Subjects monitors the research or consent process)

When the results of the research are published or discussed in conferences, no information will be included that would reveal your identity. If photographs, videos, or audiotape recordings of you will be used for educational purposes, your identity will be protected or disguised. Any information that is obtained in connection with this study and that can be identified with you will remain confidential and will be disclosed only with your permission or as required by law.

All samples are treated as anonymous, and are assigned a numbered code upon collection. No personal information is collected except the sex, ancestry, hair color, eye color and skin color of the participant. All personal information will only be accessed by the PI.

What if I am injured as a result of my participation?

In the event of injury related to this research study, you should contact your physician or the University Health Center. However, you or your third party payer, if any, will be responsible for payment of this treatment. There is no compensation and/or payment for medical treatment from Sam Houston State University for any injury you have from

participating in this research, except as may by required of the University by law. If you feel you have been injured, you may contact the researcher, Dr. Sheree Hughes-Stamm at 936-294-4359.

What are the costs for participating in this research?

There are no research costs for which the subject is responsible.

Will I be reimbursed for any of my expenses or paid for my participation in this research?

The subject will not receive payment, remuneration or reimbursement for participation in this study.

Can I withdraw or be removed from the study?

You can choose whether to be in this study or not. If you volunteer to be in this study, you may withdraw at any time without consequences of any kind. You may also refuse to answer any questions you don't want to answer and still remain in the study. The investigator may withdraw you from this research if circumstances arise which warrant doing so.

The participant can withdraw from this study at any time for any reason.

Who should I contact if I have questions?

The researcher conducting this study is Dr. Sheree Hughes-Stamm. You may ask any questions you have now. If you have questions later, you may contact the researchers at: Phone: 936-294-4359.

What are my rights as a research subject?

If you feel you have not been treated according to the descriptions in this form, or you have any questions about your rights as a research participant, you may call the Office of Research and Sponsored Programs – Sharla Miles at 936-294-4875 or e-mail ORSP at sharla_miles@shsu.edu.

You may choose not to participate or to stop your participation in this research at any time. Your decision whether or not to participate will not affect your current or future relations with the University. Non-participation in this study will not result in any sanction.

Participation is completely voluntary and you are under no obligation to participate. If you are a student, this will not affect your class standing or grades at SHSU. The investigator may also end your participation in the research. If this happens, your class standing or grades will not be affected.

If you are a staff person at SHSU, your participation in this research is in no way a part of your university duties, and your refusal to participate will not in any way affect your employment with the university, or the benefits, privileges, or opportunities associated with your employment at SHSU.

You will not be offered or receive any special consideration if you participate in this research.

Agreement to Participate

I have read (or someone has read to me) the above information. I have been given an opportunity to ask questions and my questions have been answered to my satisfaction. I agree to participate in this research.

Consent: I have read and understand the above information, and I willingly consent to participate in this study. I understand that if I should have any questions about my rights as a research subject, I can contact Dr. Sheree Hughes-Stamm at 936-294-4359 or by email at shereehs@shsu.edu. I have received a copy of this consent form.

Your name (printed):

Signature:

Date:

Assigned number:

VITA

EDUCATION

Sam Houston State University
Doctoral Candidate 2014-Present

Expected Graduation: August 2019
University of Texas at Arlington
B.S. Biology 2011-2013

Rice University
2003-2004

AWARDS

Institute for Forensic Research Training and Innovation Scholarship	2017
LTC Michael A. Lytle ’77 Academic Prize in Forensic Science	2016
“Best in Class” – Forensic Biology (SHSU)	2015
Forensic Science Scholarship (SHSU)	2014 – 2016
College of Science – Dean’s List (UTA)	January 2012 – 2013
College of Science – Dean’s List (UTA)	January 2011 – January 2012

WORK EXPERIENCE

Sam Houston State University
Graduate Assistant 2014-Present
Lab maintenance, reagent preparation, inventory of equipment, administrative duties, assisting with maintenance and troubleshooting of instruments, teaching assistant for Forensic Biology laboratories

Pappadeaux Seafood Kitchen 2005-2014
Lead bartender; Office Administrator Assistant; Banquet Captain;
Inventory and order of liquor, beer, and wine, staff development of servers and bartenders, customer service and dispute resolution, office administrator duties

RELEVANT COURSEWORK

Sam Houston State University
Forensic Biology, Advanced DNA, Molecular Biology, Introduction to Bioinformatic Tools, Forensic Statistics and Interpretation, Statistical Genetics, Forensic Laboratory Management, Pharmacogenomics, Non-human DNA Forensics, Advanced Genetics, Forensic Toxicology, Forensic Instrumental Analysis, Pattern and Physical Evidence Concepts, Trace Evidence and Microscopic Analysis, Controlled Substances

University of Texas at Arlington
Forensic Biology, Methods in Forensic Biology, Biochemistry, Instructional Techniques in Biology

RESEARCH EXPERIERNCE

Current - Sam Houston State University
Development and evaluation of miRNA panels for body fluid identification using capillary electrophoresis and massively parallel sequencing methods
Funded by the Graduate Research Fellowship in Science, Technology, Engineering, and Mathematics by the National Institute of Justice (NIJ-DN-BX-0001)

2015 (Summer) - Harris County Institute of Forensic Sciences, Houston, Texas
Validation of a procedure for recovering sperm DNA from fixed microscope slides

PUBLICATIONS

Esiri Tasker, Carrie Mayes, Madeline Roman, Mary Akosile, Sheree Hughes-Stamm, Bobby LaRue. Efficacy of "Touch" DNA Recovery and Room-Temperature Storage from Assault Rifle Magazines. Legal Medicine. (Under Review)

Esiri Tasker, Carrie Mayes, Sheree R. Hughes-Stamm. MicroFLOQ®: Collection and Direct Amplification Methods Using the GlobalFiler™ Kit for DNA Recovered from Common Pipe Bomb Components. Science and Justice. (Under Review)

Carrie Mayes, Rachel Houston, Sarah Seashols-Williams, Bobby LaRue, Sheree Hughes-Stamm. (2019). The stability and persistence of blood and semen mRNA and miRNA targets for body fluid identification in environmentally challenged and laundered samples. Legal Medicine. https://doi.org/10.1016/j.legalmed.2019.03.007

Xiangpei Zeng, Kyleen Elwick, Carrie Mayes, Jonathan L. King, Sheree Hughes-Stamm, Bruce Budowle. (2018). Assessment of impact of extraction methods on analysis of human remain samples on massively parallel sequencing success. International Journal of Legal Medicine. https://doi.org/10.1007/s00414-018-1955-9

Rachel Houston, Carrie Mayes, Jonathan L. King, Sheree Hughes-Stamm, David Gangitano. (2018). Massively parallel sequencing of 12 autosomal STRs in Cannabis sativa with the Ion™ S5. Electrophoresis. https://doi.org/10.1002/elps.201800152

Carrie Mayes, Sarah Seashols-Williams, Sheree Hughes-Stamm. (2018). A capillary electrophoresis method for identifying forensically relevant body fluids using miRNAs. Legal Medicine. Volume 30. Pages 1-4. https://doi.org/10.1016/j.legalmed.2017.10.013.

Kyleen Elwick, Carrie Mayes, Sheree Hughes-Stamm. (2018). Comparative Sensitivity and Inhibitor Tolerance of GlobalFiler® PCR Amplification and Investigator® 24plex QS Kits for Challenging Samples. Legal Medicine. Volume 32. Pages 31-36. https://doi.org/10.1016/j.legalmed.2018.01.006

Michelle Harrel, Carrie Mayes, David Gangitano, Sheree Hughes-Stamm. (2018) Evaluation Of A Powder-Free DNA Extraction Method For Skeletal Remains. Journal of Forensic Sciences. https://doi.org/10.1111/1556-4029.13749

ORAL PRESENTATIONS

2019 Rachel Houston, Carrie Mayes, Jonathan L. King, Sheree Hughes-Stamm, David Gangitano. Massively Parallel Sequencing (MPS) of 12 Autosomal Short Tandem Repeats (STRs) in Cannabis sativa. American Academy of Forensic Sciences (Baltimore, MD)

2019 Esiri Tasker, Carrie Mayes, Sheree R. Hughes-Stamm. MicroFLOQ®: Collection and Direct Amplification Methods Using the GlobalFiler™ Kit for DNA Recovered from Common Pipe Bomb Components. American Academy of Forensic Sciences (Baltimore, MD)

2018 Carrie Mayes, Michelle Harrel, Rachel Houston, Amy Sorensen Holmes, Ryan Gutierrez, Sheree Hughes-Stamm. Application of QIAGEN Workflow with Quality Sensors and Interpretation: Database and Casework Samples. QIAGEN Human Identity and Forensics Webinar. https://www.qiagen.com/us/resources/e-learning/webinars/webinars%20on-demand/human-identity-and-forensics/

2018 Carrie Mayes, Sarah Seashols-Williams, Sheree R. Hughes-Stamm. Initial evaluation of the stability and persistence of mRNA and miRNA in environmentally challenged samples. Forensic Technology Center of Excellence Webinar – Mixtures, Microhaps, and Modeling, Oh My! Cutting Edge Research in Forensic DNA Analysis in Forensic DNA Analysis from Early-Career Scientists. https:/forensiccoe.org/webinar/forensic-dna-research-early-career-scientists/

2018 Carrie Mayes, Sarah Seashols-Williams, Sheree R. Hughes-Stamm. Initial evaluation of the stability and persistence of mRNA and miRNA for body fluid identification in forensic samples. Gordon Research Seminar: Forensic Analysis of Human DNA (Newry, ME)

2018 Carrie Mayes, Sarah Seashols-Williams, Sheree R. Hughes-Stamm. Little Stains, Lots of Problems. 7th QIAGEN Investigator Forum: Young Investigator Session (San Antonio, TX)

2018 Rachel Houston, Carrie Mayes, Jonathan L. King, Sheree Hughes-Stamm, David Gangitano. Innovative molecular approaches for Cannabis sativa DNA typing. 7th QIAGEN Investigator Forum: Young Investigator Session (San Antonio, TX)

2017 Kyleen Elwick, Carrie Mayes, Michelle Harrel, Sheree Hughes-Stamm. Worlds Converge: A New Approach to Analyzing CE and MPS-based STRs. 27th Congress of the International Society for Forensic Genetics (ISFG) (Seoul, Korea)

2017 Kyleen Elwick, Carrie Mayes, Michelle Harrel, David Gangitano, Sheree Hughes-Stamm. Worlds Converge: A New Approach to Analyzing CE and MPS-based STRs. 3rd Human Identification Solutions (HIDS) Conference (Vienna, Austria)

2017 Carrie Mayes, Kyleen Elwick, Michelle Harrel, David Gangitano, Sheree Hughes-Stamm. NGM Detecting More from Skeletal Remains. 3rd Human Identification Solutions (HIDS) Conference (Vienna, Austria)

2017 Amy Sorensen, Rachel Houston, Kyleen Elwick, Carrie Mayes, Kayla Ehring, David Gangitano, Sheree Hughes-Stamm. Alternate methods for the collection, preservation, & processing of DNA samples from decomposing human cadavers; A DVI strategy. 6th QIAGEN Investigator Forum (Prague, Czech Republic)

POSTER PRESENTATIONS

2019 Carrie Mayes, Rachel Houston, Bobbly LaRue, Sarah Seashols-Williams, Sheree Hughes-Stamm. Evaluating of the stability and persistence of mRNA and miRNA for body fluid identification in forensic samples. Pittsburgh Conference (Philadelphia, PA)

2018 Ryan Gutierrez, Kari Graham, Kyleen Elwick, Carrie Mayes, Bobby LaRue. A Cost-Effective Workflow for Massively Parallel Sequencing of Drug Metabolizing Enzymes. International Symposium on Human Identification (Phoenix, AZ)

2018 Esiri Tasker, Carrie Mayes, Bobby LaRue, Sheree Hughes-Stamm. microFLOQ®: Collection and Direct Amplification Methods Using the GlobalFiler™ Kit from Common Pipe Bomb Substrates. International Symposium on Human Identification (Phoenix, AZ)

2018 Kyleen Elwick, Carrie Mayes, Xiangpei Zeng, Jonathan King, Bruce Budowle, Sheree Hughes-Stamm. Evaluation of Five Common Extraction Methods for Analysis

of Human Remain Samples on Massively Parallel Sequencing Success. 4th Human Identification Solutions Conference. (HIDS). (Rome, Italy)

2018 Carrie Mayes, Sarah Seashols-Williams, Sheree R. Hughes-Stamm. Initial evaluation of the stability and persistence of mRNA and miRNA for body fluid identification in forensic samples. Gordon Research Conference: Forensic Analysis of Human DNA (Newry, ME)

2018 Esiri Tasker, Carrie Mayes, Sheree Hughes-Stamm. Optimization of Direct Amplification Methods for DNA Samples from Common Pipe Bomb Substrates Using the GlobalFiler™ Kit. Gordon Research Seminar and Gordon Research Conference: Forensic Analysis of Human DNA (Newry, ME)

2018 Kyleen Elwick, Carrie Mayes, Xiangpei Zeng, Jonathan King, Bruce Budowle, Sheree Hughes-Stamm. Assessment of Five Common Extraction Methods for the Analysis of Human Remains using the Ion S5™ and MiSeq FGx®™ Systems. Gordon Research Seminar and Gordon Research Conference: Forensic Analysis of Human DNA (Newry, ME)

2018 Carrie Mayes, Sarah Seashols-Williams, Sheree Hughes-Stamm. A capillary electrophoresis method for identifying forensically relevant body fluids using miRNAs. Pittsburgh Conference (Orlando, FL)

2017 Carrie Mayes, Sarah Seashols-Williams, Sheree Hughes-Stamm. A capillary electrophoresis method for identifying forensically relevant body fluids using miRNAs. International Symposium on Human Identification (Seattle, WA)

2017 Michelle Harrel, Carrie Mayes, David Gangitano, Sheree Hughes-Stamm. Do we really need to crush? An alternate DNA extraction approach for bone samples. 27th Congress of the International Society for Forensic Genetics (ISFG) (Seoul, Korea)

2017 Carrie Mayes, Sheree Hughes-Stamm. Development and initial evaluation of a miRNA system for forensically relevant body fluids using capillary electrophoresis. American Academy of Forensic Sciences (New Orleans, LA)

2016 Carrie Mayes, Sheree Hughes-Stamm. Development and initial evaluation of a miRNA system for forensically relevant body fluids using capillary electrophoresis. International Symposium on Human Identification (Minneapolis, MN)

2016 Carrie Mayes, Sheree Hughes-Stamm. Development and initial evaluation of a miRNA system for forensically relevant body fluids using capillary electrophoresis.

Gordon Research Conference: Forensic Analysis of Human DNA (Waterville Valley, NH)

SKILLS AND QUALIFICATIONS

Molecular Techniques

Proficient with DNA extraction using PCIA, Chelex®, and QIAGEN® Investigator Kit; RNA extraction using AllPrep DNA/RNA Micro Kit, RNeasy Mini Kit, and the miRNeasy Mini Kit; quantification using Quantifiler® Duo, Quantifiler Trio®, Quantiplex Pro, Quantiplex Pro RGQ; amplification using Identifiler® Plus, GlobalFiler™, NGM Detect™, Investigator 24plex QS, PowerPlex® Fusion 6C PCR amplification kits; gene expression using miScript and QuantiTect® workflows; massively parallel sequencing library preparation using Precision ID Chef DL8 and the ForenSeq™ DNA Signature Prep Kit

Instrumentation

QIAcube®, QIAgility™, AutoMate Express™ Forensic DNA Extraction System, EZ1 Advanced XL, ABI Real-Time 7500 PCR System, Qubit™ 3, Rotor-Gene® Q, GeneAmp® 9700 Thermocycler, ProFlex PCR System, Applied Biosystems® 3500 Genetic Analyzer, Ion Chef™, Ion S5™, MiSeq

Software

Applied Biosystems® Genemapper ID-X, converge™, STRait Razor, R Software, vcftools, Microsoft Office

MEMBERSHIPS

Society of Forensic Science (Sam Houston State University)
American Academy of Forensic Sciences – Student Affiliate