RESEARCH ARTICLE

REVISED Rapid and inexpensive body fluid identification by RNA profiling-based multiplex High Resolution Melt (HRM) analysis [v2; ref status: indexed, http://f1000r.es/314]

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Abstract

Positive identification of the nature of biological material present on evidentiary items can be crucial for understanding the circumstances surrounding a crime. However, traditional protein-based methods do not permit the identification of all body fluids and tissues, and thus molecular based strategies for the conclusive identification of all forensically relevant biological fluids and tissues need to be developed. Messenger RNA (mRNA) profiling is an example of such a molecular-based approach. Current mRNA body fluid identification assays involve capillary electrophoresis (CE) or quantitative RT-PCR (qRT-PCR) platforms, each with its own limitations. Both platforms require the use of expensive fluorescently labeled primers or probes. CE-based assays require separate amplification and detection steps thus increasing the analysis time. For qRT-PCR assays, only 3-4 markers can be included in a single reaction since each requires a different fluorescent dye.

To simplify mRNA profiling assays, and reduce the time and cost of analysis, we have developed single- and multiplex body fluid High Resolution Melt (HRM) assays for the identification of common forensically relevant biological fluids and tissues. The incorporated biomarkers include IL19 (vaginal secretions), IL1F7 (skin), ALAS2 (blood), MMP10 (menstrual blood), HTN3 (saliva) and TGM4 (semen). The HRM assays require only unlabeled PCR primers and a single saturating intercalating fluorescent dye (Eva Green). Each body-fluid-specific marker can easily be identified by the presence of a distinct melt peak. Usually, HRM assays are used to detect variants or isoforms for a single gene target. However, we have uniquely developed duplex and triplex HRM assays to permit the simultaneous detection of multiple targets per reaction. Here we describe the development and initial performance evaluation of the developed HRM assays. The results demonstrate the potential use of HRM assays for rapid, and relatively inexpensive, screening of biological evidence.

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> **REVISED** **Amendments from Version 1**
>
> In the revised version of the article, minor modifications were made to Table 1 and Table 3, in order to include the amplicon size for each of the biomarkers. No other changes to the original version of the article were made.
>
> **See referee reports**

Introduction

Identification of the tissue source of biological material present on individuals, evidentiary items and at crime scenes can be crucial to a fuller understanding of the circumstances pertaining to a crime. However traditional protein-based body fluid identification methods use a variety of labor intensive and technologically diverse techniques that do not permit the identification of all body fluids and tissues. Thus there remains a need to develop definitive molecular based strategies for the conclusive identification of all forensically relevant biological fluids and tissues. Although protein[1–3], epigenetic DNA markers[4–10] and microRNAs[11–17] are promising examples of such a molecular based approach, messenger RNA (mRNA) profiling represents the current gold standard in this area due to the identification and development of a number of specific and sensitive mRNA assays for the identification of all of the forensically-relevant body fluids and tissues, namely blood, semen, saliva, vaginal secretions, menstrual blood and skin[13,18–36]. Importantly, messenger RNA markers are surprisingly stable in the dried state in that they can be successfully detected in environmentally impacted and partially degraded samples[20,22,25,26,37–40].

Current mRNA body fluid identification assays typically involve capillary electrophoresis (CE) or quantitative RT-PCR (qRT-PCR) platforms[13,20,24,28,29,31,32,34,35], each with its own limitations. Both platforms require the use of expensive fluorescently labeled primers or probes. CE-based assays require separate amplification and detection steps thus increasing the time required for analysis. For qRT-PCR assays, only 3 or 4 markers can be included in a single reaction since each marker requires a different fluorescent dye.

In an attempt to simplify mRNA profiling assays, and to reduce the time and cost of analysis, we have investigated alternative technology solutions for mRNA profiling, particularly High Resolution Melt analysis (HRM). HRM is a rapid and relatively cheap bioanalytical method that was initially developed for sequence variant screening (i.e. mutation analysis) but which has also found utility in single nuclear polymorphism (SNP) typing[41–44], methylation analysis[45,46], copy number variant confirmation[47–49], clone characterization[50] and as an alternative for gel electrophoresis[51]. As its name suggests, HRM is a technique that permits the identification of specific PCR products (i.e. amplicons) by their melting temperature (T_m). An amplicon's precise melting temperature is dependent on its sequence, length and the ionic strength of its environment and can be measured post-PCR to within 0.1°C with specialized software and hardware. HRM assays require only the use of unlabeled PCR primers and a single saturating intercalating fluorescent dye (e.g. Eva Green). The latter provides enhanced sensitivity by ensuring that all available double stranded DNA binding sites are saturated during the melting process. After amplification the amplicon is melted slowly by increasing the temperature and a loss of fluorescence occurs as the DNA strands separate and release the bound saturating dye into solution. By measuring the negative first derivative of fluorescence (F) with respect to temperature (T) (-dF/dT) a distinct and characteristic melt curve is obtained with the peak maximum representing the T_m. Importantly, due to the high resolution nature of the amplicon melt analysis, it is possible to perform multiplex analysis of several amplicons in one tube[52].

Here we describe the development and initial performance evaluation of singleplex and multiplex RNA-based HRM assays for the identification of the commonly encountered forensically relevant body fluids and tissues.

Methods

Body fluid samples

Body fluids were collected from volunteers using procedures approved by the University's Institutional Review Board. Informed written consent was obtained from each donor. Blood samples (10mL, Bioreclamation, Westbury, NY) (16 donors; male and female; 21–56 yrs old) were collected by venipuncture into additive-free vacutainers and 50 µl aliquots were placed onto cotton cloth and dried at room temperature. Freshly ejaculated semen (12 donors; 30–55 yrs old) was provided in sealed plastic tubes and stored frozen. After thawing, the semen was absorbed onto sterile cotton swabs and allowed to dry. Buccal samples (saliva) (18 donors; male and female; 26–60 yrs old) were collected from donors using sterile swabs by swabbing the inside of the donor's mouth. Semen-free vaginal secretions (18 donors; 20–60 yrs old) and menstrual blood (5 donors; 20–33 yrs old) were collected using sterile cotton swabs. Human skin total RNA was obtained from commercial sources: Stratagene/Agilent Technologies (Santa Clara, CA), Biochain® (Hayward, CA), Zenbio (Research Triangle Park, NC), and Zyagen (San Diego, CA). Cellular skin samples were collected by swabbing human skin or a touched object surface with a sterile water pre-moistened sterile swab. All samples were stored at -20°C or at room temperature until needed. A 50 µl stain or a single cotton swab was used for RNA isolation.

RNA isolation and quantitation

Total RNA was extracted from blood, semen, saliva, vaginal secretions, menstrual blood and skin using a manual organic RNA extraction (guanidine isothiocyanate-phenol-chloroform mixture)[28–32]. Briefly, 500 µl of pre-heated (56°C for 10 minutes) denaturing solution (4 M guanidine isothiocyanate, 0.02 M sodium citrate, 0.5% sarkosyl, 0.1 M β-mercaptoethanol) was added to a 1.5 mL Safe Lock tube extraction tube (Eppendorf, Westbury, NY) containing the stain or swab. The samples were incubated at 56°C for 30 minutes. The swab or stain pieces were then placed into a DNA IQ™ spin basket (Promega, Madison, WI), re-inserted back into the original extraction tube, and centrifuged at 14,000 rpm (16,000 × g) for 5 minutes. After centrifugation, the basket with swab/stain pieces was discarded. To each extract the following was added: 50 µl 2 M sodium acetate and 600 µl acid phenol:chloroform (5:1), pH 4.5 (Ambion by Life Technologies). The samples were placed at 4°C for 30 minutes to separate the layers and then centrifuged for 20 minutes at 14,000 rpm (16,000 × g). The RNA-containing top aqueous layer was transferred to a new 1.5 ml microcentrifuge tube, to which 2 µl

of GlycoBlue™ glycogen carrier (Ambion by Life Technologies) and 500 μl of isopropanol were added. RNA was precipitated for 1 hour at -20°C. The extracts were then centrifuged at 14,000 rpm (16,000 × g). The supernatant was removed and the pellet was washed with 900 μl of 75% ethanol/25% DEPC-treated water. Following a centrifugation for 10 minutes at 14,000 rpm (16,000 × g), the supernatant was removed and the pellet dried using vacuum centrifugation (56°C) for 3 minutes. Twenty microliters of preheated (60°C for 5 minutes) nuclease free water (Ambion by Life Technologies) was added to each sample followed by an incubation at 60°C for 10 minutes. Samples were used immediately or stored at -20°C until needed. All extracts were DNase treated to remove residual DNA using the Turbo DNA-*free*™ kit (Applied Biosystems (AB) by Life Technologies, Carlsbad, CA) according to the manufacturer's protocol. RNA extracts were quantitated with Quant-iT™ RiboGreen® RNA Kit (Invitrogen by Life Technologies, Carlsbad, CA) as previously described[28–32]. Fluorescence was determined using a Synergy™ 2 Multi-Mode microplate reader (BioTek® Instruments, Inc., Winooski, VT).

cDNA synthesis
All samples were reverse transcribed using the High Capacity cDNA Reverse Transcription kit (AB by Life Technologies) according to manufacturer's protocols. The desired total RNA input was reverse transcribed in a 20 μl RT reaction volume (standard input – 25 ng total RNA). If no quantitative value was obtained or quantitation was not performed, an aliquot of the total RNA extract was used (14.2 μl). A reverse transcription negative reaction (containing total RNA and reaction buffer but no reverse transcriptase enzyme) was performed for each sample.

High Resolution Melt (HRM) analysis
Singleplex, Duplex, Triplex Assays: HRM assays were performed using the Type-It® HRM™ PCR kit (QIAGEN, Germantown, MD). Each 25 μl reaction included: 1 × HRM master mix (contains Hot-Star Taq Plus DNA Polymerase, EvaGreen dye, and manufacturer-designated 'optimized concentrations' of Q-solution (commercial reagent, exact concentrations not specified by the manufacturer), dNTPs and MgCl₂), 2.8 μM primers (Table 1), 2 μl of RT reaction (cDNA), and RNase-free water. All assays were run on the Rotor-Gene® Q real time PCR instrument (QIAGEN), using the following cycling conditions: 95°C 5 min, followed by 45 cycles of 94°C 10 sec, 55°C 30 sec, 72°C 10 sec. HRM analysis was performed using a 65–90°C temperature range, with +0.1°C increments (90 sec of pre-melt conditioning on first step; and 2 sec for each step afterwards).

'All Body Fluids' Hexaplex Assay: HRM assays were performed using the Type-It® HRM™ PCR kit (QIAGEN, Germantown, MD). Each 25 μl reaction included: 1X HRM master mix (contains Hot-Star Taq Plus DNA Polymerase, EvaGreen dye, and optimized concentrations (commercial reagent, exact concentrations not specified by the manufacturer) of Q-solution, dNTPs and MgCl₂), 0.56 – 1.4 μM primers (Table 3) (ALAS2–0.56 μM, TGM4–0.56 μM, HTN3–1.4 μM, IL19 – 0.84 μM, MMP10 – 0.56 μM, CCL27 – 0.56 μM), 2 μl of RT reaction (cDNA), and RNase-free water. All assays were run on the Rotor-Gene® Q real time PCR instrument (QIAGEN), using the following cycling conditions: 95°C 5 min, followed by 45 cycles of 94°C 10 sec, 57°C 40 sec, 72°C 20 sec. HRM analysis was performed using a 73–90°C temperature range, with +0.1°C increments (90 sec of pre-melt conditioning on first step; 2 seconds for each step afterwards).

Results
Duplex HRM assays for body fluid identification
Development of the HRM assay. Based on our previous work with mRNA profiling[13,24,28,29,31,32], we selected one marker for each body fluid/tissue based on amplification efficiency, specificity and/or sensitivity. The fluid- and tissue-specific markers selected were ALAS2 (blood)[21,22,32], MMP10 (menstrual blood)[32], HTN3 (saliva)[26,30–32], TGM4 (semen)[26,53], IL19 (vaginal secretions)[29] and IL1F7 (skin)[27,28] (Table 1). We performed singleplex HRM assays for each of these markers to ensure that they could be uniquely identified based on their different T_ms. Samples from two donors of each body fluid of interest were used to estimate the T_m of each of the selected markers. The observed T_ms are listed in Table 1 and the melt curves for each marker are displayed in Figure 1. Derivative plots are shown, with the temperature range (70–90°C) used in the HRM assay is displayed along the x-axis and the -dF/dT value (negative first derivative of fluorescence (F) with respect to temperature (T)) is displayed along the y-axis. An analysis threshold can be set for each axis, which is represented by a horizontal (blue) line on each plot. As can be seen in Figure 1, a single melt curve was observed for

Table 1. Genes and amplicons used in singleplex, duplex and triplex body fluid HRM assays.

| Body fluid	Gene	Accession number	Amplicon size (bp)	Amplicon T$_m$		Primer sequence (5′–3′)
				°C	Range (± 3SD)	
Vaginal	IL19	NM_153758	189	81.5	81.3 – 81.7	F: AACCACGGTCTCAGGAGATG R: GAACGCCAGGAGGTTCTTG
Skin	IL1F7	NM_014439	92	83.2	82.9 – 83.5	F: CCAGTGCTGCTTAGAAGACC R: TCACCTTTGGACTTGTGTGAA
Blood	ALAS2	NM_001037967	136	85.8	85.6 – 86.0	F: TGTGTCCGTCTGGTGTAGTA R: AAACTTACTGGTGCCTGAGA
Menstrual blood	MMP10	NM_002425	227	82.2, 83.5	81.8 – 82.7, 83.0 – 84.0	F: ACAGGGAAGCTAGACACTGA R: CTGGAGAATGTGAGTGGAGT
Saliva	HTN3	NM_000200	134	76.3	75.9 – 76.7	F: GCAAAGAGACATCATGGGTA R: GCCAGTCAAACCTCCATAATC
Semen	TGM4	NM_003241	164	82.3	82.2 – 82.4	F: ATGGTGTAAAGAGGACATGGTT R: GGGAAATGCAGCAGTCCAG
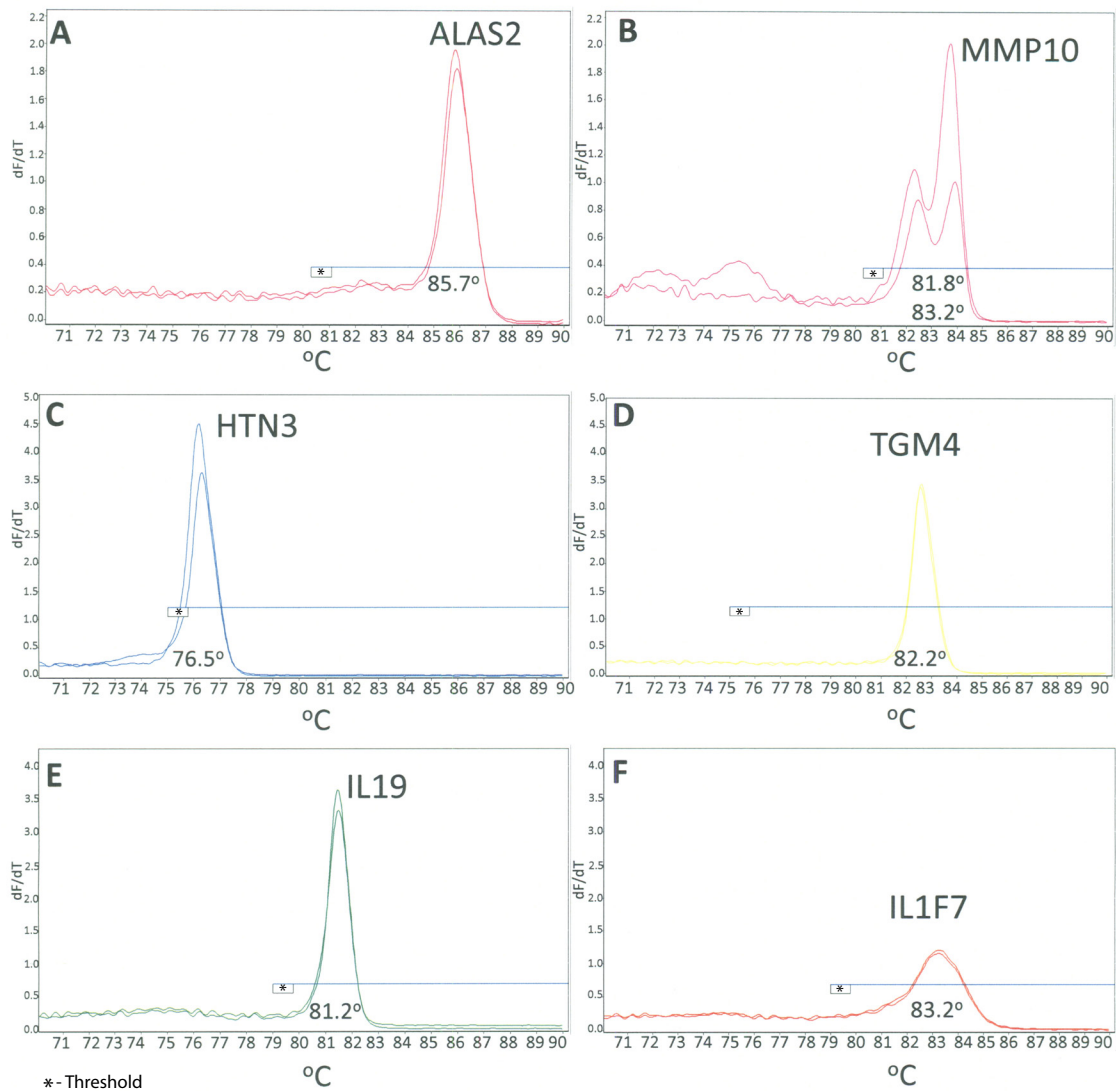
Figure 1. Singleplex HRM body fluid assays. High resolution derivative melt curve plots for individual body fluid specific markers: **A**) ALAS2 (blood); **B**) MMP10 (menstrual blood); **C**) HTN3 (saliva); **D**) TGM4 (semen); **E**) IL19 (vaginal secretions); **F**) IL1F7 (skin). The plots from two different donors are shown for each marker. The x-axis represents the temperature and the y-axis indicates the first derivative of the change of fluorescence with temperature (-dF/dT). The average T_m (°C) for each marker is displayed. The horizontal line on each plot represents the analysis threshold.

most markers with the exception of MMP10 in which two products are observed (81.8°C and 83.2°C). The same primer sequences for MMP10 used in these experiments are also used in our CE-based mRNA profiling multiplexes, where only one product is observed. Upon evaluation of the amplified sequence and comparison to different MMP genes, it was determined that the MMP10 primer set was capable of also amplifying the MMP3 gene. There are four mismatched bases in the forward primer, three of which are in the 3′end of the primer (i.e. 3 mismatches in the last 9 bases of the primer). This likely therefore causes amplification inefficiencies and results in a failure to detect the MMP3 product in some assays. The standard HRM protocol (QIAGEN Type-It® HRM™ kit)

utilizes a 55°C annealing temperature which may explain why this second product is detected using this platform. Additional work would be needed to conclusively determine if this second peak is in fact MMP3 and to determine if the primer sequences can be modified to be MMP10-specific. MMP10 is also the only marker in the body fluid set in which HRM DNA products were observed, one at 78.5°C and another at 83.1°C (Supplementary Figure 1). These were clearly distinguishable from a positive mRNA MMP10 result (82.2°C and 83.5°C). The presence of two DNA products further supports the hypothesis that MMP3 is being co-amplified, one DNA product resulting for MMP3 and MMP10 (expected DNA product size is 319 and 369 bp, respectively).

While some of the observed T_ms were similar or overlapping (Table 1), there was sufficient resolution to incorporate all markers into three separate duplex reactions: blood/menstrual blood, semen/saliva and vaginal secretions/skin. For the development of the duplex HRM assays, the primer sets for each of the two markers were incorporated into a single reaction. No other parameters of the HRM assay were modified from the original singleplex assays. For each duplex HRM assay, known samples (total n = 56–65) of each of the targeted body fluids of interest were detected (Figure 2, Table 2). The melt curves obtained during the initial testing of the duplex HRM assays are shown in Figure 2. The melt curves for each marker are shown overlaid in order to indicate their relative locations

within the duplex. For each of these single source samples, only the expected T_m(s) for the body fluid of interest was observed, with the exception of the menstrual blood samples (Figure 2A). With respect to the latter, as can be seen from the "pink" melt curves (representing the known menstrual blood samples), both MMP10 and ALAS2 were detected in the majority of samples (4/5). This is expected, as menstrual blood samples will contain varying amounts of peripheral blood, which is demonstrated by the presence of ALAS2. We observe the same co-detection in CE based mRNA profiling assays[31]. The simultaneous identification of both MMP10 and ALAS2 in individual menstrual blood samples also serves to confirm the functionality of this particular duplex assay.

* - Threshold
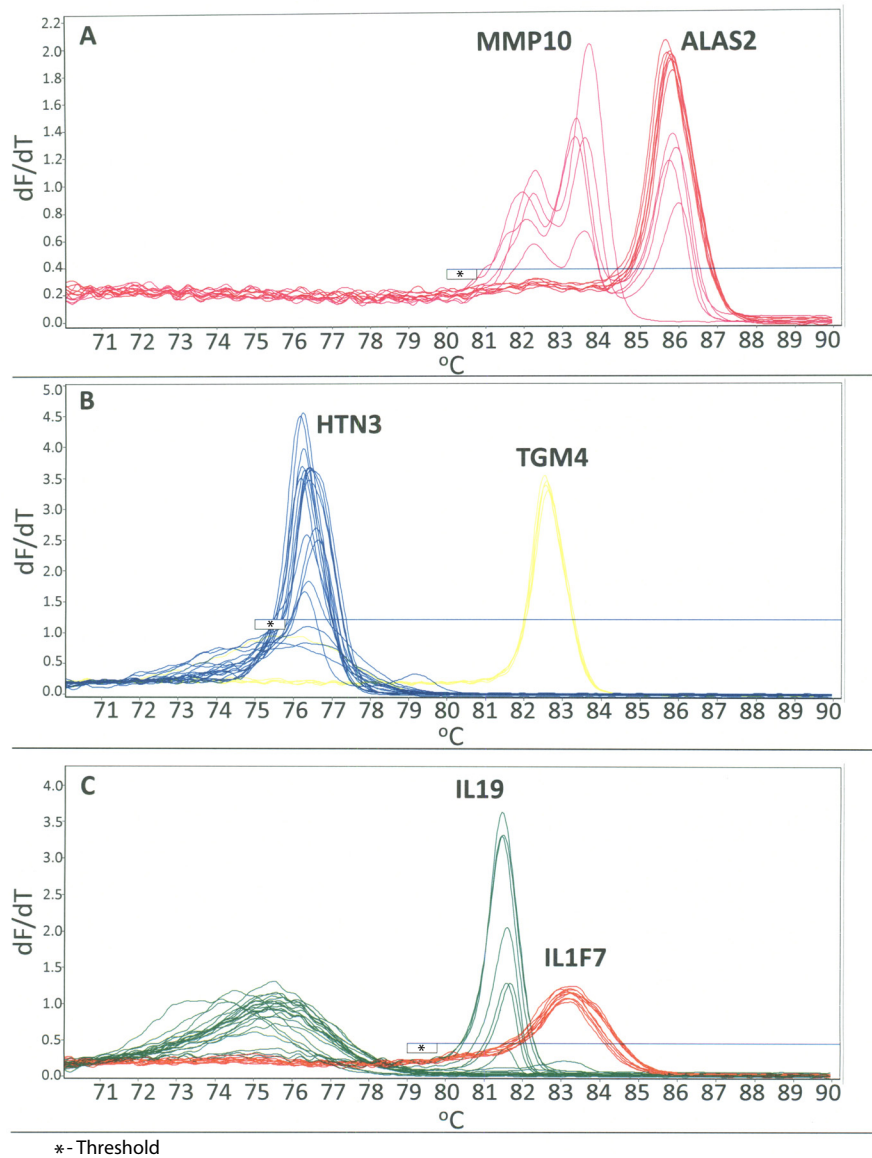
Figure 2. Duplex HRM body fluid assays. Duplex HRM assays incorporate a body fluid specific marker for each of two body fluids or tissues into a single reaction. High resolution derivative melt curve plots for the duplex assays are shown (where n = number of biological replicates (i.e. different individuals)): **A)** blood/menstrual blood (respectively: ALAS2, red, n = 8; MMP10, pink, n = 5); **B)** saliva/semen (respectively: HTN3, blue, n = 18; TGM4, yellow, n = 5); **C)** vaginal secretions/skin (respectively: IL19, green, n = 10; IL1F7, orange, n = 10). For interpretation of the reference to color, the reader is directed to the online version of the article.

Table 2. HRM duplex assay specificity. The number of samples from individuals in which the marker was successfully detected is displayed (numerator) out of the total number of individuals tested (denominator). The shading reflects the number of positive samples out of the total number tested: white – no detection; light grey 1–59%; dark grey ≥ 60%.

Body fluid/Tissue (25 ng RT)	Vaginal/Skin (n = 56)		Blood/Menstrual (n = 63)		Saliva/Semen (n = 65)	
	IL19	IL1F7	ALAS2	MMP10	HTN3	TGM4
Vaginal	6/10	0/10	0/18	4/18	0/18	0/18
Skin	0/10	10/10	0/10	0/10	0/11	0/11
Blood	0/8	0/8	7/8	0/8	0/8	0/8
Menstrual	3/5	0/5	4/5	5/5	0/5	0/5
Saliva	0/18	0/18	0/18	0/18	15/18	0/18
Semen	0/5	0/5	0/4	0/4	0/5	4/5

Table 3. Genes and amplicons used in the hexaplex body fluid HRM assay.

Body fluid	Gene	Amplicon size (base pairs)	Amplicon T_m		Primer sequence (5′–3′)
			°C	Range (± 3SD)	
Saliva	HTN3	134	76.6	76.0 – 77.2	F: GCAAAGAGACATCATGGGTA R: GCCAGTCAAACCTCCATAATC
Vaginal	IL19	109	78.6	78.0 – 79.2	F: AACCACGGTCTCAGGAGATG R: TGACATTTGGGAAGGTGTCC
Menstrual blood	MMP10	178	81.9	81.5 – 82.3	F: GGGGGTGACGTTGGTCACTTCAGCTC R: GGGGGCTGGAGAATGTGAGTGGAGT
Semen	TGM4	164	82.7	82.4 – 83.0	F: ATGGTGTAAAGAGGACATGGTT R: GGGAAATGCAGCAGTCCAG
Skin	CCL27	142	84.9	84.8 – 84.9	F: AGCACTGCCTGCTGTACTCA R: AGATGCTGCGTTGAGCCA
Blood	ALAS2	222	86.8	86.6 – 86.9	F: TGTGTCCGTCTGGTGTAGTA R: GAGTCATTGGCAACAAAGCA
DNA	--		80.6	80.2 – 80.9	NA (product originates from IL19)

*underline indicates non-template sequence addition; report amplicon size includes the non-template sequence additions

During the development of the duplex assays, we observed the appearance of broad peaks ("humps") in the lower temperature ranges for the TGM4 and IL19 melt curves in the semen/saliva and vaginal/skin duplex assays, respectively (Figure 2B and 2C). The artifacts were observed in the amplification blanks (data not shown) and are likely due to primer dimers or non-specific primer interactions. For the vaginal/skin assay, this does not interfere with data interpretation as the T_ms of these markers are at a higher temperature. Since the HRM temperature range was kept consistent for all duplex assays, the x-axis threshold can simply be set to exclude the affected temperatures from analysis. However, for the semen/saliva assay, the artifacts originating from the TGM4 melt curves are located within the HTN3 T_m region. This could affect the interpretation of the data on this assay as it could appear as a positive HTN3 result, although the peaks are abnormally broad and are distinguishable from true peaks. The intensity of the saliva sample melt curves permits us to use a suitably high x-axis threshold in order to eliminate these "humps" from analysis.

Specificity

A number of different donors (4–18 per body fluid or tissue, Table 2) of each body fluid were tested using all three duplex assays (total n = 56 (vaginal/skin), n = 63 (blood/menstrual blood, n = 65

(saliva/semen), Table 2). For the semen/saliva duplex, semen and saliva were correctly identified in a majority of samples and no cross-reactivity was observed with any of the other body fluids. For the vaginal/skin assay, vaginal secretions and skin were correctly identified in a majority of samples, and no cross-reactivity was observed for blood, semen or saliva (Table 2). As can be seen from Table 2, 3/5 menstrual blood samples evaluated were positive for IL19 (vaginal secretions) which is not surprising since menstrual blood samples are expected to contain varying amounts of vaginal secretions. However, it does indicate the need for additional interpretation guidelines for menstrual blood. For the blood/menstrual blood assay, blood and menstrual blood was correctly identified in a majority of blood and menstrual blood samples and no cross-reactivity was observed with skin, saliva and semen. However, MMP10 (menstrual blood marker) was detected in ~20% of vaginal secretion samples (4/18). While early work with MMP10 demonstrated a high degree of specificity for menstrual blood, other studies have also reported detection of MMP10 in vaginal samples[20]. Enzymes of the matrix metalloproteinase (MMP) family that play a role in the tissue remodeling that takes place during menstruation are spatiotemporally expressed in endometrial tissue[54,55]. Therefore, it is possible that increased levels of MMP10 may be present just prior to menses. The time during the reproductive cycle in which the

blood samples in this study were collected was not known. Further work will be needed to evaluate MMP10 expression levels throughout the female reproductive cycle to determine if any trends can be identified amongst numerous donors.

A number of false negative results (9/56, 16%) were observed for the body fluids or tissues of interest for the various duplex assays (Table 2). However, no false negatives were observed for skin (IL1F7 detected in all 10 donors tested) and menstrual blood (MMP10 detected in all 5 donors tested) (Table 2). The precise reason for the absence of the body fluid specific markers in these known samples is unclear. The false negative results for vaginal secretions and saliva may be explained by possible differences in input quantity. All body fluid sample extracts are quantitated to allow for standard amounts of total RNA (i.e. 25 ng) to be added to each reverse transcription reaction. However, since no validated human specific RNA quantitation method is currently available, the quantitation values obtained for body fluids with commensal bacteria such as vaginal secretions and saliva may be inaccurate with respect to estimates of the amount of human RNA in such samples. While attempts were made to normalize total RNA input into these body fluid samples, the actual amount of total human RNA may be somewhat less. This could lead to false negative results since sufficient amounts of human total RNA are not added. However, this would not be expected for blood or semen, where one false negative result was observed for each (Table 2). Further optimization of assay conditions and/or the development of a human specific RNA quantitation method might serve to reduce the occurrence of false negative results.

Mock casework

Evidentiary items of unknown body fluid/tissue origin would be analyzed using each of the three duplexes in order to determine which body fluids or tissues, or combination thereof, are present. We performed such a process during our evaluation of the body fluid duplexes by taking single source samples of known tissue provenance and testing them against the three duplex HRM assays. Interestingly the duplex HRM assays were able to successfully identify contaminated single source samples. A set of reportedly single source vaginal secretions samples were evaluated using each of the three duplex assays to check that no other cross reacting body fluids would be detected with the assay. As can be seen from Figure 3A, TGM4 was detected in two of the vaginal samples (melt curves shown in green) indicating the presence of semen. We performed additional testing of these samples using our CE-based mRNA body fluid identification multiplex and were able to confirm the presence of contaminating semen (detection of PRM2 and TGM4) in these two putative single source samples (Supplementary Figure 2).

∗ - Threshold
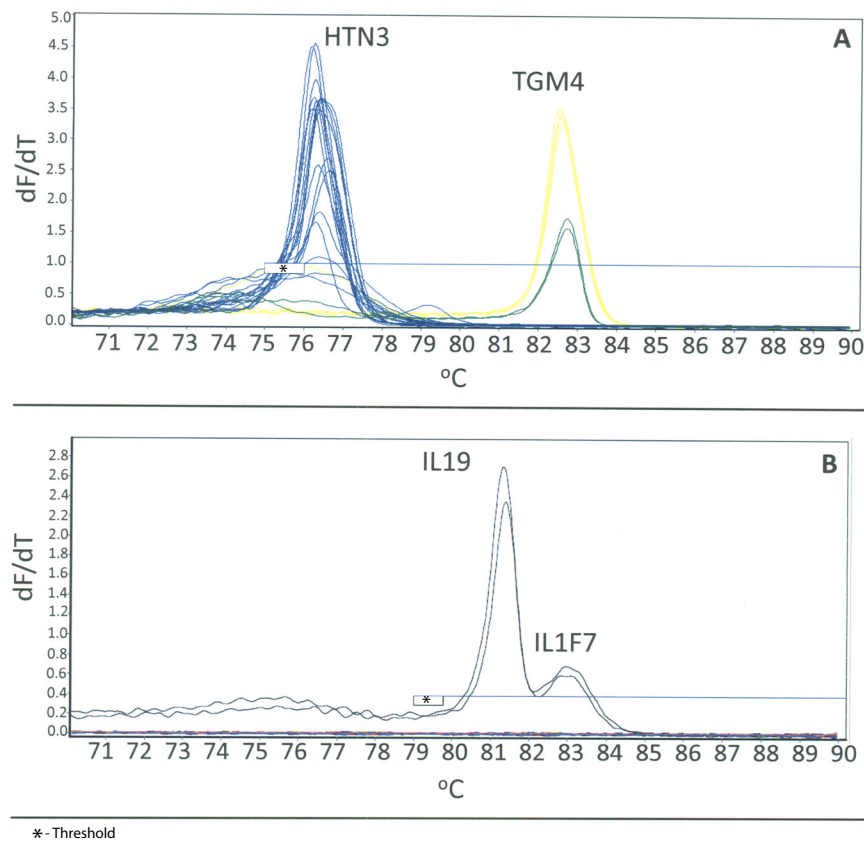
Figure 3. Performance of the saliva/semen and vaginal secretions/skin duplex HRM assays with mock casework samples. A) Saliva/Semen Assay. Detection of semen in two different vaginal samples is indicated by the presence of the TGM4 peak. A composite image of known semen and saliva samples (yellow and blue, respectively) and the two vaginal samples (green) in which semen was detected is shown. **B**) Vaginal Secretions/Skin Duplex Assay. Samples were collected from a male finger after digital penetration and from male underwear ~3 hours after intercourse. The detection of the presence of both vaginal secretions and skin is indicated by the presence of the IL19 and IL1F7 peaks in each sample.

The performance of the duplex assays with a limited number of simulated casework samples was evaluated. The mock casework samples were designed to represent possible casework scenarios including digital penetration and sexual assault (vaginal intercourse). The simulated casework samples included (i) a swab of the surface of male fingers after digital vaginal penetration of a female participant and (ii) a swab of the inside of male underwear worn 3 hours after sexual intercourse in order to attempt to detect possible vaginal secretions that might have been transferred from the penis after a sexual assault. An evaluation of potential transfer of vaginal secretions to male underwear worn after intercourse was selected to represent a sexual assault case rather than vaginal swabs since we had previously demonstrated the ability to detect semen in vaginal swabs

(described above). For both samples (male finger after digital penetration and the male underwear worn after intercourse) both vaginal secretions (IL19) and skin (IL1F7) were detected (Figure 3B). These results were consistent with the activity (i.e. behavior) of the participants prior to the collection of samples[56].

We evaluated the ability to detect blood and semen in two person body fluid mixtures as an example of a sample admixture type encountered in casework. We prepared blood-semen mixture samples using a constant amount of blood (50 µl) and decreasing amounts of semen (50 µl, 25 µl, 10 µl, and 5 µl). Each admixed sample was evaluated with the three duplex HRM assays (Figure 4). Blood (ALAS2) and semen (TGM4) were successfully detected in

∗ - Threshold
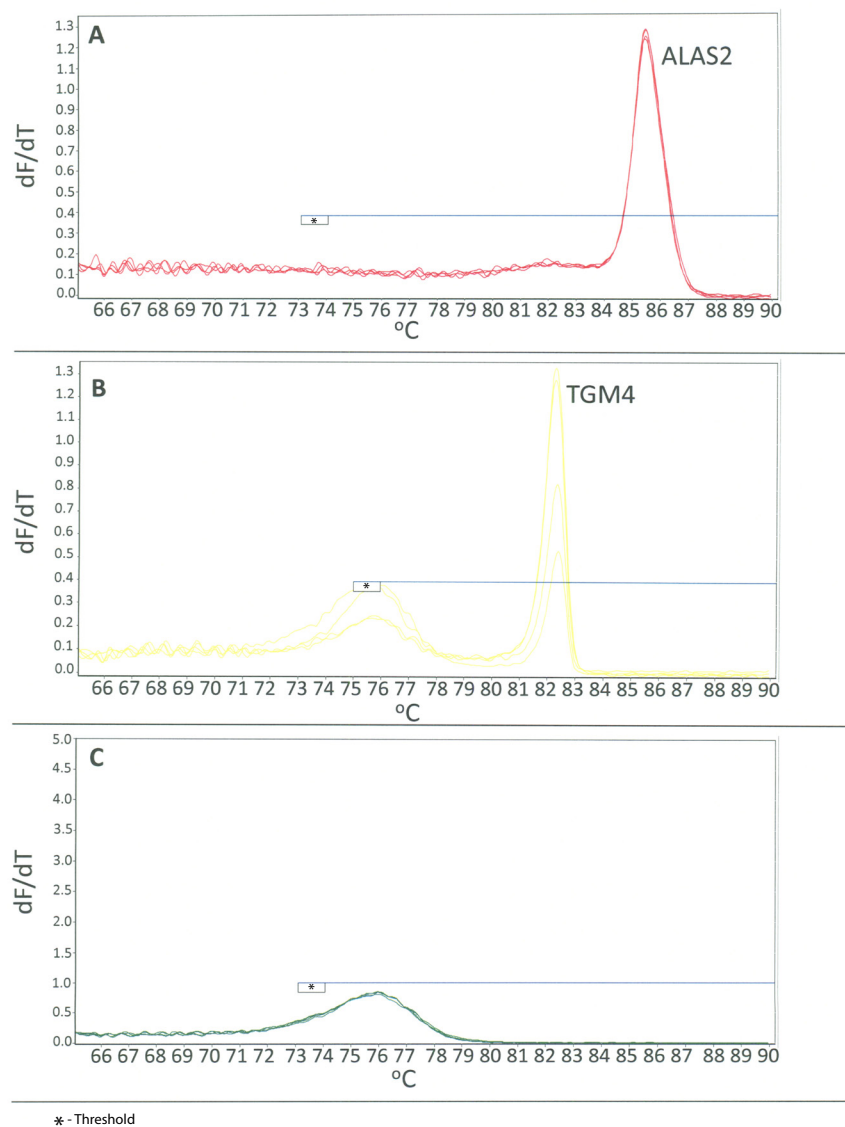
Figure 4. Mixtures. A two-body fluid mixture sample set containing different proportions of the two body fluids was created using a constant amount of blood (50 µl) and decreasing volumes of semen (50 µl, 25 µl, 10 µl, and 5 µl). The admixed samples were co-extracted and the isolated RNA (25 ng) was analyzed using each of the three duplex assays (A – blood/menstrual blood; B – saliva/semen; C – vaginal secretions/skin). Blood and semen were successfully detected, as indicated by the presence of ALAS2 (**A**) and TGM4 (**B**) in all four mixture samples.

all four admixed samples (Figure 4). The intensity of the TGM4 peaks varied among the four mixtures samples but did not seem to correlate with the known proportion of semen in the mixture. Thus further work using a more comprehensive sample set is needed to determine whether a quantitative assessment of peak heights correlate to the true marker proportions comprising a mixture.

Epithelial cell triplex HRM assay

Since epithelial cells have common functions, namely secretion, selective absorption, protection, trans-cellular transport and detection of sensation[57], finding biomarkers capable of unequivocally differentiating and identifying each of the three cell types commonly found in casework (buccal, vaginal and skin epithelia) is challenging. We have recently had success in the identification of highly specific RNA biomarkers for vaginal secretions and skin[28,29] that, in combination with well-characterized saliva markers such as HTN3 or STATH, are capable of differentiating epithelial cells.

Encouraged by the success in developing duplex HRM assays, we conceived and formulated a triplex assay for the identification of epithelial cell containing fluids and tissues (i.e. saliva, vaginal secretions and skin). The assay employs the HTN3 (saliva), IL19 (vaginal secretions) and skin (IL1F7) RNA biomarkers (Figure 5A). The melt curves from single source samples (2 donors for each fluid; for vaginal secretions, one of the two samples is a menstrual blood sample in which vaginal secretions was detected) are shown overlaid in order to demonstrate the non-overlapping location of each marker in the triplex assay. To initially test the performance of this triplex assay with forensic casework type samples, we swabbed the surface of a computer mouse and analyzed it using the triplex assay. The presence of skin was detected on the computer mouse sample as indicated by the presence of IL1F7 (Figure 5B). This preliminary indication that the sensitivity of the assay might permit the identification of touched objects augurs well for its potential use in casework.
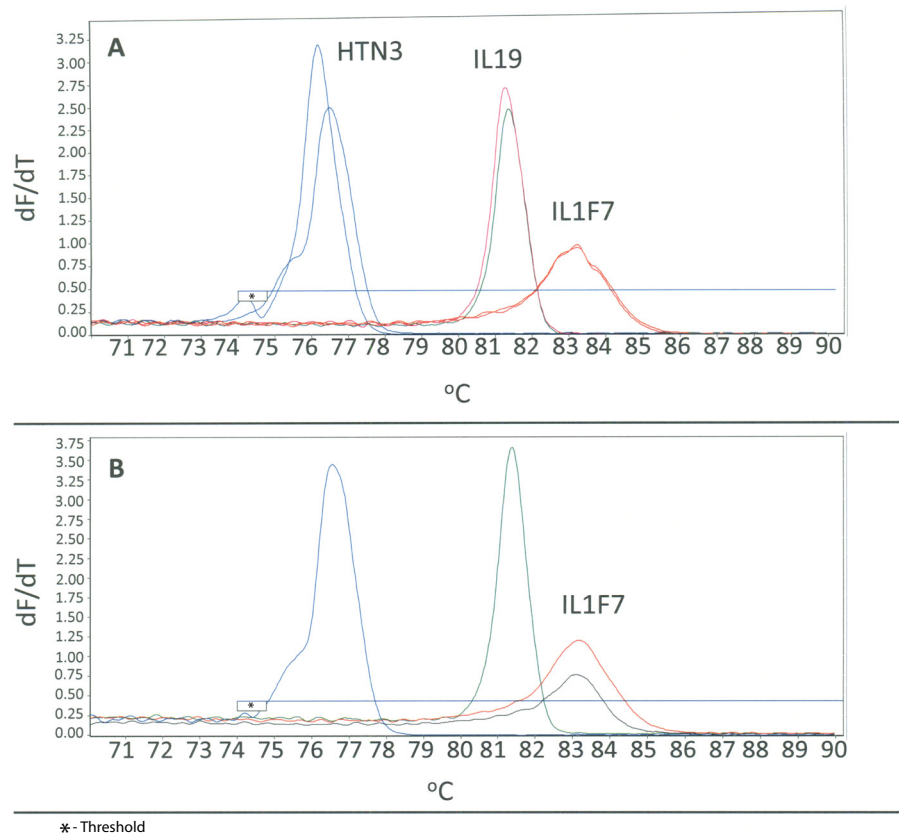
Figure 5. Epithelial cell triplex HRM assay. A Triplex HRM assay was created to permit identification of epithelial-cell containing tissues and fluids (saliva, vaginal secretions and skin). **A**) A body fluid specific marker for each of the three fluids/tissues was incorporated into a single reaction (HTN3 – saliva, IL19 – vaginal secretions, IL1F7 – skin). Each individual sample was analyzed using a single triplex reaction. Two saliva donors (blue), one vaginal secretions (green) and one menstrual blood donor (pink) and two skin donors (orange) are shown overlaid to show the relative locations of each marker in the triplex. **B**) Successful detection of skin from a swab of a computer mouse (black) using the triplex HRM assay is indicated by the presence of IL1F7. A composite image of known saliva, vaginal and skin samples (blue, green and orange, respectively) is shown as a reference.

'All body fluids' hexaplex HRM assay

The availability of a hexaplex assay that would permit a single tube identification of all of the common forensically relevant biological fluids and tissues (blood, semen, saliva, vaginal secretions, menstrual blood and skin) is desirable. The development of such an HRM assay is challenging, however, since the number of biomarkers, or sometimes the choice of biomarker, is dictated by the need to assure non-overlapping T_m values. Despite differences in amplicon size, the associated T_m values may be similar or the same depending on the particular amplicon sequences. For example, the inclusion of all biomarkers used in the singleplex, duplex and triplex assays described above into a single hexaplex system would not be possible. The T_m values of MMP10 overlap with IL19 (vaginal secretions), TGM4 (semen) and IL1F7 (skin). Nevertheless we sought to develop an 'all body fluids' hexaplex system using alternative highly specific biomarkers or primer modifications to intentionally alter the T_m value of the RNA amplicon.

The above described singleplex, duplex and triplex assays involved the use of the following markers: ALAS2 (blood), TGM4 (semen), HTN3 (saliva), IL19 (vaginal secretions), MMP10 (menstrual blood) and IL1F7 (skin). Our initial efforts were focused on attempting to include all of these biomarkers in the hexaplex system. However

attempts at suitably modifying the IL1F7 primers were unsuccessful and resulted in the requirement for an alternative biomarker for skin. We selected CCL27 as a replacement for IL1F7 based on its T_m value, sensitivity and specificity[28]. A CCL27 T_m value of 84.8°C no longer presented a conflict with MMP10 (81.9°C). However, this was close to the T_m value of ALAS2 (85.8°C). Sequence modification of the reverse primer for ALAS2 permitted a shift of the observed ALAS2 T_m value to 86.8°C and therefore resolved any potential overlap with CCL27. The use of an alternative reverse primer sequence for IL19 also resulted in a new observed average T_m value of 78.6°C and therefore resolved the conflict between MMP10 and IL19. The remaining T_m conflict was between MMP10 and TGM4. The use of a modified MMP10 forward primer sequence and the inclusion of a "GGGGG" non-template addition to the MMP10 primer set resulted in a sufficient separation of MMP10 and TGM4 T_m values (~1°C shift of the MMP10 T_m value). With these modifications a single T_m value for MMP10 was also observed. With the success of these loci and primer modifications, together with alterations to the PCR cycling parameters, we were able to develop a prototype hexaplex HRM system that was suitable for undergoing a series of preliminary developmental validation studies. The hexaplex system is shown in Figure 6A with single source samples (two donors per body fluid or tissue) overlaid to indicate the location of
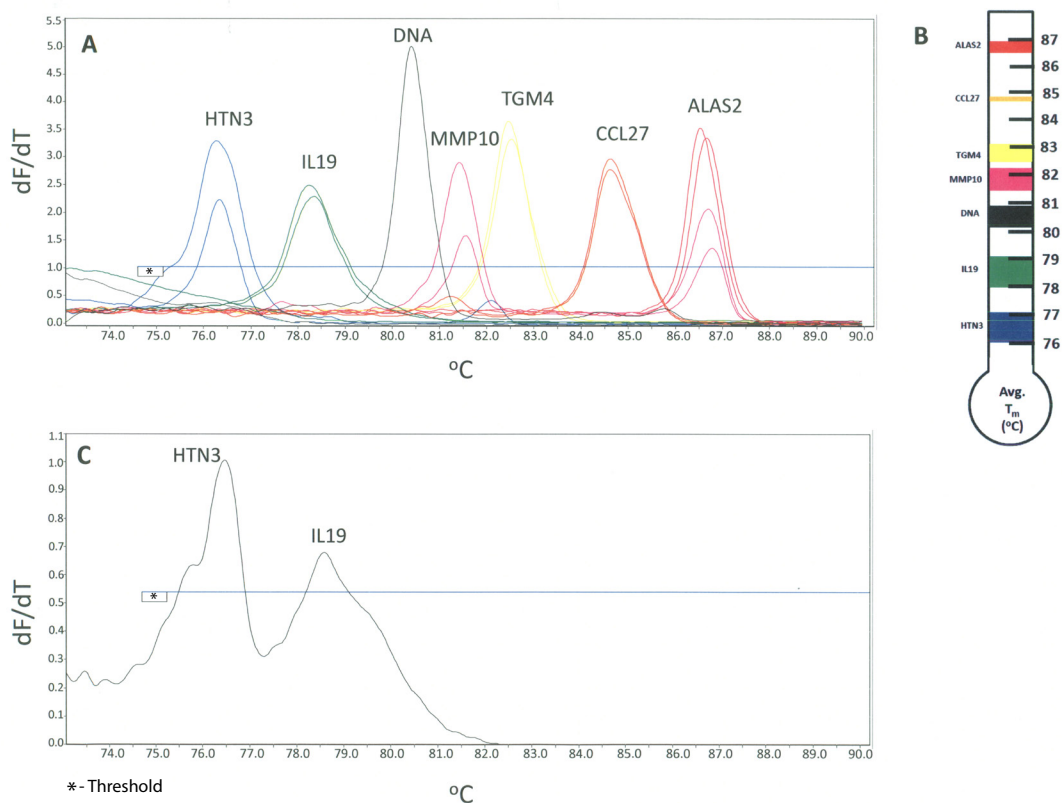
Figure 6. 'All body fluids' hexaplex HRM body fluid assay. A) Derivative melt curve plots for the hexaplex assay is shown: saliva (HTN3, blue), vaginal secretions (IL19, green), menstrual blood (MMP10, pink), semen (TGM4, yellow), skin (CCL27, orange), and blood (ALAS2, red). Two donors of each body fluid or tissue are shown. The melt plots shown are overlaid single source melt plots with all individual samples analyzed using the respective multiplex HRM assays (shown overlaid to demonstrate marker positions within the multiplex assay). The location of a peak originating from contaminating DNA (IL19) is shown in black. **B)** Hexaplex biomarker resolution as represented by the T_m (°C) range (average T_m + 3 standard deviations). **C)** Analysis of a saliva-vaginal admixture using the hexaplex. T_m values of 76.5°C (HTN3) and 78.6°C (IL19) were observed indicating the presence of saliva and vaginal secretions respectively.

each biomarker within the hexaplex. All of the incorporated markers are resolvable without any T_m overlap as indicated by measurement of the average T_m value and T_m value range (+ 3 standard deviations) (Table 3, Figure 6B). A DNA-specific product resulting from amplification with the new IL19 primer set was detected (Figure 6A, Table 3) but does not interfere with any of the included biomarkers (T_m of 80.6°C, between IL19 and MMP10 ranges). The presence of this DNA product can actually be useful in identifying samples that contain significant amounts of contaminating DNA and could therefore be used as an RNA sample quality control check for all sample types, irrespective of body fluid source. Interestingly, similar previously observed DNA products for MMP10 in the duplex assay were not apparent with the hexaplex system.

We carried out initial performance checks on the hexaplex's specificity. Single source body fluid and tissue samples (n total = 63; saliva, n = 13; vaginal secretions, n = 9; menstrual blood, n = 4; semen, n = 12; skin, n = 9; blood, n = 16) were tested. The correct biomarker was identified, except for a small number of false negative results with semen (one sample) and saliva (two samples) (Table 4). Similar to the results of specificity testing for the duplex assays, MMP10 was detected in some of the vaginal secretion samples (3/9, ~33%). Peripheral blood was detected in three of the four menstrual blood samples (Table 4), which is expected since menstrual blood samples are complex mixtures including peripheral blood and vaginal secretions. Forensic evidentiary items are frequently not single source samples and can contain admixed biological fluids. The results of hexaplex analysis of a saliva-vaginal secretion admixed sample (artificially created mixture in which 25 μl of neat saliva was added to half of a vaginal swab) are shown in Figure 6C. Both saliva (HTN3) and vaginal secretions (IL19) were correctly identified using the hexaplex system.

Table 4. 'All body fluids' hexaplex HRM assay specificity. The number of single source samples from different individuals in which the marker was successfully detected (numerator) is displayed out of the total number of individuals tested (denominator). The shading reflects the number of positive samples out of the total number tested: white – no detection, light grey 1–74%; dark grey ≥ 75%.

Body fluid/ Tissue (25 ng)	HTN3	IL19	MMP10	TGM4	CCL27	ALAS2
Saliva	11/13	0/13	0/13	0/13	0/13	0/13
Vaginal	0/9	9/9	3/9	0/9	0/9	0/9
Menstrual	0/4	0/4	4/4	0/4	0/4	3/4
Semen	0/12	0/12	0/12	11/12	0/12	0/12
Skin	0/9	0/9	0/9	0/9	9/9	0/9
Blood	0/16	0/16	0/16	0/16	0/16	16/16

N = 63

Data Sets Tm values for HRM assay specificity

4 Data Files

http://dx.doi.org/10.6084/m9.figshare.877018

Data Set 1. T_m Values for HRM duplex assay specificity.
This data set contains the individual T_m values for the specificity testing of the vaginal/skin, blood/menstrual and saliva/semen HRM duplex assay specificity presented in Table 2. Individual donors are listed according to body fluid or tissue type with the T_m values (°C) recorded under each biomarker. IL19 = vaginal biomarker, ALAS2 = blood, HTN3 = saliva biomarker, IL1F7 = skin biomarker, MMP10 = menstrual blood, TGM4 = semen biomarker, n.d. = not detected in body fluid of interest, empty cell = no detection.

Data Set 2. T_m Values for 'All Body Fluids' Hexaplex Assay Specificity.
This data set contains the individual T_m values for specificity testing of the 'all body fluids' hexaplex HRM assay presented in Table 4. Individual donors are listed according to body fluid or tissue type with the observed T_m values recorded under each biomarker. n.d. = not detected in body fluid of interest.

Discussion

Messenger RNA profiling with a battery of highly specific biomarkers can be used to positively identify all of the commonly found forensically relevant body fluids and tissues. In this work, in order to simplify mRNA profiling assays and to reduce the time and cost of analysis, we have developed a number of prototype multiplex high resolution melt (HRM) assays for the identification of blood, semen, saliva, vaginal secretions, menstrual blood and skin. With respect to critical post-cDNA reagents, the HRM assays require only the use of unlabeled PCR primers and a single intercalating saturating fluorescent dye (Eva Green). In terms of hardware and software a real time instrument with HRM capabilities is required and a number of manufacturers make such instruments. Each body-fluid specific marker can easily be identified by the presence of a distinct melt peak.

The ability to multiplex different combinations of RNA biomarkers as evidenced by the compatibility of the markers in duplex, triplex and hexaplex assays indicates that there exists an opportunity for laboratories to customize HRM assays to suit their specific needs. For example, if the intended use of the HRM assays is to rapidly screen sexual assault evidence, it may be desirable to only incorporate biomarkers for the identification of semen and vaginal secretions or, alternatively, semen, vaginal secretions and saliva. Some may want an epithelial marker assay such as the triplex that can identify and differentiate saliva, vaginal secretions and skin. However, some laboratories may want to utilize HRM assays for the identification of all forensically relevant body fluids.

We recognize that the prototype HRM assays may need to be further optimized (using modified primers, cycling conditions or loci) using a larger and more varied sample set. For example, the semen/saliva duplex assay should be further optimized in order to reduce or eliminate artifacts such as the broad peaks/humps observed in the HTN3 region arising from the TGM4 melt curve. If these artifacts cannot be eliminated it may be necessary to replace TGM4 with another suitable semen marker with a melt curve profile that does not interfere with the interpretation of HTN3 data or to replace HTN3 with another saliva marker that has a higher T_m. It may also be possible to alter the T_m of the existing markers in the assay by changing the primer location. However, any changes to primer sequences would

require additional studies to evaluate primer efficiency and to ensure that no changes to sensitivity or specificity are observed.

The principal advantages of HRM over current mRNA body fluid identification assays that employ capillary electrophoresis (CE) or quantitative RT-PCR (qRT-PCR) platforms are the not insignificant ones of cost and time. Both CE and qRT-PCR platforms require the use of expensive fluorescently labeled primers or probes whereas HRM uses unlabeled primers. CE-based assays require separate amplification and detection steps thus increasing the cost and time required for analysis. Post RNA extraction and cDNA formation, the closed tube HRM assay takes ~ 2 hours to perform which is similar to qRT-PCR assays. However for qRT-PCR assays, only 2 or 3 markers can be included in a single reaction since each marker and internal control requires a different fluorescent dye whereas HRM can multiplex at least 6 markers.

In summary, this proof of principle work describes the design and testing of a number of mRNA HRM assays that, after further validation and optimization, might prove useful in an operational casework setting. The principal advantages of HRM in terms of timeliness and cost may facilitate the technology transfer of mRNA profiling methodology into forensic casework.

Consent

Body fluids were collected from volunteers using procedures approved by the University's Institutional Review Board. Informed written consent was obtained from each donor.

Author contributions
Author Jack Ballantyne conceived the study, designed the experiments, reviewed and analyzed results. Author Erin Hanson assisted with the design of experiments, performed the laboratory work, and reviewed and analyzed results. Both authors prepared and edited the manuscript.

Supplementary material
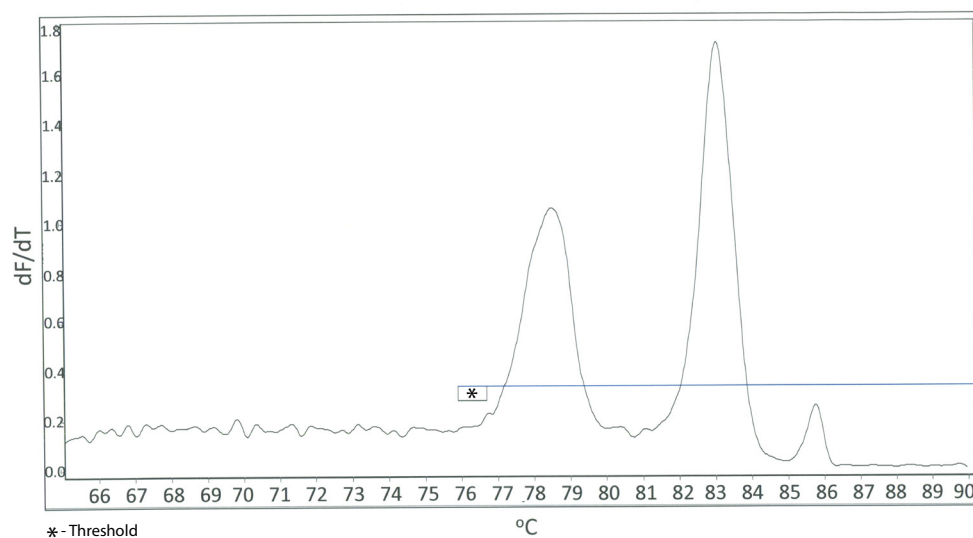
Figure S1. DNA products observed for MMP10 Singleplex HRM Assay. High resolution derivative melt curve plot for a DNA sample for the MMP10 singleplex HRM assay. The presence of two products (78.5 and 83.1°C) was observed. The x-axis represents temperature (°C) and the y-axis represents the first derivative of the change of fluorescence with temperature (-dF/dT). The horizontal line on each plot represents the analysis threshold.
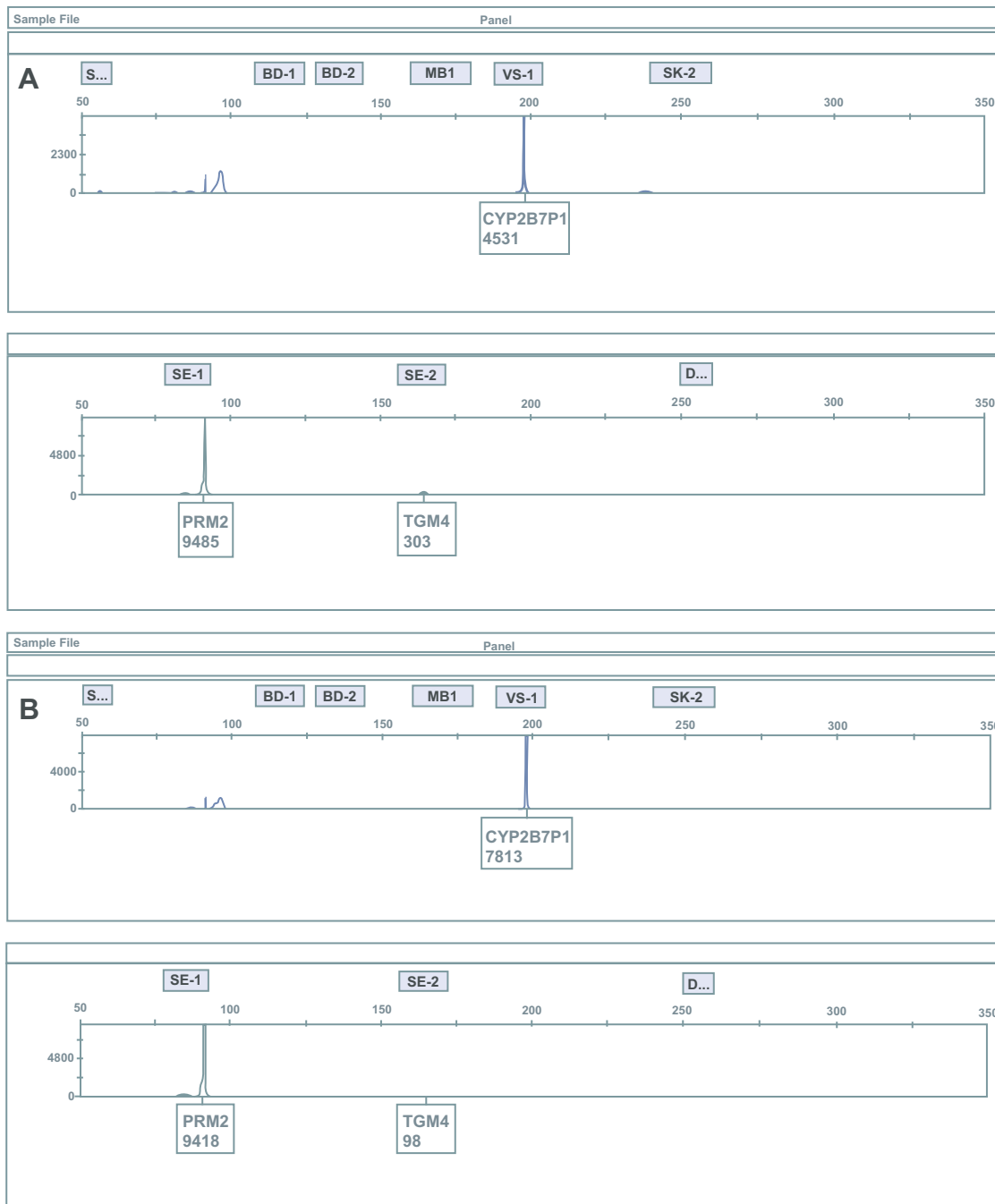
Figure S2. Detection of semen in reportedly single source vaginal samples using a capillary electrophoresis multiple mRNA profiling assay. RT-PCR products from RNA extracted from previously reported single source vaginal secretions samples were amplified in a multiplex reaction using a mRNA profiling assay for body fluid/tissue identification. The multiplex contains mRNA biomarkers for blood, semen, saliva, vaginal secretions, menstrual blood and skin. Shown here are two vaginal samples (**A** and **B**) in which semen was detected as indicated by the presence of the PRM2 and TGM4 (semen-specific biomarkers). This data is consistent with the detection of semen using the semen-saliva duplex HRM assay. The presence of vaginal secretions is indicated by the presence of CYP2B7P1. The x-axis indicates size in base pairs and the y-axis indicates relative fluorescence units (note: the y-axis scales are different for some panels due to varying signal intensities of the observed products). The grey areas represent the bins for biomarker identification. The biomarker name and relative fluorescence units are displayed.

References

1. Simons JL, Vintiner SK: **Efficacy of several candidate protein biomarkers in the differentiation of vaginal from buccal epithelial cells.** *J Forensic Sci.* 2012; **57**(6): 1585–1590.
   PubMed Abstract | **Publisher Full Text**

2. Van SK, De CM, Dhaenens M, *et al.*: **Mass spectrometry-based proteomics as a tool to identify biological matrices in forensic science.** *Int J Legal Med.* 2013; **127**(2): 287–298.
   PubMed Abstract | **Publisher Full Text** | **Free Full Text**

3. Yang H, Zhou B, Prinz M, *et al.*: **Proteomic analysis of menstrual blood.** *Mol Cell Proteomics.* 2012; **11**(10): 1024–1035.
   PubMed Abstract | **Publisher Full Text** | **Free Full Text**

4. An JH, Choi A, Shin KJ, *et al.*: **DNA methylation-specific multiplex assays for body fluid identification.** *Int J Legal Med.* 2013; **127**(1): 35–43.
   PubMed Abstract | **Publisher Full Text**

5. Choi A, Shin KJ, Yang WI, *et al.*: **Body fluid identification by integrated analysis of DNA methylation and body fluid-specific microbial DNA.** *Int J Legal Med.* 2013.
   Publisher Full Text

6. Frumkin D, Wasserstrom A, Budowle B, *et al.*: **DNA methylation-based forensic tissue identification.** *Forensic Sci Int Genet.* 2011; **5**(5): 517–524.
   PubMed Abstract | **Publisher Full Text**

7. LaRue BL, King JL, Budowle B: **A validation study of the Nucleix DSI-Semen kit--a methylation-based assay for semen identification.** *Int J Legal Med.* 2013; **127**(2): 299–308.
   PubMed Abstract | **Publisher Full Text**

8. Lee HY, Park MJ, Choi A, *et al.*: **Potential forensic application of DNA methylation profiling to body fluid identification.** *Int J Legal Med.* 2012; **126**(1): 55–62.
   PubMed Abstract | **Publisher Full Text**

9. Madi T, Balamurugan K, Bombardi R, *et al.*: **The determination of tissue-specific DNA methylation patterns in forensic biofluids using bisulfite modification and pyrosequencing.** *Electrophoresis.* 2012; **33**(12): 1736–1745.
   PubMed Abstract | **Publisher Full Text**

10. Wasserstrom A, Frumkin D, Davidson A, *et al.*: **Demonstration of DSI-semen--A novel DNA methylation-based forensic semen identification assay.** *Forensic Sci Int Genet.* 2013; **7**(1): 136–142.
    PubMed Abstract | **Publisher Full Text**

11. Courts C, Madea B: **Specific micro-RNA signatures for the detection of saliva and blood in forensic body-fluid identification.** *J Forensic Sci.* 2011; **56**(6): 1464–1470.
    PubMed Abstract | **Publisher Full Text**

12. Hanson E, Lubenow H, Ballantyne J: **Identification of forensically relevant body fluids using a panel of differentially expressed microRNAs.** *Forensic Sci Int Genet.* 2009; **2**(1): 503–504.
    Publisher Full Text

13. Hanson E, Ballantyne J: **RNA Profiling for the Identification of the Tissue Origin of Dried Stains in Forenic Biology.** *Forensic Sci Rev.* 2010; **22**(2): 145–157.
    Publisher Full Text

14. Hanson EK, Lubenow H, Ballantyne J: **Identification of Forensically Relevant Body Fluids Using a Panel of Differentially Expressed microRNAs.** *Anal Biochem.* 2009; **387**(2): 303–314.
    PubMed Abstract | **Publisher Full Text**

15. Wang Z, Luo H, Pan X, *et al.*: **A model for data analysis of microRNA expression in forensic body fluid identification.** *Forensic Sci Int Genet.* 2012; **6**(3): 419–423.
    PubMed Abstract | **Publisher Full Text**

16. Wang Z, Zhang J, Luo H, *et al.*: **Screening and confirmation of microRNA markers for forensic body fluid identification.** *Forensic Sci Int Genet.* 2013; **7**(1): 116–123.
    PubMed Abstract | **Publisher Full Text**

17. Zubakov D, Boersma AW, Choi Y, *et al.*: **MicroRNA markers for forensic body fluid identification obtained from microarray screening and quantitative RT-PCR confirmation.** *Int J Legal Med.* 2010; **124**(3): 217–226.
    PubMed Abstract | **Publisher Full Text** | **Free Full Text**

18. Bauer M, Patzelt D: **Evaluation of mRNA markers for the identification of menstrual blood.** *J Forensic Sci.* 2002; **47**(6): 1278–1282.
    PubMed Abstract

19. Bauer M, Patzelt D: **Protamine mRNA as molecular marker for spermatozoa in semen stains.** *Int J Legal Med.* 2003; **117**(3): 175–179.
    PubMed Abstract | **Publisher Full Text**

20. Haas C, Klesser B, Maake C, *et al.*: **mRNA profiling for body fluid identification by reverse transcription endpoint PCR and realtime PCR.** *Forensic Sci Int Genet.* 2009; **3**(2): 80–88.
    PubMed Abstract | **Publisher Full Text**

21. Haas C, Hanson E, Kratzer A, *et al.*: **Selection of highly specific and sensitive mRNA biomarkers for the identification of blood.** *Forensic Sci Int Genet.* 2011; **5**(5): 449–458.
    PubMed Abstract | **Publisher Full Text**

22. Haas C, Hanson E, Bar W, *et al.*: **mRNA profiling for the identification of blood--results of a collaborative EDNAP exercise.** *Forensic Sci Int Genet.* 2011; **5**(1): 21–26.
    PubMed Abstract | **Publisher Full Text**

23. Haas C, Hanson E, Kratzer A, *et al.*: **Selection of highly specific and sensitive mRNA biomarkers for the identification of blood.** *Forensic Sci. Int Genet.* 2011; **5**(5): 449–458.
    PubMed Abstract | **Publisher Full Text**

24. Haas C, Hanson E, Ballantyne J: **Capillary electrophoresis of a multiplex reverse transcription-polymerase chain reaction to target messenger RNA markers for body fluid identification.** *Methods Mol Biol.* 2012; **830**: 169–183.
    PubMed Abstract | **Publisher Full Text**

25. Haas C, Hanson E, Anjos MJ, *et al.*: **RNA/DNA co-analysis from blood stains--results of a second collaborative EDNAP exercise.** *Forensic Sci Int Genet.* 2012; **6**(1): 70–80.
    PubMed Abstract | **Publisher Full Text**

26. Haas C, Hanson E, Anjos MJ, *et al.*: **RNA/DNA co-analysis from human saliva and semen stains--results of a third collaborative EDNAP exercise.** *Forensic Sci Int Genet.* 2013; **7**(2): 230–239.
    PubMed Abstract | **Publisher Full Text**

27. Hanson E, Haas C, Jucker R, *et al.*: **Identification of skin in touch/contact forensic samples by messenger RNA profiling.** *Forensic Sci Int Genet.* 2011; **3**(1): e305–e306.
    Publisher Full Text

28. Hanson E, Haas C, Jucker R, *et al.*: **Specific and sensitive mRNA biomarkers for the identification of skin in 'touch DNA' evidence.** *Forensic Sci Int Genet.* 2012; **6**(5): 548–558.
    PubMed Abstract | **Publisher Full Text**

29. Hanson EK, Ballantyne J: **Highly specific mRNA biomarkers for the identification of vaginal secretions in sexual assault investigations.** *Sci Justice.* 2013; **53**(1): 14–22.
    PubMed Abstract | **Publisher Full Text**

30. Juusola J, Ballantyne J: **Messenger RNA profiling: a prototype method to supplant conventional methods for body fluid identification.** *Forensic Sci Int.* 2003; **135**(2): 85–96.
    PubMed Abstract | **Publisher Full Text**

31. Juusola J, Ballantyne J: **Multiplex mRNA profiling for the identification of body fluids.** *Forensic Sci Int.* 2005; **152**(1): 1–12.
    PubMed Abstract | **Publisher Full Text**

32. Juusola J, Ballantyne J: **mRNA profiling for body fluid identification by multiplex quantitative RT-PCR.** *J Forensic Sci.* 2007; **52**(6): 1252–1262.
    PubMed Abstract | **Publisher Full Text**

33. Nussbaumer C, Gharehbaghi-Schnell E, Korschineck I: **Messenger RNA profiling: a novel method for body fluid identification by real-time PCR.** *Forensic Sci Int.* 2006; **157**(2–3): 181–186.
    PubMed Abstract | **Publisher Full Text**

34. Richard ML, Harper KA, Craig RL, *et al.*: **Evaluation of mRNA marker specificity for the identification of five human body fluids by capillary electrophoresis.** *Forensic Sci Int Genet.* 2012; **6**(4): 452–460.
    PubMed Abstract | **Publisher Full Text**

35. Roeder AD, Haas C: **mRNA profiling using a minimum of five mRNA**

markers per body fluid and a novel scoring method for body fluid identification. *Int J Legal Med.* 2013; **127**(4): 707–721.
PubMed Abstract | **Publisher Full Text**

36. Visser M, Zubakov D, Ballantyne KN, *et al.*: **mRNA-based skin identification for forensic applications.** *Int J Legal Med.* 2011; **125**(2): 253–263.
PubMed Abstract | **Publisher Full Text** | **Free Full Text**

37. Kohlmeier F, Schneider PM: **Successful mRNA profiling of 23 years old blood stains.** *Forensic Sci Int Genet.* 2012; **6**(2): 274–276.
PubMed Abstract | **Publisher Full Text**

38. Setzer M, Juusola J, Ballantyne J: **Recovery and stability of RNA in vaginal swabs and blood, semen, and saliva stains.** *J Forensic Sci.* 2008; **53**(2): 296–305.
PubMed Abstract | **Publisher Full Text**

39. Zubakov D, Hanekamp E, Kokshoorn M, *et al.*: **Stable RNA markers for identification of blood and saliva stains revealed from whole genome expression analysis of time-wise degraded samples.** *Int J Legal Med.* 2008; **122**(2): 135–142.
PubMed Abstract | **Publisher Full Text** | **Free Full Text**

40. Zubakov D, Kokshoorn M, Kloosterman A, *et al.*: **New markers for old stains: stable mRNA markers for blood and saliva identification from up to 16-year-old stains.** *Int J Legal Med.* 2009; **123**(1): 71–74.
PubMed Abstract | **Publisher Full Text**

41. Intemann C, Thye T, Sieversten J, *et al.*: **Genotyping of IRGM tetranucleotide promoter oligorepeats by fluorescence resonance energy transfer.** *Biotechniques.* 2009; **46**(1): 58–60.
PubMed Abstract

42. Nguyen-Dumont T, Calvez-Kelm FL, Forey N, *et al.*: **Description and validation of high-throughput simultaneous genotyping and mutation scanning by high-resolution melting curve analysis.** *Hum Mutat.* 2009; **30**(6): 884–890.
PubMed Abstract | **Publisher Full Text** | **Free Full Text**

43. Reed GH, Wittwer CT: **Sensitivity and specificity of single-nucleotide polymorphism scanning by high-resolution melting analysis.** *Clin Chem.* 2004; **50**(10): 1748–1754.
PubMed Abstract | **Publisher Full Text**

44. Zhou L, Errigo RJ, Lu H, *et al.*: **Snapback primer genotyping with saturating DNA dye and melting analysis.** *Clin Chem.* 2008; **54**(10): 1648–1656.
PubMed Abstract | **Publisher Full Text**

45. Maat W, van der Velden PA, Out-Luiting C, *et al.*: **Epigenetic inactivation of RASSF1a in uveal melanoma.** *Invest Ophthalmol Vis Sci.* 2007; **48**(2): 486–490.
PubMed Abstract | **Publisher Full Text**

46. Worm J, Aggerholm A, Guldberg P: **In-tube DNA methylation profiling

47. Aten E, White SJ, Kalf ME, *et al.*: **Methods to detect CNVs in the human genome.** *Cytogenet Genome Res.* 2008; **123**(1–4): 313–321.
PubMed Abstract | **Publisher Full Text**

48. Bruder CE, Piotrowski A, Gijsbers AA, *et al.*: **Phenotypically concordant and discordant monozygotic twins display different DNA copy-number-variation profiles.** *Am J Hum Genet.* 2008; **82**(3): 763–771.
PubMed Abstract | **Publisher Full Text** | **Free Full Text**

49. Dobrowolski SF, Hendrickx AT, van den Bosch, *et al.*: **Identifying sequence variants in the human mitochondrial genome using high-resolution melt (HRM) profiling.** *Hum Mutat.* 2009; **30**(6): 891–898.
PubMed Abstract | **Publisher Full Text**

50. Pepers BA, Schut MH, Vossen RH, *et al.*: **Cost-effective HRMA pre-sequence typing of clone libraries; application to phage display selection.** *BMC Biotechnol.* 2009; **9**(50): 1–6.
PubMed Abstract | **Publisher Full Text** | **Free Full Text**

51. Vossen RH, Aten E, Roos A, *et al.*: **High-resolution melting analysis (HRMA): more than just sequence variant screening.** *Hum Mutat.* 2009; **30**(6): 860–866.
PubMed Abstract | **Publisher Full Text**

52. Seipp MT, Durtschi JD, Voelkerding KV, *et al.*: **Multiplex amplicon genotyping by high-resolution melting.** *J Biomol Tech.* 2009; **20**(3): 160–164.
PubMed Abstract | **Free Full Text**

53. Fleming RI, Harbison S: **The development of a mRNA multiplex RT-PCR assay for the definitive identification of body fluids.** *Forensic Sci Int Genet.* 2010; **4**(4): 244–256.
PubMed Abstract | **Publisher Full Text**

54. Dong JC, Dong H, Campana A, *et al.*: **Matrix metalloproteinases and their specific tissue inhibitors in menstruation.** *Reproduction.* 2002; **123**(5): 621–631.
PubMed Abstract | **Publisher Full Text**

55. Salamonsen LA, Woolley DE: **Matrix metalloproteinases in normal menstruation.** *Hum Reprod.* 1996; **11**(Suppl 2): 124–133.
PubMed Abstract

56. Cook R, Evett I, Jackson G, *et al.*: **A hierarchy of propositions: deciding which level to address in casework.** *Science & Justice.* 1998; **38**(4): 231–239.
Publisher Full Text

57. Alberts B, Bray D, Lewis J, *et al.*: **Molecular Biology of the Cell, 2nd, Garland Publishing, New York, NY.** 1994.
Reference Source

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Version 2

Referee Report 28 July 2014

doi:10.5256/f1000research.3928.r3892

☑ **Gustavo Gutierrez Gonzalez**, **Chiranjeevi Bodda**
Vrije Universiteit Brussel, Brussels, Belgium

The authors have satisfactorily addressed all concerns/comments we have initially raised.

We have read this submission. We believe that we have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Competing Interests: No competing interests were disclosed.

Referee Report 19 March 2014

doi:10.5256/f1000research.3928.r4195

☑ **Titia Sijen**
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I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Competing Interests: No competing interests were disclosed.

Version 1

Referee Report 19 February 2014

doi:10.5256/f1000research.3223.r3637

☑ **Titia Sijen**
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Hanson and Ballantyne describe the development of HRM-based single- and multiplex assays for the identification of forensically relevant cell types. These assays are potentially a rapid and relatively inexpensive alternative for mRNA profiling methodologies such as qRT-PCR assays and CE analysis of

fluorescently labeled RT-PCR products.

The research is scientifically sound and follows a well-designed stepwise approach. The study is focused at assay development and specificity testing. Known markers were chosen (some more studied such as HTN3, ALAS2, MMP10, TGM4 than others eg IL19, CCL27, IL1F7) that show good specificity with the body fluids tested.

The rationale, results and interpretation of the data are well-described although some figures could be improved (if possible) by using different colors: the use of pink and red to discriminate MMP10 and ALAS2 in Fig2A is somewhat challenging to the eye, and when yellow is used (Fig1D, 2B, 3A, 4B) I found some details quite hard to discern.

The authors state 'the results demonstrate the potential use of HRM assays for the screening of biological evidence' and 'we describe the development and initial performance evaluation of the developed HRM assays'. This raises the question what would be needed before HRM assays would be suited for casework?

In our laboratory, numerous cases have been analyzed using end-point RT-PCR multiplexes on both cell and organ typing detected by CE. These cases have taught us that most/many forensic casework samples will be 1) mixtures of various cell types and 2) of limited stain size. Thus, besides good specificity, sensitivity and the ability to handle (unequal) mixtures are important performance characteristics for RNA assays. The capacity to analyze unequal mixtures will depend on the analysis method. When using CE platforms for instance, high mixture components may result in pull-up signals and artifact peaks that can mask the presence of the minor components or complicate RNA profile interpretation.

Thus, I would like to invite the authors to comment on the expected performance of HRM assays when analyzing unequal mixtures.

Furthermore, as this initial study did not yet assess sensitivity, I am interested in the authors' thoughts on the sensitivity that they perceive for HRM-based RNA assays.

Overall, I compliment the authors with this study and the novelty they bring to the forensic field.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Competing Interests: No competing interests were disclosed.

Author Response 24 Feb 2014
Jack Ballantyne, University of Central Florida, USA

- **"The rationale, results and interpretation of the data are well-described although some figures could be improved (if possible) by using different colors: the use of pink and red to discriminate MMP10 and ALAS2 in Fig2A is somewhat challenging to the eye, and when yellow is used (Fig1D, 2B, 3A, 4B) I found some details quite hard to discern."**
The colors are used to aid visually distinguishing each body fluid. There are always variations between computers and printers, and therefore the original color selected in the instrument

software is not always as clearly represented. We also labeled each biomarker so that its location would be clearly indicated on each diagram. We will keep color selection in mind for future publications.

- **"The authors state 'the results demonstrate the potential use of HRM assays for the screening of biological evidence' and 'we describe the development and initial performance evaluation of the developed HRM assays'. This raises the question what would be needed before HRM assays would be suited for casework?"**

A full developmental validation in accordance with SWGDAM guidelines would be required. This would include a more comprehensive evaluation of sensitivity, specificity, reproducibility and performance with mixtures and mock casework samples.

In our laboratory, numerous cases have been analyzed using end-point RT-PCR multiplexes on both cell and organ typing detected by CE. These cases have taught us that most/many forensic casework samples will be 1) mixtures of various cell types and 2) of limited stain size. Thus, besides good specificity, sensitivity and the ability to handle (unequal) mixtures are important performance characteristics for RNA assays. The capacity to analyze unequal mixtures will depend on the analysis method. When using CE platforms for instance, high mixture components may result in pull-up signals and artifact peaks that can mask the presence of the minor components or complicate RNA profile interpretation.

- **"Thus, I would like to invite the authors to comment on the expected performance of HRM assays when analyzing unequal mixtures**."

A blood-semen mixture, with decreasing amounts of semen in each mixture, was evaluated using the duplex assays. Both body fluids were correctly identified in each of these mixtures. Successful detection of both components of a saliva-vaginal admixed sample was also observed for the hexaplex assay. While these were not large-scale comprehensive mixture studies, we are hopeful that the HRM assays will permit the ability to identify all components in admixed body fluid samples. Similar to the experience of the referee with CE based assays, It is likely that some mixture types will be challenging to define because of the particular mixture composition or because one or more of the components are present as a minor fraction. In our experience with CE as well, highly abundant biomarkers tend to dominate detection and can mask the presence of a minor component. It will be interesting to see with future studies whether the use of the HRM assays will permit an improved detection of minor component fluids or tissues. So while more comprehensive mixtures studies are needed (and will be included as part of a full developmental validation), we are hopeful that the successful initial results presage the efficacy of HRM assays to perform reasonably well with mixtures. [See also the response to the first referee report].

- **"Furthermore, as this initial study did not yet assess sensitivity, I am interested in the authors' thoughts on the sensitivity that they perceive for HRM-based RNA assays."**

We performed initial sensitivity studies for each of the HRM duplex assays. The sensitivity of each of the duplex assays was evaluated by testing a range of input total RNA, from 15pg to 100ng (15pg, 25pg, 50pg, 100pg, 250pg, 500pg. 1mg, 5ng, 10ng, 25ng, 50ng and 100ng). For the blood/menstrual blood duplex assay, both biomarkers (ALAS2 and MMP10) were detected using picogram quantities of input total RNA (consistently detected using 250 pg or more; variable below 250pg (positive results for both biomarkers with 15 and 50pg input, but not 25 and 100pg)). For the saliva/semen assay, both biomarkers (HTN3 and TGM4) were detected using as little as 25pg input. Using 15pg input, HTN3 was also detected but not TGM4. Therefore the sensitivities of

these two duplex assays appear to be highly sensitive, requiring only picogram levels of input total RNA. Additionally, it should be noted that the total RNA input amounts listed represent the amount used in the RT reaction. Detection of product in the real time assays is performed with 1/10th of the RT input amount (2ml cDNA/20ml reaction). For the vaginal secretions/skin duplex assay, 5ng of input RNA (still only 500pg for detection) was required for IL19. Skin was successfully detected with as little as 100pg total RNA RT input. Since only one donor was tested for this initial sensitivity work, the actual sensitivity limits need to be determined with additional testing. For vaginal secretions, since no human specific RNA quantitation method is currently available, there is always a potential for the obtained RNA quantitation value to be artificially higher due to the presence of endogenous bacteria. Therefore, the total RNA input used for these samples may not be fully accurate which will then affect the sensitivity results. But overall, these assays appear to require picogram levels of input total RNA, which would make them suitable for use with forensic, samples.

An initial sensitivity check for the hexaplex assay was also performed (again only one donor per fluid; 5 pg to 25 ng range of total RNA input into the RT reaction). The body fluids were detected down to varying lower input limits: blood (ALAS2) - 25 pg; menstrual blood (MMP10) - 250 pg; skin (CCL27) - 1ng; semen - 5ng; vaginal secretions – consistently with 1 ng or more input (variable detection in the 5pg to 1ng range); saliva (HTN3) – not determined at this time.

While we performed this preliminary sensitivity evaluation, it was not included in the manuscript since only one donor was evaluated. More comprehensive sensitivity studies, particularly for the hexaplex system, will be included in a future publication describing a comprehensive testing and evaluation of the HRM assay.

Competing Interests: No competing interests were disclosed.

Referee Report 09 January 2014

Gustavo Gutierrez Gonzalez, **Chiranjeevi Bodda**
Vrije Universiteit Brussel, Brussels, Belgium

Hanson & Ballantyne report in this article a novel use for High Resolution Melt analysis of RNA samples in order to identify single or mixed human body fluids (and tissues), namely vaginal secretions, skin, blood, menstrual blood, saliva, or semen using a series of molecular biomarkers: IL19, IL1F7 or CCL27, ALAS2, MMP10, HTN3, and TGM4, respectively. Identification of these body fluids/tissues can be of crucial importance in forensic sciences and criminal cases. The methodology appears to be quite specific and versatile, and according to the authors, less expensive and time-consuming compared to similar mRNA-based platforms such as quantitative real-time polymerase chain reaction or capillary electrophoresis. Remarkably, the technique can allow the identification of up to 6 body fluids in one single reaction (i.e., hexaplex assay).

The paper is very well written, scientifically sound and their results are very clear and adequately interpreted and discussed. I support its approval in the journal F1000 Research. I also have several comments to be eventually addressed:

1. Would there be a way to differentiate between blood and menstrual blood samples? Would that be something important to achieve?

2. Have the authors used (or planned to use) this technique with samples such as sweat, urine, or hair? Additionally, could the technique differentiate human from animal samples (using the same biomarkers)?

3. Can the authors give a little bit more information about the size of the amplicons generated by PCR for the different biomarkers employed and also about the design of the primers employed?

4. How reproducible is the assay for one sample coming from an individual?

5. We noticed that when you compare tables 2 and 4, there is a significant improvement of the specificity using the IL19 marker in the hexaplex assay. As mentioned by the authors, they have used a different reverse primer for IL19 in the hexaplex assay. We wonder whether this new reverse primer can also improve the specificity for IL19 to differentiate vaginal secretions from menstrual blood also in single, duplex, or triplex analyses.

6. Regarding the possible amplification of MMP3 by MMP10 primers in the menstrual blood samples under certain experimental conditions. I think it could be important to test that this is indeed the case; maybe by designing MMP3 specific primers that confirm this particular peak or eventually sequencing the products that were generated.

7. Finally, I got the impression from the experiments in which the authors mixed blood with decreasing amounts of semen that the technique does not necessarily reflect quantitative trends. Is that indeed the case? Could quantitation be ultimately important in a forensic case?

We have read this submission. We believe that we have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Competing Interests: No competing interests were disclosed.

Author Response 29 Jan 2014

Jack Ballantyne, University of Central Florida, USA

- **Would there be a way to differentiate between blood and menstrual blood samples? Would that be something important to achieve?**

The described hexaplex assay has a biomarker for both blood and menstrual blood. Therefore they will be distinguished using this assay. However, menstrual blood will also contain varying amounts of peripheral blood and therefore both the blood (ALAS2) and menstrual blood (MMP10) biomarkers may be present in menstrual blood samples. If the sample is strictly peripheral blood, only the ALAS2 (blood) biomarker would be present and the menstrual blood (MMP10) marker would be absent. This can be important in some cases where intimate contact is implied or suggested based on the circumstances of the case and a suspect argues that it is blood from the nose of the victim, for example, during an assault but not a sexual assault. The identification of menstrual blood as the source would indicate more intimate contact. Additionally during sexual assault investigations, peripheral blood could indicate that trauma had taken place to the female

reproductive system, and therefore a determination that the blood present is not simply menstrual blood from normal menstruation could be probative.

- **Have the authors used (or planned to use) this technique with samples such as sweat, urine, or hair? Additionally, could the technique differentiate human from animal samples (using the same biomarkers)?**

We have not yet used this technique with other types of samples such as sweat, urine or hair. The HRM approach described here could certainly be utilized for the identification of other forensically relevant materials if suitable biomarkers were identified. This could also include internal organs and tissues tissues such as brain, heart, etc. This work was focused on those body fluids and tissues commonly found at the crime scene, but the approach could certainly be used for other fluids and tissues in the future.

- **Can the authors give a little bit more information about the size of the amplicons generated by PCR for the different biomarkers employed and also about the design of the primers employed?**

All of the primers were designed using Primer 3 software. Primers were designed to either span exon-exon boundaries (no DNA amplification) or designed to be in separate exons (so that the DNA product size would be different than the RNA product size due to the inclusion of an intron). Tables 1 and 3 have been updated to include amplicon size and also the exon location of the primers.

- **How reproducible is the assay for one sample coming from an individual?**

The assay is highly reproducible within an individual. Typically in a single experiment the Tm curves from the same individual are exactly superimposable upon one another The donors used in this study were also used repeatedly throughout testing, with both separate RNA extractions and multiple RT-PCR's from the same individual extract being employed. No significant differences in Tm were observed.

- **We noticed that when you compare tables 2 and 4, there is a significant improvement of the specificity using the IL19 marker in the hexaplex assay. As mentioned by the authors, they have used a different reverse primer for IL19 in the hexaplex assay. We wonder whether this new reverse primer can also improve the specificity for IL19 to differentiate vaginal secretions from menstrual blood also in single, duplex, or triplex analyses.**

Menstrual blood samples will, by nature of the source of the material, contain varying amounts of vaginal secretions. Therefore IL19 in a singleplex assay still will never provide sufficient resolution to distinguish between a vaginal and menstrual sample, as both could contain vaginal secretions. While every menstrual blood sample may not contain sufficient levels of vaginal material to result in IL19 detection, vaginal secretions will still be present in such samples to a varying extent. Therefore a menstrual blood-specific biomarker is typically needed to definitively identify the presence of menstrual blood. A duplex reaction with IL19 and MMP10 for example would permit a determination of vaginal secretions and menstrual blood. Vaginal secretions only samples should have only IL19, whereas menstrual blood samples could contain only MMP10 or a mixture of MMP10 and IL19.

- **Regarding the possible amplification of MMP3 by MMP10 primers in the menstrual blood samples under certain experimental conditions. I think it could be important to test that this is indeed the case; maybe by designing MMP3 specific primers that**

confirm this particular peak or eventually sequencing the products that were generated.

It is certainly of interest to determine whether the additional product is in fact MMP3 and to determine if specific MMP10 and MMP3 primer sets could be distinguished. This will be the subject of future work, although it is important to note that this uncertainty does not interfere with the accurate interpretation of the body fluid results using the current assay.

- **Finally, I got the impression from the experiments in which the authors mixed blood with decreasing amounts of semen that the technique does not necessarily reflect quantitative trends. Is that indeed the case? Could quantitation be ultimately important in a forensic case?**

In our evaluation so far of data from the HRM assays, the height of the melt peak, somewhat surprisingly, does not appear to correlate with the amount of analyte. More in depth studies need to be performed in order to determine possible reasons for this. It should be noted that the melt peak is the first derivative of fluorescence with temperature. It might be the case that other transforms of the data other than the first derivative might prove to be better correlated with analyte input.

The ability to make a relative quantitative assessment of the amount of each body fluid present in an unknown sample would be useful. The RNA quantitation method we employ, namely the binding of the fluorescent dye RiboGreen, provides an overall assessment of the amount of RNA present, but cannot provide quantitative information regarding the amount of each fluid present if the sample contains more than one body fluid. While a quantitative assessment is not critical for a determination of the presence or absence of a body fluid, a quantitative assessment could be provide important probative information for admixed body fluid samples.

Competing Interests: No competing interests were disclosed.