Development of RNA profiling tools and the implementation in forensic casework

Lindenbergh, P.A.

Publication date
2014

Document Version
Final published version

Citation for published version (APA):

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: https://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.
Chapter 3

RNA cell typing and DNA profiling of mixed samples: can cell types and donors be associated?

Science and Justice (2013) 53:261-269

Joyce Harteveld
Alexander Lindenbergh
Titia Sijen
Abstract

Forensic samples regularly involve mixtures, which are readily recognised in forensic analyses. Combined DNA and mRNA profiling is an upcoming forensic practice to examine donors and cell types from the exact same sample. From DNA profiles individual genotypes may be deconvoluted, but to date no studies have established whether the cell types identified in corresponding RNA profiles can be associated with individual donors. Although RNA expression levels hold many variables from which an association may not be expected, proof of concept is important to forensic experts who may be cross examined about this possible correlation in court settings. Clearly, the gender-specificity of certain body fluids (semen, vaginal mucosa, menstrual secretion) can be instructive. However, when donors of the same gender or gender-neutral cell types are involved, alternatives are needed. Here we analyse basic two-component mixtures (two cell types provided by different donors) composed of six different cell types, and assess whether the heights of DNA and RNA peaks may guide association of donor and cell type. Divergent results were obtained; for some mixtures RNA peak heights followed the DNA results, but for others the major DNA component did not present higher RNA peaks. Also, variation in mixture ratios was observed for RNA profiling replicates and when different donor couples gave the same two body fluids. As sample degradation may affect the two nucleic acids and/or distinct cell types differently (and thus influence donor and cell type association), mixtures were subjected to elevated temperature or UV-light. Variation in DNA and RNA stability was observed both between and within cell types and depended on the method inducing degradation. Taken together, we discourage to associate cell types and donors from peak heights when performing RNA and DNA profiling.
Introduction

Recent developments have supplied forensic researchers with mRNA profiling systems that enable inference of a variety of human cell types such as blood, semen, saliva, menstrual secretion, vaginal mucosa and skin [1–7]. Efforts have not only focused on finding and validating markers, but in addition useful multiplexes were developed which allows simultaneous detection of the various cell types. Gradually, RNA assays have been introduced for forensic casework, which generates the need to develop interpretation strategies and guidelines [8] extending to the combined assessment of DNA and RNA profiling results. When for instance multiple cell types are detected, these can relate to multiple donors and in those cases it can be useful to infer ‘who donated what cells’ and associate the DNA profiles of individual donors to observed cell types (although the situation can be more complex as one donor can donate several cell types and one cell type can be donated by multiple individuals). The involvement of gender-specific body fluids (such as semen, vaginal mucosa or menstrual secretion) is helpful when samples have two donors of different genders. However, biological grounds to associate donors and cell types are lacking when donors of the same gender or gender-neutral cell types are implicated.

In this study we assessed whether the strengths of the DNA and RNA signals can be used to associate donor and cell type in mixtures containing two cell types of different donors. For the DNA part, it is well established that the quantities present for each mixture component are converted to the profiles, based on which mixture deconvolution methods [9–14] have been developed that resolve the DNA profiles of the individual donors. Mixture deconvolution is easier with DNA of good quality and sufficient quantity, when the potential number of contributors is limited and the mixture ratio is easily estimated from the electropherogram. Factors such as peak heights, thresholds (detection and stochastic), heterozygote peak height balance, (locus-specific) stutter filters and allele frequencies are considered. For the RNA part, the underlying biology is more complex as different levels exist for each particular mRNA. This effect is somewhat compensated during the construction of RNA multiplexes by selecting a fluorescent label of appropriate strength and adapting primer concentrations for each marker. Analogous to the DNA typing methods, mRNA profiling assays translate quantitative aspects as higher peaks are obtained when more templates are present, although the linearity of mRNA assays may suffer from the additional cDNA synthesis step and the general use number of amplification cycles [1–4]. RNA results are further affected by mRNA expression level differences that can occur for different individuals and with changes in physiological condition. When questioning forensic scientists on
their intuitive views on the strength of RNA signals for cell types having a (very) low contribution to a mixture, the answers carried ambiguity. On one hand, minute contributions giving few alleles in the DNA profiles were expected to give low or no RNA signals. On the other hand, it was not expected that the mixture ratios in the DNA and RNA profiles were the same or similar. Regardless the expectation, forensic reporting officers were keen on acquiring experimental substantiation in order to have a proof of this concept as in court settings experts may be enquired about this possible correlation.

Thus, we took an empirical approach to assess whether peak height data in DNA and RNA profiles can be used to associate donor and cell type for a set of designed mixtures containing two cell types of different donors. The mixture set involved six cell types (blood, saliva, semen, skin, vaginal mucosa and menstrual secretion) that were combined in three informative ratios prior to RNA/DNA co-extraction. For one of the mixture types (saliva with blood) the effects of different donors, replicate RNA analyses and various degradation-inducing conditions were tested as well. This experimental procedure will either reveal a strategy to associate donor and cell type or demonstrate that such an association cannot be made safely. As the use of mRNA assays in casework is imminent, we feel it is appropriate to address such issues now as these affect interpretation and case reporting.

Materials and methods

Preparation of mixed samples

Donors gave informed consent and provided reference DNA profiles. Peripheral blood was collected using a finger prick (Accu-chek, Softclix Pro, Roche Diagnostics GmbH, Germany). Saliva was collected in 50 mL tubes. Vaginal mucosa (also known as vaginal fluid) and menstrual secretion (also known as menstrual blood) were collected using a dry cotton swab (Deltalab, Barcelona, Spain). Semen from a fertile donor was taken from storage at −20 °C. To collect skin, cotton cloths were rubbed extensively over hands and forehead after which the cell material was gathered using tape lifts [15].

The first experiment involved two-component mixtures based on six cell types. For each cell type, one donor was used as we preferred to include several mixture ratios for each mixture. We set out to have each mixture in three set ratios (representing the DNA mixture ratio). To achieve this, a large volume of lysate was
prepared for each individual cell type of which a small aliquot was sampled for DNA content. Based on these DNA contents, lysate aliquots were mixed to the designed set-up and processed further. Consequently, mixtures of cell types were used in the extraction process. This process in more detail: 30 µL blood, 120 µL saliva, 5 µL semen, a portion of a swab containing vaginal mucosa, a portion of a swab containing menstrual secretion and tape lifts from 12 cotton cloths containing skin were each added up to a volume of 1450 µL using Lysis Binding Buffer (mirVana™, Applied Biosystems™, Ambion®, Austin, TX, USA) and lysed by a two-step proteinase K (20 mg/mL, QIAGEN Benelux B.V., Venlo, The Netherlands) incubation: first a 2 h incubation at 56 °C using a 20 µL aliquot proteinase K followed by a 30 minutes (min) incubation at 56 °C using a 30 µL aliquot proteinase K. This extensive lysis procedure was invoked by the relatively large contribution of specimen in the lysate which was captured in swabs for some of the samples. Then, the samples were centrifuged for 10 min. To assess how much DNA resides in one microliter of lysate, an aliquot of 20 µL lysate was taken and processed by adding 280 µL Lysis Binding Buffer and performing DNA and RNA extractions according to the RNA/DNA co-isolation procedure described in [4]. The remaining lysate was stored at −80 °C (we have established that storage at −80 °C does not affect DNA or RNA yield or integrity, results not shown). After quantification of the DNA fraction, the amount of DNA residing in one microliter of lysate was calculated. Origin of donor and cell type and integrity of DNA and RNA were confirmed by NGM profiling of the DNA fraction and RNA cell typing of the RNA fraction. Details for DNA and RNA profiling are described in the next section. For each cell type, the volume needed to yield 20 ng or 100 ng DNA after RNA/DNA co-extraction was determined. For each combination of cell types, three mixtures of lysates were prepared after thawing the stored lysate mixtures were added up to 300 µL with Lysis Binding Buffer and subjected to RNA/DNA co-extraction, DNA quantification, NGM genotyping and RNA cell typing as described below.

The mixtures for the next experiments were prepared by mixing specified volumes of neat blood and saliva. The different donor experiments involved three blood and three saliva donors (adding up to nine donor sets) and mixtures consisted of 5 µL blood and 15 µL saliva. One of the donor sets represented the same blood and saliva specimens of these two individuals were also used in the degradation experiments. Degradation was induced by a 30 minutes (min) incubation at 37 °C.
RNA cell typing and DNA profiling of mixed samples

using either liquid state samples (mixtures of 2 µL blood and 30 µL saliva) or dried samples (mixtures of 2 µL blood and 6 µL saliva). For the preparation of dried samples for each individual stain 2 µL blood and 6 µL saliva were pre-mixed in a tube prior to spotting all fluid on a small textile cutting (textile consists of 50% cotton, 50% viscose). Stains were dried overnight.

Temperature-induced degradation of liquid state samples involved storage in a closed 1.5 mL tube at various temperatures (−80 °C, 4 °C, 20 °C or 37 °C) for various days (1, 4 or 7). After the set number of days, samples were transferred to −80 °C until RNA/DNA co-extraction was started. To degrade the dried samples by increased temperature and higher humidity, the stains were placed in an open tube sited in a sealed container having a layer of water. To prevent condensates falling into the tubes a sloping plastic shield was placed in the lid of the container. The container was placed in an incubator (HIR10M Grant Boekel, VWR International B.V., Amsterdam, The Netherlands) set at 37 °C and samples were removed after 2, 4, 7, 10, 14 and 20 days and stored at −80 °C until RNA/DNA co-extraction was started. Degradation by UV-light was achieved by exposing dried stains to 254 nm UV-light in a CL-1000 UV CrossLinker (UVP, Upland, USA) at 0.9 J/cm² for 0, 30, 120, 240 and 360 min.

For RNA/DNA co-extraction, samples were added up to 300 µL Lysis Binding Buffer and processed as described in [4] except that the elution volumes were reduced when samples held 2 µL blood and 6 µL saliva: 50 µL was used to elute the DNA columns and 40 µL to elute RNA columns. Next, the extracts were subjected to DNA quantification, NGM genotyping and RNA profiling as described below.

DNA and RNA profiling

For RNA/DNA co-isolation we used the protocol described by Lindenbergh et al. [4]. Analysis of the DNA fractions started with determining the human-specific genomic DNA concentrations. The Alu repeat system previously described by Nicklas and Buel [16] was used through real-time PCR on an ABI 7900HT real-time PCR system (AB). Minor adjustments were made to the protocol described in [15] as 200 nM Alu forward primer and DYZ5 reverse primer were used. DNA profiling used the AmpFISTR® NGM™ PCR Amplification Kit (AB, Foster City, Texas, USA) and 0.5 ng DNA template (in case of a high enough DNA concentration). Amplified DNA fragments were separated and detected by injection on a 3130xl ABI Prism® Genetic Analyzer™ (AB) having performance optimised polymer 4 (POP-4) (AB) as separation matrix. DNA profile analysis used Genemapper ID-X version 1.1.1 (AB) with a detection threshold of 50 RFU [4].
The steps for RNA analysis include treatment with DNase, cDNA synthesis, multiplex reverse transcriptase (RT)-PCR, PCR product purification and injection on a 3130xl capillary electrophoresis instrument (AB) having performance optimised polymer 7 (POP-7) (AB) as separation matrix. Details for these procedures are described in [4]. RNA profiles were analysed using GeneMapper ID-X using a 50 RFUs detection threshold which is lower than that described in [4]. The 50 RFU threshold facilitated the detection of minor component peaks in the unbalanced mixtures. Since designed mixtures were analysed, true peaks and low-level noise signals are evident. In the cDNA reaction an RNA input of 10 µL was used in all experiments (which represents RNA amounts well below the maximum RNA capacity in the cDNA reaction that is at least 2 µg according to the manufacturer). For the multiplex RT-PCR, inputs of 0.2, 0.5, 1, 2 or 5 µL cDNA were used, depending on the experiment. In case of saturated (flat top) RNA signals, RT-PCR products were diluted 50-fold in water and re-injected (this procedure is validated in-house). In all experiments negative controls were included where reverse transcriptase was omitted from the cDNA reaction. These negative controls never showed signals. For RT-PCR a 14-plex was prepared that represented a selection of the markers described earlier [4]: 18S-rRNA was included as housekeeping marker; HBB, CD93, AMICA1 indicated blood; STATH and HTN3 saliva; PRM1 and SEMG1 semen, CDSN and LOR skin, HBD1 and MUC4 vaginal mucosa and MMP7 and MMP11 menstrual secretion (blood and vaginal mucosa markers are co-expressed with menstrual secretion but do not mark this body fluid). Since HBD1 performs suboptimal in multiplex [8] additional singleplex HBD1 RT-PCRs were performed.

Mixture analysis in DNA and RNA profiles

DNA profiles showing all alleles of both donors were used for analysis, except in the degradation experiments. From the genotypes of the donors, the non-shared alleles for each donor were identified in each mixture and the average peak height (RFU) of these alleles was determined. Homozygous alleles were counted as two alleles. For allele drop-outs (occurring for severely degraded samples), a peak height of zero was used. The donor mixture ratio in these DNA profiles was calculated by dividing the average peak height of the non-shared alleles of donor 1 by that of donor 2.

Due to the absence of an RNA quantification method in our procedure, in most experiments several RNA profiles were generated by applying different cDNA inputs [4,8]. From this set of profiles, one informative RNA profile, detecting non-saturated signals for the appropriate cell type-specific markers, was selected for each
mixture. When studying replicates, four of such profiles were prepared using the same cDNA input. For the cell types involved in the mixture, the average peak height per cell type was determined (in RFU). The markers considered were $HBB$ and $CD93$ for blood ($AMICA1$ has relatively low signals and can drop out especially in mixtures in which blood is the minor component), $STATH$ and $HTN3$ for saliva, $PRM1$ for semen (the $SEMG1$ marker was below detection level in most amplifications, which may be due to the donor), $CDSN$ and $LOR$ for skin, $MUC4$ and $HBD1$ for vaginal mucosa (for $HBD1$ the average peak height of two singleplex RT-PCRs was taken) and $MMP7$ and $MMP11$ for menstrual secretion. When a marker signal was lacking (for some mixtures containing vaginal mucosa and in the degradation experiments) a peak height of zero RFU was used in the analysis. In the degradation experiments for each degradation series RNA profiles were obtained from CE injection of both undiluted and diluted RT-PCR products as the undiluted products gave flat top peaks for some markers for the less degraded samples. Within a degradation series, the peak heights for a mixture component were preferably all derived from the diluted injection series. If peaks dropped below detection threshold upon diluted injection (occurring for the more degraded samples), the profiles of the undiluted injections were used for that mixture component in that degradation series. Then, for the flat top peaks, calculated heights were used for which the peak height in the diluted profile was multiplied with the ratio undiluted/diluted height determined for a marker giving non-saturated values in both the diluted and undiluted profile of that amplification (which was mostly $CD93$). This correction relies on the general knowledge that the ratio between marker peaks is not affected by differences in CE injections (e.g. settings or amounts of PCR product injected) when peaks are non-saturated. The cell type mixture ratio in the RNA profiles was calculated by dividing the average peak height values for cell type 1 and cell type 2.

**Results and discussion**

**Mixture ratios in DNA and RNA profiles of various two-component mixtures**

To assess whether peak height data of DNA and RNA profiles can be used to associate donor and cell type, various two-component mixtures having different cell types from distinct donors were generated. Currently, six different cell types are identified by forensic RNA assays [4]: blood, saliva, semen, skin, vaginal mucosa and menstrual secretion. Theoretically, one can prepare 15 two-component mixtures for these cell types, but two of these mixtures (namely vaginal mucosa with menstrual
secretion and blood with menstrual secretion) do not present specific RNA markers for one of the cell types, as vaginal mucosa and blood are co-expressed in menstrual secretion. Thus, 13 informative mixtures can be prepared. Preparation of these mixtures in three different ratios (1:1, 1:5 and 5:1, based on DNA mixture ratios) would result in 39 mixtures, but due to relatively low cell yields for skin, 34 mixtures were completed (Table 1). When the mixture ratios between both donors were determined in the DNA profiles (one profile per mixture; mixture ratio derived from peak heights of the non-shared alleles; genotypes of the donors known), they were in the range of the designed DNA ratios (Table 1), corroborating our experimental approach. After RNA profiling, a single RNA profile was taken for each mixture (showing non-saturated peaks for all defined cell type markers; except for HBD1 for which the data of two singleplex PCRs were used), and the ratio between the average peak heights for cell types 1 and 2 was determined to estimate the cell type mixture ratio (Table 1).

From the donor and cell type mixture ratios it was determined which donor (defined by the cell type he or she provided) and which cell type represented the major contributor in the DNA and RNA profiles, respectively (Table 1). For 10 of the 34 mixtures a discrepancy was observed for the identity of the major component in the two types of profiles (indicated in bold in Table 1). Next, the mixture ratio observed for the RNA profiles was divided by mixture ratio observed for the DNA profiles (Table 1). When this value is close to one, the cell type and donor mixture ratios coincide. For some mixtures (e.g. semen with blood, saliva with blood and saliva with semen) these mixture ratios appear to agree (illustrative DNA and RNA electropherograms (epgs) are shown in Fig. 1A–C). It is conceivable that this correspondence is donor and/or donation-dependent as body fluids are a collection of various cell types that not all express all markers and the actual composition may vary for donors and/or donations. For instance in blood the leukocytes express CD93 and the red blood cells HBB while the abundance of these cell types is affected by infections and immune diseases. For semen, PRM1 is expressed in spermatozoa (thereby corresponding to the fertility level of the donor) and SEMG1 in seminal fluid. Here only PRM1 signals were regarded (due to drop-out of the SEMG1 signal in all amplifications) which may have promoted the correspondence between donor and cell type ratios because of the concurrence of PRM1 expression and DNA in the spermatozoa. For the other mixtures (especially mixtures with skin or vaginal cells), the DNA profile-based and RNA profile-based ratios are different (Table 1 and illustrative epgs in Fig. 1D–E). This may be due to an apparent lower sensitivity of the vaginal mucosa markers and higher sensitivity of the skin markers in the cell typing assay relative to the heights of the peaks obtained with DNA-profiling.
Table 1. Observed mixture ratios between donors (DNA) and cell types (RNA) in two-component mixtures. When a discrepancy for the major component in DNA and RNA profiles is observed the component is presented in bold.

<table>
<thead>
<tr>
<th>Cell type 1</th>
<th>Cell type 2</th>
<th>Designed donor ratio</th>
<th>Observed donor ratio</th>
<th>Observed cell type ratio</th>
<th>DNA major</th>
<th>RNA major</th>
<th>Cell type ratio to donor ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Semen</td>
<td>Blood</td>
<td>0.2</td>
<td>0.2</td>
<td>0.4</td>
<td>Blood</td>
<td>Blood</td>
<td>2.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.0</td>
<td>0.6</td>
<td>0.5</td>
<td>Blood</td>
<td>Blood</td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5.0</td>
<td>3.4</td>
<td>2.5</td>
<td>Semen</td>
<td>Semen</td>
<td>0.7</td>
</tr>
<tr>
<td>Saliva</td>
<td>Blood</td>
<td>0.2</td>
<td>0.2</td>
<td>0.3</td>
<td>Blood</td>
<td>Blood</td>
<td>1.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.0</td>
<td>0.7</td>
<td>0.5</td>
<td>Blood</td>
<td>Blood</td>
<td>0.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5.0</td>
<td>3.4</td>
<td>1.9</td>
<td>Saliva</td>
<td>Saliva</td>
<td>0.6</td>
</tr>
<tr>
<td>Skin</td>
<td>Blood</td>
<td>0.2</td>
<td>0.4</td>
<td>3.2</td>
<td>Blood</td>
<td>Skin</td>
<td>8.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.0</td>
<td>1.6</td>
<td>5.4</td>
<td>Skin</td>
<td>Skin</td>
<td>3.4</td>
</tr>
<tr>
<td>Vaginal</td>
<td>Blood</td>
<td>0.2</td>
<td>0.2</td>
<td>0.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Blood</td>
<td>Blood</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.0</td>
<td>0.9</td>
<td>0.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Blood</td>
<td>Blood</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5.0</td>
<td>3.3</td>
<td>0.7</td>
<td>Vaginal</td>
<td>Blood</td>
<td>0.2</td>
</tr>
<tr>
<td>Saliva</td>
<td>Semen</td>
<td>0.2</td>
<td>0.3</td>
<td>0.2</td>
<td>Semen</td>
<td>Semen</td>
<td>0.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.0</td>
<td>0.9</td>
<td>1.0</td>
<td>Semen</td>
<td>Equal</td>
<td>1.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5.0</td>
<td>3.3</td>
<td>2.6</td>
<td>Saliva</td>
<td>Saliva</td>
<td>0.8</td>
</tr>
<tr>
<td>Skin</td>
<td>Semen</td>
<td>0.2</td>
<td>0.7</td>
<td>2.2</td>
<td>Semen</td>
<td>Skin</td>
<td>3.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.0</td>
<td>2.8</td>
<td>7.4</td>
<td>Skin</td>
<td>Skin</td>
<td>2.6</td>
</tr>
<tr>
<td>Menstrual</td>
<td>Semen</td>
<td>0.2</td>
<td>0.2</td>
<td>0.03</td>
<td>Semen</td>
<td>Semen</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.0</td>
<td>1.0</td>
<td>0.9</td>
<td>Equal</td>
<td>Semen</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5.0</td>
<td>6.4</td>
<td>1.0</td>
<td>Menstrual</td>
<td>Equal</td>
<td>0.2</td>
</tr>
<tr>
<td>Vaginal</td>
<td>Semen</td>
<td>0.2</td>
<td>0.2</td>
<td>0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Semen</td>
<td>Semen</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.0</td>
<td>0.9</td>
<td>0.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Semen</td>
<td>Semen</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5.0</td>
<td>3.2</td>
<td>0.5</td>
<td>Vaginal</td>
<td>Semen</td>
<td>0.2</td>
</tr>
<tr>
<td>Skin</td>
<td>Saliva</td>
<td>0.2</td>
<td>0.7</td>
<td>4.4</td>
<td>Saliva</td>
<td>Skin</td>
<td>6.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.0</td>
<td>3.0</td>
<td>12.2</td>
<td>Saliva</td>
<td>Saliva</td>
<td>0.2</td>
</tr>
<tr>
<td>Menstrual</td>
<td>Saliva</td>
<td>0.2</td>
<td>0.3</td>
<td>0.06</td>
<td>Equal</td>
<td>Saliva</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.0</td>
<td>1.0</td>
<td>0.4</td>
<td>Menstrual</td>
<td>Saliva</td>
<td>0.2</td>
</tr>
<tr>
<td>Vaginal</td>
<td>Saliva</td>
<td>0.2</td>
<td>0.2</td>
<td>0.8</td>
<td>Saliva</td>
<td>Saliva</td>
<td>4.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.0</td>
<td>1.1</td>
<td>0.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Vaginal</td>
<td>Saliva</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5.0</td>
<td>6.0</td>
<td>0.4</td>
<td>Vaginal</td>
<td>Saliva</td>
<td>0.1</td>
</tr>
<tr>
<td>Menstrual</td>
<td>Skin</td>
<td>1.0</td>
<td>0.4</td>
<td>0.01</td>
<td>Skin</td>
<td>Skin</td>
<td>0.0</td>
</tr>
<tr>
<td>Vaginal</td>
<td>Skin</td>
<td>5.0</td>
<td>7.1</td>
<td>0.2</td>
<td>Menstrual</td>
<td>Skin</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.0</td>
<td>0.5</td>
<td>0.4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Vaginal</td>
<td>Skin</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5.0</td>
<td>4.1</td>
<td>0.2</td>
<td>Vaginal</td>
<td>Skin</td>
<td>0.1</td>
</tr>
</tbody>
</table>

<sup>a</sup> The ratios 1:5, 1:1 and 5:1 are given as 0.2, 1.0 and 5.0.

<sup>b</sup> Drop out of both vaginal markers.

<sup>c</sup> Drop out of one vaginal marker.
Fig. 1. DNA profile sections and overlay RNA profile epgs for a selection of the mixtures (mixture type and designed ratio are indicated on the left). For the DNA profiles a single locus is shown for which the donors do not share alleles (both donors are homozygous in E and F). The marker bins for the RNA profiles are presented above and the cell type below the epgs. The DNA peaks and RNA signals for the first component are marked with pink asterisks; those for the second component are marked with black asterisks. In the RNA profiles only the signals included in the cell type ratio calculations (see Materials & methods section) are marked. (A–C, F) Mixtures for which DNA and RNA profiles indicate the same component as major contributor; (D–E) mixtures for which a different major component is observed in the DNA and RNA profiles; (E–F) two ratios for the same mixture showing that the proportional contribution of the saliva component (indicated with black asterisks) decreases both in the DNA and RNA profiles.
Although for several mixtures the identity of the major component in the DNA and RNA profiles did not correspond, the proportional contributions of the mixture components may be used to infer which cell type associates to which donor. This rationale relates to a scenario in which two samples have the same components but in different ratios. After all, donor and/or donation-dependent variation is not an issue when the relation between distinct mixed stains carrying the same components (i.e. same cell types from the same donors) is regarded. In this study, each mixture was prepared in two or three ratios. To assess the relation for the proportional contributions, the donor and cell type mixture ratios were plotted for each mixture (Fig. 2). Separate plots were generated for each subset of the mixtures carrying one of the six cell types. For most mixture types the presence of relatively more cell material (ratios designed to go up from 0.2 to 1.0 and 5.0) resulted in stronger signals for both the corresponding donor alleles and appropriate cell type markers (increasing lines in Fig. 2). These were mixtures carrying blood, semen, saliva or skin (Fig. 2A–D) and to some extent menstrual secretion (Fig. 2F, increasing lines of low slope). Mixtures containing vaginal mucosa showed a fluctuating pattern (Fig. 2E) indicating that the relative strengths of cell type signals in the RNA profiles do not follow the mixture ratios at the DNA level. The potential of using proportional contribution is illustrated by the skin with saliva mixture for which two ratios were completed; one mixture having a DNA mixture ratio of 0.7 (skin to saliva; the saliva donor is therefore the major component in the DNA profile), and another one having a DNA mixture ratio of 3.0 (which means that the skin donor represents the major contributor in the DNA profile) (Table 1). Although the skin to saliva ratios derived from the RNA profiles deviate from these values (as they are 4.4 and 12.2 respectively, which means that the skin signals are the major component in both mixtures), it is clear that the skin-derived signals are relatively higher in the second mixture (Fig. 1E–F). Thus, donors and cell types may be associated from assessing which alleles in the DNA profile have higher peaks when the signals for a certain cell type in the RNA profile increase. Evidently, a significant difference in the ratio of both components in both stains is needed (for instance from 0.7 to 3.0 – as in the example above – and not from 0.7 to 0.8).

Effects of different donors and replicate RNA profiling for saliva with blood mixtures

While the first experiment aimed to examine a wide range of mixtures in different ratios using a single donation for each cell type, the next experiment set out to elaborate on effects of different donors and replicate RNA analyses when using a single mixture type.
Fig. 2. Graphical representations of observed mixture ratios between donors (based on DNA profiles) and cell types (based on RNA profiles) in two-component mixtures. In the panels, subsets of the mixtures are shown: (A) mixtures with blood, (B) mixtures with semen, (C) mixtures with saliva, (D) mixtures with skin, (E) mixtures with vaginal mucosa, and (F) mixtures with menstrual secretion. Each mixture is shown twice using the same colour. Plots use different X- and Y-axis scales. To show the relation between the mixture ratios within each mixture, the data points are connected.
As the saliva with blood mixture showed fairly good coincidence for the mixture ratios in the DNA and RNA profiles, this mixture was selected for further analyses. Saliva and blood show variation in their composition for different donors; for instance in saliva the microbial flora varies for different individuals [17,18] and in blood the amounts of white and red blood cells depend on infection and immune diseases. Nine sets of saliva with blood mixtures were prepared for which three donors gave saliva and three other donors gave blood. DNA profiling was performed once; RNA profiling was done in quadruplicate. NGM DNA profiles carry 15 STR markers to derive the donor ratio, while in the RNA profiles only two markers per cell type are regarded, and this may lead to greater variation between RNA profiling data when replicates are performed. In addition, RNA analyses use 33 amplification cycles and DNA profiling by NGM 29, which suggests that RNA profiles are based on less template molecules, which can lead to more peak height variation. Again, the mixture ratios between donors and between cell types (saliva to blood) were determined. The results are summarised in Fig. 3 and epgs for two of the mixtures are shown in Fig. 4. Variation is observed both between donor sets and for the replicate RNA analyses (Fig. 3). For most donor sets, the donor and cell type analyses indicate the same major component, and the data points reside well in the white areas of Fig. 3 (white areas highlight same major component in DNA and RNA profiles, grey areas indicate different major component, some data points lay around 1 which reflects approximately equal contributions). However, for one donor set all data points reflect a discrepancy for the major component in the donor and cell type analyses, as all brown dots are located in the grey areas of Fig. 3 (also apparent from the epgs shown in Fig. 4A). We infer that the combination of donors whose samples get mixed in an evidentiary trace can affect whether donor and cell type are correctly associated upon DNA profiling and RNA cell typing. For the replicate analyses, some replicate sets show a similar pattern regarding the peak heights of expressed markers (Fig. 4A), while large differences occur for other replicate sets (Fig. 4B, replicates II and IV). Concomitantly, calculated cell type ratios were found to vary nearly 9-fold (Fig. 3, orange dots, highest dot has a saliva/blood ratio of 1.52, the lowest one of 0.17). We cannot identify a specific source for the large variation that is occurring between some RNA profiling replicates, which implies that one needs to anticipate that variation may happen.

Effect of sample degradation on DNA and RNA in saliva with blood mixtures

Forensic evidentiary samples are often not only mixed but also degraded from exposure to UV-light, high humidity, increased temperatures and/or microbes. DNA and RNA have a different chemical composition and structure
Fig. 3. Graphical representations of mixture ratios between donors (based on DNA profiles) and cell types (based on RNA profiles) in saliva with blood mixtures of different donor couples, each indicated by distinct colours. The data points in (A) represent the individual data for the four replicates of RNA profiling that were performed and in (B) the average RNA ratio for the replicates and the standard deviation are presented. Data points in the grey-coloured areas represent data sets that indicate a different component as major in the DNA and in the RNA profiles.

and may therefore degrade at different rates. DNA and/or RNA degradation may further vary for different cell types since distinct types of cells have very different builds. In mixed samples this could lead to different degradation rates for the various components, and we examined this effect in saliva with blood mixtures. Degradation was induced by three strategies. The first strategy involved exposure of liquid state samples to different temperatures (−80 °C (which is regarded untreated), 4 °C, 20 °C or 37 °C) for various days (1, 4 or 7), and this method reflects an extended version of rapid degradation processes that may occur immediately upon stain deposition at a crime scene. The second strategy used specimens dried on textile that were placed at 37 °C and high humidity for various days (0, 2, 4, 7, 10, 14 or 20), which simulates crime scene sample exposure to hot summer days. For the third strategy samples spotted on textiles were irradiated with UV-light for a range of minutes (0, 30, 120, 240 or 360), thereby mimicking sun light exposition. Although crime scene samples may experience combinations of these conditions, this was not tested. Duplicate samples were used for each condition (except for the untreated samples for which six or four specimens were used). After exposure, samples were processed and subjected to DNA and RNA analyses. From the DNA quantification results and the mixture ratio between the two donors (derived from the DNA profiles) the DNA yield per component was
RNA cell typing and DNA profiling of mixed samples

Fig. 4. DNA profile sections and overlay RNA profile epgs for two donor sets comprising saliva with blood mixtures. DNA profiling was done once and a locus having four allele calls representing the two different donors is shown. For RNA profiling four replicates were performed that are marked I, II, III and IV. The marker bins for the RNA profiles are presented above and the cell type below the epgs where * marks a dye blob in the green channel and ** marks a by-product for the 18S-rRNA positive control marker when some over-amplification occurs [4]. The DNA peaks and RNA signals for the blood component are marked with pink asterisks; those for the saliva component are marked with black asterisks. (A) Epgs for the mixture indicated by brown dots in Fig. 3. For this donor combination the major components in the DNA and RNA profiles do not coincide and the RNA replicates show limited peak height variation as a similar pattern is seen in all four replicates. (B) Epgs for the mixture indicated by orange dots in Fig. 3 (DNA and RNA profiles indicate same major component). Here the RNA replicates show much variation and saliva peaks vary from 90 RFU (STATH peak in replicate II) to 6722 RFU (HTN3 peak in replicate IV).
calculated. The average yield for each treatment was divided by the average yield for the untreated samples and this value represents the percentage saliva or blood DNA remaining upon a treatment. To infer the percentage remaining RNA for saliva and blood, RNA profiles were generated with the exact same cDNA inputs for all specimens in a degradation series. Then, the average peak height per cell type for each treatment was determined and divided by that for the untreated samples. For each degradation strategy the percentages remaining saliva or blood DNA and RNA are plotted in Figure 5 (for clarity some data points are not shown but these follow the observed trends).

Fig. 5. DNA and RNA results for saliva and blood in samples exposed to various degradation-inducing conditions as indicated on the X-axis. DNA data are presented by filled bars (blue for saliva, red for blood); RNA data are shown as hatched bars (blue for saliva, red for blood). Data indicate percentage remaining DNA or RNA per body fluid (compared to samples regarded untreated). Results are based on duplicate samples (except for untreated samples which are based on six or four samples for the liquid state or dried specimens respectively) and standard deviations are presented.

All three strategies induce degradation of both DNA and RNA, and less nucleic acids remain with longer exposure times. Interestingly, each method appears to affect the assayed components differently (Fig. 5): when the saliva–blood mixtures are kept in liquid state at elevated temperatures, saliva DNA and blood RNA appear especially affected (Fig. 5). When dried stains are exposed to 37 °C at high humidity, the RNA of both cell types gets eliminated eventually while DNA of both components is remaining (although saliva DNA appears somewhat more stable than blood DNA). UV-light exposures induce reduction of all assayed components (with saliva RNA the least affected) and even after 360 min no component is fully gone (Fig. 5). Thus, the mechanisms underlying degradation seem different for each degradation method: UV-light is known to cause nicks or strand breaks in nucleic acids, while degradation in the
liquid state samples may derive from microbial or enzymatic components in saliva (as blood degrades much less when not mixed with saliva and the level of degradation varies when saliva specimens from different donors are used, results not shown). In all cases, both the smaller and the larger amplicons in the DNA profiles were affected, although we had expected “ski-slope” DNA profiles for UV-light exposed samples [19]. No marker-specific effects were seen: for instance when blood RNA degrades both mRNAs expressed in leukocytes (CD93) and mRNAs specific to erythrocytes (HBB) were affected. The different responses of the various components to the degradation-inducing conditions can have an effect on the association of donor and cell type from DNA and RNA profiles: when liquid samples were incubated at elevated temperatures saliva DNA and blood RNA disappear most rapidly. As a consequence, distinct major components are observed in the DNA and RNA profiles of the longer exposed samples while the same majors were seen in the untreated samples. Illustrative epgs are shown in Figure 6.

Fig. 6. DNA profile sections and RNA profile overlay epgs for liquid saliva with blood mixtures exposed to elevated temperatures. For the DNA profiles a locus having four allele calls representing the two different donors is shown. For the RNA profiles two epgs are shown which are obtained from CE injection of diluted and undiluted RT-PCR products (the latter represents standard injection conditions). Upon undiluted injection saturated peaks are obtained for the conditions with less degradation and here diluted injection presents informative RNA profiles. When degradation proceeds, informative RNA profiles are obtained from the undiluted injections whereas the diluted injections show very low signals. The DNA peaks and RNA signals for the blood component are marked with pink asterisks; those for the saliva component are marked with black asterisks.
Concluding remarks

In the forensic context, RNA profiling for the inference of cell types is generally accompanied by DNA profiling. In this study we assessed whether the results from both types of profiles can be combined to associate cell types and donors. This could be highly valuable for the interpretation of a case and therefore we considered it important to establish whether it is “inadvisable to do so” or whether one would “dispose of useful information” if one would refrain from doing so. We used straightforward two-component mixtures containing different cell types from distinct donors in two or three ratios. Although in several instances the major component in the DNA profile had indeed provided the cell type showing the highest peaks in the RNA profile, this was not strict. Factors that were found to have a role are: 1) the type of cells involved in the mixture, 2) the donor combination, 3) the replicate of the RNA profile and 4) conditions inducing degradation. The source that established degradation (UV-light or elevated temperature for liquid or humid specimens) influenced which component(s) (saliva DNA, saliva RNA, blood DNA or blood RNA) were most affected. In addition, we examined whether donor and cell type association could be made from the proportional contributions in two samples having the same components in different ratios. This approach may hold potential unless vaginal mucosa or menstrual secretion is involved. These two body fluids have the advantage that they are gender-specific, which can guide donor association in mixtures consisting of two contributions by different genders.

Acknowledgements

The authors are grateful to all volunteers who donated samples for this work. We thank Corina Benschop, Hinda Haned and Laurens Grol for the assistance with the manuscript. Role of funding: this study was supported by a grant from the Netherlands Genomics Initiative (NGI)/Netherlands Organization for Scientific Research (NWO) within the framework of the Forensic Genomics Consortium Netherlands (FGCN).
References

Chapter 3

