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A multiplex (m)RNA-profiling system for the forensic identification of body fluids and contact traces

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ABSTRACT

In current forensic practice, information about the possible biological origin of forensic traces is mostly determined using protein-based presumptive testing. Recently, messenger RNA-profiling has emerged as an alternative strategy to examine the biological origin. Here we describe the development of a single multiplex mRNA-based system for the discrimination of the most common forensic body fluids as well as skin cells. A DNA/RNA co-isolation protocol was established that results in DNA yields equivalent to our standard in-house validated DNA extraction procedure which uses silica-based columns. An endpoint RT-PCR assay was developed that simultaneously amplifies 19 (m)RNA markers. This multiplex assay analyses three housekeeping, three blood, two saliva, two semen, two menstrual secretion, two vaginal mucosa, three general mucosa and two skin markers. The assay has good sensitivity as full RNA profiles for blood, semen and saliva were obtained when using $\geq$0.05 μL body fluid starting material whereas full DNA profiles were obtained with $\geq$0.1 μL. We investigated the specificity of the markers by analysing 15 different sets of each type of body fluid and skin with each set consisting of 8 individuals. Since skin markers have not been incorporated in multiplex endpoint PCR assays previously, we analysed these markers in more detail. Interestingly, both skin markers gave a positive result in samplings of the hands, feet, back and lips but negative in tongue samplings. Positive identification (regarding both DNA- and RNA-profiling) was obtained for specimens stored for many years, *e.g.* blood (28 years-old), semen (28 years-old), saliva (6 years-old), skin (10 years-old) and menstrual secretion (4 years-old).

The described approach of combined DNA- and RNA-profiling of body fluids and contact traces assists in the interpretation of forensic stains by providing information about not only the donor(s) that contributed to the stain but also by indicating which cell types are present.

1. Introduction

Next to DNA-typing results, knowledge about the cellular origin of crime-related biological stains can be of significant importance for the reconstruction of the events at a crime scene. Conventional methods of body fluid identification, like the tetramethylbenzidine test, hexagon-obti and RSID-blood for blood stains [1,2], the PSA and semenogelin test for semen [3] and the amylase tests (Phadebas or RSID-saliva) for saliva [4] are protein or enzyme-based, presumptive in nature and not always human-specific. Most of these methods rely on a colour-forming reaction which can be difficult to interpret, especially when dealing with coloured extracts or samples containing very little amounts of target material.

Within forensic genetics, messenger RNAs (mRNAs) have increasingly gained popularity regarding their potential to distinguish between human body fluids and other forensically relevant tissues [5–10]. Alternative methods for cell-typing include tissue-specific miRNAs, DNA methylation [11,12] and microbial markers [13]. miRNAs are small (20–24 nucleotides) regulatory RNAs which are strongly associated with members of a class of proteins called Argonautes [14], which makes them very stable and advantageous when dealing with degraded forensic samples [15,16]. Also epigenetic DNA methylation markers have been described that can differentiate between some tissue types [11,12]. Both miRNA and DNA methylation markers seem promising, but still in its infancy as for instance more markers are needed to discriminate the forensic range of body fluids. The use of microbial markers has been suggested for the identification

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of especially vaginal mucosa [13,17–19]. However, it is not yet established whether the same microbes also occur on skin surfaces that are in close proximity of, or in contact with the vaginal microbial flora, such as skin surfaces of the hands, groin or penis. For these reasons, we regard tissue-specific mRNA analysis as the most versatile cell-typing approach.

mRNA-profiling has evolved from a singleplex PCR technique to a multiplex RT-PCR platform, providing expression of data on multiple genes simultaneously. mRNA-profiling is readily combined with DNA-genotyping since RNA and DNA can be obtained from the exact same sample [6,9,20,21]. The different multiplexes that have been developed include markers for venous blood, saliva, semen, vaginal epithelia and menstrual secretion [9,10,13], and their selection was mainly based on the function described in literature [9,10] or the tissue-specific expression as reported in expression databases [22]. Dedicated whole-genome expression array analysis in samples from forensically relevant body fluids that were stored for various time intervals has previously shown to deliver stable mRNA markers useful for forensic tissue identification [23,24]. Skin is an additional forensically important cell type. Recently three mRNA transcripts (*LOR*, *CDSN* and *KRT9*) were reported to show high expression in skin samples relative to other forensically relevant cell types [25]. The addition of skin markers to an RNA-based cell-typing multiplex would increase the practical forensic value of the assay for two reasons: (1) a more complete view on all cell types present in an evidentiary trace is established which is important because skin cells are expected to occur in many crime scene samples and (2) an indication for the presence of contact DNA can be obtained. The most often used method to show the presence of contact traces is through dactyloscopic fingerprint analysis but also other microscopical and immunocytological techniques [26] have been described which can identify skin cells. Fingerprint visualisation methods do not apply to all types of substrates, and some can have negative effects on the nucleic acids in the skin cells [27] while others can introduce contamination [28]. To efficiently and objectively assess the biological origin of a forensic evidentiary trace, a single profiling assay was developed that assays both the forensically relevant body fluids and skin cells.

2. Methods

2.1. Sample collection

Body fluids and tissues from eight individuals were collected with their informed consent. Ten, 5, 1 and 0.5 μL blood, semen and saliva were collected on cotton swabs (Deltalab, Barcelona, Spain). The 1/10, 1/20, 1/100 and 1/1000 body fluid dilutions were prepared in phosphate buffered saline. Blood was collected through a finger prick (Accu-chek, Softclix Pro, Roche Diagnostics GmbH, Germany). Vaginal mucosa and menstrual secretions (day two of the menstrual cycle) were collected on cotton swabs. Skin samples were collected from the palm of the hand, the middle of the back and on the sole of the foot by means of a damp cotton swab or omniswab (Whatman Inc, United Kingdom). Additional skin samples from fingers were obtained by fiercely rubbing a piece of cotton for 15 s and by tape lift according to de Bruin et al. [29]. Mixtures of skin and saliva were collected by swabbing a plastic cup after a drink simulation and by swabbing the palm of the hand after transfer of 10 μL saliva. Mixtures of blood and skin were obtained by swabbing finger prick areas. All samples were dried overnight at room temperature and processed immediately or stored at −80 °C.

2.2. Aged stains

Blood and semen were spotted on cotton cloth and stored at room temperature. After 28 years, areas of ~0.5 cm^2 in size were cut out for analysis (courtesy A.D. Kloosterman). Used items like jewellery, musical instruments and pacifiers that were left untouched at room temperature for 6–20 years, were swabbed according to standard procedures using a water-hydrated swab. Other samples include stamps on postal cards stored for 3–12 years, buccal swabs stored for 10 years and menstrual secretion stains stored for 4 years, all kept dry and dark at a constant humidity and at room temperature.

2.3. DNA/RNA co-isolation

In order to deviate as little a possible from the current certified in-house procedures for DNA isolations, a DNA/RNA co-isolation protocol was developed incorporating the widely used QIAamp DNA mini Kit (QIAGEN Benelux B.V., Venlo, The Netherlands) and the *mir*Vana miRNA Isolation Kit (Applied Biosystems™, Ambion®, Austin, TX, USA). Cells were lysed in 300–600 μL Lysis Binding Buffer (*mir*Vana miRNA Isolation Kit, Ambion® (MRIKA)) plus 20–40 μL proteinase K (20 mg/mL, QIAamp DNA mini Kit, Qiagen (QDKQ)) and incubated for 2 h at 56 °C. Following addition of 1/10 volume homogenate additive (MRIKA) and 10 min incubation on ice, the lysate was transferred to a shredder column (QIAGEN Benelux B.V., Venlo, The Netherlands) to separate the carrier material from the lysate. DNA was separated from the RNA by transfer of the lysate to a QIAamp DNA column and 1 min centrifugation at 8000 rpm. The RNA containing flow-through was processed first because of the fragile nature of RNA while the DNA columns were stored at 4 °C until further processing. Next, 300–600 μL phenol/chloroform (pH 4.5, MRIKA) was added to the RNA fraction, mixed for 1 min and centrifuged for 5 min at 10,000 rpm. The aqueous phase was carefully collected and absolute ethanol was added to a final concentration of 55.5%. The lysate/ethanol mixture was applied to an RNA filter cartridge (MRIKA), centrifuged ~15 s at 10,000 rpm and washed once with 700 μL wash buffer 1 (MRIKA) and twice with 500 μL wash buffer 2/3 (MRIKA). The RNA was eluted in 60 μL of nuclease-free water (Ambion®) pre-heated to 95 °C.

The DNA columns were washed once with 500 μL wash buffer 1 and 500 μL wash buffer 2 (QDKQ). The DNA was eluted in 100 μL of a 25% AE solution (QDKQ), pre-heated to 70 °C.

Standard DNA extractions were performed using the QIAamp DNA mini Kit (QIAGEN Benelux B.V., Venlo, The Netherlands) according to the manufacturer's protocol.

2.4. DNase treatment

The RNA extracts were treated with 4 U DNase (TURBO DNA *free*™, Ambion®) for 30 min. DNase was inactivated using Inactivation Buffer (TURBO DNA *free*™, Ambion®) according to the manufacturer's protocol.

2.5. RNA integrity

RNA integrity was assessed by an Agilent 2100 Bioanalyzer using the RNA 6000 pico or the 6000 nano kit according to the manufacturer's protocol. For both kits, 1 μL of RNA was used.

2.6. cDNA synthesis

cDNA was synthesized using the RETROscript® Kit (Ambion®) in a total volume of 20 μL. The reaction contained a maximum volume of 10 μL RNA extract, random decamers (5 μM), RT-buffer (1×), dNTPs (0.5 mM of each), RNase Inhibitor (10 U) and MMLV-RT (100 U). For each sample a negative control was included to which no MMLV-RT was added. The Quantifiler® human genomic DNA (gDNA) quantification was used as an indicator to determine input in the cDNA synthesis reaction. While the amount of genomic DNA in a