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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

cell pellet and incubated for 30–60 min at 56 °C. Both the non-sperm fraction (NF) and sperm fraction (SF) were subjected to DNA extraction according to the above mentioned QIAamp mini column protocol (QIAGEN, Venlo, The Netherlands).

2.4. RNA analysis

The steps for RNA analysis are previously described [4] and include cDNA synthesis, 19-plex reverse transcriptase (RT)-PCR, PCR product purification, injection on a capillary electrophoresis instrument and analysis using GeneMapper ID-X version 1.1.1 (AB). In agreement with the suggestion made by Lindenbergh et al. [4] we used a standard RNA input of 10 μL in the cDNA reaction and a series of inputs in the multiplex RT-PCR, namely 0.01, 0.05, 0.1, 0.2, 0.5, 1, 3.5 and 7.5 μL cDNA. For the three most informative cDNA inputs a HBD1 single-plex RT-PCR was performed as previously described [4].

To infer body fluid identity the following marker signals (besides the three housekeeping markers 18S-rRNA, ACTB and GAPDH) are expected: three markers for blood (HBB, CD93, AMICA1), five markers for saliva (STATH and HTN3 indicating saliva and KRT4, KRT13 and SPRR2A marking mucosa in general), two markers for semen (PRM1 and SEMG1 with PRM1 for fertile men only), two markers for skin (CDSN and LOR, but low signals for the general mucosa markers may occur as well), five markers for vaginal mucosa (the three general mucosa markers plus HBD1 and MUC4 with specificity for vaginal mucosa) and ten markers for menstrual secretion (MMP7 and MMP11 for menstrual secretion plus the three blood and five vaginal markers).

2.5. DNA analysis

Human-specific genomic DNA concentrations of extracts isolated with the DNA/RNA co-isolation were determined through real-time PCR using *Alu* repeats as previously described by Nicklas and Buel [12] with minor adjustments for the primer concentrations as 200 nM *Alu* forward primer and DYZ5 reverse primer were used. Concentrations of DNA extracts obtained through the standard protocol were determined with Quantifiler® Duo (AB) according to the manufacturer's recommendations.

The steps to generate DNA profiles are previously described [4] and include PCR amplification using the AmpFℓSTR® NGM™ PCR Amplification Kit (AB, Foster City, TX, USA), injection on a capillary electrophoresis instrument and analysis using GeneMapper ID-X (AB).

3. Unbiased assessment of RNA profiling results

At the Netherlands Forensic Institute (NFI), specialised teams perform different aspects of the forensic analyses. Trace examiners investigate, photograph and sample evidentiary items and perform microscopic analyses and presumptive tests. Type 1 DNA analysts extract DNA from scene samples, quantify the extracted DNA and set-up the amplification reactions. Type 2 DNA analysts run the PCRs, analyse the amplified products by capillary electrophoresis and perform DNA profile analysis using specialist software. Reporting officers (ROs) combine the data from the trace examinations and DNA profiling and evaluate the outcomes to those for other evidentiary traces in the case and reference data. The results of the presumptive tests can take the following format: 'positive', 'negative' or 'invalid'. The results of microscopic examination include details on the approximate number of sperm heads observed per field. DNA genotyping results are available to the ROs as electropherogram (EPG) prints or can be viewed (not modified) in GeneMapper ID-X. RNA profiling data are generated by RNA researchers (RRs), and these data can be presented to ROs either as RNA profiles (in the form of EPGs) or in a results table (analogous to the results of the presumptive tests). Since the interpretation of RNA profiles is relatively complex for reasons given in the introduction, and because a set of RNA profiles is obtained from the serial cDNA input approach [4], experience is needed to interpret the RNA profiles. Therefore, RNA profile interpretation is, in our view, best performed by RRs, rather than ROs. Thus, at the NFI, RRs both analyse (meaning mRNA marker calling) and interpret (meaning body fluid inference) generated RNA profiles. This approach has two consequences. Firstly, the RRs should have no prior knowledge about the case as this may induce subjective judgement during interpretation and secondly, the interpretation of the RNA profiles has to be presented to the ROs in a standardised format. If ROs need advice whether or not RNA profiling could be constructive to a case, an RR can be asked to give an opinion however a second RR should then carry out the RNA profile interpretation.

A standard RNA results table was designed that presents categorised evaluations for the different cell types represented in the 19-plex [4]. Although the multiplex can distinguish semen of sterile and fertile men, this is not scored distinctly as this would overlook low fertility. Six different scoring categories are used (Fig. 1A): the obvious categories "observed" and "not observed" are supplemented with "observed and fits with", "sporadically observed and fits with", "sporadically observed, no reliable statement possible" and "non-specific due to high cDNA input". Absence of signals is translated to the category "not observed". The term "observed" is used when cell type signals are present in at least half of the positions possible. This procedure begins with the selection of a set of informative RNA profiles from the profiles obtained by the serial input approach (optionally supplemented with replicate analyses for the most informative input). Informative profiles need to show signals for housekeeping genes. Although housekeeping markers are universally expressed across various tissue types, the amount expression per tissue varies, resulting in distinct profiling characteristics: while 18S-rRNA has general high expression, ACTB expression varies for the different cell types (relatively strong in skin, blood and menstrual secretion) and GAPDH is relatively low in saliva or degraded samples. These features are taken into account when determining whether a profile is informative. Then, for each cell type it is determined how often a signal (of good peak morphology and above detection threshold) is present (x) relative to the times a signal could have occurred (n). This latter value "n" depends on the number of RNA profiles that is regarded and the number of markers for a cell type in the multiplex. This guideline is analogous to the strategy described by Benschop et al. [13]. An observed cell type is categorised "observed and fits with" if it is co-expressed with another cell type. Examples are blood concurring with menstrual secretion, vaginal mucosa with menstrual secretion and general mucosa with saliva, vaginal mucosa, menstrual secretion and (in low levels) skin. As a consequence, mixtures of, for instance, blood and menstrual secretion will only be categorised as such when the blood component gives much stronger signals than the menstrual secretion components, otherwise blood will be regarded as "observed and fits with". "Much stronger signals" refers to prominent peaks for the co-expressed body fluid (*e.g.* blood) in the lower cDNA input profiles and signals for the complex body fluid (*e.g.* menstrual secretion) only appearing in profiles with higher cDNA inputs. When cell type signals are present in less than half of the positions possible (x is above zero but below $n/2$) we use the term "sporadically observed". Again, co-expression issues are regarded and translated to scoring the results as "sporadically observed, no reliable statement possible" or "sporadically observed and fits with". As a result of the serial cDNA input approach, RNA profiles can show overloading with higher inputs [4]. Since

1A

Cell Type	Observed	Observed and fits	Sporadically obs. and fits	Not observed	Sporadically obs. not reliable	Non specific due to high input
Mock Case 1						
blood					x	
menstrual secretion	x					
vaginal mucosa		x				
mucosa		x				
saliva				x		
semen				x		
skin					x	
Mock Case 2						
blood						x
menstrual secretion				x		
vaginal mucosa	x					
mucosa		x				
saliva				x		
semen	x					
skin	x					
Mock Case 3						
blood	x					
menstrual secretion				x		
vaginal mucosa					x	
mucosa	x					
saliva						x
semen	x					
skin	x					
Mock Case 4						
blood						x
menstrual secretion				x		
vaginal mucosa	x					
mucosa		x				
saliva				x		
semen				x		
skin	x					
Mock Case 5						
blood				x		
menstrual secretion				x		
vaginal mucosa				x		
mucosa		x				
saliva	x					
semen				x		
skin	x					
Mock Case 6						
blood				x		
menstrual secretion				x		
vaginal mucosa				x		
mucosa		x				
saliva				x		
semen	x					
skin	x					
Mock Case 7[a]						
blood	x					
menstrual secretion				x		
vaginal mucosa				x		
mucosa			x			
saliva				x		
semen				x		
skin	x					

[a] Data presented for bloodstain one. Highly similar results were obtained for bloodstain two.

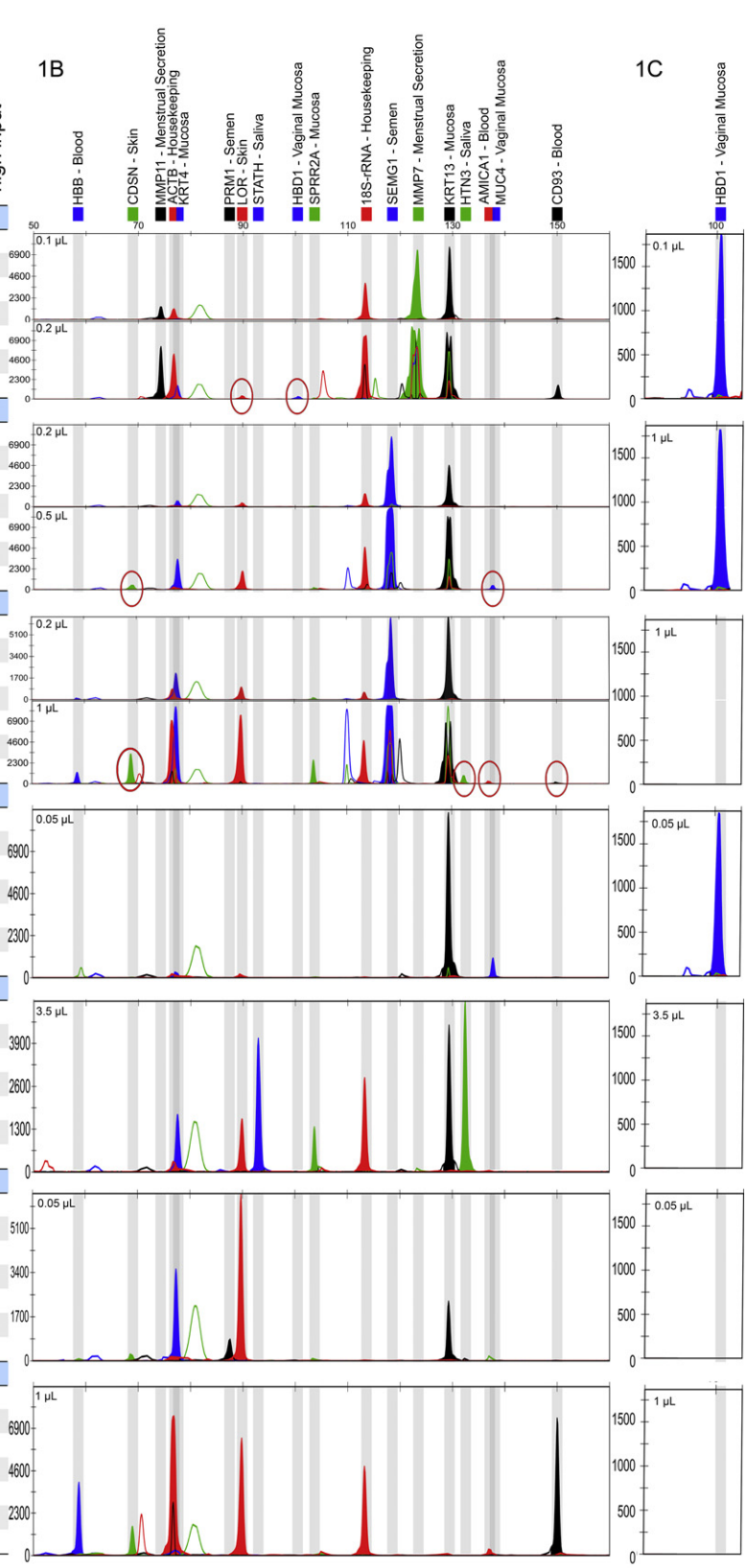

Fig. 1. RNA results for the seven mock cases presented per case. Panel (A) shows the RNA results table, panel (B) presents overlay electropherograms (EPGs) selected to be illustrative for the case and panel (C) shows HBD1 single-plex EPGs. For interpretation, all the informative RNA profiles from the serial input approach were considered. In the left upper corner, the cDNA input for the shown profile is indicated (serial input range involved eight amounts between 0.01 and 7.5 μL). For case 1, five profiles were informative (inputs 0.05–1 μL), for case 2 four (inputs 0.1–1 μL), for case 3 four (inputs 0.1–1 μL), for case 4 four (inputs 0.1–1 μL), for case 5 two (inputs 3.5 μL and 7.5 μL), for case 6 two (inputs 0.05 μL and 0.5 μL) and for case 7 five (inputs 0.1–3.5 μL). The red circles indicate peaks that become apparent in the higher inputs for the cases where two EPGs are shown. Results for the GAPDH marker (at 260 bp) are not shown.

RNA markers show variation in signal strength, sometimes profiles with one or two saturated peaks are included in the subset of informative profiles that is used to derive the results table. Around these saturated markers, increased base-line noise and amplification by-products (usually few nucleotides shorter) may occur, which can result in non-specific signals (of correct morphology) in surrounding marker bins. Also, the high input may reveal the occurrence of spurious or sporadic transcripts for genes not specific to the body fluids present in the sample. To allow for translation of these signals, the category "non-specific due to high input" is introduced in the RNA results table. For some signals it may be difficult to choose between the categories "sporadically observed, no reliable statement possible" and "non-specific due to high input", but for other signals the categorisation will be evident. The impact of both categories is somewhat different: "sporadically observed, no reliable statement possible" aims to translate low-level components and "non-specific due to high cDNA input" exists to capture spurious signals. By this approach, all signals observed by RRs are translated to the ROs.

4. Assessment of mock cases by conventional and RNA/DNA methodology

In order to compare the outcomes of RNA cell typing (using the previously described 19-plex [4]) and conventional methods for presumptive body fluid inference, seven mock cases were designed and prepared in duplicates. The design of each mock case is shown in Table 1. The samples were also submitted to DNA profiling. The RNA results table with six different scoring categories, described in the previous paragraph, was used to capture the interpretation of the RNA profiling results. The interpretation table as shown in Fig. 1, is based on all informative RNA profiles that were obtained in the serial input approach. Profiles with insufficient housekeeping expression or profiles showing excessive over-amplification were excluded from interpretation (as illustrated in Supplementary Fig. S1 for mock case 3). The RNA profiles in Fig. 1 are illustrative to the cases and do not represent all data underlying the scorings in the results table.

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fsigen.2012.09.003.

The first mock case assessed a menstrual secretion stain (Table 1). Using RNA profiling, peaks indicating the presence of menstrual secretion, vaginal mucosa and general mucosa were observed (Fig. 1B). These results are indicative of the presence of menstrual secretion. The presence of vaginal mucosa was verified by HBD1 single-plex analysis (Fig. 1C). Sporadic signals for the blood markers were observed which is not unusual for menstrual secretion specimens [4]. A signal for skin was observed once in the set of the RNA profiles and therefore also skin was scored as "sporadically observed, no reliable statement possible". The presumptive tests indicated the presence of blood (Table 2), which is expected for a menstrual secretion stain. Full, single donor DNA profiles concordant with the female donor were obtained with both methodologies (Table 2).

The samplings in the second and third case have a similar basis (intercourse stain) with additional blood for case 3 (Table 1). For both cases, the RNA profiles indicated the presence of vaginal mucosa, skin and semen (Fig. 1). No PRM1 expression was observed (but strong SEMG1 signals) indicating involvement of a possibly sterile male, which is not scored as such (see previous section). For mock case 3 the signal for vaginal mucosa was only observed sporadically and could not be reproduced with HBD1

Table 2
Results for the seven mock cases (as described in Table 1 using standard presumptive testing combined with DNA profiling and the DNA/RNA co-extraction methodology. For some cases standard extraction was replaced by differential extraction resulting in a SF and NF. DNA quantifications are presented as well.

Mock case	Method and fraction	DNA quant[a]		DNA typing result[b]	Presumptive tests[c]					
		Total	Male		TB	Sperm heads	RSID semen	PSA	Phosphatesmo	RSID saliva
1	Standard	51.24	0.000	♀: full	pos	neg	–	–	neg	–
	RNA/DNA	5.294	0.000	♀: full						
2	Standard-SF	0.362	0.010	♀: full, ♂: 3/22	–	neg	pos	–	pos	–
	Standard-NF	23.39	0.288	♀: full, ♂: 1/22						
	RNA/DNA	31.417	0.129	♀: full, ♂: 2/22						
3	Standard-SF	0.348	0.004	♀: full, ♂: 0/22	pos	neg	pos	–	pos	–
	Standard-NF	4.040	0.082	♀: full, ♂: 1/22						
	RNA/DNA	22.982	0.082	♀: full, ♂: 2/22						
4	Standard	1.680	0.033	♀: full, ♂: 0/21	neg	–	–	–	–	neg
	RNA/DNA	2.713	0.003	♀: full, ♂: 1/21						
5	Standard-dry[d]-SF	0.006	0.000	♀: full, ♂: 0/15	neg	–	–	–	–	pos
	Standard-dry-NF	0.048	0.000	♀: full, ♂: 0/15						
	RNA/DNA-dry	0.090	0.000	♀: full, ♂: 4/15						
6	Standard-SF	0.128	0.178	♀: full, ♂: 17/17	neg	pos (1–3 hpf)[e]	–	–	–	–
	Standard-NF	3.430	0.044	♀: full, ♂: 0/17						
	RNA/DNA	5.032	0.116	♀: full, ♂: 12/17						
7	Standard-stain one	0.528	0.750	$♂_B$: full, $♂_S$: 12/15, $♂_{BS}$: 2/16[f]	pos	–	–	–	–	–
	RNA/DNA-stain one	0.341	0.315	$♂_B$: full, $♂_S$: 2/15, $♂_{BS}$: 1/16						
	Standard-stain two	0.629	0.906	$♂_B$: 0/17, $♂_S$: 6/15, $♂_{BS}$: full	pos	–	–	–	–	–
	RNA/DNA-stain two	0.665	0.546	$♂_B$: 1/17, $♂_S$: 4/15, $♂_{BS}$: full						

[a] In ng/μL, for the standard method the Quantifiler Duo kit was used while for the RNA/DNA procedure the *Alu* repeat assay was applied.
[b] In all cases, complete profiles (32 of 32 NGM alleles) were obtained for the major contributor (full), homozygous alleles are counted as two. For incomplete minor (male) profiles, the observed number of non-shared alleles from the total number of non-shared alleles is indicated.
[c] Positive (pos) and negative (neg) test results are indicated. Dashes (–) refer to not done. For the grey rows, results are not applicable.
[d] The wet and dry swab generated with the double swab technique [10] were processed separately. For the dry swab, around five times higher DNA concentrations were obtained. Thus, results for the wet swab are not shown.
[e] Heads per field.
[f] Three male donors contributed different cell types; blood (B), skin (S) and blood and skin (BS).

single-plex profiling like for mock case 2. Therefore, in mock case 3, mucosa is scored "observed" and vaginal mucosa "sporadically observed, no reliable statement possible", while in mock case 2, vaginal mucosa is scored "observed" and mucosa "observed and fits". In the profiles with SEMG1 and KRT13 overloading, sporadic signals for blood (mock case 2) or saliva (mock case 3, Fig. 1B, second EPG) were seen that are scored as "non-specific due to high cDNA input". In addition, mock case 3 yielded positive results for blood (Fig. 1B). RNA profiles of all dilutions including interpretation are shown in Supplementary Fig. S1. The presumptive tests for mock case 2 gave an indication for the presence of seminal fluid while for mock case 3 presence of seminal fluid and peripheral blood was indicated (Table 2). For the conventional strategy, differential extraction was performed (as both cases mimicked sexual assault cases) but no enrichment for male donor alleles was observed in the SF (Table 2). Actually, in all of the DNA extracts (that is both the NF and SF and also the DNA extract from the RNA/DNA co-extraction procedure) of both mock cases (two and three) only a limited number of alleles of low peak heights for the male donor (Table 2) were observed which agrees with a vasectomised donor.

Examination of the fingernails present in mock case 4 (Table 1) gave only cell origin information upon RNA analysis (Table 2, Fig. 1), and indicated the presence of vaginal mucosa and skin (plus a "non-specific due to high cDNA input" blood signal). The DNA profiles that were generated from DNA prepared by either the standard or the DNA/RNA extraction method, originated from the female donor with the exception of one non-shared allele that belonged to the male donor in the profile obtained after co-extraction (Table 2).

Additional information regarding the cellular origin of the stain was also obtained with RNA profiling in mock case 5 (male skin licked by female, Table 1). In addition to the identification of saliva with both methods, RNA profiling was able to comment on the presence of skin cells (Table 2, Fig. 1). Since this mock case mimicked a penile swab with the possible presence of semen, differential extraction was carried out with the conventional method (to separate possibly strong semen signals from relatively low non-semen signals which may correspond to the victim). Both the SF and NF DNA profiles showed only alleles belonging to the female contributor. In the DNA profiles from the RNA/DNA co-extraction, four additional alleles were observed which corresponded to the male donor whose skin was sampled (Table 2).

In mock case 6 (semen stain on victim's leg, Table 1) the presence of semen was clear with the conventional approach as sperm heads were observed upon microscopic inspection. Also with RNA profiling semen is scored as "observed" even though only signals for PRM1 were seen. Apparently the scoring guideline (regard a cell type observed when $x \geq n/2$) works even when a donor does not show expression for one of the markers. RNA cell typing also indicated the presence of skin and general mucosa. The heights of the general mucosa peaks are consistent with the co-expression signals for skin [4] and mucosa was therefore scored as "observed and fits" (Fig. 1). For the female donor full DNA profiles were obtained in all fractions (NF, SF, DNA from RNA/DNA co-extraction). Alleles of the male contributor were also represented in the SF (mixed DNA profile containing all alleles of both the male and female contributor in approximately a one to one ratio) and in the DNA profile following the RNA/DNA co-extraction method (around two-third of the male alleles is detected, Table 2).

Analyses of the two adjacent bloodstains in mock case 7 indicated the possible presence of blood with the presumptive testing and blood and skin with RNA profiling (Fig. 1, Table 2). Sporadic co-expression of general mucosa markers was also observed. Three males contributed to this case. In the DNA profiles, these three donors were detected up to different levels, irrespective of the method employed. In bloodstain one, donor B (the male donating only blood) was present as a clear major component while for donor BS (who contributed both blood and skin) just few non-shared alleles were observed. In bloodstain two, the reverse was seen: full profiles for donor BS and no or few non-shared alleles for donor B. For donor S (the male contributing only skin to the sample) several (four to twelve) non-shared alleles were observed in both bloodstains (Table 2). These observations are consistent with the preparation of the samples as donors S and BS rubbed the textile after which blood of donors B and BS were deposited as adjacent spots. Only by combining the DNA data from both the bloodstains, the presence of three contributors of DNA on the textile could be inferred.

Overall, RNA profiling was found to provide additional insight when menstrual secretion, vaginal mucosa and skin samples were involved, as no presumptive tests are available to detect these types of cells. For five of the seven cases all the cell types present in the trace samples were inferred with RNA profiling (which was not the case using presumptive testing due to the design of the cases). For cases 3 and 6, no reliable signals were obtained for the markers indicating the presence of vaginal mucosa (neither in the HBD1 single-plex). This presents an issue for case 6 but is irrelevant for case 3 as the question of interest involved deciding whether the stain was peripheral or menstrual blood (Table 1). Thus, for all the mock cases except mock case 6, information on the forensic question as formulated in Table 1 was obtained and supported to variable degrees by the DNA results as for several but not all contributors full DNA profiles were obtained.

The detection of skin mRNA signals mostly had a confirmatory role: for all cases in which skin areas were sampled, skin marker signals were observed. On the other hand, the detection of skin material did not have a high incriminating value for any of the mock cases. In the DNA profiles a variable number of alleles corresponding to the skin donor were detected. This may have been influenced by the amount of DNA shed by the various skin donors [14], or the fact that the samples were mixtures with DNA-rich body fluids by which skin donor alleles may have been overshadowed. Besides a confirmatory role, skin mRNA signals may explain sporadic alleles observed in DNA profiles, as sporadic skin cells can be readily envisaged in crime scene samples.

For the mock sexual assault samples (in cases 2, 3, 5 and 6), differential extraction was applied within the standard methodology to separate sperm and non-sperm DNA. Only in mock case 6, a strong enrichment for male alleles was observed in the SF. This is due to the fact that a fertile donor of semen was used in mock case 6 while the rest of the mock cases had a vasectomised donor. For such cases a future integration of differential extraction into the RNA/DNA co-isolation procedure would present an improvement of the methodology.

5. Reporting RNA profiling results

For the forensic report, the RO regards both the genotyping data and the RNA profiling results (assuming informative DNA and RNA results were obtained) and formulates separate conclusions for both types of data (Table 3A presents an example for mock case 4). DNA analyses aims to provide information at a source level, such as who contributed the cellular material to the trace. Therefore, obtained DNA profiles are compared to a DNA database or to reference DNA profiles. Sometimes, the DNA data offers a hint on the trace type; for instance high DNA quantification results may be more likely for body fluid stains than for contact traces or secondarily transferred cell material. Also the number of donors can be inferred from the DNA profiles [15]. RNA profiling assesses which cell types may be present in an evidentiary trace. When multiple cell types are observed, it is most often uncertain

Table 3
Exemplary conclusions on DNA and RNA level (A), sets of Hp and Hd propositions on origin (B) or activity level (C) for mock case 4.

A	Research question	DNA results	RNA results
	Vaginal mucosa on suspect's hands?	♀ Profile, RMP[a] less than one in a billion One non-shared allele matching suspect	RNA profile fits pattern of a mixture of vaginal mucosa and skin cells

B	Set	Hp	Hd	Assumption(s)	Hp over Hd[b]
	A	Vaginal cells from victim	Skin from victim	Victim donated cell material (DNA match)	Results are much more probable under Hp than Hd (positive vaginal markers)
	B	Vaginal cells from victim	Skin from victim and vaginal cells from girlfriend	Victim donated cell material (DNA match and 1-person profile) Vaginal cells are present (positive RNA markers)	Results are much more probable under Hp than Hd (found DNA profile matches victim)

C	Set	Hp	Hd	Hp over Hd[b]
	A	Suspect penetrated victim's genitals with fingers	Suspect touched victim's skin while dancing	Results are much more probable under Hp than Hd
	B	Suspect penetrated victim's genitals with fingers	Suspect touched victim's skin while dancing and suspect penetrated girlfriend's genitals with fingers	Results are much more probable under Hp than Hd
	C	Suspect penetrated victim's genitals with fingers	Suspect touched the crotch of panties worn by victim	Results are more probable under Hp than Hd

[a] RMP, random match probability.
[b] The verbal strength is chosen for illustrative purposes.

whether these have originated from the same donor or from multiple donors (as an exception, gender-specific cell types: *e.g.* semen and vaginal mucosa signals will have originated from different donors).

In the next step, the RO combines the obtained DNA and RNA results in one conclusion with the aim to infer which cell type may belong to which donor. The sensitivities of RNA and DNA profiling are approximately in the same range [4], although for skin cells, RNA profiling can be more sensitive than DNA profiling and vaginal mucosa markers can drop out while female DNA profiles are obtained. For the combined interpretation of DNA and RNA results it can be helpful to formulate sets of propositions. The findings (*e.g.* number of donors, gender of the donors) can justify the assumptions that accompany the propositions. Examples of sets of prosecution hypotheses (Hp) and defence hypotheses (Hd) on origin level for mock case 4 are presented in Table 3B.

Integrated inference of donor and cell type can assist an assessment at activity level *i.e.* assess the probability of different actions in having led to the deposition of the cellular material. Not only RNA profiling, but also presumptive testing provides cell type information and can therefore assist activity level analysis, albeit that RNA cell typing provides more detailed information as more cell origins are assessed (simultaneously). Examples of activity level hypotheses are given in Table 3C, again for mock case 4. Evidently, the RO does not formulate these hypotheses him or herself; the initiative lies with the prosecution (or defence lawyers). To capture the expert belief regarding the strength of the conclusions, a verbal scale [16] may be the best approach to explain the results in an informative manner to the courts of law.

6. Concluding remarks

RNA profiling is increasingly recognised as a supplementary method for body fluid identification. RNA profiling has clear advantages over conventional methods: a larger range of body fluids can be examined simultaneously and high sensitivity and specificity for human origins [1,2,4]. On the other hand, RNA profiling is more labour-intensive and thus more costly. Implementation of RNA profiling (or any other novel forensic methodology) in forensic casework goes beyond its technical aspects, such as the preparation, analysis, labelling and storage of DNA and RNA fractions and the corresponding (RT-)PCR products. In addition, strategies need to be developed to maintain the chain of custody and accommodate unbiased forensic interpretation [17]. In this study, we present a general approach that ensures unbiased interpretation of RNA results: (1) all analysts executing the case remain uninformed about the context of the case, (2) RNA/DNA co-isolation is performed by an RR after which (3) DNA extracts are analysed by DNA analysts while (4) the RNA profiling is performed by the RR who (5) interprets the obtained RNA profiles by clear guidelines and generates for each evidentiary trace a results table that uses six different scoring categories. With this approach the RR focuses on the RNA results and does not receive cognitive bias from either the DNA results or the forensic context of the case. The scoring guidelines and results table aim to interpret RNA profiling results objectively and apply to any RNA multiplex. The actual way RNA profiling is implemented in forensic casework will depend on the organisation within a forensic laboratory and the procedure described here may be of assistance.

For each evidentiary trace, two types of results are presented to an RO: DNA genotyping and RNA profiling results. The RO proceeds to considering the coherence of all results (also those of other samplings and items) within the case and comparison against reference profiles. Following these assessments, three types of conclusions can be formulated in the expert report: DNA conclusions, RNA conclusions and hypotheses-driven conclusions based on the combined results.

For vaginal mucosa, DNA profiling can be more sensitive than RNA profiling (full DNA profiles but only general mucosa indicated), and further optimisation of the 19-plex for this tissue appears needed. Bacterial species specific to vaginal environments may be helpful [18] although these microbes may also occur on body sites that are in contact or in proximity with the vagina [19], which could lead to the false identification of vaginal mucosa in skin samplings such as penile swabs. For skin cells, RNA profiling can be more sensitive than DNA profiling. In this latter case the information on the biological origin cannot be deduced to a specific donor. Nevertheless, skin RNA signals can serve several purposes during forensic case assessment: it (1) can confirm the sampling of skin areas, (2) can indicate skin contact when signals for other body fluids are absent (in these cases low template DNA profiling [13] may be applied to extract individualising information) or (3) explain sporadic contamination in DNA profiles from occurrence of a low number of skin cells (which may be encountered in many crime scene specimens). A growing practice in expert reporting relates to addressing activity level questions. Detailed cell type

information as provided by RNA cell typing, will aid this development.

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