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Final Report: Who and How: Comprehensive RNA-based BodyfluID Assay to Provide Context to a Recovered DNA Profile

ABSTRACT

This report is the final technical report and summarizes the work performed for the full project period.

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Biological Evidence

Who and How: Comprehensive RNA-Based BodyFluID Assay to Provide Context to a Recovered RNA Profile

FINAL REPORT

August 25, 2015

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FOREWARD

Current body fluid identification methods use a variety of labor intensive and technologically diverse techniques that do not permit the identification of all frequently encountered body fluids such as saliva, vaginal secretions, menstrual blood or skin [1]. Proper identification of the biological material present might be crucial to the investigation and prosecution of a criminal offense and a misrepresentation of the nature of the evidence can have undue influence on the perception of the circumstance of the crime. Therefore it is critical that novel strategies for the conclusive identification of forensically relevant biological fluids be developed. One such approach is the use of mRNA profiling. Terminally differentiated cells, whether they comprise blood monocytes or lymphocytes, ejaculated spermatozoa, epithelial cells lining the oral cavity or epidermal cells from the skin become such during a developmentally regulated program in which certain genes are turned off (i.e. are transcriptionally silent) and others are turned on (i.e. are actively transcribed and translated into protein) [2]. This produces a pattern of gene expression that is unique to each cell type not only evidenced by the specific mRNAs present but also their relative abundance. Thus if it was possible to determine the type and abundance of mRNAs in a stain or tissue sample recovered at the crime scene it would be possible to definitively identify the tissue or body fluids present. Such an approach would offer a number of advantages over conventional methods for body fluid identification including: (i) the ability to perform parallel tests for numerous markers of a **single** body fluid in a single assay format, (ii) the ability to perform parallel tests for **different** body fluids in a single assay format, (iii) a definitive identification of body fluids for which presently no specific tests exist. Numerous studies have been reported in current forensic literature to support the use of an RNA profiling approach for identification of forensically relevant body fluids and tissues in routine casework [3-24].

In addition to the ability to conclusively identify all forensically relevant biological fluids, it would be desirable, if not critical, for the developed mRNA body fluid identification system to be seamlessly compatible with current DNA typing technology. Without the development of compatible DNA and RNA isolation methods, separate samplings of biological stains would need to be taken. In the case of heterogeneous mixtures of different body fluids, separate sampling of these mixed stains from different "geographical" locations of the stains to isolate DNA and RNA could result in a misleading estimate of the ratio of the body fluids present and, in extreme cases, even fail to detect one of the contributors. The simultaneous analysis of DNA and RNA should permit not only the identification of the donor of the stain, but also provide an indication of what kind of activity may have led to the deposition of the DNA.

The aims of the current work were to custom-develop for DFSC casework use: (i) a DNA/RNA co-extraction isolation protocol suitable for dried biological evidence material (including trace amounts); (ii) a highly specific and robust mRNA body fluid multiplex system that permits the simultaneous identification of all forensically relevant biological fluids and tissues (blood, semen, saliva, vaginal secretions, menstrual blood and skin) in a single assay. Based upon our prior experience with DNA/RNA co-isolation methods, we re-evaluated the most promising in terms of the quantity and quality of the nucleic acids recovered and developed a highly robust, semi-automated DNA/RNA co-isolation method for casework operational use. Using highly specific gene candidates for the identification of forensically relevant biological fluids and tissues, custom capillary electrophoresis (CE) and high resolution melt (HRM) assays

were developed and fully validated. The results of this study demonstrate the high sensitivity and specificity of the developed BodyfluID assays and their suitability for use in operational casework for the identification of forensically relevant body fluids and tissues.

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III. STATEMENT OF THE PROBLEM

Increasingly, conventional methods for body fluid identification are being supplanted by the facile and routine identification of human DNA in the sample. Proponents of this approach argue that the presence of human DNA from a particular individual is sufficient for most purposes and that the cell type from which the DNA originated is inconsequential or that present methods are non-specific. While the ability to bypass conventional body fluid identification tests is appealing due to a perceived increase in operational efficiency, there are a number of case scenarios where body fluid identification *per se* would provide important probative evidence. For example, consider a sexual assault on a female victim with an object (recovered from the suspect) where the victim's DNA is recovered from the object. He could claim that the victim handled the item during a casual encounter and this explains why her DNA was present. However, the significance of this evidence would increase if the DNA could be shown to originate from vaginal cells, a circumstance that would be consistent with a sexual encounter but not with casual handling. Currently there are no routinely used means by which to distinguish the presence of vaginal secretions or skin. Therefore, the inability to conclusively identify the context in which a recovered DNA profile was deposited may allow perpetrators to exploit the uncertainty of the origin of this evidence.

Therefore, in the current work we leveraged our extensive knowledge of body-fluid specific biomarkers [25-28], multiplex [7,16,17] and DNA/RNA co-isolation strategies [25,27] to develop a custom-built, casework-ready body fluid identification system that is compatible with the DFSC DNA analysis pipeline. Specifically, the aims of the current work were to custom-develop for DFSC casework use: (i) a DNA/RNA co-extraction isolation protocol suitable for dried biological evidence material (including trace amounts); (ii) a highly specific and sensitive mRNA body fluid multiplex system that permits the simultaneous identification of all forensically relevant biological fluids (blood, semen, saliva, vaginal secretions, menstrual blood and skin) in a single CE-based assay (which we term for convenience 'BodyfluID'). During the course of this project, we also developed three highly sensitive and specific high resolution (HRM) melt assays: 1) blood-menstrual blood assay, 2) sexual assault (identification of semen, saliva and vaginal secretions) and 3) a comprehensive hexaplex assay (identification of blood, semen, saliva, vaginal secretions, menstrual blood and skin).

IV. RESULTS

A. Development of an optimized DNA/RNA Co-Isolation Method

The first aim of the current work was to develop a DNA/RNA co-extraction protocol suitable for dried biological evidence material. The development of a DNA/RNA co-extraction method was intended to be useful in cases of heterogeneous mixtures of different body fluids. Separate sampling of these mixed stains from different "geographical" locations of the stains to isolate DNA and RNA could result in a misleading estimate of the ratio of the body fluids present and, in extreme cases, even fail to detect one of the contributors. The simultaneous analysis of DNA and RNA should permit not only the identification of the donor of the stain, but also provide an indication of what kind of activity may have led to the deposition of the DNA. A DNA/RNA co-extraction approach also serves to reduce sample consumption since separate samplings of biological stains would not need to be taken.

We have extensive experience with DNA/RNA co-extraction methods, particularly a manual/organic based method with spin column purification and the automatable commercially available AllPrep DNA/RNA Micro kit (QIAGEN). The DFSC DNA laboratory personnel indicated that they do not readily use organic or manual extractions and therefore the AllPrep DNA/RNA Micro extraction kit was selected as the most suitable method for use in the current work. Using the AllPrep DNA/RNA Micro co-extraction kit, samples are first lysed in a highly denaturing guanidine-isothiocyanate–containing buffer, which immediately inactivates DNases and RNases to ensure isolation of intact DNA and RNA. The lysate is then passed through an AllPrep DNA spin column. This column, in combination with the high-salt buffer, selectively and efficiently binds genomic DNA. The column is washed and purified and ready-to-use DNA is then eluted. Ethanol is added to the flow-through from the AllPrep DNA spin column to provide appropriate binding conditions for RNA, and the sample is then applied to an RNeasy MinElute spin column, where total RNA binds to the membrane and contaminants are efficiently washed away. High-quality RNA is then eluted. The AllPrep DNA/RNA Micro co-extraction can be performed manually or can be semi-automated on the 12-sample QIACube. Using the QIAcube, 10 samples can be processed at a time (two spaces are needed during DNA isolation due to pre-heating of the elution buffer). We evaluated various protocol parameters (e.g. lysis times and temperatures, the use of additives (e.g. carrier RNA)) in order to develop an optimized co-extraction method that would work with relevant sample types (blood, semen, saliva, vaginal secretions, menstrual blood and skin ('touch DNA' samples)).

The developed protocol involves an initial 1-hour lysis with Buffer RTL Plus (with 2M DTT added). The lysis step is performed on the QIACube. Following a brief 3-min centrifugation step with the swab/stain piece in a spin basket, the lysate is placed back on QIACube for DNA isolation. The DNA column is placed into position two of the rotor adaptor (Figure 1), which requires removal of the column lid. After DNA isolation, the same rotor adaptor is used for RNA isolation. A separate elution tube and RNAeasy column are placed into the rotor adaptor (Figure 1) and the flow through from the DNA isolation serves as the sample for the RNA isolation steps. The entire optimized AllPrep DNA/RNA co-extraction procedure takes approximately 2.5-3 hours for completion. Following DNA and RNA isolation, each fraction can then be processed using DNA and RNA specific workflows (Figure 2). As with standard DNA analysis, the DNA fraction is quantitated using real time PCR, amplified using an

STR amplification kit (AmpFlSTR Identifiler Plus was used in this study, 28 amplification cycles, 12.5 μl reaction volume) and CE detection of the amplified products. The RNA fractions are treated with DNase to remove any residual DNA and are then quantitated using RiboGreen (Invitrogen by Life Technologies). Total RNA is then reverse transcribed and analyzed using with the developed CE 10-plex multiplex or the three developed multiplex high resolution melt (HRM) assays. The HRM analysis combines amplification and detection into a single reaction and therefore require less time to perform than the CE multiplex (~8 hours vs ~14.5 hours, Figure 2). Each of the developed RNA profiling assays is described below.

The optimized AllPrep DNA/RNA co-extraction method was successfully used to isolate DNA and RNA of sufficient quantity and quality from all forensically relevant biological fluids and tissues (blood, semen, saliva, vaginal secretions, menstrual blood and skin), admixed body fluid samples and a range of mock casework and environmentally compromised samples.

B. Development of the 10-plex Capillary Electrophoresis Multiplex System

The second aim of the current work was to develop a highly specific and sensitive mRNA body fluid multiplex system that permits the simultaneous identification of all forensically relevant biological fluids (blood, semen, saliva, vaginal secretions, menstrual blood and skin) in a single CE-based assay (which we termed for convenience 'BodyfluID'). It was our recommendation that at least two biomarkers per body fluid or tissue, when possible, be used to identify the presence of each body fluid. In the current work, we leveraged our extensive knowledge of mRNA biomarkers in order to select the most highly sensitive and specific biomarkers for each of the target body fluids and tissues. Initially, two multiplex systems were developed: a 10-plex system and a 12-plex system, with the 12-plex multiplex containing two housekeeping genes, beta-2-microglobulin (B2M) and 18S rRNA. For both multiplex systems, the following biomarkers were included: blood – ANK1 and ALAS2; semen – SEMG1 and PRM2; saliva – HTN3 and STATH; vaginal secretions – CYP2B7P1; menstrual blood – MMP10 and LEFTY2; and skin – LCE1C. A single biomarker was included for vaginal secretions as not other candidate with suitable specificity was available. A single biomarker was selected for skin as LCE1C is the most abundant candidate identified for touch DNA samples.

During development of the CE multiplex system, it became apparent that the 10-plex would be the better of the two alternative multiplex systems. The forensic community has not come to a consensus on whether it is necessary to include a housekeeping gene in multiplex systems. The 12-plex system was not selected due to the following reasons: 1) there is currently no housekeeping gene that is equally expressed amongst all body fluids and tissues, 2) due to the high abundance of housekeeping genes in some body fluids and tissues, they tend to titrate out critical PCR reagents and therefore affect the overall performance and efficiency of the multiplex and 3) the presence of body fluids markers with no housekeeping genes present has been observed and therefore could effect the ability to identify body fluids genuinely present in the sample.

The design of the 10-plex CE system is shown in Figure 3. The multiplex is a 5-dye system using FAM, VIC, NED and PET labeled biomarkers (G5 module) and LIZ-500 as the internal size standard. The multiplex was designed such that there would be little to no interference between channels as a result of pull-up from any of the included biomarkers. The primer sequences and primer mix formulation are provided in Table 1. Representative electropherograms for blood, semen, saliva, vaginal secretions, menstrual blood and skin are

shown in Figure 4. For semen, SEMG1 is a seminal fluid biomarker that should be detected in both vasectomized and non-vasectomized males. PRM2 is a sperm-specific biomarker and therefore should only be detected in non-vasectomized males. For menstrual blood, in addition to the two menstrual blood specific biomarkers (MMP10, LEFTY2), blood and vaginal secretions biomarkers may also be present as they are normal constituents of menstrual blood.

Primers for each biomarker are designed to be present in separate exons. DNA amplification products, if present, can be distinguished from RNA products due to size differences resulting from amplification of introns. Due to the small size of some introns, three DNA products are present in the analysis size range for the multiplex, two on the FAM (blue channel) and one of the VIC (green) channel (Figure 5). These products are separate from all biomarkers in the multiplex and therefore do not interfere with analysis if present. These products are useful indicators of residual DNA contamination, which may affect overall assay performance. This can alert a user to the need for a possible second DNase treatment of the sample.

A full developmental validation has been performed for the CE 10-plex system. The results of this validation will be described in Section IV-D below.

C. Development of the Multiplex High Resolution Melt (HRM) Assays

In addition to the originally proposed CE multiplex system, we also additionally included the development of three multiplex high resolution melt (HRM) assays as potential 'rapid screening' assays. The HRM assays are based on the ability to distinguish the amplification products from body fluid specific gene candidates based on different melt temperatures. These real time PCR based assays utilize unlabeled PCR primers and therefore significantly reduce the cost of analysis. Additionally, the amplification and melt curve analysis are performed in a single ~2 hour reaction and therefore reduces the length of time needed for analysis. The three HRM assays developed include the following: blood-menstrual blood (Table 2, Figure 6), sexual assault (Table 3, Figure 7) and a comprehensive hexaplex assay (Table 4, Figure 8). The blood-menstrual blood HRM assay contains two biomarkers each for blood (ALAS2, ANK1) and menstrual blood (MMP10, LEFTY2). The sexual assault HRM assay (colloquially the 'sex-plex' assay) contains one biomarker each for saliva (HTN3) and vaginal secretions (CYP2B7P1) and two biomarkers for semen (SEMG1 and PRM2). The hexaplex assay contains one biomarker each for blood (ANK1), semen (SEMG1), saliva (HTN3), vaginal secretions (CYP2B7P1), menstrual blood (LEFTY2) and skin (LCE1C).

A full developmental validation study has been performed for each of the HRM multiplex systems. The results of the validation experiments will be described in Section IV-D below.

D. Developmental Validation of the CE and HRM Assays

1. Sensitivity

To evaluate the sensitivity of the CE and HRM assays, a range of input total RNA (50 pg to 15 ng) for each body fluid (blood, semen, saliva, vaginal secretions and menstrual blood) was analyzed. Skin was not analyzed in this same manner as touch DNA samples often have low to undetected quantitation values. Therefore, the ability of the assays to detect skin (i.e. touch DNA samples) will be addressed separately.

CE 10-plex

The sensitivity results for the CE 10-plex are provided in Table 5. For each body fluid, three donors were evaluated, with the exception of semen in which four donors were evaluated in order to include a vasectomized male in the study. As can be seen from Table 5, picogram-level sensitivity was observed for most biomarkers. MMP10 was detected in all donors using as little as 100 pg of input total RNA and was detected in two of the three donors using only 50 pg of total input RNA. The saliva biomarkers were the least sensitive but still detectable with as little as 1 ng of input total RNA. The blood-specific mRNA biomarker sensitivity of one of the blood donors is shown in Figure 9. ALAS2 was detected for all input levels with product peaks clearly visible and well above detection thresholds. ANK1 was detected down to an input of 250 pg of total RNA.

The results of the sensitivity study were used to determine the optimal input for the CE 10-plex. Reasonably consistent results were obtained when an input of 5 ng or more was used with the occasional failure to detect one or two donors for certain biomarkers (i.e. SEMG1 10 ng and STATH 5 ng; Table 5). However, when 15 ng of total RNA was used, positive detection of all biomarkers for all donors was achieved. This input level was also consistent with HRM sensitivity studies and would therefore permit a single RT reaction to be used with all four multiplex assays.

The results of the sensitivity study demonstrate that even though a 15 ng total RNA input was selected for the multiplex system, successful detection of biomarkers would still be possible for samples in which that amount of RNA was not obtained.

Blood-Menstrual Blood HRM Assay

The sensitivity results for the Blood-Menstrual Blood HRM assays are provided in Table 6. For each body fluid, three donors were tested at each input level. As was observed with the CE 10-plex, picogram-level sensitivity was observed for each of the biomarkers. The least sensitive biomarker was ALAS2 with successful detection of all three donors using as little as 500 pg of input total RNA. All other biomarkers had at least one donor detected using the lowest input level of 50 pg. Representative melt plot assays for one of the blood donors is shown in Figure 10. While an optimal input level for this multiplex could be lower than 15 ng (~5 ng total RNA input), the optimal input was kept at 15 ng to be consistent with the other multiplex systems.

Sexual Assault HRM Assay

The sensitivity results for the Sexual Assault HRM assays are provided in Table 6. For each body fluid, three donors were tested at each input level with the exception of semen in which a fourth donor (vasectomized male) was included. For this semen donor, only SEMG1 detection was expected. Picogram-level sensitivity was again observed for each of the biomarkers, with the exception of CYP2B7P1 in which reliable results were obtained with input levels of 1 ng or above. The semen biomarkers were both detected in all donors with as little as 50 pg of input total RNA. Therefore the true sensitivity of these biomarkers is likely to be well below 50 pg. A representative melt plot assays for one of the semen donors is shown in Figure 10 indicating successful detection of both biomarkers for each input level. While an optimal input level for this multiplex could be lower than 15 ng (~5 ng total RNA input), the optimal input was kept at 15 ng to be consistent with the other multiplex systems.

Hexaplex HRM Assay

The sensitivity results for the Hexaplex HRM assay is provided in Table 7. For each body fluid, three donors were tested at each input level with the exception of semen in which a fourth donor (vasectomized male) was included. Picogram-level sensitivity was observed for SEMG1 and HTN3. However, a nanogram-level input was needed for reliable biomarker detection for ANK1, CYP2B7P1 and LEFTY2. Representative melt plots for one of the saliva and vaginal secretions donors is shown in Figure 11. An overall input level of 15 ng was optimal for this multiplex system.

Comparison

An overall comparison of the sensitivities of the body fluid specific biomarkers amongst the four multiplex systems is provided in Table 8. This sensitivity comparison may be useful in selection of the most suitable detection assay for low-level samples. A picogram-level sensitivity is possible for all biomarkers using at least one multiplex system, with the exception of STATH (saliva) in which 5 ng of total RNA input is needed. STATH is the only biomarker only found in the CE multiplex system and not in one of the HRM assays.

<u>*2. Specificity*</u>

In order to evaluate the specificity of body fluid specific biomarkers in each of the multiplex systems, we evaluated numerous donors of each of the different body fluids to identify potential cross reactivity, including various tissue samples as well as nasal secretion samples (an uncommonly encountered body fluid recently evaluated and reported in the forensic literature [29]).

Body Fluid Samples

For each of the multiplex systems, 10–20 donors of each body fluid or tissue were evaluated with the exception of menstrual blood for which only 7-8 donors were available. For skin, twenty touch DNA samples were obtained and tested (a swab of headphones, computer mouse, backpack or purse strap and car steering wheel from five different donors). The results of the specificity for each multiplex is provided in Table 9.

For the CE 10-plex, no cross reactivity was observed for any of the included biomarkers. The detection of blood and vaginal secretions biomarkers in menstrual blood samples is not unexpected cross-reactivity as they are normal constituents of menstrual blood. The presence of skin was detected in three of the fifteen skin samples. This is likely a genuine detection of skin in these samples as contamination with skin can occur during collection of these samples from outer external genital areas or from the hands of donors as gloves are not typically worn during donor collections. False negative results were obtained for several biomarkers. The multiplex systems are designed to reduce (or even eliminate to the extent possible) false positive results and therefore the occasional false negative result is observed. For PRM2, only thirteen of the fourteen samples were expected to be positive as one donor tested was a vasectomized male.

For the blood-menstrual blood assay, no cross reactivity was observed for ANK1, ALAS2 or LEFTY2. MMP10 was detected in menstrual blood samples but also in four of the thirteen vaginal secretions samples. Detection of MMP10 in vaginal samples has been observed and reported by other research groups [4]. The time of collection in the female reproductive cycle is not reported for a majority of vaginal secretion samples and therefore it is possible that

increased levels of MMP10 expression are observed just prior to or just after menstruation resulting in its detection in vaginal secretions samples. As a result of the occasional detection of MMP10 in vaginal secretions samples, a definitive identification of the presence of menstrual blood requires the presence of MMP10 and LEFTY2.

For the sexual assault HRM assay, no cross reactivity was observed for any of the included biomarkers. Only one false negative result was observed for SEMG1 and PRM2 and CYP2B7P1.

For the hexaplex HRM assay, no cross reactivity was observed for any of the included biomarkers. Skin was detected in three of the seven menstrual blood donors but again is likely due to the genuine presence of skin in these samples.

Tissue Samples

In addition to body fluid samples, a number of tissue total RNA samples (trachea, kidney, ovary, adipose, placenta, spleen, liver, esophagus, small intestine, lung, colon, thymus, bladder, thyroid, cervix, skeletal muscle, brain and heart) were evaluated. These samples were obtained from commercial companies as highly purified total RNA. Therefore, in order to confirm the specificity results obtained here the extraction of tissue samples using the developed AllPrep DNA/RNA co-extraction method would be required.

The results for the tissue specificity study for the CE 10-plex are provided in Table 10. None of the body fluid biomarkers were detected in kidney, ovary, adipose, esophagus, colon, thymus, bladder, thyroid, cervix, skeletal muscle, brain or heart. The red blood cell marker ANK1 was detected in spleen, which is not surprising since this tissue is involved in the recycling of red blood cells. Placenta tissue had both blood and menstrual blood biomarkers. This is not unexpected as this is a reproductive tissue. The presence of STATH, CYP2B7P1 and MMP10 was detected in trachea. This expression profile is not typical of any of the forensically relevant body fluids, including in admixed samples, and therefore could possibly be distinguished. CYP2B7P1 was detected in liver, small intestine and lung tissues and therefore could not be distinguished from vaginal secretions solely with this multiplex system. Since only a single sample was evaluated initially, a second and different sample for each of these tissues was evaluated (Table 11). CYP2B7P1 was not detected in the additional liver sample, but was detected in the second lung and small intestine sample. It may be possible to distinguish lung tissue with a recently developed mRNA profiling multiplex for the identification of organ tissue [19]. Additional development of such tissue identification multiplexes may result in the ability to fully distinguish vaginal secretions from these two tissue types. Additionally, the identification of novel highly specific vaginal secretions biomarkers may also aid in differentiation of these tissue types from vaginal secretions. As stated above, additional testing of actual dried tissue samples using the developed co-extraction protocol would also be required to determine if cross-reactivity with these tissues is still observed. Therefore, while current reporting guidelines will recognize the possibility of the presence of these tissues based on detection of CYP2B7P1, we fully expect to be able to fully and definitively distinguish these tissue types with alternative or additional assays that distinguish unique patterns of gene expression across all tissues.

The results for the tissue specificity study for the blood-menstrual blood assay are provided in Table 12. The blood biomarkers were not observed in a majority of the tissue samples with the exception of spleen in which ANK1 was again detected. The menstrual blood biomarkers were observed in reproductive tissues (ovary and placenta) which is therefore not

entirely unexpected. MMP10 was also detected in trachea tissue. However, no additional trachea samples were available for confirmation of that result.

The results for the tissue specificity study for the sexual assault HRM assay are provided in Table 13. As observed with the CE 10-plex, CYP2B7P1 was detected in the liver, small intestine and lung samples. SEMG1 was observed in the bladder sample but this was not observed with CE 10-plex multiplex and therefore can be distinguished from semen using a combination of these assays. The Tm value of this product is also slightly shifted ~0.5^{o}C from the expected or average Tm for this biomarker.

The results for the tissue specificity study for the hexaplex HRM assay are provided in Table 14. Again LEFTY2 was detected in reproductive tissues (ovary and placenta), ANK1 detected in spleen and SEMG1 detected in bladder.

Any potential cross reactivity has been incorporated into interpretation guidelines for each multiplex system and as stated above will likely improve as further advances in mRNA profiling assays are made.

Nasal Secretions

Several studies have reported the presence of STATH in nasal secretions samples [29]. We therefore included an evaluation of nasal secretions samples in the specificity studies in this validation. STATH is only present in the CE 10-plex and therefore these samples were only evaluated using CE (the 12-plex assay was actually used since it was still being evaluated at the time of testing of these samples). Nasal secretions samples were obtained from four donors. STATH was detected in two of these four samples. The presence of STATH only in the absence of HTN3 has not been observed for any saliva sample tested in the current work or in our prior experience. Therefore, the presence of STATH only could indicate the possible presence of nasal secretions.

3. Species Specificity

Comprehensive species specificity testing was possible for blood biomarkers only as samples of other body fluids from animals were largely not available. A few cat and dog saliva samples were available and will be presented in the 'case type samples' section below (Section IV-D-6).

Blood samples from 18 animals and 10 primates were tested using the CE 10-plex, blood-menstrual blood HRM and hexaplex HRM assays. The sexual assault HRM assay does not contain blood biomarkers and therefore was not included in this part of the study. The results of the initial species specificity study are provided in Table 15. For the CE 10-plex, the blood biomarkers were detected in a majority of the primate samples (7/10 samples for ALAS2 and 5/10 samples for ANK1). ANK1 was not detected in any of the animal samples indicating the human/primate specificity of this biomarker. ALAS2 was detected in the rabbit, ferret and cat sample only. For the blood-menstrual blood sample, ALAS2 was again detected in a majority of primate samples as well as the rabbit sample. The cat sample was not positive for ALAS2 using this assay. ANK1 was not detected in any of the animal samples and only in two of the primate samples (chimp and black howler monkey). The hexaplex only contains ANK1 and the high human/primate specificity was again confirmed for ANK1 using the hexaplex assay.

The only cross reactivity in the animal samples was for ALAS2 which was detected in the rabbit, ferret and cat samples. Since the initial testing included only one sample of each of

these animals, additional ferret and cat samples were evaluated (eight additional ferret samples and one additional cat sample, Table 16). Additional rabbit samples were not available. As can be seen from the results in Table 16, ALAS2 was not detected in the eight other ferret samples tested using the CE 10-plex, the blood-menstrual blood or hexaplex assays. Therefore only one of nine ferret samples was positive for ALAS2. ALAS2 was also not detected in the second cat sample. Therefore cross reactivity with these species appears to be sporadic.

4. Stability

To address the stability of the developed CE and HRM multiplex assays, body fluid samples exposed to various temperatures, incubation times and environmental insults (heat, light, humidity and rain) were tested. Additionally, the stability in expression of vaginal secretions and menstrual blood biomarkers were evaluated using samples collected during female reproductive cycles in both menstruating and menopausal female samples. These samples were not extracted with the AllPrep co-extraction method but with a manual organic RNA extraction (extracted previously and stored in the laboratory at -20°C).

Environmentally Compromised Samples

To evaluate the stability of the blood biomarkers, blood samples exposed to various temperatures, incubation times and environmental insults were tested (Table 17). These conditions included exposure to room temperature, 37°C and 56°C for 1 and 2 years, outside storage with varying combinations of heat, light humidity, and rain, as well as storage in a car trunk and back seat (extreme high temperatures). These samples were tested with the CE 10-plex, the blood-menstrual blood HRM assay and the hexaplex HRM assay. As can be seen from Table 17, the blood biomarkers were detected in many of the blood samples tested but were not detected in samples that included exposure to rain (sun and shade samples). This is not unexpected as rain can not only wash away biological materials but result in increased growth of microorganisms which are detrimental to nucleic acids, both DNA and RNA. Surprisingly, the blood biomarkers were detected in all of the car and trunk samples (1-2 weeks storage) for each multiplex assay. These samples were exposed to extreme heat demonstrating a high degree of stability of these biomarkers.

Semen samples were exposed to similar conditions as described above for blood: room temperature, 37°C and 56°C for 1 and 2 years and outside storage exposed to heat, light, humidity and rain. The semen samples were evaluated using the CE 10-plex and sexual assault HRM assays (Table 18). As can be seen from Table 18, PRM2 was detected in almost every sample for both multiplex assays. This includes samples stored outside exposed to heat, light, humidity and rain. SEMG1 was also detected in a majority of samples but was more affected by exposure to rain than PRM2. These results demonstrate stability of the semen biomarkers.

Similar sample sets as described for blood and semen were tested for saliva (Table 19). The saliva markers were not as readily detected using the CE 10-plex and outside storage appeared to be detrimental to them (exposure to heat, light, humidity and rain). Saliva possesses endogenous bacteria and therefore it is expected that increased amounts of microbial growth will occur with these samples. However, the saliva biomarkers were detected in significantly more samples using the sexual assault HRM assay demonstrating increased sensitivity of this multiplex system.

Vaginal secretions samples were also exposed to room temperature, 37°C and 56°C storage for 1 and 2 years as well as exposure to outdoor conditions (heat, light, humidity and

rain). These samples were tested using the CE 10-plex, sexual assault and hexaplex HRM assays (Table 20). The vaginal secretions samples were affected more significantly by heat and outdoor conditions than the other body fluids. As with saliva, endogenous bacteria are present in vaginal samples and therefore increased amounts of microbial growth may have occurred. CYP2B7P1 was nevertheless still detected in several samples.

In addition to the samples described above, additional semen and saliva samples that had been exposed to environmental insults as well as samples deposited onto other substrates (i.e. denim) were available for testing. These samples included semen and saliva stored outside (heat, light, humidity), at 37°C (3 months) and 56°C (1 month) and on denim (room temperature for 6 months). These samples were co-extracted using the AllPrep DNA/RNA co-extraction kit (lysis was increased to 3 hours due to the protocol used in the study in which they were first analyzed). The results from the testing of these samples with the CE 10-plex, sexual assault HRM and hexaplex assays are provided in Table 21. The semen biomarkers were detected in all semen samples for all three assays, with the exception of the semen sample stored outside for 7 days (no biomarkers detected in this sample for any of the three assays). HTN3 was detected in the saliva samples stored outside as well as at 37°C (3 months) for both the sexual assault HRM and hexaplex HRM assays. The saliva sample on denim was also detected using the hexaplex HRM assay. HTN3 and STATH were not readily detected in these samples using the CE 10-plex, again consistent with better sensitivity achievable with the HRM assays.

Despite the false negative results obtained for some samples, overall the results of the environmentally compromised sample studies demonstrate the robust nature of the developed multiplex systems. Definitive body fluid identification was possible for challenging and compromised body fluid samples.

Female Reproductive Cycles

To further evaluate the stability of the vaginal secretions and menstrual blood biomarkers, expression of these biomarkers was evaluated in 25-28 day reproductive cycles from two menstruating (day 1 as the start of menstruation) and one menopausal female. These samples were extracted previously using a manual organic RNA extraction. Re-collection of these sample sets was not possible and therefore the non co-extracted extracts were tested. The samples were tested using the CE 10-plex (Table 22), the blood-menstrual blood HRM assay (Table 23) and the sexual assault HRM assay (Table 24).

Using the CE 10-plex, CYP2B7P1 was detected in a majority of samples for the menstruating females and in all samples for the menopausal female (Table 22). There do not appear to be any hormonal related expression changes but rather due to the age of the extracts. For the first menstruating female, menstrual blood biomarkers were detected in days 2 and 3 of menstruation. For the second menstruating female, the menstrual blood biomarkers were detected in all four days of reported menstruation. MMP10 was also detected in cycle days 5 and 6. It is possible that light menstrual blood was still present on these days but not readily noticeable to the donor.

Using the blood-menstrual blood HRM assay (Table 23), menstrual blood biomarkers were again detected for donor 1 on days 2 and 3 of menstruation and days 1-4 for donor 2 (although LEFTY2 was only detected on days 1 and 2).

Using the sexual assault HRM assay (Table 24), CYP2B7P1 was detected in a majority of samples with some sporadic false negative results as observed with the CE 10-plex. Again,

none of the negatives appear to be hormonally regulated (no obvious trend with time during the cycle).

5. Mixtures

Two-donor admixed fluids (RNA extraction only)

All samples evaluated in previous studies involved the use of single source body fluid samples. The ability of the multiplex systems to identify the presence of more than one body fluid in admixed samples was next evaluated. Two-fluid admixed body fluid samples had been previously prepared and extracted using a manual organic RNA extraction. For all mixtures, the first fluid was deposited (50 µl for liquid samples (blood, semen, saliva) and a 1/2 swab for vaginal secretions and menstrual blood) and dried and then the second fluid (liquid) was added on top of the dried stain. The admixed sample was then extracted. The mixtures included: blood-semen, blood-saliva, saliva-semen, vaginal-semen, menstrual blood-semen, vaginal-saliva and menstrual blood-saliva. For each of the liquid samples added, they were added in decreasing volumes (50, 25, 10 and 5 µl) for each mixture type. The samples were tested using the CE 10-plex and all three HRM assays.

The results from the CE 10-plex analysis of the admixed body fluid samples are provided in Table 25. Each biomarker is represented by a "+" for each body fluid (e.g. for blood ANK1 and ALAS2 would be represented by a ++). For each mixture containing blood, both biomarkers were detected in each sample. ALAS2 was also detected for menstrual blood mixtures. For semen, PRM2 was detected in a majority of samples and only absent in two samples (vaginal-semen-25 µl and menstrual blood-semen 50 µl) in which no semen markers were detected. SEMG1 was not as readily detected as PRM2 but was present in some samples in each semen mixture type with the exception of the saliva-semen mixture. Saliva was successfully detected in all blood-saliva mixtures but was not detected in a majority of the mixtures with vaginal secretions or menstrual blood. This is likely due to the high RNA yield of these body fluids. Dilutions were made based on an overall quantitation and therefore saliva RNA (typically less abundant than other body fluids) will have been highly diluted. Additionally, endogenous bacteria in both of the body fluids in these mixtures were present causing an artificially higher quantitation value. CYP2B7P1 was detected in all mixtures containing vaginal secretions or menstrual blood. Menstrual blood markers were detected in all but one of the menstrual blood mixtures (menstrual blood-semen 50 µl). This mixture was negative for both menstrual blood and semen biomarkers and therefore indicative of a sample issue. A representative electropherogram for the detection of both body fluids in an admixed sample is shown in Figure 13. This blood-saliva mixture is comprised of 50 µl of each body fluid and as can be seen both blood and both saliva biomarkers were detected.

All mixtures were tested using the blood-menstrual blood HRM assay (Table 26) as well. All mixtures not containing blood or menstrual blood were negative. Blood was detected in all mixtures containing blood. Menstrual blood was detected in each of the menstrual blood-saliva mixture samples and in a majority of the menstrual blood-semen mixtures.

All mixtures were tested using the sexual assault HRM assay (Table 27). The detection of saliva was better using this assay compared to the CE multiplex. The same blood-saliva and saliva-semen samples were positive for saliva using this assay as was observed for the CE 10-plex, however two vaginal-saliva and all four menstrual blood-saliva samples were positive using the sexual assault HRM assay. For the CE 10-plex, none of the vaginal-saliva and only one

of the menstrual blood-saliva assays were positive. For semen, PRM2 was detected in all semen containing mixtures with the exception of the menstrual blood-semen 50 µl which was previously determined to be a sample issue. SEMG1 was detected in a majority of samples with the exception of the saliva-semen mixtures in which it was not detected in any of the four samples. The same result was obtained with the CE 10-plex. CYP2B7P1 was detected in all but one sample. A representative melt plot for a saliva-semen admixture is shown in Figure 14. For this sample, 5 µl of semen was added to a dried 50 µl saliva sample. As can be seen from this figure, both saliva (HTN3) and semen (PRM2 and SEMG1) were successfully detected.

All mixtures were tested with the hexaplex HRM assay (Table 28). HTN3 was again detected in more of the saliva admixtures compared to the CE 10-plex (similar to the results observed using the sexual assault HRM assay). SEMG1 was detected in all blood-semen and menstrual blood-semen mixtures (with the exception of the 50 µl sample which was previously determined to be a sample issue). It was detected in two samples each of the saliva-semen and vaginal-semen mixtures. ANK1 was again detected in all blood-containing mixtures. CYP2B7P1 was detected in all vaginal-saliva samples but not in the vaginal-semen mixtures. LEFTY2 was detected in a majority of the menstrual blood-semen and menstrual blood-saliva samples.

Despite some differences between assays, overall the results of the initial two-fluid mixture study demonstrate the ability of each system to successfully detect multiple body fluids from an individual sample. All of the mixtures tested in the initial mixture study were not co-extracted. We therefore wanted to evaluate additional admixed samples using the full BodyfluID system including the AllPrep DNA/RNA co-extraction. We focused on semen-vaginal secretions mixtures since these best represent sexual assault evidence. The mixtures were prepared by adding liquid semen to ¼ dried vaginal samples in decreasing volumes (25, 10, 5 and 1 µl). One of the semen donors was a vasectomized male. The mixtures were tested with the CE 10-plex and sexual assault HRM assays (Table 29).

For the CE 10-plex, semen biomarkers were detected in two of the three mixture sets, with SEMG1 not detected in any of the samples with the vasectomized male donor. CYP2B7P1 was detected in two out of four samples for two of the donor sets and in all four samples for the third donor set. Using the sexual assault HRM, SEMG1 was detected in a majority of samples including the vasectomized male set (detected in three out of four samples). PRM2 was detected in the samples from the two non-vasectomized male donor sample sets. CYP2B7P1 was also detected in all but two samples amongst the three donor sets. Figure 15 shows the DNA and RNA profiling results from a vaginal-semen (5 µl of liquid semen, ¼ vaginal swab). As can be seen an admixed DNA profile is obtained. Using the CE 10-plex, semen (PRM2 and SEMG1) and vaginal secretions (CYP2B7P1) were identified. Similarly, both body fluids were successfully detected using the sexual assault HRM assay as well. Figure 16 shows another vaginal-semen (5 µl of liquid semen, ¼ vaginal swab) mixture sample. For this sample, a single source female profile was obtained for the DNA fraction and only vaginal secretions was detected using the CE 10-plex. However, when the sample was tested with the sexual assault HRM assay SEMG1 (semen) was also identified. This is another example of the high sensitivity of the developed HRM assays.

The results of this study demonstrate the ability of both multiplex systems to detect both components in admixed body fluid samples but also demonstrate the improved sensitivity and performance of the HRM assays.

6. Case Type Samples
A variety of casework type samples were tested, including 100 touch DNA samples and various mock casework samples.

Touch DNA Samples
In order to evaluate the ability of the CE 10-plex and hexaplex HRM assays to identify skin in touch DNA samples, 100 touch DNA samples were tested. Twenty samples from five different donors were collected and included samples collected from the following items: cups, water/drink bottle, computer mouse, door handle/knob, cell phone, pen, keyboard, headphones, phone, key, care steering wheel, refrigerator door handle, microwave button, backpack or purse trap, hair brush handle, TV remote, silverware, toothbrush handle and then a swab of a finger and arm. The same sample types were collected for each donor for consistency. The samples were all co-extracted using the AllPrep DNA/RNA co-extraction. For the CE 10-plex, slightly modified conditions were employed (increase to 2 µl of cDNA from 1 µl and increase to 35 cycles from 33). These modifications were developed specifically to optimize results for touch DNA samples but could also be used for any low level samples.

The RNA profiling results for both the CE 10-plex and the hexaplex HRM assay are provided in Table 30. LEC1C was successfully detected in 44% of samples for both the CE 10-plex and hexaplex HRM assays. Interestingly, the positive results are not identical for both systems with some samples positive for both multiplex systems, with others only positive for one. For 83 of the 100 samples, undetectable RNA quantitation values were obtained. For those with a detectable quantitation, values ranged from 0.1 to 7.7 ng/µl (average ~3.3 ng/µl). Therefore, skin was still detected in numerous samples with undetectable RNA quantitation values.

Since the touch DNA samples were co-extracted, the DNA fractions were also tested. Table 31 contains the DNA and RNA profiling results for all 100 touch DNA samples. A DNA profile (1 or more allele) was not obtained for only 12 of the samples (therefore 88% success rate for DNA profiling, albeit with varying degrees of probity). For four of these samples, skin (LCE1C) was still detected. However, approximately 30 of the 88 samples had 14 alleles or less and therefore may not be highly probative profiles. ~50% of the samples had probative DNA profiles (more similar to the 44% success rate for RNA profiling). Admixed DNA profiles were only observed for 6 of the 100 samples.

Mock Casework Samples
Over the past several years, a series of six collaborative exercises for the evaluation of RNA profiling have been performed by the European DNA Profiling Group (EDNAP), co-organized by the Institute of Legal Medicine at the University of Zurich and the University of Central Florida [5,6,8-10]. These studies were designed to allow laboratories to evaluate the potential use of mRNA profiling, with a majority of laboratories having little to no RNA experience. Participating laboratories (sixteen or more) analyzed unknown-to-participant samples prepared by the organizer (Zurich) and included both target and non-target body fluid samples and in some cases admixed samples. Laboratories were provided primer mixes and recommended protocols. Laboratories were also asked to include additional casework or mock casework samples. A summary of the subjects of each of the six studies is provided in Table 32. From these studies, co-extracted 'unknown' samples were available in our laboratory from the

semen, saliva and vaginal secretions studies. Therefore we evaluated these 'mock casework' samples using the CE and HRM multiplex systems developed in the current study.

The results from the testing of these samples with the CE 10-plex are provided in Table 33. As can be seen for the first E3-semen/saliva sample set, the correct body fluid was identified for each of the expected samples with the exception of the chewing gum sample in which saliva was not identified. Semen and saliva were successfully detected in a saliva-semen admixture in which 5 µl and 1 µl, respectively, of each body fluid were present. A dog buccal swab was included in the study and none of our saliva biomarkers were detected, perhaps due to differences in the dog versus human transcripts. For the second E3-semen/saliva sample set, the correct body fluid was successfully identified in five samples including a blood-saliva mixture in which 1 and 2 µl, respectively, of each body fluid were present. In this sample set, a cat buccal swab was tested and again no saliva biomarkers were detected. For the E5-vaginal sample set, vaginal secretions was successfully detected in all samples containing vaginal secretions with the exception of the sanitary towel which was negative for the presence of menstrual blood as well. These samples included worn underpants and fresh and aged vaginal swabs. CYP2B7P1 was detected (minor peak) in a urine swab but it is not possible to exclude the possibility that vaginal secretions were present in this sample.

The same samples were tested with the blood-menstrual blood HRM assay (Table 34). Only a small number of these samples contained blood, but the blood biomarkers were successfully detected in each of these samples (3 µl blood on swab, 2 µl saliva/1 µl blood mixture). ANK1 was detected on the ¼ vaginal swab. This was not observed for the CE multiplex. However, the HRM assays are more sensitive and therefore it is possible (likely?) that a trace amount of blood is present in this sample.

The samples were also tested with the sexual assault HRM assay (Table 35). Saliva, semen and vaginal secretions were successfully detected in a majority of the saliva-, semen- and vaginal secretions-containing samples. Semen was detected in the '3 µl semen inside latex glove sample' that was not detected using the CE 10-plex.

The samples were also tested with the hexaplex HRM assay (Table 36). Saliva and semen were successfully detected in a majority of saliva-containing samples. Blood was successfully detected in the two samples containing blood. Vaginal secretions was not as successful using the hexaplex HRM assay, as is expected since the sensitivity of the hexaplex assay is not as good as the other HRM assays.

The results of the touch DNA and mock casework samples demonstrate the suitability of use of the developed RNA multiplex assays (CE and HRM) for use in routine casework. Body fluids were successfully identified in trace body fluid samples including admixed samples.

7. Precision and Accuracy

To assess the precision and accuracy of the CE and HRM assays, three donors were tested five times each (both biological and technical replicates). Metric averages (base pair size (CE); Tm value (HRM assays)) and variation (standard deviations) were calculated for each of the biomarkers in each multiplex system. These values were used as the basis for bin development for marker calling in each multiplex system.

The results for the CE 10-plex are provided in Table 37. As can be seen, highly reproducible results were obtained with either a 0.0 or 0.1 standard deviation for all biomarkers.

The results for the HRM assays are provided in Table 38. Again, highly reproducible results were obtained for all samples with standard deviations of only 0.0, 0.1 or 0.2 for all biomarkers.

E. Differential DNA/RNA Co-Extraction

Previously, semen-vaginal secretions mixtures were extracted using the AllPrep DNA/RNA co-extraction method. As was seen in Figure 15, an admixed DNA profile is often obtained as there is no separation of sperm and non-sperm fractions prior to analysis. For the DNA analysis of sexual assault evidence, it is routine to separate sperm and non-sperm fractions by a differential extraction in order to try and obtain single source DNA profiles of the contributors. Current RNA profiling methods are not fully compatible with differential extractions. We therefore began the development of a differential DNA/RNA co-extraction method using a modified AllPrep DNA/RNA co-extraction. A differential co-extraction method requires separation of sperm and non-sperm fractions prior to DNA and RNA isolation.

We evaluated various lysis buffers, lysis temperatures and times as well as various storage temperatures for the non-sperm lysate. We have developed a preliminary differential co-extraction protocol using diluted ATL buffer which is the current differential extraction lysis buffer used by the DFSC laboratory. The differential co-extraction protocol uses a quick 15-minute lysis with diluted ATL buffer. The sample is then centrifuged to pellet sperm cells and the non-sperm cell fraction is then transferred to a separate tube. The non-sperm cell fraction is stored during the subsequent one-hour sperm cell lysis (start of the developed AllPrep DNA/RNA co-extraction). After sperm cell lysis, the non-sperm and sperm cell fractions are then both ready for DNA and RNA isolation. A DNA and RNA sample is then obtained for both the sperm and non-sperm cell fractions. Preliminary results suggest that, with further optimization, a successful differential co-extraction method can be developed.

The prototype differential co-extraction protocol has been tested with simulated sexual assault mixtures (liquid semen added to dried vaginal swabs). Figures 17 and 18 show examples of semen-vaginal secretions mixtures that were extracted with the differential co-extraction protocol. In the example shown in Figure 17, a single source DNA profile of the female donor was obtained for the F1 non-sperm fraction and semen (PRM2) was identified using the CE 10-plex. Unexpectedly the vaginal secretions marker CYP2B7P1 was not detected. A single source DNA profile of the male donor was obtained for the F2 sperm fraction and PRM2 (semen) was identified using the CE 10-plex. This example highlights one of the issues with the current protocol – the failure to identify vaginal secretions RNA (CYP2B7P1) in the non-sperm fraction. However, it also demonstrates the successful separation of sperm and non-sperm fractions with single source DNA profiles obtained for both donors. Additionally, semen was correctly identified which is critical. While not ideal, it is not necessarily critical that vaginal secretions be identified particularly if the sample is a vaginal sample. In the example shown in Figure 18, vaginal secretions (CYP2B7P1) was successfully identified in the F1 non-sperm fraction. PRM2 was also present indicating some premature lysis of sperm cells during the initial lysis step, which is not unexpected. As a result of this premature lysis, some male alleles can be seen in the DNA profile obtained for this fraction. The major contributor is the female donor and the major contributor profile is discernible. For the sperm fraction, semen (PRM2) was identified and a single source male DNA profile was obtained.

These two examples demonstrate limited success of the differential co-extraction protocol and indicates the need for additional work in order to resolve a few critical issues: 1) failure to

detect CYP2B7P1 consistently in non-sperm fractions, 2) the need to detect SEMG1 in sperm fractions and 3) prevention of premature lysis into the non-sperm fraction in order to eliminate the presence (even if minor) of the male donor in the non-sperm fraction. However, overall the current prototype protocol permits successful single source STR profiles to be obtained which was the main goal of this protocol. Further optimization will hopefully improve RNA stability and biomarker detection.

V. CONCLUSIONS

The aims of the current work were to custom-develop for DFSC casework use: (i) a DNA/RNA co-extraction isolation protocol suitable for dried biological evidence material (including trace amounts); and (ii) a highly specific and robust mRNA body fluid multiplex system that permits the simultaneous identification of all forensically relevant biological fluids and tissues (blood, semen, saliva, vaginal secretions, menstrual blood and skin) in a single assay. As described here, we have achieved both aims of the project and have developed a comprehensive RNA-based body fluid identification system for use by the DFSC laboratory. This would possibly be the first implementation of such an approach in the U.S.

The DFSC body fluid identification system includes a DNA/RNA co-extraction method as well as highly specific and sensitive CE and HRM RNA profiling multiplex systems that permit an identification of all of the major forensically relevant body fluids and tissues. The developmental validation demonstrated the suitability of use of the BodyfluID system with a variety of casework type samples, admixed body fluid samples and environmentally compromised samples. A full technical protocol manual was also created and provided to the DFSC laboratory. Technology transfer of the protocols and assays has begun.

Despite the success of the developed RNA profiling assays, continued optimization and further development of enhanced RNA profiling assays may further support workflows of operational crime laboratories. This includes the full development of the differential co-extraction method as well as efforts to improve overall 'sample to detection' times for more high-throughput sample analysis.

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VII. TABLES

Table 1.Primer Sequences (top) and Primer Mix Composition (bottom) for the CE 10-plex assay

Primer sequences

Biomarker (Gene)	Primer sequence (5'-3')
ANK1	F: FAM-CAGCTGCTCCTCCTCAAG
	R: GCTGCTCTCAGAAAGCTGGTA
ALAS2	F: FAM-TGTGTCCGTCTGGTGTAGTA
	R: AAACTTACTGGTGCCTGAGA
PRM2	F: VIC-GGCGCAAAAGACGCTCC
	R: GCCCAGGAAGCTTAGTGCC
SEMG1	F: NED-TCGGTAACCATGTGAAAGGA
	R: CGGGAGTCTGCATCTCAGAA
STATH	F: NED-TCATCAGTAACTGCAGGACA
	R: AGGCAAGTATCCTGAAACAA
HTN3	F: NED-TTGCTTTAATCTTGGCTCTC
	R: GCGGTATGACAAATGAGAAT
CYP2B7P1	F: FAM-TCCTTTCTGAGGTTCCGAGA
	R: TTTCCATTGGCAAAGAGCAT
MMP10	F: FAM-TGACGTTGGTCACTTCAGCTC
	R: CTGGAGAATGTGAGTGGAGT
LEFTY2	F: PET-GCCCACGTGAGGGCCCAGTATGTAGT
	R: GGTGTGTGCTGGCCTCCGACGC
LCE1C	F: GCTGAAGGACCCTGTGCT
	R: CAGGACATCTTGGTGGCG

Primer mix

Biomarker	Conc (μM) stock primer	Vol (μl) F, R primers	Final conc (μM)
ANK1	200	5.0	1
ALAS2	100	0.45	0.045
PRM2	100	0.25	0.025
SEMG1	100	0.3	0.03
STATH	200	6.0	1.2
HTN3	200	4.5	0.9
CYP2B7P1	100	1.5	0.15
MMP10	100	0.75	0.075
LEFT2	100	4.0	0.4
LCE1C	100	0.3	0.03
Nuc. Free H$_2$O	NA	53.9	NA

100μl sufficient for 80 reactions

Table 2.Primer Sequences (Top) and Primer Mix Composition (Bottom) for the Blood-Menstrual Blood HRM Assay

Biomarker	Tm (°C)	Primer Sequence (5'-3')
MMP10	81.5, 82.0	F: GGGGGTGACGTTGGTCACTTCAGCTC
		R: GGGGGCTGGAGAATGTGAGTGGAGT
ANK1	83.2	F: GGCATGCCCTATTCTGTG
		R: CTTAGAAGCCAGATGCAAGC
ALAS2	86.1	F: TGTGTCCGTCTGGTGTAGTA
		R: AAACTTACTGGTGCCTGAGA
LEFTY2	89.0	F: GCCCACGTGAGGGCCCAGTATGTAGT
		R: GGTGTGTGCTGGCCTCCGACGC

Biomarker	Conc (µM) stock primer	Vol (µl) F, R primers	Final conc (µM) in assay
MMP10	40	7	0.0085
ANK1	40	10	0.121
ALAS2	40	8	0.097
LEFTY2	40	8	0.097

Table 3. Primer Sequences (Top) and Primer Mix Composition (Bottom) for the Sexual Assault HRM Assay

Biomarker	Tm (°C)	Primer Sequence (5'-3')
HTN3	77.0	F: GCAAAGAGACATCATGGGTA
		R: GCCAGTCAAACCTCCATAATC
SEMG1	78.7	F: TCGGTAACCATGTGAAAGGA
		R: TGAAACTACACGGGAGTCTGC
PRM2	84.4	F: GGCGCAAAAGACGCTCC
		R: GCCCAGGAAGCTTAGTGCC
CYP2B7P1	86.6	F: TCCTTTCTGAGGTTCCGAGA
		R: TTTCCATTGGCAAAGAGCAT

Biomarker	Conc (µM) stock primer	Vol (µl) F, R primers	Final conc (µM) in assay
HTN3	40	10	0.0077
SEMG1	40	12	0.092
PRM2	40	12	0.092
CYP2B7P1	40	18	0.1385

Table 4. Primer Sequences (Top) and Primer Mix Composition (Bottom) for the Hexaplex HRM Assay

Biomarker	Tm (°C)	Primer Sequence (5'-3')
HTN3	76.7	F: GCAAAGAGACATCATGGGTA
		R: GCCAGTCAAACCTCCATAATC
SEMG1	78.5	F: TCGGTAACCATGTGAAAGGA
		R: TGAAACTACACGGGAGTCTGC
LCE1C	81.7	F: GCTGAAGGACCCTGTGCT
		R: CAGGACATCTTGGTGGCG
ANK1	83.1	F: GGCATGCCCTATTCTGTG
		R: CTTAGAAGCCAGATGCAAGC
CYP2B7P1	86.4	F: TCCTTTCTGAGGTTCCGAGA
		R: TTTCCATTGGCAAAGAGCAT
LEFTY2	89.0	F: GCCCACGTGAGGGCCCAGTATGTAGT
		R: GGTGTGTGCTGGCCTCCGACGC

Biomarker	Conc (μM) stock primer	Vol (μl) F, R primers	Final conc (μM) in assay
HTN3	40	12	0.0552
SEMG1	40	14	0.0644
LCE1C	40	15	0.0690
ANK1	40	14	0.0644
CYP2B7P1	40	20	0.0920
LEFTY2	40	12	0.0552

Table 5. CE 10-plex Sensitivity

10-plex									
Input (ng)	Blood (n=3)		Semen (n=4*)		Saliva (n=3)		Vaginal (n=3)	Menstrual Blood (n=3)	
	ANK1	ALAS2	PRM2	SEMG1	STATH	HTN3	CYP2B7P1	MMP10	LEFTY2
15	+++	+++	+++-	++++	+++	+++	+++	+++	+++
10	+++	+++	+++-	+-++	+++	+++	+++	+++	+++
5	+++	+++	+++-	++++	-+-	+++	+++	+++	+++
1	+++	+++	+++-	--++	---	-+-	+++	+++	+-+
0.5	+++	+++	+++-	--++	---	---	-++	+++	++-
0.25	-++	-++	+-+-	--++	---	---	-++	++-	++-
0.1	---	-++	+-+-	----	---	-+-	-++	+++	+--
0.05	+--	+++	+---	----	---	---	---	++-	---

Each donor represented by a "+" (biomarker detected) or a "-" (biomarker not detected)
Dark grey > 1000 RFUs; **Medium grey** 500 – 999 RFUs; **Light grey** 50 – 499 RFUs; **No color** < 50 RFUs

Table 6. Blood-Menstrual Blood and Sexual Assault HRM Multiplex Sensitivity

Blood-Menstrual Blood (4-plex)				
Input (ng)	**Blood (n=3)**		**Menstrual Blood (n=3)**	
	ANK1	ALAS2	MMP10	LEFTY2
	83.0°C	86.2°C	82.3°C	89.2°C
15	+++	+++	+++	+++
10	+++	+++	+++	+++
5	+++	+++	+++	+++
1	+++	+++	+++	+-+
0.5	+++	+++	+++	+++
0.25	+++	--+	+++	++-
0.1	-++	---	+++	+-+
0.05	--+	---	++-	+--

Sexual Assault Multiplex (4-plex)			
Input (ng)	**Semen (n=4)**		**Saliva (n=3)**	**Vaginal (n=3)**
	SEMG1	PRM2	HTN3	CYP2B7P1
	78.6°C	84.2°C	76.8°C	86.7°C
15	++++	+++-	+++	+++
10	++++	+++-	+++	+++
5	++++	+++-	+++	+++
1	++++	+++-	+++	-++
0.5	++++	+++-	--+	--+
0.25	++++	+++-	++-	---
0.1	--++	+++-	---	---
0.05	++++	+++-	---	---

*4th semen donor included was a vasectomized male; only SEMG1 expected for this donor

Each donor represented by a "+" (biomarker detected) or a "-" (biomarker not detected). **Dark grey -** 3/3 donors; **Medium grey**- 2/3 donors; **Light grey**- 1/3 donors; **No color**- no donors detected. Average Tm (°C) values are provided below each biomarker name.

Table 7. Hexaplex HRM Assay Sensitivity

Input (ng)	Blood (n=3) ANK1 83.3°C	Semen (n=4) SEMG1 78.9°C	Saliva (n=3) HTN3 77.1°C	Vaginal (n=3) CYP2B7P1 86.5°C	Menstrual Blood (n=3) LEFTY2 89.1°C	Skin LCE1C
15	+++	++++	+++	+++	+++	NT
10	+++	++++	+++	+++	+++	NT
5	+++	++++	+++	+++	-+-	NT
1	++-	++++	+++	-++	+--	NT
0.5	-+-	+-++	+++	-+-	+--	NT
0.25	---	-+++	++-	---	+--	NT
0.1	---	--++	+--	---	-+-	NT
0.05	---	-+++	+-+	---	--+	NT

Each donor represented by a "+" (biomarker detected) or a "-" (biomarker not detected)
Dark grey 3/3 donors; **Medium grey** 2/3 donors; **Light grey** 1/3 donors; **No color** no donors detected

Table 8. Comparison of Biomarker Sensitivity within Each Multiplex Assay

Body Fluid	Biomarker	CE 10-plex	BD-MB	Sex-plex	Hexaplex
Blood	ANK1	50 pg	50 pg		500 pg
	ALAS2	50 pg	250 pg		
Semen	PRM2	50 pg		50 pg	
	SEMG1	250 pg		50 pg	50 pg
Saliva	HTN3	1 ng		250 pg	50 pg
	STATH	5 ng			
Vaginal	CYP2B7P1	100 pg		500 pg	500 pg
Menstrual	MMP10	50 pg	50 pg		
	LEFTY2	100 pg	50 pg		50 pg

Table 9. Specificity - CE 10-plex and HRM Multiplex Assays

The specificity of each multiplex assay (CE 10-plex, blood-menstrual blood HRM, sexual assault HRM and hexaplex HRM) was assessed by an evaluation of numerous donors of each body fluid and tissue for each system. For each assay, the number of individual body fluid donors positive each biomarker out of the total number of donors tested is shown. For the skin samples, twenty touch samples were evaluated (a swab of headphones, computer mouse, backpack or purse strap and car steering wheel from five different donors).

CE-10plex

Body Fluid	ANK1	ALAS2	PRM2	SEMG1	STATH	HTN3	CYP2B7P1	MMP10	LEFTY2	LCE1C
Blood	14/14	14/14								
Semen			13/14	13/14						
Saliva					8/11	11/11				
Vaginal Secretions							15/15			3/15
Menstrual Blood	3/8	6/8					7/8	7/8	6/8	
Skin (Touch)										9/20

BD-MB

Body Fluid	ANK1	ALAS2	MMP10	LEFTY2
Blood	15/15	15/15	0/15	0/15
Menstrual Blood	3/7	4/7	7/7	6/7
Saliva	0/12	0/12	0/12	0/12
Semen	0/11	0/11	0/11	0/11
Vaginal Secretions	0/13	0/13	4/13	0/13
Skin	0/20	0/20	0/20	0/20

S. Assault

Body Fluid	HTN3	SEMG1	PRM2	CYP2B7P1
Saliva	12/12	0/12	0/12	0/12
Semen	0/15	14/15	13/15	0/15
Vaginal Secretions	0/11	0/11	0/11	10/11
Skin	0/20	0/20	0/20	0/20
Blood	0/12	0/12	0/12	0/12
Menstrual Blood	0/9	0/9	0/9	7/9

Hexaplex

Body Fluid	HTN3	SEMG1	LCE1C	ANK1	CYP2B7P1	LEFTY2
Saliva	14/14	0/14	0/14	0/14	0/14	0/14
Semen	0/14	13/14	0/14	0/14	0/14	0/14
Skin	0/20	0/20	8/20	0/20	0/20	0/20
Blood	0/12	0/12	0/12	11/12	0/12	0/12
Vaginal Secretions	0/14	0/14	0/14	0/14	11/14	0/14
Menstrual Blood	0/7	0/7	3/7	1/7	3/7	6/7

Table 10. Tissue Specificity – CE 10-plex Multiplex

The specificity of the CE 10-plex multiplex system was further evaluated with a panel of 18 tissue samples. The tissue samples were purchased as total RNA pools from Life Technologies. The RFU values of any detected products are included in the table. Reference samples for each body fluid (blood, semen, saliva, vaginal secretions, menstrual blood and skin) are provided.

Tissue	Blood		Semen		Saliva		Vaginal	Menstrual		Skin
	ANK1	ALAS2	PRM2	SEMG1	STATH	HTN3	CYP2B7P1	MMP10	LEFTY2	LCE1C
Trachea					1189		282	1219		
					180.31		197.99	168.29		
Kidney										
Ovary										
Adipose										
Placenta	112	244						343	141	
	113.59	134.36						168.15	127.24	
Spleen	112									
	113.58									
Liver							710			
							197.95			
Esophagus										
Small Intestine							723			
							197.96			
Lung							1588			
							197.97			
Colon										
Thymus										
Bladder										
Thyroid										
Cervix										
Skeletal Muscle										
Brain										
Heart										
Blood	718	2369								
Semen			9333	3390						
Saliva					3289	8204				
Vaginal							9038			222	539
Menstrual	250	1870					1857	9058	5805	404
Skin								6956		6453

Table 11. Tissue Specificity, Additional Donors – CE 10-plex Multiplex

Expression of CYP2B7P1 was evaluated in an additional liver, small intestine and lung sample. These total RNA samples were obtained from Biochain and therefore are different than those used in original testing. The RFU values of any detected products are included in the table. The results from the original tissue sample is shown with the additional testing of the Biochain tissue samples provided below the original results for each tissue.

Tissue	Blood		Semen		Saliva		Vaginal	Menstrual		Skin
	ANK1	ALAS2	PRM2	SEMG1	STATH	HTN3	CYP2B7P1	MMP10	LEFTY2	LCE1C
Liver							710			
Liver - Biochain										
Lung							1588			
Lung - Biochain							709			
Small Intestine							723			
Sm. Intestine - Biochain							213			

Table 12. Tissue Specificity – Blood Menstrual Blood HRM Assay

The specificity of the blood-menstrual blood HRM assay was further evaluated with a panel of 18 tissue samples. The tissue samples were purchased as total RNA pools from Life Technologies. The Tm (^{o}C) values of any detected products are included in the table. Reference samples for each body fluid (blood and menstrual blood) are provided.

Tissue (1ng input)	MMP10 82.3°C	ANK1 83.0°C	ALAS2 86.2°C	LEFTY2 89.2°C
Trachea	81.9			
Kidney				
Ovary				88.7
Adipose				
Placenta	81.6			88.7
Spleen		82.7		
Liver				
Esophagus				
Small Intestine				
Lung				
Colon				
Thymus				
Bladder				
Thyroid				
Cervix				
Skeletal Muscle				
Brain				
Heart				
Blood		83.1	86.2	
Menstrual Blood	82.2	83.0	86.0	89.1

Table 13. Tissue Specificity – Sexual Assault HRM Assay

The specificity of the sexual assault HRM assay was further evaluated with a panel of 18 tissue samples. The tissue samples were purchased as total RNA pools from Life Technologies. The Tm (^{o}C) values of any detected products are included in the table. Reference samples for each body fluid (saliva, semen and vaginal secretions) are provided.

Sexual Assault (4-plex)			
Tissue (1ng input)	HTN3 76.8°C	SEMG1 78.6°C	PRM2 84.2°C	CYP2B7P1 86.7°C
Trachea				
Kidney				
Ovary				
Adipose				
Placenta				
Spleen				
Liver				86.2
Esophagus				
Small intestine				86.2
Lung				86.2
Colon				
Thymus				
Bladder		78.2		
Thyroid				
Cervix				
Skeletal Muscle				
Brain				
Heart				
Semen		78.7	84.4	
Saliva	76.7			
Vaginal				86.6

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Table 14. Tissue Specificity – Hexaplex HRM Assay

The specificity of the hexaplex HRM assay was further evaluated with a panel of 18 tissue samples. The tissue samples were purchased as total RNA pools from Life Technologies. The Tm (^{o}C) values of any detected products are included in the table. Reference samples for each body fluid (blood, semen, saliva, vaginal secretions, menstrual blood and skin) are provided.

Tissue	Hexaplex (6-plex)					
(1ng input)	HTN3	SEMG1	LCE1C	ANK1	CYP2B7P1	LEFTY2
	77.1°C	78.9°C	81.5°C	83.3°C	86.5°C	89.1°C
Trachea						
Kidney						
Ovary						88.8
Adipose						
Placenta						88.9
Spleen				83.0		
Liver						
Esophagus						
Small Intestine						
Lung						
Colon						
Thymus						
Bladder		78.1				
Thyroid						
Cervix						
Skeletal Muscle						
Brain						
Heart						
Blood				83.4		
Semen		78.7				
Saliva	76.7					
Vaginal					86.5	
Menstrual				83.1		89.0
Skin			81.7			

Table 15.Species Specificity (Blood) – CE 10-plex Multiplex

Blood samples from 18 animals and 10 primates were tested to evaluate the species specificity of the blood biomarkers (ANK1, ALAS2) in the CE 10-plex, Blood-Menstrual HRM, and hexaplex HRM assays. The RFU values for any observed products (ANK1, ALAS2) are provided for the CE assays. The Tm value for any observed products (ANK1, ALAS2) are provided for the HRM assays. Positive results are highlighted in red.

	Sample	CE		HRM-BD-MB		HRM-6plex
		ANK1	ALAS2	ANK1	ALAS2	ANK1
Animal	Alligator					
	Duck					
	Frog					
	Mouse					
	Opposum					
	Rabbit		1239		85.1	
	Rooster					
	Guinea Pig					
	Turtle					
	Cat		592			
	Cow					
	Deer					
	Dog					
	Goat					
	Horse					
	Pig					
	Sheep					
	Ferret		231			
Primate	Crab eating macaque	999	9043		86.0	
	Baboon	81	5106		86.0	
	Chimp	331	9027	83.0	86.1	83.3
	Spot-Nosed Guenon	7929	9176		85.6	
	Spider Monkey					
	Black Howler Monkey		3022	82.3	85.1	
	Pig tailed Macaque	917	8911		86.0	
	Brown Lemur					
	Rhesus Monkey					
	African Green Monkey		129			

Table 16. Species Specificity (Blood) – Additional cat and ferret samples

Additional ferret (8 additional, ferret 2-9) and cat (1 additional, cat 2) blood samples were tested to evaluate the species specificity of the blood biomarkers (ANK1, ALAS2) in the CE 10-plex, Blood-Menstrual HRM, and hexaplex HRM assays. The RFU values for any observed products (ANK1, ALAS2) are provided for the CE assays. The Tm value for any observed products (ANK1, ALAS2) are provided for the HRM assays. Positive results are highlighted in red.

CE

Sample	Description	Blood		Semen		Saliva		Vaginal	Menstrual		Skin
		ANK1	ALAS2	PRM2	SEMG1	STATH	HTN3	CYP2B7P1	MMP10	LEFTY2	LCE1C
ferret 1	dark eyed white		231								
ferret 2	cinnamon										
ferret 3	chocolate										
ferret 4	siamese										
ferret 5	sable										
ferret 6	albino										
ferret 7	silver mitt										
ferret 8	sable blaze										
ferret 9	silver										
cat 1	?		592								
cat 2	domestic short hair										
Blood	positive control										

BD–MB

Sample	Description	MMP10	ANK1	ALAS2	LEFTY2
ferret 1	dark eyed white				
ferret 2	cinnamon				
ferret 3	chocolate				
ferret 4	siamese				
ferret 5	sable				
ferret 6	albino				
ferret 7	silver mitt				
ferret 8	sable blaze				
ferret 9	silver				
cat 1	?				
cat 2	domestic short hair				
Blood	positive control		83.0	86.0	

Additional ferret and cat samples tested – No detection

Hexaplex

Sample	Description	HTN3	SEMG1	LCE1C	ANK1	CYP2B7P1	LEFTY2
ferret 1	dark eyed white						
ferret 2	cinnamon						
ferret 3	chocolate						
ferret 4	siamese						
ferret 5	sable						
ferret 6	albino						
ferret 7	silver mitt						
ferret 8	sable blaze						
ferret 9	silver						
cat 1							
cat 2	domestic short hair						
Blood	positive control				83.1		

Table 17. Stability (Environmentally Compromised Samples) - Blood

Blood samples exposed to various temperatures and incubation times, as well as heat, light, humidity and rain (outside samples) were evaluated with the CE 10-plex and blood-menstrual blood (BD-MB) and hexaplex HRM assays. The results from each assay are provided: CE – RFU values recorded; HRM – Tm values recorded. Red cells indicate a positive blood result; grey cells indicate a negative result.

	Sample #	Sample	CE		BD-MB HRM		Hexaplex
			ANK1	ALAS2	ANK1	ALAS2	ANK1
Blood	1	M1 room temp 1 year	4342	5792	83.3	86.4	83.4
	2	M1 room temp 2 yr	951	153	83.3	86.4	83.5
	3	M1 37oC 1 yr	688	1825	83.3	86.4	83.4
	4	M1 37oC 2 yr	984	1086	83.4		83.5
	5	M1 56oC 1 yr	573	477	83.4		
	6	M1 56oC 2 yr	350				
	7	F2 room temp 1 year	1898	8215	83.2	86.3	83.4
	8	F2 room temp 2 yr		125			
	9	F2 37oC 1 yr					
	10	F2 37oC 2 yr				86.4	
	11	F2 56oC 1 yr	167	178		86.3	
	12	F2 56oC 2 yr	314	575	83.4	86.2	
	13	FS1 patio 1 month	9068	9077	83.1	86.3	83.4
	14	FS1 patio 2 months	3087	3969	83.2	86.3	83.5
	15	FSI patio 3 months	140	1374	83.3	86.4	
	16	FS1 patio 4 months	2754	3397	83.2	86.3	83.5
	17	SFS1 sun 1 week					
	18	FS1 sun 1 month					
	19	FS1 sun 2 months					
	20	FS1 shade 1 month					
	21	FS1 shade 2 months					
	22	FS1 shade 6 months					
	23	CM24 car back seat 1 week	9216	8106	83.2	86.3	83.4
	24	FM3 car back seat 1 week	9113	9125	83.1	86.3	83.4
	25	CM24 car trunk 2 weeks	8942	8999	83.2	86.4	83.4
	26	FM3 car trunk 2 weeks	8840	9017	83.1	86.3	83.4

M1, F2, FS1, CM24, FM3 are sample name designations. Patio samples were exposed to heat and humidity but protected from direct sunlight and rain. Sun samples were exposed to heat, humidity, rain and direct sunlight. Shade samples were exposed to heat, humidity and rain but mainly protected from direct sunlight.

Table 18. Stability (Environmentally Compromised Samples) - Semen
Semen samples exposed to various temperatures and incubation times, as well as heat, light, humidity and rain (outside samples) were evaluated with the CE 10-plex and the sexual assault and hexaplex HRM assays. The results from each assay are provided: CE – RFU values recorded; HRM – Tm values recorded. Yellow cells indicate a positive semen result; grey cells indicate a negative result. Outside covered – exposed to heat, light, humidity; patio – exposed to heat and humidity; sun – direct sunlight, heat, humidity and rain; shade – non-direct sunlight, heat, humidity and rain.

Samples			CE 10plex		HRM - S.Assault.	
			SEMG1	PRM2	SEMG1	PRM2
Semen	1	M1 room temp 1 year	1448	9485	78.7	84.5
	2	M1 room temp 2 yr	889	9516	78.7	84.5
	3	M1 37oC 1 yr	1291	9469	78.7	84.5
	4	M1 37oC 2 yr	460	8467	78.8	84.6
	5	M1 56oC 1 yr	346	7706	78.6	84.4
	6	M1 56oC 2 yr	1199	9150	78.7	84.4
	7	M2 room temp 1 year	409	4156		84.4
	8	M2 room temp 2 yr	204	945		84.4
	9	M2 37oC 1 yr	106	913	78.9	84.5
	10	M2 37oC 2 yr		244	78.8	84.5
	11	M2 56oC 1 yr		45		84.5
	12	M2 56oC 2 yr	397	1932	78.8	84.6
	13	M2 outside covered 1 day	685	6981	78.8	84.6
	14	M2 outside covered 3 days		102		84.6
	15	M2 outside covered 1 week		2604		84.6
	16	M1 outside covered 3 days	64	1342		84.6
	17	SE UV > 1 yr			78.9	84.5
	18	JSE sun 1 day	81	1453		84.5
	19	JSE sun 2 days	90	3675	78.8	84.5
	20	JSE sun 12 days				
	21	JSE sun 1 month				
	22	JSE patio 1 day	704	9454	78.9	84.6
	23	JSE patio 2 days	120	1385	78.9	84.6
	24	JSE patio 12 days		267		84.7
	25	JSE patio 1 month		2338	78.9	84.7
	26	JSE shade 1 day	278	7973	79.0	84.6
	27	JSE shade 2 days		3197	78.8	84.5
	28	JSE shade 12 days		524	78.7	84.6
	29	JSE shade 1 month				84.7

M1, M2 and JSE are sample name designations. Patio samples were exposed to heat and humidity but protected from direct sunlight and rain. Sun samples were exposed to heat, humidity, rain and direct sunlight. Shade samples were exposed to heat, humidity and rain but mainly protected from direct sunlight.

Table 19. Stability (Environmentally Compromised Samples) - Saliva

Saliva samples exposed to various temperatures and incubation times, as well as heat, light, humidity and rain (outside samples) were evaluated with the CE 10-plex and the sexual assault and hexaplex HRM assays. The results from each assay are provided: CE – RFU values recorded; HRM – Tm values recorded. Blue cells indicate a positive saliva result; grey cells indicate a negative result. Outside covered – exposed to heat, light, humidity; outside uncovered – exposed to sunlight, heat, humidity and rain; patio – exposed to heat and humidity; sun – direct sunlight, heat, humidity and rain; shade – non-direct sunlight, heat, humidity and rain.

	Samples	CE 10plex STATH	CE 10plex HTN3	HRM - S.Assault. HTN3	HRM - S.Assault. HTN3
1	M2 room temp 1 year		229		77.3
2	M2 room temp 2 yr				77.4
3	M2 37oC 1 yr		1300	76.9	77.4
4	M2 37oC 2 yr				77.5
5	M2 56oC 1 yr	311		76.8	77.5
6	M2 56oC 2 yr		172		77.6
7	F2 room temp 1 year	314	391	76.9	77.5
8	F2 room temp 2 yr				77.5
9	F2 37oC 1 yr		237		77.8
10	F2 37oC 2 yr				
11	F2 56oC 1 yr				
12	F2 56oC 2 yr				
13	M2 outside covered 1 day				77.8
14	M2 outside uncovered 1 day				
15	M2 outside uncovered 3 days				77.6
16	F2 outside covered 1 day				
17	FS1 sun 1 day				
18	FS1 sun 2 days				
19	FS1 sun 1 week			76.5	77.8
20	FS1 sun 1 month				77.5
21	FS1 Shade 1 day	7062	9569	76.7	77.4
22	FS1 shade 2 days	2957	9224	76.7	77.5
23	FS1 shade 1 month				
24	FS1 patio 1 day	3606	9638		77.3
25	FS1 patio 2 days	7098	9607	76.5	77.3
26	FS1 patio 1 week	3505	2281	76.5	77.2
27	FS1 patio 1 month				

(Saliva)

M2, F2, and FS1 are sample name designations. Patio samples were exposed to heat and humidity but protected from direct sunlight and rain. Sun samples were exposed to heat, humidity, rain and direct sunlight. Shade samples were exposed to heat, humidity and rain but mainly protected from direct sunlight.

Table 20. Stability (Environmentally Compromised Samples) – Vaginal secretions

Vaginal secretions samples exposed to various temperatures and incubation times, as well as heat, light, humidity and rain (outside samples) were evaluated with the CE 10-plex and the sexual assault and hexaplex HRM assays. The results from each assay are provided: CE – RFU values recorded; HRM – Tm values recorded. Green cells indicate a positive saliva result; grey cells indicate a negative result. Outside covered – exposed to heat, light, humidity; outside uncovered – exposed to sunlight, heat, humidity and rain.

		Samples	CE 10plex CYP2B7P1	HRM Sex. Assault. CYP2B7P1	Hexaplex CYP2B7P1
Vaginal	1	F1 room temp 1 year	739	86.6	86.7
	2	F1 room temp 2 yr	204		
	3	F1 37oC 1 yr	1345	86.7	86.7
	4	F1 37oC 2 yr	580		
	5	F1 56oC 1 yr			
	6	F1 56oC 2 yr			
	7	F2 room temp 1 year			
	8	F2 room temp 2 yr			
	9	F2 37oC 1 yr			
	10	F2 37oC 2 yr			
	11	F2 56oC 1 yr			
	12	F2 56oC 2 yr			
	13	F1 outside covered 1 day	388		
	14	F2 outside covered 1 day	146		
	15	F2 outside covered 3 days			
	16	F2 outside covered 1 week			
	17	F2 outside covered 1 month			
	18	F2 outside uncovered 1 day			
	19	F2 outside uncovered 1 month			

*F1 and F2 are sample name designations. Outside covered samples were exposed to heat, light and humidity but protected from rain. Outside uncovered samples were exposed to heat, light, humidity and rain.

Table 21. Stability (Environmentally Compromised Samples) - Additional Samples

Semen and saliva samples exposed to various temperatures and incubation times, as well as heat, light, humidity and rain (outside samples) or deposited onto denim were evaluated with the CE 10-plex and blood-menstrual blood (BD-MB) and hexaplex HRM assays. The results from each assay are provided: CE – RFU values recorded; HRM – Tm values recorded. Shading indicates positive detection of body fluid biomarkers (yellow - semen and blue - saliva). Grey shading indicates no biomarker detection.

CE

Sample	Blood ANK1	Blood ALAS2	Semen PRM2	Semen SEMG1	Saliva STATH	Saliva HTN3	Vaginal CYP2B7P1	Menstrual MMP10	Menstrual LEFTY2	Skin LCE1C
semen outside covered 1 day			2238	96						
semen outside covered 7 day										
semen 37oC 3m			9268	2528						
semen 56oC 1m			9027	1783						
saliva outside covered 1 day										
saliva outside covered 7 day										
saliva 37oC 3m						82				
saliva 56oC 1m										
semen denim RT 6m			9406	232						
saliva denim RT 6m										

Additional UCF Environmental Samples

Sex-plex

Sample	HTN3 76.8	HTN3 77.1	SEMG1 78.6	PRM2 84.2	CYP2B7P1 86.7
semen outside covered 1 day			78.6	84.3	
semen outside covered 7 day					
semen 37oC 3m			78.6	84.4	
semen 56oC 1m			78.6	84.4	
saliva outside covered 1 day		77.1			
saliva outside covered 7 day		77.0			
saliva 37oC 3m	76.4	77.1			
saliva 56oC 1m					
semen denim RT 6m			78.6	84.4	
saliva denim RT 6m					

Hexaplex

Sample	HTN3 76.5	HTN3 77.1	SEMG1 78.9	LCE1C 81.5	ANK1 83.3	CYP2B7P1 86.5	LEFTY2 89.1
semen outside covered 1 day			79.0				
semen outside covered 7 day							
semen 37oC 3m			79.0				
semen 56oC 1m			79.0				
saliva outside covered 1 day		77.1					
saliva outside covered 7 day		77.2					
saliva 37oC 3m	76.6	77.2					
saliva 56oC 1m				82.0			
semen denim RT 6m			78.9				
saliva denim RT 6m		77.1					

48

44

Table 22. Detection of Vaginal Secretions in Female Reproductive Cycles - CE 10plex

Vaginal swabs were collected each day during a 28-day (left) or a 25-day (middle) reproductive cycle from two menstruating female donors, with day 1 as the start of menstruation. Menstruation was reported to be on days 1-4 (day 4 light menstruation was reported). Vaginal swabs were collected each day during a 28-day period from a menopausal female donor (right). The samples were analyzed using the CE 10-plex multiplex assay. RFU values of CYP2B7P1 (vaginal), MMP10 and LEFTY2 (menstrual blood), if detected, are shown. Green and pink shading represents detection of the biomarker. Grey and white cells represent no detection.

Donor 1 - menstruating

Day of Cycle	Menstruation reported?	Vaginal CYP2B7P1	Menstrual MMP10	Menstrual LEFTY2
1	yes	447		
2	yes		2829	221
3	yes	327	9055	848
4	yes (light)			
5	no	573		
6	no	122	161	
7	no			
8	no			
9	no			
10	no	225		
11	no	1131		
12	no	547		
13	no	844		
14	no	2369		
15	no	2427		
16	no	689		
17	no	800		
18	no	686		
19	no	2648		
20	no			
21	no	4648		
22	no	2213		
23	no	633		
24	no	376		
25	no	2542		
26	no	1131		
27	no	8901		
28	no	9012		

Donor 2 - menstruating

Day of Cycle	Menstruation reported?	Vaginal CYP2B7P1	Menstrual MMP10	Menstrual LEFTY2
1	yes	1195	9102	7506
2	yes	5652	5788	254
3	yes	3424	903	
4	yes (light)	9072	5216	103
5	no	9124	3421	
6	no	9075	2553	
7	no	218		
8	no	8865		
9	no	2966		
10	no	596		
11	no	769		
12	no	439		
13	no			
14	no			
15	no	8764		
16	no			
17	no	9084		
18	no	8297		
19	no	9097		
20	no	9093		
21	no	9078		
22	no	9085		
23	no	8839		
24	no	8848		
25	no	418		

Donor 3 - menopausal

Day of Cycle	Vaginal CYP2B7P1	Menstrual MMP10	Menstrual LEFTY2
1	8857		
2	9102		
3	1479		
4	8882		
5	8859		
6	9097		
7	9178		
8	9007		
9	121		
10	3621		
11	4025		
12	9092		
13	8166		
14	7119		
15	9109		
16	9093		
17	7811		
18	9099		
19	9041		
20	9088		
21	1623		
22	1998		
23	9111		
24	7937		
25	8453		
26	8300		
27	8834		
28	8847		

Table 23. Detection of Menstrual Blood in Female Reproductive Cycles (Menstruating Females) - Blood Menstrual Blood HRM

Vaginal swabs were collected each day during a 28-day (left) or a 25-day (right) reproductive cycle, with day 1 as the start of menstruation. Menstruation was reported to be on days 1-4 (day 4 light menstruation was reported). The samples were analyzed using the blood-menstrual blood HRM assays. Tm (^{o}C) values of detected biomarkers (and DNA) are recorded. Pink shading represents detection of a menstrual blood biomarker. Red shading represents detection of a blood biomarker. Grey cells represent no detection.

Day of cycle	Menstrual blood reported?	MMP10		ANK1	ALAS2	LEFTY2
1	yes					
2	yes	81.9		83.0		89.0
3	yes	81.9				89.0
4	yes (light)					
5	no					
6	no					
7	no					
8	no					
9	no					
10	no					
11	no					
12	no					
13	no					
14	no					
15	no					
16	no					
17	no					
18	no					
19	no					
20	no					
21	no					
22	no					
23	no					
24	no					
25	no					
26	no					
27	no					
28	no					

Day of cycle	Menstrual blood reported?	DNA		MMP10	ANK1	ALAS2	LEFTY2
1	yes			82.0			88.9
2	yes			82.0			88.9
3	yes			82.2			
4	yes (light)			82.2			
5	no	78.9	80.3	82.3	83.1		
6	no	78.5	80.3	82.2			
7	no	79.0	80.4				
8	no	79.0	80.4				
9	no	79.0	80.4				
10	no	79.0	80.4				
11	no	79.1	80.6				
12	no						
13	no	79.1	80.6				
14	no	79.2	80.6				
15	no	79.0	80.4				
16	no	79.1					
17	no	79.0	80.5				
18	no	78.9	80.5				
19	no	78.9	80.5				
20	no	78.8	80.5				
21	no	79.0	80.5				
22	no	79.0	80.5				
23	no	79.0	80.4				
24	no	79.0	80.4				
25	no	79.0	80.4				

Table 24. Detection of Vaginal Secretions in Female Reproductive Cycles - Sexual Assault HRM

Vaginal swabs were collected each day during a 28-day (left) or a 25-day (middle) reproductive cycle from two menstruating female donors, with day 1 as the start of menstruation. Menstruation was reported to be on days 1-4 (day 4 light menstruation was reported). Vaginal swabs were collected each day during a 28-day period from a menopausal female donor (right). The samples were analyzed using the sexual assault HRM assay. Tm (^{o}C) values of CYP2B7P1 (vaginal) if detected are shown. Green shading represents detection of the biomarker. Grey cells represent no detection.

Day of cycle	Menstrual blood reported?	CYP2B7P1	Day of cycle	Menstrual blood reported?	CYP2B7P1	Donor	Day of cycle	CYP2B7P1
1	yes	86.3	1	yes	86.5		1	86.5
2	yes		2	yes	86.4		2	86.4
3	yes	86.3	3	yes	86.5		3	86.4
4	yes (light)		4	yes (light)	86.4		4	86.4
5	no	86.4	5	no	86.4		5	86.4
6	no		6	no	86.4		6	86.5
7	no		7	no			7	86.4
8	no		8	no	86.4		8	86.4
9	no		9	no	86.5		9	
10	no	86.3	10	no	86.4		10	
11	no	86.3	11	no	86.5		11	86.5
12	no	86.4	12	no			12	86.4
13	no	86.4	13	no			13	86.4
14	no	86.4	14	no		Donor FM3	14	86.5
15	no	86.4	15	no	86.4		15	86.4
16	no	86.4	16	no			16	86.3
17	no	86.4	17	no	86.4		17	86.4
18	no	86.3	18	no	86.4		18	86.4
19	no	86.3	19	no	86.4		19	86.4
20	no		20	no	86.3		20	86.4
21	no	86.5	21	no	86.4		21	86.5
22	no	86.4	22	no	86.3		22	86.4
23	no	86.5	23	no	86.4		23	86.4
24	no		24	no	86.4		24	86.4
25	no	86.5	25	no			25	86.4
26	no	86.4					26	86.5
27	no	86.4					27	86.5
28	no	86.4					28	

Table 25. Detection of Body Fluids in Two Fluid Admixtures (CE-10plex)

Two donor (fluid) admixtures were created by placing varying volumes (50, 25, 10 and 5μl) of a second liquid body fluid on top of a dried stain. The dried component is listed first in each mixture with the liquid component listed second. The presence of biomarkers for each of the body fluids is represented by a "+" symbol. Two "+" signs indicate the presence of both biomarkers for that body fluid: blood – ANK1, ALAS2; semen – PRM2, SEMG1; saliva – STATH, HTN3; menstrual blood – MMP10, LEFTY2. Only one biomarker is present in the multiplex for vaginal secretions (CYP2B7P1) and skin (LCE1C) and therefore only one "+" is possible. A "-" indicates the failure to detect biomarkers for the body fluid. The "+" and "-" are only listed for the body fluids in which a positive result was expected.

Mixture	Vol (μl) liquid	Blood	Semen	Saliva	Vaginal	Menstrual	Skin
BD-SE	50	++	+-				
	25	++	+-				
	10	++	++				
	5	++	+-				
BD-SA	50	++		++			
	25	++		++			
	10	++		++			
	5	++		++			
SA-SE	50		+-	--			
	25		+-	-+			
	10		+-	--			
	5		+-	-+			
VS-SE	50		++		+		
	25		--		+		
	10		+-		+		
	5		+-		+		
MB-SE	50		--			--	
	25		+-			+-	
	10		+-			++	
	5	-+	++			++	
VS-SA	50			--	+		
	25			--	+		
	10			--	+		
	5			--	+		
MB-SA	50	-+		--	+	++	
	25	-+		--	+	+-	
	10	-+		++	+	++	
	5	-+		--	+	++	

Table 26. Analysis of Two-Fluid Admixed Body Fluid Samples (Blood-Menstrual Blood HRM Assay)

For each mixture, the first body fluid listed was a dried stain to which the second body fluid (underlined) in liquid form was placed on the dried stain thus creating admixed body fluid samples. For the dried stains, blood samples were 50µl bloodstains and for all other body fluids ½ swabs were used. For the liquid body fluid added, we evaluated four different volumes (50µl, 25µl, 10µl and 5µl). Tm (^{o}C) values of detected biomarkers are recorded. Pink shading represents detection of a menstrual blood biomarker. Red shading represents detection of a blood biomarker. Grey cells represent no detection.

Mixture	Vol(µl) - liquid	MMP10		ANK1	ALAS2	LEFTY2
BD-SE	50			83.0	86.1	
	25			83.0	86.1	
	10			82.9	86.1	
	5			82.9	86.1	
BD-SA	50			83.0	86.2	
	25			83.0	86.2	
	10			83.0	86.3	
	5			83.0	86.1	
SA-SE	50					
	25					
	10					
	5					
VS-SE	50					
	25					
	10					
	5					
MB-SE	50					
	25	81.7	82.2			
	10	81.6	82.0			89.1
	5	81.5	82.0	83.0		89.1
VS-SA	50					
	25					
	10		81.1			
	5					
MB-SA	50		82.1			89.1
	25*		82.0			89.0
	10	81.9				89.1
	5	81.8		82.9		89.0

BD = blood, SE = semen, SA = saliva; VS = vaginal secretions; MB = menstrual blood; SK = skin

** present, but below threshold

Table 27. Analysis of Two-Fluid Admixed Body Fluid Samples (Sexual Assault HRM Assay)

For each mixture, the first body fluid listed was a dried stain to which the second body fluid (underlined) in liquid form was placed on the dried stain thus creating admixed body fluid samples. For the dried stains, blood samples were 50µl bloodstains and for all other body fluids ½ swabs were used. For the liquid body fluid added, we evaluated four different volumes (50µl, 25µl, 10µl and 5µl). Tm (^{o}C) values of detected biomarkers (and DNA) are recorded. Blue shading represents detection of saliva (HTN3). Yellow shading represents detection of semen (SEMG1, PRM2). Green shading represents detection of vaginal secretions (CYP2B7P1). White cells represent no detection. Grey cells represent no detection in samples where that biomarker would have been expected.

Mixture	Vol(µl) - liquid	HTN3		SEMG1	PRM2	CYP2B7P1
BD-SE	50			78.5	84.2	
	25			78.6	84.3	
	10			78.6	84.3	
	5			78.5	84.2	
BD-SA	50	76.2	76.9			
	25	76.3	77.0			
	10	76.2	77.0			
	5	76.2	76.9			
SA-SE	50		(grey)		84.2	
	25		76.9		84.3	
	10		(grey)		84.3	
	5		77.0		84.3	
VS-SE	50			78.5	84.2	86.5
	25*			78.0	84.3	(grey)
	10			78.6	84.2	86.5
	5			(grey)	84.2	86.5
MB-SE	50			(grey)	(grey)	
	25			78.5	84.2	
	10			78.5	84.2	
	5			78.5	84.2	
VS-SA	50		76.9			86.4
	25		76.6			86.3
	10		(grey)			86.4
	5		(grey)			86.3
MB-SA	50		76.6		84.2	86.4
	25		76.5			86.4
	10		76.7			86.5
	5		76.5			86.3

BD = blood, SE = semen, SA = saliva; VS = vaginal secretions; MB = menstrual blood; SK = skin

*VS-SE-25 – low, broad peaks at SEMG1 and CYP2B7P1 but below the 0.2 threshold used.

Table 28.Detection of Body Fluids in Two Fluid Admixtures (Hexplex HRM Assay)

Two donor (fluid) admixtures were created by placing varying volumes (50, 25, 10 and 5µl) of a second liquid body fluid on top of a dried stain. The dried component is listed first in each mixture with the liquid component listed second. The presence of biomarkers for each of the body fluids is represented by a "+" symbol. A "-" indicates a failure to detect the biomarker for the body fluid. The "+" and "-" are only listed for the body fluids in which a positive result was expected.

Mixture	Vol (µl) liquid	Saliva HTN3	Semen SEMG1	Skin LCE1C	Blood ANK1	Vaginal CYP2B7P1	Menstrual LEFTY2
BD-SE	50		+		+		
	25		+		+		
	10		+		+		
	5		+		+		
BD-SA	50	+			+		
	25	+			+		
	10	+			+		
	5	+			+		
SA-SE	50	-	-				
	25	+	+				
	10	-	-				
	5	+	+				
VS-SE	50		+			-	
	25		-			-	
	10		+			-	
	5		-			-	
MB-SE	50		-				-
	25		+				-
	10		+				+
	5		+				+
VS-SA	50	+				+	
	25	+				+	
	10	-				+	
	5	-				+	
MB-SA	50	+					+
	25	+					-
	10	+					+
	5	+					+

Table 29. Detection of Body Fluids in Vaginal-Semen Admixed Samples

Vaginal secretions-semen admixed samples were prepared by adding 25, 10, 5 and 1μl of liquid semen to ¼ dried vaginal swabs. Three different donor sets were used (no overlapping donors between sets). One of the semen donors (SE4vs) was from a vasectomized male. The results of the CE 10plex and HRM sexual assault and hexaplex assays are shown. For CE results, RFU values are provided. For HRM results, Tm values (^{o}C) are provided. Colors indicate positive results: green – vaginal secretions, yellow – semen. Grey cells indicate negative results or no detection of the biomarker. VS = vaginal secretions, SE = semen.

CE

Sample	Blood		Semen		Saliva		Vaginal	Menstrual		Skin	
	ANK1	ALAS2	PRM2	SEMG1	STATH	HTN3	CYP2B7P1	MMP10	LEFTY2	LCE1C	
VS24-SE4vs-25											
VS24-SE4vs-10							8505			443	
VS24-SE4vs-5							6023				
VS24-SE4vs-1											
VS4-SE22-25			9329	513				170			
VS4-SE22-10			4538	204							
VS4-SE22-5			2765	87			800				
VS4-SE22-1			150				201				
VS2-SE9-25			1132	147			1114				
VS2-SE9-10			116	231			1477				
VS2-SE9-5			72				1104				
VS2-SE9-1							579				

Sex-plex

Sample	HTN3		SEMG1	PRM2	CYP2B7P1
VS24-SE4vs-25			78.7		86.5
VS24-SE4vs-10			78.7		86.5
VS24-SE4vs-5			78.6		86.5
VS24-SE4vs-1					
VS4-SE22-25			78.7	84.3	
VS4-SE22-10			78.5	84.2	86.5
VS4-SE22-5			78.6	84.3	86.6
VS4-SE22-1			78.7	84.3	86.6
VS2-SE9-25			78.8	84.3	86.7
VS2-SE9-10			78.7	84.3	86.6
VS2-SE9-5				84.4	86.7
VS2-SE9-1				84.4	86.7

- ¼ vaginal swab
- Liquid semen added 25, 10, 5, 1μl
- Co-extracted

Table 30. Identification of Skin in Touch DNA Samples (CE 10-plex)

100 touch DNA samples (twenty samples from five different donors) were extracted with the AllPrep Micro DNA/RNA co-extraction kit. The ability to identify skin in the touch DNA samples was evaluated using the CE 10-plex system with slightly modified conditions (35 amplification cycles (from 33) and 2 µl cDNA (from 1 µl)). The results from CE and HRM (hexaplex) analysis are shown. RFU values are listed for CE results and Tm values (^{o}C) are listed for HRM results. Peach cells indicate positive skin detection and grey cells indicate negative results (no skin detection).

Sample	T1 (female)		T2 (male)		T3 (female)		T4 (female)		T5 (female)	
	CE	HRM	CE	HRM	CE	HRM	CE	HRM	CE	HRM
cup		81.8		81.5		81.6			556	
water/drink bottle	804		106					81.7		
computer mouse	686						280	82.0		81.9
door handle/knob	385				459	81.9	312		216	
cell phone			1453	81.6	260		495	81.9	2106	
pen										
keyboard	549	81.8		81.7	178		1368	82.0		
headphones	1026	81.7								
phone	267		148	81.6	346	81.6				82.1
key	223					81.9	139			
steering wheel			3038	81.8			182		343	81.9
refrigerator door handle	689		143		211	81.3			1666	81.8
microwave buttons			483		250	81.8				81.8
backpack/purse strap	126	81.7	2643	81.8						81.5
hair brush handle		81.9			1784	81.7	394	82.1	1021	81.5
tv remote	283							81.8		
silverware	91							81.6		81.5
tooth brush handle	116	81.9			336	81.7				81.9
fingers	274	81.9			1199	81.6		81.5	254	81.8
arm				81.2		81.9	692	82.0		81.4

Success rate (by donor) N=20	T1		T2		T3		T4		T5	
	CE	HRM	CE	HRM	CE	HRM	CE	HRM	CE	HRM
	13/20	7/20	7/20	7/20	9/20	10/20	8/20	9/20	7/20	11/20
	65%	35%	35%	35%	45%	50%	40%	45%	35%	55%

Overall (N=100)	CE	HRM
	44%	44%

Table 31. DNA and RNA Analysis of 100 Touch DNA Samples

100 touch DNA samples (twenty samples from five different donors) were extracted with the AllPrep Micro DNA/RNA co-extraction kit. The results from CE and HRM (hexaplex) analysis are shown. RFU values are listed for CE results and Tm values (^{o}C) are listed for HRM results. Peach cells indicate positive skin detection and grey cells indicate negative results (no skin detection). The number of alleles observed in the DNA profiles for each of the touch samples is also shown. The single source or admixed nature of the profile is also indicated for each sample.

Sample	T1 (female) RNA - skin		T1 (female) DNA	
	CE	HRM	# alleles	mixture?
cup	-	+	30	no
water/drink bottle	+	-	30	no
computer mouse	+	-	30	no
door handle/knob	+	-	5	no
cell phone	-	-	30	no
pen	-	-	30	no
keyboard	+	+	30	no
headphones	+	+	30	no
phone	+	-	30	no
key	+	-	18	no
steering wheel	-	-	29	no
refrigerator door handle	+	-	11	no
microwave buttons	-	-	2	no
backpack/purse strap	+	+	29	no
hair brush handle	-	+	28	no
tv remote	+	-	30	no
silverware	+	-	30	no
tooth brush handle	+	+	28	no
fingers	+	-	21	no
arm	-	-	22	no

Sample	T3 (female) RNA - skin		T3 (female) DNA	
	CE	HRM	# alleles	mixture?
cup	-	+	3	no
water/drink bottle	-	-	0	no
computer mouse	-	-	30	no
door handle/knob	+	+	30	no
cell phone	+	-	30	no
pen	-	-	15	no
keyboard	+	-	12	no
headphones	-	-	1	no
phone	+	+	43	yes
key	-	+	11	no
steering wheel	-	-	1	no
refrigerator door handle	+	+	1	no
microwave buttons	+	+	20	no
backpack/purse strap	-	-	13	no
hair brush handle	+	+	0	no
tv remote	-	-	1	no
silverware	-	-	13	no
tooth brush handle	+	+	30	no
fingers	+	+	12	no
arm	-	+	30	no

Sample	T5 (female) RNA - skin		T5 (female) DNA	
	CE	HRM	# alleles	mixture?
cup	+	-	37	yes
water/drink bottle	-	-	30	no
computer mouse	-	+	0	no
door handle/knob	+	-	30	no
cell phone	+	-	30	no
pen	-	-	13	no
keyboard	-	-	40	yes
headphones	-	-	28	no
phone	-	+	31	yes
key	-	-	0	no
steering wheel	+	+	11	no
refrigerator door handle	+	+	21	yes
microwave buttons	-	+	18	no
backpack/purse strap	-	+	27	no
hair brush handle	+	+	6	no
tv remote	-	-	29	no
silverware	-	+	30	no
tooth brush handle	-	+	29	no
fingers	+	+	30	no
arm	-	+	5	no

Sample	T2 (male) RNA - skin		T2 (male) DNA	
	CE	HRM	# alleles	mixture?
cup	-	+	20	no
water/drink bottle	+	-	0	no
computer mouse	-	-	12	no
door handle/knob	-	-	3	no
cell phone	+	+	6	no
pen	-	-	1	no
keyboard	-	+	23	yes
headphones	-	-	0	no
phone	+	+	1	no
key	-	-	22	no
steering wheel	+	+	1	no
refrigerator door handle	+	-	30	no
microwave buttons	+	-	10	no
backpack/purse strap	+	+	2	no
hair brush handle	-	-	0	no
tv remote	-	-	0	no
silverware	-	-	28	no
tooth brush handle	-	-	0	no
fingers	-	-	28	yes
arm	-	+	10	no

Sample	T4 (female) RNA - skin		T4 (female) DNA	
	CE	HRM	# alleles	mixture?
cup	-	-	0	no
water/drink bottle	-	+	4	no
computer mouse	+	+	37	yes
door handle/knob	+	-	29	yes
cell phone	+	+	21	?
pen	-	-	10	no
keyboard	+	+	31	yes
headphones	-	-	18	no
phone	-	-	23	no
key	+	-	9	no
steering wheel	+	-	29	no
refrigerator door handle	-	-	8	no
microwave buttons	-	-	0	no
backpack/purse strap	-	-	28	no
hair brush handle	+	+	20	no
tv remote	-	+	30	no
silverware	-	+	0	no
tooth brush handle	-	-	30	no
fingers	-	+	24	yes
arm	+	+	14	no

Legend:

not detected

> 30 alleles (mixture)
full profile (30/30)
probative (21-29/30)
moderate probative (15-20/3...)
5-14 alleles/30
1-4 allele
0 alleles

54

Table 32. Summary of the EDNAP Collaborative Studies on RNA Profiling Assays for Body Fluid Identification

The body fluid or tissue of interest for each of the six EDNAP studies is indicated.

Exercise	Subject
1	Blood
2	Blood
3a/b	Semen/Saliva
4	Menstrual, Housekeeping
5	Vaginal
6	Skin (touch)

Table 33.Detection of the Body Fluid/Tissue Source of Origin of Mock Casework samples – CE 10-plex

Mock casework samples from three collaborative EDNAP mRNA profiling exercises were tested to determine if the body fluid of origin could be detected using the CE 10-plex system. Descriptions of each sample are provided, as well as the input (ng) amount of total RNA used in the RT reaction. If a biomarker was detected, the RFU value is recorded. Colored cells indicate positive results for each body fluid: red – blood, yellow – semen, blue – saliva, green – vaginal secretions, peach – skin.

	Sample	Blood		Semen		Saliva		Vaginal	Menstrual		Skin	
		ANK1	ALAS2	PRM2	SEMG1	STATH	HTN3	CYP2B7P1	MMP10	LEFTY2	LCE1C	
E3-SE/SA	2ul semen on swab			2854	366							
	3ul of human blood on swab	1499	6230									
	3ul saliva on recycling tissue						153					
	5ul azospermic semen on swab				1784							
	5ul saliva/1ul semen mix on swab			689	170	249	727					
	1/4 chewed chewing gum											
	1/4 buccal swab (dog)											
	1/4 buccal swab					1382	2780					
	3ul semen on white textile			99								
	5ul saliva on pad					112	522					
	5ul saliva on paper											
	3ul azospermic semen on pad				227							
E3-SE/SA	1/4 vaginal swab							8309			1991	3247
	5ul saliva on glass slide											
	2ul semen on toilet paper											
	5ul saliva/1ul semen mix on swab					121	83					
	1/4 buccal swab (cat)											
	2ul saliva on swab/1ul blood	493	1641			113						
	licked plastic spoon						95				154	
	3ul semen inside latex glove											
E5 -VS	1/4 vaginal swab (2 yrs old)							9201			786	2270
	5x5 mm from white worn underpant (fresh)							280			708	1294
	1/2 swab urine							200				
	1/2 swab vaginal, pregnant (fresh)							9037				1896
	1/4 vaginal swab (5 yrs old)							3313				905
	1/2 vaginal swab (fresh)							4776			232	457
	1/2 buccal swab (fresh)					461	928					
	5x5mm sanitary towel (fresh)											488

Unknown samples from EDNAP 3/5 studies

Table 34. Detection of the Body Fluid/Tissue Source of Origin of Mock Casework samples – Blood-Menstrual Blood Assay

Mock casework samples from three collaborative EDNAP mRNA profiling exercises were tested to determine if the body fluid of origin could be detected using the Blood-menstrual blood HRM assay. Descriptions of each sample are provided. If a biomarker was detected, the Tm value is recorded. Colored cells indicate positive results for each body fluid: red – blood. White cells indicate no result obtained (no peak detected).

	Sample	MMP10		ANK1	ALAS2	LEFTY2
		81.8	82.3	83.0	86.2	89.2
E3-SE/SA	2ul semen on swab					
	3ul of human blood on swab			83.3	86.5	
	3ul saliva on recycling tissue					
	5ul azospermic semen on swab					
	5ul saliva/1ul semen mix on swab					
	1/4 chewed chewing gum					
	1/4 buccal swab (dog)					
	1/4 buccal swab					
	3ul semen on white textile					
	5ul saliva on pad					
E3-SE/SA	5ul saliva on paper					
	3ul azospermic semen on pad					
	1/4 vaginal swab			83.5		
	5ul saliva on glass slide					
	2ul semen on toilet paper					
	5ul saliva/1ul semen mix on swab					
	1/4 buccal swab (cat)					
	2ul saliva on swab/1ul blood			83.4	86.5	
	licked plastic spoon					
	3ul semen inside latex glove					
E5 -VS	1/4 vaginal swab (2 yrs old)					
	5x5 mm from white worn underpant (fresh)					
	1/2 swab urine					
	1/2 swab vaginal, pregnant (fresh)					
	1/4 vaginal swab (5 yrs old)					
	1/2 vaginal swab (fresh)					
	1/2 buccal swab (fresh)					
	5x5mm sanitary towel (fresh)					

Table 35. Detection of the Body Fluid/Tissue Source of Origin of Mock Casework samples – Sexual Assault HRM Assay

Mock casework samples from three collaborative EDNAP mRNA profiling exercises were tested to determine if the body fluid of origin could be detected using the sexual assault assay. Descriptions of each sample are provided. If a biomarker was detected, the Tm value is recorded. Colored cells indicate positive results for each body fluid: blue – saliva, yellow – semen, green – vaginal secretions. White cells indicate no result obtained (no peak detected). Grey cells indicate a negative result for biomarkers that were expected to be present given the origin of the sample.

	Sample	HTN3		SEMG1	PRM2	CYP2B7P1
		76.8	77.1	78.6	84.2	86.7
E3-SE/SA	2ul semen on swab			78.6	84.3	
	3ul of human blood on swab					
	3ul saliva on recycling tissue		77.1			
	5ul azospermic semen on swab			78.5		
	5ul saliva/1ul semen mix on swab	76.8		76.7	84.4	
	1/4 chewed chewing gum	(grey)	(grey)			
	1/4 buccal swab (dog)					
	1/4 buccal swab		77.0			
	3ul semen on white textile			(grey)	84.5	
	5ul saliva on pad		77.2			
E3-SE/SA	5ul saliva on paper	(grey)				
	3ul azospermic semen on pad			79.0		
	1/4 vaginal swab					86.6
	5ul saliva on glass slide					
	2ul semen on toilet paper			(grey)		
	5ul saliva/1ul semen mix on swab	76.9		78.8	84.0	
	1/4 buccal swab (cat)					
	2ul saliva on swab/1ul blood	(grey)				
	licked plastic spoon		77.2			
	3ul semen inside latex glove			(grey)	84.6	
E5 -VS	1/4 vaginal swab (2 yrs old)					86.5
	5x5 mm from white worn underpant (fresh)					(grey)
	1/2 swab urine					
	1/2 swab vaginal, pregnant (fresh)					86.6
	1/4 vaginal swab (5 yrs old)					86.5
	1/2 vaginal swab (fresh)					86.6
	1/2 buccal swab (fresh)	76.9				
	5x5mm sanitary towel (fresh)					(grey)

Table 36. Detection of the Body Fluid/Tissue Source of Origin of Mock Casework samples – Hexaplex HRM Assay

Mock casework samples from three collaborative EDNAP mRNA profiling exercises were tested to determine if the body fluid of origin could be detected using the hexaplex assay. Descriptions of each sample are provided. If a biomarker was detected, the Tm value is recorded. Colored cells indicate positive results for each body fluid: red – blood, blue – saliva, yellow – semen, green – vaginal secretions. White cells indicate no result obtained (no peak detected). Grey cells indicate a negative result for biomarkers that were expected to be present given the origin of the sample.

Sample	HTN3 76.5	HTN3 77.1	SEMG1 78.9	LCE1C 81.5	ANK1 83.3	CYP2B7P1 86.5	LEFTY2 89.1
E3-SE/SA							
2ul semen on swab			79.0				
3ul of human blood on swab					83.5		
3ul saliva on recycling tissue	(blue)	77.2					
5ul azospermic semen on swab			79.0				
5ul saliva/1ul semen mix on swab	(blue)	77.1	78.1				
1/4 chewed chewing gum	(grey)	(grey)					
1/4 buccal swab (dog)							
1/4 buccal swab	(blue)	77.1					
3ul semen on white textile			(grey)				
5ul saliva on pad	(blue)	77.2					
E3-SE/SA							
5ul saliva on paper	(grey)						
3ul azospermic semen on pad	(grey)		79.0				
1/4 vaginal swab				81.9		86.8	
5ul saliva on glass slide	(grey)						
2ul semen on toilet paper			(grey)				
5ul saliva/1ul semen mix on swab	(blue)	77.1	79.1				
1/4 buccal swab (cat)							
2ul saliva on swab/1ul blood	76.3	(blue)			83.7		
licked plastic spoon		77.5					
3ul semen inside latex glove			(grey)				
E5 -VS							
1/4 vaginal swab (2 yrs old)				81.7		86.5	
5x5 mm from white worn underpant (fresh)				81.9		(grey)	
1/2 swab urine							
1/2 swab vaginal, pregnant (fresh)						86.6	
1/4 vaginal swab (5 yrs old)				81.6		(grey)	
1/2 vaginal swab (fresh)						(grey)	
1/2 buccal swab (fresh)	76.8	(blue)		82.0			
5x5mm sanitary towel (fresh)				81.9		(grey)	

Table 37. CE 10-plex Assay Reproducibility

To evaluate the reproducibility in the base pair size of each biomarker, cDNA (RTs) from three or four donors (four only in the case of skin; two skin tissue total RNA samples and two touch DNA samples) were analyzed five times each. All replicates were amplified at the same time. The average base pair size and standard deviation for each donor is shown as well as the overall averages for each biomarker (all replicates together). Semen donor 3 is a vasectomized male and therefore only SEMG1 was detected. NT = not tested.

Donor		Blood		Semen		Saliva		Vaginal	Menstrual		Skin	
		ANK1	ALAS2	PRM2	SEMG1	STATH	HTN3	CYP2B7P1	MMP10	LEFTY2	LCE1C	
Donor 1	Average	113.6	134.3	91.6	86.1	180.3	225.1	198.0	168.1	127.3	60.6	62.6
	Std Dev	0.2	0.1	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0	0.1
Donor 2	Average	113.6	134.3	91.5	86.1	180.2	225.1	198.0	168.1	127.3	60.6	62.6
	Std Dev	0.1	0.1	0.2	0.0	0.0	0.0	0.1	0.2	0.1	0.0	0.0
Donor 3	Average	113.7	134.3		86.2	180.2	225.1	198.0	168.1	127.3	60.7	62.7
	Std Dev	0.0	0.1		0.1	0.0	0.0	0.0	0.1	0.1	0.1	0.0
Donor 4	Average	NT	NT	NT	NT	NT	NT	NT	NT	NT	60.7	62.7
	Std Dev										0.1	0.0
Overall	Average	113.7	134.3	91.5	86.1	180.2	225.1	198.0	168.1	127.3	60.6	62.6
	Std Dev	0.1	0.1	0.1	0.1	0.0	0.0	0.1	0.1	0.1	0.1	0.0

Table 38. HRM Assay Reproducibility

To assess reproducibility, five replicates each of three donors were evaluated (all replicates taken from the same RT). The average Tm value and standard deviation (st. dev) for each donor (average of five replicates) was calculated. An overall biomarker average Tm (and standard deviation) was also calculated (shown at bottom) (average of all 15 samples (3 donors x 5 replicates). Tm (^{o}C) values of detected biomarkers are recorded. Colored shading highlights the average Tm for each biomarker.

Blood-Menstrual

Donor		MMP10		ANK1	ALAS2	LEFTY2
Donor 1	Average		81.9	83.1	86.2	89.0
	St. Dev		0.1	0.1	0.1	0.0
Donor 2	Average		82.1	83.0	86.1	89.0
	St. Dev		0.0	0.0	0.0	0.0
Donor 3	Average	81.5	82.0	83.1	86.2	89.0
	St. Dev	0.0	0.0	0.1	0.0	0.0
Overall	Average	81.5	82.0	83.1	86.2	89.0
	St. Dev	0.0	0.1	0.1	0.1	0.0

Sexual Assault

Donor		HTN3	SEMG1	PRM2	CYP2B7P1
Donor 1	Average	77.1	78.5	84.3	86.6
	Std Dev	0.1	0.0	0.0	0.1
Donor 2	Average	77.1	78.9	84.6	86.5
	Std Dev	0.1	0.0	0.1	0.0
Donor 3	Average	76.9	78.6	84.3	86.6
	Std Dev	0.1	0.1	0.0	0.1
Donor 4	Average	NT	78.6	--	NT
	Std Dev		0.1		
Overall	Average	77.0	78.7	84.4	86.6
	Std Dev	0.1	0.2	0.1	0.1

Hexaplex

Donor		HTN3	SEMG1	LCE1C	ANK1	CYP2B7P1	LEFTY2
Donor 1	Average	76.6	78.5	81.9	83.0	86.3	88.9
	Std Dev	0.1	0.0	0.2	0.0	0.1	0.0
Donor 2	Average	76.6	78.5	81.7	83.1	86.4	89.0
	Std Dev	0.0	0.1	0.1	0.1	0.1	0.0
Donor 3	Average	76.9	78.6	81.7	83.3	86.4	89.0
	Std Dev	0.0	0.1	0.1	0.0	0.0	0.1
Overall	Average	76.7	78.5	81.7	83.1	86.4	89.0
	Std Dev	0.1	0.1	0.2	0.1	0.1	0.1

Figure 1. QIACube Rotor Adaptor Set-Up For AllPrep DNA/RNA Co-Extraction
For DNA isolation (left), the elution tube is placed into the position 3 (lid into the L3 position) of the rotor adaptor (shown in images on the left). The lid to the AllPrep DNA spin column is removed and the column is placed into position 2 (shown in images on the left). Following DNA isolation, the same rotor adaptor is used for RNA isolation with the flow-through from the DNA isolation serving as the sample for the subsequent RNA steps. The elution tube is again placed into position 3 (lid into the L3 position) and the RNAeasy® MinElute® spin column is placed into position 1 (lid into position L1).
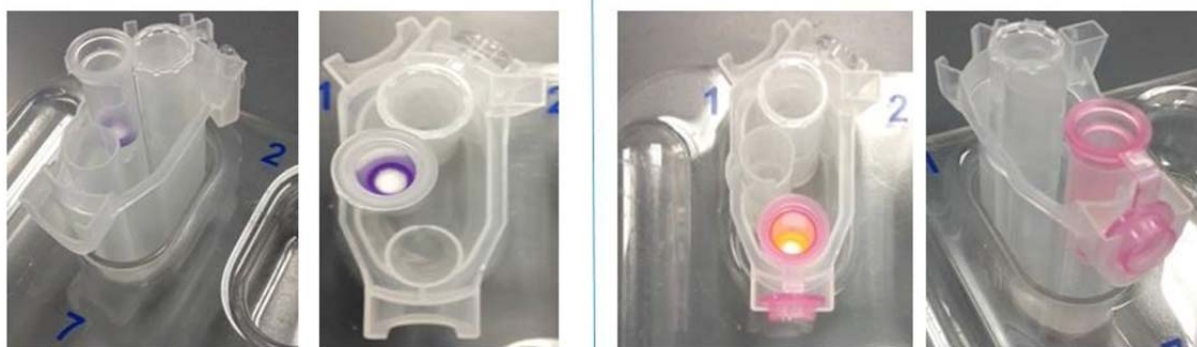
Figure 2. Workflow Summary for the RNA BodyfluID Assays
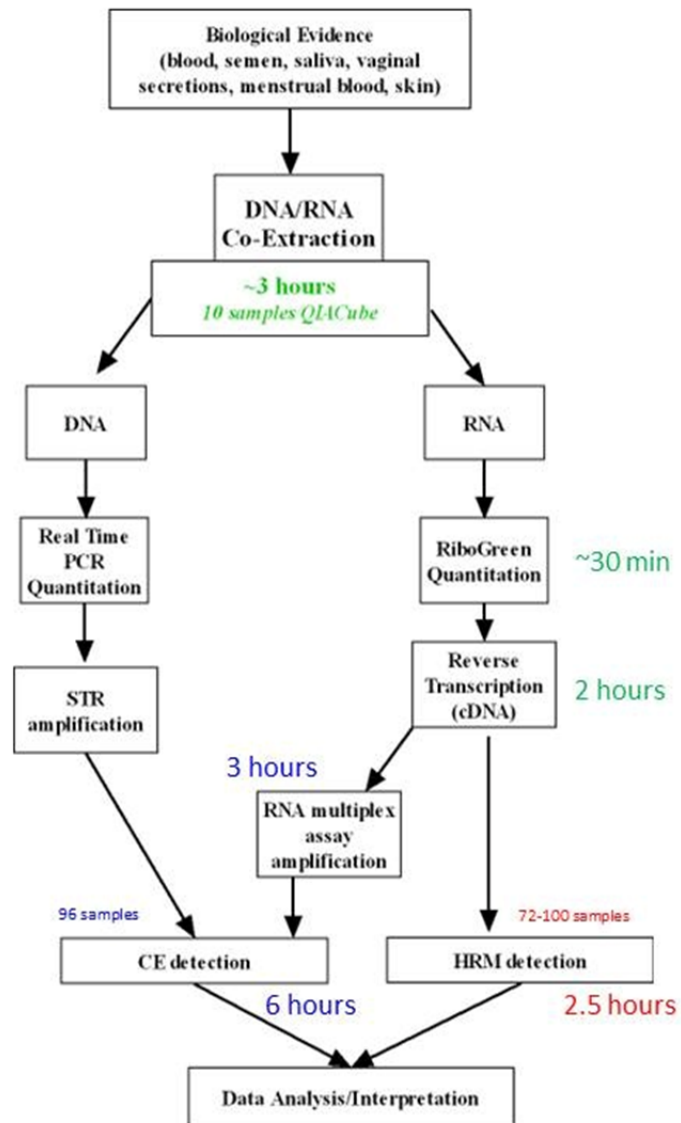
Figure 3. CE 10-plex multiplex.

The design of the CE 10-plex multiplex system is shown. The colored squares represent biomarker location within the multiplex. The size of the box is not representative of the size range for the amplification products as only a single peak is obtained for each biomarker. The boxes are sized to fit the text accordingly. The expected base pair (bp) size of each product is listed below each biomarker. LIZ-500 is used as the size standard for this multiplex.
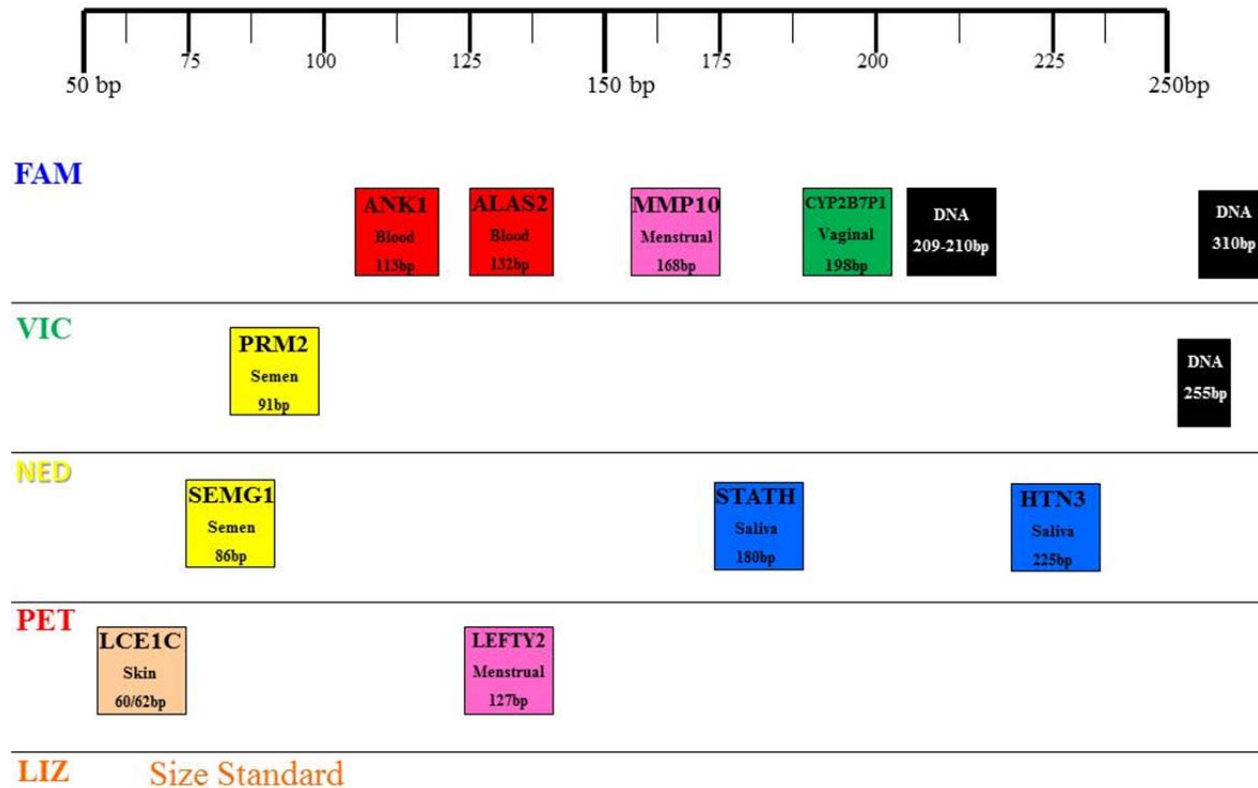
Figure 4. Representative Electropherograms for Body Fluids and Tissues Using the CE 10-plex.

Representative electropherograms for each of the target body fluids and tissues in the CE 10-plex are shown: blood (ANK1, ALAS2), semen (PRM2, SEMG1), saliva (STATH, HTN3), vaginal secretions (CYP2B7P1), menstrual blood (MMP10, LEFTY2) and skin (LCE1C). For menstrual blood, blood and vaginal secretions biomarkers may also be present as these body fluids are present in menstrual blood. Grey boxes represent biomarker bins. X-axis: size in base pairs (bp); Y-axis: relative fluorescence units (RFUs).

Body Fluid	mRNA biomarker (Gene)	Dye label, base pair (bp) size
Blood	ANK1	FAM, 113 bp
	ALAS2	FAM, 132 bp
Semen	PRM2	VIC, 91 bp
	SEMG1	NED, 86 bp
Saliva	STATH	NED, 180 bp
	HTN3	NED, 225 bp
Vaginal Secretions	CYP2B7P1	FAM, 198 bp
Menstrual Blood	MMP10	FAM, 168 bp
	LEFTY2	PET, 127 bp
Skin	LCE1C	PET, 60/62 bp
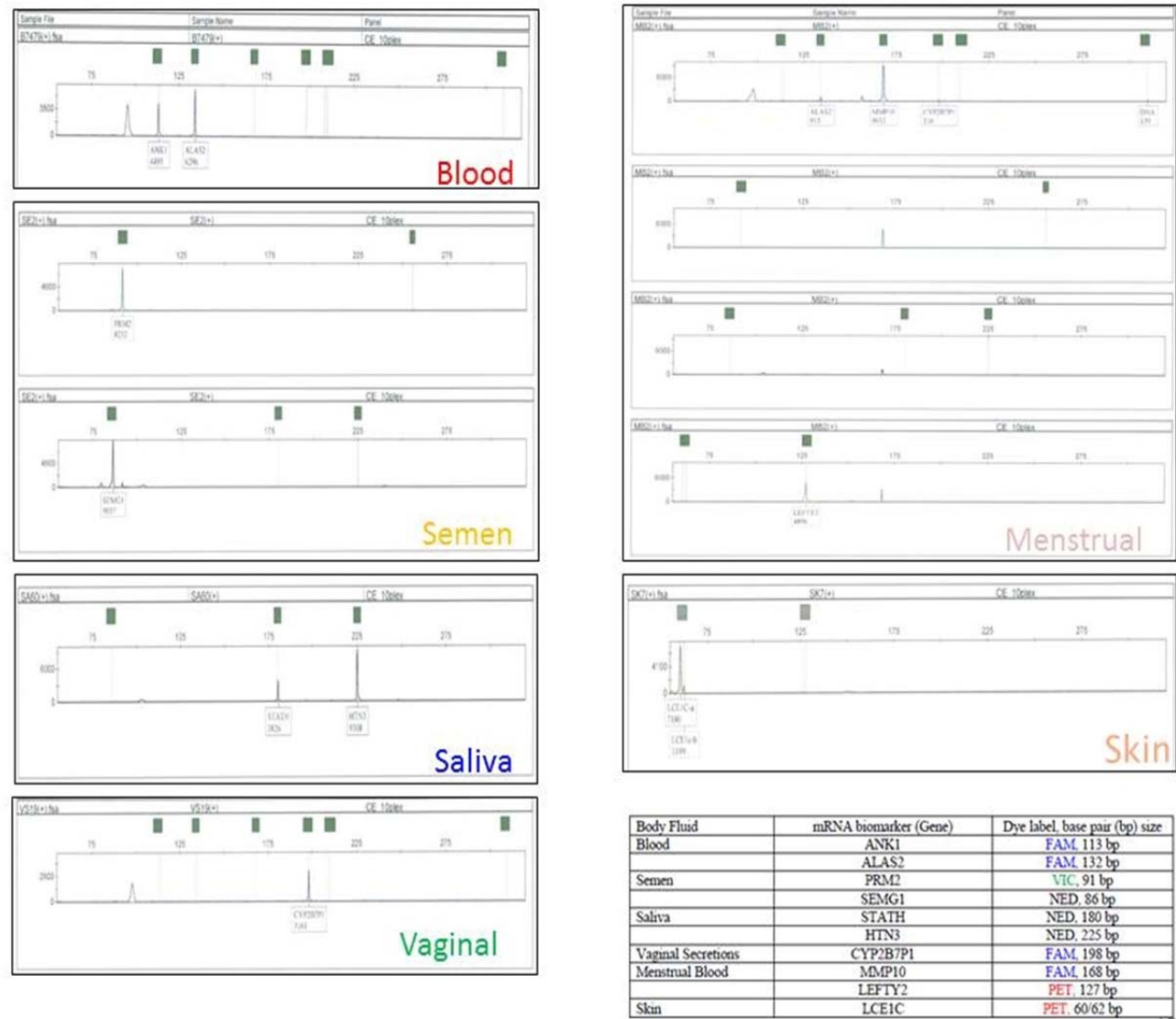
Figure 5. DNA Amplification Products in the CE 10-plex

Several DNA amplification products are observed within the analysis window of the CE10-plex. These products are due to the presence of smaller introns. They do not interfere with biomarker analysis. The location of the DNA products on the FAM (two products) and the VIC (one product) channels are shown. Their location relative to the other body fluid and tissue biomarkers are shown. X-axis: size in base pairs (bp); Y-axis: relative fluorescence units (RFUs).
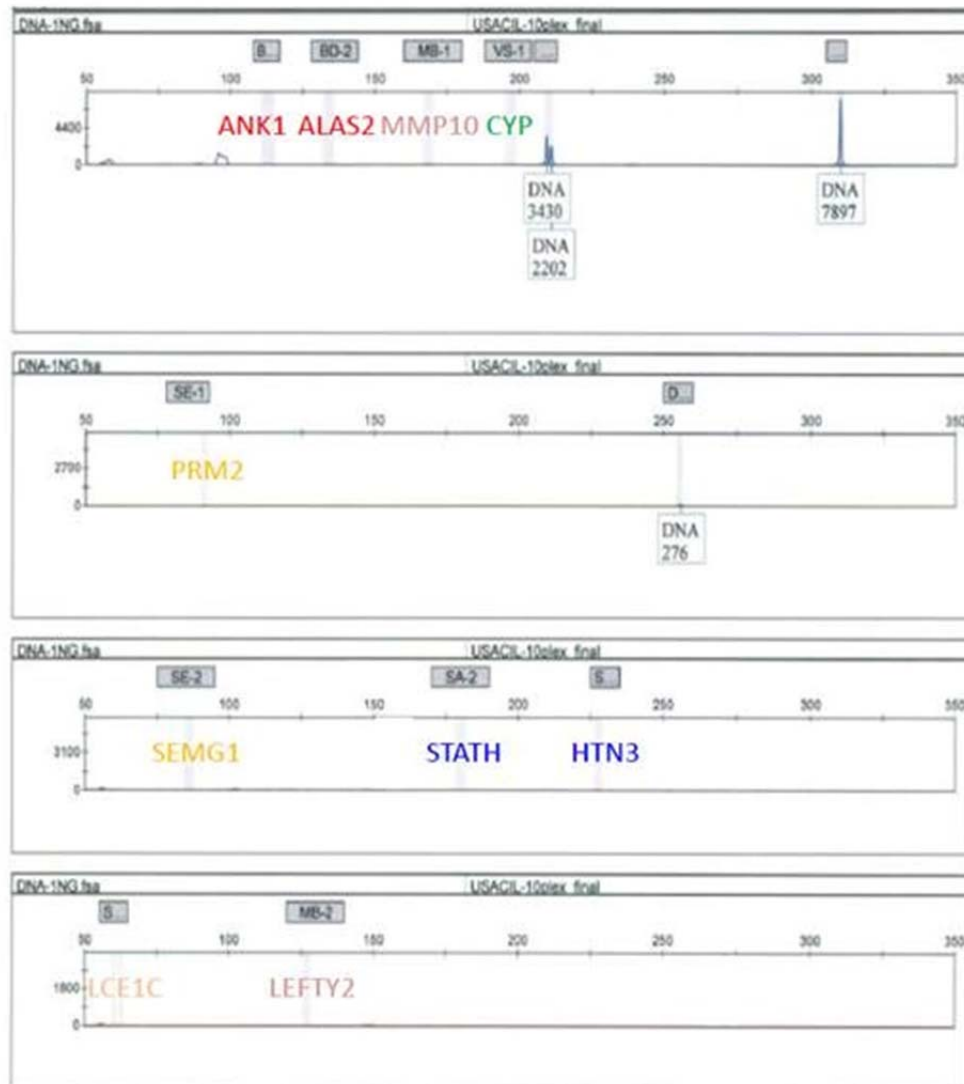
Figure 6. Blood-Menstrual Blood HRM Assay

The blood-menstrual blood HRM assays contains two biomarkers each for the identification of blood (ANK1, ALAS2) and menstrual blood (MMP10, LEFTY2). Single source blood (red) and menstrual blood (pink) samples are shown overlaid to indicate relative locations of the biomarkers within the multiplex assay. The horizontal line represents the peak detection threshold. X-axis: Tm (^{o}C); Y-axis: -dF/dT.
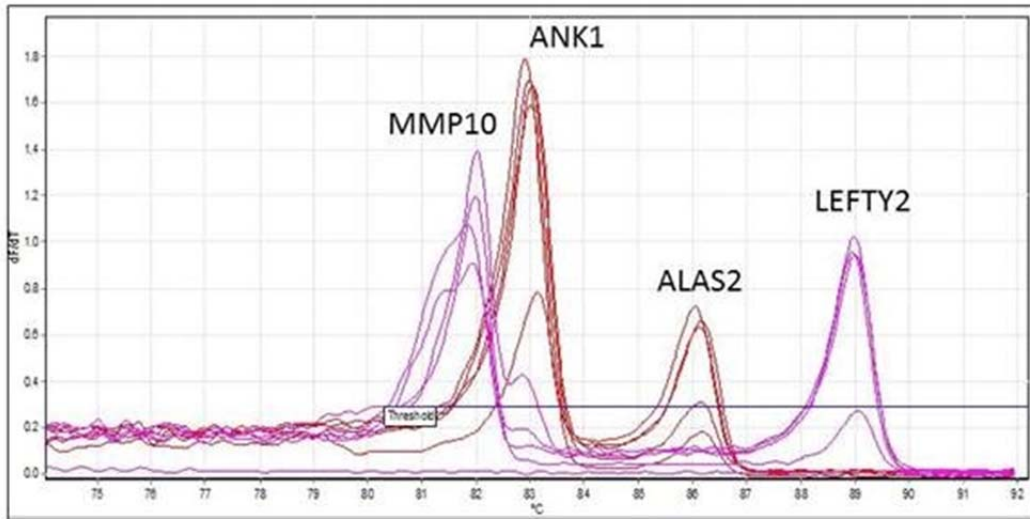
Figure 7. Sexual Assault ('Sex-Plex') HRM Assay

The sexual assault HRM assays contains two biomarkers for the identification of semen (seminal fluid specific SEMG1 and sperm specific PRM2) and one biomarker each for the identification of saliva (HTN3) and vaginal secretions (CYP2B7P1). Single source saliva (blue), semen (yellow) and vaginal secretions (green) samples are shown overlaid to indicate relative locations of the biomarkers within the multiplex assay. X-axis: Tm (^{o}C); Y-axis: -dF/dT.
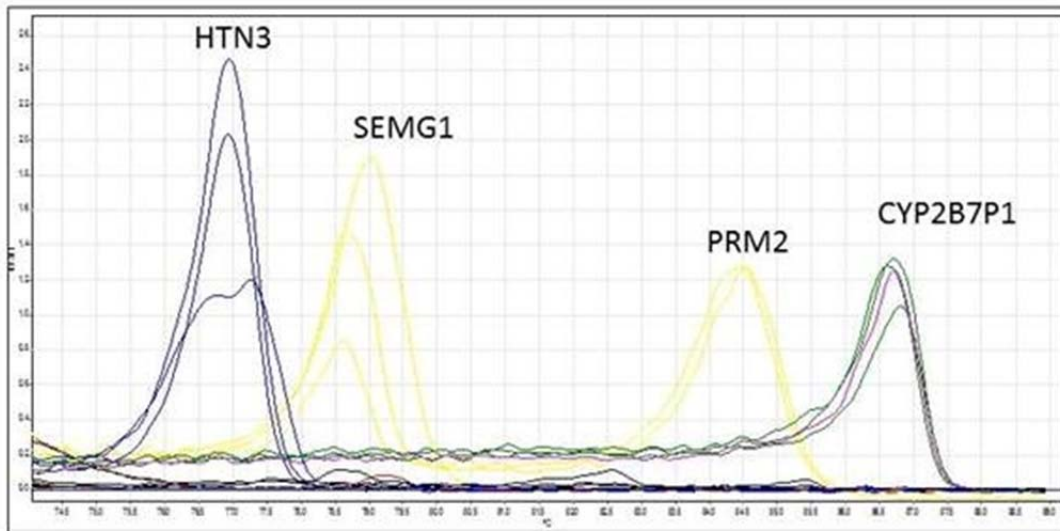
Figure 8. Hexaplex HRM Assay

The hexaplex HRM assays contains one biomarker each for the identification of saliva (HTN3), semen (SEMG1), skin (LCE1C), blood (ANK1), vaginal secretions (CYP2B7P1) and menstrual blood (LEFTY2). Single source saliva (blue), semen (yellow), skin (orange), blood (red), vaginal secretions (green), and menstrual blood (LEFTY2) samples are shown overlaid to indicate relative locations of the biomarkers within the multiplex assay. The horizontal line represents the peak detection threshold. X-axis: Tm (^{o}C); Y-axis: -dF/dT.
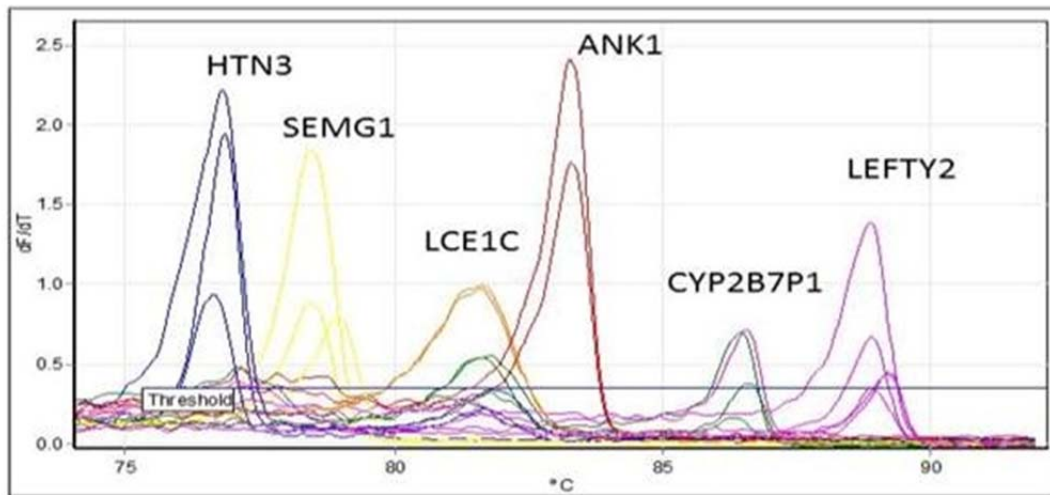
Figure 9. Sensitivity of the Blood Biomarkers in the CE 10-plex

The sensitivity of the blood biomarkers in the CE 10-plex was evaluated using a range of input total RNA (0.05ng to 15ng). As can be seen, ALAS2 was detected for all input amounts and ANK1 was detected with as little as 0.25ng. For each input amount, the blood biomarkers on the blue channel are shown. The RFUs and bp size are listed below each biomarker. X-axis: size in base pairs (bp); Y-axis: relative fluorescence units (RFUs).
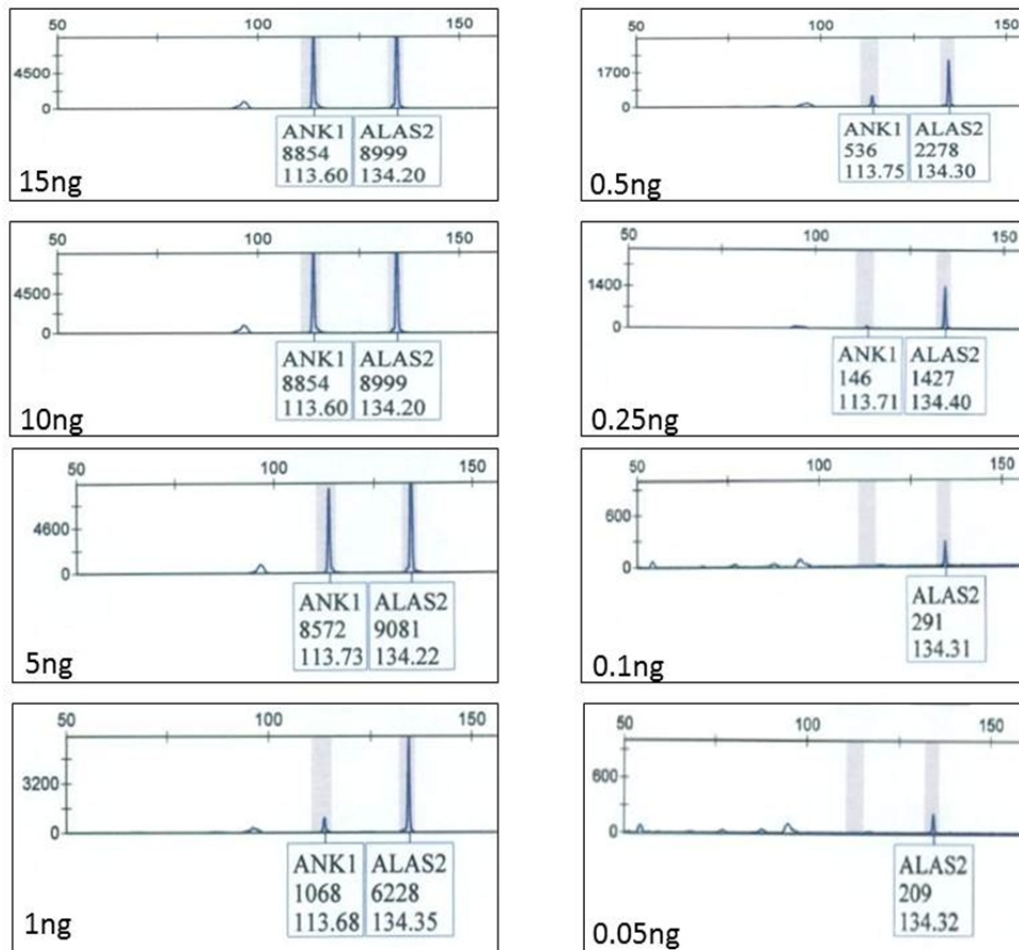
Figure 10. Sensitivity of the Blood-Menstrual Blood and Sexual Assault HRM Assays

The sensitivity of the HRM multiplex assays was evaluated using a range of input total RNA (0.05ng to 15ng). Representative melt plots for the blood-menstrual blood HRM assay (blood sample shown, red/left) and sexual assault HRM assay (semen sample shown, yellow/right). The observed Tm values for each of the input levels are listed. Colored shading represents positive detection of the biomarkers. Grey shading indicates no detection. X-axis: Tm (^{o}C); Y-axis: -dF/dT.

Blood donor 1
BD-MB HRM

Semen donor 3
Sexual Assault HRM
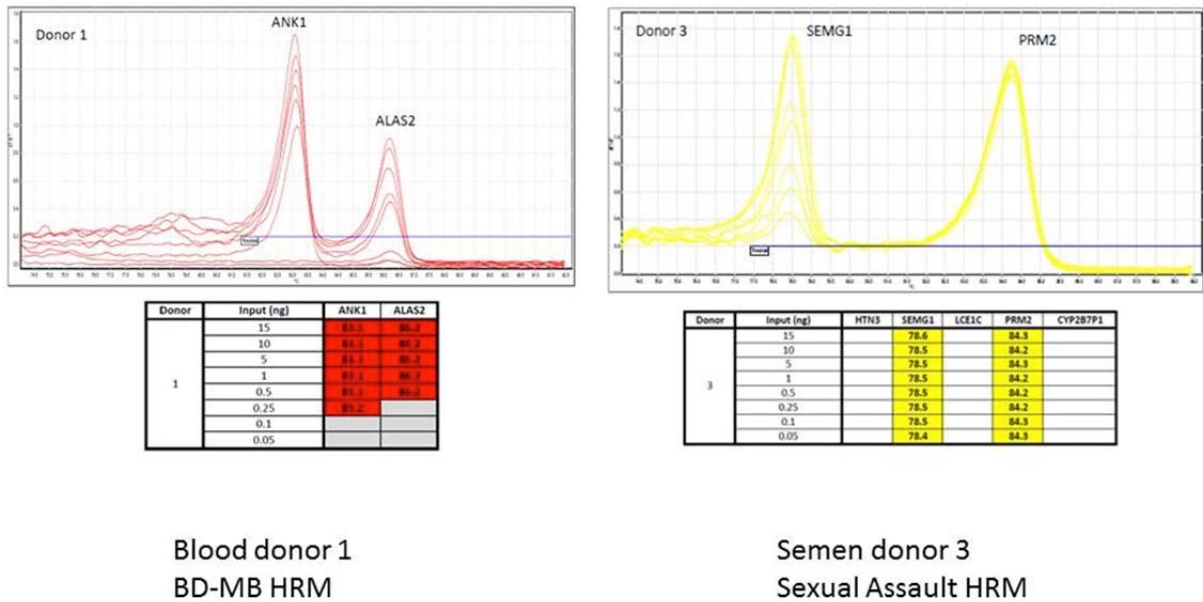
Figure 11. Sensitivity of the Hexaplex HRM Assay

The sensitivity of the hexaplex HRM multiplex assay was evaluated using a range of input total RNA (0.05ng to 15ng). Representative melt plots for the saliva (blue, left) and vaginal (green, right) samples are shown. The observed Tm values for each of the input levels are listed. Colored shading represents positive detection of the biomarkers. Grey shading indicates no detection. X-axis: Tm (^{o}C); Y-axis: -dF/dT.

Saliva donor 1
Hexaplex HRM

Vaginal donor 2
Hexaplex HRM

Figure 12. RNA Expression Profile for Nasal Secretions (CE)

The RNA expression profile for a nasal secretions sample is shown (CE 12-plex, 15ng total RNA input). STATH is the only body-fluid specific biomarker detected in this sample. Housekeeping genes B2M and 18S-rRNA were also detected. **Note: The 12-plex CE multiplex system was only used in early stages of the project but was used here as it was still used at the time of analysis for this sample. The RFUs and bp size are listed below each biomarker. X-axis: size in base pairs (bp); Y-axis: relative fluorescence units (RFUs).
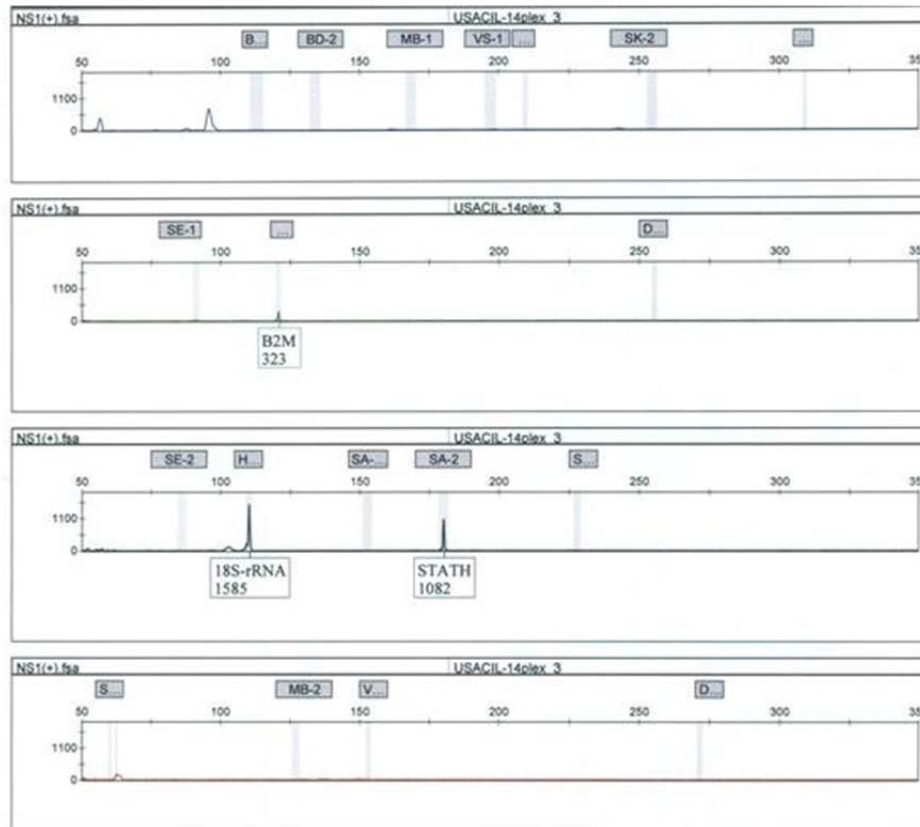
Figure 13. Detection of Body Fluid Components in a Two-Fluid Admixed Body Fluid Sample (CE 10-plex)

An admixed blood-saliva (50μl liquid saliva dried on a 50μl bloodstain) was co-extracted with the AllPrep DNA/Micro kit. The presence of both blood (ANK1, ALAS2) and saliva (STATH and HTN3) were detected using the CE 10-plex. The RFUs and bp size are listed below each biomarker. X-axis: size in base pairs (bp); Y-axis: relative fluorescence units (RFUs).
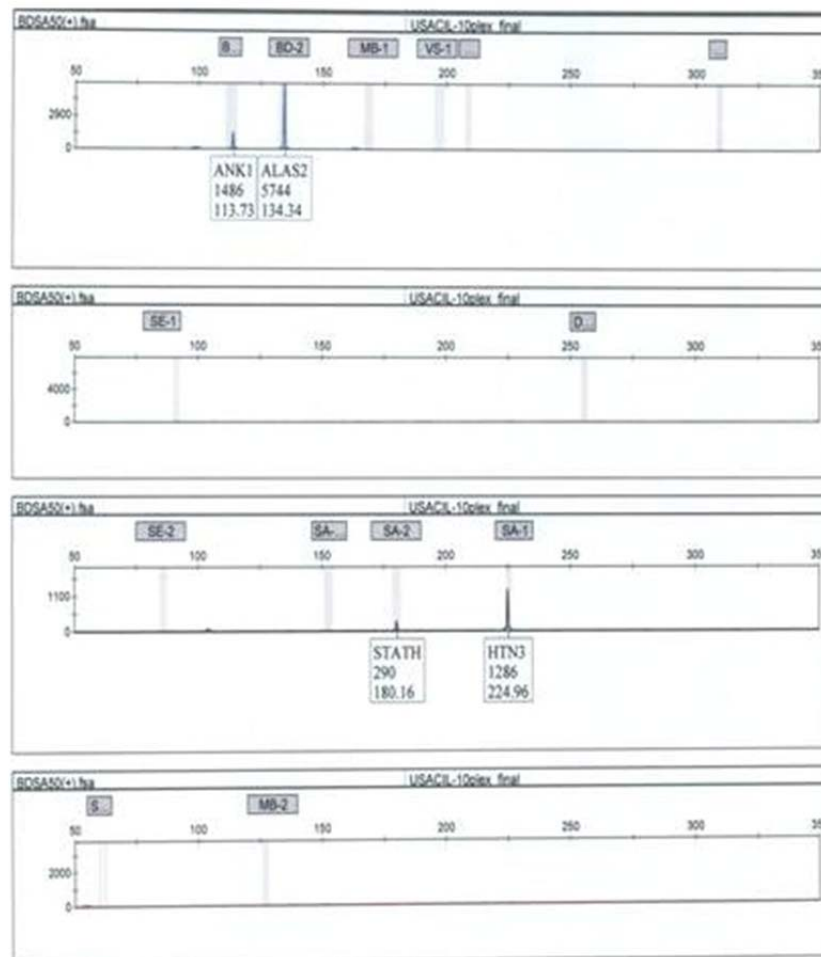
Blood-Saliva (50μl)

Figure 14. Detection of Body Fluid Components in an Two-Fluid Admixed Body Fluid Sample (Sexual Assault HRM)

The sexual assault HRM assay contains four biomarkers for the identification of saliva (HTN3), semen (PRM2 and SEMG1) and vaginal secretions (CYP2B7P1). The top panel shows melt plots for a saliva, semen and vaginal secretions single source sample to show the location of each biomarker. The bottom panel shows the analysis of a saliva-semen (5 μl semen added to a dried buccal swab). Saliva (HTN3) and semen (PRM2 and SEMG1) were successfully detected in this mixture sample. X-axis - Tm values (^{o}C); Y-axis represents dF/dT.

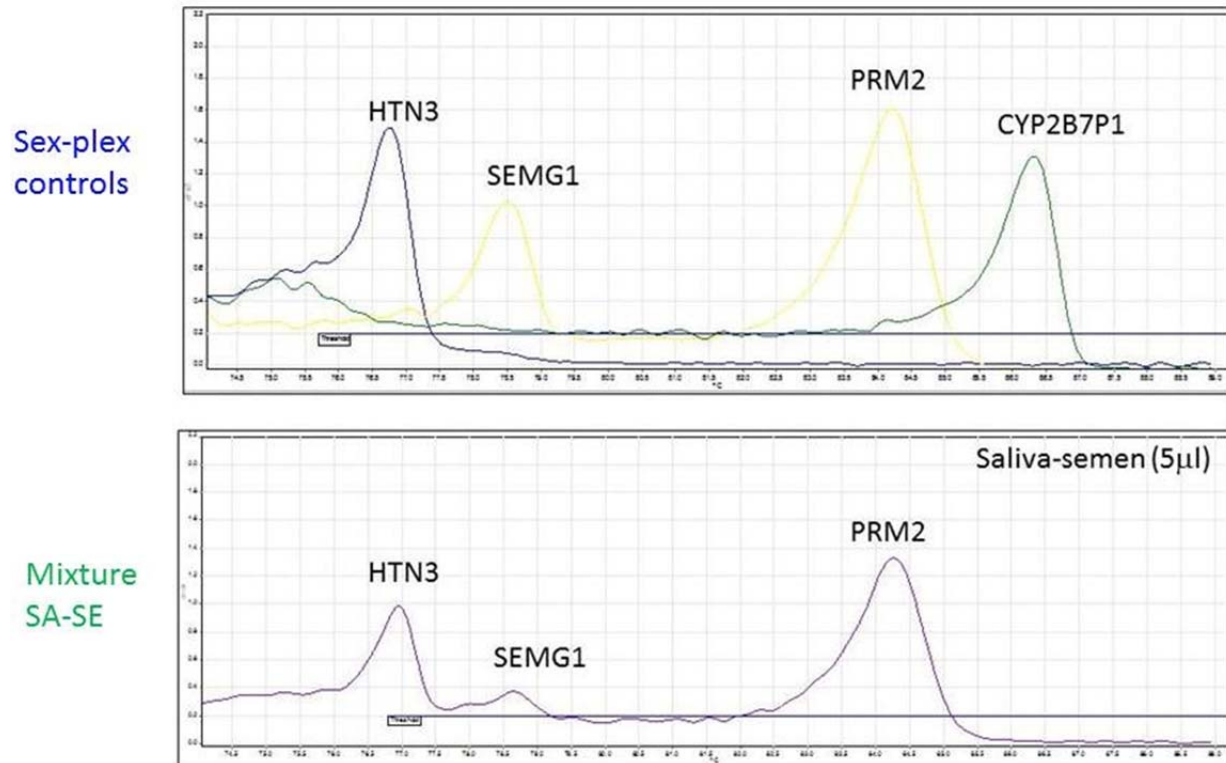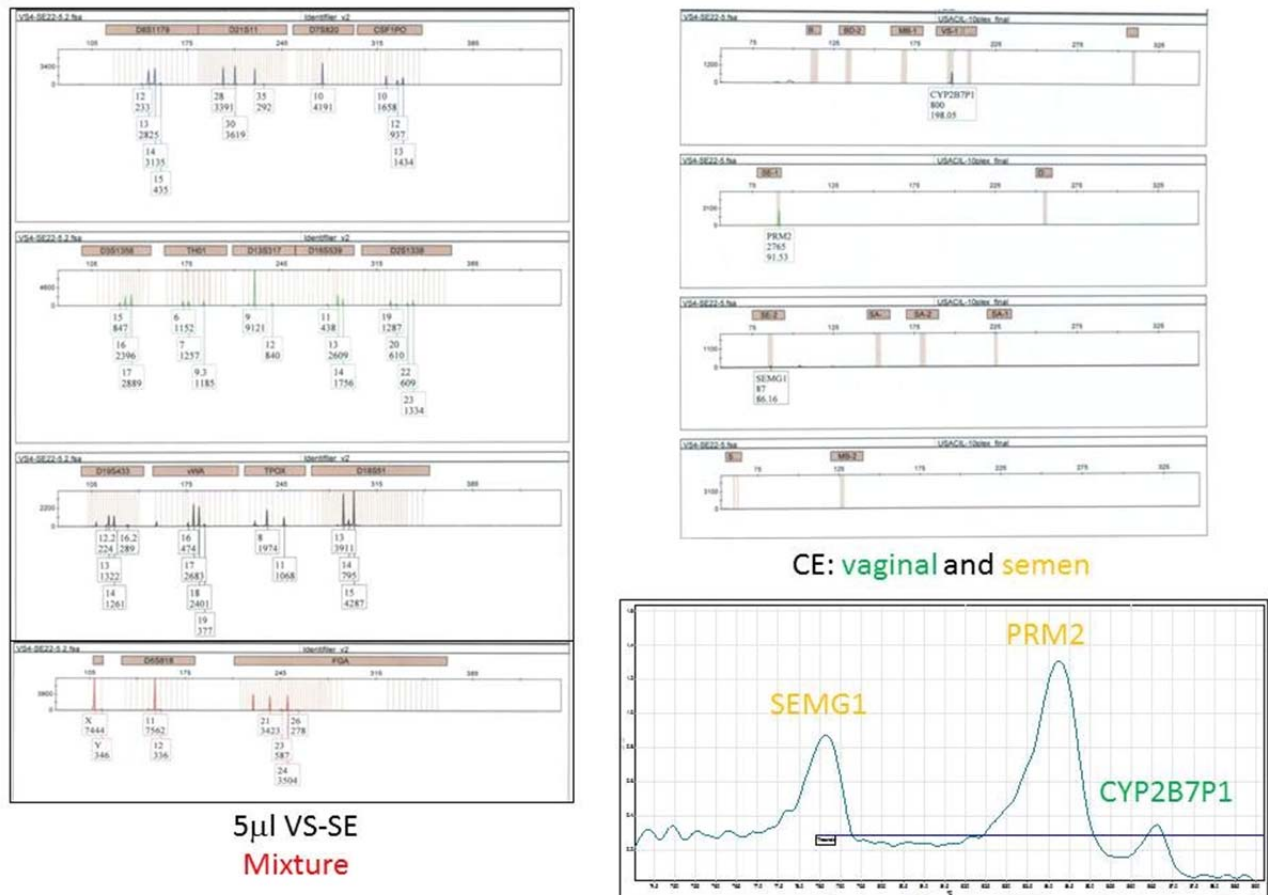**Figure 15. DNA and RNA Profiling of a Simulated Vaginal-Semen Mixture (Example 1)**

A vaginal secretions-semen admixed sample was prepared by adding 5 µl of liquid semen to ¼ dried vaginal swabs. The results of the DNA profiling (Identifiler Plus, left), CE 10-plex (right, top) and HRM sexual assault assay (right, bottom) are shown. For CE results, allele number or biomarker and RFU values are provided. X-axis: size in base pairs, Y-axis: relative fluorescence units (RFUs). For the HRM assay: X-axis - Tm values (^{o}C), Y-axis: -dF/dT. VS = vaginal secretions, SE = semen.
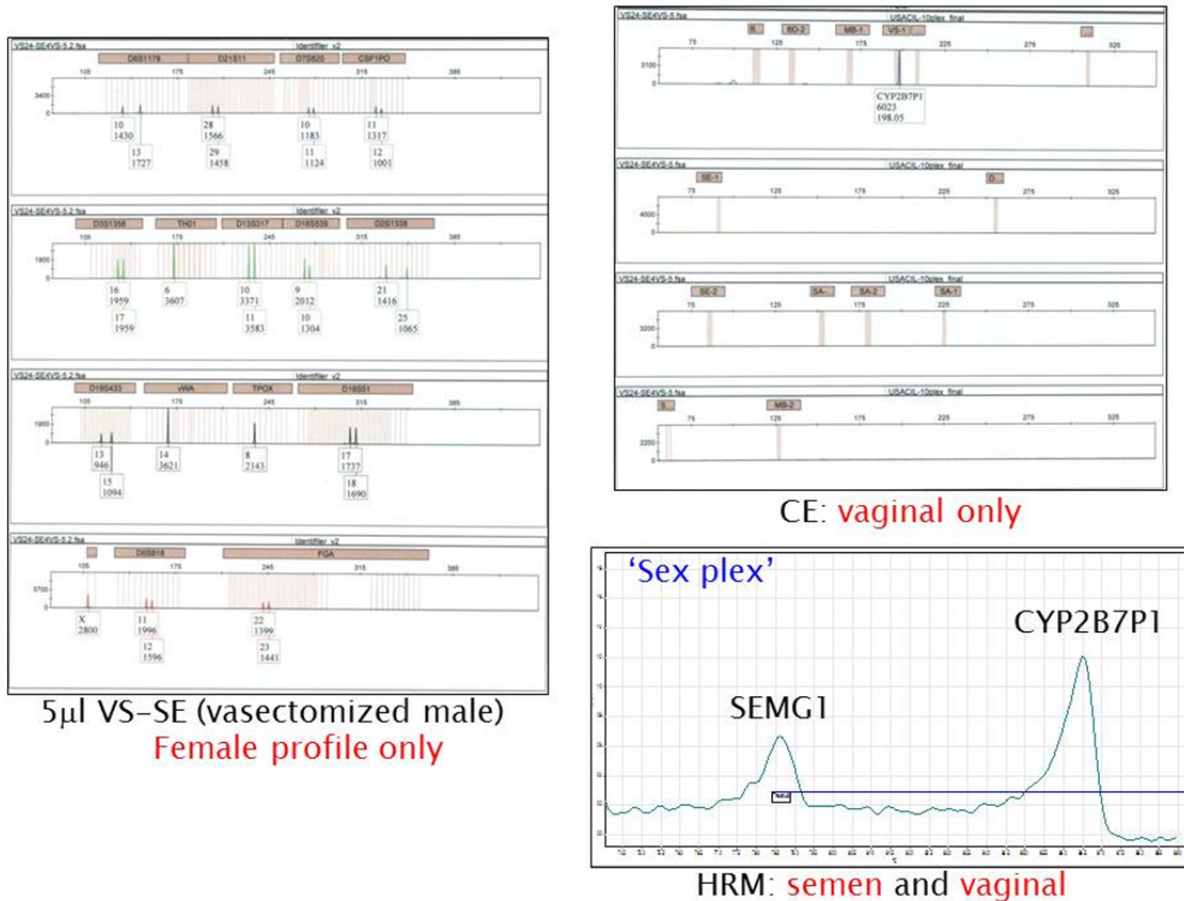

5µl VS-SE
Mixture

CE: vaginal and semen

Figure 16. DNA and RNA Profiling of a Simulated Vaginal-Semen Mixture (Example 2)

A vaginal secretions-semen admixed sample was prepared by adding 5 µl of liquid semen to ¼ dried vaginal swabs. The results of the DNA profiling (Identifiler Plus, left), CE 10-plex (right, top) and HRM sexual assault assay (right, bottom) are shown. For CE results, allele number or biomarker and RFU values are provided. X-axis: size in base pairs, Y-axis: relative fluorescence units (RFUs). For the HRM assay: X-axis - Tm values (^{o}C), Y-axis: -dF/dT. VS = vaginal secretions, SE = semen.

5µl VS–SE (vasectomized male)
Female profile only

CE: vaginal only

HRM: semen and vaginal

Figure 17. DNA and RNA Profiling of a Differentially Co-Extracted Vaginal-Semen Mixture (Example 1)

A vaginal secretions-semen admixed sample was prepared by adding 25 µl of liquid semen to ½ of a dried vaginal swab. The sample was then differentially co-extracted (separating non-sperm and sperm fractions prior to DNA and RNA analysis). The CE 10-plex (top panels) was used to determine if semen (PRM2, SEMG1) and vaginal secretions (CYP2B7P1) could be identified. Semen/sperm (PRM2) was detected in both the F1 (non-sperm) and sperm fractions. Vaginal secretions (CYP2B7P1) was not detected. The DNA fractions were amplified using Identfiler Plus (bottom panels) and full single source donor profiles were obtained. X-axis: size in base pairs, Y-axis: relative fluorescence units (RFUs).
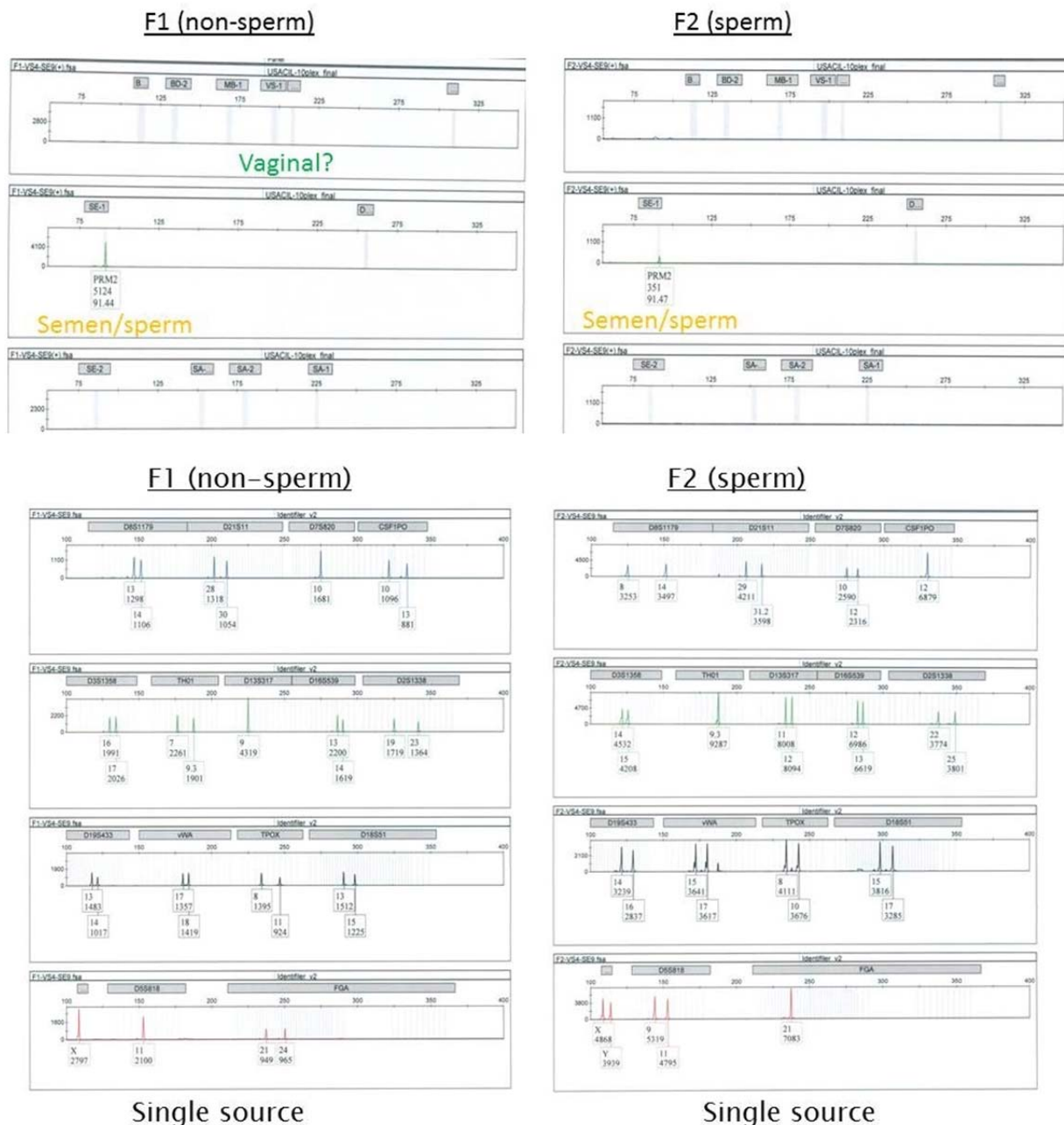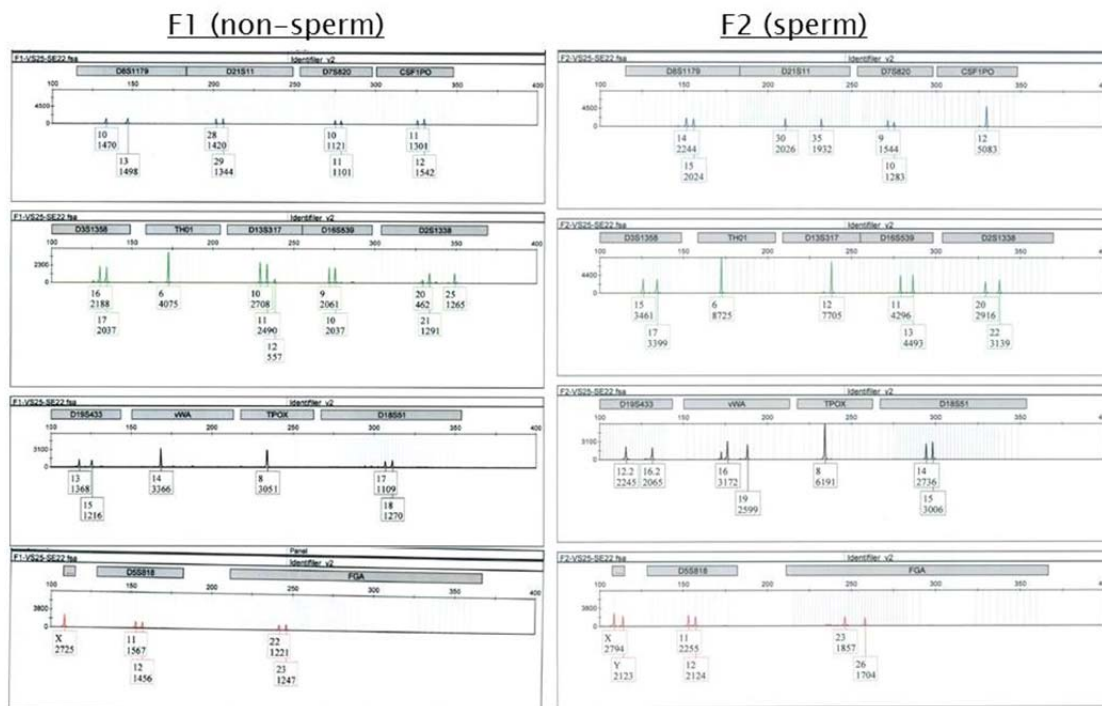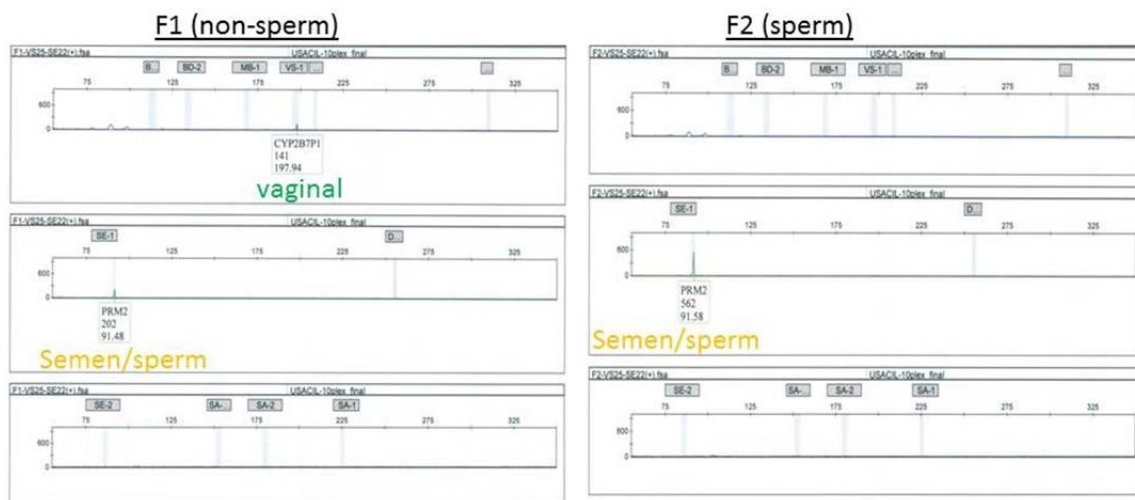
Figure 18. DNA and RNA Profiling of a Differentially Co-Extracted Vaginal-Semen Mixture (Example 2)

A vaginal secretions-semen admixed sample was prepared by adding 25 µl of liquid semen to ½ of a dried vaginal swab. The sample was then differentially co-extracted (separating non-sperm and sperm fractions prior to DNA and RNA analysis). The CE 10-plex (top panels) was used to determine if semen (PRM2, SEMG1) and vaginal secretions (CYP2B7P1) could be identified. Semen/sperm (PRM2) was detected in both the F1 (non-sperm) and sperm fractions. Vaginal secretions (CYP2B7P1) was detected in the F1 (non-sperm) fraction. The DNA fractions were amplified using Identfiler Plus (bottom panels) and full single source donor profiles were obtained. X-axis: size in base pairs, Y-axis: relative fluorescence units (RFUs).