mRNA profiling of mock casework samples: Results of a FoRNAP collaborative exercise

Salzmann, Andrea Patrizia ; Bamberg, Malte ; Courts, Cornelius ; Dorum, Guro ; Gosch, Annica ; Hadrys, Thorsten ; Hadzic, Gavrilo ; Neis, Maximilian ; Schneider, Peter M ; Sijen, Titia ; van den Berge, Margreet ; Wiegand, Peter ; Haas, Cordula

Abstract: In recent years, forensic mRNA profiling has increasingly been used to identify the origin of human body fluids. By now, several laboratories have implemented mRNA profiling and also use it in criminal casework. In 2018 the FoRNAP (Forensic RNA Profiling) group was established among a number of these laboratories with the aim of sharing experiences, discussing optimization potential, identifying challenges and suggesting solutions with regards to mRNA profiling and casework. To compare mRNA profiling methods and results a collaborative exercise was organized within the FoRNAP group. Seven laboratories from four countries received 16 stains, comprising six pure body fluid / tissue stains and ten mock casework samples. The laboratories were asked to analyze the provided stains with their in-house method (PCR/CE or MPS) and markers of choice. Five laboratories used a DNA/RNA co-extraction strategy. Overall, up to 11 mRNA markers per body fluid were analyzed. We found that mRNA profiling using different extraction and analysis methods as well as different multiplexes can be applied to casework-like samples. In general, high input samples were typed with high accuracy by all laboratories, regardless of the method used. Irrespective of the analysis strategy, samples of low input or mixed stains were more challenging to analyze and interpret since, alike to DNA profiling, a higher number of markers dropped out and/or additional unexpected markers not consistent with the cell type in question were detected. It could be shown that a plethora of different but valid analysis and interpretation strategies exist and are successfully applied in the Forensic Genetics community. Nevertheless, efforts aiming at optimizing and harmonizing interpretation approaches in order to achieve a higher consistency between laboratories might be desirable in the future. The simultaneous extraction of DNA alongside RNA showed to be an effective approach to identify not only the body fluid present but also to identify the donor(s) of the stain. This allows investigators to gain valuable information about the origin of crime scene samples and the course of events in a crime case.

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Research paper

mRNA profiling of mock casework samples: Results of a FoRNAP collaborative exercise

Andrea Patrizia Salzmann [a], Malte Bamberg [b], Cornelius Courts [c], Guro Dørum [a], Annica Gosch [c], Thorsten Hadrys [d], Gavrilo Hadzic [e], Maximilian Neis [f], Peter M. Schneider [f], Titia Sijen [g], Margreet van den Berge [g], Peter Wiegand [b], Cordula Haas [a,*]

[a] Zurich Institute of Forensic Medicine, University of Zurich, Switzerland
[b] Institute of Legal Medicine, University Hospital, University of Ulm, Germany
[c] Institute of Forensic Medicine, University Hospital of Schleswig-Holstein, Kiel, Germany
[d] Bavarian State Criminal Police Office, Institute of Forensic Sciences, Munich, Germany
[e] National Forensic Laboratory, Police, Slovenia
[f] Institute of Legal Medicine, Faculty of Medicine, University Hospital, University of Cologne, Germany
[g] Netherlands Forensic Institute, The Hague, the Netherlands

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ABSTRACT

In recent years, forensic mRNA profiling has increasingly been used to identify the origin of human body fluids. By now, several laboratories have implemented mRNA profiling and also use it in criminal casework. In 2018 the FoRNAP (Forensic RNA Profiling) group was established among a number of these laboratories with the aim of sharing experiences, discussing optimization potential, identifying challenges and suggesting solutions with regards to mRNA profiling and casework. To compare mRNA profiling methods and results a collaborative exercise was organized within the FoRNAP group. Seven laboratories from four countries received 16 stains, comprising six pure body fluid / tissue stains and ten mock casework samples. The laboratories were asked to analyze the provided stains with their in-house method (PCR/CE or MPS) and markers of choice. Five laboratories used a DNA/RNA co-extraction strategy. Overall, up to 11 mRNA markers per body fluid were analyzed. We found that mRNA profiling using different extraction and analysis methods as well as different multiplexes can be applied to casework-like samples. In general, high input samples were typed with high accuracy by all laboratories, regardless of the method used. Irrespective of the analysis strategy, samples of low input or mixed stains were more challenging to analyze and interpret since, alike to DNA profiling, a higher number of markers dropped out and/or additional unexpected markers not consistent with the cell type in question were detected. It could be shown that a plethora of different but valid analysis and interpretation strategies exist and are successfully applied in the Forensic Genetics community. Nevertheless, efforts aiming at optimizing and harmonizing interpretation approaches in order to achieve a higher consistency between laboratories might be desirable in the future. The simultaneous extraction of DNA alongside RNA showed to be an effective approach to identify not only the body fluid present but also to identify the donor(s) of the stain. This allows investigators to gain valuable information about the origin of crime scene samples and the course of events in a crime case.

1. Introduction

The identification of human body fluids in biological trace material can crucially contribute to the stains' contextualization and thus provide essential information for the investigation and the prosecution of criminal offences [1]. Therefore, analysis of biological evidence typically begins with a screening for the presence of body fluids. In the last decades new techniques for the identification of body fluids have been developed, including forensic RNA analysis [2–11], tissue specific methylation [12–16], microbial forensics [17–21] and proteomics [22, 23]. Since each body fluid or tissue type is associated with cells exhibiting a typical transcriptome for that cell type, the detection of specific

* Corresponding author at: University of Zurich, Zurich Institute of Forensic Medicine, Forensic Genetics, Winterthurerstrasse 190/52, CH-8057 Zurich, Switzerland.
E-mail address: cordula.haas@irm.uzh.ch (C. Haas).

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RNA transcripts can be used to identify the presence of a body fluid or tissue type.

In recent years, several mRNA markers have been identified for the forensically most relevant body fluids / tissue types encountered in criminal casework (blood, menstrual blood, nasal and vaginal secretion, saliva, semen, skin) [24–36]. The classical workflow of mRNA profiling includes RNA extraction, DNase treatment, reverse transcription, marker-specific endpoint PCR and capillary electrophoresis (CE) or detection by qPCR [4]. To reduce sample consumption and time, RNA can be co-extracted along with DNA from the same sample. This approach enables the simultaneous identification of the body fluid donor by STR profiling and the identification of the tissue / fluid source of origin by mRNA profiling [37,38]. Although RNA degrades more quickly in post-mortem tissues and *ex-vivo* than DNA (e.g. due to the chemical structure, RNases present in the environment, humidity and UV-radiation), mRNA has been demonstrated to be more stable than formerly supposed depending on the storage conditions [32,35,39]. In a series of collaborative exercises, the European DNA Profiling Group (EDNAP) evaluated the performance, robustness and reproducibility of different mRNA markers for the identification of blood, saliva, semen, vaginal secretion, menstrual blood and skin [40–44]. For many laboratories this was a good opportunity to get acquainted with the method and gain experience. Furthermore, the European Forensic Genetics Network of Excellence (EUROFORGEN-NoE) examined the interpretation of RNA profiling data [45]. In the meantime, mRNA profiling has been implemented and accredited according to ISO 17025 in several laboratories, is regularly used in casework and accepted by courts.

Expression of body fluid- or cell-type specific transcripts is typically abundant in the respective body fluid / tissue, but not necessarily absent in others. Many forensic mRNA markers are also detected at low frequencies in non-target body fluids. Roeder et al. [34] developed a scoring system which minimizes the chances of misidentification of a sample due to marker expression in a non-target body fluid. Each marker is weighed according to its specificity for a certain body fluid. For a specific sample the body fluid specific values of the detected mRNA markers are summed up to produce a body fluid score. Threshold scores are then determined for the identification of each body fluid. Lindenbergh et al. [46] proposed a scoring method based on replicate analyses, which allows an unbiased interpretation of mRNA results in casework. The replicates are used to determine how often a signal is present (observed number of markers) relative to the times a signal could have occurred (maximum number of markers, which could have been detected per body fluid). Depending on the result of this ratio, a body fluid is reported as "observed", "not observed" or "sporadically observed". De Zoete et al. [47] proposed a probabilistic approach to identify body fluids using naive Bayes and multinomial logistic regression. Dørum et al. [48] built a probabilistic model, which incorporates quantitative information (MPS read counts) instead of only the presence/absence of markers. Recently, Iacob et al. [49] introduced a machine learning prediction model that incorporates probabilistic information based on MPS data.

In the last decade, massively parallel sequencing (MPS) technologies have constantly evolved, leading to a decrease in operational costs and an increase in throughput [50]. By now, several MPS kits for forensic applications (autosomal STRs, Y- and X-STRs, identity, phenotypic and ancestry SNPs, as well as whole mtDNA) and different platforms are commercially available and are used for casework [51]. Besides, DNA sequencing and targeted RNA sequencing has been introduced in forensic stain analysis. Hanson et al. [52] developed an MPS assay for the identification of body fluids using 33 tissue-specific mRNA markers on the Illumina MiSeq platform. The assay was then tested within the EUROFORGEN-NoE and EDNAP groups [53]. The results of this collaborative mRNA MPS exercise suggest that mRNA sequencing is a reliable body fluid identification method, which could be added to the repertoire of forensic MPS panels.

In 2018 the FoRNAP group was established as a community of laboratories using mRNA profiling in casework with the aim of sharing experiences, discussing optimization potential and suggesting solutions with regards to casework. A collaborative exercise among the FoRNAP laboratories was organized by the Zurich Institute of Forensic Medicine, Switzerland. The aim was to compare mRNA profiling methods and results by analyzing a set of mock casework samples. The laboratories were asked to analyze the provided stains with their own methods (PCR/CE or MPS) and markers. Here we present the results of the seven participating laboratories.

2. Material and methods

2.1. Participants and samples

Seven laboratories from Germany, the Netherlands, Slovenia and Switzerland participated in this collaborative exercise. All participants are accredited according to ISO 17025 and actively employ forensic RNA analysis in casework.

The organizing laboratory (Zurich Institute of Forensic Medicine, Switzerland) prepared 16 stains, comprising six pure body fluid / tissue stains and mock casework samples (Table 1). Body fluids / tissues were collected from healthy volunteers with their informed consent. The sampling was approved by the local ethics commission KEK (declaration of no objection No. 24-2015). Thirteen single source and three mixed stains were prepared as depicted in Table 1. After preparation, all samples were dried at room temperature for at least 12 h. The composition of the stains was not disclosed to the participants. At the organizing laboratory, sample preparation and experimental part were done by different persons; the experimenter had no knowledge of the stain composition. The samples were not tested by the organizing laboratory prior to shipment and were sent at ambient temperature by courier to the different laboratories. All parcels were received within two days. Participants were asked to store the samples at room temperature in the dark until further processing.

2.2. RNA and DNA analyses

The participating laboratories were asked to analyze the provided stains using their standard mRNA profiling protocol, as well as the interpretation and reporting guidelines, they are using in casework. Four laboratories (Labs 1, 3, 4, 7) used an RNA/DNA co-extraction approach, while two laboratories (Labs 2 and 5) extracted RNA only. Lab 6 used a co-extraction as well as an RNA only extraction approach, and we will refer to their results as Lab 6a and Lab 6b, respectively. A summary of the different RNA and DNA analysis methods used is displayed in Tables 2 and 3. An overview of the markers used for mRNA profiling is shown in Table 4.

Most laboratories analyzed mRNA markers for the identification of blood, saliva, semen, vaginal secretion, menstrual blood and skin. Lab 4 only used markers for saliva, menstrual blood and vaginal secretion and Lab 7 did not use markers for skin. In addition, some laboratories used markers for housekeeping genes (Labs 1, 3, 5 and 7), gender (Labs 2, 3, 5 and 7) and nasal secretion (Labs 3 and 7). Six laboratories employed a PCR/CE based method (Labs 1, 3, 4, 5, 6a and 7), while laboratories 2 and 6b analyzed the stains using an MPS approach on the Illumina MiSeq/FGx platform. The PCR/CE based laboratories used different RNA amplification strategies. Three laboratories performed replicate analyses (Labs 1, 6a and 7). Laboratory 1 performed duplicate RT analyses and PCR replicates with alternative input volumes when deemed necessary. Laboratory 6a used different body fluid / tissue specific primer multiplexes to confirm the results of the initial screening multiplex. Laboratory 7 amplified each cDNA four times. The remaining five participants performed single analyses.

Different strategies were employed by the laboratories to analyze the DNA (Table 3). Two laboratories repeated the PCR if deemed necessary based on the quality of the profiles (Labs 6a and 7), while three

Table 1
Overview of the 16 stains prepared by the organizing laboratory.

	Body fluid(s)	Age of body fluid	Substrate	Sample processing	Classification
1	25 µL saliva	fresh	swab	whole swab/piece	pure / high input
2	nose bleed blood	2 years	tissue (0.5 × 0.5 cm)	whole swab/piece	low input
3	saliva	fresh	tea spoon	swab the mouth part of the spoon and use whole swab for extraction	low input
4	menstrual blood	1 year	1/4 swab	whole swab/piece	pure / high input
5	finger print	fresh	microscope slide (finger unwashed)	swab the upper side ("Superfrost" and "5" visible) of glass surface	low input
6	skin collected from forearm	fresh	swab (forearm unwashed)	whole swab/piece	pure / low input
7	5 µL blood + 5 µL semen	1 year (blood), 3.5 years (semen)	swab	whole swab/piece	mixed
8	vaginal secretion	fresh	1/4 swab	whole swab/piece	pure / high input
9	menstrual blood	fresh	cotton panty (ca. 0.5 × 0.5 cm)	whole swab/piece	high input
10	vaginal secretion + 10 µL semen	fresh (vaginal secretion), 4 years (semen)	Finger of latex glove (vaginal secretion on outside, semen on inside)	swab, extract and analyze outer and inner parts separately	high input
11	saliva (child) on forearm (mother)	fresh	swab (forearm unwashed)	whole swab/piece	mixed
12	25 µL semen	3.5 years	swab	whole swab/piece	pure / high input
13	1 µL blood	1 year	cord (unwashed)	whole swab/piece	low input
14	vaginal secretion + 5 µL semen	fresh (vaginal secretion), 5 years (semen)	1/4 swab	whole swab/piece	mixed
15	25 µL blood	1 year	swab	whole swab/piece	pure / high input
16	5 µL semen (azoospermic)	2 years	cellulose	cut out area where stain is suspected to be	low input

laboratories did not perform replicate analyses (Labs 1, 3 and 4). In addition, laboratory 4 generated Y-STR profiles (Yfiler™ Plus Kit, Thermo Fisher Scientific) for the stains. The profiles were searched in the YHRD database, using the ancestry information function on the YHRD website (https://yhrd.org) [54]. In the end the participating laboratories were asked to fill in a questionnaire describing what methods they used, the quantification results (RNA and optionally DNA), the mRNA profiling and optionally STR analysis results and a tentative interpretation.

2.3. Compilation of RNA and DNA results

The composition of the stains was not disclosed to the participants during the runtime of the exercise. The compilation of the results was performed at the Zurich Institute of Forensic Medicine. Based on the body fluid / tissue input amount and the RNA/DNA quantification results, stains were classified into high input stains (No. 1, 4, 8, 9, 10.1, 10.2, 12, 15), low input stains (No. 2, 3, 5, 6, 13, 16) and mixed stains (No. 7, 11, 14).

Within the RNA results, markers were categorized into 1) correctly identified, 2) missing and 3) additional markers (not expected in the stain). Graphs showing the percentage of the average peak height / read count were generated per stain. In addition, results were analyzed per laboratory and per method (CE/MPS) as well as per marker. The laboratories applied their own thresholds to call a marker (Table 2). Markers not meeting this specified threshold were omitted from the analysis. For menstrual blood samples (stains 4 and 9), blood, vaginal secretion, and menstrual blood markers were regarded as possible markers. Menstrual blood had to be reported to be present in order for the result to be correct. For nose bleed (stain 2), blood markers and nasal secretion markers (if used by the analyzing laboratory) were taken into account. For the azoospermic semen sample (stain 16), only markers for seminal fluid were considered as correct markers.

Within the DNA results, a locus was marked as correct, when all required alleles were present (see Table 3 for thresholds). Results were depicted as ratios, based on the number of correctly typed and missing loci.

3. Results

3.1. RNA and DNA extraction

The phenol/chloroform based RNA extraction method used by Laboratory 6b produced the highest RNA yields in all but two stains, when considering the six laboratories that quantified the RNA (Fig. 1). Samples containing vaginal secretion or menstrual blood showed the highest RNA concentrations, while the fingerprint on the glass slide and 1 µL of blood on a piece of cord provided the lowest RNA concentrations (Fig. 1).

Of the five laboratories that analyzed the DNA, the DNA/RNA co-extraction strategy using the QIAamp DNA mini kit (Qiagen) in combination with the mirVana miRNA isolation kit (Thermo Fisher Scientific) resulted in the highest DNA concentrations (Labs 1, 3, 7). Overall, stains containing menstrual blood, vaginal secretion or semen (with the exception of the azoospermic semen stain 16) showed the highest DNA yields (Fig. 1). The DNA concentrations were lowest in skin samples (finger print, skin collected by rubbing lower arm), 1 µL of blood on piece of cord and 5 µL azoospermic semen on cotton swab.

3.2. mRNA profiling

The numbers of correctly typed, missing and additional markers per laboratory and per stain are displayed in Fig. 2. Overall, high input samples (No. 1, 4, 8, 9, 10.1, 10.2, 12, 15) showed higher numbers of correctly identified markers as well as lower numbers of missing markers compared to the low input samples (No. 2, 3, 5, 6, 13, 16) and mixed samples (No. 7, 11, 14).

Fig. 3a and 3b give an overview of specific and unspecific markers observed in each stain and for each laboratory. Since the laboratories analyzed different markers, the figures display marker category rather than the individual markers. The bars show the sum of peak heights / read counts for all markers specific to a body fluid category, relative to the total sum of peak heights / read counts for the sample. Housekeeping / reference gene markers were not included in the calculation of the total peak height / read counts per sample. For the laboratories with replicates, the average percentage is shown with standard errors indicated on the bars. Note that STATH is included in the saliva marker category only, although two laboratories reported it as a combined nasal and saliva marker. In most of the high input stains (Fig. 3a) the body fluid specific markers were identified correctly. The exceptions are stain 15 in which the blood markers were not detected by Lab 6a, and stain 9 where Lab 1 and Lab 6b did not detect the menstrual blood markers. In the low input and mixture stains (Fig. 3b) all laboratories detected the blood markers in stain 2 (nose blood), while in stain 13 only four laboratories found blood. Semen markers were detected in all stains where semen was present, with one exception. In the pure skin sample (stain 6), four out of

Table 2
Overview of the RNA analysis and interpretation methods used by the different laboratories. *Laboratories 3 and 4 did not quantify the RNA extract [+]according to MPS protocol.

	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6a	Lab 6b	Lab 7
Extraction method	Thermo Fisher mirVana miRNA isolation kit, Qiagen QIAamp DNA mini kit	Promega Maxwell RSC miRNA tissue kit	Thermo Fisher mirVana miRNA isolation kit, Qiagen QIAamp DNA mini kit	Thermo Fisher RNAQUEOUS-MICRO KIT	Promega Maxwell RSC miRNA tissue kit	Qiagen AllPrep RNA/DNA co-extraction kit	Organic extraction (Phenol/ chloroform)	Thermo Fisher mirVana miRNA isolation kit, Qiagen QIAamp DNA mini kit
Quantification method	Promega Quantifluor RNA System	Promega Quantifluor RNA System	*	*	Promega Quantifluor RNA System	Promega Quantifluor RNA System (HS assay)	Promega Quantifluor RNA System (HS assay)	Thermo Fisher Qubit RNA HS assay
RT Kit	Thermo Fisher High Capacity cDNA Reverse Transcription Kit	Illumina TruSeq Targeted RNA Kit	Thermo Fisher RetroScript Kit	Thermo Fisher High Capacity cDNA Reverse Transcription Kit	Bioline SensiFAST cDNA-Synthese Kit	Ambion Superscript III Kit	Illumina TruSeq Targeted RNA Kit	Promega GoScript
RT enzyme	Multiscribe Reverse Transkriptase	New England BioLabs ProtoScript II Reverse Transcriptase	MMLV-RT	MultiScribe Reverse Transcriptase	Bioline SensiFAST cDNA-Synthese Kit	Superscript III	New England BioLabs ProtoScript II Reverse Transcriptase	GoScript Reverse Transcriptase
RNA input into RT reaction	optimal 1 ng/µl in 20 µL reaction volume	3 µL in 10 µL reaction volume	10 µL in 20 µL reaction volume	10 µL in 20 µL reaction volume	10 µL in 20 µL reaction volume	8 µL in 20 µL reaction volume	50 ng or if 50 ng not reached 3 µL in 10 µL reaction volume	10 µL in 20 µL reaction volume
cDNA input into PCR	up to 7.5 µL	+	serial input: 0.2 µL - 1.0 µL - 4.0 µL	10 µL	up to 7.5 µL	2.5 µL	+	serial input: 0.1 µL - 1.0 µL - 3.75 µL
Platform	Genetic Analyzer 3500	MiSeq FGx	Genetic Analyzer 3130*xl*	Genetic Analyzer 3500	Genetic Analyzer 3130	Genetic Analyzer 3130*xl*	MiSeq FGx	Genetic Analyzer 3130
Threshold to call a peak	150 rfu	50–10'000 reads, depending on marker	150 rfu	100 rfu	200 rfu (all markers except skin), 100 rfu (skin)	50 rfu	500 reads	150 rfu
No of markers analyzed	16	28	21	11	18	30	33	19
Replicate analyses	Duplicate RT reaction, followed by further replicates with alternative input volume when necessary	no	no	no	no	Screening mulitplex followed by body fluid specific mulitplexes	no	Four replicates per stain
Interpretation strategy	Interpretation based on combined results of replicate anaysis. Interpretation matrix with guidelines taking marker-specific information (e.g. whether a certain marker is known to possibly be co-expressed in a different body fluid) into consideration.	Defined read count per marker has to be reached to call a marker present. Interpretation done only based on read count values. No probabilistic/ prediction approach used.	Interpretation based on the combined results of two multiplexes with in general a body fluid observed when ≥50 % of the markers detected.	Marker is present, when the threshold of 100 rfu is reached. No interpreation tool was used.	Marker are present, when the threshold of 200 rfu (100 rfu for skin) is reached. If housekeeping markers were under 1000 rfu, the threshold was reduced to 50 RFU. No interpretation tool applied.	Marker is present, when the threshold of 50 rfu is reached. No interpretation tool applied.	500 reads have to be reached to call a marker present. Interpretation done only based on read count values. No probabilistic/ prediction approach used.	Scoring according to Lindenberg et al. [46]: $x = n/2$, $x =$ observed number of markers, $n =$ maximum number of markers, which could have been detected per body fluid. Body fluid is reported as "observed" ($x \geq n/2$), "not observed"($x = 0$) or "sporadically observed, not reliable" ($0 < x < n/2$).

six laboratories detected skin markers, while five laboratories detected skin in the saliva-skin mixture (stain 11). The saliva markers had few unspecific reads, however Labs 2 and 7 did not detect saliva in stain 3, and Lab 3 did not detect saliva in stain 11. Some variation can be seen for the laboratories with replicates.

3.3. Observation of additional mRNA markers

An overview of the detected additional markers per laboratory and per stain is depicted in Figs. 2, 3a, 3b and Table 5. High input samples (No. 1, 4, 8, 9, 10.1, 10.2, 12, 15) show a higher number of additional markers than low input samples (No. 2, 3, 5, 6, 13, 16). Mixed samples

Table 3
Overview of the DNA analysis and interpretation methods used by the different laboratories to generate STR profiles. *no DNA extraction was performed $^{+}$Quantification method according to Lindenbergh et al [55].

	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6a	Lab 6b	Lab 7
Extraction method	Qiagen QIAamp DNA mini kit, Ambion mirVana miRNA isolation kit	*	Qiagen QIAamp DNA mini kit, Ambion mirVana miRNA isolation kit	Qiagen EZ1 Advanced XL	*	Qiagen AllPrep RNA/DNA Co-Extraction kit	*	Qiagen QIAamp DNA mini kit, Ambion mirVana miRNA isolation kit
Quantification method	Promega PowerQuant (7500 fast)	*	Alu-repeat based quant $^{+}$	Thermo Fisher Quantifiler Trio DNA Quantification Kit	*	Promega Quantifluor ONE dsDNA	*	Thermo Fisher Quantifiler Human DNA Quantification Kit
STR kit	Promega PPL ESX 17 fast	*	Thermo Fisher AmpF*ℓ*STR NGM	Thermo Fisher GlobalFiler, Thermo Fisher Y-filer plus	*	Thermo Fisher NGM Select	*	Thermo Fisher NGM Select and NGM Detect
DNA input into PCR	0.5 ng/µl in 25 µL reaction volume	*	aiming at 500 pg	1−10 µl	*	1−10 µl	*	350−400 pg
Genetic Analyzer	3500	*	3130*xl*	3500	*	3130*xl*	*	3130
Threshold to call a peak	50 rfu	*	50 rfu	100 rfu	*	50 rfu	*	50 rfu
Replicate analyses	DNA-input into PCR based on quantification results. No replicate amplifications.	*	no	no	*	DNA-input into PCR based on quantification results. If neccessary PCR was repeated with adjusted input volume.	*	Replicates if necessary based on quality of profiles
Interpretation strategy	Allele is present, when the threshold of 50 rfu is reached.	*	Allele is present, when the threshold of 50 rfu is reached.	Allele is present, when the threshold of 100 rfu is reached.	*	Allele is present, when the threshold of 50 rfu is reached.	*	Allele is present, when the threshold of 50 rfu is reached.

Table 4
Summary of mRNA markers used by the different laboratories. Brackets behind the markers indicate by how many laboratories the marker was used. $^{+}$STATH is used to detect saliva and nasal secretion, *marker used to detect male gender, **marker used to detect female gender.

Blood	Semen	Saliva	Nasal	Vaginal	Menstrual	Skin	Ref. Gene	Gender
ALAS2 (7)	KLK3 (7)	HTN1 (3)	BPIFA1 (2)	CYP2A6 (2)	LEFTY2 (1)	CCL27 (3)	ACTB (4)	RPS4Y1* (4)
AMICA1 (1)	PRM1 (6)	HTN3 (7)	STATH^{+} (2)	CYP2B7P1 (8)	MMP10 (6)	CDSN (3)	18S-rRNA (4)	XIST**(3)
ANK1 (3)	PRM2 (3)	MUC7 (1)		DKK4 (2)	MMP11 (7)	COL17A1 (2)		
CD3G (3)	SEMG1 (6)	PRB3 (2)		FAM83D (2)	MMP7 (8)	IL1F7 (1)		
CD93 (3)	SEMG2 (1)	PRB4 (2)		HBD1 (1)	MMP3 (1)	IL37 (2)		
HBA (1)	TGM4 (3)	PRH2 (2)		MUC4 (6)	SFRP4 (1)	KRT77 (2)		
HBB (5)		STATH^{+} (8)		MYOZ1 (5)	STC1 (2)	LCE1C (4)		
PBGD (1)				Lcris (2)		LCE1D (1)		
SPTB (3)				Lgas (2)		LCE2D (1)		
				Ljen (2)		LOR (1)		
						SERPINA12 (2)		

(No. 7, 11, 14) show several additional markers across all participating laboratories. Skin markers were the most frequently observed additional markers. These markers were identified in 11 out of 16 samples, although the samples did not purposefully comprise skin. Blood and vaginal secretion markers were also detected unexpectedly in some samples. Laboratories reporting replicate results (Labs 1, 6a and 7) tended to show a higher number of additional markers compared to the remaining participants, because with each replicate performed chances of detecting additional markers increased. Thus it depends on the interpretation guidelines how these observations are assessed.

3.4. Assessment of the body fluid / tissue specific mRNA markers

Three to six mRNA markers per body fluid / tissue have been analyzed by three or more laboratories and are displayed in Supplementary Fig. 1. Most blood markers were very specific (they did not appear in non-target body fluids), but not very sensitive (they were not detected). Saliva, semen, vaginal secretion and menstrual blood markers were detected at very high numbers, with only a few missing markers in the respective stains. The skin markers LCE1C and CDSN were readily detected, but they also showed up in other body fluids.

3.5. STR profiling

Five laboratories used a DNA/RNA co-extraction approach and generated DNA profiles in addition to mRNA profiling. The compilation of the DNA results showed that the high input stains (No. 1, 4, 8, 9, 10.1, 10.2, 12, 15) were typed without mistakes; only in two stains a single dropout was observed (Table 6). Even within the low input samples (No. 2, 3, 5, 6, 13, 16) full DNA profiles were obtained for most stains. The skin samples, stain 5 (finger print on glass slide) and stain 6 (skin collected by swabbing forearm), were most challenging. Stain 5 was typed with >75 % of correctly typed loci by only two laboratories, while for stain 6 four laboratories reported >75 % of correctly typed loci. Some laboratories reported up to eight different alleles per locus in stains 5 and 6 (data not shown). Within the mixed stains (No. 7, 11, 14) almost full DNA profiles were generated for stains 7 and 14 (including all alleles of the two contributors). Stain 11 (saliva on skin) was challenging, only one laboratory reported >75 % of correctly typed loci, while four laboratories typed <50 % of the loci. Poor STR profiling results were in line with low DNA concentrations. The lowest DNA concentrations were obtained for stains 5, 6 and 11 (Fig. 1).

Fig. 1. RNA and DNA concentrations (log 10 (ng/μl)) shown per stain. Six laboratories quantified RNA, while five laboratories quantified DNA. Each symbol corresponds to one measurement, and each laboratory is identified by a different shaped/colored icon. Stains 1, 4, 8, 9, 10.1, 10.2, 12, 15 are high input samples, stains 2, 3, 5, 6, 13, 16 are low input samples, stains 7, 11, 14 are mixtures.

3.6. Interpretation

Participating laboratories were asked to fill in a questionnaire where information about the interpretation of the stains (body fluid(s) / tissue of origin, number of contributors and gender) was collected. The interpretation was done using the laboratories' standard interpretation rules and/or guidelines, based on all obtained results. Results are depicted in Table 7 for mRNA and Table 8 for STR profiling. In the high input category (stains 1, 4, 8, 9, 10.1, 10.2, 12, 15) most body fluids were identified correctly by mRNA profiling and the number and gender of the contributors was retrieved from DNA and/or RNA profiling. Only two mRNA results were incorrect or showed no result and two gender assignments were incorrect. For the identification of body fluids the main issue for a correct interpretation was the detection of additional mRNA markers.

The low input stains (No. 2, 3, 5, 6, 13, 16) were more difficult to infer. Stains 2 and 16 were interpreted correctly by all laboratories. Two laboratories analyzed markers for nasal mucosa, though stain 2 (nose

Fig. 2. RNA results per laboratory and per stain. The number of markers (y-axis) is displayed per stain as number of correct (blue), missing (orange) and additional markers (grey) on the x-axis. Two laboratories did not test for all body fluids; therefore, the respective stains were omitted from the graphs. *All three seminal fluid markers were detected below threshold. Nevertheless, semen was reported to be present in the sample.

bleed blood) did not show any nasal mucosa markers. Four laboratories stated correctly that sample 16 (azoospermic semen) only contains markers for seminal fluid and might originate from an azoospermic donor. Stain 3 (saliva on tea spoon), stain 5 (finger print on glass), stain 6 (skin from forearm) and stain 13 (1 µL blood on cord) were challenging samples. About half of the laboratories identified the body fluids in stain 3, 6 and 13 correctly, whereas stain 5 was correctly typed by two participants. For stains 5 and 6 (touch samples) the number of contributors and gender were difficult to identify. Only one laboratory assigned the number of contributors and the gender of the donors of stain 6 correctly.

In the mixed stain category (No. 7, 11, 14) at least one of the two body fluids was identified correctly by most laboratories. Some laboratories reported additional body fluids besides the correct ones. In stain 7 (blood + semen) semen was identified by all laboratories, but the blood component was identified only by two laboratories. However, all five laboratories that performed DNA profiling detected the two contributors. Both components in mixed stain 11 (saliva + skin) were correctly identified by four laboratories, another three laboratories identified one of the two components. Based on the DNA results only two out of five laboratories detected the two contributors. For stain 14 (vaginal secretion + semen) both components were identified by all laboratories, but four laboratories reported additional body fluids. Based on DNA results and/or gender markers seven out of eight laboratories detected the two contributors.

One laboratory also generated Y-STR profiles for the stains and identified the donors to originate from Western Europe, which is correct.

3.7. CE versus MPS

When comparing the conventional PCR/CE method (Labs 1, 3, 4, 5,

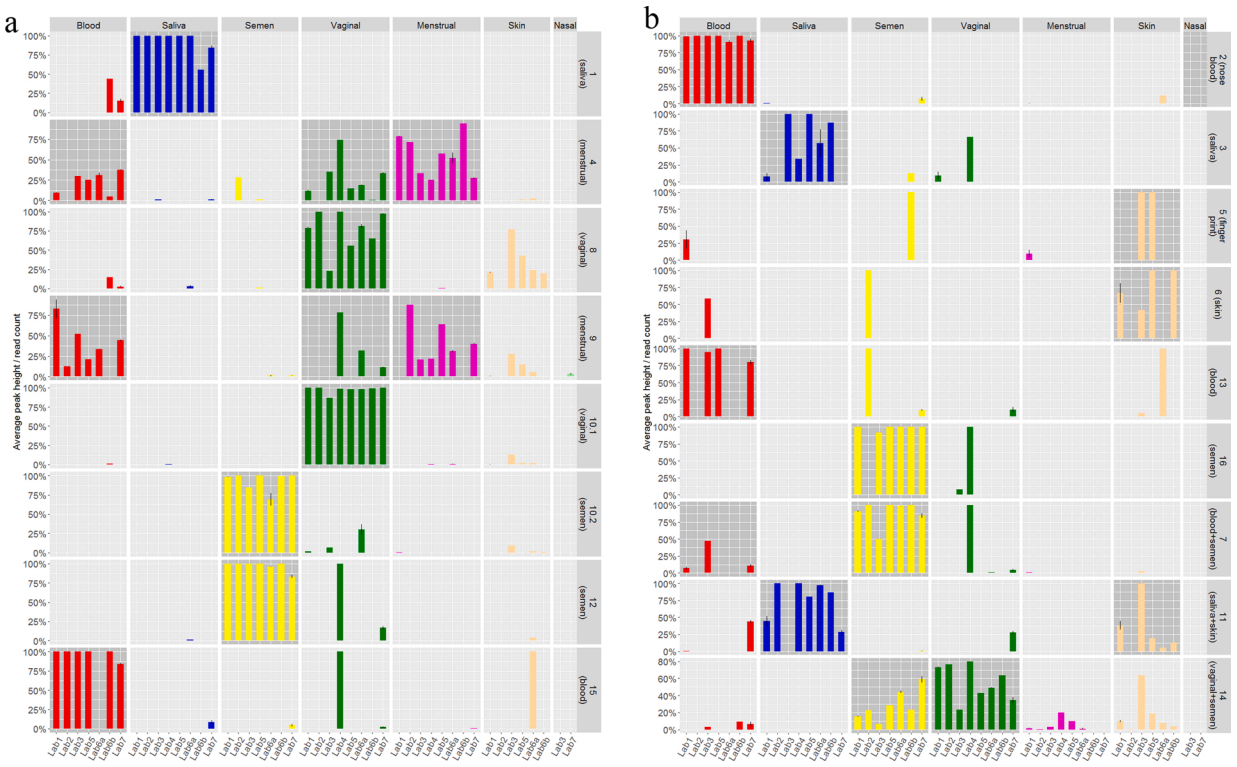

Fig. 3. a: High input stains: comparison of peak heights / read counts in each marker category (blood, saliva, semen, vaginal secretion, menstrual blood and skin) relative to the peak height / read count sum for the sample. Labs 1, 6a and 7 had replicates, and the error bars represent variation between replicates as ±1 standard error about the mean. Lab 4 only tested for saliva, vaginal secretion and menstrual blood. The unspecific peaks for vaginal secretion in stains 12/15 may appear disproportionately large due to the lack of body fluid specific markers. Lab 7 did not test for skin. Only labs 3 and 7 had nasal secretion markers.
b: Low input and mixed stains: comparison of peak heights / read counts in each marker category (blood, saliva, semen, vaginal secretion, menstrual blood, skin) between labs. The y-axis shows the percentage of peak heights/read counts attributed to a marker type, relative to the peak height / read count sum for the stain. Labs 1, 6a and 7 had replicates, and the error bars represent variation between replicates as ±1 standard error about the mean. Lab 4 only tested for saliva, vaginal secretion and menstrual blood. The unspecific peaks for vaginal secretion in stains 16/7 may appear disproportionately large due to the lack of body fluid specific markers. Lab 7 did not test for skin. Only labs 3 and 7 had nasal secretion markers.

6a, 7) and the MPS approach (Labs 2, 6b), it becomes evident that the number of correctly identified RNA markers tends to be lower in the samples analyzed by MPS (Fig. 3). Nevertheless, MPS laboratories were not lagging behind in correctly identifying the body fluids in the high input stains. One exception is stain 9 (menstrual blood), where no result was reported by one MPS participant. Low input samples were more challenging to analyze. No results or not correctly identified body fluids were reported from laboratories employing MPS as well as CE/PCR.

4. Discussion

Seven laboratories within the FoRNAP group, that are using mRNA profiling in casework, participated in this exercise. They analyzed a set of 16 mock casework samples using their standard mRNA profiling protocol (PCR/CE or MPS based). The aim of this study was to compare mRNA profiling methods including RNA/DNA co-extraction strategies and the resulting outcomes. To evaluate the difficulties faced when working with casework samples, the organizing laboratory prepared 13 single source stains ranging from high input (e.g. 25 μL of saliva on a cotton swab) to low input (e.g. 1 μL of blood on a piece of cord) and three mixtures.

When comparing the RNA concentrations between the participants, it was striking that higher RNA concentrations did not lead to better results. This could be explained by differences between the protocols used by the participating laboratories and the resulting quality of the RNA. In addition, residual phenol-chloroform might inhibit the downstream sample analysis. Another factor might be an overestimation of the human or non-human (bacterial) RNA content. On a side note, Grabmüller et al. [56] reported that comparing RNA quantification values obtained with different quantification methods is difficult. This is not relevant in our study, since all participants used fluorescence-based nucleic acid quantification methods (Qubit or Quantus fluorometer) for total RNA quantification.

With mRNA profiling, almost all body fluids of the high input samples were correctly identified. Stains of low input and mixtures were more challenging to analyze and interpret. However, low input samples were correctly identified by more than half of the laboratories, while at least one of the body fluids of each sample in the mixed stain category was reported correctly by most participants. Other studies reported that in mixed body fluid samples usually one of the body fluids is dominating [48,53,57]. This is probably dependent both on the mixture proportions and the types of body fluids involved. Therefore, it has to be kept in mind that for mixtures containing body fluids / tissues with generally low RNA amounts, e.g. blood, semen and skin, this component might be missed due to overrepresentation of the dominant body fluid. As a consequence, it should be considered to include a respective caveat for the interpretation of casework samples to clarify that the failure to detect a particular body fluid does not categorically exclude the presence of this body fluid in the original sample.

Skin samples proved most difficult to analyze, both for RNA and DNA profiling. Skin cells of the outermost layer of the epidermis are dead, keratinized and lack a nucleus. Van den Berge et al. [36] showed that for skin samples RNA profiling is more sensitive than DNA profiling, while the opposite is true for blood, menstrual blood, saliva and semen. Samples containing low amounts of blood (stains 7, 13, 15) were also difficult to analyze and interpret. Especially stain 7 (5 μL of semen mixed

Table 5

Correctly typed and additional RNA markers per stain and laboratory. Samples are sorted into high input stains (No. 1, 4, 8, 9, 10.1, 10.2, 12, 15), low input stains (No. 2, 3, 5, 6, 13, 16) and mixed stains (No. 7, 11, 14). Additional markers are subdivided into 1) skin markers, 2) markers which could be co-expressed in the body fluid (e.g. menstrual blood markers in vaginal secretion), due to sampling artefacts (e.g. blood markers in saliva or vaginal secretion samples) or can be explained by sexual intercourse (e.g. semen marker in vaginal secretion or menstrual blood samples) and 3) where cross-reactivity is suspected. [+]Lab 4 only used markers for saliva, menstrual blood and vaginal secretion. [++]Lab 7 did not use markers for skin. [+++] STATH was not used by Lab 2 as marker for nasal secretion but for saliva only. Since it has been described as marker for nasal secretion, it is listed in the category "co-expression possible".

Stain	Body Fluid	Lab 1				Lab 2				Lab 3				Lab 4			
		Correctly typed marker	Skin marker	Co-expression possible	Possible cross-reactivity	Correctly typed marker	Skin Marker	Co-expression possible	Possible cross-reactivity	Correctly typed marker	Skin Marker	Co-expression possible	Possible cross-reactivity	Correctly typed marker	Skin Marker	Co-expression possible	Possible cross-reactivity
Stain 1	saliva	STATH, HTN3	-	-	-	PRB3, PRH2, STATH	-	-	-	STATH	-	-	-	HTN3, STATH	-	-	-
Stain 4	menstrual blood	HBB, ALAS2, MUC4, MMP10, MMP7, MMP11	-	-	-	ALAS2, DKK4, MMP10, MMP7, MMP3, STC1	-	PRM2	-	HBB, ALAS2, CD93, MYOZ1, MUC4, MMP7, MMP10, MMP11		-	STATH	MYOZ1, CYP2B7P1, MUC4, Lcris, Lgas, MMP7, MMP11, HBD1	-	-	-
Stain 8	vaginal secretion	MUC4, CYP2B7P1	LCE1C, CDSN	-	-	CYP2A6, CYP2B7P1, DKK4, FAM83D	-	MMP10	-	MYOZ1, MUC4, CYP2B7P1	CDSN, LOR	-	-	MYOZ1, CYP2B7P1, MUC4, Lcris, Lgen, HBD1	-	-	-
Stain 9	menstrual blood	HBB	CDSN	-	-	ALAS2, ANK1, MMP10, MMP7, MMP3, STC1	-	-	-	HBB, MMP7	CDSN, LOR	-	-	CYP2B7P1, MUC4, Lcris, Lgas, MMP7, MMP11, HBD1	-	-	-
Stain 10.1	vaginal secretion	MUC4, CYP2B7P1	CDSN	-	-	CYP2B7P1, DKK4, FAM83D		MMP7	-	MYOZ1, MUC4, CYP2B7P1	CDSN, LOR	-	-	MYOZ1, CYP2B7P1, MUC4, Lcris, Lgas, HBD1	-	MMP7, MMP11	HTN3
Stain 10.2	semen	PRM1, KLK3, SEMG1		-	MUC4, MMP11	PRM2	-	-	-	PRM1	LOR	-	MYOZ1	+	-	-	-
Stain 12	semen	SEMG1, PRM1, KLK3	-	-	-	PRM2, SEMG1, TGM4, KLK3	-	-	-	KLK3, PRM1	-	-	-	+	-	-	HBD1
Stain 15	blood	HBB, ALAS2	-	-	-	ANK1	-	-	-	HBB	-	-	-	+	-	-	Lgas
Strain 2	nose blood	HBB, ALAS2		STATH[+++]	MMP11	ALAS2, CD3G	-	-	-	HBB, ALAS2, CD93	-	-	-	+	-	-	-
Stain 3	saliva	HTN3	-	-	MUC4	-	-	-	-	HTN3, STATH	-	-	-	HTN3, STATH	-	-	MYOZ1, MUC4, HBD1
Stain 5	finger print	-		-	HBB, MMP10	-	-	-	-	CDSN, LOR	-	-	-	+	-	-	-
Stain 6	skin	CDSN, LCE1C	-	-	-	-	-	-	PRM2	CDSN, LOR		-	HBB	+	-	-	-
Stain 13	blood	HBB	-	-	-	-	-	-	PRM2	HBB	LOR	-	-	+	-	-	-
Stain 16	semen (azospermic)	KLK3	-	-	-	-	-	-	-	SEMG1, KLK3		-	MYOZ1	+	-	-	HBD1
Stain 7	blood +semen	HBB, ALAS2, SEMG1, PRM1, KLK3	-	-	MMP10, MMP11, MMP7, MUC4	PRM2, SEMG1, TGM4		-	PRB4, STATH	HBB, SEMG1, KLK3, PRM1	LOR	-	-	+	-	-	MUC4
Stain 11	saliva + skin	STATH, HTN3, CDSN, LCE1C	-	-	HBB	STATH	-	-	-	CDSN, LOR	-	-	-	HTN3 +	-	-	-
Stain 14	vaginal secretion + semen	SEMG1, PRM1, KLK3, MUC4, CYP2B7P1	CDSN, LCE1C	MMP10, MMP7, MMP11	-	PRM2, SEMG1, CYP2A6, CYP2B7P1, DKK4, FAM83D	COL14A1	MMP7	-	MYOZ1, MUC4, CYP2B7P1, PRM1	CDSN, LOR	HBB, CD93, MMP7, MMP11	-	MYOZ1, CYP2B7P1, MUC4, Lcris, HBD1 +	-	MMP7, MMP11	-

Stain	Body Fluid	Lab 5				Lab 6a				Lab 6b				Lab 7			
		Correctly typed marker	Skin marker	Co-expression possible	Possible cross-reactivity	Correctly typed marker	Skin Marker	Co-expression possible	Possible cross-reactivity	Correctly typed marker	Skin Marker	Co-expression possible	Possible cross-reactivity	Correctly typed marker	Skin Marker	Co-expression possible	Possible cross-reactivity
Stain 1	saliva	STATH, HTN3	-	-	-	HTN3, HTN1, MUC7, STATH	-	-	-	HTN1, HTN3	-	AMICA1, CD93	-	STATH, HTN3	-	HBB, CD93	-
Stain 4	menstrual blood	HBB, ALAS2, MUC4, MYOZ1, CYP2B7P1, MMP7, MMP11, STC1	CDSN	KLK3	-	MMP11, HBA, ALAS2, MMP10, MYOZ1, CYP2B7P1, MUC4, MMP7, HBB, CD3G	LCE1C, LCE2D, IL1F7	-	STATH	LEFTY2, MMP10, MMP11, MMP7, ALAS2, AMICA1, CD93, DKK4	-	PRM1, PRM2	-	HBB, ALAS2, CD93, MUC4, MYOZ1, MMP7, MMP10, MMP11	-	-	STATH
Stain 8	vaginal secretion	MUC4, MYOZ1, CYP2B7P1	LCE1C, CDSN	KLK3 MMP7	-	MYOZ1, CYP2B7P1, Lcris, MUC4, Ljen	LCE1C, LCE1D, LCE2D	-	MUC7	CYP2B7P1, DKK4, FAM83D, CYP2A6	LCE1C	AMICA1, CD93	-	MUC4, CYP2B7P1, MYOZ1	-	CD93	-
Stain 9	menstrual blood	HBB, MMP7	LCE1C, CDSN	-	-	MYOZ1, MMP11, HBA, MUC4, ALAS2, CYP2B7P1, MMP10, Lcris, Lgas, MMP7, HBB, CD3G, ANK1, PRGD, SPTB	LCE1C	SEMG1	-	-	-	-	-	HBB, ALAS2, CD93, MUC4, CYP2B7P1, MMP7, MMP10, MMP11	-	SEMG1	BPIFA1
Stain 10.1	vaginal secretion	MUC4, MYOZ1, CYP2B7P1	CDSN	-	-	MYOZ1, CYP2B7P1, Lgas, MUC4	LCE2D	MMP10	-	CYP2B7P1, DKK4, FAM83D	-	AMICA1	-	MUC4, MYOZ1	-	-	-
Stain 10.2	semen	SEMG1, PRM1, KLK3	-	-	-	PRM2, PRM1, SEMG1	LCE1C	-	Lgas, MUC4	PRM1, PRM2	CCL27	-	-	PRM1, KLK3	-	-	-
Stain 12	semen	SEMG1, PRM1, KLK3	-	-	-	PRM1, PRM2, SEMG1, TGM4, KLK3	LCE1C, CCL27	-	MUC7	SEMG1, SEMG2, TGM4, PRM1, PRM2	-	-	-	SEMG1, PRM1, KLK3	-	-	MUC4, MYOZ1
Stain 15	blood	HBB	-	-	-	-	LCE1C	-	-	CD93	-	-	-	HBB, ALAS2	-	-	KLK3, MYOZ1, MMP10, STATH
Strain 2	nose blood	HBB, ALAS2	-	-	-	HBA, ALAS2, HBB	LCE1C	-	-	ALAS2, AMICA1	-	-	-	HBB, ALAS2	-	-	PRM1
Stain 3	saliva	STATH, HTN3	-	-	-	HTN1	-	-	-	HTN3	-	-	PRM1	-	-	-	-
Stain 5	finger print	CDSN, LCE1C	-	-	-	-	-	-	-	-	-	-	PRM1, PRM2	++	-	-	-
Stain 6	skin	CDSN, LCE1C	-	-	-	-	-	-	-	LCE1C	-	-	-	++	-	-	-
Stain 13	blood	HBB	-	-	-	-	LCE1C	-	-	-	-	-	-	HBB	-	-	KLK3, PRM1, MYOZ1
Stain 16	semen (azospermic)	SEMG1, KLK3	-	-	-	TGM4, SEMG1	-	-	-	TGM4	-	-	-	SEMG1, KLK3	-	-	-
Stain 7	blood +semen	SEMG1, PRM1, KLK3	-	-	-	PRM2, SEMG1, PRM1, TGM4		-	MUC4	PRM1, PRM2, SEMG1, SEMG2, TGM4	-	-	-	SEMG1, PRM1, KLK3, HBB	-	-	MUC4
Stain 11	saliva + skin	STATH, HTN3, LCE1C, CDSN	-	-	-	HTN3, HTN1, MUC7, STATH, LCE1C, LCE2D	-	-	-	LCE1C, HTN3	-	-	-	STATH, HTN3 ++	-	-	MUC4 MYOZ1, KLK3, HBB, CD93
Stain 14	vaginal secretion + semen	SEMG1, PRM1, KLK3, MUC4, MYOZ1, CYP2B7P1	LCE1C, CDSN	MMP7, MMP11, STC1,	-	CYP2B7P1, Lcris, PRM2, SEMG1, MYOZ1, MUC4, PRM1, TGM4	LCE1C	MMP11	-	CYP2B7P1, DKK4, FAM83D, CYP2A6, SEMG1, PRM1, PRM2	LCE1C	AMICA1, CD93	-	PRM1, KLK3, CYP2B7P1, MYOZ1, MUC4	-	-	-

with 5 μL of blood) and stain 13 (1 μL of blood on a piece of cord) were challenging. Several blood markers were not detected in these stains, except for the very sensitive blood marker HBB. An explanation might be that the blood used to prepare the stains has been stored frozen (−20 °C) for about one year in EDTA coated sampling tubes. Several studies found that expression levels of different mRNA species change over time when stored *ex-vivo* for more than 24 h and that storing samples at low temperatures does not prevent degradation [58]. It could be shown that freezing whole blood samples [58] or adding EDTA dramatically reduces RNA quality when stored for a longer period of time [59]. The cellular origin of stain 9 (menstrual blood on cotton panty) was also difficult to determine. Two laboratories did not detect any menstrual blood markers, but only reported blood as present. The sample was collected on day 3 of the menstrual cycle. It is known that menstrual blood comprises several tissue types such as blood, degraded endometrial tissue and epithelial cells from the vaginal lumen [4,60]. The blood content of menstrual blood is only about 30–50 % of the total flow volume. A study by Hanson et al. [52] indicated that the cellular composition changes across the course of menses. On days 1 and 2 of the menstrual cycle, menstrual blood markers were detected at a very high level, from day 3 on, these markers then decreased, while the opposite trend was observed for the vaginal secretion markers. The highest expression of blood markers was observed on day 4 of a 6-day menstrual cycle. No specific nasal secretion stain was included in the exercise to test the nasal mucosa markers (BPIFA, STATH). Stain 2 (nose bleed blood on a tissue) could have shown nasal mucosa markers but did not, probably because it was a low input and old stain.

When comparing the performance of MPS and CE/PCR based methods the number of correctly identified RNA markers tends to be lower in the samples analyzed by MPS. However, it has to be kept in

Table 6
Number of correctly typed STR loci. For single source stains, loci were marked as correct when the correct alleles were present (irrespective of additional alleles). For the mixed stains, loci were marked as correct, when all alleles of both contributors were present. Dark and light green boxes represent 100 % and >75 % correctly identified loci, respectively. Orange and red boxes represent 50 % – 75 % or <50 % correctly typed, respectively. One laboratory did not analyze the inner and outer side of stain 10 separately, but as a mixed profile of two persons.

Stains	Lab 1	Lab 3	Lab 4	Lab 6a	Lab 7
1	17/17	16/16	17/17	17/17	17/17
4	17/17	16/16	17/17	17/17	17/17
8	17/17	16/16	16/17	17/17	17/17
9	17/17	16/16	17/17	17/17	17/17
10.1	17/17	16/16	16/17	17/17	17/17
10.2	17/17	16/16		17/17	17/17
12	17/17	16/16	17/17	17/17	17/17
15	17/17	16/16	17/17	17/17	17/17
2	17/17	16/16	17/17	17/17	17/17
3	16/17	16/16	17/17	17/17	17/17
5	1/17	16/16	-	4/17	16/17
6	11/17	16/16	14/17	17/17	17/17
13	17/17	16/16	17/17	17/17	17/17
16	17/17	16/16	16/17	17/17	17/17
7	17/17	16/16	17/17	11/17	17/17
11	13/17	5/16	5/17	6/17	5/17
14	15/17	15/16	17/17	16/17	17/17

mind that MPS laboratories analyzed more markers per body fluid, probably including markers that are expressed at lower levels. Nevertheless, laboratories using MPS were not lagging behind when analyzing high input stains. The analysis of mixed and low input stains was challenging for all participants, irrespective of the method. The MPS protocol requires substantial amounts of RNA. The library preparation protocol used by Hanson et al. [52] and Ingold et al. [53] for example needs 50 ng of RNA for the degraded sample protocol. Depending on the sample type this amount is difficult to reach when working with forensic samples. In turn, this may lead to low read counts posing a genuine challenge for the interpretation of the data. This highlights the need for further improvement of library preparation and sequencing protocols. However, promising results have been obtained for body fluid identification not only by the present study but also by others [52,53].

Several additional (not expected) RNA markers were detected in the provided stains. This could be due to real contaminations or sampling artefacts. E.g. in stain 10 (finger of latex glove, outside vaginal secretion 10.1, inside semen 10.2) the detection of vaginal markers on the inside by two laboratories can be ascribed to sample processing. Nevertheless, the semen markers were detected as major component, whereas vaginal markers were the minor component. The detection of semen markers in vaginal secretion or menstrual blood samples could be due to sexual intercourse. In this regard, no information was collected from the body fluid donors. The detection of sporadic blood markers in non-blood samples might be due to an invasive sampling procedure, e.g. cotton swabs inserted into the vagina might have caused micro lesions in the mucosa (stains 8, 10.1). In sample 14 (vaginal secretion + semen), markers for menstrual blood and blood were detected in addition to the vaginal markers. These vaginal secretion samples were collected on day 8 of the menstrual cycle and could explain the high detection rate of markers for menstrual blood and blood. In 11 out of the 16 samples, skin markers were detected although the samples did not comprise skin. Touching skin during sample collection cannot be avoided (e.g. nose bleed blood on a piece of tissue, blood collection by finger prick, or vaginal secretion and menstrual blood collection on cotton swabs) and this could be the reason for the wide-ranging detection of the very sensitive skin markers. Since skin is a ubiquitous source of cells in any forensic scenario, the presence of skin markers must be interpreted very cautiously in the context of a given case.

Another explanation why additional RNA markers were detected, is that some mRNA markers might be affected by cross-reactivity. Body fluid- or cell-type specific mRNA markers have been selected based on their high expression levels in target tissues. Nevertheless, transcription might not be completely absent in other tissues. Therefore, background expression in non-target tissues remains possible. The skin markers (e.g. LCE1C) are very sensitive and may well be expressed in low levels in other body fluids. In a study by van den Berge et al. [45] cross-reactivity of skin markers (LOR and to lesser extent CDSN) were reported for vaginal mucosa samples. Saliva, vaginal secretion, menstrual blood and nasal secretion are derived from mucous membranes, which show a very similar composition; therefore such mRNA markers are prone to cross-reactivity. The detection of HBD1 in semen could be due to its expression in the germ line from pachytene spermatocytes to late spermatids [61]. In addition, the peptide can also be present in ejaculated spermatozoa and seminal plasma. MUC4 was shown to cross-react with semen and saliva [33]. Besides, it cannot be excluded that different primer designs and capillary electrophoresis instruments used by the participants may have led to differences in assay specificity and sensitivity. This might have had an influence on the detection of additional markers.

In general, the detection of additional markers poses a challenge when it comes to the interpretation of the results as body fluids or tissues not being present might be reported as observed. A good strategy to cope with variable expression and cross-reactivity is the use of interpretation and reporting guidelines. We mentioned the scoring systems [34,46] and probabilistic approaches [47,48] to minimize the chances of misidentification of a sample due to marker expression in a non-target body fluid. Nevertheless, there is still an ongoing discussion how to interpret mRNA data and present the evidence in court. In this study, one laboratory applied the scoring method based on 4 replicate analyses [46]. Another participant applied a different replicate strategy by analyzing the stains first with a screening multiplex containing primers for all body fluids, followed by body fluid specific multiplexes to confirm the results, but did not use an interpretation tool. A third laboratory performed RT and PCR replicate analyses. The other laboratories did not perform replicates or use an interpretation tool. This plethora of analyses and

Table 7
Interpretation of the mRNA results by the laboratories. Samples are sorted into high input stains (No. 1, 4, 8, 9, 10.1, 10.2, 12, 15), low input stains (No. 2, 3, 5, 6, 13, 16) and mixed stains (No. 7, 11, 14). Green shaded boxes represent correctly identified body fluids, light green coloring indicates the detection of additional body fluids besides the correct one, light red boxes represent stains, where markers are missing and therefore the body fluid was not correctly identified, red boxed depict no or incorrect results. In the mixed stain category yellow-green boxes identify samples where only one of the two body fluids in the stain was detected. $^{+}$Laboratory 4 only used markers for saliva, menstrual blood and vaginal secretion $^{++}$Laboratory 7 did not use markers for skin $^{+++}$Laboratory 1 and 6a reported possible RNA transfer between inside and outside during sampling. $^{++++}$Laboratory 1 reported to have swabbed both sides of the glass slide and thus indicated that results might be a contamination *Markers were detected under the read count threshold **Body fluid reported as possibly present in the stain. ***In routine casework, laboratory 4 does not report the detection of menstrual blood, even though markers for menstrual blood are present. Instead only vaginal secretion is reported to be present.

	Body Fluid	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6a	Lab 6b	Lab 7
Stain 1	saliva	saliva	saliva	saliva	saliva	saliva	saliva	blood, saliva	saliva
Stain 4	menstrual	menstrual, blood, vaginal	menstrual	blood, saliva, vaginal, menstrual	vaginal***	blood, menstrual, vaginal, skin	menstrual, saliva, skin	menstrual, semen, skin	menstrual, blood
Stain 8	vaginal	vaginal, skin	vaginal	vaginal, skin	vaginal	vaginal, skin	vaginal, saliva, skin	blood, vaginal, skin	vaginal
Stain 9	menstrual	blood	menstrual	blood, skin	vaginal***	menstrual, vaginal, blood, skin	menstrual, skin, semen**	no result	menstrual/blood
Stain 10.1	vaginal	vaginal	vaginal	vaginal, skin	vaginal	vaginal, skin	vaginal, skin	blood, vaginal	vaginal
Stain 10.2	semen	semen, vaginal+++	semen	skin, semen	+	semen	semen, vaginal+++	semen, skin	evidence for semen
Stain 12	semen	semen	semen	semen	+	semen	semen, saliva, skin	semen	semen
Stain 15	blood	blood	blood	blood	+	blood	skin	blood	evidence for blood
Stain 2	nose blood	blood	blood	blood	+	blood	blood, skin	blood	blood
Stain 3	saliva	no result	vaginal*/semen*	saliva	saliva, vaginal	saliva	saliva	saliva, semen	no result
Stain 5	finger print	blood++++	vaginal*/semen*	skin	+	skin	no result	semen	++
Stain 6	skin	skin	semen*	skin	+	skin	no result	skin	++
Stain 13	blood	blood**	semen*	blood, skin	+	blood	skin	no result	evidence for blood/semen
Stain 16	semen (azoospermic)	semen** (fluid)	semen*	semen (fluid)	+	semen (fluid)	semen (fluid)	semen (fluid)	semen
Stain 7	blood + semen	blood, semen, vaginal**, menstrual**	semen	blood, semen, skin	+	semen	semen	semen	semen
Stain 11	saliva + skin	saliva, skin	saliva	skin	no result	saliva, skin	saliva, skin	saliva, skin	saliva++ (evidence for blood, vaginal)
Stain 14	vaginal + semen	semen, vaginal, menstrual, skin	semen, vaginal	blood, vaginal, menstrual, semen, skin	vaginal+	semen, menstrual, vaginal, skin	vaginal, semen, skin	blood, semen, vaginal, skin	semen, vaginal

interpretation strategies leads also to differences in reporting of the results. This is exemplified by stain 9 (menstrual blood). Laboratories 6a and 7 correctly reported menstrual blood to be present. In addition, both participants detected the semen marker SEMG1. Laboratory 6a reported semen to be possibly present, while laboratory 7 did not report semen. The reason for this lays in different strategies to interpret the results. Laboratory 6 first used a screening multiplex, which contained primers for all body fluids, followed by body fluid specific multiplexes to confirm the results. A marker was called present, when 50 rfu were reached. A scoring tool was not used. In contrast, laboratory 7 used the scoring method ($x = n/2$) developed by Lindenbergh et al. [46], which is based on replicate analyses. The replicates are used to determine how often a signal is present (x = observed number of markers) relative to the times a signal could have occurred (n = maximum number of markers, which could have been detected per body fluid). Depending on the result of this ratio, a body fluid is reported as "observed" ($x \geq n/2$), "not observed"($x = 0$) or "sporadically observed, not reliable" ($0 < x < n/2$). Out of the maximal 12 possible peaks for semen, SEMG1 was detected in two replicates, scored as "sporadically observed, not reliable" and therefore not reported. We found that laboratories performing replicate analyses tended to show a higher number of additional markers compared to the remaining participants, because with each replicate chances of detecting additional markers are increased. The use of an interpretation tool such as the scoring method mentioned here is a good way to overcome this problem. Alternatives to the scoring methods are probabilistic models that apply quantitative information to predict the body fluids [48,49]. These methods consider the co-expression of all markers rather than just presence/absence of individual markers, and can assign a probability to each body fluid.

For a reliable assessment of the body fluid specific mRNA markers there is not enough data in this study, but our results reflect somehow the intuitive rating of markers. E.g. blood and semen markers are very specific in the high input stains, and they do not appear regularly in non-target body fluids. The skin markers are very sensitive, but they also show up in other body fluids. Mucous membranes (like saliva, vaginal secretion and menstrual blood) have a very similar composition, therefore the respective mRNA markers tend to cross-react with other mucous membranes.

DNA profiling of the 16 samples was straight forward. The high input stains (No. 1, 4, 8, 9, 10.1, 10.2, 12, 15) were typed easily, only in two stains a single dropout was observed. Even within the low input samples (No. 2, 3, 5, 6, 13, 16) full DNA profiles were obtained for most stains. Some laboratories reported mixed DNA profiles for the skin samples (stains 5 and 6), although they were collected from a single person. The donors were advised not to wash hands and arms before sample collection. Therefore, the additional detected alleles most likely originate from somebody touching the donor's arms or from surfaces, which have been touched by the donor prior to sample collection. This stands in line with findings by Szkuta et al. [62] who reported that DNA which was transferred from one individual's hand to another by handshake could be detected on different surfaces several hours post-handshake. A similar result was obtained by van den Berge et al. [63] who showed that background cellular material, which is unrelated to the case being analyzed, can be detected on any public or private item. In addition,

Table 8
Results for number of contributors (NoC) and gender identification by the laboratories, based on DNA and/or RNA analysis. Green represents correctly identified NoC and gender, orange indicates missing contributor or gender, red represents wrong results, white indicates not reported result. Laboratories 2, 5, 6b performed no DNA analyses but used gender specific RNA markers and gender specific body fluids to infer the gender of the contributors. *transmission error. Gender was correctly typed during STR analysis but reported as male instead of female **read counts for markers were reported to be below the detection threshold, therefore, gender determination was reported to be unreliable.

	Real		Lab 1		Lab 2		Lab 3		Lab 4		Lab 5		Lab 6a		Lab 6b		Lab 7	
Stain	NoC	Gender	NoC	Gender	NoC	Gender	NoC	Gender	NoC	Gender	NoC	Gender	NoC	Gender	NoC	Gender	NoC	Gender
1	1	f	1	f	-		1	f	1	m*	-	-	1	f	-	-	1	f
4	1	f	1	f	-	f	1	f	1	f	-	-	1	f	-	f	1	f
8	1	f	1	f	-	f	1	f	1	f	-	-	1	f	-	f	1	f
9	1	f	1	f	-	f	1	f	1	f	-	-	1	f	-	-	1	f
10.1	1	f	1	f	-	f	1	f	2	major f, minor m	-	-	1	f	-	f	1	f
10.2	1	m	1	m	-	m	1	m			-	m	1	m	-	m	1	m
12	1	m	1	m	-	m	1	m	1	m	-	m	1	m	-	m	1	m
15	1	f	1	f	-	m	1	f	1	f	-	-	1	f	-	-	1	f
2	1	m	1	m	-	m	1	m	1	m	-	m	1	m	-	-	1	m
3	1	f	1	f	-	m/f?**	1	f	1	f	-	-	1	f	-	-	1	f
5	1	f	?	?	-	f/m?**	-	-	-	-	-	-	1?	f?	-	-	>3	f > m
6	1	f	2	2 f	-	m	2	f	-	-	-	-	1	f	-	-	>3	f > m
13	1	m	1	m	-	m	1	m	1	m	-	-	1	m	-	-	1	m
16	1	m	1	m	-	m	1	m	1	m	-	m	1	m	-	m	1	m
7	2	m+f	2	?	-	m	2	f+m	2	major m, minor f	-	m	min 2	m+f	-	m	2	m (+f?)
11	2	m+f	2	?	-		1	m	1	m	-	-	min 2	m+f?	-	-	1	m
14	2	m+f	2	?	-	m/f	2	f+m	-	major f, minor m	-	m	min 2	m+f	min 2	m+f	2	f+m

sample composition might vary between samples sent to participants in this study. Within the mixed stains, stain 11 (mixture of saliva and skin of mother and son) was the most difficult sample to interpret on the DNA level. The son having donated saliva was the major contributor of the DNA profile, while for the mother many alleles had dropped out. Mother and son share one allele per locus and therefore a minor contributor adding 0–1 alleles is even more challenging to detect.

In summary, we found that mRNA profiling and RNA/DNA co-extraction strategies using different analysis methods can successfully be performed and applied to casework-like samples. Participating laboratories were provided with a variety of different samples ranging from very high to very low input amounts. In general, high input stains were typed with high accuracy by all laboratories, regardless of the method used. Samples of low input or mixed stains were more challenging to analyze and interpret since a higher number of markers dropped out and/or additional unexpected markers not consistent with the cell type in question were detected, irrespective of the analysis strategy. Nevertheless, it could be shown that a plethora of different but valid analysis and interpretation strategies exist and are successfully applied in the Forensic Genetics community. This allows investigators to gain valuable information about the origin of crime scene samples and the course of events in a crime case. We would also like to highlight that the aim of this study was not to harmonize forensic RNA analysis across countries and laboratories, but to show that several different and valid interpretations strategies are being applied, where each laboratory decides in its own way whether to report the presence of a body fluid based on the markers detected. Nonetheless, efforts aiming at optimizing and harmonizing interpretation approaches in order to achieve a higher consistency between laboratories might be desirable in the future.

A continuative question is whether cell types and donors can be associated in a mixture. Two studies discourage from associating donor and cell type based on peak heights / read counts when performing combined RNA and DNA analyses [64,65]. Coding SNPs (cSNP) are found within body fluid specific mRNA transcripts and could directly link a body fluid to its donor [66]. A proof-of-concept study by Ingold et al. showed promising results when it came to linking a body fluid to its donor [65], although to increase discrimination power more SNPs are needed. A collaborative exercise was organized within the EUROFORGEN / EDNAP groups to test the performance of a 35-plex body fluid identification assay and a 35-plex cSNP assay. The results indicated that cSNPs can provide the investigator with valuable information about the donor / body fluid association [57].

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Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.fsigen.2020.102409.

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