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Development of RNA Profiling Tools and the Implementation in Forensic Casework

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Summary

In this thesis we have explored the use of mRNA profiling applications as tools for human body fluids and organ tissue identification of biological material found on crime scenes. The chapters cover the developmental processes and validation aspects of two RT-PCR multiplex assays; one aiming at body fluids and skin, and the second identifying organ tissues. In addition, detailed information is provided on the analysis of mixed RNA profiles, interpretation guidelines necessary for the implementation of RNA profiling in forensic laboratory and the prevalence of specific cell types on public objects.

In the current forensic framework, methods used to obtain information on the cellular origin of stains found on crime scene samples are mostly based on histological and immunological principles. These methods have limited sensitivity and are presumptive, which means that definitive statements on the biological origin cannot be given. Around thirteen years ago, mRNA profiling emerged as a possible alternative method for body fluid identification. Some tissue specific mRNA markers for common body fluids like blood, semen and saliva were identified but an all-in-one system for the simultaneous amplification of these markers was not yet developed. In **Chapter 1** we describe the development and validation of an end-point multiplex RT-PCR assay with CE detection for the simultaneous detection of blood, saliva, semen, menstrual secretion, vaginal mucosa and skin. To accommodate the profiling of both mRNA and DNA, a co-isolation procedure was developed combining a DNA and an RNA isolation method. The performance of the co-isolation procedure was compared with the current in-house standard DNA isolation protocol used at the NFI and found to approach the DNA yields. Next, 19 markers were selected from literature studies and combined to form a single multiplex assay. The 19-plex analyses three housekeeping, three blood, two saliva, two semen, two menstrual secretion, two vaginal mucosa, three general mucosa and two skin markers. Through analysis of 15 different sets of body fluid and skin samples (8 individuals each), we found that the combination of markers used to identify the different tissue types yielded RNA profiles specific for each of the investigated tissues. Although differences in peak heights between different markers for the same tissue were obtained, this did not negatively affect the result of RNA profiling. The sensitivity of the 19-plex was assessed by analysis of a dilution series and proved sensitive as full RNA profiles could be obtained from a minimum of 0.05 µL of blood, saliva and semen whereas 0.1 µL of these body fluids was necessary to obtain full DNA profiles. The ability to detect mRNA in old and possibly degraded biological material was also assayed using samplings from objects, which were stored up to 28 years. RNA profiles could successfully be generated for, amongst others, saliva present on a 6-year-old pacifier and a 10-year-old buccal swab, skin on jewellery and musical instruments stored up to 12 years and menstrual secretion on a 4-year-old bed sheet. We also investigated whether RNA could be analysed in stored extracts. DNA extracts from ten mock casework samples and 24 fresh flow-through fractions (collected after binding of DNA on a DNA column), all of known cell type, were subjected to RT-PCR analysis. Positive RNA profiling results were obtained in only five instances, which indicates that by use of commonly used forensic DNA isolation methodologies, RNA will degrade and thus will rarely be analysable. From February 2010 up to September 2013, this assay has been applied in 57 cases analysing 340 samples.

Next to the technical aspects of new methodologies, additional consideration has to be given to profiling strategies and the means by which forensic results are to be interpreted. In **Chapter 2**, we describe the development of a stepwise strategy for obtaining the most optimal RNA profiling result for a case, free of any form of cognitive bias, suitable for any mRNA based multiplex assay. This strategy mainly involved the repetitive analysis of different amounts of cDNA with scoring rules based on the $x=n/2$ rule for each tissue type represented in the multiplex. A scoring table was designed in which profiling results are summarised through assigning one of six scoring categories to each tissue assessed by the multiplex. The procedure was evaluated using seven mock cases particularly suitable for RNA profiling. Samples from each mock case were investigated using six conventional methods for presumptive body fluid inference and these test results were compared those obtained by to the RNA/DNA profiling methodology. For all investigated mock cases the results

from the presumptive tests concurred with the RNA profiles. Also, concordant DNA profiles were obtained. Compared to the results of the presumptive tests, RNA profiling was found to be extra informative for case samples containing vaginal mucosa, menstrual secretion and skin cells. Since for these tissues presumptive tests are not available, RNA profiling adds a new and highly useful layer of information. Next, we contemplated on how to report combined DNA and RNA profiling data to the judiciary or other applicants. Using the results from one of the mock cases (representing clipped fingernails containing vaginal mucosa) we showed that exemplary conclusions on the DNA and RNA level could be made and that formulation of prosecution and defence hypotheses on the origin level as well as on activity level can be useful. A verbal scale was found to be informative to indicate the strength of the formulated conclusions.

Chapter 3 describes a study on DNA and RNA profiling of straightforward two-component mixtures to investigate how donors and cell types can be associated. Mixed profiles occur frequently in forensic casework and association between donors and cell types may be informative for case interpretation. We investigated whether the peak height data of DNA and RNA profiles could be used to infer these associations. For this purpose, 34 basic two-component mixtures (two cell types provided by different donors) composed of six different cell types were prepared in three different ratios (1:1, 1:5 and 5:1) based on DNA mixture ratios and subjected to DNA and RNA profiling. For RNA profiling, a 14-plex was used composed of markers described in Chapter 1. When comparing observed ratios to designed ratios, deviating results were obtained. For some mixtures containing semen with blood, saliva with blood and saliva with semen, corresponding mixture ratios between DNA and RNA peak heights were found. For the majority of the other mixtures, the major DNA component did not correspond to the higher RNA peaks in the RNA profile. Next, the effect of different donors and replicate profiling as well as the effects of sample degradation on mixture ratios were assessed using nine sets of saliva with blood mixtures. The different donor sets showed variable results between mixture ratios indicating that these can affect correct association between DNA profiling and RNA cell typing. Also in a quadruplicate analysis, replicate sets showed large differences in peak heights and calculated cell type ratios for which a specific reason cannot be identified. To investigate the effects of degradation, samples were degraded by means of temperature, high humidity and UV-light. Following DNA profiling and RNA cell typing we inferred that each degradation-inducing method affected DNA and RNA profiles differently and that methods had dissimilar effects on the association of donor and cell type. We conclude the chapter by discouraging the use of peak height data from DNA and RNA profiles for the association of different donors and cell types unless gender-specific cell types and two donors of different genders are involved.

Another topic of interest during is the criminalistic value of DNA and RNA profiles. Next to cell material deposited on an object during the act of a crime, redundant biological material with no link to the criminal event can be present which can complicate reconstruction of the events that took place. In **Chapter 4** we investigated the prevalence of human cell material using DNA and RNA analyses. With RNA analyses, the multiplex described in Chapter 1 supplemented with a sensitive new skin marker was used for 60 samplings (ten different objects, six samples each). The objects were anticipated to be frequently handled by various persons. Samples were collected using DNA-free adhesive tape lifts. For 54 of the 60 samples measurable DNA yields were obtained using a highly sensitive human-specific quantitation method, which is the Alu repeat system. The majority of the DNA profiles of all 60 samples, generated using NGM DNA profiling, resulted in DNA profiles with allele calls under the stochastic threshold. Generally, two or more contributors were involved and samples with more than one contributor tended to have higher DNA yields. RNA cell typing results (performed in quadruplicate and analysed using the method described in Chapter 2) were obtained for nearly all samples and in which skin was regarded observed in 93% of the samples. An additional cell type (besides skin) was observed in 13% of the samples and involved vaginal mucosa or saliva. Signals for blood, menstrual secretion and semen were not observed in the sample set. When examining the relation between observed cell type and DNA yield it was shown that samples containing saliva or vaginal mucosa contained relatively high DNA yields but also skin alone can

provide high DNA quantities. Some objects tend to carry more cellular material such as (paper) money.

In a forensic setting, biological material found on a crime scene is not restricted to body fluids and skin but can also involve organ tissues especially with violent crimes. In **Chapter 5** we explored whether the approach of using a combined DNA/RNA extraction and multiplex analysis described in Chapter 1 for body fluids, could also be applied to organ tissues. Using the BIOGPS expression database, 41 candidate mRNA markers for the identification of brain, lung, liver, skeletal muscle, heart, kidney and skin, were selected. The capacity of these markers to differentiate between these organs was determined using five selection rounds analysing an extensive set of test samples which involved 36 excised autopsy specimens, 49 frozen tissue sections, 20 commercially available RNAs from different human tissues. After selection, 14 markers were found to be tissue-specific and were incorporated in an endpoint RT-PCR multiplex together with additional control markers to yield a final 17-plex. The markers in this 17-plex were specific for their target tissue and sensitive as picogram amounts of organ tissue were sufficient for successful RNA profiling. The 17-plex was in addition able to successfully analyse samples from a blind test set (20 single source and mixed samples, prepared from autopsy material) and samples from a mock abdominal stabbing. Despite that some autopsy samples were degraded (based on DNA profiling), full RNA profiles could be generated from which it can be inferred that the multiplex can manage degraded samples. The results of RNA organ typing also allowed interpretation through the approach described in Chapter 2. This multiplex assay has also been applied in casework and from November 2012 up to September 2013, 15 cases (33 samples) have been assessed.

The methods, which have been developed and used in this thesis are aimed to provide the forensic community a new reliable framework to assess and report the biological origin of forensic specimens. This study provides a detailed insight in the most important technical aspects of this methodology and shows that RNA profiling is fit for purpose.