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Implementation of RNA profiling in forensic casework

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ABSTRACT

An essential aspect for forensic methods is the prevention of cognitive (confirmation, expectation or motivational) bias. While implementing RNA profiling in casework, we developed a stepwise procedure for unbiased assessment in which: (1) the RNA researcher who generates DNA/RNA fractions and performs RNA profiling, remains uninformed about the context of the case and (2) presents RNA profiling results that are derived by clear guidelines in a results table that uses six different scoring categories, (3) the DNA fractions are processed and analysed by DNA analysts following the standard routine after which (4) reporting officers interpret the DNA profiles and establish the relation to the RNA results which is succeeded by (5) collating all generated results in the case and formulating conclusions in expert reports. The scoring guidelines and results table have a general purpose and can apply to any RNA multiplex. This procedure was applied in a comparative study encompassing seven mock cases designed to be especially interesting for body fluid identification by RNA profiling. Samples were prepared in duplicates and subjected to either presumptive testing combined with standard DNA typing or RNA/DNA co-extraction followed by RNA and DNA profiling. For all cases, the results from presumptive testing and RNA profiling agreed to the level of details the tests can give and concordant DNA results were obtained. RNA profiling was especially useful when (1) menstrual secretion and peripheral blood needed to be distinguished, (2) presence of vaginal mucosa was questioned or (3) presence of skin cells was informative. For forensic reports, we propose to use sets of hypotheses evaluated by the conclusions obtained with DNA and RNA analyses.

1. Introduction

Recent developments in forensic mRNA profiling systems have allowed the simultaneous inference of a variety of human cell types from small amounts of samples [1–4]. In addition to body fluids such as blood, semen, saliva, menstrual secretion and vaginal mucosa, the presence of skin cells can also be determined [4–7]. Since more cell types can be examined, RNA profiling complements the existing detection methods of body fluids which are mainly serology-based and presumptive in nature. In forensic genetic analyses, the highest priority often goes to establishing the possible contributor(s) of DNA to an evidentiary trace. As a consequence, RNA profiling is incorporated into a DNA/RNA co-assessment strategy that generates both a DNA and an mRNA profile from the same stain [4]. Next, RNA profiling needs to be implemented in forensic casework, which involves assessing: (1) when RNA profiling is opportune (also considering the time, costs and laboratory organisation that is needed), (2) how unbiased interpretation of results is warranted [8] and (3) how RNA results can be explained in expert reports to the judiciary.

The organisation of the process at a forensic laboratory determines when the decision to perform RNA profiling is to be taken. When RNA is isolated using a DNA column flow-through [9] these fractions can be collected and stored until RNA profiling is required. When RNA/DNA co-isolation occurs *via* a distinct extraction procedure [4], the decision for RNA profiling needs to be made in advance. Both the markers incorporated in the RNA test and the forensic question determines the added value of RNA profiling over conventional presumptive tests.

The analysis of RNA profiles requires different expertise to DNA profile interpretation as mRNA profiles exhibit features not commonly observed in DNA profiles. Firstly, RNA amounts vary between cell types. In addition, peaks for distinct markers for the same body fluid differ in heights (or may drop out) due to the different expression levels for the specific mRNAs and to the regulation of mRNA by biological, physiological or environmental factors (for instance in case of a vasectomy no spermatozoa-specific signals will be observed). This imbalance and the absence of an accurate human-specific RNA quantification system to regulate RNA input can result in over-amplified peaks, bleed-through signals and amplification artefacts. Also, dye blobs are

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more frequently observed in these non-commercial RNA profiling multiplexes. Furthermore, variable levels of degradation may be present for these single-stranded RNA molecules due to different degrees of intra molecular base pairing (facilitated by A–U, G–C and G–U pairing). Using this knowledge and experience, RNA researchers are able to correctly infer which body fluids are present in the analysed sample. The coherence between the DNA and RNA profiling results for an evidentiary trace is established by a reporting officer who also regards the forensic case in its entirety. Finally, the conclusions are translated to a court report to be used by the judiciary.

In this paper we propose a procedure that accommodates unbiased analysis and interpretation of RNA profiles. Furthermore we compare the results of seven mock casework samples upon analysis by standard methodology (involving presumptive testing) and a DNA/RNA co-profiling approach. Finally, we contemplate on how to formulate conclusions in expert reports.

2. Methods

2.1. Mock case sample preparation

Mock case traces were prepared to mimic crime scene biological evidence. The researchers who prepared the samples were not involved in sample analysis. Donors gave informed consent and presented reference DNA profiles. Mock case 1 consisted of a dried menstrual secretion stain on underpants. Mock case 2 involved female underpants worn after sexual intercourse with a vasectomised male individual. For mock case 3, peripheral blood from the female donor was added to an excised part of the same item used for mock case 2. Peripheral blood from the donor was collected after a finger prick (Accu-chek, Softclix Pro, Roche Diagnostics GmbH, Germany). For mock case 4, a vaginal mucosa sample from a donor was collected using a dry cotton swab (Deltalab, Barcelona, Spain) and transferred to fingernail clippings of a male donor. For mock case 5, the arm of a male donor was licked by a female donor and the licked area was sampled using the double swab technique [10]. For mock case 6, cell material from a vaginal swab (collected with a dry swab) was transferred to a skin swab (collected with a water-moistened swab) of the same female by rubbing the swabs together. To this swab (containing a combination of skin cells and vaginal material), 5 μL of semen sampled from a fertile donor was added. For mock case 7, two male donors rubbed a piece of textile extensively. This was followed by the addition of 2.5 μL of blood donated by one of these males and 2.5 μL blood taken from of a third different male. The blood samples were placed as adjacent spots on the fabric. Each blood spot was excised and processed as a separate evidentiary trace. A summary of the design of each mock case and the hypothetical research question underlying case design can be found in Table 1.

Fingernail samples were prepared in duplicate, swabs were halved and textiles split in two apparently equal portions after which all were stored at room temperature until required. One duplicate was used for conventional analysis (presumptive testing and DNA typing) and the other duplicate was submitted to RNA/DNA profiling. Clearly the duplicates will not be exact replicates and this fact may affect outcomes such as the ratio of female to male DNA observed at the quantification step.

2.2. Presumptive tests

For each mock case the relevant presumptive tests were performed. RSID-semen tests (Galantos Genetics, Mainz, Germany), PSA tests (Seratec, Goettingen, Germany) and microscopic analysis for the presence of spermatozoa were performed as described previously [11]. The phosphatesmo KM test (Macherey-Nagel GmbH & Co. KG, Düren, Germany) was performed according to the manufactures recommendations. RSID-saliva tests (Galantos Genetics, Mainz, Germany) were performed according to the manufacturer's protocol. TB testing for blood was performed by transferring biological material to a with water-moistened filter paper, to which one drop of tetrabase solution (0.5% tetrabase (Sigma–Aldrich, Zwijndrecht, The Netherlands) in 10% acidic acid (Merck, Schiphol-Rijk, The Netherlands)) was added. One drop of barium peroxide solution (5% BaO_2 (Sigma–Aldrich, Zwijndrecht, The Netherlands) in 10% acidic acid) was added next and colour formation was judged according to in-house validated criteria.

2.3. RNA and DNA isolation

For DNA/RNA co-isolation we used the protocol described by Lindenbergh et al. [4]. RNA extracts were treated with DNase as previously described [4]. Standard DNA isolations were performed using QIAamp mini columns (QIAGEN, Venlo, the Netherlands), according to the provided protocol. Minor modifications were made regarding DNA elution as two sequential elution steps using 50 μL pre-heated (70 °C) 25% AE-buffer were applied to yield 100 μL DNA extract.

Differential extraction was performed to separate sperm DNA from non-sperm DNA. Cells were released from the sample by incubating in phosphate buffered saline (PBS) buffer (or RSID-semen or RSID-saliva extraction buffer) for one hour at 700 rpm at room temperature. For mild cell lysis, 20 μL proteinase K (20 mg/mL, QIAGEN, Venlo, The Netherlands) were added and incubated for one hour at 56 °C. The lysate was cleared using a QIA-shredder column (QIAGEN, Venlo, The Netherlands) and centrifugation at 2 min at 11,000 rpm. Not-lysed cells were pelleted by centrifugation for 5 min at 13,200 rpm. The supernatant, containing the non-sperm fraction (NF), was transferred to a new collection tube and stored at 4 °C until further processing. The remaining sperm pellet (sperm fraction, SF) was washed 3–4 times using 500 μL ATL buffer (QIAGEN, Venlo, The Netherlands) and followed by a 5 min 13.200 rpm centrifugation step each time. Next, 300 μL ATL buffer, 10 μL proteinase K and 10 μL 1 M DTT were added to the

Table 1
Description of the seven mock cases.

Case	Hypothetical research question	Description evidentiary item
1	Blood or menstrual secretion on clothing?	Menstrual secretion on textile
2	Semen in woman's underpants?	Vaginal mucosa and semen (vasectomised male) on textile
3	Blood or menstrual secretion in intercourse stain in underpants?	Vaginal mucosa and peripheral blood of one female donor and semen (vasectomised male) on textile
4	Vaginal mucosa on suspect's hands?	Swab from clipped fingernails containing vaginal mucosa
5	Saliva present in penile swab?	Double swab from a male's skin containing female saliva
6	Vaginal mucosa present in semen stain on female leg?	Swab from skin with vaginal mucosa of one female donor and semen (from a fertile male)
7	What donors and cell types are present in bloodstains found on clothing?	Two bloodstains from different males on textile containing skin of one of these males and from another male