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Advancing forensic RNA typing: On non-target secretions, a nasal mucosa marker, a differential co-extraction protocol and the sensitivity of DNA and RNA profiling

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ABSTRACT

The forensic identification of human body fluids and tissues by means of messenger RNA (mRNA) profiling is a long studied methodology that is increasingly applied to casework samples. Previously, we have described an mRNA multiplex system that targets blood, saliva, semen, menstrual secretion, vaginal mucosa and skin (Lindenbergh et al. and van den Berge et al.) [1–2]. In this study we consider various topics to improve this mRNA profiling system or its use and adapt the method accordingly. Bodily secretions that may be encountered at a crime scene whilst not targeted by the multiplex—*id est* nasal mucosa, sweat, tears, faeces and urine-were examined for false positive signals. The results prompted us to identify a nasal mucosa marker that allows the discrimination of nasal mucosa from saliva or vaginal mucosa and nosebleed blood from peripheral blood. An updated version of the multiplex was prepared to which the nasal mucosa marker was added and in which markers for semen, vaginal mucosa and blood were replaced. *Lactobacillus* markers were regarded unsuitable as replacement for vaginal mucosa mRNA markers because of background signals on penile swabs that appeared devoid of female DNA. Furthermore, we provide approaches to deal with highly unbalanced mixtures. First, a differential extraction protocol was incorporated into a co-extraction protocol to allow DNA and RNA analysis of separated non-sperm and sperm fractions. In a second approach, besides the standard multiplex, a customized multiplex is used which excludes markers for prevailing cell types. This allows the use of lower cDNA inputs for the prevailing cell types and higher inputs for cell types that appear masked. Additionally, we assessed the relation between the percentage of alleles or markers detected in DNA or RNA profiles when decreasing sample amounts are analysed. While blood, saliva, semen and menstrual secretion show the trend that DNA profiling is more sensitive than RNA profiling, the reverse is seen for skin and variable results occur for vaginal and nasal mucosa. Lastly, we show that replicates are useful for interpretation of RNA data, as variations can be found even for true technical replicates. Increased numbers of replicates (over four) do, however, not cancel out the impact of this variation on data interpretation. Overall, the results of this study further forensic RNA profiling.

1. Introduction

Messenger RNA (mRNA) profiling for the purpose of human body fluid and organ tissue identification has been investigated intensively in the last decade [1–11]. The majority of mRNA profiling systems focus on the identification of body fluids most commonly encountered at a crime scene like blood, saliva, vaginal mucosa, menstrual secretion and semen. Other bodily secretions like nasal mucosa and faeces are generally not targeted by mRNA profiling systems as these cell types are less frequently relevant in forensics [10]. Awareness of possible cross-reactivity of mRNA markers to these secretions is, however, important to optimise data interpretation. In case of cross-reactivity, the addition of a marker specific to the cross-reacting cell type may be opportune. Studies describe, for example, false positive signals for vaginal mucosa and saliva mRNA markers in nasal mucosa [12–13]. Thus we assessed the level of cross-reactivity with our marker system and searched for a nasal mucosa-specific marker to aid in the distinction of nasal mucosa, saliva and vaginal mucosa. mRNA markers specific to vaginal mucosa are known to be particularly

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difficult to find as many of the target genes are expressed in a broader range of mucous membrane-enriched areas [14]. A suggested alternative approach for the identification of vaginal mucosa is by use of bacterial markers such as *Lactobacillus* species [15–19]. The suitability of bacterial markers for this purpose has been questioned as they are reported to respond also in non-vaginal samples [20]. Evidentiary samples frequently examined for the presence of vaginal mucosa are penile swabs and fingernail samples. We assessed the presence of *Lactobacillus* species on penile swabs lacking a detectable female DNA source. Furthermore we searched for ways to facilitate mRNA analysis of samples with highly deviating cell type ratios such as sexual assault samples comprising sperm and (female) epithelial cells. Separate analysis of DNA in the non-sperm (NF) and sperm fraction (SF) is commonly achieved by use of differential extraction. We explored the possibility of incorporating co-extraction of RNA into the differential extraction procedure so that next to DNA, RNA of the NF and SF can be analysed separately. In addition, we describe a method to perform differential analysis of RNA extracts post extraction. Over-amplification of markers for prevailing cell types can interfere with the detection of markers for underlying cell types. We assessed for the possibility of generation informative RNA profiles for underlying cell types by analysing markers for prevailing and underlying cell types in separate multiplexes. Furthermore, we examined the relation between the sensitivities of RNA and DNA profiling in single source samples of various donors, which is useful when interpreting both the DNA and the RNA results for a sample. Some of these samples were used to assess the effect of adding RNA profiling replicates in the RNA data interpretation system that uses a $x = n/2$ guideline [29].

2. Materials and methods

2.1. Sample collection

A set of control samples for body fluids and tissues commonly encountered in forensic cases was taken from a previous study [1]. This control set includes blood, saliva, vaginal mucosa, menstrual secretion, semen and skin samplings from four individuals. The four semen samples represent two samples from fertile and two samples from vasectomised donors.

Fresh nasal mucosa samples from 22 individuals were taken from both nostrils, using a single cotton swab per nostril. Seven of these individuals suffered from a cold. A total of 11 nosebleed samples from six donors were collected on tissue paper. Sweat, tear and urine samples from 10 donors were collected on cotton swabs while attempting to avoid skin contact. Each donor contributed all three body fluids. Swabs were air-dried and stored at room temperature in the dark until used. Faeces samplings were taken from specimens that had been stored at −20 °C. A total

Table 1
Primer sequences for the different mRNA markers residing in the Cell-typer (V3) multiplex.

Marker name	Tissue	[primer] μM	Forward primer (5′–3′) Reverse primer (5′–3′)	Size (bp)	Dye	Reference
ALAS2	Blood	0.04	TTCTGCACCAGAAGGACTCAGCC[a] TAAATCTCGCACCCTGGCAGGATC	103	FAM™	[b]
CD93	Blood	0.25	ACCAGTACAGTCCGACAC TTGCTAAGATTCCAGTCCAG	151	NED™	[1]
HBB	Blood	0.035	GCACGTGGATCCTGAGAAC ATGGGCCAGCACACAGAC	61	FAM™	[1]
HTN3	Saliva	0.2	GCAAAGAGACATCATGGGTA GCCAGTCAAACCTCCATAATC	134	VIC®	[1]
STATH	Saliva/nasal mucosa	0.3	TTTGCCTTCATCTTGGCTCT CCCATAACCGAATCTTCCAA	93	FAM™	[1]
BPIFA1	Nasal mucosa	0.2	CAAGTGAATACGCCCCTGGTCG GAATGGGTGCAGTCACCAAGGAC	131	PET™	[b]
KLK3	Seminal fluid	0.05	GACGTGGATTGGTGCTGCACC CTTCTCGCACTCCCAGCCTC	64	PET™	[b]
SEMG1	Seminal fluid	0.8	GGAAGATGACAGTGATCGT CAACTGACACCTTGATATTGG	121	FAM™	[1]
PRM1	Spermatozoa	0.3	AGACAAAGAAGTCGCAGAC TACATCGCGGTCTGTACC	91	NED™	[1]
CYP2B7P1	Vaginal mucosa	0.8[c]	AGTCTACCAGGGATATGGCATG CTATCAGACACTGAGCCTCGTCC	146	VIC®	[2]
MUC4	Vaginal mucosa	0.8	CTGCTACAATCAAGGCCA AAGGGAAGTTCTAGGTTGAC	141	FAM™	[1]
MYOZ1	Vaginal mucosa	0.8	GGGTTGGTGAGACAGGATCA TTTTCCCATGGGGAAATATAGGT	88	VIC®	[b]
MMP7	Menstrual secretion	0.8	GAACAGGCTCAGGACTATCTC TTAACATTCCAGTTATAGGTAGGCC	127	VIC®	[1]
MMP10	Menstrual secretion	0.1[c]	GCATCTTGCATTCCTTGTGCTGTTG GGTATTGCTGGGCAAGATCCTTGTT	107	VIC®	[2]
MMP11	Menstrual secretion	0.4	CAACCGACAGAAGAGGTTCG GAACCGAAGGATCCTGTAGG	76	NED™	[1]
CDSN	Skin	0.6	CTGGCTGGTCTCCTCCTG GGGTCCTTACAAGGGTCTGA	71	VIC®	[1]
LCE1C	Skin	0.02	TGTGACCCCGCTCCTGAATCCG CTTGGGAGGGCACTTGGGGGTG	99	NED™	[2]
ACTB	Housekeeping	0.2	TGACCCAGATCATGTTTGAG CGTACAGGGATAGCACAG	75	PET™	[1]
18S-rRNA	Housekeeping	0.025	CTCAACACGGGAAACCTCAC CGCTCCACCAACTAAGAACG	110	PET™	[1]

[a] Underlined nucleotides are 5′ tails added to improve multiplex spacing.
[b] Developed for this study using Ensembl and NCBI primer blast [25,26].
[c] Primer concentrations lowered compared to [2].