Advancing forensic RNA profiling

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Chapter 9

A male and female RNA marker to infer sex in forensic analysis

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Abstract

In forensics, DNA profiling is used for the identification of the donor of a trace, while messenger RNA (mRNA) profiling can be applied to identify the cellular origin such as body fluids or organ tissues. The presence of male cell material can be readily assessed by the incorporation of Y-chromosomal markers in quantitation or STR profiling systems. However, no forensic marker exists to positively identify female cell material; merely the presence of female DNA is deduced from the absence of a Y peak, or unbalanced X-Y signals at the Amelogenin locus or unbalanced response of the total and Y-specific quantifier. The presence of two X-chromosomes in female cells invokes dosage compensation, which is achieved through inactivation of one of the X-chromosomes in females. Since this process involves specific RNA molecules, identification of female cellular material may be possible through RNA profiling. Additionally, male material may be identified through RNAs expressed from the Y-chromosome. RNAs preferentially expressed in either sex were assessed for their potential to act as sex markers in forensic RNA assays. To confirm sex-specificity, body fluids and organ tissues of multiple donors of either sex were tested. Additionally, sensitivity of the markers and the suitability of positively identifying male-female mixtures were assessed and degraded samples were used to assess performance of the markers in forensic settings. The addition of sex-specific markers is of added informative value in any RNA profiling system and both markers were incorporated into existing RNA assays that either target body fluids or organs. These are the first forensic assays that enable positive identification of female cellular material.

Introduction

In forensics, DNA profiling is generally used for the identification of the donor of a trace, in which the sex (referring to the genetic origin of sex) of the donor is generally deduced from the response of the single copy gene Amelogenin (AMEL) gene that is located on the X and the Y sex chromosomes [1,2]. AMEL-based sex determination methods rely on the simultaneous amplification of differently sized X- and Y-chromosome targets and use the presence or absence of the Y-signals to determine sex [1–3]. The AMEL-Y can drop-out because of low-level DNA input or primer binding site mutations which can lead to misinterpretation of males as females [1–4]. The most recently developed STR (Short Tandem Repeats) kits circumvent this issue as they carry additional Y-chromosomal STRs markers [3,5–7]. There are, however, currently no forensic markers available to positively identify female cell material. Basically the absence or relatively low peak height of the AMEL-Y or Y-STR signals is used to determine female sex [1–3,5], which is more difficult to apply in male-female mixtures. On the DNA level the X-chromosome can therefore not be used to distinguish between female and male DNA. Female-specific markers may, however, be found on RNA or protein level as certain cellular processes are specific to females. Since forensic analyses may involve all kinds of body fluids or organ tissues, we aim for a process occurring in all diploid female cells such as the process leading to X-chromosome inactivation. In mammals, the inactivation of one X-chromosome provides functional X-chromosomal dosage equivalence between XY males and XX females. Identification of female cellular material may therefore be possible via targeting cellular products involved in X-chromosome inactivation. Since X-chromosome inactivation is an RNA-induced process (see the description of the process below), RNA markers seem more promising than protein-based markers.

Messenger RNA (mRNA) profiling has been increasingly applied in the last decade for the inference of body fluids [8–15] and organ tissues [16,17] residing in an evidentiary trace. RNA profiling is generally performed alongside DNA profiling via DNA/RNA co-extraction of a stain. mRNA profiling, however, currently lacks the possibility of providing information regarding the donor of a trace. The incorporation of sex-biased genes into presently employed mRNA profiling systems could provide additional information concerning the donors' sex. Female- and male-specific RNA markers have previously been described for use mainly in evolutionary biology, medical- and clinical genetics [18,19] such as to examine sex differences in cancer genetics [20,21], neuropsychiatric disorders [22] or human diseases [20,23]. In forensics, no applications have been described.

For this study, two markers with global sex-biased expression [24] were assessed, namely XIST and RPS4Y1. XIST (X Inactive Specific Transcript), the candidate female RNA marker, is a long non-coding RNA (lncRNA) involved in X-chromosome

inactivation (XCI) [25]. This process leads to the random silencing of one of the two X-chromosomes in females, resulting in an active and an inactive X (Xa and Xi, respectively), in order to provide X-chromosome dosage equivalence in males and females [26–28]. XCI is controlled by the X-chromosome inactivation centre (Xic) which is involved in counting the number of X-chromosomes to be inactivated, selecting and initiating the XCI and the in cis spread of the inactivation on the entire Xi [29,30]. The Xic contains four non-translated RNA genes, namely JPX, FTX, XIST and TSIX [27,28]. Both JPX and FTX escape XCI and show a female-specific role in the up-regulation of the expression of XIST [31,32]. Expression of these two genes, however, appears not fully female-specific [28,33]. XIST expression is initiated in early embryogenesis and is expressed exclusively from the Xic of the Xi. XIST RNA is not translated, although it is spliced, capped and polyadenylated. The largest human XIST transcript is 19 kb and remains in the nucleus where it "coats" the Xi to inactivate the chromosome. Males do not have dosage compensation, which makes XIST expression female-specific [34]. XIST transcription in male cells has only been described in the germ cells prior to meiosis [30,35]. The other marker candidate, TSIX, is transcribed antisense to XIST and is a negative regulator of XIST. TSIX is therefore expressed prior to inactivation and from both X-chromosomes; its expression is repressed once inactivation is established [29,30]. Consequently, XIST was the selected female marker candidate in this study.

The majority of male-specific genes are located on the Y-chromosome, such as DBY, SMCY, UTY, RPS4Y, and USP9Y [22,24,36]. Y-chromosome encoding ribosomal protein RPS4Y1 (Ribosomal protein S4, Y-linked 1) [36,37] was selected, as this gene is described to have the highest average expression difference between females and males [24] and is ubiquitously expressed in human tissues [36,37].

In this study we assessed the functionality of incorporating these sex-specific RNA markers in currently employed mRNA multiplexes [15,17], which can provide new possibilities in forensic analyses.

Materials and methods

Sample collection

Primer concentration optimization of the sex markers was performed using a set of control samples originating from the FirstChoice® Human Total RNA Survey Panel (Ambion® by Life Technologies, Carlsbad, USA). This panel contains total RNA pools of at least three donors for various human tissues and details on donor sex are provided by the manufacturer. The samples selected for use in this study contained material of solely male or solely female donors, *i.e.* colon, prostate, trachea, testes and spleen of

male donors and cervix, skeletal muscle, placenta and ovary of female donors. RNA pools come in a concentration of 1 µg/µL and they were processed so that after reverse transcription a cDNA input corresponding to 5 pg RNA was used in the PCR (which is also the amount of human RNA used to optimise the Organtyper [16]).

Single donor samples from various body fluids and organ tissues previously described in Refs. [15–17] were used to confirm sex-specificity of the candidate markers. These samples include 30 single donor body fluids from ten male and 20 female donors comprising four samples each for blood, saliva, vaginal mucosa, menstrual secretion, fertile semen, skin, nasal mucosa, plus two sterile semen samples obtained from vasectomised donors. The organ tissue sample set includes a total of 21 single donor autopsy organ tissues comprising brain, lung, liver, skeletal muscle, heart, kidney and skin tissues of one male and two female donors.

Mixed samples were analysed to confirm detection of both sex markers in case of male-female mixtures and to assess marker drop-out of the two sex-specific markers when the male-female ratios are unbalanced. This set includes seven blood:blood mixtures with varying inputs of male and female contribution, *i.e.* male-female ratios of 1:0, 0:1, 1:1, 1:½ , ½:1, 1:⅕ and ⅕ :1. The mixtures were prepared by combining cDNAs of single donor male and female blood extracts, the described ratios do therefore not relate to DNA ratios.

The applicability of the sex markers to (severely) degraded samples was assessed by analysing a set of ten exhumed organ samples. These samples have previously been used in a study which aimed to search for trends in nucleic acid degradation and the post mortem interval [17]. This exhumed organ sample set comprises ten organ tissue samples originating from four females and six males which had been exhumed after burial times ranging from four to over 40 years.

Lastly, the added value of the sex-specific RNA markers in forensic settings is presented through the analysis of a male nosebleed sample. This sample has been used in a previous study [15] and could then, based on mRNA profiling results, not be specified as such due to co-expression of various body fluid markers [15].

The autopsy and exhumed organ specimens have been used in previous studies and carry the necessary approvals [16,17]. The body fluid samples used for this study were collected with informed consent of the voluntary donors whose cell material was used.

Marker selection and primer design

Sex-specific markers were selected from RNA sequencing data generated by the Genotype-Tissue Expression (GTEx) project as described in Ref. [24]. Two markers, namely between the two sexes XIST (female) and RPS4Y1 (male), showing highest average expression differences in females versus males were selected from a list of

global sex-biased expressed genes [24].

XIST and RPS4Y1 primer sets were developed using Ensembl and NCBI primer blast [38,39], with at least one of the primers covering an exon–exon junction. Primers were designed in such a way that amplification products would fit in two existing mRNA profiling multiplexes, namely the Cell-typer [15] and Organtyper [16,17] multiplexes. Details on primer sequences of the two sex markers are shown in Table 1.

Preliminary tests were performed to assess marker perfor- mance, fragment sizing and optimise primer concentrations. Initially, both sex markers and housekeeping marker 18S-rRNA [16,17] were combined in a triplex and applied to samples containing material of solely male or female donors (FirstChoice® Survey Panel, see "Sample collection" section). Primer concentrations of the sex markers were optimized by assessing a range of concentrations (0.2, 0.4 and 0.8 µM) while 18S-rRNA remained in its standard concentration (0.015 µM [9,17]). The optimal primer concentration for both XIST and RPS4Y1 was set on 0.2 µM, after which both sex markers were combined into the Cell-typer [15] and the Organtyper [17] multiplex.

Table 1 Primer sequences for the two sex-specific markers.

Marker name	Tissue	[primer] µM	Forward primer (5'-3') / Reverse primer (5'-3')	Size (bp)	Dye
XIST	Female	0.2	ATTTTAACTGATCCCATTGAAGATACCACGC [a]	83	
			TCAGAATGTCCAAGAGGAGCCTAAGG		PET™ [b]
RPS4Y1	Male	0.2	TGGAAGAGGCAAAGTACAAGTTGTGC	63	NED™
			GGATTCCCTTCACTCCCACAGTAAT		

[a] Underlined nucleotides are 5' tails added to obtain the desired product size.
[b] Reverse primer labelled.

RNA profiling

DNA/RNA co-extraction, DNase treatment, DNA quantification, ethanol precipitation and reverse transcription were performed as described in Ref. [8]. RNA extracts were ethanol-precipitated prior to reverse transcription when the total DNA yield of a sample was below 1 ng and processed as described in Ref. [9].

The reverse transcription protocol was adjusted when it became apparent that sex marker XIST was poorly amplified in the Cell-typer [15] and Organtyper [17] multiplexes. To this aid, an unlabelled version of the XIST reverse primer presented in Table 1, referred to as XIST RT-primer, was added during the first step of the reverse transcription protocol, in which Random Decamers (5 µM per reaction) are combined with RNA extracts and denatured. Random Decamers and XIST RT-primer concentrations were optimized to a final concentration of 4.7 µM and 0.625 µM per reaction, respectively (after considering a range of 2.5–4.8 µM Random Decamers and 5–0.3 µM XIST RT-primer). The input of RNA extract (maximum 10 µL) remained unadjusted. No negative effects were observed by this change in protocol, while the

average peak height of XIST signals was boosted an approximate fivefold (data not shown).

PCR amplification and product detection for all RNA analyses were performed according to standardized protocols [8]. PCR products were purified [8] prior to detection via a 3130XL Genetic Analyzer (Life Technologies). Purified amplification products were analysed using POP-4 separation matrix (Life Technologies) and 3 kV, 10 s injection settings. Profile analysis was performed using Genemapper ID-X version 1.1.1 (Life Technologies) with a detection threshold of 150 relative fluorescence units (rfu).

RNA data interpretation was performed according to the "$x=n/2$" guidelines and four PCR replicates per sample as described in [40]. This method compares the number of observed (x) to the number of theoretically possible peaks (n) in all replicates. A cell type is scored "observed" when at least half of the possible peaks are observed ($x \geq n/2$), denoted "sporadically observed" when less than half of the possible peaks are observed ($0 < x < n/2$) and scored "not observed" when no peaks are detected ($x = 0$).

DNA profiling

DNA profiles were generated using the AmpFℓSTR® NGM™ Amplification Kit (Life Technologies) using a maximum of 500 pg DNA input based on quantification as described in [8]. PCR products were separated according to standardized protocols [8] using a 3130XL Genetic Analyzer (Life Technologies) with POP-4 (Life Technologies) separation matrix. Profile analysis was performed using Genemapper ID-X version 1.1.1 (Life Technologies) using a detection threshold of 50 rfus.

Results and discussion

Specificity

After selection of one candidate sex-specific RNA marker for each sex, primer sets were designed and tested on a set of control samples (sections "Sample collection", "Marker selection and primer design") with positive results. The assay conditions were optimized (sections "Marker selection and primer design", "RNA profiling") after which both markers were incorporated into the Cell-typer and Organtyper multiplexes (now referred to as Sex-Cell-typer and Sex-Organ- typer). Sex-specificity of the markers was assessed using single donor body fluids (n=30, four technical replicates each) and organ tissues (n=21, four technical replicates each). Single donor contribution was confirmed for all samples via NGM profiling, except the semen donations from vasectomised males, that lack spermatozoa and DNA (data not shown).

An overview of the specificity results using the single donor body fluids and organ tissues is shown in Table 2. Female sex was correctly scored "observed" in 97% of

the female samples (one sample scored "sporadically observed") and male sex was correctly scored "observed" in 94% of the male samples (one sample resulted in a "not observed" scoring). This includes the detection of the male sex marker in semen samples from vasectomised donors, for which no DNA-profile could be obtained using standard NGM profiling (data not shown). The detection of RPS4Y1 in azoospermic semen can be explained as RPS4Y1 is a ribosomal RNA marker, expression is therefore not restricted to the spermatozoa, and is thus also expressed in seminal fluid (alike the KLK3 and SEMG1 marker included in the Cell-Typer [15]). Furthermore, no incorrect "observed" scorings of female marker XIST in male samples or male marker RPS4Y1 in female samples occurred, indicating the markers are sex-specific with no false positive scoring.

Table 2 **XIST and RPS4Y1 RNA profiling results after analysing single donor body fluids (*n*=30) and organ tissues (*n*=21). Interpretation of the sex markers is performed using the "*x*=*n*/2" guidelines and four PCR replicates per sample. Shown are the number of female and male samples analysed for the specified body fluids and organ tissues. (Light) green marking indicate the correct "(sporadically) observed" scoring of markers, missed marker signals are indicated by a grey marking.**

Single donor	Female donor (*n*)	Male donor (*n*)	XIST correctly "observed"	XIST "sporadically observed"	RPS4Y1 incorrectly "observed"	RPS4Y1 correctly "observed"	RPS4Y1 "sporadically observed"	XIST incorrectly observed
Body fluids			Female samples			Male samples		
Blood	3	1	100%			100%		
Saliva	3	1	100%			0%		
Vaginal mucosa	4	-	100%			-		
Menstrual secretion	4	-	100%			-		
Semen*	-	6	-			100%		
Skin	3	1	100%			100%		
Nasal mucosa	3	1	100%			100%		
# samples	20	10						
Organ tissues			Female samples			Male samples		
Brain	2	1	100%			100%		
Lung	2	1	100%			100%		
Liver	2	1	100%			100%		
Skeletal muscle	2	1	100%			100%		
Heart	2	1	100%			100%		
Kidney	2	1	50%	50%		100%		
Skin	2	1	100%			100%		
# samples	14	7						

*Four fertile semen and two sterile semen samples.

Example overlay RNA profiling electropherograms after analysing single donor body fluids and organ tissues are presented in Figure 1. Shown is the positioning of the sex markers in the Sex-Cell- typer and Sex-Organtyper multiplexes and the correct detection of the sex markers in male and female samples. It has been described in medical research, such as cancer studies, that expression of XIST is correlated with the development of some cancers [20,21,35], leading to the detection of female marker XIST in male cancer cells. In forensic scenarios this may be relevant, for example when bullets are analysed that potentially perforated cancerous organ tissues.

To investigate this cross-reactivity a set of 28 biopsy samples, most likely holding cancer cells as they are taken from patients in hospitals, was analysed using the Sex-Organtyper multiplex. These biobank samples include 14 male and 14 female donors divided over four samples each for brain, lung, liver, skeletal muscle, heart, kidney and skin tissue. Male marker RPS4Y1 was detected, as expected, only in samples obtained

from male donors. Female marker XIST was detected in 50% of the male samples, which, based on four technical replicates per sample, resulted in an "observed" scoring for female cells in 43% of the analysed male donor samples and the "sporadically observed" scoring in one male sample (data not shown). This may be explained by the dysregulation of XIST in cancer cells, for which the expression of long, abundantly expressed non-coding transcripts is described to be altered [21]. Unfortunately, we cannot retrieve the detailed information on which sample was positively described to be cancerous.

Mixture analysis

Next, a set of blood:blood mixtures (n=7, four technical replicates each) with varying contributions of male and female material were analysed using the Sex-Cell-typer multiplex to confirm detection of both sex markers in case of male-female mixtures and to assess marker drop-out of the two sex-specific markers when the male-female ratios are unbalanced. Illustrative electropherograms are presented in Figure 2 and show the detection of male marker RPS4Y1 in each of the mixtures containing male RNA, and female marker XIST detection in samples containing female RNA. As can be seen from the varying signal heights of the male and female marker, sample input does not go hand in hand with peak height. This befits the use of 33 amplification cycles and has previously been described in Ref. [41].

Degraded samples

The performance of the sex markers on degraded samples was assessed on a set of exhumed organ tissues [17]. Using the Sex- Organtyper multiplex, sex was correctly scored "observed" for 80% of the samples; the remaining two samples resulted in a "sporadically observed" scoring. This includes the positive infer- ence of sex for a skin sample which had been buried up to 40 years, indicating that the sex markers can successfully be applied to degraded samples. Again, no false positive scorings occurred (Table 3).

Application in casework

The added value of the sex-specific RNA markers in forensic casework becomes clear when applied to a sample in which, without sex markers, the combination of detected mRNA markers could lead to incorrect assumptions. Recently, the detection of vaginal mucosa markers, normally linked to the presence of female cell material, has been described in expirated blood, nosebleed blood and nasal mucosa samples, regardless of the sex of the donor [15]. For example, when analysing a male donor

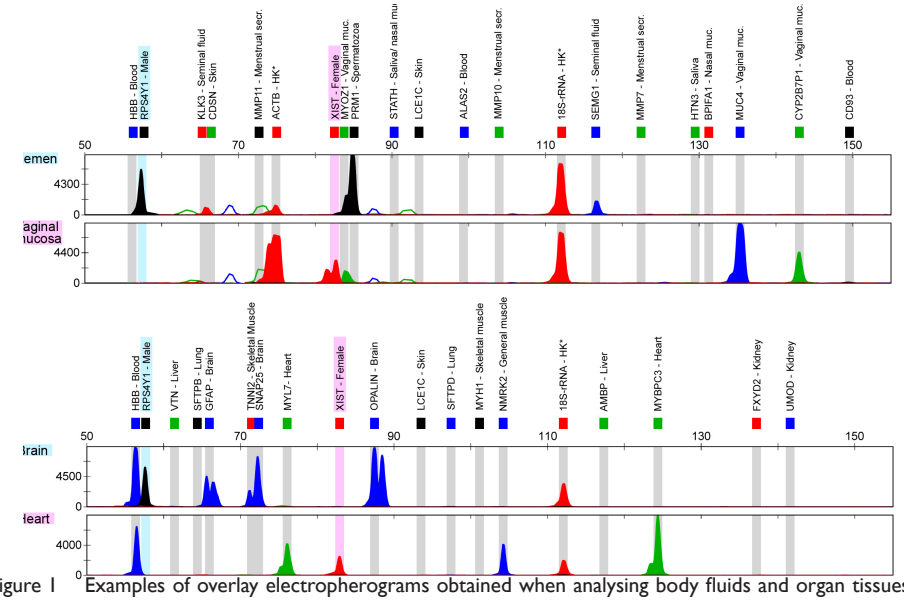

Figure 1 Examples of overlay electropherograms obtained when analysing body fluids and organ tissues from a single donor of either sex using the Cell-typer and Organtyper multiplexes supplemented with a male and a female sex marker. Highlighted in light-blue are the male sex marker RPS4Y1 and the male samples. The female marker and female samples are indicated by a pink highlight. *HK= housekeeping.

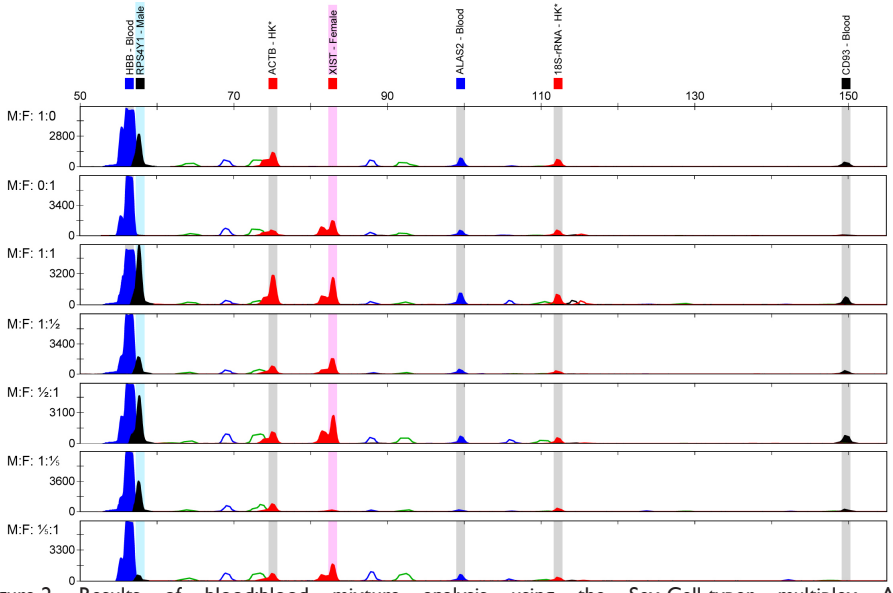

Figure 2 Results of blood:blood mixture analysis using the Sex-Cell-typer multiplex. A series of samples with varying ratios of male:female (M:F) contributions were analysed to assess the markers' suitability to detect both markers simultaneously. Highlighted in light-blue are the male sex marker RPS4Y1 and the male samples. The female marker XIST and female samples are indicated by a pink highlight. *HK= housekeeping. Only the bins for the responding RNA markers are shown.

Table 3 XIST and RPS4Y1 profiling results after analysing exhumed human organ tissues (*n*=10), which had been buried between 4 and up to 40 years. For three of the samples the exact burial times were unknown (# years buried indicated by "-"). Interpretation of the sex markers is performed using the "*x*=*n*/2" guidelines and four PCR replicates per sample. Green marking indicate the correct scoring of the target marker, light green markings indicate the "sporadic" detection of the target marker. No non-target markers were detected in non-target tissues (indicated in green).

Degraded samples	# years buried	Sex	Correctly "observed"	"Sporadically observed"	Incorrectly "observed"
Exhumed tissues			Target marker		Non-target
Kidney	4y6m	M	Y		N
Liver	4y10m	M	Y		N
Brain	5y2m	M	Y		N
Liver	5y6m	M		Y	N
Kidney	6y11m	M	Y		N
Brain	30y4m	F	Y		N
Skin	40y4m	F	Y		N
Heart	-	M	Y		N
Skin	-	F		Y	N
Skeletal muscle	-	F	Y		N

*Y= yes, N=no

nosebleed sample, blood, saliva, vaginal mucosa and menstrual secretion markers are detected, as shown in Figure 3A. A nasal mucosa marker has previously been developed and incorporated in an updated version of the Cell-typer multiplex [15] to aid in the distinction of a nosebleed samples from a mixture of blood, saliva and vaginal mucosa (Figure 3B). The addition of sex markers now confirms the male sex of the donor (Figure 3C). Thus, the addition of sex-specific RNA markers aids the inference that the sample involves a nosebleed sample from a male donor rather than a mixture of cells from a male and a female donor.

Concluding remarks

This study describes the incorporation of sex-specific RNA markers in currently employed forensic mRNA profiling multiplexes to simultaneously identify the sex of the donor and the cell type of the material in an evidentiary trace. The use of sex-specific markers has been described by various studies, mostly in medical context, but is new to forensics [18,22,23]. The Sex-Cell-typer and Sex-Organtyper multiplexes were successfully applied to single donor samples, mixtures and degraded samples. No false positive results were obtained, except for the XIST marker in biopsy samples that are likely tumorous. Thus, when cell material derives from an individual carrying cancerous cells in the targeted tissue, the data regarding the female marker should be interpreted carefully as long non-coding RNAs, such as XIST, are known to have dysregulated gene expression in tumour cells. We realise, however, that the health status of individuals involved in a crime will often be unknown. Additionally, certain syndromes such as Klinefelter (*e.g.* 47,XXY) and Triple X syndrome (47,XXX) will lead

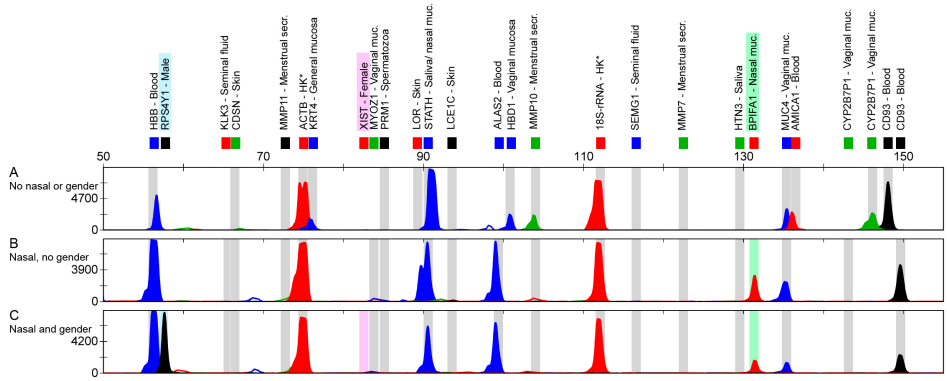

Figure 3 The added value of the Sex-Cell-typer multiplex shown by the analysis of a male nosebleed sample using three versions of the Cell-typer multiplex. A: Analysis using the Cell-typer multiplex as described in Ref. [9]. Results indicate a blood, saliva, vaginal mucosa, menstrual secretion mixture, which cannot be classified as a male nosebleed sample due to the absence of nasal mucosa and sex markers. B: The addition of a nasal mucosa marker (Ref. [15]) allows for the classification as a nosebleed sample. C: The addition of sex-specific RNA markers allows for inference of a nosebleed sample originating from a male donor. (Note that some markers have been replaced or spaced differently during the development of the multiplexes. Presence of bins under marker names indicate the presence of the respective marker per analysis.)

to the inactivation of multiple X-chromosomes and therefore increased XIST expression, while females with Tuner syndrome (45,X) will show no XIST expression as no X-chromosome inactivation occurs [42,43]. Syndromes due to skewed X-chromosome inactivation (non-random choice regarding which X is inactivated) are not expected to affect XIST marker detection [44]. The incorporation of sex-specific RNA markers provides the first overlapping information in DNA and RNA profiles.

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