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Major Goals: The aims of the current work were to custom-develop for DFSC casework use: (i) a differential DNA/RNA co-extraction isolation protocol for the separation and analysis of non-sperm and sperm fractions in sexual assault evidence (i.e. prior to DNA profiling and identification of body fluid of origin); and (ii) a rapid RNA direct lysis method for higher throughput screening of forensic biological evidence (blood, semen, saliva, vaginal secretions and menstrual blood).

Accomplishments: The goals of the current project were to custom-develop for DFSC casework use: (i) a differential DNA/RNA co-extraction isolation protocol for the separation and analysis of non-sperm and sperm fractions in sexual assault evidence (i.e. prior to DNA profiling and identification of body fluid of origin); and (ii) a rapid RNA direct lysis method for higher throughput screening of forensic biological evidence (blood, semen, saliva, vaginal secretions and menstrual blood). All aims of the project were successfully completed.
The results from the validation studies demonstrate the successful use of EZ1 DNA waste fractions for the recovery for RNA for body fluid identification. The method can be applied to both non-differential and differential extractions. It is fully compatible with existing DFSC DNA protocols and therefore does not require additional validation of DNA protocols. The results of this study were presented at the 21st Triennial Meeting of the International Association of Forensic Sciences (2017) and we are currently working with QIAGEN to develop a user-defined protocol that will be available on their website.
The rapid RNA analysis method was developed that permits an ~3-hour 'sample-to-detection' time. The rapid lysis, Nucelospin RNA (Macherey-Nagel), permits the extraction of 8-48 samples or 96-samples in ~1 hour or less. In addition to timeliness, this extraction has the potential to be automated. Additionally, the methods developed here for the rapid RNA analysis can also be applied to the RNA EZ1 method as well in order to improve the timeliness of that method as well. The Nucleospin RNA purification can be used to recover RNA from the EZ1 waste fractions as well.
To aid in the analysis and interpretation of the HRM assays, we have evaluated the ScreenClust software (QIAGEN).
The technology transfer of all methods and protocols developed in the current study has been completed. Comprehensive protocol manuals for both the EZ1 RNA and rapid RNA analysis method have been prepared and provided to DFSC. Reagents and consumables have also been provided for initial in-house testing by DFSC. RotorGene run template files and bin sets have also been created and transferred: 1) EZ1 – standard sexual assault and blood-menstrual blood HRM assays, 2) Rapid RNA (Nucleopsin) – rapid HRM assays (blood-menstrual blood, sexual assault, 5-fluid 6-plex) and standard HRM assays (blood-menstrual blood and sexual assault). We have conducted an on-site one day training for the rapid RNA analysis methods and will continue to provide

support to DFSC regarding these methods as needed.
We are hopeful that the results of the current work will aid in the implementation of RNA profiling for body fluid identification.

Training Opportunities: Nothing to Report

Results Dissemination: The results of this study were presented at the 21st Triennial Meeting of the International Association of Forensic Sciences (2017) and we are currently working with QIAGEN to develop a user-defined protocol that will be available on their website.

Honors and Awards: Nothing to Report

Protocol Activity Status:

Technology Transfer: All developed protocols have been transferred to the DFSC laboratory including protocol manuals and initial reagents and consumables for in-house testing. On site training was performed on rapid RNA analysis methods.
There were no patents, inventions or licenses developed during the study.

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Biological Evidence

Optimization of RNA Body FluID Assays: Differential Co-Extraction for Sperm Analysis and Rapid Direct Lysis for High-Throughput Evidence Screening

FINAL REPORT

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FOREWARD

Conventional body fluid identification methods use a variety of labor intensive, technologically diverse techniques that do not permit the ability to positively identify frequently encountered body fluids or tissues such as vaginal secretions, menstrual blood or skin. Since the nature of the biological material present might be crucial to the investigation and prosecution of a criminal offense, more specific body fluid identification methods are required. One such approach to body fluid identification is the use of mRNA profiling which utilizes a body fluid or tissue specific pattern of gene expression to definitively identify the tissue or body fluids present. Messenger RNA detection assays permit the positive identification of all forensically relevant biological fluids and tissues (blood, semen, saliva, vaginal secretions and skin) in a single assay format with numerous highly specific biomarkers employed for each body fluid and tissue[1-30]. Messenger RNA profiling has been adopted into operational casework use in New Zealand, the Netherlands and Germany. We have recently completed the development and validation of a comprehensive RNA-based body fluid identification system for the DFSC laboratory for possibly 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.

Despite the success of RNA profiling strategies for body fluid identification, continued development work is needed to fully support current workflows of operational crime laboratories. Current RNA profiling methods are not yet fully compatible with differential extraction workflows for single source DNA profiling of sexual assault samples. RNA/DNA co-extraction methods are designed to recover all DNA in one fraction and therefore do not separate sperm and non-sperm fractions prior to DNA isolation. A significant portion of the evidence examined by many operational crime laboratories comprises sexual assault samples and therefore the ability to seamlessly integrate a differential extraction strategy with RNA based body fluid identification systems profiling systems is desirable to encourage routine and widespread usage of the RNA technology. Additionally, the length of time required to perform the RNA profiling assays is not currently optimal for high-throughput evidence screening. Thus improvements to overall ‘sample to detection’ times as well as the number of samples to be evaluated simultaneously (e.g. 96 well formats) would be highly beneficial.

In order to address these workflow issues, the aims of the current work were to custom-develop for DFSC casework use: (i) a differential DNA/RNA co-extraction isolation protocol for the separation and analysis of non-sperm and sperm fractions in sexual assault evidence (i.e. prior to DNA profiling and identification of body fluid of origin); and (ii) a rapid RNA direct lysis method for higher throughput screening of forensic biological evidence (blood, semen, saliva, vaginal secretions and menstrual blood). All aims of the project were successfully completed. The results from each aim are described below.

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III. STATEMENT OF THE PROBLEM

Messenger RNA (mRNA) profiling is now an established alternative molecular based approach for body fluid identification that permits the identification of all frequently encountered body fluids including blood, semen, saliva, vaginal secretions, menstrual blood and skin. Identification of these body fluids and tissues can be achieved in a single assay format with numerous biomarkers for the definitive identification of target body fluids and tissues. The advantages of RNA-based profiling assays have resulted in their use internationally in operational crime laboratories. Despite the implementation of these assays, further development and optimization is needed in order to fully support current workflows of casework laboratories. The development of a differential DNA/RNA co-extraction method is needed in order to permit single source DNA profiling of sexual assault evidence (i.e. separation of sperm and non-sperm fractions). Additionally, improvements to the overall timeliness of 'sample to detection' times will serve to assist laboratories with high-throughput requirements. In order to address these workflow issues, modifications to existing established methodologies should be possible.

In order to address these above mentioned workflow issues, the aims of the current work were to expand upon the existing DFSC RNA profiling portfolio to custom-develop for DFSC casework use: (i) a differential DNA/RNA co-extraction isolation protocol for the separation and analysis of non-sperm and sperm fractions in sexual assault evidence (i.e. prior to DNA profiling and identification of body fluid of origin); and (ii) a rapid RNA direct lysis method for higher throughput screening of forensic biological evidence (blood, semen, saliva, vaginal secretions and menstrual blood) (Figure 1).

IV. RESULTS

A. Development of a differential DNA/RNA co-extraction process for the separation of sperm and non-sperm cells for the analysis of sexual assault evidence

1. Evaluation of EZ1® 'pre' and 'post' fractions

   The first aim of the current work was to improve upon the existing differential DNA/RNA co-extraction isolation protocol developed in previous work. This involved the use of the QIAGEN AllPrep® Micro DNA/RNA co-extraction kit. However, during the initial project kick-off meeting, there was a discussion on developing a method to utilize the current DFSC EZ1® DNA Investigator® (QIAGEN) DNA extraction method to recover RNA. The potential use of DNA extraction 'waste' for the recovery of RNA has been demonstrated by other laboratories (ex: ESR [31], waste fractions from their DNA IQ (Promega) extraction). If RNA could be recovered from the EZ1® waste fractions, this would result in a DNA/RNA co-isolation from individual samples without any change to

existing DNA protocols. This would eliminate the need for validation of DNA protocols as would have been required using the previously developed QIAGEN AllPrep® Micro DNA/RNA co-extraction. Since DFSC non-differential and differential EZ1® DNA Investigator® protocols were available, if successful this approach would accomplish the goal of a differential DNA/RNA co-extraction method.

For the non-differential EZ1® DNA extraction, a one-hour lysis (56ºC, 900rpm) was performed using diluted ATL buffer, proteinase K and DTT (for semen-containing samples only). Following the lysis, the samples were extracted using the large volume protocol and 50 µL TE for elution. For the differential EZ1® DNA extraction, a one-hour lysis (56ºC, 900 rpm) was also performed using diluted ATL buffer and proteinase K. The QIAcube® separation and wash protocols were used to separate the sperm (F2) and non-sperm fractions (F1). An additional lysis was performed for the F2 sperm fraction and F3 substrate fractions using buffer G2, proteinase K and DTT for 10 minutes (70ºC, 900rpm). The F1 non-sperm fraction was extracted using the large volume protocol and 50 µL TE for elution. The F2 sperm and F3 substrate fractions were extracted using the trace protocol and 50 µL TE for elution. Initial EZ1® extractions were performed by DFSC until an EZ1® instrument could be procured for in-house experiments. All protocols were performed according to DFSC protocols, with the exception of the addition of carrier RNA for large volume protocol extractions. We were unaware that this was added routinely by DFSC and therefore all experiments performed in-house at UCF did not include carrier RNA. It was determined that this would not have a significant impact on the work since this protocol would mainly be used for differential extractions where the trace protocol is used for the F2 sperm fractions.

The initial fractions collected during the EZ1® DNA Investigator® extraction for possible recovery of RNA included: 1) "pre" fraction – 10 µL collected (with and without buffer MTL added, which is part of the EZ1® large volume extraction protocol), 2) "post" fraction EZ1® cartridge lane 1 (large volume protocol) or EZ1® cartridge lane 3 (trace protocol) (Figure 2), 3) "post" fraction remaining sample + MTL buffer (Figure 2). Cartridge lane 2 waste was also initially tested but was not successful and therefore not included in further studies. We also additionally tested combined "post" fractions (EZ1® cartridge lane 1 (or 3) and remaining sample + MTL) to potentially improve RNA yield since the waste fractions were now combined.

Waste fractions were purified using the QIAGEN RNeasy® MinElute® Clean Up kit. This kit is an easy-to-use silica spin column method that can be performed manually or on the QIACube®. Briefly, the protocol includes initial flow-through steps to bind the sample to the silica column, followed by washes with 500 µL buffer RPE, 500 µL 80% ethanol, a 5-min centrifuge step to dry the columns and elution using 16 µL of RNase-free water (~14 µl recovered). The standard protocol for the RNeasy® MinElute® Clean Up kit recommends the use of only 100 or 200 µL of starting material. While these volumes of waste fraction were tested (and ultimately used), initial studies involved the use of the full waste fraction volume. For the "post" samples this volume ranged from 600 µL (remaining sample + MTL) to ~1 mL (EZ1® cartridge lane 1 or 3). For the combined "post" fractions, this volume was ~1.6 mL. The use of the larger volumes was possible but required additional flow-through steps (from ~5 to 13 flow-through steps) with each flow-through step requiring only a 15 sec centrifugation step. The standard

RNeasy® MinElute® Clean Up kit protocol was followed for all samples following the initial flow-through steps.

The purified RNA samples were then DNase treated (Turbo DNase, ThermoFisher, 30 min at 37ºC) followed by enzyme inactivation (10 min at 75ºC). No quantitation was performed and 8 µL of the purified RNA extracted was added to the reverse transcription reaction (SuperScript IV VILO RT, ThermoFisher) which is a ~25 min reaction. Some early work was performed using the ABI High Capacity RT kit (~2.5 hours), but a majority of experiments were performed using the SuperScript IV VILO RT kit. High-resolution melt (HRM) analysis was performed using the existing sexual assault (Figure 3) and blood-menstrual blood assays (Figure 4) (from phase I of the work; previous study). The only modification was that both assays could now be performed using the same Rotor-Gene® protocol using the assay conditions for the blood-menstrual blood assay.

The initial DFSC sample set included blood, saliva and menstrual blood samples extracted using the non-differential EZ1® DNA extraction and semen samples extracted using the differential EZ1 DNA extraction. Table 1 lists the different fractions collected from each sample type (both "pre" and "post" samples as previously described). To initially test the ability to recover RNA from EZ1® waste fractions, we evaluated the non-differentially extracted blood, saliva and menstrual blood samples. The results from this initial testing are provided in Tables 2 (non-differential extraction) and 3 (differential extraction). For the blood samples (two donors), both blood biomarkers (ANK1 and ALAS2), were detected in most fractions, with the exception of EZ1® cartridge lane 2 in which the biomarkers were not detected for either donor or the 10 µL pre without MTL sample for donor 1. For saliva (two donors), HTN3 was detected in all samples except for again the EZ1® cartridge lane 2 fractions. No results were obtained for the menstrual blood "pre" samples (two donors, no detection of MMP10 or LEFTY2). It was subsequently determined that the source for the menstrual blood samples was a commercial company which we have also previously used and not obtained successful results with any of their menstrual blood samples. Therefore, the "post" fractions for these samples were not tested. The results from the differentially extracted semen samples are shown in Table 3. Semen markers (SEMG1 and PRM2) were both detected in the F1 non-sperm fraction using both "pre" and "post" samples, with the exception again of the EZ1® cartridge lane 2 for both donors and one "pre" 10 µL with MTL sample for one of the donors. The presence of SEMG1 is expected as seminal fluid would be present in the non-sperm fraction and PRM2 (sperm) is also expected due to premature lysis of sperm cells into the non-sperm fraction. PRM2 is extremely abundant and therefore will be detected even with trace levels of sample. For the F2 sperm fractions, PRM2 was detected which is as expected since this is a sperm-specific marker. Again, RNA was not detected in the EZ1® cartridge lane 2 samples. For the F3 substrate fraction, PRM2 (sperm) was detected in most samples. Based on these initial results, we proceeded with testing of all fractions except the EZ1® cartridge lane 2 fraction as it was not successful.

Since these samples were not extracted in-house at UCF, we next performed in-house EZ1® non-differential (one donor each of blood, menstrual blood, saliva, semen and vaginal secretions) and differential (two donors sets; ¼ vaginal swab – 20 µL semen mixtures) extractions. The number of fractions was reduced to one "pre" (10 µL after

initial lysis but before addition of MTL buffer) and two "post" (remaining sample + MTL after extraction and EZ1® cartridge lane 1 or 3 waste). Extraction blanks were added to this experiment (not available in the initial experiment). The results from the HRM analysis of these samples are provided in Tables 4 (non-differential extraction) and 5 (differential extraction). As can be seen from these results, RNA was successfully detected in all samples. For the non-differential single source samples, all expected biomarkers were detected from "pre" and "post" fractions, with the exception of ALAS2 in the "pre" sample for blood. This experiment demonstrated the ability to obtain RNA in EZ1® waste from semen, vaginal secretions and menstrual blood. The differential extraction performed in this experiment included vaginal-semen mixtures (two donor sets) in order to test the full differential protocol and evaluate the ability to detect vaginal secretions in these samples. As can be seen in Table 5, CYP2B7P1 (vaginal secretions) was detected in all samples for the F1 non-sperm fraction as expected. It was also detected in the EZ1® cartridge lane 3 samples for the F3 substrate samples for both donors. SEMG1 was detected in all F1 non-sperm fractions for donor set 1 and in one "pre" and both "post" samples for donor set 2. PRM2 (sperm) was detected in the F2 sperm fractions for all samples for donor set 2. It was only detected in the EZ1 cartridge lane 3 waste for donor set 1. PRM2, and occasionally SEMG1), were detected still in the F3 substrate fraction.

Overall, the results of the initial testing were highly successful. Therefore, optimization and validation experiments for the EZ1® RNA recovery protocol were next performed. The DNA from each of the EZ1® extractions performed in the study was also tested. However since no validation of this portion of the assay was required since it was the current DFSC protocol, the DNA results will not be described in detail in the report. DNA of sufficient quantity was obtained from all samples with suitable quality to permit autosomal STR profiles to be obtained.

2. Non-differential EZ1® extraction

Method Summary

Initial testing included the previously described fractions: 1) "pre" – 10 µL after initial lysis, 2) "post" - remaining sample + MTL buffer and 3) "post" – EZ1® cartridge lane 1. During initial validation, we also included a combined post sample (2 + 3) in order to maximize RNA recovery. While successful, it was decided that the "pre" samples would not be ideal as it would remove sample upfront and result in the need for possible re-validation of DNA protocols. Additionally, the combined "post" fraction was also successful but was determined not to be ideal due to the large number of initial flow-through steps required as a result of the large sample volume. We next evaluated the use of 100 and 200 µl aliquots from the "post" waste fractions since these volumes were included in the manufacturer's suggested protocol.

To evaluate the use of the 100 and 200 µL aliquots, we tested two donors of each body fluid. The results from this experiment are provided in Table 6. The expected biomarkers for each body fluid were successfully detected in both "post" sample types and for both donors. Examples of the HRM melt plot results for blood (blood-menstrual blood HRM assay) and semen (sexual assault HRM assay) are shown in Figures 5 and 6, respectively. The only exceptions were the 100 µL aliquots for menstrual blood and the

100 µL EZ1® cartridge lane 1 sample for one of the two donors in each set. Since the 200 µl aliquots were more successful, all subsequent validation experiments were performed using 200 µL aliquots. The 200 µL taken from the EZ1® cartridge lane 1 (large volume protocol) or lane 3 (trace protocol) was decided to be more optimal for routine use and therefore selected as part of the standard protocol. All subsequent validation studies involved the use of 200 µL aliquots taken from the EZ1® cartridge lane 1 or 3 (although for some experiments the remaining sample + MTL fractions were also tested since it was before the final decision on the waste fraction to use was made). Additionally, a custom QIACube® protocol was created in order to provide a semi-automated purification protocol instead of the manual RNeasy® MinElute® Clean Up kit protocol. A custom protocol needed to be created to permit the use of the 200 µL starting material since the standard RNeasy® MinElute® Clean Up kit QIACube® protocol uses only 100 µL of starting material.

Swab/Stain Size

To evaluate the sensitivity of the developed method in terms of size of stain or swab needed for analysis, we tested the following sample sizes for both swab and body fluid stains: 1) swabs (buccal, vaginal and menstrual; 3 donors each) – ½, ¼ and 1/8th swab; 2) dried stains prepared from liquid samples (saliva, semen, blood; 3 donors each) – 25, 10, 5 and 1 µL stains; 3) dried 50 µL stains (semen (1 donor) and blood (2 donors) – ½, ¼, 1/8 and 1/16th stain. A summary of the results from the stain and swab size study is shown in Table 7. For the dried saliva (buccal), vaginal secretions and menstrual blood swabs, the expected biomarkers were detected in each of the three donors for all swab sizes (1/2, 1/4 and 1/8th). For the 50 µL dried bloodstains (cotton cloth), ANK1 and ALAS2 (blood biomarkers) were detected in both donors for the ½, ¼, and 1/16th stain sizes. The blood biomarkers were only detected in one of the two donors for the 1/8th size stain. Since the biomarkers were detected in the smaller 1/16th stain size, it is unclear why the biomarkers were not detected for one of the 1/8th stain size. For semen, similar results were obtained with PRM2 (sperm) detected in all samples regardless of stain size and SEMG1 detected in all stain sizes except for the 1/8th stain. For the dried stains prepared from liquid fluids, the expected biomarkers were detected in all samples with the exception of ANK1 not detected for one of the three donors for a 10 and 5 µL stain size. It is important to note that all expected biomarkers were detected for the 1 µL stain size demonstrating the relatively high sensitivity of the developed method.

Mixtures

Any developed forensic body fluid identification assay needs to be able to identify the presence of multiple body fluids in an individual sample. In order to evaluate the ability of the EZ1® RNA recovery method to detect body fluid admixtures, we prepared several two-fluid mixtures using a dried stain/swab with a liquid body fluid added in varying volumes. For the dried stain portion of the mixture, a ½ 50 µL bloodstain, ½ buccal swab, ¼ vaginal swab or ¼ menstrual blood swab was used. To these dried stains, liquid semen or saliva was added in 10, 5 or 1 µL volumes and allowed to dry. The results from the testing of the two-fluid admixed samples are provided in Table 8. Two donors sets for each mixture type were tested.

For the mixtures in which semen was added as a second body fluid (top set of Table 8), the presence of the body fluid from the original dried body fluid portion of the mixture was successfully detected in all donors sets. Semen, the liquid component added to the dried stain, was successfully detected in most samples including when only 1 μL of semen was added. For the saliva-semen and vaginal-semen mixtures, semen in 1 μL quantities was only detected in one of the two donor sets. However, generally these results demonstrate the ability to detect two-fluid mixtures even when the minor component is present in very small quantities.

For the mixtures in which saliva was added as a second body fluid (bottom section of Table 8), the presence of the body fluid from the original dried body fluid portion of the mixture was successfully detected in all donors sets. More variation was observed in the identification of saliva compared to the semen mixtures. This is not unexpected due to several factors: 1) saliva has more variable RNA levels than semen, 2) the viscosity of saliva makes pipetting small volumes sometimes difficult, 3) there are endogenous bacteria present in saliva not present in semen and 4) the abundance of the saliva biomarkers are lower than that of semen biomarkers. These factors may contribute to the additional variability in successful detection of saliva. However, despite these factors, saliva could still be identified in a large number of samples. When added in 10 μL volumes, saliva was detected in both donor sets for each mixture type. When added in 5 μL volumes, saliva was detected in one or both donor sets for each mixture type. When saliva was added in 1 μL volumes, it was detected in both donor sets for blood-saliva mixtures and in one of the two donor sets for vaginal-saliva mixtures. It was not detected in either donor set for the semen-saliva or menstrual-saliva sample sets. However, again overall these results demonstrate the ability to detect two-fluid mixtures even when the minor component is present in very small quantities.

Substrate and Temperature

All of the previous experiments were performed using body fluid samples dried on cotton swabs or cloth. We next evaluated whether there were any effects from samples dried on other substrates such as denim, polyester or paper. We also evaluated whether exposure to varying temperatures would have an adverse effect on body fluid identification.

We evaluated blood and semen samples (50 μL aliquots) dried onto denim, polyester and paper that had been stored at room temperature for 1 month, 6 months and 1 year (Table 9). For semen, SEMG1 and PRM2 were detected in a majority of samples with the exception of SEMG1 in denim samples stored at room temperature for 1 and 6 months. Both biomarkers were detected for all polyester and paper samples, with the exception of SEMG1 for one of the two donors on paper for 1 year. For blood, ALAS2 was detected in all samples on denim whereas ANK1 was only detected in one of the two donors for the denim samples. Both biomarkers were detected for all paper samples, with the exception of ANK1 for one of the two donors for 1 year. More variability was observed for bloodstains deposited onto polyester. ALAS2 was detected in a majority of samples, but ANK1 was only detected in the 6 month samples. Overall, the inability to efficiently remove the sample from the substrate during the initial lysis was observed for many of the denim and polyester samples. This could account for some of the negative results that were observed rather than interference from a particular substrate.

Saliva and vaginal secretions samples (swabs; 2 donors of each body fluid) were exposed to 22ºC, 37ºC and 56ºC temperatures for 1 week and 1 month (Table 10). Saliva (HTN3) was detected in all samples regardless of the temperature or exposure time. Vaginal secretions (CYP2B7P1) was also detected in a majority of samples, with the exception of 1 week samples at 22ºC and 56ºC in which it was detected in only one of the two samples tested. However, since the biomarker was detected at the longer time point of 1 month for all samples, it demonstrates the ability to successfully identify body fluids using the developed protocol in environmentally (temperature) exposed samples.

Blood stains (cotton cloth) that had been stored in a car trunk for 2 weeks and a car back seat for 1 week were also available for testing. These samples were in the car during a Florida summer and therefore exposed to high temperatures and humidity. ALAS2 was detected in both the car trunk and car back seat samples and ANK1 was detected in the car seat sample as well.

Large Volume Purifications

With only 200 µL aliquots used, ~800 µL of the EZ1® cartridge lane 1 or 3 waste was still available for subsequent analysis if needed. Since we had previously used the full volume for purification, we wanted to evaluate whether the purification of the remaining 800 µL of waste fraction would result in positive biomarker detection for those samples in which a negative result was obtained using the original 200 µL aliquot. Overall between the stain size and mixture studies, 198 samples were tested (this value included some remaining sample + MTL fraction testing), only 15 samples (~7.6%) were tested with the large volume purification protocol. Only 7 of the 15 samples (~3.5%) were truly negative with the remaining 8 samples having one of the two expected biomarkers present (presence of that body fluid could still be inferred). Of the 7 negative samples, only 1 (1/198 = 0.5%) resulted in body fluid identification after large volume purification. For the 8 samples with one of the two expected biomarkers, the large volume purification resulted in detection of the second biomarker for six of samples. Overall, it was determined that large volume purification was not necessary as it resulted in little additional biomarker recovery. However, it may be possible to utilize this protocol for samples in which only one of the two expected biomarkers is present. These samples, with the presence of one biomarker, would indicate the presence of the body fluid (and sometimes could be conclusive on its own) whereas a true negative would not need to be further tested.

Freeze-Thaw and 4ºC Storage

All EZ1® cartridge lane 1 or 3 samples are stored at -20ºC until needed. Therefore, each sample is thawed at least once before analysis. Since all samples in the validation experiments (including mixtures and small sample sizes) were tested in this manner, there was no initial indication that freeze-thawing would have an adverse effect on the results. Each waste fraction is ~1 mL and therefore four 200 µL aliquots can be taken from the same sample. To further test the possible effects from freeze-thawing, we tested two donors of each body fluid, using four purifications with re-freezing and thawing in between each purification. The results from this study are shown in Table 11. As can be seen biomarkers were successfully detected down to the 4th aliquot for saliva, semen and menstrual blood. For blood, ANK1 and ALAS2 were detected in the 2nd and

3rd aliquots tested, but not the 4th (ALAS2 present for one of the two samples only). Similarly for vaginal secretions, CYP2B7P1 was not detected n the 4th aliquot. Therefore, the waste fractions were stable through several freeze-thaws and it would be unlikely that most samples would be required to be tested three or four times.

To avoid any possible effect from -20ºC storage and to better accommodate storage capabilities of laboratories, we also evaluated the stability of the waste fractions when stored at 4ºC rather than -20ºC. We evaluated 4ºC storage for days, weeks and months length of time (Table 12). As can be seen from Table 12, storage for ~1 week at 4ºC was successful and could be suitable if needed. However, the recommendation would be no more than 1 week at 4ºC due to the loss of some biomarker detection over weeks – months storage, particular for saliva and vaginal secretions.

3. Differential EZ1® extraction

Method Summary

Initial testing included the previously described fractions: 1) "pre" – 10 µL after initial lysis, 2) "post" - remaining sample + MTL buffer and 3) "post" – EZ1® cartridge lane 1. During initial validation, we also included a combined post sample for the F1 non-sperm fractions in order to maximize RNA recovery. Very little sample remained in the "remaining sample + MTL buffer" fraction for the F2 sperm and F3 non-sperm samples since the trace protocol was used. For the differential extraction, "post" fractions led to better biomarker detection (i.e. biomarkers detected in a larger number of samples). Since the "pre" samples were not as successful and would not be ideal as it would remove sample upfront and result in the need for possible re-validation of DNA protocols, they were excluded from further analysis. Additionally, the combined "post" fraction was also successful but was determined not to be ideal due to the large number of initial flow-through steps required as a result of the large sample volume. Similar to the non-differential extraction, we tested 100 and 200 µL aliquots for the waste fractions from differential EZ1® extraction and 200 µL aliquots from the EZ1® cartridge lane 1 or 3 waste was suitable for us. This would keep the protocols the same between the differential and non-differential extractions which is ideal. To further evaluate the use of the EZ1® RNA recovery protocol for differentially extracted samples, we tested vaginal-semen and vaginal-semen-saliva mixtures.

Vaginal-semen mixtures

Five sets of vaginal-semen mixtures were prepared using different donor pairs. For each donor set, ¼ vaginal swab was used with liquid semen added in 10, 5 and 1 µL aliquots and allowed to dry. 200 µL aliquots of the purified RNA recovered from the EZ1® cartridge lane 1 (F1 non-sperm fraction) and lane 3 (F2 sperm and F3 substrate) samples were tested. The F3 substrate fractions were tested simply to evaluate biomarker detection in this fraction. It is possible that analysis of the F3 substrate fraction would not be required, but testing would follow standard DFSC protocols regarding this fraction. The results from this study are provided in Table 12. Examples of the melt plots obtained for the F1 non-sperm and F2 sperm fractions are shown in Figure 7. For the F1 non-sperm fractions, CYP2B7P1 was detected in all samples except for one donor set in the

10 μL semen mixture. SEMG1 was also detected in all samples for four of the five donors. We have observed an absence of SEMG1 for the one donor in other RNA assays so this was likely a sample issue. PRM2 was detected in four of the five donor sets with the 5th set with semen from a vasectomized male and therefore PRM2 was not expected. For the F2 fractions, PRM2 was detected in all samples. SEMG1 and CYP2B7P1 could also be detected in this fraction as well. There was significant detection of all biomarkers with the F3 substrate fractions, indicating that re-extraction from the substrate after the initial lysis could result in additional biomarker detection. Interestingly, the F1 fraction alone could be used for identification of semen and saliva for most samples. Therefore, it might be possible to test the F1 fraction first and if all biomarkers are present testing of the F2 sperm and F3 substrate fractions would not be needed. If semen was not detected in the F1 non-sperm fraction then the F2 sperm fraction could then be tested.

Vaginal-semen-saliva mixtures

Five sets of vaginal-semen-saliva mixtures were prepared using different donor pairs. For each donor set, ¼ vaginal swab was used, with 5 μL liquid semen, and liquid saliva added in 25, 10, 5 and 1 μL aliquots and allowed to dry. 200 μL aliquots of the purified RNA recovered from the EZ1® cartridge lane 1 (F1 non-sperm fraction) and lane 3 (F2 sperm and F3 substrate) samples were tested. The results from this study are provided in Table 13. All three body fluids could be detected in the F1 non-sperm fractions. Saliva was not detected in the F1 non-sperm fractions when added in 1 μL volumes. For the F2 sperm fractions, PRM2 was detected for all samples except one donor set (1 μL saliva mixture). Saliva was readily detected in the F2 fraction with detection even in one of the 1 μL mixture samples. All three body fluids were also still present in the F3 substrate fractions. Saliva (HTN3) was detected in two donor sets for the 1 μL volume. It may be necessary to test all three fractions to detect trace amounts of saliva, since it was present in F2 sperm and F3 substrate fractions.

B. Development of a rapid analysis method for high-throughput screening of forensic biological evidence

1. Development of Rapid HRM Body Fluid Identification Assays

The previously developed HRM assays (blood-menstrual blood and sexual assault) required ~2.5 hours to complete. In order to develop a rapid analysis system for high-throughput screening the run length time needed to be reduced. Additionally we also wanted to develop a new rapid 5-fluid 6-plex HRM assay that contained biomarkers for blood, semen (both SEMG1 and PRM2), saliva, vaginal secretions and menstrual blood.

For the blood-menstrual blood and sexual assault assays, we used the original full-length assays and then modified the existing conditions including primer concentrations, reaction mix and cycling conditions. No changes were made to the biomarkers or primer sequences. For the new rapid blood-menstrual blood and rapid sexual assault HRM assays, the run time is ~86 minutes and both assays can be run on the same program. The reaction volume was reduced to 20 μL with 2 μL of cDNA added in the reaction. For the new rapid 5-fluid 6-plex the same biomarkers as the rapid blood-

menstrual blood and sexual assault assays were used: HTN3 (saliva), SEMG1 and PRM2 (semen), ANK1 (blood), CYP2B7P1 (vaginal secretions) and LEFTY2 (menstrual blood). The same primer sequences were used for all biomarkers except for ANK1 in which a different primer set was needed to eliminate overlapping Tm value ranges. A representative melt plot for the new rapid 5-fluid 6-plex is shown in Figure 8.

Detailed protocols for all three rapid assays have been provided in the comprehensive protocol manual for the project. These assays were designed for use with the rapid extraction methods (see below) but could be used with the EZ1® RNA method developed as well.

2. Rapid Extraction Methods

The goal of the rapid RNA assay was ~3 hours or less from 'sample to detection' time. This does not necessarily include time for sample cutting as that is required for any assay. However, once the samples are available the assay should only take 3 hours to perform from extraction to detection. This includes the lysis/extraction, DNase treatment, RT reaction and HRM assay (amplification and detection).

We evaluated two different rapid RNA extraction methods: the QIAGEN RNeasy RNA kit and the Macherey-Nagel Nucleospin® RNA kit. Both extractions require the use of a vacuum manifold for the flow-through steps. While RNA was successfully isolated using the QIAGEN RNeasy kit, the vacuum manifold did not work as well and it was necessary to push down on the plates in order to a suitable seal to be obtained. This is not ideal as it could lead to contamination. The vacuum manifold for the Macherey-Nagel Nucleospin® kit worked well and the kit is available in 8-strip or 96-well plate formats. For the 8-strip format, up to 6 strips can be used at a time thereby giving the laboratory flexibility in the number of samples to be extracted at one time. Filter strips are also available for both formats that permit recovery of the full lysate volume separate from the substrate thereby resulting in maximum sample recovery. Another benefit to the Nucleospin® RNA kits is that they can be automated. The manufacturer can be contacted for scripts and other considerations for adapting to certain workstations. Figure 9 shows the vacuum apparatus set up as well as examples of the 8-strip and 96-well plate formats.

The overall extraction process is relatively simple and is similar to other silica-column based extractions. A small piece of stain is added to the filter strip or plate. Three-hundred microliters of lysis buffer (with DTT added) is added to each well. The samples are incubated at room temperature for 5 min at 400 rpm. The strips or plates are vacuum filtered collecting the lysate in tubes with the stain/swab piece remaining in the filter strip/plate. The lysate is then bound to the column, de-salted, washed and then vacuum dried. Each step requires on a quick vacuum filtration step (~20 sec) except for the drying step which is 3 minutes. The purified RNA is eluted using 50 µL nuclease-free water.

Subsequent to the rapid lysis, the samples are DNase treated using the ezDNase supplied with the SuperScript IV VILO RT kit. The ezDNase treatment is only 2 min and is performed in the same 0.2 mL PCR tube or 96-well plate as the subsequent RT fraction. The RT reaction mix is added to the DNased-sample and the RT is performed which is ~25 minutes. This process in combination with the rapid HRM assays developed fit within the proposed 3-hour 'sample to detection' time frame (lysis – ~1 hour, DNase/RT - ~30 min, HRM - ~86 min). The validation of the developed RNA analysis

method is described below. All validation studies were performed using the 8-strip format.

3. Validation of the Rapid RNA Analysis Method

Single Source Samples

To initially evaluate the developed rapid RNA analysis method, single source samples were tested including samples both on cotton swabs as well as dried stains (cotton cloth). For the swab samples, cuttings from both the swab tip and side of the swab were tested. One donor was tested in duplicate. For the dried stains on cotton cloth, two samplings from the same stain were tested. Each sample was tested with the rapid sexual assault, rapid blood-menstrual blood and rapid 5-fluid 6-plex assays (Table 14). For all three rapid HRM assays, no false positive results were observed. The expected biomarkers were detected for body fluid samples across all three assays with a small number of false negative results obtained. The same sample sets were used with all three rapid HRM assays and the results were relatively consistent with the exception of saliva samples using the rapid 5-fluid 6-plex assay. HTN3 (saliva) was only detected in 12 of the 22 samples, whereas 21 of the 22 samples were positive using the rapid sexual assault assay. Minor modifications were made to the 5-fluid 6-plex (finalized assay is what is provided in the comprehensive protocol manual provided to DFSC) and the saliva samples were re-tested. This resulted in the detection of saliva in 18 of the 22 samples. This was an improvement compared to the original testing. The 6-plex systems do not seem as robust as the smaller 4-biomarker assays and could account for the small difference in success rate for saliva samples between these two assays. Overall there was no difference observed between sampling of the tip and side of swabs.

Stain Size

The rapid RNA extraction requires the use of only a small portion of swab or stain (e.g. swab tip). In order to try to provide a quantitative assessment of the stain size, we prepared 1, 2 and 3 μL stains of semen and saliva, as well as saliva-semen mixtures (1:1 mixtures by volume, 2 and 4 μL stains). The full stain was used in the rapid RNA analysis method and the results of this study are shown in Table 15 (rapid sexual assault assay). As can be seen from the results, successful detection of both semen and saliva was obtained with as little as a 1 μL sample. More false negatives were observed for the 2 μL saliva-semen mixtures. However, successful detection of both body fluids was possible with the 4 μL mixture stains. Representative melt plots from the 1μL single source and 2 μL mixture samples are shown in Figure 10.

Temperature and Substrate

We next evaluated whether there were any effects from samples dried on other substrates such as denim, polyester or paper. We also evaluated whether exposure to varying temperatures would have an adverse effect on body fluid identification. The previously described temperature and substrate samples were available for testing with the rapid RNA analysis method: saliva and vaginal secretions samples (swabs; 2 donors

of each body fluid) exposed to 22ºC, 37ºC and 56ºC temperatures for 1 week and 1 month (Table 16). As can be seen from Table 16, semen was detected in 1 month – 1 year room temperature samples on denim, polyester and paper using the rapid RNA analysis method and the rapid sexual assault HRM assay. Saliva was detected in all samples after exposure to 22ºC and 37ºC for 1 week and 1 month. Saliva was only detected in two of the four samples exposed to 56ºC for 1 month. This is an extremely high temperature and not likely one that would be routinely encountered in forensic operations. For vaginal secretions, CYP2B7P1 was detected in all samples except for two of the four samples exposed to 37ºC for 1 month. Overall, there did not appear to be any adverse effects from substrate or temperature but a larger study would be needed before a final assessment of assay performance with environmentally challenged samples could be made.

Mixtures

As discussed previously, any developed forensic body fluid identification assay needs to be able to identify the presence of multiple body fluids in an individual sample. In order to evaluate the ability of the rapid RNA analysis method to identify multiple components in a body fluid admixture, we prepared saliva-semen, semen-saliva and vaginal secretions-semen mixtures. For the saliva-semen mixtures, 5 and 1 µL of liquid semen was dried into a dried buccal swab. For the semen-saliva mixtures, 5 and 1 µL of liquid saliva was dried onto a swab containing 10 µL semen (dried). For both the semen-saliva and saliva-semen mixtures, the tip of the swab was used for analysis. For the vaginal-semen mixtures, 5 and 1 µL of semen was added to a ~1/8th dried vaginal swab. The results from the mixture study are provided in Table 17. Each of the mixtures was tested with the rapid sexual assault and rapid 5-fluid 6-plex. For the saliva-semen mixtures, both saliva and semen were detected in all samples even when only 1 µL of semen was present. For the vaginal-semen mixtures, semen was detected in all samples including when only 1 µL of semen was present. Vaginal secretions was detected mainly in the mixtures with 1 µL of semen and for an as yet unknown reason was not detected in any of the mixtures where 5 µL of semen was used.

C. Additional Studies

1. Rapid purification of EZ1® waste

The original EZ1® RNA recovery protocol included the use of the QIAGEN RNeasy® MinElute® Clean Up kit for purification of the EZ1® waste fractions. While this purification method can be completed in a relatively short amount of time and in a semi-automated manner (QIACube®, 12 samples at a time), it is not a high-throughout purification method. Each sample must be purified using individual spin columns rather than in 8-strip or 96-well plate format as with the rapid RNA assay (Nucleospin® 8/96 RNA). Since a successful Nucleospin® RNA isolation and purification method was developed and worked well with small portions of dried biological fluid stains, we wanted to evaluate whether it could be used for potential routine, and more rapid, purification of EZ1® waste fractions.

Since the starting material for the EZ1® waste fractions was liquid rather than the usual solid stain or swab piece, we first needed to evaluate whether it was necessary to

add the initial lysis buffer (RA1) with DTT or if this step could be skipped since cell lysis had likely already occurred. One sample of each body fluid was tested with and without the initial lysis buffer/DTT. Following this step, all steps in the developed Nucleospin® protocol were followed. Semen (both PRM2 and SEMG1) and saliva were detected in both sets using the rapid sexual assault HRM, both with and without the initial lysis buffer/DTT step. Menstrual blood was detected only when the lysis buffer/DTT was omitted (using the rapid blood-menstrual blood HRM assay). Blood and vaginal secretions were not detected in either set.

While this approach was not initially successful for all body fluids, we wanted to try to optimize the method to see if the results could be improved. The initial experiments used only 200 µL aliquots of the EZ1® fraction waste since that is the volume used in the RNeasy® MinElute® Clean Up kit purification from these samples. We decided to try a larger volume of waste fraction to determine if that would improve sensitivity and permit the detection of all body fluids. For this experiment, we used the full ~900 µL of the EZ1® cartridge waste and purified it using the developed Nucleospin® protocol. We omitted the initial lysis (lysis buffer/DTT and 5 min incubation) due to the large volume. For this experiment we analyzed the samples using the standard sexual assault and standard blood-menstrual blood assays in order to maximize efficiency and sensitivity for initial experiments. Using this approach, we were able to detect saliva, semen and vaginal secretions using the standard sexual assault assay. Two donors of each body fluid were tested and both semen and saliva samples were positive and one of the two vaginal samples was positive. However, the results were not as successful for blood and menstrual-blood. Both ANK1 and ALAS2 were detected in one of the two blood samples. The other blood sample was negative for both markers. For menstrual blood, MMP10 was not detected in either sample but LEFTY2 was detected in one of the two samples. Interestingly, ANK1 was detected in both menstrual blood samples.

Overall, while not completely successful, these initial experiments indicate the potential for the Nucleospin® purification method to be used with the EZ1® waste fractions for RNA recovery. The method could be optimized to improve the results, but further experiments were not possible within the time frame of this project.

2. ScreenClust Software

The current analysis of HRM data includes the use of a 0.2 threshold. Any peaks above the threshold are called. However, there are many instances in which peaks below this threshold are still clearly visible. Therefore, there is some room for peaks to be called below this threshold at the analysts' discretion. This is not ideal as it introduces subjectivity into peak calls and differences between analysts are possible. In order to aid in the interpretation of HRM 'peaks', we evaluated the QIAGEN Rotor-Gene® ScreenClust HRM software. The software is intended to be used to process HRM data files generated on the Rotor-Gene® Q instrument and its purpose is to allow for automated calling of 'genotypes' using either supervised or unsupervised data sets. Data analysis using this software involves the following steps: 1) normalization, 2) generation of a residual plot, 3) principal component analysis and 4) clustering. Despite the number of steps, the analysis is performed quite rapidly.

To use the software, a .rex file from the Rotor-Gene® instrument with HRM data is opened and the first step within the software is to select which samples to include in

the analysis and the temperature range to use. This is helpful in the event that two different HRM assays are included in the same run. The analysis will likely have to be performed separately for each HRM assay so there is no temperature overlap that could result in inaccurate analyses. Once the samples to include are selected, the software provides the option of proceeding with supervised (if known controls are available) or unsupervised (no controls and the software determines the number of clusters, etc).

In order to evaluate the software, we re-ran additional Nucelospin 8 RNA body fluid samples (single source, section 3). We tried to include at least 8 donors of each target body fluid and 3-4 donors of each non-target body fluid. We included the additional donors for each body fluid so that a known cluster could be defined using three samples (the minimum number suggested by the software manual; probabilities and typicalities won't be generated without the minimum number of controls) with additional samples not in the known cluster to determine if they would be identified correctly by the software.

The results from the supervised analysis of the blood-menstrual blood data set are provided in Figure 11. The principal component plots are displayed on the left (note: ellipses are a visual aide provided by the software to give users an idea of how clusters are oriented but they do not cover the entire area of the cluster so some samples will be positioned outside of the ellipses but still identified as part of that cluster) and the results table on the right. The results table lists each sample included in the analysis, the cluster the software assigned each sample, the typicality and the probabilities. The software uses established methods of statistical analysis for each sample, indicating the probability that a sample belongs to a particular cluster and how well it belongs in the cluster (typicality). There were three clusters created – blood, menstrual and negative. For each cluster three samples were randomly selected as the known samples. For the negative group, we selected one semen, one saliva and one vaginal sample so that all non-target body fluid samples would cluster into the 'negative' group. Each of the samples selected as 'knowns' is indicated by an asterisk in the results table (Figure 11). Using the defined known clusters, all of the other samples were classified correctly, with the exception of the amp blank (AB). One of the vaginal samples, #25 VS35-T, was identified as 'menstrual'. This vaginal sample had MMP10 present which is why it was classified in the 'menstrual' cluster. The data point is separated from the other menstrual samples in the cluster but is still classified in that cluster (the data point can be seen in each of the principal component plots as green square within or near the blue ellipse for the menstrual cluster). The amplification blank in this study was very unusual and had a very large, broad peak on the melt plot (data not shown). This peak would not have been identified as a biomarker and upon evaluation of the original melt data would have been excluded from analysis. The ScreenClust software automatically selects any NTCs (no template controls) for exclusion in the analysis if they are defined as such in the Rotor-Gene® run file. We included it in the analysis here in order to determine how the software would handle such a sample. This sample, since it was identified as menstrual by the software, demonstrates the need to carefully review the melt data using the regular analysis prior to using the ScreenClust software (although negative controls are typically excluded from ScreenClust analysis). Based on the analysis performed here, ten control samples would need to be run in each blood-menstrual blood HRM assay run: three blood (to define the 'blood' known cluster), three menstrual blood (to define the 'menstrual'

known cluster), one each of semen, saliva and vaginal secretions (to define the 'negative' cluster) and an amp blank (as a negative control for the HRM run itself but would be excluded from ScreenClust analysis). Since an HRM run can have 72 samples, ~14% would be needed for controls.

The results from the supervised analysis of the sexual assault HRM data are provided in Figure 12. There were four clusters created for this analysis: semen (using three known semen samples), saliva (using three known saliva samples), vaginal secretions (using three known vaginal secretions samples) and 'negative' (using one known blood sample and two known menstrual blood samples). As can be seen from the results, all of the body fluid samples were correctly identified by the software. We included the amp blank (AB) sample in this data set as well and it was correctly identified as 'negative' since there were no peaks observed in the melt plot for this sample. It is interesting to note that the two menstrual samples selected as 'knowns' for the negative group were identified by the software as 'vaginal'. This of course is a correct classification as they do contain CYP2B7P1 (vaginal secretions). Therefore, the only 'negative' sample for this assay is blood and three blood samples will likely be used to construct the 'negative' cluster for this assay. Based on the analysis performed here, 13 control samples would need to be run in each sexual assault HRM assay run: three semen (to define the 'semen' known cluster), three saliva (to define the 'saliva' known cluster), three vaginal (to define the 'vaginal' known cluster), three blood (to define the 'negative' cluster) and an amp blank (as a negative control for the HRM run itself but would be excluded from ScreenClust analysis). Since an HRM run can have 72 samples, ~18% would be needed for controls.

While the results from the initial testing of the software were quite promising, it is unlikely that the software would be useful for mixtures in its present state and therefore further evaluation of the software was not performed. For the software to be used for mixtures, 'known' samples of each mixture type would have to be included in the same run as single source samples and the casework samples. It is unlikely that the number of controls needed would be suitable for routine use. It is possible that with further development of the software (if QIAGEN was interested in doing so) that it could be suitable for use with existing HRM assays to aid in the interpretation of HRM results.

3. Biomarker Specific Thresholds

Since modifications to the QIAGEN ScreenClust software were not possible during the current project, we evaluated an alternative method for HRM melt data interpretation using biomarker specific thresholds. This would aid in not only the interpretation of below threshold peaks but also reduce the amount of non-specific calls that are picked up by the software. For evaluation of this type of approach, we focused on the EZ1® RNA recovery method for the standard sexual assault HRM assay. The same process described here would need to be performed for each assay for each extraction method. The thresholds may be similar between extractions but due to slight shifts in Tm values, it would be necessary to evaluate the data for each extraction type. An additional limitation of this approach is that the threshold may change for admixed samples compared to single source samples. All samples types would need to be evaluated to determine if suitable universal biomarker thresholds could be established or if they would

need to be different between single and multi-source samples. Since this approach was not evaluated until late in the project, we have focused solely on single source samples.

In order to initially evaluate potential biomarker specific thresholds, we analyzed data in each biomarker Tm range over several runs using both positive and negative samples. Figures 13-15 show representative melt data collected for saliva (HTN3, Figure 13), vaginal secretions (CYP2B7P1, Figure 14) and semen (both SEMG1 and PRM2, Figure 15). As can be seen in Figure 13, for saliva (HTN3) positive samples, the threshold could be set relatively low at 0.1 since there is no baseline noise to consider in this Tm region when an HTN3 peak is present. This same threshold would be appropriate as well after examining assay blanks and negative controls (e.g. extraction blanks). However, when other body fluid samples (semen and vaginal secretions) are run on the assay, while no peak is observed in the HTN3 Tm range the baseline becomes raised slightly and a 0.3 – 0.35 threshold would be needed in order to avoid software calls in this area. Using a 0.3 – 0.35 threshold would not cause a false negative result for any of the saliva samples used in this example. Therefore, if sufficient data was reviewed and no samples would fall below the 0.3 – 0.35 threshold resulting in a false negative, then that threshold could be sufficient for use with HTN3 in the standard sexual assault HRM assay for the EZ1® RNA samples. Alternatively, it might be possible to use a 0.35 threshold, with the 0.1 – 0.34 range as inconclusive or at the analysts discretion. Validation data would need to be used to evaluate any data falling within this region in order to determine suitable thresholds. If an inconclusive or analysts discretion range is not ideal, then the threshold would have to be set to eliminate any false positive calls and reduce the number of false negative calls as much as possible.

Interestingly, for CYP2B7P1 (Figure 14), a 0.1 threshold looks suitable across all sample types evaluated. The baseline in this region for all non-vaginal samples (saliva, semen, assay blanks and negative controls) is relatively smooth and below the 0.1 threshold. The use of this lower threshold would aid in the identification of smaller peaks such as the one shown in the top left panel. This peak falls around 0.16 and would not be called using the previously used universal 0.2 threshold.

For semen, there are slight differences in thresholds between SEMG1 and PRM2 for semen samples. As can be seen in Figure 15, a 0.1 threshold could be used for PRM2 while a 0.2 threshold is needed for SEMG1 since the baseline for a few semen samples in which PRM2 only was detected are slightly raised in the SEMG1 Tm region. For both saliva and negative and assay control samples, a 0.1 threshold was sufficient for both biomarkers. However, for both markers the baseline samples for vaginal (CYP2B7P1 positive) samples was slightly raised in the Tm ranges leading up to CYP2B7P1 including those ranges for both SEMG1 and PRM2. As a result of this raised baseline, a 0.25 threshold would be needed. Using this data collectively, a 0.25 threshold could be applied which would be sufficient to eliminate baseline noise. A inconclusive or analysts discretion range from 0.1 – 0.25 could be employed for PRM2 if it was determined that the false negative rate was too high using this higher threshold.

Overall, the use of the biomarker specific thresholds is complicated and may not ultimately result in an easily employed finite threshold value. Again, thresholds for single source and admixed samples would likely be needed. The baseline between consecutive markers is often raised if both biomarkers are present, especially if the biomarkers are closer together. Additionally, the software threshold line can currently only be set to a

specific starting Tm value. The threshold then continues for the rest of the Tm range. The use of biomarker specific thresholds would not eliminate software calls from baseline levels in other biomarker Tm ranges since it would have to be reset when looking at each biomarker. It would be ideal if the Tm range could be set around each biomarker bin, with the data only evaluated in each of those regions and evaluated individually to determine which biomarkers were present. In this event specific thresholds could be set to each of those regions making the analysis much easier.

All of the approaches evaluated in this project are ad hoc methods using existing software in an attempt to fit that software to the needs of the project. Ultimately it is likely that modified software may be needed in order to fully implement more robust threshold and peak calling metrics. Some of the approaches described here hopefully will provide a potential next step in HRM data analysis as an improvement over an arbitrarily set 0.2 threshold for all data. Any measures employed need to ensure that false positive calls are eliminated while reducing the amount of false negative calls as much as possible.

V. CONCLUSIONS

The goals of the current project were to custom-develop for DFSC casework use: (i) a differential DNA/RNA co-extraction isolation protocol for the separation and analysis of non-sperm and sperm fractions in sexual assault evidence (i.e. prior to DNA profiling and identification of body fluid of origin); and (ii) a rapid RNA direct lysis method for higher throughput screening of forensic biological evidence (blood, semen, saliva, vaginal secretions and menstrual blood). All aims of the project were successfully completed.

The results from the validation studies demonstrate the successful use of EZ1® DNA waste fractions for the recovery for RNA for body fluid identification. The method can be applied to both non-differential and differential extractions. It is fully compatible with existing DFSC DNA protocols and therefore does not require additional validation of DNA protocols. The results of this study were presented at the 21st Triennial Meeting of the International Association of Forensic Sciences (2017) and we are currently working with QIAGEN to develop a user-defined protocol that will be available on their website.

The rapid RNA analysis method was developed that permits an ~3-hour 'sample-to-detection' time. The rapid lysis, Nucelospin® RNA (Macherey-Nagel), permits the extraction of 8-48 samples or 96-samples in ~1 hour or less. In addition to timeliness, this extraction has the potential to be automated. Additionally, the methods developed here for the rapid RNA analysis can also be applied to the RNA EZ1® method as well in order to improve the timeliness of that method as well. The Nucleospin® RNA purification can be used to recover RNA from the EZ1® waste fractions as well.

To aid in the analysis and interpretation of the HRM assays, we have evaluated the ScreenClust software (QIAGEN).

The technology transfer of all methods and protocols developed in the current study has been completed. Comprehensive protocol manuals for both the EZ1® RNA and rapid RNA analysis method have been prepared and provided to DFSC. Reagents and consumables have also been provided for initial in-house testing by DFSC. Rotor-Gene® run template files and bin sets have also been created and transferred: 1) EZ1® – standard sexual assault and blood-

menstrual blood HRM assays, 2) Rapid RNA (Nucleopsin®) – rapid HRM assays (blood-menstrual blood, sexual assault, 5-fluid 6-plex) and standard HRM assays (blood-menstrual blood and sexual assault). We have conducted an on-site one day training for the rapid RNA analysis methods and will continue to provide support to DFSC regarding these methods as needed.

We are hopeful that the results of the current work will aid in the implementation of RNA profiling for body fluid identification.

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VII. TABLES

Table 1.Summary of DFSC-Provided EZ1® DNA Extraction Waste Fractions

Log Number	Sample ID	Sample description
BF0001	BD1	blood donor 1 LV quarter swab
BF0002	BD2	blood donor 2 LV quarter swab
BF0003	SA1	saliva donor 1 LV quarter swab
BF0004	SA2	saliva donor 2 LV quarter swab
BF0005	MBT3129	menstrual blood donor T3129 half swab
BF0006	MBT3994	menstrual blood donor T3994 half swab
BF0007.1	SET4286F1	semen donor T4286F1 quarter swab
BF0007.2	SET4286F2	semen donor T4286F2 quarter swab
BF0007.3	SET4286F3	semen donor T4286F3 quarter swab
BF0008.1	SET3035F1	semen donor T3035 quarter swabF1
BF0008.2	SET3035F2	semen donor T3035 quarter swabF2
BF0008.3	SET3035F3	semen donor T3035 quarter swabF3

Tube Label	waste description	protocol reference	additonal samples
BF0001-1	10ul w/o buffer MTL	removed following step 4	BF0002/BF0003/BF0004
BF0001-2	10ul w/ buffer MTL	removed following step 16	BF0002/BF0003/BF0004
BF0001-3	sample + MTL after extraction	removed following step 19	BF0002/BF0003/BF0004
BF0001-4	EZ1 Cartridge Lane 1	removed following step 19	BF0002/BF0003/BF0004
BF0001-5	EZ1 Cartridge Lane 2	removed following step 19	BF0002/BF0003/BF0004

Tube Label	waste description	protocol reference	additonal samples
BF0007.1-1	10uL after epi lyse before QiaCube wash	removed following step 3	BF0008.1
BF0007.1-2	10uL epi fraction w/o buffer MTL	removed following step 9	BF0008.1
BF0007.1-3	10ul epi fraction w/ buffer MTL	removed following step 21	BF0008.1
BF0007.1-4	sample +MTL after extraction	removed follwing step 23	BF0008.1
BF0007.1-5	EZ1 Cartridge Lane 1	removed follwing step 23	BF0008.1
BF0007.1-6	EZ1 Cartridge Lane 2	removed following step 23	BF0008.1

Tube Label	waste description	protocol reference	additonal samples
BF0007.2-1	10uL semen fraction after lysis	removed following step 26	BF0008.2
BF0007.2-2	sample after extraction	removed following step 40	BF0008.2
BF0007.2-3	EZ1 Cartridge lane 2	removed following step 40	BF0008.2
BF0007.2-4	EZ1 Cartridge Lane 3	removed following step 40	BF0008.2

Tube Label	Tube Label	Tube Label	Tube Label
BF0007.3-1	10uL substrate fraction after lysis	removed following step 26	BF0008.3
BF0007.3-2	sample after extraction	removed following step 40	BF0008.3
BF0007.3-3	EZ1 Cartridge lane 2	removed following step 40	BF0008.3
BF0007.3-4	EZ1 Cartridge Lane 3	removed following step 40	BF0008.3

Table 2. HRM Results From Single-Source Non-Differentially Extracted EZ1® "Pre" and "Post" Waste Fractions

Colored cells represent detection of the expected biomarker (red- blood, blue – saliva, pink – menstrual blood). Grey cells represent samples in which the expected biomarkers were not detected. Blood and menstrual samples were tested with the Blood-Menstrual Blood HRM assay and the saliva samples were tested with the Sexual Assault HRM Assay.

Sample		Description	HRM				
			ANK1	ALAS2	HTN3	MMP10	LEFTY2
Blood	BF001-1	10 ul w/o MTL					
	BF001-2	10 ul w/ MTL					
	BF001-3	sample + MTL after extraction					
	BF001-4	EZ1 cartridge lane 1					
	BF001-5	EZ1 cartridge lane 2					
	BF002-1	10 ul w/o MTL					
	BF002-2	10 ul w/ MTL					
	BF002-3	sample + MTL after extraction					
	BF002-4	EZ1 cartridge lane 1					
	BF002-5	EZ1 cartridge lane 2					
Saliva	BF003-1	10 ul w/o MTL					
	BF003-2	10 ul w/ MTL					
	BF003-3	sample + MTL after extraction					
	BF003-4	EZ1 cartridge lane 1					
	BF003-5	EZ1 cartridge lane 2					
	BF004-1	10 ul w/o MTL					
	BF004-2	10 ul w/ MTL					
	BF004-3	sample + MTL after extraction					
	BF003-4	EZ1 cartridge lane 1					
	BF003-5	EZ1 cartridge lane 2					
Menstrual	BF005-1	10 ul w/o MTL					
	BF005-2	10 ul w/ MTL					
	BF006-1	10 ul w/o MTL					
	BF006-2	10 ul w/ MTL					

Table 3. HRM Results From Single-Source Differentially Extracted EZ1® "Pre" and "Post" Waste Fractions

Colored cells represent detection of the expected biomarker (yellow - semen). Grey cells represent samples in which the expected biomarkers were not detected. Semen samples were tested with the Sexual Assault HRM Assay.

Sample		Fraction	Description	HRM	
				SEMG1	PRM2
Semen	BF007.1-1	Non sperm (pre)	10ul after epi lyse before QIAcube wash		
	BF008.1-1		10ul after epi lyse before QIAcube wash		
	BF007.1-2		10ul epi fraction w/o MTL		
	BF008.1-2		10ul epi fraction w/o MTL		
	BF007.1-3		10ul epi fraction w/MTL		
	BF008.1-3		10ul epi fraction w/MTL		
	BF007.1-4	F1 Nsp (post)	sample + MTL (F1 non-sperm)		
	BF008.1-4		sample + MTL (F1 non-sperm)		
	BF007.1-5		EZ1 cartridge lane 1 (F1 non-sperm)		
	BF008.1-5		EZ1 cartridge lane 1 (F1 non-sperm)		
	BF007.1-6		EZ1 cartridge lane 2 (F1 non-sperm)		
	BF008.1-6		EZ1 cartridge lane 2 (F1 non-sperm)		
	BF007.2-1	F2 Sp (post)	10ul semen fraction after lysis		
	BF008.2-1		10ul semen fraction after lysis		
	BF007.2-2		sample + MTL (F2 sperm)		
	BF008.2-2		sample + MTL (F2 sperm)		
	BF007.2-3		EZ1 cartridge lane 2 (F2 sperm)		
	BF008.2-3		EZ1 cartridge lane 2 (F2 sperm)		
	BF007.2-4		EZ1 cartridge lane 3 (F2 sperm)		
	BF008.2-4		EZ1 cartridge lane 3 (F2 sperm)		
	BF007.3-1	F3 Subst (post)	10ul substrate fraction after lysis		
	BF008.3-1		10ul substrate fraction after lysis		
	BF007.3-2		sample + MTL (F3 substrate)		
	BF008.3-2		sample + MTL (F3 substrate)		
	BF007.3-3		EZ1 cartridge lane 2 (F3 substrate)		
	BF008.3-3		EZ1 cartridge lane 2 (F3 substrate)		
	BF007.3-4		EZ1 cartridge lane 3 (F3 substrate)		
	BF008.3-4		EZ1 cartridge lane 3 (F3 substrate)		

Table 4. HRM Results From Single-Source In-House Non-Differentially Extracted EZ1® "Pre" and "Post" Waste Fractions

Colored cells represent detection of the expected biomarker (red- blood, pink – menstrual blood, blue – saliva, yellow – semen and green – vaginal secretions). Grey cells represent samples in which the expected biomarkers were not detected. The number recorded in each biomarker column is the observed Tm (°C) value. Blood and menstrual samples were tested with the Blood-Menstrual Blood HRM assay and the semen, saliva and vaginal samples were tested with the Sexual Assault HRM Assay.

Blood-Menstrual Blood HRM						
Sample	Fraction	Description	MMP10	ANK1	ALAS2	LEFTY2
Blood B7479	1	10ul after initial lysis		83.25		
	2	sample + MTL after extraction		82.55	85.73	
	3	EZ1 cartridge lane 1		82.55	85.75	
Menstrual Blood MB1-d2	1	10ul after initial lysis	81.78		85.83	
	2	sample + MTL after extraction	81.52		85.63	88.73
	3	EZ1 cartridge lane 1	81.53		85.67	88.7

Sexual Assault HRM						
Sample	Fraction	Description	HTN3	SEMG1	PRM2	CYP
Saliva SA1	1	10ul after initial lysis	76.25			
	2	sample + MTL after extraction	76.22			
	3	EZ1 cartridge lane 1	76.17			
Semen SE15	1	10ul after initial lysis		78	83.85	
	2	sample + MTL after extraction		77.92	83.82	
	3	EZ1 cartridge lane 1		77.97	83.9	
Vaginal VS32	1	10ul after initial lysis				86
	2	sample + MTL after extraction				85.78
	3	EZ1 cartridge lane 1				85.82

Table 5. HRM Results From Single-Source In House Differentially Extracted EZ1® "Pre" and "Post" Waste Fractions

Colored (and 'X'd') cells represent detection of the expected biomarker (yellow – semen, green – vaginal secretions). Grey cells represent samples in which the possible or expected biomarkers were not detected. The semen, saliva and vaginal samples were tested with the Sexual Assault HRM Assay.

Fraction		Sample	Donor set 1			Donor set 2		
			SEMG1	PRM2	CYP	SEMG1	PRM2	CYP
F1	1	10ul after initial lysis	X	X	X	X	X	X
	2	10ul after QIACube sep/lysis	X	X	X		X	X
	3	remaining sample + MTL (after extraction)	X	X	X	X	X	X
	4	EZ1 cartridge lane 1	X	X	X	X	X	X
F2	1	10ul after lysis					X	
	2	remaining sample (after extraction)					X	
	3	EZ1 cartridge lane 3		X			X	
F3	1	10ul after lysis				X	X	
	2	remaining sample (after extraction)					X	
	3	EZ1 cartridge lane 3		X	(WEAK)	X	X	X

Table 6.HRM Results From 100 and 200 µl Aliquots of EZ1® Extraction Waste Fractions

Fractions: 1 – 100 µL EZ1® cartridge lane 1, 2 – 200 µL EZ1® cartridge lane 1, 3 – 100 µL remaining sample + MTL buffer, 4 – 200 µL remaining samples + MTL buffer. Colored cells represent detection of the expected biomarkers (red – blood, pink – menstrual blood, yellow – semen, blue – saliva, green – vaginal secretions). The number of donors with positive results out of the two donors tested is listed within each cell.

Sample	Fraction	Blood-Menstrual Blood			
		MMP10	ALAS2	ANK1	LEFTY2
Blood	1		2/2	2/2	
	2		2/2	2/2	
	3		2/2	2/2	
	4		2/2	2/2	
Menstrual Blood	1	2/2	1/2	2/2	2/2
	2	2/2	2/2	2/2	2/2
	3	2/2	1/2	2/2	1/2
	4	2/2	2/2	2/2	2/2

Sample	Fraction	Sexual Assault			
		HTN3	SEMG1	PRM2	CYP2B7P1
Semen	1		2/2	2/2	
	2		2/2	2/2	
	3		2/2	2/2	
	4		2/2	2/2	
Saliva	1	2/2			
	2	2/2			
	3	2/2			
	4	2/2			
Vaginal	1				1/2
	2				2/2
	3				2/2
	4				2/2

Table 7. Evaluation of Stain-Swab Size on RNA Recovery from EZ1® Non-Differentially Extracted Body Fluid Samples

The number of samples in which the biomarker was detected out of the number of donors tested is shown. Colored cells represent positive results in at least 1 sample (blue – saliva, green - vaginal secretions, pink – menstrual blood, maroon and red – blood, yellow – semen). CYP = CYP2B7P1.

Dried Swabs

Body Fluid	Marker	No. of Donors	Swab Size		
			1/2	1/4	1/8
Saliva	HTN3	3	3/3	3/3	3/3
Vaginal	CYP	3	3/3	3/3	3/3
Menstrual	MMP10	3	3/3	3/3	3/3
	LEFTY2		3/3	3/3	3/3

Dried Stain (50 µl)

Body Fluid	Marker	No. of Donors	Stain Size			
			1/2	1/4	1/8	1/16
Blood	ALAS2	2	2/2	2/2	1/2	2/2
	ANK1		2/2	2/2	1/2	2/2
Semen	SEMG1	1	1/1	1/1	0/1	1/1
	PRM2		1/1	1/1	1/1	1/1

Liquid stains

Body Fluid	Marker	No. of Donors	Stain Size			
			25ul	10ul	5ul	1ul
Blood	ALAS2	2	3/3	3/3	3/3	3/3
	ANK1		3/3	2/3	2/3	3/3
Semen	SEMG1	3	3/3	3/3	3/3	3/3
	PRM2		2/2	2/2	2/2	2/2
Saliva	HTN3	3	3/3	3/3	3/3	3/3

Table 8. HRM Analysis of Two-Fluid Admixed Samples from RNA Recovered From EZ1® Cartridge Lane 1 'Waste'

Two-fluid admixtures were prepared by adding a liquid body fluid (semen or saliva) in 10, 5 or 1 µL aliquots on top of an already dried body fluid stain (blood – ½ 50 µL stain, saliva – ½ buccal swab, vaginal – ¼ vaginal swab, menstrual blood – ¼ menstrual blood swab). The first body fluid listed in each mixture represents the dried body fluid stain and the second body fluid listed is the liquid body fluid added second. Colored cells represent the successful detection of the body fluid (red – blood, blue – saliva, green – vaginal, pink – menstrual blood and yellow – semen). The number of samples in which the body fluid was detected out of the number tested is indicated in each cell.

Mixture	Vol. of Liquid Fluid	Body Fluid 1	Body Fluid 2
blood-semen	10	2/2	2/2
	5	2/2	2/2
	1	2/2	2/2
saliva-semen	10	2/2	2/2
	5	2/2	2/2
	1	2/2	1/2
vaginal-semen	10	2/2	2/2
	5	2/2	2/2
	1	2/2	1/2
menstrual-semen	10	2/2	2/2
	5	2/2	2/2
	1	2/2	2/2
blood-saliva	10	2/2	2/2
	5	2/2	2/2
	1	2/2	2/2
semen-saliva	10	2/2	2/2
	5	2/2	1/2
	1	2/2	0/2
vaginal-saliva	10	2/2	2/2
	5	2/2	1/2
	1	2/2	1/2
menstrual-saliva	10	2/2	2/2
	5	2/2	1/2
	1	2/2	0/2

Table 9. HRM Analysis of Blood and Semen Samples Deposited onto Denim, Polyester and Paper and Stored at Room Temperature for 1 month – 1 year

Blood and semen samples (50 µL) deposited onto denim, polyester and paper were evaluated using the blood-menstrual blood HRM assay and the sexual assault HRM assay, respectively. The number of samples in which the biomarker was detected out of the number tested is listed in each cell. Colored cells (red – blood, yellow – semen) indicate biomarker detection in both donors. Light color cells indicate detection of the biomarkers in one of the two donors. Grey cells indicate no detection of the biomarker.

Substrate	Exposure	Time	Blood		Semen	
			ANK1	ALAS2	SEMG1	PRM2
Denim	Room Temp	1m	1/2	2/2	0/2	2/2
		6m	1/2	2/2	0/2	2/2
		1yr	1/2	2/2	2/2	2/2
Polyester	Room Temp	1m	0/2	2/2	2/2	2/2
		6m	2/2	2/2	2/2	2/2
		1yr	0/2	1/2	2/2	2/2
Paper	Room Temp	1m	2/2	2/2	2/2	2/2
		6m	2/2	2/2	2/2	2/2
		1yr	1/2	2/2	1/2	2/2

Table 10. HRM Analysis of Saliva, Vaginal Secretions and Blood Exposed to High Temperatures for 1 week – 1 month

Blood samples (50 µL) left in a car trunk (2 weeks) and a car back seat (1 week) were tested using the blood-menstrual blood HRM assay. Saliva and vaginal secretions swabs exposed to 22ºC, 37ºC and 56ºC temperatures for 1 week and 1 month were tested using the sexual assault HRM assay. The number of samples in which the biomarker was detected out of the number tested is listed in each cell. Colored cells (blue – saliva, green – vaginal secretions, red - blood) indicate biomarker detection in both donors. Light color cells indicate detection of the biomarkers in one of the two donors. Grey cells indicate no detection of the biomarker.

Saliva		HTN3
Room Temp	1 wk	2/2
	1 m	2/2
37oC	1 wk	2/2
	1 m	2/2
56oC	1 wk	2/2
	1 m	2/2

Vaginal		CYP2B7P1
Room Temp	1 wk	1/2
	1 m	2/2
37oC	1 wk	2/2
	1 m	2/2
56oC	1 wk	1/2
	1 m	2/2

Blood		ANK1	ALAS2
Car Trunk	2 wk	0/1	1/1
Car Seat	1 wk	1/1	1/1

Table 11. Evaluation of Possible Effects from Multiple Freeze-Thawing of the EZ1® Cartridge Waste

Four 200 µL aliquots of EZ1® cartridge waste were tested with a freeze-thaw in between each analysis. Results after the 2nd, 3rd and 4th freeze-thaw are shown (all samples were positive after the first freeze-thaw cycle). The detection of a biomarker is indicated by colored cells (blue – saliva, yellow – semen, green – vaginal secretions, pink – menstrual blood and red – blood) as well as the Tm (°C) value within each cell.

Sample		# Freeze-Thaw (total)	HTN3	SEMG1	PRM2	CYP2B7P1
SA1-10	10ul liquid saliva (dried)	2	76.83 (HTN3)			
		3	76.57 (HTN3)			
		4	76.67 (HTN3)			
SA10-1.2	1/2 buccal swab	2	76.78 (HTN3)			
		3	76.65 (HTN3)			
		4	76.63 (HTN3)			
SE16-10	10ul liquid semen (dried)	2		78.35 (SEMG1)	84.05 (PRM2)	
		3		78.23 (SEMG1)	84.00 (PRM2)	
		4		78.25 (SEMG1)	83.97 (PRM2)	
SE22-1.2	1/2 semen swab	2		78.35 (SEMG1)	84.05 (PRM2)	
		3		78.57 (SEMG1)	84.15 (PRM2)	
		4		78.45 (SEMG1)	84.05 (PRM2)	
VS2-1.2	1/2 vaginal swab	2				86.28 (CYP2B7P1)
		3				86.30 (CYP2B7P1)
		4				
VS4-1.2	1/2 vaginal swab	2				86.30 (CYP2B7P1)
		3				86.25 (CYP2B7P1)
		4				

Sample		# Freeze-Thaw (total)	MMP10	ANK1	ALAS2	LEFTY2
MB1-1.2	1/2 menstrual swab	2	81.75 (MMP10)		85.77 (ALAS2)	88.80 (LEFTY2)
		3	81.83 (MMP10)		85.78 (ALAS2)	88.90 (LEFTY2)
		4	not enough sample left for 4th freeze-thaw test			
MB2-1.2	1/2 menstrual swab	2	81.98 (MMP10)	82.80 (ANK1)	85.80 (ALAS2)	88.85 (LEFTY2)
		3	81.95 (MMP10)	82.77 (ANK1)	85.80 (ALAS2)	88.85 (LEFTY2)
		4	81.90 (MMP10)			88.85 (LEFTY2)
BD1-10	10ul liquid blood (dried)	2		82.97 (ANK1)	86.05 (ALAS2)	
		3		82.95 (ANK1)	86.05 (ALAS2)	
		4				
B7482-1.2	1/2 50ul bloodstain	2		82.93 (ANK1)	86.03 (ALAS2)	
		3		82.97 (ANK1)	86.03 (ALAS2)	
		4			85.98 (ALAS2)	

Table 12. HRM Analysis of RNA Recovered from EZ1® Waste of Differentially Extracted Vaginal-Semen Mixtures

Five sets of vaginal-semen mixtures were analyzed using the EZ1® differential extraction and RNeasy® MinElute® Clean Up recovery method. The mixtures were prepared using a ¼ vaginal swab with semen added in 10, 5 and 1 µL aliquots. The detection of a biomarker is indicated by colored cells (semen – yellow, vaginal secretions - green). The number of samples in which the biomarker was detected out of the number tested is listed in each cell.

Fraction	Vol Semen (ul)	SEMG1	PRM2*	CYP2B7P1
F1 non-sperm	10	4/5	4/4	4/5
	5	4/5	4/4	5/5
	1	4/5	4/4	5/5
F2 sperm	10	4/5	4/4	2/5
	5	3/5	4/4	4/5
	1	2/5	4/4	3/5
F3 substrate	10	3/5	4/4	3/5
	5	4/5	4/4	3/5
	1	2/5	4/4	4/5

*One semen donor vasectomized so PRM2 expected in only 3 of the 4 samples

Table 13. HRM Analysis of RNA Recovered from EZ1® Waste of Differentially Extracted Vaginal-Semen-Saliva Mixtures

Five sets of vaginal-semen-saliva mixtures were analyzed using the EZ1® differential extraction and RNeasy® MinElute® Clean Up recovery method. The mixtures were prepared using a ¼ vaginal swab, 5 µL of semen and saliva added in 25, 10, 5 and 1 µL aliquots. The detection of a biomarker is indicated by colored cells (saliva – blue, semen – yellow, vaginal secretions - green). The number of samples in which the biomarker was detected out of the number tested is listed in each cell.

Fraction	Vol Saliva (ul)	HTN3	SEMG1	PRM2*	CYP2B7P1
F1 non-sperm	25	5/5	5/5	4/4	5/5
	10	4/5	5/5	4/4	5/5
	5	5/5	5/5	4/4	5/5
	1	0/5	3/5	4/4	5/5
F2 sperm	25	5/5	2/5	4/4	1/5
	10	5/5	2/5	4/4	1/5
	5	5/5	3/5	4/4	2/5
	1	1/5	2/5	3/4	1/5
F3 substrate	25	4/5	3/5	4/4	1/5
	10	3/5	3/5	4/4	3/5
	5	4/5	3/5	4/4	3/5
	1	2/5	3/5	4/4	4/5

*one vasectomized male so only 4/4 possible

Table 14. HRM Analysis of Single Source Body Fluid Samples Extracted Using the Rapid RNA Assay

The detection of a biomarker is indicated by colored cells (saliva – blue, semen – yellow, vaginal secretions – green, menstrual blood – pink, blood - red). The number of samples in which the biomarker was detected out of the number tested is listed in each cell. The top panel contains the results from the rapid sexual assault HRM assay, the middle panel contains the results from the rapid blood-menstrual blood assay and the bottom panel contains results from the rapid 5-fluid 6-plex.

Sexual assault	# Donors	HTN3	SEMG1	PRM2	CYP2B7P1
Semen	10		18/22	20/20	
Saliva	10	21/22			
Vaginal	8				17/18
Blood	10				
Menstrual Blood	6				12/14

BD-MB	# Donors	MMP10	ANK1	ALAS2	LEFTY2
Blood	10		11/11	11/11	
Menstrual Blood	6	12/14		7/14	8/14
Semen	10				
Saliva	10				
Vaginal	8	1/18		1/18	

6plex	# Donors	HTN3	SEMG1	ANK1	PRM2	CYP2B7P1	LEFTY2
Semen	10		21/22		20/20		
Saliva	10	12/22					
Vaginal	8					14/18	
Blood	10			11/11			
Menstrual Blood	6			5/14		6/14	8/14

Table 15.HRM Analysis of 1-3 µL Saliva and Semen Stains and 2-4 µL Saliva-Semen Mixtures Using the Rapid RNA Analysis Method (Rapid Sexual Assault HRM Assay)

The detection of a biomarker is indicated by colored cells (saliva – blue, semen – yellow). The number of samples in which the biomarker was detected out of the number tested is listed in each cell.

Semen	SEMG1	PRM2
1ul	2/3	2/3
2ul	2/3	2/3
3ul	2/3	2/3

Saliva	HTN3
1ul	4/6
2ul	4/5
3ul	5/5

Saliva-Semen (1:1 by volume)	HTN3	SEMG1	PRM2
2ul	4/8	5/8	5/8
4ul	4/4	4/4	4/4

Table 16. HRM Analysis of Semen, Saliva and Vaginal Samples on Different Substrates and Exposed to Different Temperatures Using the Rapid RNA Analysis Method (Rapid Sexual Assault HRM Assay)

The detection of a biomarker is indicated by colored cells (saliva – blue, semen – yellow, green – vaginal secretions). The number of samples in which the biomarker was detected out of the number tested is listed in each cell.

Body Fluid	Substrate	Room Temp	SEMG1	PRM2
Semen	Denim	1m	2/2	2/2
		6m	2/2	2/2
		1yr	2/2	2/2
	Polyester	1m	2/2	2/2
		6m	2/2	2/2
		1yr	2/2	2/2
	Paper	1m	2/2	2/2
		6m	2/2	2/2
		1yr	2/2	2/2

Body Fluid	Temp	Time	HTN3
Saliva	Room Temp	1 wk	4/4
		1 m	4/4
	37oC	1wk	4/4
		1 m	4/4
	56oC	1 wk	4/4
		1 m	2/4

Two donors each; tip and side of swab tested

Body Fluid	Temp	Time	CYP2B7P1
Vaginal	Room Temp	1 wk	2/2
		1 m	2/2
	37oC	1wk	4/4
		1 m	2/4
	56oC	1 wk	2/2
		1 m	2/2

Table 17. HRM Analysis of Two Fluid Admixtures Using the Rapid RNA Analysis Method (Rapid Sexual Assault HRM Assay)

The detection of a biomarker is indicated by colored cells (saliva – blue, semen – yellow, green – vaginal secretions, grey – no detection). The number of samples in which the biomarker was detected out of the number tested is listed in each cell.

Mixture		Rapid 6plex		Rapid Sexual Assault	
		SA	SE	SA	SE
Buccal swab	5ul semen	5/5	5/5	5/5	5/5
	1ul semen	5/5	5/5	5/5	5/5

Mixture		Rapid 6plex		Rapid Sexual Assault	
		SA	SE	SA	SE
Semen 10ul swab	5ul saliva	2/5	5/5	2/5	5/5
	1ul saliva	2/5	5/5	3/5	5/5

Mixture		Rapid 6plex		Rapid Sexual Assault	
		VS	SE	VS	SE
Vaginal swab (~1/8th)	5ul semen	0/5	5/5	0/5	5/5
	1ul semen	4/5	5/5	3/5	5/5

VIII. FIGURES

Figure 1. Schematic Overview of Current Work

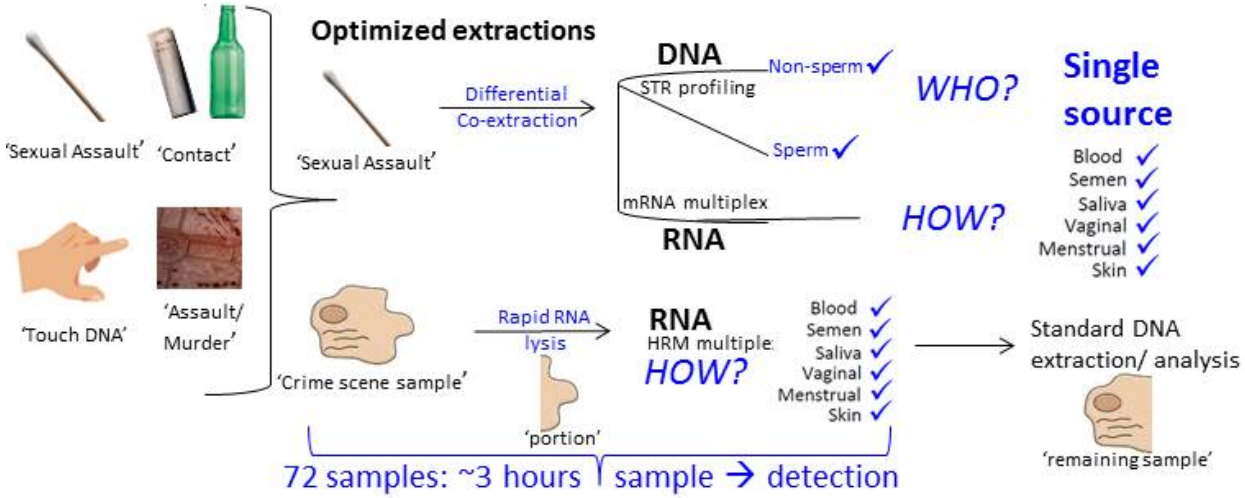


Figure 2. EZ1® DNA Extraction "Waste" Fractions Evaluated For RNA Recovery

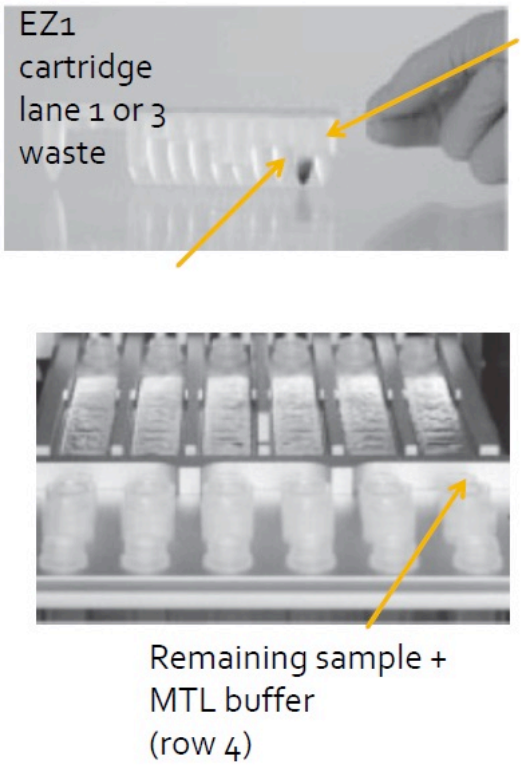
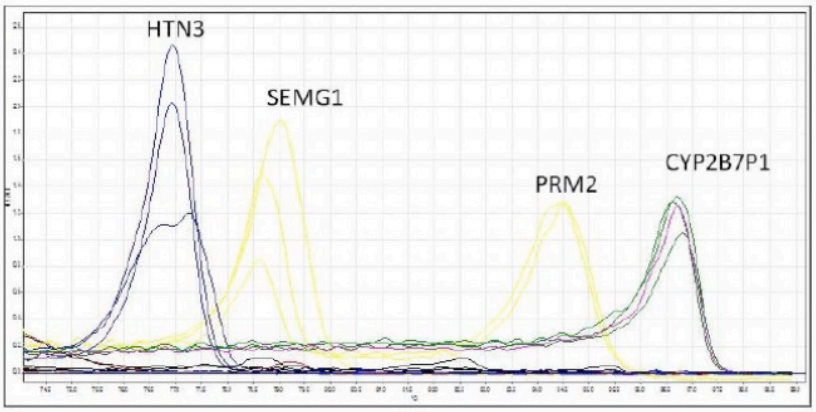


Figure 3. Sexual Assault HRM Assay

The sexual assault HRM assay contains one biomarker for the identification of saliva (HTN3) and vaginal secretions (CYP2B7P1) and two markers for the identification of semen (SEMG1, seminal fluid) and PRM2 (sperm specific). Single source body fluid samples (blue – saliva, green – vaginal secretions, pink – menstrual blood, yellow – semen) are shown overlaid to indicate marker position within the assay. The primer sequences, primer concentration, Rotor-Gene® Q cycling parameters and the expected Tm (°C) value for all markers are also shown. X-axis – temperature; Y-axis: dF/dT.



Biomarker	Tm (°C)
HTN3	77.0
SEMG1	78.7
PRM2	84.4
CYP2B7P1	86.6

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 primers	Vol (µl) R primers	Final conc (µM) in assay
HTN3	40	10	10	0.077
SEMG1	40	12	12	0.092
PRM2	40	12	12	0.092
CYP2B7P1	40	18	18	0.1385

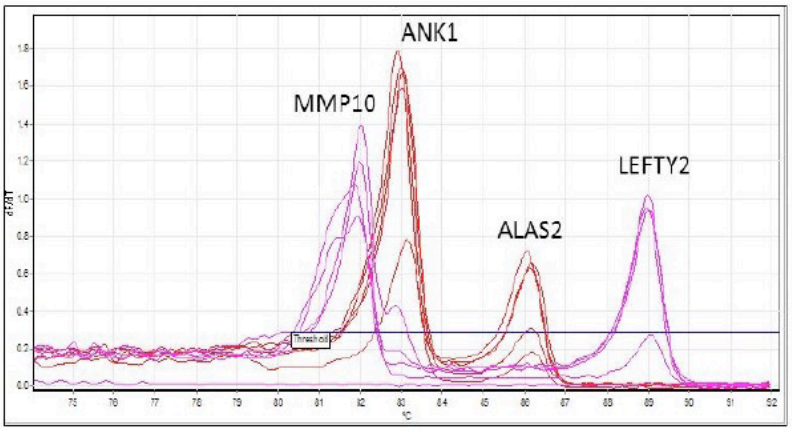
95°C 5 min
45 cycles: 95°C 10 sec
57°C 40 sec
72°C 25 sec

HRM: 74 – 92°C

(Program length ~2 hours 15 min)

Figure 4. Blood-Menstrual Blood HRM Assay

The blood-menstrual blood HRM assay contains two markers for the identification of blood (ANK1, ALAS2) and menstrual blood (MMP10, LEFTY2). Single source body fluid samples (red - blood, pink – menstrual blood) are shown overlaid to indicate marker position within the assay. The primer sequences, primer concentration, Rotor-Gene® Q cycling parameters and the expected Tm (°C) value for all markers are also shown. X-axis – temperature; Y-axis: dF/dT.



Biomarker	Tm (°C)
MMP10	81.5, 82.0
ANK1	83.2
ALAS2	86.1
LEFTY2	89.0

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 primers	Vol (µl) R primers	Final conc (µM) in assay
MMP10	40	7	7	0.085
ANK1	40	10	10	0.121
ALAS2	40	8	8	0.097
LEFTY2	40	8	8	0.097

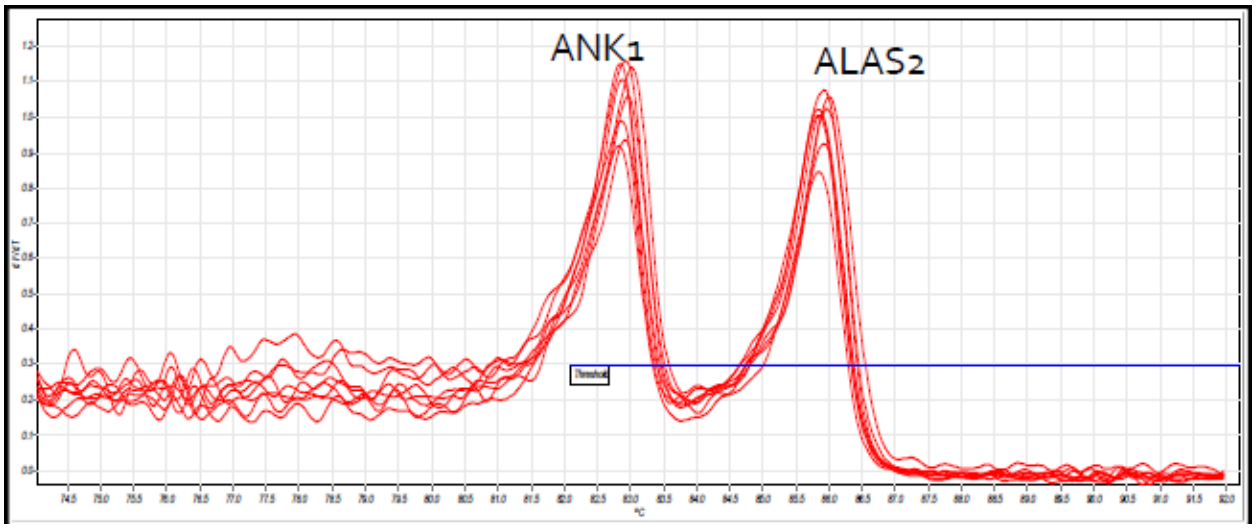
95°C 5 min
45 cycles: 95°C 10 sec
57°C 40 sec
72°C 25 sec

HRM: 74 – 92°C

(Program length ~2 hours 15 min)

Figure 5. HRM Melt Plots from 100 and 200 µL "Post" Waste Samples from EZ1® DNA Non-Differential Blood Extractions

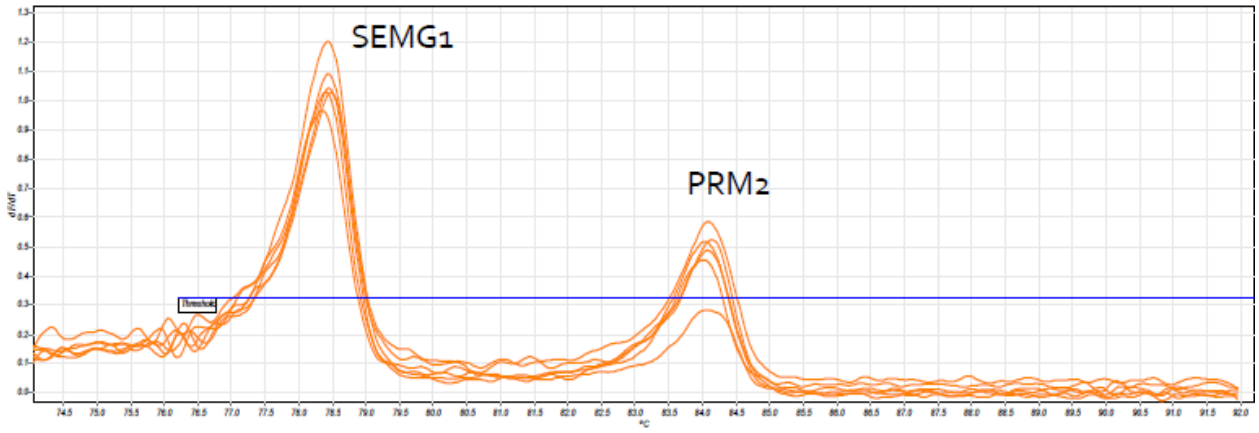
The blood-menstrual blood HRM assay contains two markers for the identification of blood (ANK1, ALAS2) and menstrual blood (MMP10, LEFTY2). Single source body fluid samples (red - blood) are shown overlaid. The blue horizontal line represents the detection threshold. The Tm (°C) values for each sample are provided in the red colored cells indicating the positive detection of the biomarker. X-axis – temperature; Y-axis: dF/dT.



Sample	Fraction	MMP10	ANK1	ALAS2	LEFTY2
B7490-1	100ul EZ1 cartridge lane 1		82.8	85.9	
B7482-1	100ul EZ1 cartridge lane 1		82.9	85.8	
B7490-2	200ul EZ1 cartridge lane 1		82.9	85.9	
B7482-2	200ul EZ1 cartridge lane 1		82.9	85.9	
B7490-3	100ul remaining sample + MTL		82.9	85.9	
B7482-3	100ul remaining sample + MTL		82.9	85.9	
B7490-4	200ul remaining sample + MTL		83.0	86.0	
B7482-4	200ul remaining sample + MTL		83.0	86.0	

Figure 6. HRM Melt Plots from 100 and 200 µL "Post" Waste Samples from EZ1® DNA Non-Differential Semen Extractions

The sexual assault HRM assay contains one biomarker for the identification of saliva (HTN3) and vaginal secretions (CYP2B7P1) and two markers for the identification of semen (SEMG1, seminal fluid) and PRM2 (sperm specific). Single source body fluid samples (orange - semen) are shown overlaid. The blue horizontal line represents the detection threshold. The Tm (°C) values for each sample are provided in the yellow colored cells indicating the positive detection of the biomarker. X-axis – temperature; Y-axis: dF/dT.



Sample	Fraction	HTN3	SEMG1	PRM2	CYP2B7P1
SE2-1	100ul EZ1 cartridge lane 1		78.4	84.1	
SE16-1	100ul EZ1 cartridge lane 1		78.5	84.2	
SE2-2	200ul EZ1 cartridge lane 1		78.4	84.1	
SE16-2	200ul EZ1 cartridge lane 1		78.5	84.1	
SE2-3	100ul remaining sample + MTL		78.5	84.1	
SE16-3	100ul remaining sample + MTL		78.5	84.2	
SE2-4	200ul remaining sample + MTL		78.4	84.0	
SE16-4	200ul remaining sample + MTL		78.4	84.0	

Figure 7. HRM Melt Plots from RNA Recovered from EZ1® Cartridge Waste from Differentially Extracted Vaginal-Semen Mixtures

The sexual assault HRM assay contains one biomarker for the identification of saliva (HTN3) and vaginal secretions (CYP2B7P1) and two markers for the identification of semen (SEMG1, seminal fluid) and PRM2 (sperm specific). The locations of the markers are indicated by the vertical lines. The blue horizontal line represents the detection threshold. X-axis – temperature; Y-axis: dF/dT. Top panel – F1 non-sperm fractions from a vaginal-semen mixture set in which semen was added to a ¼ vaginal swab in 10, 5 and 1 µL aliquots. Bottom panel – F2 sperm fractions from the same sample set.

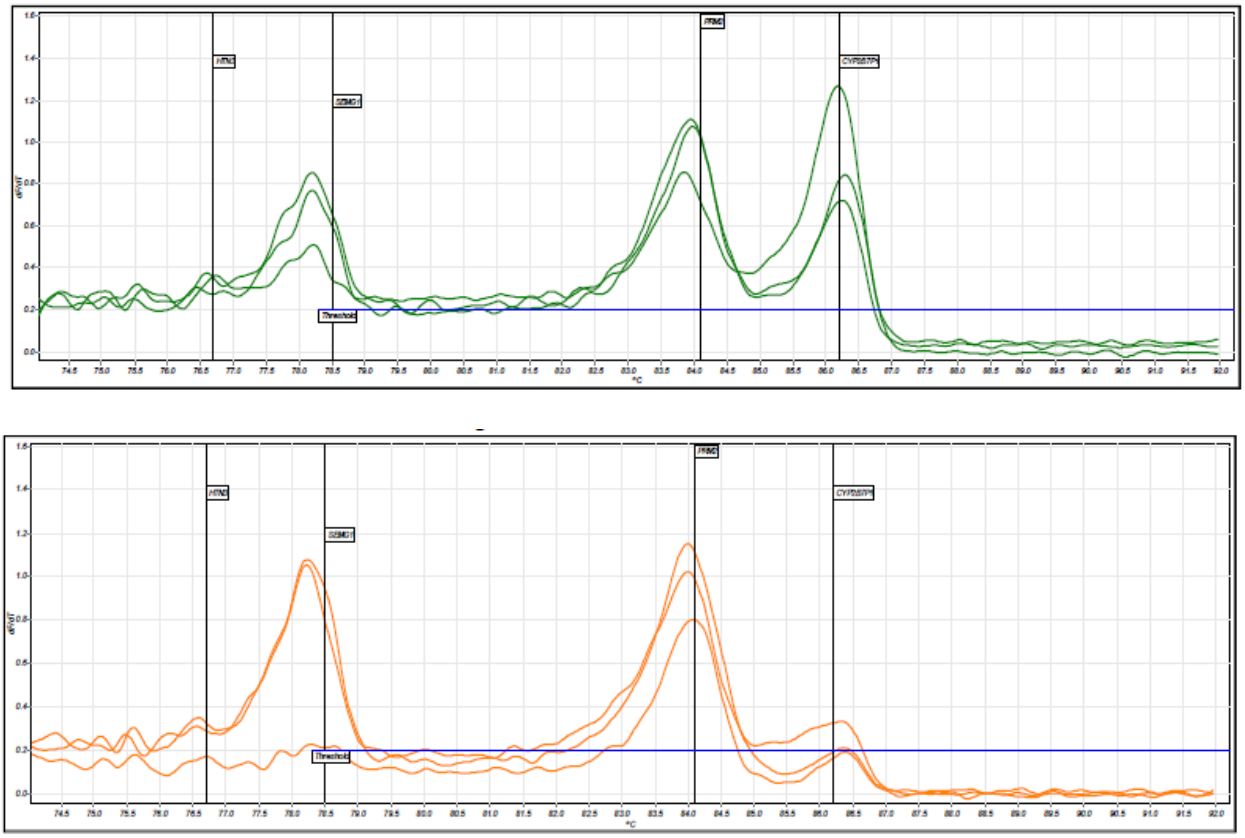


Figure 8. HRM Melt Plot for the New Rapid 5-fluid 6-plex HRM Assay

The rapid 5-fluid 6-plex HRM assay contains one biomarker each for saliva (HTN3, blue), blood (ANK1, red), vaginal secretions (CYP2B7P1, green) and menstrual blood (LEFTY2, pink) as well as two biomarkers for semen (SEMG1 and PRM2, orange). Single source body fluid samples are shown overlaid to indicate biomarker position within the assay. The blue horizontal line represents the detection threshold. The Tm (ºC) values for each sample are provided in the yellow colored cells indicating the positive detection of the biomarker. X-axis – temperature; Y-axis: dF/dT.

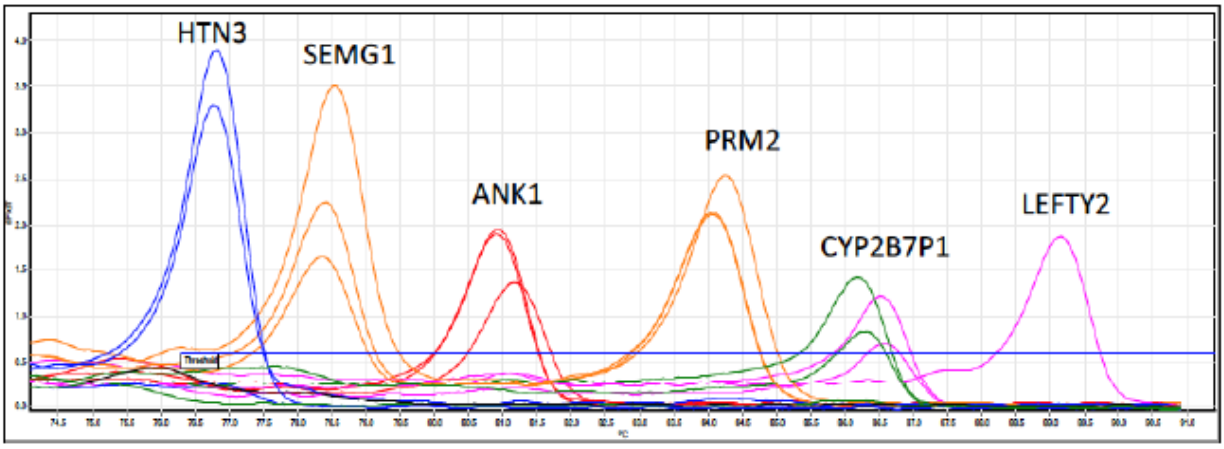


Figure 9. Nucleospin® RNA Extraction Kit (Macherey-Nagel)

The Macherey-Nagel Nucleospin® 8/96 RNA kit permits the rapid lysis of body fluid samples with RNA recovery in ~1 hour or less. Shown here is the vacuum manifold set up (left) as well as examples of the 8-strip and 96-well formats.

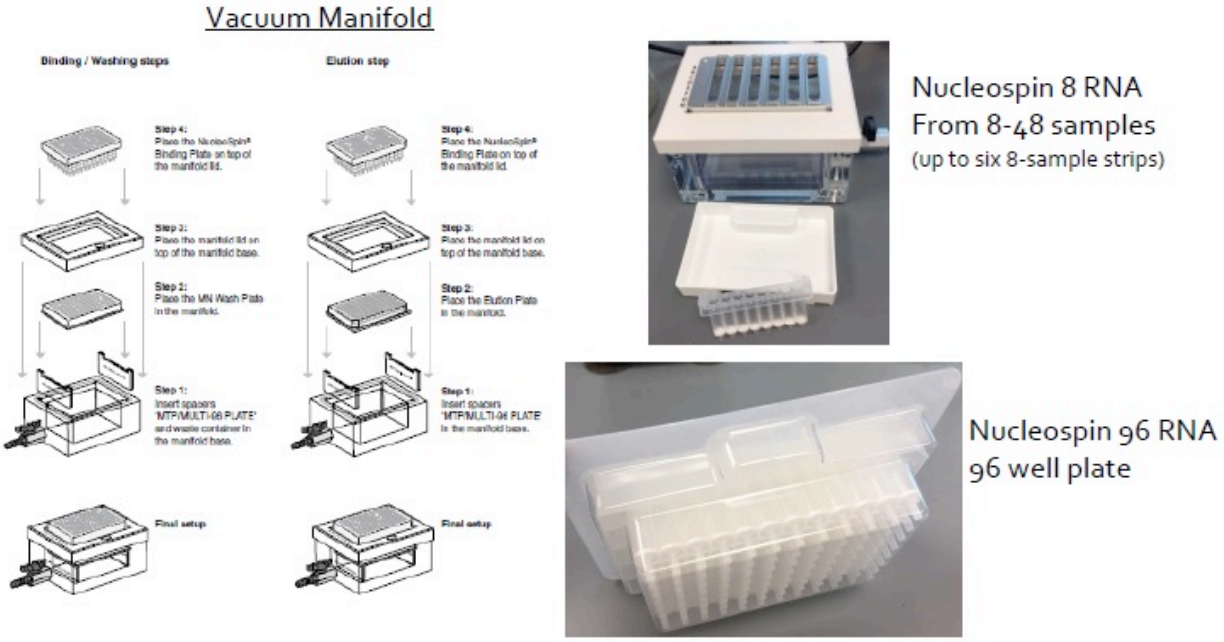


Figure 10. HRM Melt Plots (Rapid Sexual Assault Assay) from 1 µL Single Source Saliva and Semen Samples and 2 µL Saliva-Semen Mixtures Using the Rapid RNA Analysis Method

The rapid sexual assault HRM assay contains one biomarker for the identification of saliva (HTN3) and vaginal secretions (CYP2B7P1) and two markers for the identification of semen (SEMG1, seminal fluid) and PRM2 (sperm specific). The blue horizontal line represents the detection threshold. X-axis – temperature; Y-axis: dF/dT.

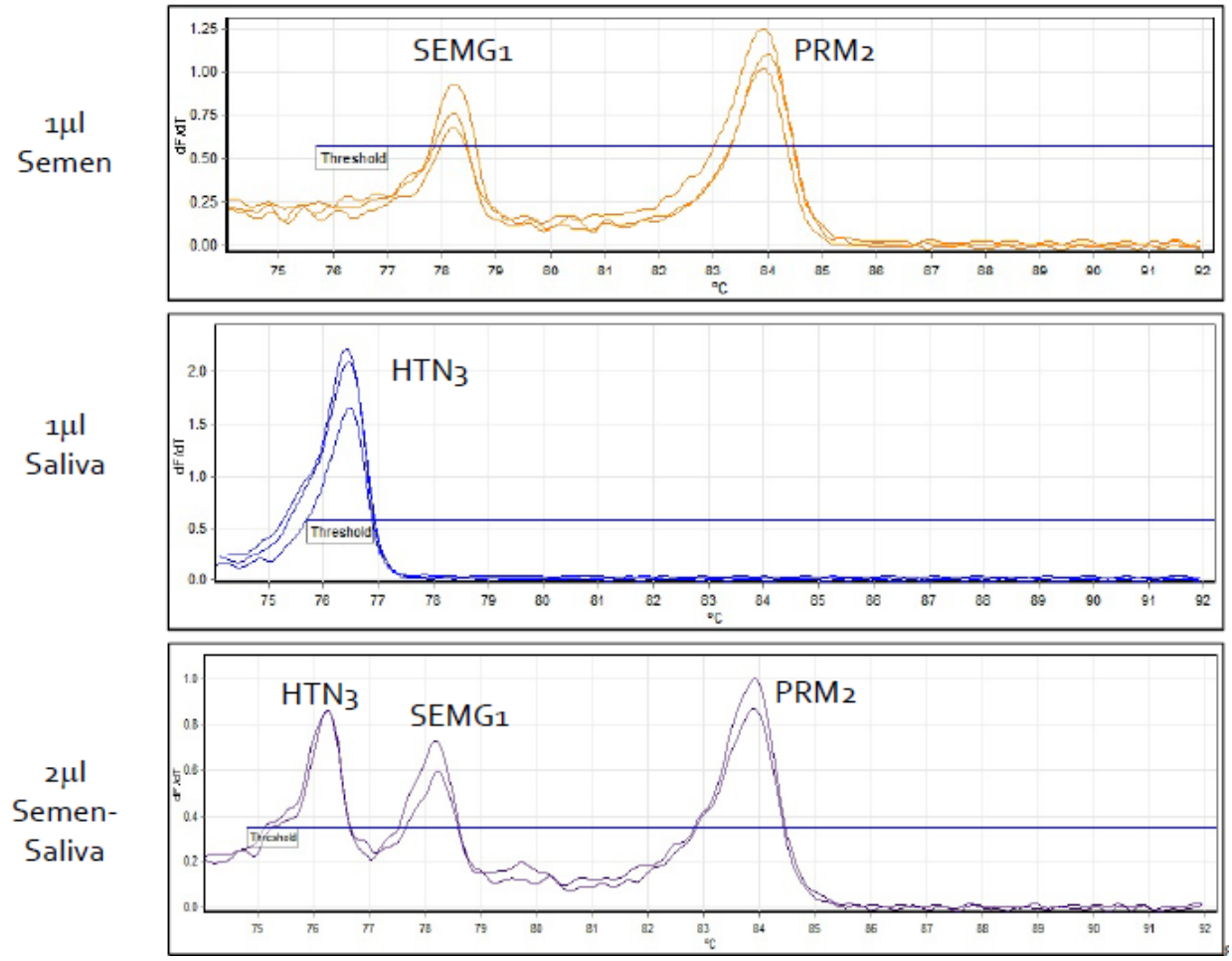
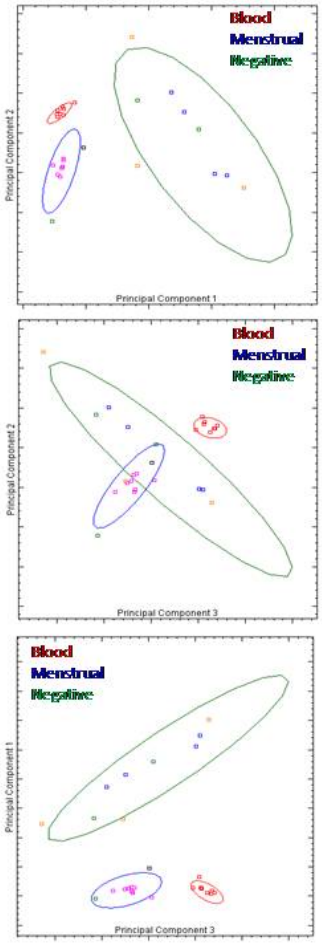


Figure 11. ScreenClust Software Analysis of the Blood-Menstrual Blood HRM Assay Using the Supervised Analysis Mode

The QIAGEN Rotor-Gene® ScreenClust HRM software was used to analyze HRM data the blood-menstrual blood HRM assay (Nucleopin 8 RNA samples were used for the analysis). The software is intended to be used to process HRM data files generated on the Rotor-Gene® Q instrument and its purpose is to allow for automated calling of 'genotypes' using either supervised or unsupervised data sets. Data analysis using this software involves the following steps: 1) normalization, 2) generation of a residual plot, 3) principal component analysis and 4) clustering. Provided below are raw principal component analysis plots and the analysis results table. Individual sample data points are represented by open squares (red – blood, menstrual – pink, green – vaginal secretions, orange – semen, blue – saliva). Supervised analysis mode was used with 'blood', 'menstrual' and 'negative' clusters defined using three known samples within each cluster (samples indicated with an * in the table). The negative cluster consisted of known semen, saliva and vaginal secretions samples so that all non-target body fluid samples would be represented in the "negative" cluster. Clusters are indicated on the principal component analysis plots. Typicality indicates how well the sample belongs to its assigned cluster.



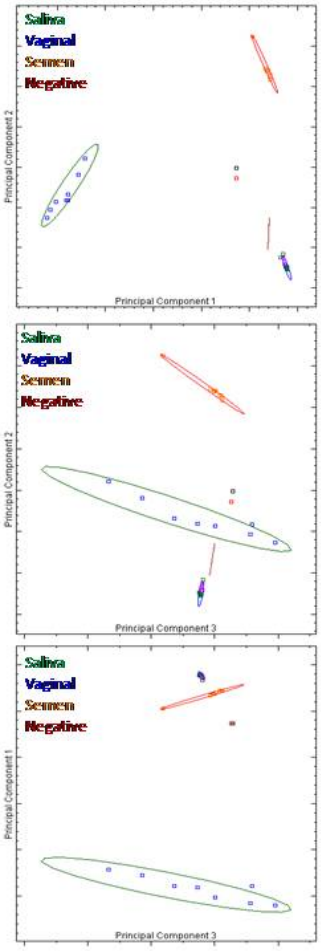
Blood-Menstrual Blood HRM

ID	Name	Cluster	Typicality	Probabilities		
				blood	menstrual	negative
1	MB2-S *	menstrual	0.93268778	0.0000	1.0000	0.0000
2	MB13-S *	menstrual	0.79936558	0.0000	1.0000	0.0000
3	MB14-S *	menstrual	0.76493084	0.0000	1.0000	0.0000
4	MB15-S	menstrual	0.78724285	0.0000	1.0000	0.0000
5	MB17-S	menstrual	0.09190494	0.0000	1.0000	0.0000
6	MB1-S	menstrual	0.98360968	0.0000	1.0000	0.0000
7	MB1-D-S	menstrual	0.90608308	0.0000	1.0000	0.0000
8	MB2-T	menstrual	0.95573798	0.0000	1.0000	0.0000
9	B7486 *	blood	0.46490055	1.0000	0.0000	0.0000
10	B7478	blood	0.09842483	0.9999	0.0001	0.0000
11	B7494 *	blood	0.95818777	1.0000	0.0000	0.0000
12	B7484 *	blood	0.63711850	1.0000	0.0000	0.0000
13	B7478	blood	0.18683162	1.0000	0.0000	0.0000
14	B7483	blood	0.41630197	1.0000	0.0000	0.0000
15	B7485	blood	0.66513256	1.0000	0.0000	0.0000
16	B7476	blood	0.81962049	1.0000	0.0000	0.0000
17	SA71-T *	negative	0.91412778	0.0000	0.0000	1.0000
18	SA72-T	negative	0.54873432	0.0000	0.0000	1.0000
19	SA73-T	negative	0.71850947	0.0000	0.0000	1.0000
20	SA74-T	negative	0.82157714	0.0000	0.0000	1.0000
21	SE16-cl *	negative	0.33602315	0.0000	0.0000	1.0000
22	M14-T	negative	0.08038083	0.0000	0.0000	1.0000
23	VS37-T	negative	0.81618405	0.0000	0.0000	1.0000
24	VS36-T *	negative	0.35734659	0.0000	0.0000	1.0000
25	VS35-T	menstrual	0.05217164	0.0000	1.0000	0.0000
26	AB	menstrual	0.03448455	0.0000	1.0000	0.0000
27	SE22-cl	negative	0.18705505	0.0000	0.0000	1.0000

* 3 blood known
* 3 menstrual known
* 3 negative (one SE, SA, VS)

Figure 12. ScreenClust Software Analysis of the Sexual Assault HRM Assay Using the Supervised Analysis Mode

The QIAGEN Rotor-Gene® ScreenClust HRM software was used to analyze HRM data the sexual-assault HRM assay (Nucleopin 8 RNA samples were used for the analysis). The software is intended to be used to process HRM data files generated on the Rotor-Gene® Q instrument and its purpose is to allow for automated calling of 'genotypes' using either supervised or unsupervised data sets. Data analysis using this software involves the following steps: 1) normalization, 2) generation of a residual plot, 3) principal component analysis and 4) clustering. Provided below are raw principal component analysis plots and the analysis results table. Individual sample data points are represented by open squares (red – blood, menstrual – pink, green – vaginal secretions, orange – semen, blue – saliva). Supervised analysis mode was used with 'semen', 'saliva', 'vaginal' and 'negative' clusters defined using three known samples within each cluster (samples indicated with an * in the table). The negative cluster consisted of known blood and menstrual blood samples so that all non-target body fluid samples would be represented in the "negative" cluster. Clusters are indicated on the principal component analysis plots.



Sexual Assault HRM

				Probabilities			
ID	Name	Cluster	Typicality	semen	vaginal	saliva	negative
29	SA71-T *	saliva	0.40814955	0.0000	0.0000	1.0000	0.0000
30	SA72-T *	saliva	0.78093615	0.0000	0.0000	1.0000	0.0000
31	SA73-T	saliva	0.42905956	0.0000	0.0000	1.0000	0.0000
32	SA74-T	saliva	0.47585140	0.0000	0.0000	1.0000	0.0000
33	SA2-S *	saliva	0.52810672	0.0000	0.0000	1.0000	0.0000
34	SA10-S	saliva	0.44288040	0.0000	0.0000	1.0000	0.0000
35	SA13-S	saliva	0.87274320	0.0000	0.0000	1.0000	0.0000
36	SA69-S	saliva	0.10444208	0.0000	0.0000	1.0000	0.0000
37	SE22-cl *	semen	0.35757025	1.0000	0.0000	0.0000	0.0000
38	SE16-cl *	semen	0.57694219	1.0000	0.0000	0.0000	0.0000
39	M14-T	semen	0.56386756	1.0000	0.0000	0.0000	0.0000
40	SE19-T	semen	0.58606302	1.0000	0.0000	0.0000	0.0000
41	SE16-S	semen	0.58888298	1.0000	0.0000	0.0000	0.0000
42	SE15-S	semen	0.44434702	1.0000	0.0000	0.0000	0.0000
43	SE14-S	semen	0.39862828	1.0000	0.0000	0.0000	0.0000
44	SE4vs-S *	semen	0.23391401	1.0000	0.0000	0.0000	0.0000
45	VS37-T *	vaginal	0.33345410	0.0000	1.0000	0.0000	0.0000
46	VS36-T *	vaginal	0.85841880	0.0000	1.0000	0.0000	0.0000
47	VS35-T *	vaginal	0.80204293	0.0000	1.0000	0.0000	0.0000
48	VS29-T	vaginal	0.78449110	0.0000	1.0000	0.0000	0.0000
49	VS8-S	vaginal	0.03045985	0.0000	1.0000	0.0000	0.0000
50	VS4-S	vaginal	0.87919670	0.0000	0.0000	0.0000	1.0000
51	VS2-S	vaginal	0.74468265	0.0000	1.0000	0.0000	0.0000
52	VS1-S	vaginal	0.08704420	0.0000	1.0000	0.0000	0.0000
53	MB2-S *	vaginal	0.46255157	0.0000	1.0000	0.0000	0.0000
54	MB13-S *	vaginal	0.64014800	0.0000	1.0000	0.0000	0.0000
55	MB14-S	vaginal	0.54776073	0.0000	1.0000	0.0000	0.0000
56	B7486 *	negative	0.80125196	0.0000	0.0000	0.0000	1.0000
59	AB	negative	0.80125196	0.0000	0.0000	0.0000	1.0000

* 3 semen known
* 3 saliva known
* 3 vaginal known
* 3 negative (2 MB, 1 BD)

Even with MB's used as known for negative cluster, still ID'd as vaginal

Figure 13. Evaluation of Possible HTN3-Specific Threshold for Use With EZ1® RNA Samples Analyzed with the Standard Sexual Assault HRM Assay

Previously obtained HRM data (standard sexual assault HRM assay) for single source saliva, vaginal and semen samples was evaluated in order to determine if a biomarker-specific threshold could be established for HTN3 for the RNA EZ1® samples. The top panels represent one run with data evaluated from single source saliva samples (blue), assay blanks and negative controls (grey and blank), single source vaginal samples (green) and single source semen samples (orange). The bottom panel contains data from a second run. For each panel, x-axis: Tm value (°C), y-axis: dF/dT.

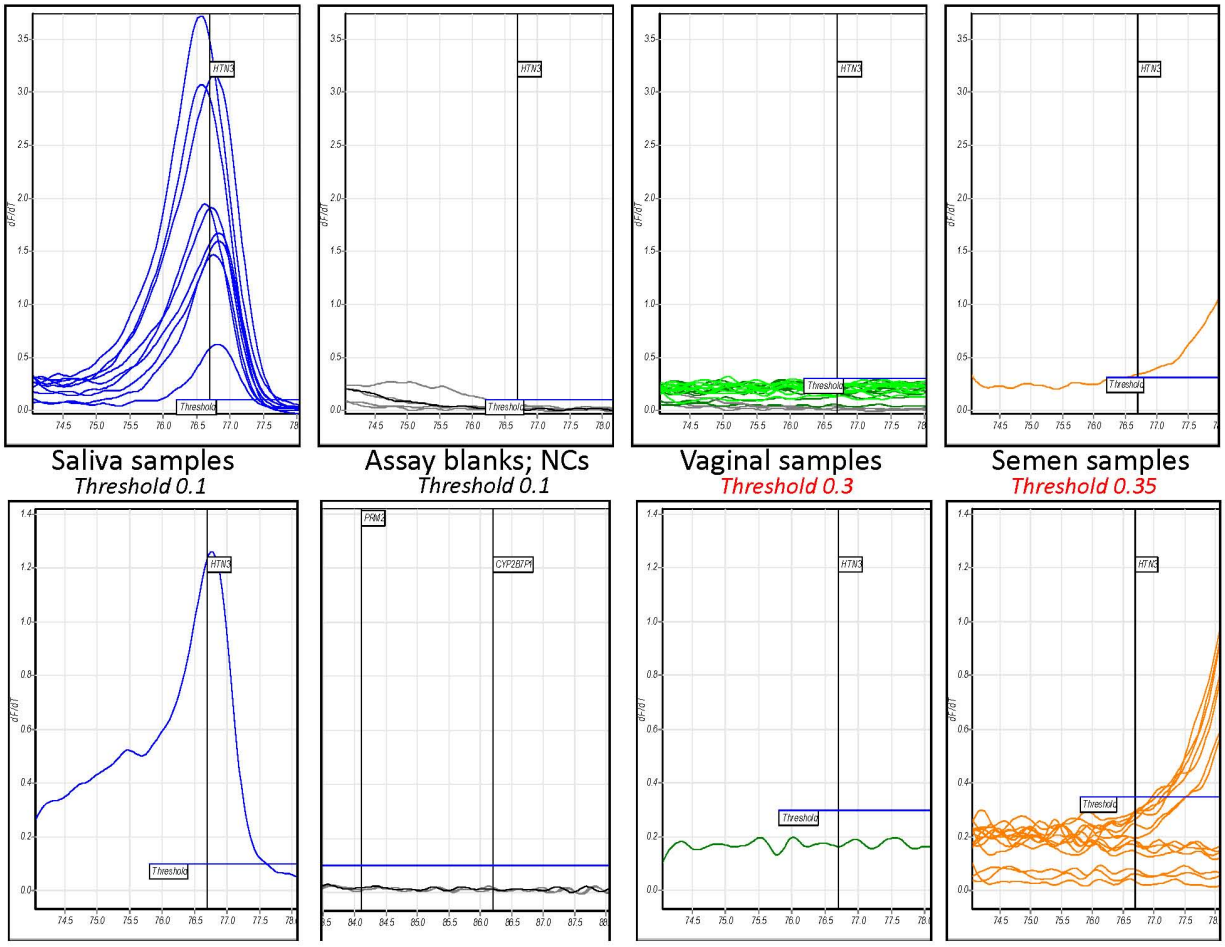


Figure 14. Evaluation of Possible CYP2B7P1-Specific Threshold for Use With EZ1® RNA Samples Analyzed with the Standard Sexual Assault HRM Assay

Previously obtained HRM data (standard sexual assault HRM assay) for single source saliva, vaginal and semen samples was evaluated in order to determine if a biomarker-specific threshold could be established for CYP2B7P1 for the RNA EZ1® samples. The top panels represent one run with data evaluated from single source saliva samples (blue), assay blanks and negative controls (grey and blank), single source vaginal samples (green) and single source semen samples (orange). The bottom panel contains data from a second run. For each panel, x-axis: Tm value (°C), y-axis: dF/dT.

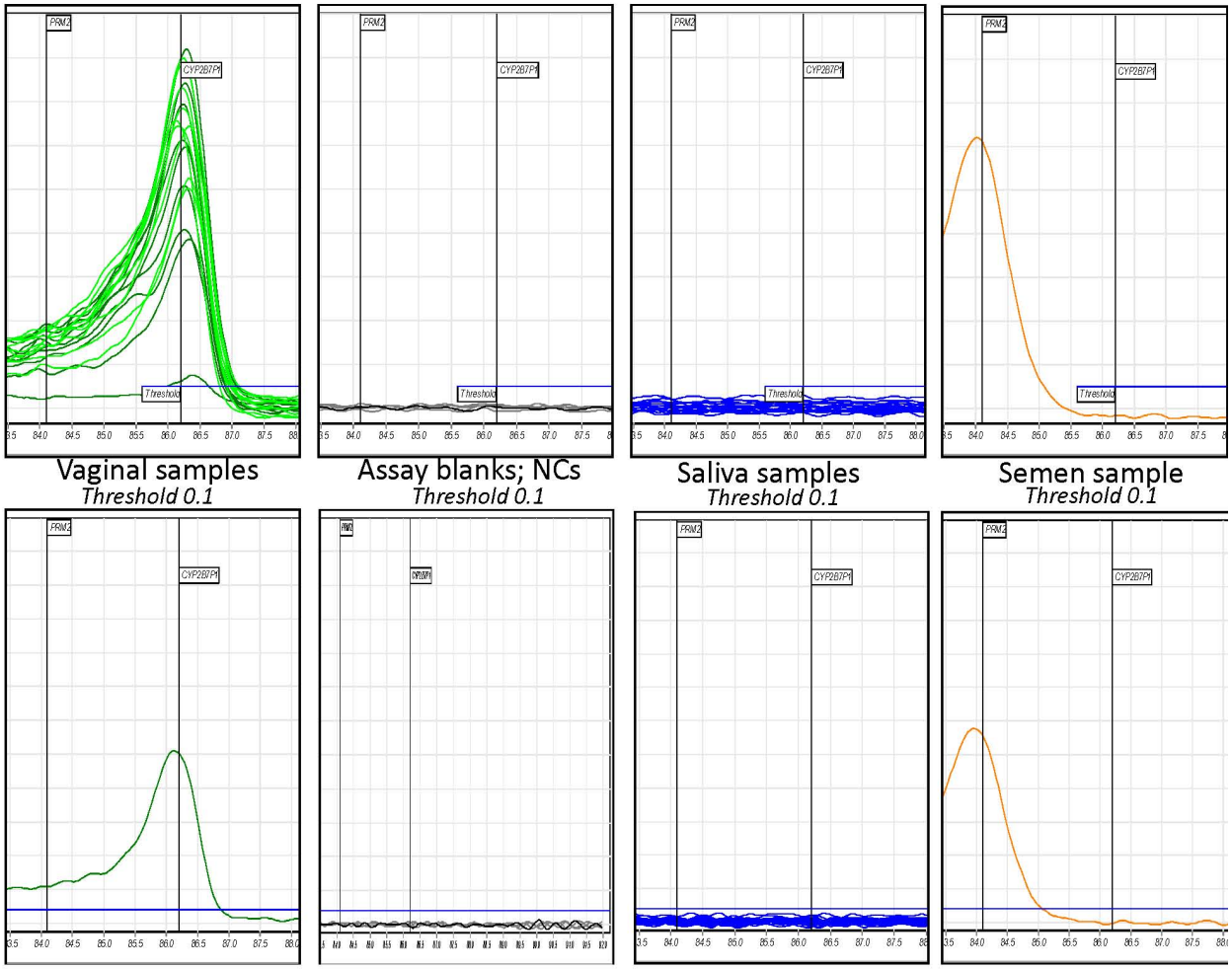


Figure 15. Evaluation of Possible SEMG1 and PRM2-Specific Threshold for Use With EZ1® RNA Samples Analyzed with the Standard Sexual Assault HRM Assay

Previously obtained HRM data (standard sexual assault HRM assay) for single source saliva, vaginal and semen samples was evaluated in order to determine if a biomarker-specific threshold could be established for SEMG1 and PRM2 for the RNA EZ1® samples. The panels (Top row – SEMG1, bottom row – PRM2) represent one run with data evaluated from single source saliva samples (blue), assay blanks and negative controls (grey and blank), single source vaginal samples (green) and single source semen samples (orange). The bottom panel contains data from a second run. For each panel, x-axis: Tm value (°C), y-axis: dF/dT.