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Stability and Variation within Donors of miRNA Markers for Body Fluid Identification

Kelsey R. Price
Virginia Commonwealth University

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Kelsey Price

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Director: Sarah J. Seashols-Williams, PhD.

Seashols-Williams Research Laboratory Department of Forensic Science Virginia Commonwealth University

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Abstract

Traditional enzymatic tests for body fluid identification are prone to false negatives, false positives, and several body fluids do not have confirmatory or reliable presumptive tests. Therefore, molecular-based tests may be more reliable, such as messenger RNA (mRNA), microbial DNA, or microRNA (miRNA) assays.

miRNAs are small, noncoding RNAs whose main function *in-vivo* is the regulation of protein expression by selectively suppressing the translation of their corresponding mRNAs. They also lack a poly-A tail, and because of this, their small size, and their association with other molecules such as the RISC protein complex, they are more robust than other RNAs, and more easily detected in compromised samples. miRNAs are also present in DNA extracts.

The overall purpose of this project is to test the robustness of an optimized miRNA panel as part of the developmental validation process. This panel was tested within individuals over a biological cycle, and samples that have been exposed to either a heat, chemical, UV, or environmental treatment. Detection of these markers was evaluated using reverse-transcription quantitative PCR (RT-qPCR). Following RT-qPCR, the differential expression values (ΔCq) were calculated and input into a quadratic discriminate analysis model for body fluid classification.

Significant differences were present in the detection of these markers within the same donor in saliva and blood, however, all body fluids except for blood and urine exhibited low classification rates compared to previous population studies, especially menstrual blood, which was often incorrectly classified as vaginal fluid. Blood and urine that were treated with heat both showed classification rates similar to previous population studies. In contrast, semen and saliva both showed a low correct classification rate. The classification rate of each body fluid after either a chemical or UV treatment depended on both the body fluid and the marker. Dish soap, 1:10 bleach and full-strength bleach treatment impacted the detection of miRNA markers in semen. Bleach affected the detection in saliva, glacial acetic acid affected detection in urine, and all treatments except UV affected the detection of markers in blood, although not enough to affect the classification rate. The environmentally treated samples were all correctly classified in blood and urine, however, neither semen nor saliva were correctly classified in any of the samples due to failure to amplify. Overall, the classification rates in compromised samples were similar to population studies in blood and urine and were lower in semen and urine.

In future studies, this assay could benefit from adding markers of different types, such as microbial DNA markers, and using high throughput sequencing to increase its multiplexing ability, which would decrease the required analyst time. Although vaginal fluid, saliva, and semen showed relatively low classification rates, the other body fluids tested were resistant to degradation and showed stable detection within a donor. This assay could prove to be a reliable, time efficient method for identifying any forensically relevant body fluid.

Keywords: body fluid identification, microRNA, miRNA stability, forensic science

Introduction:

DNA evidence is a valuable forensic tool that can place persons involved in a crime at the scene or tie them to evidence [1,2]. However, body fluid identification is still important for corroborating testimony and lending additional information about the specific events of an alleged crime, especially in violent crimes, such as homicide or sexual assault [1,2]. However, the enzymatic based serological tests currently used in forensic laboratories are mostly considered to be presumptive and exhibit limited specificity and sensitivity [2]. For example, Luminol, one of the most sensitive presumptive tests for blood, displays cross reactivity with bleach [2,3]. This is especially problematic since bleach is a common cleaning chemical in crime scenes [3]. Additionally, serological tests are only able to detect one body fluid at a time and can negatively affect downstream processes such as PCR, which can contribute to unnecessary consumption of sample [1]. An alternate light source (ALS) can be used to test for multiple body fluids at once, however, this approach cannot be used to distinguish between the fluids that fluoresce, which is comprised of all body fluids with the exception of blood [4]. Furthermore, there are several body fluids which do not have confirmatory tests or reliable presumptive tests available at all, such as perspiration, vaginal fluid, and menstrual blood [1].

These limitations indicate a need for more accurate body fluid identification assays which can accurately identify all forensically relevant body fluids and do not contribute to unnecessary sample consumption. Several molecular and spectroscopic based body fluid identification methods have been investigated, including assays utilizing mass spectrometry, Raman spectroscopy, Fourier-Transform Infrared Spectroscopy (FT-IR), microbial DNA, DNA methylation, messenger RNA (mRNA), and microRNA (miRNA). Mass spectrometry is a promising technique that can identify the presence of any body fluid in one test by measuring the

specific mass-to-charge ratio of the peptides that make up proteins found in the different body fluids [5]. This technique exhibits high sensitivity across all forensically relevant body fluids; however, this can be problematic when analyzing evidence samples, especially in cases of sexual assault [5]. For example, prostate specific antigen (PSA), a highly abundant protein in semen, can be detected using this technique in the urine of adult males, and women taking oral contraceptives, which could result in false positives for semen [5,6]. Further, mass spectrometry also requires a potentially time-consuming protein extraction and expensive instrumentation which is not typically found in forensic DNA laboratories, making the implementation of this technique difficult [5,7].

Alternatively, Raman spectroscopy and FT-IR are both non-destructive confirmatory assays that test for all body fluids simultaneously [4,8-11]. Raman spectroscopy utilizes a laser to scan the surface of a sample and returns a spectral "fingerprint" for each component that is based on the distinct pattern of Raman light scattered from each molecule [4,8,9]. Raman spectroscopy can also accurately identify body fluids contaminated with sand, dust, and soil, which would make the identification of samples from outdoor crime scenes much more reliable [12]. The biggest limitation of this technique is that it has been shown to misclassify substances which exhibit autofluorescence, such as semen [13,14]. This could be problematic since semen is a forensically relevant body fluid, especially in sexual assault cases. FT-IR is very similar to Raman spectroscopy except that it uses radiation to measure the stretching and bending of the bonds between atoms rather than measuring patterns of scattered light [9,11]. Similar to the other spectral techniques discussed, FT-IR results in a unique pattern, which must then be compared to a library of known samples for identification [4,5,8,9]. However, FT-IR also necessitates

expensive new instrumentation, since this is not a common technique used in forensic biology laboratories [9].

More recently, molecular assays for body fluid identification have been proposed, as they are often more sensitive and specific than current serological methods. For example, microbial DNA analysis uses the microbiota found in different areas of the body to identify forensically relevant body fluids [15-17]. This approach tests for the presence of specific bacterial ribosomal DNA sequences, which are differentially present in specific body fluids [16,17]. Similar to the tests previously discussed, microbial DNA assays can test for multiple body fluids at once, but there are some limitations [16]. While the vagina, mouth, nose, skin, and stool have the most consistent microbiomes between individuals, microbiomes are not static within a person as they can change based on disease status, weight, and body area. This could be problematic for creating assays which are applicable to a variety of unknown individuals [16]. Finally, the most significant hurdle for body fluid identification using microbial DNA is that this method has mostly been successful in body fluids that contain high levels of bacteria, such as saliva and vaginal fluid. This method has not been as successful in more sterile body fluids such as blood, semen, and urine [15,16].

Methods analyzing DNA methylation patterns have also been developed and are based on tissue-specific differentially methylated regions (tDMRs). tDMRs are regions of chromosome that are methylated differently depending on the tissue [15,16,18]. Methylation of these areas occurs at different levels for each tissue and helps to regulate gene expression [18]. Unfortunately, these methylation levels have been shown to vary between individuals and to be affected by both age and environment [16]. Furthermore, the method requires double-stranded high molecular weight DNA, which is not ideal for forensic analysis since many evidentiary

samples exhibit some level of DNA degradation due to environmental factors commonly found at a crime scene [16,18].

Another molecular method uses mRNA analysis to evaluate the abundance of tissue-specific mRNA markers in biological fluids using qPCR and has been extensively researched in the forensic community as a molecular serological assay [16,19]. Although no such method has been implemented in the United States, there are currently protocols in use for casework in Europe, New Zealand, and Australia [16,19]. Unfortunately, this method consumes more evidence sample than the previous techniques discussed above by requiring an additional RNA extraction step. Due to this limitation, there may not be enough evidence sample available to perform this assay. mRNA also has variable marker expression within individuals, especially in menstrual blood and vaginal fluid, which can be forensically important in certain cases [16,19]. Additionally, due to its relatively large size, mRNAs can begin to degrade just seven days after deposition, which is not ideal for accurate body fluid identification in forensic casework [19,20].

Because of the limitations associated with using mRNAs for body fluid identification, assays which utilize microRNAs (miRNAs) have been developed. MicroRNAs are small, non-coding RNAs that range from 21 to 24 nucleotides long. Because of their size, they are less susceptible to degradation as compared to longer mRNAs [20,21]. MicroRNAs are primarily found in the cytoplasm and are either bound in a RISC protein complex or encapsulated in a lipid exosome, which further contributes to their robustness [21,22]. MicroRNAs are highly conserved across species, are more abundant than mRNAs, and are protected from RNases *in vivo* [16,21,22,23]. Their primary function *in-vivo* is the regulation of protein expression by either selectively suppressing the translation of certain mRNAs or tagging them for degradation [21,22]. If the miRNA is exactly complementary to a specific region on the mRNA, then the

strand will be degraded, alternatively, if a miRNA is only partially complementary, it will prevent the translation of that mRNA [22]. Similar to mRNA, many miRNAs are differentially expressed in body fluids, and the expression levels of these diagnostic markers can be evaluated to identify forensically relevant body fluids [16,21,20,24]. MicroRNAs can be co-extracted with DNA using several commonly used DNA extraction methods without the need for an extra DNase treatment step, which reduces time and sample consumption [24-26].

The Seashols-Williams laboratory at Virginia Commonwealth University has created and optimized a miRNA panel capable of identifying forensically relevant body fluids [24-27]. Initially, high-throughput sequencing was used to identify a panel of differentially expressed diagnostic candidates (miR-200b, miR-1246, miR-320c, miR-10b, miR-26b, and miR-891a) and two ubiquitously expressed normalization markers (let-7g and let-7i). The expression levels of these markers were validated using blood, semen, saliva, urine, vaginal fluid, menstrual blood, and perspiration [24,27]. The normalized expression levels of these markers were used to predict the presence of body fluids [25]. The ability of this panel to identify body fluids was evaluated by inputting the differential expression values into a ten-fold cross validated quadratic discriminate analysis (QDA) model, which was created using the statistical software R version 4.0.2 (R Foundation for Statistical Computing, Vienna, Austria). The full miRNA model correctly classified 77.9% of blood, menstrual blood, feces, urine, saliva, semen, and vaginal fluid samples; however, this correct classification rate increased to 93.3% after removing two of the body fluid specific markers, miR-26b and miR-1246 [25,27]. Following these results, the expression levels were investigated within a single donor over a biological cycle in RNA extracts. The correct classification rates observed for blood, feces, urine, and vaginal fluid were

comparable to that of the population studies mentioned above [25,27]. In contrast, the classification rates of saliva, semen, and menstrual blood were lower [25,27].

To create a more easily implemented assay for forensic DNA laboratories, the Williams-Seashols laboratory assessed the ability of the model to identify body fluids from DNA extracts, which resulted in an overall correct classification rate of 88.0% in the body fluids tested above. Three more markers were added to the panel (miR-412, miR-141, and miR-205) to increase the correct classification rate of the model, specifically for saliva and vaginal fluid. After these changes were made, the correct classification rate for saliva increased from 66% to 86% and in vaginal fluid, increased from 66% to 74%. The model achieved an overall correct classification rate of 91.4% [25,27] (Table 1). The markers in the final optimized panel were miR-200b, miR-320c, miR-10b, miR-412, miR-141, miR-205, miR-891a, and two normalization markers, let-7g and let-7i [27].

The blood target used in this panel was miR-200b. The saliva specific marker used in this panel, miR-205, has been posited to indicate the presence of epithelial cells [34]. Epithelial cells can be present in saliva samples, especially those collected by buccal swabs, and they can also be present in skin, vaginal fluid, and menstrual blood samples [34]. This marker has also been shown to be stimulated differently due to the gustatory response and changes in metabolism in response to food [34]. MircoRNA-10b was the urine specific target in this panel, and the feces marker was miR-320c. The semen specific marker was miR-891a, and miR-141 and miR-412 were the two menstrual blood markers in this panel.

Most miRNA stability assays thus far have focused on blood samples, presumably because blood has been the most consistently classified body fluid using miRNAs thus far [24,27-31]. The stability of miRNA in both aged and treated samples has been investigated by

several groups, although this area of study is relatively new and requires much more investigation before being applied to real casework samples [28-31]. Sauer et al. investigated the detectability of miRNAs in venous blood, saliva, menstrual blood, vaginal fluid, and semen which were aged at room temperature for varying time periods up to 36 years [28]. They found that all markers tested in these samples were still detectable at similar levels compared to non-aged samples [28].

The detection of miRNAs has also been studied in forensically relevant body fluids in several studies [29-31]. Blood has been found to be stable under both dry and humid environmental conditions over time [29]. Fang et al tested the stability of dried bloodstains under several different conditions, including treatment with heat or sodium hypochlorite, and the effect of several freeze/thaw cycles on liquid blood [30]. They found that the expression of miRNA was stable after storage at an elevated temperature, but both treatment with sodium hypochlorite and freeze/thaw cycles consistently decreased the detection of blood specific miRNAs [30]. Mayes et al. studied the effects of environmental treatment and laundering on both blood and semen and demonstrated that although body fluid specific markers could still be detected after washing and drying, their normalized values were different compared to untreated controls [31]. However, more thorough investigation is needed as a limited number of samples was used and several of these studies were performed on liquid body fluids, which is not what would typically by found in casework [31].

The Seashols-Williams laboratory also tested the stability of miRNA from RNA extracts in blood, saliva, urine, and semen after compromising treatments [26]. The treatments used in this study were heat, various chemical treatments, Ultraviolet (UV) light, or environmental treatment [26]. They found that none of the body fluids tested showed decreased let-7g

detectability when treated with UV light or glacial acetic acid, however, the detection of let-7g in blood, semen, and saliva decreased after treatment with a common detergent, which is an easily accessible chemical for crime scene cleanup. Saliva, blood, and semen showed similar decreases in detectability after full strength bleach treatment, and saliva detectability was also negatively affected by treatment with a 1:10 bleach dilution. In this study, body fluids were also treated at multiple timepoints with either 55°C or 95°C up to 24 hours. There was no significant reduction in detectability in let-7g in any body fluid tested, except at the 2-hour timepoint for blood at both temperatures and the 4 and 24-hour timepoints for semen at 95°C. No significant decreases in detectability in miR-200b, miR-891a, miR-10b, miR-26b, let-7g, or let-7i were observed in an of the body fluids tested after exposure in the environmental chamber, which mimicked the typical Virginia summer day [26].

Prior to implementation in a crime laboratory environment, it is essential to investigate the impact of possibly degrading treatments, as well as any variation in expression of the miRNA markers within an individual. It is not uncommon for crime scene samples to be partially degraded, due to either environmental factors or attempted crime scene cleanup. Sample degradation could potentially affect the ability of our model to correctly classify body fluids. Evidentiary samples can also come any time in an individual's biological cycle, i.e., a menstrual blood sample could have originated during any day of the menstrual cycle. Since variable marker expression within individuals has been shown to affect the ability of mRNA assays to identify body fluids, this is a concern which needs to be addressed in miRNAs [16,19]. These studies are necessary to test whether this assay is applicable on real crime scene samples and on a variety of unknown individuals. The overall goal of this project was to assess whether any degradation caused by these treatments or possible marker variation within individuals would affect the

ability of the QDA model to correctly classify the body fluids tested. The correct classification rate was calculated by dividing the number of samples in a set that were correctly classified with at least a 50% confidence divided by the total number of samples tested. The desired correct classification rate is minimally 80%, ideally 90% for implementation into the forensic workflow.

Materials & Methods

Sample Collection & Deposition

All samples were collected from donors of varying ethnicity, gender, and age through an approved Institutional Review Board Human Subjects Research Protocol (VCU- HM2000293) (Table 2).

Samples were collected over various biological times (Table 3) to evaluate variation of miRNA expression within donors. Blood, menstrual blood, vaginal fluid, feces, saliva, urine, and semen from three different donors were used in this study (Table 3). All samples were collected or deposited onto sterile cotton swabs. Blood was collected via finger prick with a Unistick® 3 Normal lancet (Owen Mumford Ltd., Woodstock, UK). Saliva was collected by rotating a swab on the inside of the donor's check. Vaginal fluid and menstrual blood were collected by inserting a swab two to three inches into the vagina and rotating along the vaginal wall for full coverage. Feces were collected onto a cotton swab while the donor was defecating. Urine and semen were collected in a sterile collection cup and stored at -20°C before deposition of 100 µL (urine) or 50 µL (semen) onto the swab. Prepared swabs were stored at -20°C until DNA isolation.

Heat, Chemical, & UV stability

Blood, semen, saliva, and urine from 3 donors per body fluid were used in this study. Blood was collected into a Vacutainer® containing EDTA (Beckton, Dickinson & Company, Franklin Lakes, NJ, USA) and inverted for 15 seconds before depositing 50 µL onto a sterile

cotton swab. Urine, semen, and saliva were collected into a sterile collection cup, and 50 μL (semen, saliva) or 100 μL (urine) was deposited onto sterile cotton swabs. The swabs were dried at room temperature for 42 hours and then stored at -20°C until treatment, which was performed within 72 hours of drying. Heat treated samples were exposed to either 55°C or 95°C for 0.5, 1, 2, 4, or 24 hours. For the chemically treated samples, 100 μL of either 1:10 (87 mM) or full-strength (870 mM) sodium hypochlorite, dish soap (Dawn Ultra Dishwashing Liquid, Proctor & Gamble, Cincinnati, OH, USA), or glacial acetic acid (GAA) (pH 2.5, 17.4 M) were deposited onto the prepared swabs and dried for 72 hours. UV light treated samples were exposed to 4 hours of 302 nm light using the UVP high-performance ultra-violet transilluminator (UVP, Upland, CA, USA). After treatment, all swabs were stored at -20°C until DNA isolation.

Environmental Chamber Stability

This assay utilized previously prepared samples of dried blood, semen, saliva, and urine which had been deposited onto a cotton swatch from Layne et al. using a single donor for each body fluid. These samples were stored at -80°C for seven years, prior to use in this study. Samples were exposed to treatment in a Q-sun Xe-3 Environmental Chamber (Q-Lab Corporation, Westlake, OH, USA) at the Federal Bureau of Investigation Research Laboratory. The Environmental chamber controlled for temperature, humidity, and a 24-hour light/dark cycle to imitate a summer day in Virginia (Table 4). The samples were removed from the chamber at 48-hour intervals up to 14 days and stored at -80°C until punches were taken [26]. Using a biopsy punch, 4 mm punches were taken from the remaining cotton swatches and stored at -80°C until DNA isolation.

DNA Isolation

MicroRNA taken from DNA extracts were used in this study. MicroRNA and DNA was isolated from whole swabs using the QIAgen DNA Investigator Kit® on the QIAcube (Qiagen, Valencia, CA, USA) and the manufacturer's protocol for forensic casework samples as previously described in [25]. Final elution volumes were as follows: 30 µL for saliva, blood, menstrual blood, semen, and vaginal fluid, 50 µL for feces, and 20 µL for urine.

Reverse Transcription Quantitative PCR

Reverse transcription was performed on the samples using the Proflex PCR System (Thermo Fisher Scientific, Waltham, MA, USA) and the qScript miRNA cDNA Synthesis Kit (QuantaBiosciences, Gaithersburg, MD, USA) following a protocol previously described by the Seashols-Williams laboratory [25,26]. The miRNA assay primers used for all targets were purchased from Integrated DNA Technologies (IDT, Coralville, IA, USA) (Table 5). Quantitative PCR was performed using the QuantStudio™ 6 Flex Real-Time PCR System (Life Technologies, Carlsbad, CA, USA) and the qScript MicroRNA quantitation system (QuantaBiosciences). All reagent blanks were tested, and a no-template control was included in each qPCR plate. The reverse transcription reaction and singleplex quantitative PCR followed a protocol previously described by Lewis et al [25].

Data Analysis

Raw data was analyzed at a Cq threshold of 0.015 in the QuantStudio™ Real-Time PCR v1.3 (Thermo Fisher Scientific) and exported into Microsoft Excel (Microsoft Corporation, Redmond, WA, USA). The Cq values were normalized by calculating the ΔCq ($\Delta Cq = Cq_{target} - Cq_{(avg\ of\ let\text{-}7g\ and\ let7i)}$). All normalized Cq values, except for the environmental chamber assessment, were tested for significant relationships between marker levels in either untreated controls (heat stability assessment), or compared to other donation timepoints (variation within

donors assessment), using linear regression. No statistical analysis was performed for the environmental chamber or chemical/UV treated samples. JMP® Statistical Software v14.2.0 (SAS Institute, Cary, NC, USA) and an alpha of $\alpha = 0.05$ was used. Calculated differential expression values were analyzed in the previously developed QDA model [33]. A sample was considered correctly classified if the QDA model showed at least a 50% classification confidence.

Results & Discussion

Heat Treatment

The robustness of miRNA markers in blood, semen, saliva, and urine was tested over a period of 24 hours at a temperature exposure of either 55°C or 95°C. MicroRNA levels in blood proved to be highly resistant to degradation over all heat treatments and the ΔCq values were consistent throughout all treatments, and there did not appear to be different trends between 55°C or 95°C treatment (Fig. 1). The data also showed no significant relationship between the normalized ΔCq values and treatment time among markers at either temperature when compared to untreated controls (Fig. 1). These findings are similar to those of Fang et al., which also demonstrated the robustness of miRNA markers in blood at elevated heat conditions [30]. Our observations are also consistent with the findings from Layne et al., who performed a similar stability study but with RNA extracts and other studies which have shown the robustness of blood under heat treatment [26,31]. The overall correct classification rate in the blood samples was 97% (Table 6).

In contrast, semen, saliva, and urine proved to be affected by treatment. Although there was no significant relationship between ΔCq values and treatment time in semen samples, there was a slight downwards trend after 4 hours of treatment at 95°C (Fig.2). The correct

classification rate in these samples was 57.6%, which is lower than the previous population data, which was 90.0% (Table 6). The ΔCq values across treatment times appeared to be stable at both 55°C and 95°C in saliva samples, and there was no significant relationship found between treatment time and ΔCq value (Fig. 3). Surprisingly, saliva showed the lowest overall classification rate of all the heat-treated samples at 48.5% (Table 6). This suggests that the saliva marker, miR-205 may not be accurate enough to identify saliva at elevated temperature. The ΔCq values for urine samples treated with 55°C heat appeared to be similar at all timepoints compared to the untreated controls (Fig. 4*a*), while the samples exposed to 95°C heat showed a downwards trend in ΔCq values in miR-200b, miR-891a, miR-412 and miR-205 (Fig. 4*b*). There was a significant relationship found between miR-200b ΔCq values and treatment time at 95°C ($p=0.0433$, $r^2=0.2315$). Despite this, the overall correct classification rate of the urine heat-treated samples were 84.8%, which is comparable to the classification rate in the previous population data, which was 80.4% (Table 6).

Chemical & UV Treatment

The detectability of the tested miRNA markers after chemical or UV treatment seemed to be dependent on both the treatment and the body fluid, since different fluids were more susceptible to degradation from different treatments (Fig. 5-8). Blood appeared to be most affected by treatment with dish soap, GAA, 1:10 bleach, and full-strength bleach treatment in all markers except miR-200b, while the detection of miR-200b was most affected by only dish soap and full-strength bleach (Fig 5). The detection of the markers tested were not affected by UV treatment (Fig 5). Similar to the heat-treated samples and findings from Layne et al., chemical or UV treatment did not affect the correct classification rate of blood samples (Table 5) [26].

Semen was most greatly impacted by the application of dish soap, 1:10 bleach dilution, or full-strength bleach (Fig. 6). Markers miR200b, miR10b, and miR205 appeared to be the most affected by these treatments compared to the other markers tested (Fig 6). These differences are reflected in the low correct classification rate of 66.7%, which is lower than the previous population rate of 90.0% (Table 7). The low classification rate is supported by findings from Mayes et al., which found differing ΔCq values after laundering with a detergent [31].

Saliva showed greater sensitivity to UV and GAA treatment in miR200b (Fig. 7). Saliva also showed degradation after full strength bleach treatment in all markers except for miR200b, including the two endogenous reference genes (let-7g and let-7i) (Fig. 7). Due to these differences, saliva exhibited a low overall classification rate of 33.3%, which is the lowest of all the chemical/UV treated samples and indicates that miR-205 may not be sufficient to identify saliva (Table 7).

Finally, urine samples treated with chemical or UV treatments appeared to be the least affected in most of its markers compared to the other body fluids tested (Fig. 8). Both miR141 and let-7i were impacted by GAA and 1:10 bleach treatment, but not full-strength bleach treatment (Fig. 8). These results differ from the preliminary results in Layne et al., which found that urine was not significantly affected by these treatments [26]. The overall correct classification rate of this set of urine samples was 77.8%, which is similar to the previous population classification rate of 80.4% (Table 7).

Overall, these findings were concordant with Mayes et al., in which it was observed that miRNA abundances within degraded samples can be disproportionally affected by compromising treatments [31].

Environmental Chamber Stability

Exposure to controlled heat, light/dark cycle, and humidity appeared to have the least effect on marker expression levels in both blood and urine, compared to semen and saliva, which reflects the stability of these two body fluids seen in other compromising conditions (Fig. 9-12) [26,30,31]. There was a slight downward trend in the ΔCq values in all markers in the blood samples (Fig 9). This did not affect the correct classification rate in blood samples, since it was 100%, which is comparable to the previous population rate, 95.0% (Table 8).

Unlike the blood samples, urine did not appear to be affected by environmental chamber treatment, and showed no downward trend in ΔCq values in any markers across any treatment times (Fig. 10). The correct classification rate in these samples was 87.5%, which is consistent with the data, and comparable to the previous population rate of 80.4% (Table 8)

Semen samples exposed to treatment in the environmental chamber showed a downward trend in the detection of miR-200b and miR-412 across treatment times, although the semen specific marker, miR-891a, appeared to be unaffected (Fig. 11). It is important to note that the ΔCq value for the semen specific marker, miR-891a, in the untreated control is out of the normal range for semen samples in DNA extracts according to previous unpublished work completed by the Seashols-Williams laboratory (Fig. 11). This difference could explain the 0.0% correct classification rate of these samples, and it also means that this data is inconclusive, since there is no reliable control to compare the treated samples to (Table 8).

Saliva showed no distinct trend in detection of the tested markers, but the saliva specific miR-205 marker, as well as miR-200b, and miR-412 was out of the normal ΔCq range established by previous unpublished work by the Seashols-Williams laboratory, Valentine et al., and what is seen in the untreated controls for the other sample sets in this study (Fig. 12) [27]. These differences explain the 0.0% correct classification value seen with the saliva samples in

this assay (Table 8). Similar to the semen samples, the data for the saliva samples is also inconclusive since there is no reliable untreated control to compare the treated samples to. The most likely explanation for the state of these samples is that they are seven years old, and have gone through several freeze/thaw cycles, which, according to Fang et al., has been shown to affect the detection of miRNA in liquid blood samples [30].

The compromised samples in this study only tested blood, semen, saliva, and urine (Table 9). While these are all forensically important body fluids, other fluids, such as menstrual blood, vaginal fluid, and feces, should be tested in the future under similar conditions in order to understand their limitations.

Variation within Donors

The expression levels of the miRNA markers in each body fluid were measured to observe whether there was a change over a biological cycle or period of time. Blood did not show any difference in the expression of the markers tested (Fig. 13). All of the blood samples in this set were correctly classified by the QDA model, which is similar to the rate seen in the population data, which was 98.0% (Table 10).

There did not appear to be any change in the ΔCq values of the markers tested in the menstrual blood samples (Fig. 14). Surprisingly, none of the menstrual blood samples were correctly classified, which is much lower than the previous population rate of 66.0% (Table 10). Instead, they were mostly classified as vaginal fluid, which has historically been a common issue with menstrual blood identification since both menstrual blood and vaginal fluid pass through the vagina [16,19].

Feces also did not show any change in the detection of the miRNA markers tested across the donation period (Fig. 15). Despite this, the correct classification rate of these samples was

77.8%, which is lower than the population rate which was 98.0% (Table 10). The data suggests that although there was no significant relationship found between donation day and marker expression in the feces samples, there was still enough variation to affect the ability of the model to correctly classify the sample.

Saliva showed no overall trend in the expression of markers across the sampling period, but there was some non-significant variation seen between samples taken upon waking, before eating, and after eating (Fig. 16). The correct classification rate of the saliva samples in this set was 7.4%, which is much lower than the previous population rate of 86.0% (Table 10). The low classification rate could be caused by differences in metabolism and stimulation of different salivary glands prior to and after eating a meal [32].

The expression of the markers tested in the urine samples did not change much throughout the donation period, however, the variation between individuals was much lower than the other body fluids tested (Fig. 17). There was a significant relationship found between donation day and the expression of miR891a ($p=0.0315$, $r^2=0.2578$), miR141 ($p=0.0247$ $r^2=0.2774$), and miR412 ($p=0.0189$, $r^2=0.2988$). These relationships were not enough to affect the correct classification rate of the urine samples, which was 94.4%, which is higher than the previous population rate, 80.4% (Table 10).

Semen showed some variation in marker expression, however, none of these differences were statistically significant (Fig. 18). The ability of the model to classify these samples still appeared to be affected by this variation since they were only correctly classified 77.8% of the time, which was lower than the previous population study rate, which was 90.0% (Table 10).

Vaginal fluid was the longest biological cycle tested and did not show any differences in expression over the cycle (Fig. 19). There was some non-significant variability between the

donors tested, which was also seen in the previous population study, which was performed in an unpublished study in the Seashols-Williams laboratory. This variation does not seem to have contributed to the classification rate of 76.9%, which was comparable to the rate seen in the population study, 74% (Table 10).

Conclusions

This study investigated the stability and variation within donors of an optimized panel of miRNA for body fluid identification, as well as their ability to be correctly classified by a QDA model. Heat treatments seemed to have a greater impact on the miRNA expression levels of semen and saliva compared to blood and urine. This trend continued with the chemical and UV treatments, however, this decrease in detectability proved to be treatment, body fluid, and marker dependent. Overall, the correct classification rates for the blood and urine samples were similar to those of the previous population study performed by the Seashols-Williams laboratory, unlike semen and saliva, which were much lower. While the data from the environmental chamber assay was inconclusive due to the unreliability of the control samples, the blood and urine samples still showed similar correct classification rates to the previous population study even after being stored at -80°C for seven years and undergoing several freeze/thaw cycles (Table 8). The effect of both time and multiple freeze/thaw cycles must be further investigated before this or a similar assay is implemented into casework. Marker expression across a biological cycle of single individuals appeared to affect the correct classification rate in saliva, semen, feces, and menstrual blood more than the other body fluids tested (Table 10). However, all body fluids have lower rates than in the previous population study except for vaginal fluid, urine ,and blood.

In conclusion, the expression of the miRNA markers was still detectable and demonstrated body fluid specificity in DNA extracts in blood and urine after compromising

treatments, however, all other body fluids exhibited concerning variability of expression within donors and decreased detectability after compromising treatments. In future studies, additional miRNA markers could be added to the panel to increase the correct classification rate of these body fluids, specifically saliva, semen, vaginal fluid, and menstrual blood. It may also be beneficial to explore adding non-miRNA markers to the panel. The Seashols-Williams laboratory has begun adding microbial DNA markers, which may be more accurate than miRNA markers alone. This is because microbial DNA assays show promising results with vaginal fluid and saliva, two body fluids which show low correct classification rates with only miRNA markers [15]. As more markers are added to the panel, targeted high-throughput sequencing may be considered instead of qPCR, since it would allow this assay to be performed more quickly while simultaneously evaluating many markers of different origins. This could also help to overcome the low classification rates. Overall, this assay shows great promise to become a highly sensitive body fluid identification method in the forensic DNA workflow, assuming that these issues can be overcome.

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Table 1. Correct classification rates for DNA population samples previously tested by the Seashols-Williams laboratory [33].

Body Fluid	n	Correct Classification	Classification as another BF	Classification as Other
Blood	49	98.0%	2.0%	0.0%
Menstrual Blood	50	66.0%	34.0%	0.0%
Feces	50	98.0%	2.0%	0.0%
Saliva	50	86.0%	14.0%	0.0%
Semen	50	90.0%	10.0%	0.0%
Urine	46	80.4%	19.6%	0.0%
Vaginal Fluid	50	74.0%	26.0%	0.0%
Overall	345	91.4%	9.0%	0.0%

Table 2. Sample data including self-reported demographic information of individuals included in the stability and variation within donors assays

	Blood	Menstrual Blood	Feces	Urine	Saliva	Semen	Vaginal Fluid
Individual donors	6	3	3	7	7	7	3
Sex							
Female	5	3	2	5	6	0	3
Male	1	0	1	2	1	7	0
Age group (years)							
<18	1	0	0	0	0	0	0
18-30	4	3	3	7	7	6	3
31-50	1	0	0	0	0	1	0
>50	0	0	0	0	0	0	0
Unreported	0	0	0	0	0	0	0
Ethnicity							
Caucasian	6	3	3	4	5	5	2
African American	0	0	0	1	2	2	0
Hispanic	0	0	0	1	0	0	1
Asian	0	0	0	1	0	0	0
Admixed	0	0	0	0	0	0	0
Other	0	0	0	0	0	0	0

Table 3. Sampling method for donors included in the assay addressing the variation of marker expression within donors over a biological cycle or period of time [33].[1]

Body Fluid	# of Donors	Samples Collected
Blood	3	3 donations within a 7-day period
Menstrual Secretions	3	5-7-day donations*
Feces	3	3 donations within a 7-day period
Urine	3	6 donations over a 3-day period: upon waking and afternoon
Saliva	3	3 donations/day for 3 days: upon waking, before eating, after eating
Semen	3	3 donations within a 30-day period
Vaginal Secretions	3	>21-day donations*

[1] Exact number of donations is dependent on donor's menstruation cycle

Table 4. Environmental Chamber Parameters on the Q-sun Xe-3 Environmental Chamber as according to Layne et al. [26].[2]

Step	Function	Irradiance (W/m^2)	BP Temp* (°C)	Air Temp (°C)	Humidity (%)	Time (h)
1	Light	0.34	45	35	50	3
2	Light	0.68	52	35	50	4
3	Light	0.34	45	35	50	3
4	Dark	—	—	32	50	14
5	Final Step, or Go to Step 1 (repeat for up to 14 days)					

[2] The black panel temperature (BP Temp) is the Temperature of a sensor on the same level as the samples.

Table 5. Sequences for all miRNA primers used for RT-qPCR.

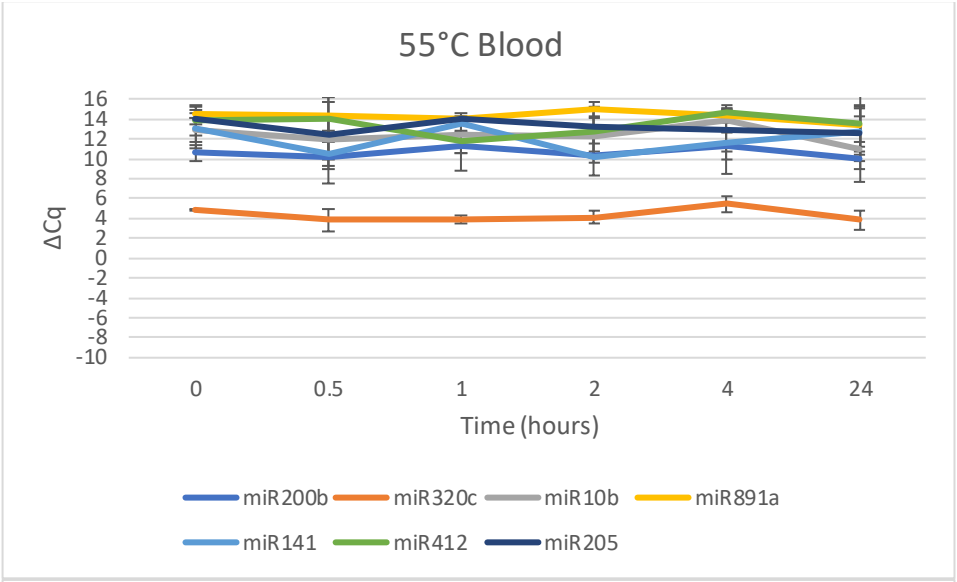
Quanta PN	ID	Accession	Human miRNA Sequence	Primer sequence
HSLET-0007G-5P	hsa-let-7g-5p	MIMAT0000414	UGAGGUAGUAGUUUGUACAGUU	CCGAGCTGAGGTAGTAGTTTGTAC
HSLET-0007I	hsa-let-7i-5p	MIMAT0000415	UGAGGUAGUAGUUUGUGCUGUU	CGTTCTGAGGTAGTAGTTTGTGCT
HSMIR-0010B	hsa-miR-10b-5p	MIMAT0000254	UACCCUGUAGAACCGAAUUUGUG	CGTACCCTGTAGAACCGAATTTGT
HSMIR-0141-5P	hsa-miR-141-5p	MIMAT0004598	CAUCUUCCAGUACAGUGUUGGA	TCCAGTACAGTGTTGGAAAAA
HSMIR-0200B-5P	hsa-miR-200b-5p	MIMAT0004571	CAUCUUACUGGGCAGCAUUGGA	CTTACTGGGCAGCATTGGAA
HSMIR-0205-5P	hsa-miR-205-5p	MIMAT0000266	UCCUUCAUUCCACCGGAGUCUG	TCCTTCATTCCACCGGAGTC
HSMIR-0320C	hsa-miR-320c	MIMAT0005793	AAAAGCUGGGUUGAGAGGGU	AAAGCTGGGTTGAGAGGGT
HSMIR-0412	hsa-miR-412-3p	MIMAT0002170	ACUUCACCUGGUCCACUAGCCGU	CCTGGTCCACTAGCCGTAAA
HSMIR-0891A	hsa-miR-891a-5p	MIMAT0004902	UGCAACGAACCUGAGCCACUGA	CGAACCTGAGCCACTGAAA

Table 6. Correct classification rates for samples treated with either 55°C or 95°C heat compared to previous population classification rates [33].

Body Fluid	n	Correct Classification	Classification as another BF	Classification as Other	Previous Population Classification
Blood	33	97.0%	3.0%	0.0%	98.0%
Semen	33	57.6%	42.4%	0.0%	90.0%
Saliva	33	48.5%	51.5%	0.0%	86.0%
Urine	33	84.8%	6.1%	0.0%	80.4%

Figure 1 (a-b). Average ΔCq values of blood samples that were exposed to 55°C or 95°C for up to 24 hours. n=33

A.



B.

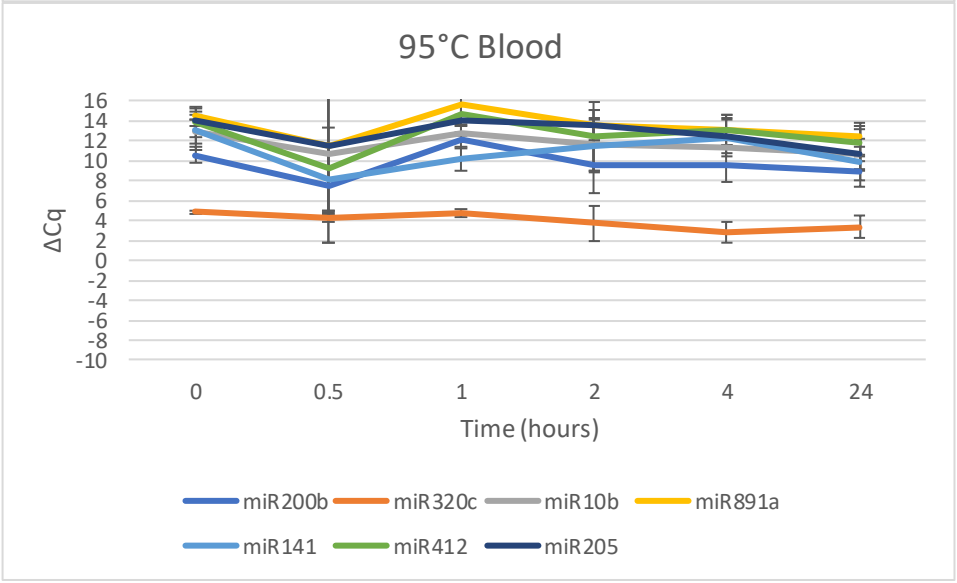
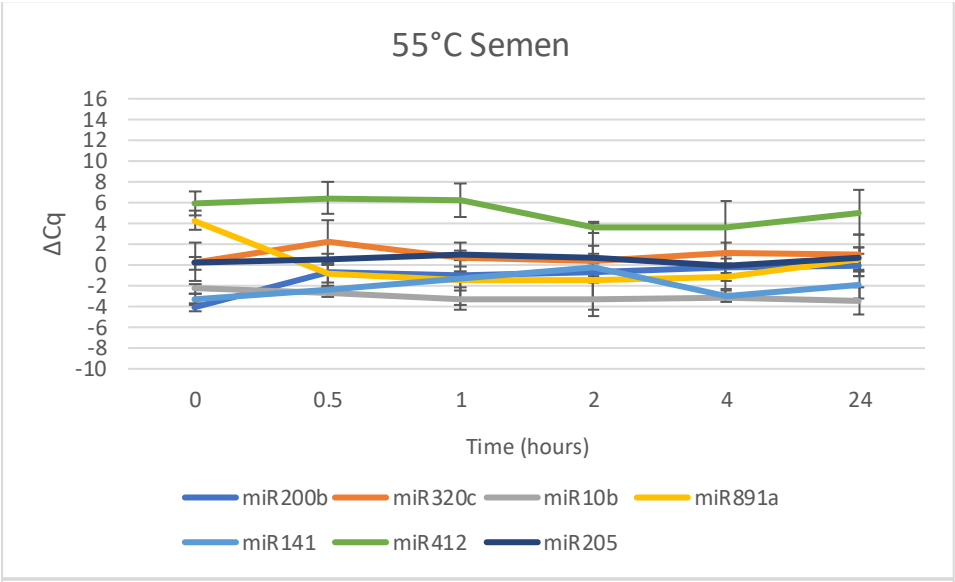


Figure 2 (a-b). Average ΔCq values of semen samples that were exposed to 55°C or 95°C for up to 24 hours. n=33

A.



B.

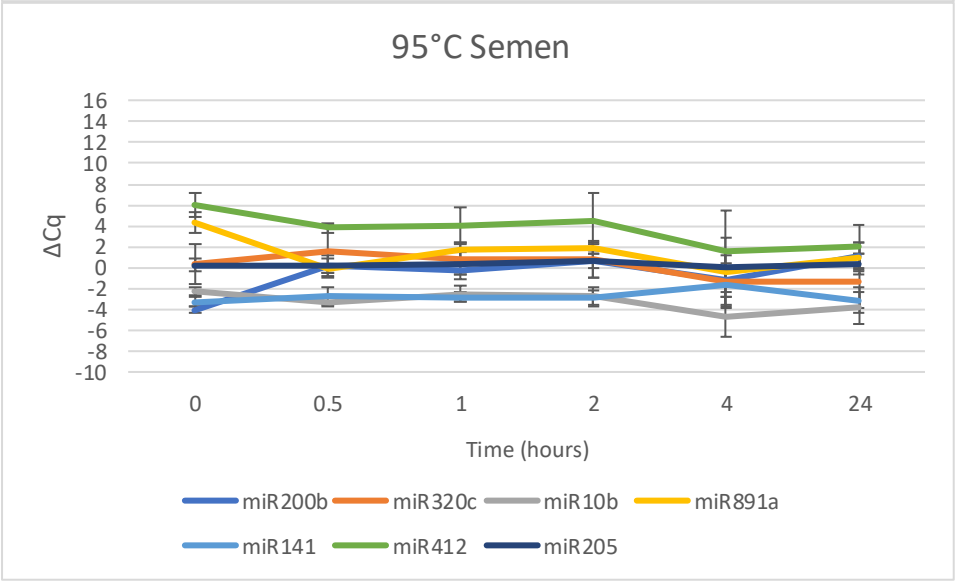
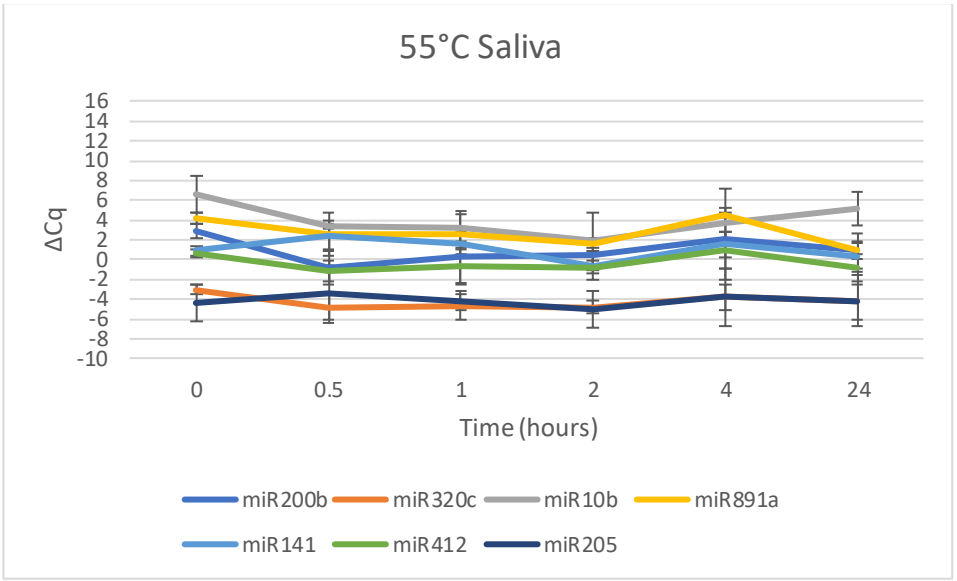


Figure 3 (a-b). Average ΔCq values of saliva samples that were exposed to 55°C or 95°C for up to 24 hours. n=33

A.



B.

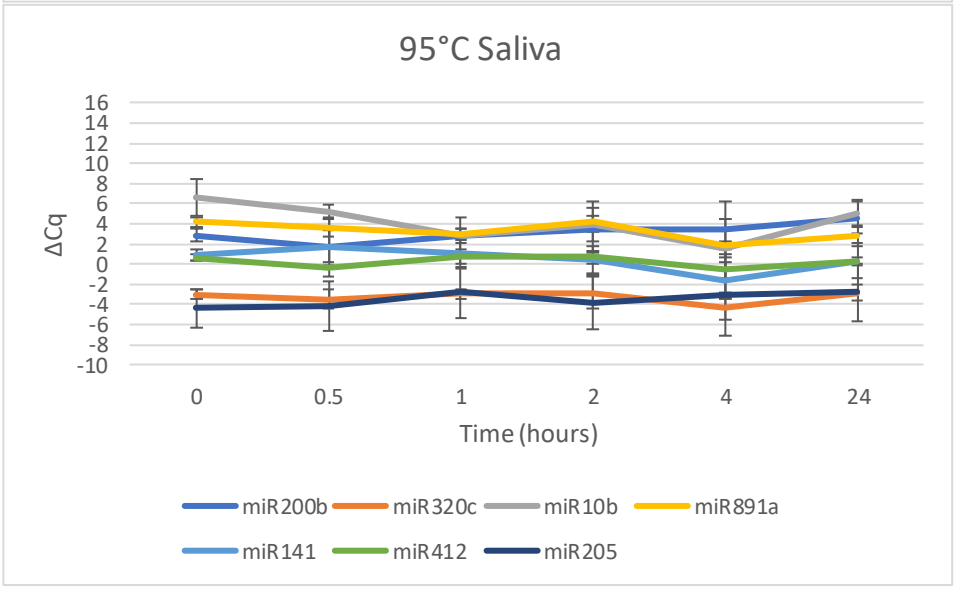
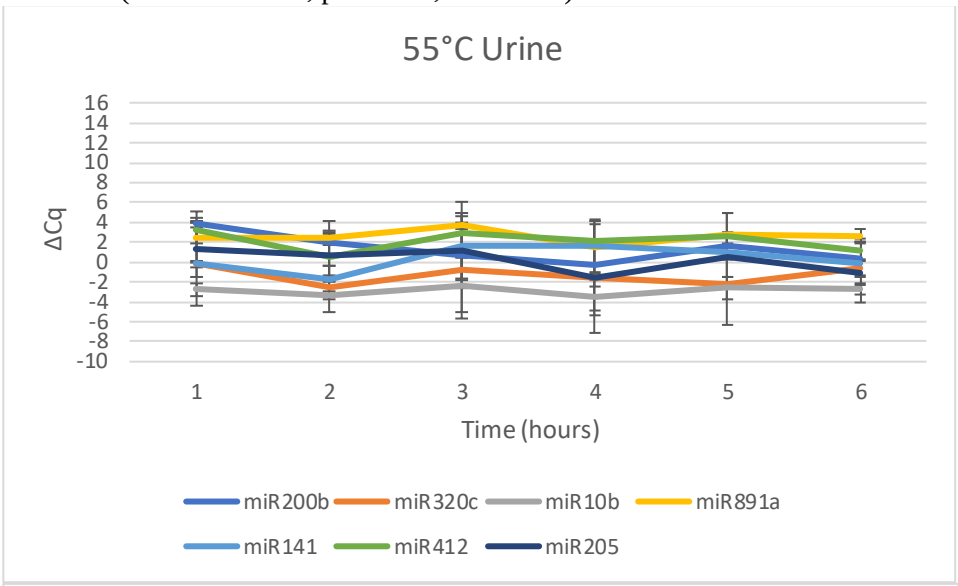


Figure 4 (a-b). Average ΔCq values of urine samples that were exposed to 55°C or 95°C for up to 24 hours. n=33 (miR200b 95°C, p=0.0433, r^2=0.2315)

A.



B.

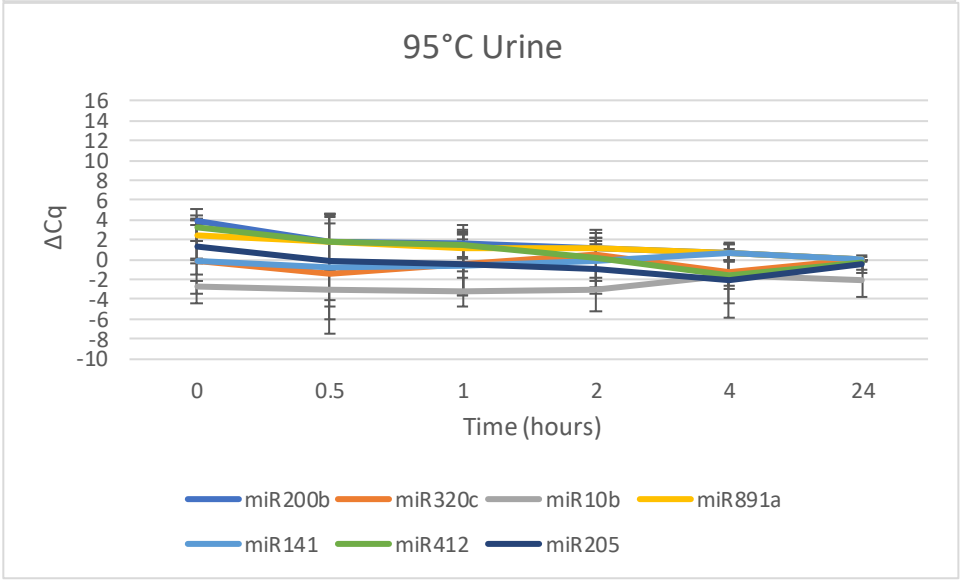


Table 7. Correct classification rates for samples treated with a chemical or UV treatment compared to previous population classification rates [33].

Body Fluid	n	Correct Classification	Classification as another BF	Classification as Other	Previous Population Classification
Blood	18	100.0%	0.0%	0.0%	98.0%
Semen	18	66.7%	33.3%	0.0%	90.0%
Saliva	18	33.3%	61.1%	3.0%	86.0%
Urine	18	77.8%	22.2%	0.0%	80.4%

Figure 5. Average ΔCq values of blood samples treated with either a chemical or UV exposure. n=18

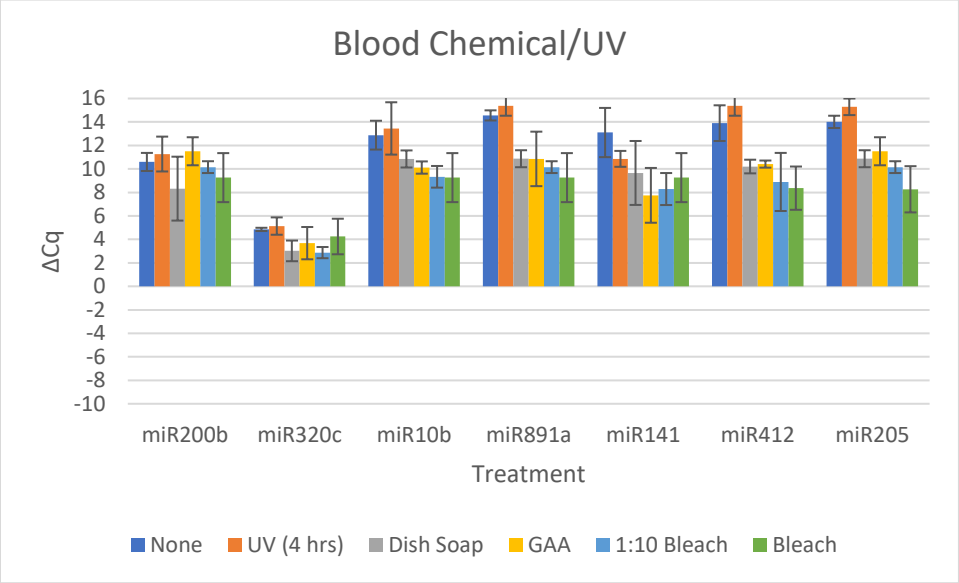


Figure 6. Average ΔCq values of semen samples treated with either a chemical or UV exposure. n=18

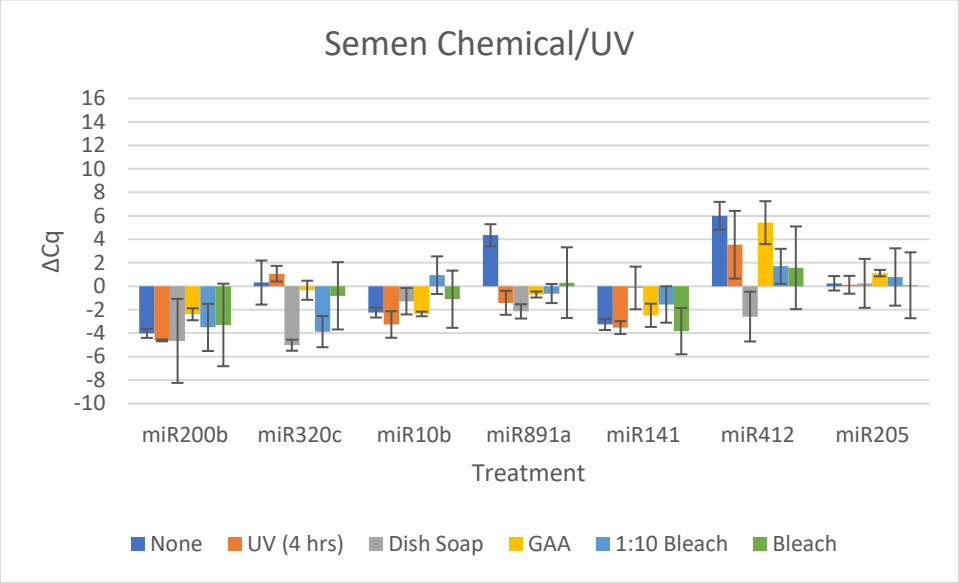


Figure 7. Average ΔCq values of saliva samples treated with either a chemical or UV exposure. n=18

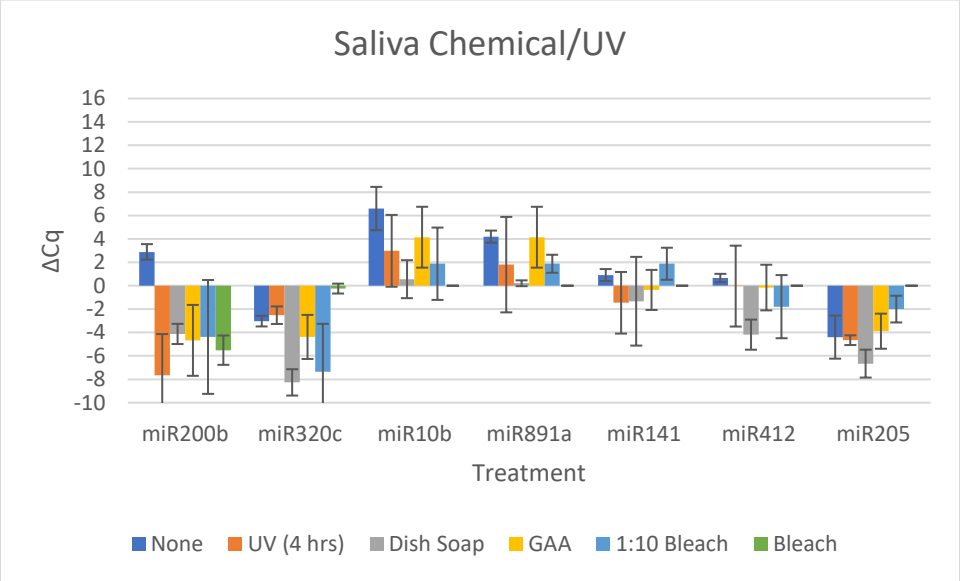


Figure 8. Average ΔCq values of urine samples treated with either a chemical or UV exposure. n=18

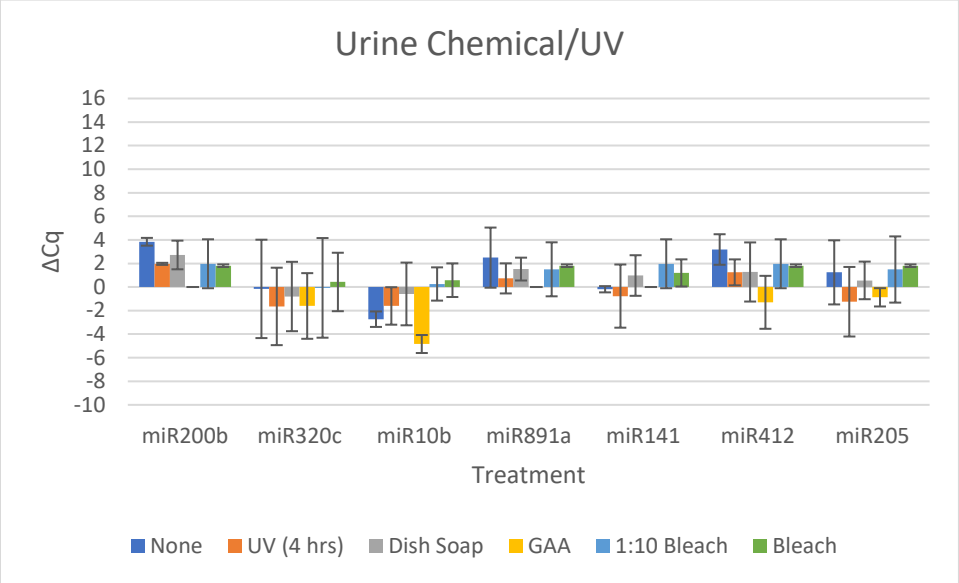


Table 8. Correct classification rates for samples treated with up to 14 days in the Q-sun Xe-3 Environmental Chamber as according to Layne et al. [26] compared to previous population classification rates [33].

Body Fluid	n	Correct Classification	Classification as another BF	Classification as Other	Previous Population Classification
Blood	8	100.0%	0.0%	0.0%	98.0%
Semen	8	0.0%	0.0%	0.0%	90.0%
Saliva	8	0.0%	0.0%	0.0%	86.0%
Urine	8	87.5%	12.5%	0.0%	80.4%

Figure 9. Average ΔCq values of blood samples that were exposed to 0-14 days in a Q-sun Ce-3 Environmental Chamber. n=8

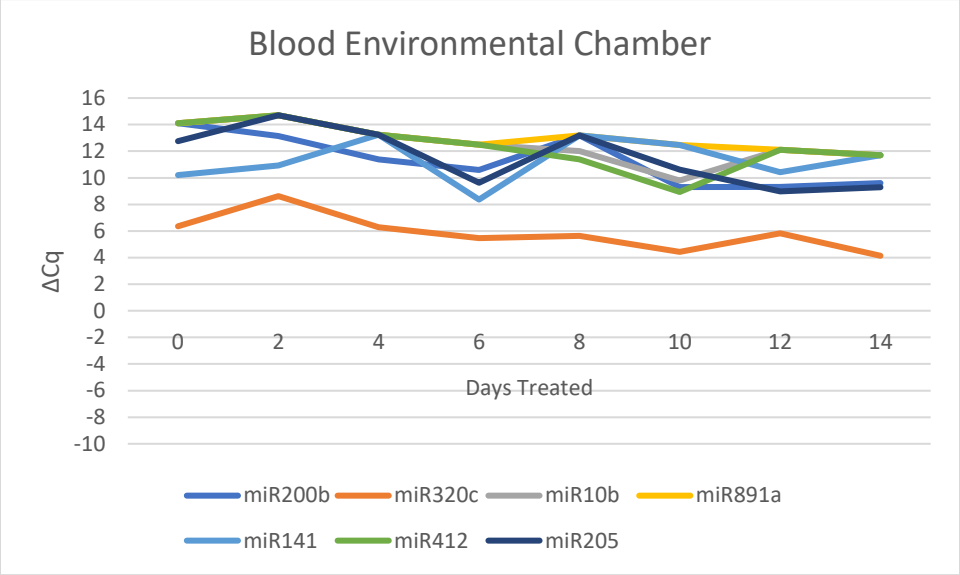


Figure 10. Average ΔCq values of urine samples that were exposed to 0-14 days in a Q-sun Ce-3 Environmental Chamber. n=8

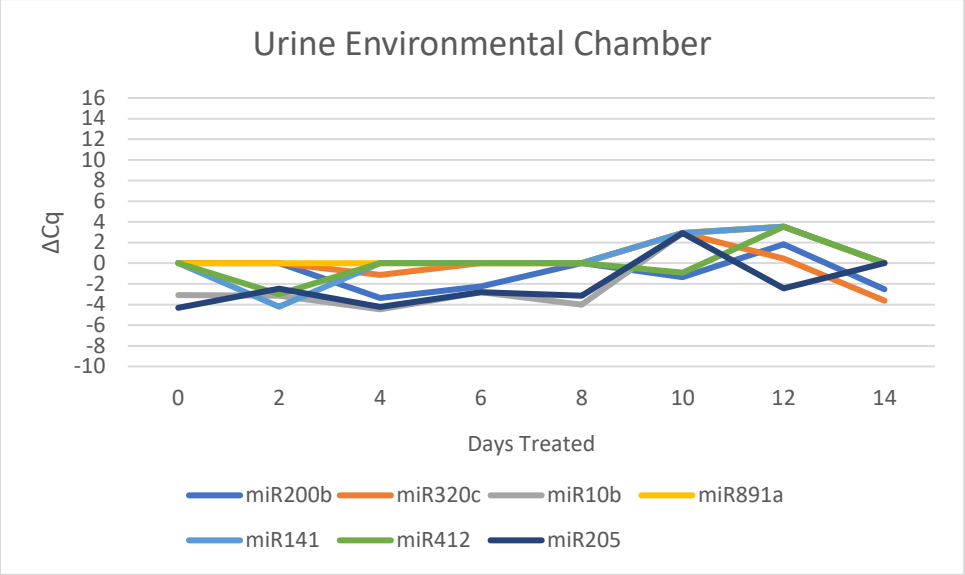


Figure 11. Average ΔCq values of semen samples that were exposed to 0-14 days in a Q-sun Ce-3 Environmental Chamber. n=8

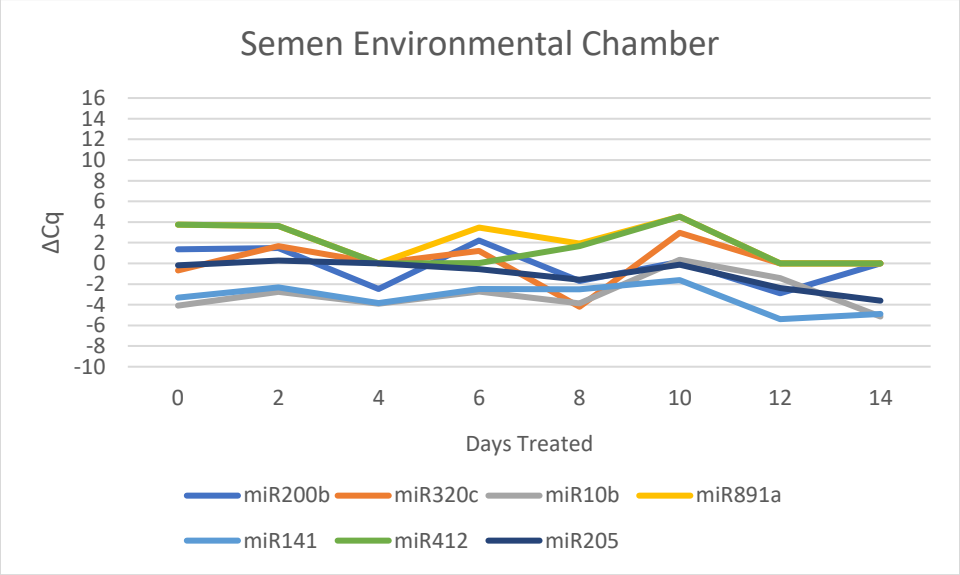


Figure 12. Average ΔCq values of saliva samples that were exposed to 0-14 days in a Q-sun Ce-3 Environmental Chamber. n=8

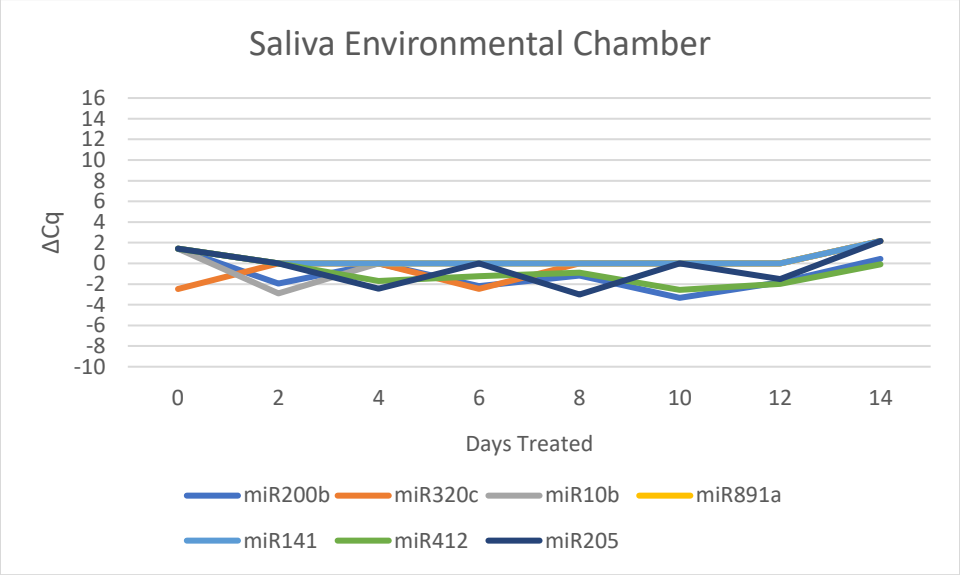


Table 9. Overall correct classification rates of all treated samples compared to previous population classification rates [33].

Body Fluid	n	Correct Classification	Classification as another BF	Classification as Other	Previous Population Classification
Blood	59	98.3%	1.6%	0.0%	98.0%
Semen	59	52.5%	47.5%	0.0%	90.0%
Saliva	59	38.5%	60.1%	1.7%	86.0%
Urine	59	83.0%	16.0%	0.0%	80.4%

Table 10. Correct classification values for samples included in the variation within donors sample set.

Body Fluid	n	Correct Classification	Classification as another BF	Classification as Other	Previous Population Classification
Blood	9	100.0%	0.0%	0.0%	98.0%
Menstrual Blood	17	5.9%	94.1%	0.0%	66.0%
Feces	9	77.8%	22.2%	0.0%	98.0%
Saliva	27	7.4%	92.6%	0.0%	86.0%
Semen	9	77.8%	22.2%	0.0%	90.0%
Urine	18	94.4%	5.6%	0.0%	80.4%
Vaginal Fluid	65	76.9%	23.1%	0.0%	74.0%

Figure 13. Average ΔCq values from donors sampled over the course of a biological cycle in blood. n=9

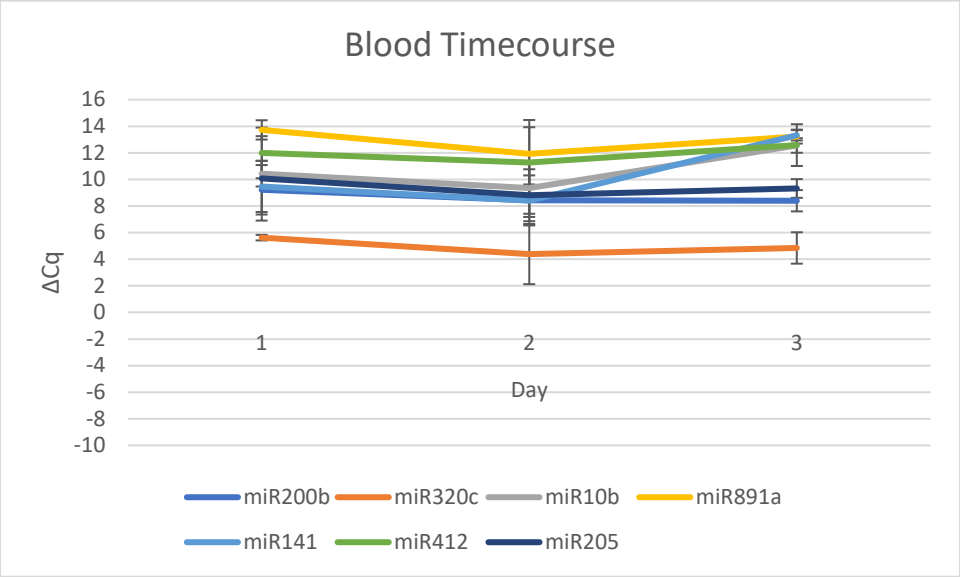


Figure 14. Average ΔCq values from donors sampled over the course of a biological cycle in menstrual blood. n=17

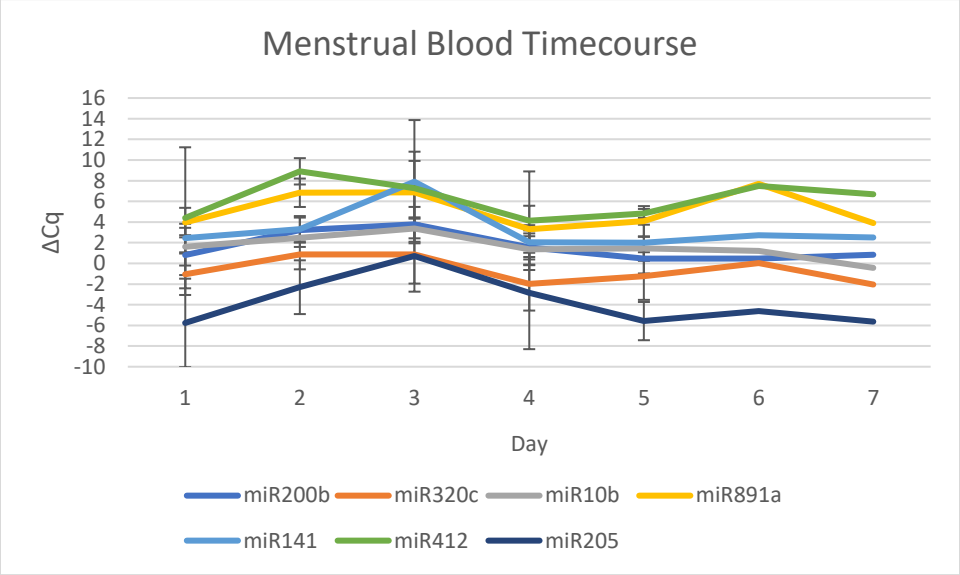


Figure 15. Average ΔCq values from donors sampled over the course of a biological cycle in feces. n=9

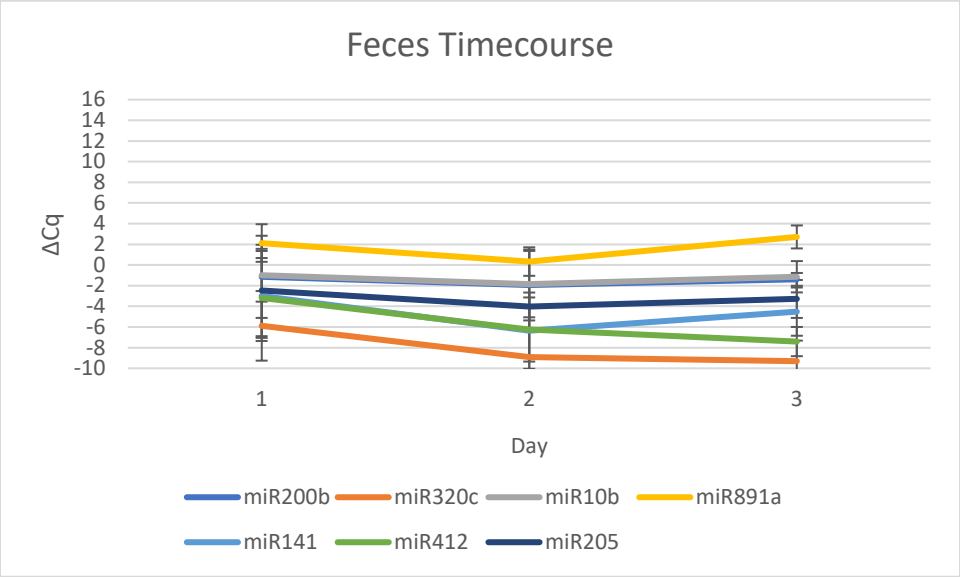


Figure 16. Average ΔCq values from donors sampled over the course of a biological cycle in saliva. n=27

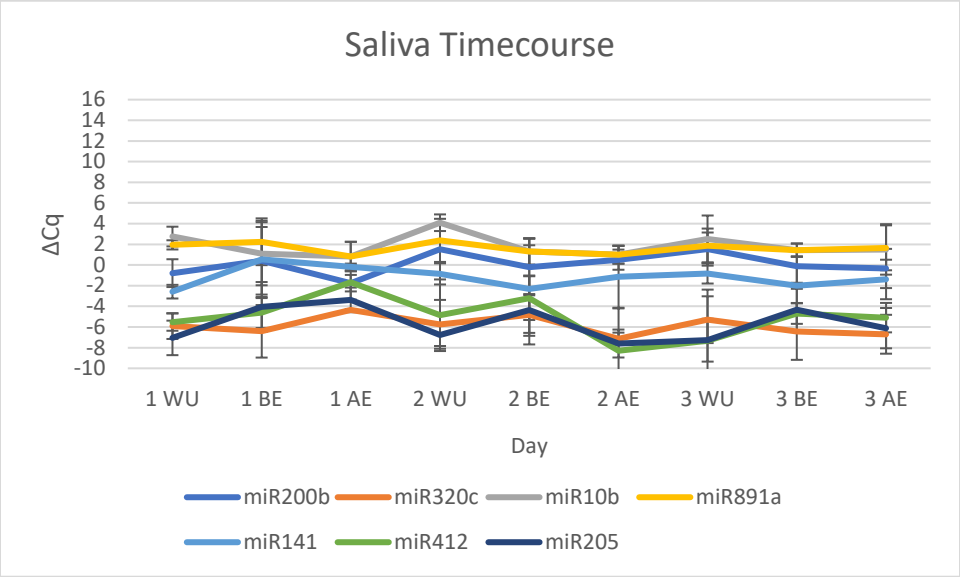


Figure 17. Average ΔCq values from donors sampled over the course of a biological cycle in urine. n=18 (miR891a, p=0.0315, r^2=0.2578, miR141, p=0.0247 r^2=0.2774, miR412 p=0.0189, r^2=0.2988)

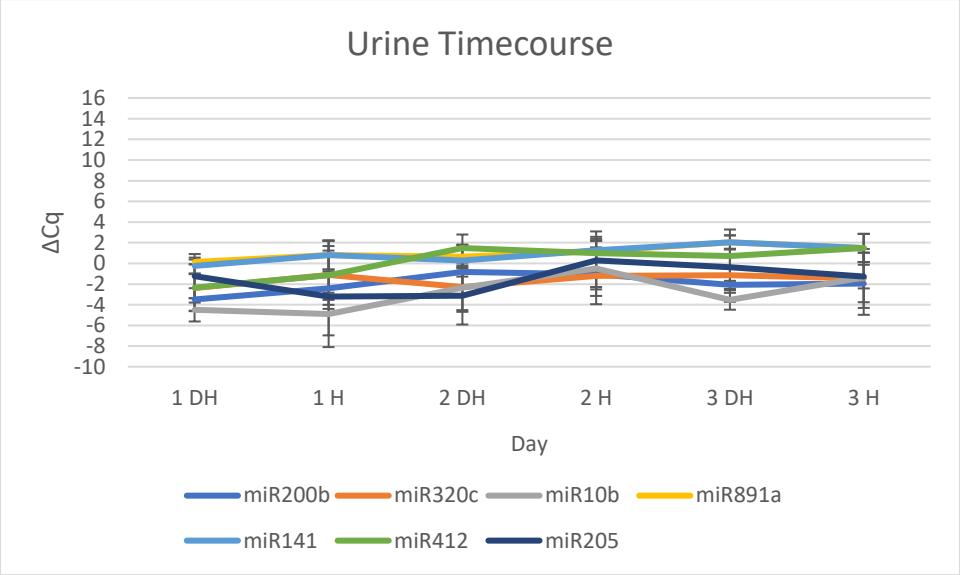


Figure 18. Average ΔCq values from donors sampled over the course of a biological cycle in semen. n=9

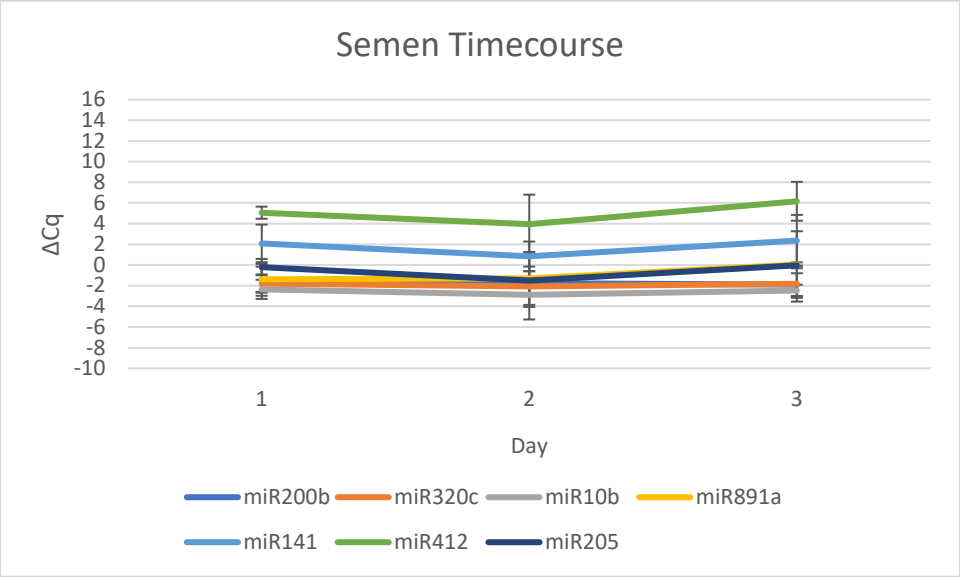
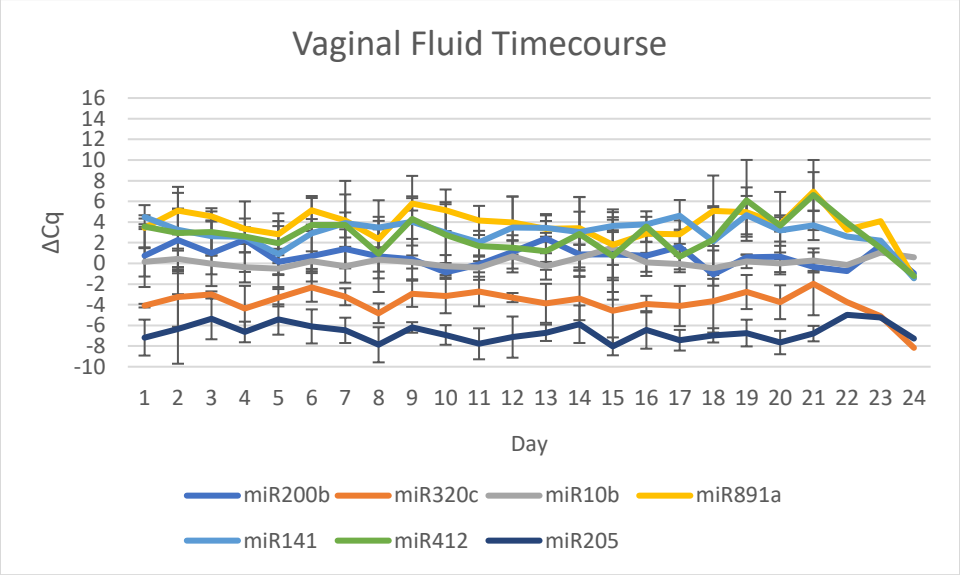


Figure 19. Average ΔCq values from donors sampled over the course of a biological cycle in vaginal fluid. n=65



Vita

Kelsey Price was born in Kennewick, Washington and graduated from Pasco Senior High School in Pasco, Washington in 2013. She attended Washington State University where she graduated cum laude and earned her Bachelor of Science degree in Neuroscience with a minor in Biology. After graduating, she worked full-time as a Research Technician for Dr. Brian Kraemer's neurodegenerative research laboratory, where she worked on projects for mouse embryo implantation and forward genetic screens in *C. elegans*. She then enrolled in Virginia Commonwealth University Department of Forensic Science graduate program in the forensic biology track. Since the summer of 2021, she has been working on a research project in the Seashols-Williams laboratory on body fluid identification using micro RNA targets. During her time at VCU, she served as the secretary of the Forensic Science Graduate Organization.