Aus dem Institut für Rechtsmedizin
der Universität zu Köln
Direktor: Universitätsprofessor Dr. med. M. A. Rothschild

Identification of forensically relevant body fluids and tissues using RNA markers

Inaugural-Dissertation zur Erlangung der Würde
eines doctor rerum medicinalium
der Hohen Medizinischen Fakultät
der Universiät zu Köln

vorgelegt von
Miriam Nicole Sirker
aus Köln

promoviert am 23. November 2018

Gedruckt mit Genehmigung der Medizinischen Fakultät der Universität zu Köln im Dezember 2018.

Hundt Druck GmbH, Zülpicher Straße 220, 50937 Köln

Dekan: Universitätsprofessor Dr. med. Dr. h. c. Th. Krieg

1. Berichterstatter: Universitätsprofessor Dr. rer. nat. P. M. Schneider

2. Berichterstatter: Privatdozent Dr. med. T. Streichert

Erklärung

Ich erkläre hiermit, dass ich die vorliegende Dissertationsschrift ohne unzulässige Hilfe Dritter und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe; die aus fremden Quellen direkt oder indirekt übernommenen Gedanken sind als solche kenntlich gemacht.

Bei der Auswahl und Auswertung des Materials sowie bei der Herstellung des Manuskriptes habe ich Unterstützungsleistungen von folgenden Personen erhalten:

Universitätsprofessor Dr. rer. nat. P. M. Schneider

Dr. rer. nat. I. Gomes

Weitere Personen waren an der geistigen Herstellung der vorliegenden Arbeit nicht beteiligt. Insbesondere habe ich nicht die Hilfe einer Promotionsberaterin/eines Promotionsberaters in Anspruch genommen. Dritte haben von mir weder unmittelbar noch mittelbar geldwerte Leistungen für Arbeiten erhalten, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertationsschrift stehen.

Die Dissertationsschrift wurde von mir bisher weder im Inland noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde vorgelegt.

Erklärung zur guten wissenschaftlichen Praxis:

Ich erkläre hiermit, dass ich die Ordnung zur Sicherung guter wissenschaftlicher Praxis und zum Umgang mit wissenschaftlichem Fehlverhalten (Amtliche Mitteilung der Universiät zu Köln AM 24/2011) der Universität zu Köln gelesen habe und verpflichte mich hiermit, die dort genannten Vorgaben bei allen wissenschaftlichen Tätigkeiten zu beachten und umzusetzen.

Köln, den 12.05.2018 Unterschrift: .....

Alle dieser Arbeit zugrunde liegenden Experimente wurden nach Anleitung durch Universitätsprofessor Dr. rer. nat. P. M. Schneider sowie durch Dr. rer. nat. I. Gomes von mir selbst durchgeführt.

Danksagung

An erster Stelle möchte ich mich ganz herzlich bei Prof. Dr. P. M. Schneider bedanken: für die Überlassung des spannenden Promotionsthemas und für die Möglichkeit der Mitwirkung an dem Forschungsprojekt *European Forensic Genetics Network of Excellence* - EUROFORGEN-NoE. Darüber hinaus möchte ich mich für Herrn Schneiders professionelle und warmherzige Unterstützung, seine stetige Hilfs- und Diskussionsbereitschaft sowie sein mir entgegengebrachtes Vertrauen bedanken. Die letzten Jahre meines Ausbildungsweges wurden dadurch maßgeblich bereichert und wahrlich unvergesslich.

Weiterhin möchte ich mich bei der gesamten Arbeitsgruppe der Abteilung für forensische Molekulargenetik des Instituts für Rechtsmedizin für die tolle Zusammenarbeit bedanken: Conny Schmitt, Magdalena Bogus, Gabi Förster, Andrea Mucha, Theresa Groß, Iva Gomes, Karin Schuster und Jan Fleckhaus – es war schön ein Teil von euch gewesen zu sein. Besonderen Dank möchte ich an dieser Stelle Kerstin Schöbel aussprechen, für ihre fachliche und moralische Unterstützung im Laboralltag sowie ihre Freundschaft.

Ebenfalls bedanken möchte ich mich bei Jennifer Beck für ihre aufmunternden und motivierenden Worte. Die gemeinsamen Abende sorgten für die nötige Ablenkung und einen „frischen Kopf".

Außerdem möchte ich mich bei Benjamin Weing bedanken. Du hast mir, vor allem in der Endphase meiner Promotion, den Rückhalt und die Kraft zum Durchhalten gegeben.

Meiner lieben Familie, insbesondere Oma Lore, möchte ich für ihre Liebe und Unterstützung in den letzten Jahren danken. Ihr habt mir meine akademische Laufbahn erst ermöglicht, habt mich motiviert und hattet immer mindestens ein offenes Ohr für mich.

Meiner Familie

Table of contents

List of tables

List of figures

Glossary

ALS	alternate light source
AP	acid phosphatase
BKA	Bundeskriminalamt
CE	capillary electrophoresis
CNRQ	calibrated normalized relative quantity
Cq	quantification cycle
cSNP	coding region SNP
DAD	DNA-Analyse Datei
DNA	deoxyribonucleic acid
DVI	disaster victim identification
EDNAP	European DNA Profiling Group
ENFSI	European Network of Forensic Science Institutes
EPG	electropherogram
ESS	European standard set
FRET	fluorescence resonance energy transfer
GOI	gene of interest
HBB	hemoglobin beta
HTN3	histatin 3
Indel	insertion deletion polymorphism
IRC	inter run calibrator
ISFG	International Society of Forensic Genetics
LMG	leucomalachite green
LR	likelihood ratio

miRNA	micro ribonucleic acid
MPS	massive parallel sequencing
mRNA	messenger ribonucleic acid
mtDNA	mitochondrial DNA
NDNAD	UK national DNA database
NFI	Netherlands Forensic Institute
NGS	next generation sequencing
PCR	polymerase chain reaction
PRM1 + 2	protamine 1 and 2
PSA	prostate-specific antigen
qPCR	real-time quantitative PCR
RFLP	restriction fragment length polymorphism
RM	rapidly mutating
RNA	ribonucleic acid
RNase	ribonuclease
RSID	Rapid Stain Detection
SBE	single-base extension
siRNA	small interfering RNA
SNP	single nucleotide polymorphism
SPTB	beta-spectrin
StaRs	transcript stable regions
STATH	statherin
STR	short tandem repeat
VNTR	variable number of tandem repeats

1 Introduction

Wherever he steps, whatever he touches, whatever he leaves, even unconsciously, will serve as a silent witness against him. Not only his fingerprints or his footprints, but his hair, the fibers from his clothes, the glass he breaks, the tool marks he leaves, the paint he scratches, the blood or semen he deposits or collects. All of these and more bear mute witness against him. This is evidence that does not forget. It is not confused by the excitement of the moment. It is not absent because human witnesses are. It is factual evidence. Physical evidence cannot be wrong, it cannot perjure itself, it cannot be wholly absent. Only human failure to find it, study and understand it, can diminish its value.

Paul L. Kirk reciting Edmond Locard

Known as pioneer of forensic science and "Sherlock Holmes of France", Edmond Locard (1877-1966) formulated "*Locard´s exchange principle*". Basic idea of this concept is that any action of an individual is not possible without leaving a trace, e.g., fingerprints, hairs, skin cells or different kinds of bodily fluids, and, at the same time, evidence material will be taken away from the scene. Physical materials will thus always be exchanged (Kirk, 1953). According to this principle, it should therefore be possible to associate persons that may have come in contact with the crime scene with encountered physical evidence.

At a scene of crime, the objectives of forensic investigators are more or less the same in every case: detecting, documenting and collecting physical evidence aiming to find as many pieces of the investigative puzzle as possible in order to form a picture of

what happened. Does the blood on the floor belong to the victim, or to the offender? Where exactly can crime-related traces be found? Only in the bathroom, or spread over several rooms? Is the encountered stain actually blood, or could it be possible that the red-brownish spot on the carpet or the wallpaper is another kind of (trace) material? Is it human blood, or does it belong to an animal? Those questions are crucial to reconstruct the course of events and to clarify all ambiguities regarding the circumstances leading to the final picture documented by the forensic investigators. It is highly important to obtain as much detailed information as possible in order to either i) associate a person (or objects) with a scene, other persons or other objects, or ii) to exclude a falsely accused person of being involved in a crime.

In former times, crime scene investigations were restricted to comparatively large and visible stains. Hairs could be examined and compared microscopically for color, damages or morphological structures, or blood could be analyzed determining blood group polymorphisms (early 20th century). An unambiguous identification of a certain individual using mentioned methods was, however, not possible, and, due to the low evidentiary value, these methods do not find judicial use anymore. Nowadays, one of the most important tools to find a match between crime scene evidence and one or more suspect(s) is the recovery of biological material containing deoxyribonucleic acid (DNA) in order to create a (practically) unique genetic profile. For that purpose, theoretically, only a few cells are sufficient. Skin flakes, e.g., obtained from tape lifts or latent fingerprint residues, can successfully be analyzed (Balogh *et al.*, 2003; H. Schneider *et al.*, 2011; Schulz and Reichert, 2002; van Oorschot and Jones, 1997).

The increased sensitivity of methods enables forensic investigators to associate minute amounts of trace evidence material with a particular individual, and therefore it is feasible to establish a link between a person and a crime. However, these new technological possibilities can also cause issues for the interpretation of encountered DNA traces, especially regarding their passive transfer (see section 1.1.2), or DNA mixtures of different individuals. Furthermore, the intensified usage of genetic information can evoke serious challenges for the legal system due to the public fear of possible intrusion into a person´s genetic privacy.

In section 1.1, the most important advances, especially regarding the development of molecular genetic techniques, and the determination of suitable markers, are described to give an overview about the milestones that have been achieved in the history of forensic genetics. Challenges are addressed which may arise dealing with trace amounts of DNA: contamination and secondary transfer; both important issues due to the increasing sensitivity of investigative methods. In sections 1.2 and 1.3 the main subject of this thesis, the identification of forensically relevant human body fluids and tissues, will be taken up again.

1.1 The evolution of forensic genetics: a historical outline

Forensic genetics is, compared to other disciplines in the field, a comparatively young branch of forensic science. As the name implies, it deals with questions relevant for forensics that can be answered with methods and techniques based upon genetics. In this context, the individualization of biological material is one of the chief aims in forensic practice, e.g., to attribute trace evidence found at a crime scene to a suspicious person or to exclude this person from being a trace donor in order to clarify a felony or a homicide. Another important application is the identification of human remains. The latter can comprise the unambiguous identification of an unrecognizable, decomposed body, or the identification of mass disaster victims (disaster victim identification, DVI), e.g., after accidents involving public transport, terrorist attacks, or natural catastrophes (Budowle *et al.*, 2005; Lessig *et al.*, 2011; Prinz *et al.*, 2007). Other important points, besides the branch of criminalistics, are kinship or paternity testing and monitoring hematopoietic stem cell engraftment after bone marrow transplantation (Bader *et al.*, 2004; Khan *et al.*, 2004).

Over the last century, the development of biological and genetic techniques for forensic purposes has rapidly and drastically increased. From today´s perspective, parentage testing based upon anthropological comparisons, e.g., of eye or hair colors, only have historical significance. The discovery of human blood groups in 1901

(Landsteiner, 1901), the clarification of their heredity 10 years later (Dungern and Hirschfeld, 1910), and their final introduction into kinship investigation by Fritz Schiff and Georg Strassmann in the 1920s (Schiff, 1924; Schiff and Adelsberger, 1924; Strassmann, 1927) started an era where hemogenetics was used for forensic purposes (Geserick and Wirth, 2012). In following years, other polymorphisms in serum proteins, such as the HP (haptoglobin) or the GC (vitamin D binding protein) system (Geserick, 2012), polymorphisms in leucocyte antigens, e.g., the HLA system (Dausset, 1958; Van Rood and Van Leeuwen, 1963), or polymorphisms in erythrocyte enzymes (Hopkinson *et al.*, 1963) were described and used for nearly half a century to clarify the majority of kinship cases (Geserick, 2012). Compared to nowadays used marker types (see section 1.1.1), serological blood groups have only little evidentiary value due to their limited discrimination capacities and are practically not used for legal applications anymore. Instead, genetic markers, which exhibit an increased appearance of allele variants per locus (see section 1.1.1.1) and associated high power of discrimination, are studied at DNA level. Thus, the typing potential changed from phenotype to genotype level and from low to more complex marker types taking advantage of the human genome´s polymorphic diversity.

Several fundamental technological milestones have been achieved leading from physical to genetic fingerprints using DNA for an unambiguous identification of a person. Today, some findings, such as the discovery of restriction enzymes (Arber and Linn, 1969; Dussoix and Arber, 1965; Meselson and Yuan, 1968) or the invention of gel electrophoresis for the size-dependent separation of biological molecules, are considered as a matter of course. Back then, however, they opened gates for further developments of increasingly complex methodologies using discovered genetic tools and widened the scope for numerous subject-specific applications.

A big step towards DNA profiling was the detection of restriction fragment length polymorphisms (RFLPs) (Kan and Dozy, 1978). For that purpose, DNA samples of interest were digested specifically by the use of bacterial restriction enzymes and the resulting fragments were then separated according to their length performing agarose gel electrophoresis (Butler, 2009). For their detection, the size-fractioned nucleic acids were denatured and subsequently transferred to a filter membrane and exposed to

complementary labeled DNA hybridization probes; a molecular technique called Southern blot (Southern, 1975). Performing described methodological procedure, two individuals can be differentiated from each other if the length of the detected fragments vary, e.g., due to the loss of a restriction site (see Fig. 1, left site; loss of *r2* leads to a longer fragment *a* spanning from restriction site *r1* to *r3* compared to the shorter fragment *A* spanning from *r2* to *r3*). Sequence polymorphisms (also see section 1.1.1.2) may also cause the loss of such restriction sites.

In 1985, Sir Alec Jeffreys and his discovery of hypervariable loci, also known as minisatellites, proverbially revolutionized the field of forensic molecular genetics (Jeffreys *et al.*, 1985a). He found that certain regions of the DNA are composed of repeated satellite sequences, which also cause length polymorphisms (see Fig. 1, right site; fragment *A*, containing four repeat units, is shorter compared to fragment *a*, containing nine repeat units). One core unit is in the range of eight to 100 bp in length (Tautz, 1993), which are tandemly repeated reaching total allele length of 400 to 1000 bp (Butler, 2011). The detection of those variable number of tandem repeats (VNTRs) polymorphisms using described methods coined the concept of genetic fingerprinting (Jeffreys *et al.*, 1985b).

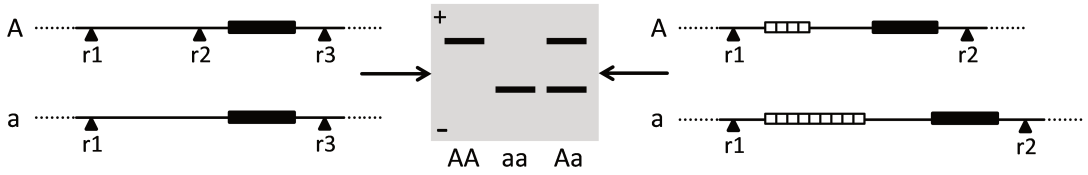


Fig. 1 Restriction fragment length polymorphisms (RFLPs)

Restriction fragment length polymorphisms (RFLPs) can be caused by two different mechanisms: firstly, due to the loss of a restriction site *r* (depicted on the left site), or, secondly, due to variable number of repeats (VNTRs) in between two restriction sites (depicted on the right site; white boxes). In both cases, a treatment with restriction enzymes results in DNA fragments of different sizes (*A* and *a*), which are separated electrophoretically (depicted in the middle), blotted to a filter membrane, and visualized using specific probes complementary against specific DNA sequences (indicated by the thicker black line). The generated band pattern can be used to differentiate between individuals.

The analysis of minisatellite markers was the first DNA profiling technique used to process forensically relevant stain material in order to create individual-specific multi-band patterns, or phrased elsewise, DNA fingerprints (Gill *et al.*, 1985). Due to somatic and germline stability of these polymorphisms (Gill, 1985) it was also possible

to perform paternity testing (Jeffreys, 1985b). Over the years, the analysis of minisatellites using multi-locus probes (Ali *et al.*, 1986) or specific VNTR single-locus probes (Wong *et al.*, 1986) was, however, displaced from forensic practice due to several reasons. On the one hand, the described method requires several micrograms of DNA (Jeffreys, 1985b), which are often not available in connection with forensic casework samples. On the other hand, it is not possible to determine unambiguously which of the generated VNTR band pairs correspond to discrete alleles. These issues were partly overcome by the use of the nowadays famous method polymerase chain reaction (PCR), firstly reported by Kary Mullis and Randall Saiki (Mullis and Faloona, 1987; Saiki *et al.*, 1986). This method provided an enormous increase in sensitivity, also allowing the analysis of minute amounts of DNA (Jobling and Gill, 2004). From a technological point of view, PCR was, however, not the ideal method to analyze VNTRs due to their comparatively large fragment length and the risk for allelic dropout caused by preferential amplification of smaller DNA sections (Tully *et al.*, 1993). Concerning this matter, research took its course towards describing more suitable markers. Since the eukaryotic genome is scattered with tandemly reiterated DNA sequences (Ellegren, 2004), it was only a matter of time to discover and recognize the usability of microsatellite markers, also known as short tandem repeats (STRs) (Edwards *et al.*, 1991; Litt and Luty, 1989; Weber and May, 1989).

The core repeat units of STRs are two to seven bp in length and alleles reach a size of 100 to 400 bp (Butler, 2011) (also see section 1.1.1.1). Compared to VNTRs, smaller STRs represent the best candidates meeting the criteria required for forensic applications: they are highly variable, meaning that multiple variants can be found in a given population, their mutation rate is low, and their inheritance is independent due to separated (or widely spaced) chromosomal locations (Butler, 2011). A further achievement in the development of molecular-genetic techniques was the combined analysis of several STR loci in one simultaneously performed reaction. Multiplex PCRs (Kimpton *et al.*, 1993) offer advantages over singleplex analyses regarding both expenditure of time and costs. Consequently, this method found its way into forensic genetics and is nowadays routinely used to generate DNA profiles of high uniqueness. Data do not contain personal genetic information from coding DNA sequences but

from non-coding regions permitting their legally admissible use for, e.g., criminal investigations, inheritance or immigration cases.

Recent developments are concerned with DNA sequencing techniques, better known as next generation sequencing (NGS) methods. They enable the high throughput analysis of genetic sample material compared to the slowness of the commonly used sequencing approach by Sanger (Sanger *et al.*, 1977), where only a few thousand nucleotides can be sequenced in a week. In contrast to the Sanger method, which uses fluorescently labeled terminating nucleotides in a PCR reaction followed by gel electrophoresis, NGS systems pertinently revolutionized sequencing approaches, e.g., concerning population genetic or biomedical research studies, by rendering the possibility of analyzing human genomes in a single run in a matter of days.

In the following section 1.1.1, the most important and commonly used marker types for forensic genetic applications are reported.

1.1.1 Markers used for forensic purposes

Markers used for forensic genetic applications can roughly be divided into two classes. The first class encompasses markers located on autosomal chromosomes (markers without lineage specificity), and the second class is located either on the gonosomal chromosomes X or Y, or on the mitochondrial genome (markers with lineage specificity). Due to their particular inheritance patterns, these markers can be utilized for different specific areas of application.

1.1.1.1 Short tandem repeats (STRs)

Probably the most popular and important marker type used in forensic genetics are STRs, which, nowadays, have a firm place in routine work. For most forensic relevant questions their analysis represents the standard examination procedure including DNA extraction from the cells, amplification of certain STR loci using multiplexed PCR assays, fragment separation utilizing capillary electrophoresis (CE) and fluorescence detection. In general, STRs can be categorized into groups according to the length of

their core repeat units (two to seven bp). The number of these repeats eventually determines the actual size of a certain locus. Beside the categorization of markers according to the length of their repeat units, they can also be classified pursuant to their repeat patterns into simple, compound and complex repeats (Urquhart *et al.*, 1994). The typing results are usually recorded as DNA fragment lengths or "alleles" indicating the number of core repeats (P.M. Schneider, 1997).

The nomenclature of autosomal STRs was determined by the International Society of Forensic Genetics (ISFG)[1] and the European DNA Profiling (EDNAP) group[2] to ensure a consistent designation of molecular genetic systems (Bär *et al.*, 1997; Gill *et al.*, 1997). Tetranucleotide repeats have become the most attractive STR markers due to their low stutter characteristics (Walsh *et al.*, 1996), their high polymorphy (existence of many different allele variants per system due to susceptibility for copy number variation polymorphisms), and their simple electrophoretic separation (Butler, 2011). Located in non-informative areas of the genome, STRs can be used for human identification purposes without violating a person´s genetic privacy.

In 1997, 13 core STR loci plus amelogenin (for sex determination) were chosen as a result of a community-wide project in the United States (Butler, 2006), meeting the criteria for human identification applications, i.e., separate chromosomal locations to ensure independent inheritance, low mutation rate, high heterozygosity, and multiplexing capacity (Carracedo and Lareu, 1998). Besides other STRs, they are included into a variety of commercial forensic STR kits. To support law enforcement DNA databases, the primary marker set has been expanded from 13 to 20 STRs in January 2017[3].

In Europe, a comparable development has taken place. In 1998, four STR loci were chosen by an Interpol initiative to build a register containing DNA information about sexual offenders: TH01, vWA, FGA and D21S11. Markers had been recommended as suitable for standardization by the EDNAP group and the European Network of

[1] https://www.isfg.org (last access at 14th April 2018)

[2] https://www.isfg.org/EDNAP (last access at 14th April 2018)

[3] https://www.fbi.gov/services/laboratory/biometric-analysis/codis (last access at 7th May 2018)

Forensic Science Institutes (ENFSI)[4] based on a series of collaborative exercises throughout Europe (P.M. Schneider and Martin, 2001). One year later, the nowadays known as the European standard set (ESS) of loci was expanded by D3S1358, D8S1179 and D18S51 (Martin *et al.*, 2001), and by D10S1248, D22S1045, D2S441, D1S1656, and D12S391 a short time later. In Germany, marker SE33 has been additionally integrated into the core markers set from the beginning. It is the most polymorphic STR locus, which considerably increases the power of discrimination of the ESS.

As part of the harmonization procedure, national databases were introduced, the first one in the United Kingdom in 1995 (UK national DNA database, NDNAD), in order to identify donors of biological traces recorvered from crime scenes by comparing these with DNA profiles from convicted offenders. The German database *DNA-Analyse Datei* (DAD) was launched later on in 1998 and is maintained by the Federal Criminal Police Office (Bundeskriminalamt, BKA) ever since. The extension of the ESS ensured a stronger power of discrimination and therefore a lower statistical chance of a false-positive match (Gill *et al.*, 2006, 2015).

Besides human identification purposes, STR profiling can also be applied for kinship analysis. Most human cells contain a diploid chromosome set and therefore two copies of each autosomal genetic marker: one maternal and one paternal. Different variants of these copies can be found, also termed alleles. If two identical alleles are present, the cell or organism is called a homozygote. If two different alleles are present, it is called heterozygote. In cases of a questionable paternity, maternity, or another familial relationships, the analysis of these STR variants can bring clarification by determining shared alleles, e.g., between a child and an alleged father (see Fig. 2) and calculating a cumulative likelihood ratio (LR) using population specific allele frequencies for the examined STR loci. This LR compares two hypotheses: 1) the alleged father is the biological father of the child and 2) the alleged father is unrelated to the child.

[4] http://enfsi.eu/ (last access at 14th April 2018)

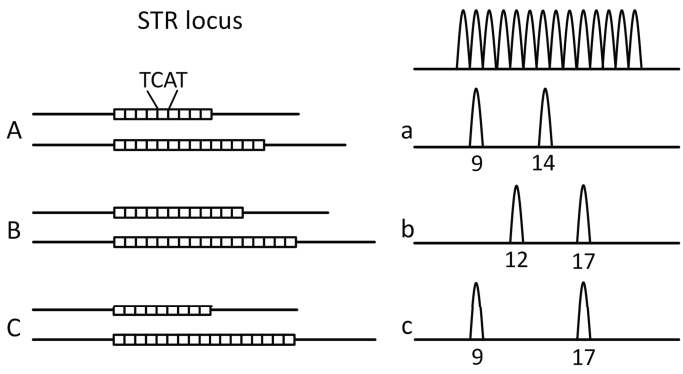


Fig. 2 Scheme of an exemplary STR locus and corresponding EPG of three different individuals
An exemplary STR locus is depicted on the left site of the picture. The core repeat unit consists of the nucleotide sequence TCAT that is tandemly repeated nine and 14 times, respectively, in individual A. Individual B shows 12 and 17 repeat units, and individual C nine and 17. In the corresponding scheme of an electropherogram (EPG), the allele peaks of individuals A, B and C are presented (a, b and c), as they would appear after capillary electrophoretic fragment separation (right site). Results are compared to an allelic ladder (depicted in the top row), an artificial mixture of common alleles of a certain STR locus. In the illustrated case, individual A (or B) could be the mother, and individual B (or A) could be the father of individual C (allele 9 could have been maternally inherited and allele 17 could have been paternally inherited).

A lineage-specific type of STRs is named according to its location on the Y-chromosome: the Y-STRs. They can be uses for different forensic relevant questions. In complex sexual assault cases, e.g., where only DNA mixtures of the female victim and the male perpetrator have been recovered as evidence, difficulties can arise once the portion of female DNA is significantly higher than the male DNA portion. The male allele peaks can be repressed and/or masked by the female allele peaks in the resulting electropherogram (EPG) (see Fig. 3, left site) hindering their detection (Butler, 2011).

Analyzing Y-STRs allows testing exclusively for the male part of the DNA mixture, enabling the identification of the perpetrator (see Fig. 3, right site). Since the Y-chromosome is passed unchanged from individual to individual in male lineages, it is possible to identify male members of a certain family, which can also be helpful in deficiency cases where, e.g., the potential father of a child is missing (Roewer *et al.*, 1992). The Y-specific inheritance pattern can therefore also be helpful clarifying historical questions, e.g., like the identification of the Romanov children (Coble *et al.*, 2009; Gill *et al.*, 1994).

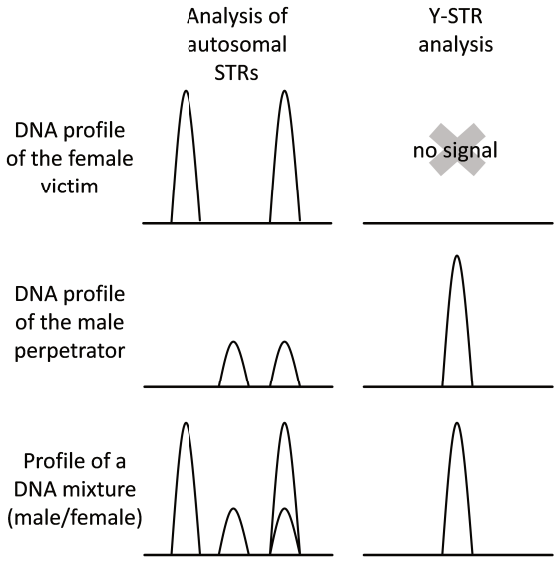


Fig. 3 Comparison of using autosomal or Y-STR analysis in male/female DNA mixtures
Using autosomal STRs (in the scheme, a single STR locus is depicted as an example) it is possible to generate unambiguous DNA profiles both for females and males, e.g., when samples are obtained from a victim and an alleged perpetrator as reference material. A mixture of male and female DNA, as it occurs in sexual assault cases, can result in less informative DNA profiles, e.g., due to a masking effect of allele peaks. In comparison, analyzing Y-STRs the possibility is given to independently and specifically type the male DNA portion in a mixture because the female DNA is not amplified. Thus, female peaks cannot interfere with male peaks enabling the generation of a clear Y-STR profile. The picture was slightly modified from Butler (2011).

However, obvious limitations are implicated when more than one male of the same lineage can be considered as potential perpetrators of a crime. To enable the possibility of differentiating between male individuals of the same genealogical tree, scientists introduced rapidly mutating (RM) Y-STRs to the forensic community (Ballantyne *et al.*, 2014). Some of these RM Y-STRs are already included into commercially available kits for the use in forensic casework, kinship analysis and population genetic studies (Purps *et al.*, 2014). Although these markers show increased locus-specific mutation rates, a clear distinction between two male individuals of a line is, however, not necessarily guaranteed.

1.1.1.2 Single nucleotide polymorphisms (SNPs)

Sequencing the human genome, it was found that a typical genome differs from the reference genome at 4.1 to 5 million sites (The 1000 Genomes Project Consortium, 2015). Approximately 99 % of those variants are single nucleotide polymorphisms

(SNPs) and insertion deletion polymorphisms (indels) (see section 1.1.1.3). In total, more than 80 million SNPs could be characterized (Consortium, 2015). They can be found both in coding and non-coding regions of the DNA (Frazer *et al.*, 2007; Wheeler *et al.*, 2008).

Nowadays, the analysis of SNPs is only supportively performed due to methodical advantages over the analysis of STRs. Using the SNaPshot method, a single-base extension (SBE) assay with standard CE as detection system, comparably short DNA regions (approximately 60 to 115 bp) can be amplified. Due to the small size of these segments it is possible to analyze degraded sample material (Phillips, 2012), e.g., obtained from decomposed tissue of crime case investigations (Sanchez *et al.*, 2006). In order to achieve a comparable power of discrimination, like the routinely used 10 to 15 STRs, it is necessary to analyze about 50 polymorphic SNPs (Jobling, 2004). Today, NGS methods enable the analysis of, e.g., 128 SNP markers for ancestry determination in one assay (Eduardoff *et al.*, 2016).

A further example for the use of lineage-specific markers is the investigation of mitochondrial DNA (mtDNA) sequence polymorphisms, e.g., the analysis of certain SNPs. Due to the particular inheritance pattern of mtDNA, small circular DNA molecules of 16569 bp located in the mitochondria (Anderson *et al.*, 1981), it is possible to compare siblings and maternal relatives as the mitochondria are passed on only from mothers to their children (Hutchison *et al.*, 1974), which is, i.e., interesting for genealogical studies (Butler and Levin, 1998; Parson, 2009). Furthermore, the examination of challenging biological material (i.e., ancient DNA recovered from archaeological and historical skeletal material), or strongly degraded DNA (i.e., recovered from disaster victims) can be performed by the analysis of mtDNA when no DNA profile, or solely a partial DNA profile could be generated analyzing autosomal STRs (Butler, 1998). There are at least several hundred mtDNA copies located in each cell, which drastically increases the chance to obtain results from samples with low genomic DNA content like teeth, old bones or hair (Budowle *et al.*, 2003; Jobling, 2004).

1.1.1.3 Insertion deletion polymorphisms (indels)

Indels are, compared to multiallelic STRs, binary length polymorphisms, representing a type of marker settled between STRs and SNPs. As the name implies, they result from isolated insertion or deletion events where short DNA motifs (one or more nucleotides) are spontaneously incorporated or deleted from the genome sequence during germline DNA replication. Resulting alleles can thus be classified as "short" or "long". By now, more than 2000 biallelic indels are characterized and a multiplex has been developed for a simultaneous analysis of 38 autosomal indels (Pereira *et al.*, 2009a, 2009b).

1.1.2 Challenges for forensic investigators regarding trace DNA samples

"DNA is everywhere in the environment. A DNA profile cannot be interpreted under the strict confines of "Locard´s exchange principle" as there are several alternative transfer methods other than direct "contact". DNA can be transferred from one place to another—either by other people or in aerosol suspension, as house dust, composed of dead-skin-cells…" (Gill, 2014)

Prosecutors, scientists and judges are often faced with criminal cases where only minor amounts of evidentiary cell material are available for investigative procedures; biological traces which are potentially invisible for the human eye. The evaluation of those results therefore represents a challenging endeavor due to numerous issues that can arise processing trace DNA samples. For the reasons discussed in the paragraph below, Locard´s exchange principle is not applicable to all forensic cases as it may stoke expectations leading to a false objectivity, also known as a phenomenon called confirmation bias (Gill, 2014).

Over the last 25 years, especially after the amplification method PCR has been introduced to the scientific community, the sensitivity of DNA profiling techniques increased remarkably (reported in section 1.1). Nowadays, only a few cells are ideally necessary to create a DNA profile (Gill *et al.*, 2000) disclosing new possibilities for forensic scientists; in particular since it has been demonstrated that trace DNA can

persist on different surfaces for several weeks depending upon factors like surface type, humidity, or light exposure (Raymond *et al.*, 2009). However, there is a parallel increase in the uncertainty of association (Gill, 2014). The DNA profile itself can only provide information for the identification of the person who deposited a biological stain, but it does not explain when or how the cellular material was left behind (Fonneløp *et al.*, 2016; Gill, 2014), neither it reveals the information about its biological source, e.g., can the generated DNA profile be associated with semen, or does it originate from saliva or skin flakes? Contamination events can therefore represent a serious issue as the transfer of biological material occurs easily and in most of all cases without intention, especially if (almost) invisible traces are present, such as skin cells on touched surfaces. To aid understanding the complexity of (contamination) events that can cause misleading trace DNA evidence different hierarchical levels of interpretation were defined (Gill, 2014). The first level, the sub-source level, refers to the DNA profile itself, independent of its origin. The source-level is ranking above the sub-source level and deals with the question if the DNA profile can be associated with a certain biological source, e.g., a bloodstain. The activity-level directly associates the DNA profile with the crime event: if semen was detected, sexual assault can be assumed. The question of guilt or innocence is discussed at the highest level (Gill, 2014). Therefore, the basic idea of Locard's exchange principle that "any action of an individual is not possible without leaving a trace" is, in general, correct in terms of its applicability. A perpetrator will leave blood behind in case of an injury, e.g., when he cuts himself at a broken window glass pane during a burglary. In this case, a DNA profile generated from a visible stain like blood can directly be associated with the crime event itself. In contrast, touch traces are much more difficult to evaluate and the exchange principle is therefore not as easy to apply. In crime scenarios where only small quantities of cell material, like a few skin flakes, are involved, secondary transfer and (background) contamination always need to be considered, in particular when public places are in focus of forensic investigations. The sensitivity of today´s methods, which allow obtaining information from just a few cells, means that the detection of a DNA profile has to be interpreted with great caution, as its source can be ambiguous. The determination of the biological source of an obtained profile is fundamental as it may change the outcome of a criminal investigation and hence the future of a defendant´s life.

1.2 Conventional approaches for forensic body fluid identification

Contemplating about forensic investigations, most people immediately think of bloody homicides, fingerprints that unambiguously match a suspect, and full DNA profiles that enable the police to send the suspect directly to prison. Due to the so-called CSI effect, which represents a quite modern phenomenon arisen from crime television shows like *CSI: Crime Scene Investigation*, the idea of forensic practice work is often exaggerated and in large parts highly unrealistic. In reality, evidentiary material needs to be secured with great care according to a standardized protocol, a strict chain of custody needs to be maintained to ensure the admissibility for legal proceedings, and exhibits need to be delivered to an accredited forensic laboratory for analyses; all steps realized by responsible investigating authorities. The final analysis of samples, executed by trained laboratory technicians, is not performed within a few minutes, but can take up to a few hours, days or even weeks depending on the extent of the examinations required. Furthermore, a DNA profile, which can also be partial, is not the only trace evidence that can clarify a crime. Fingerprints, footprints, hair, fabric fibers, plant material, seeds, pollen and body fluids also tell the story of what happened at a crime scene, and can help to complete the picture about the course of events that took place at the scene of crime (An *et al.*, 2012).

The identification of a certain body fluid or tissue type can serve as key evidence in different legal issues regarding the question of guilt and its clarification in order to support a claim or to exonerate a suspect from being involved in a criminal act. An example are sexual assault cases where DNA could be secured from a child´s bed sheet, pajamas or underwear, and a connection to a person could be established, e.g., to the father or another male family member: if the biological origin of the DNA is not evident, one could argue that it may have originated from shedded skin cells, and that the suspect did not harm the child; in case of a positive confirmation that the DNA originated from semen, the argumentation and explanations would be a lot more difficult for the defendant. A further example showing the importance of determining a body fluid´s origin is the differentiation of venous and menstrual blood. If a woman accuses a man of having her raped, it is crucial for the prosecution of the case to

distinguish the type of blood. It could be possible that the woman submits underwear with spots of menstrual blood as evidence, claiming it derives from a vaginal injury caused by the pretended rape. An identification of the blood type could therefore be helpful to convict a person of making a false testimony.

With current conventional methods it is possible to identify blood, saliva, semen, and urine. These tests are of presumptive (preliminary evaluation of evidence material regarding the likelihood of the presence of a certain body fluid) or confirmatory nature. If the result of the preliminary test is positive, a confirmatory test may additionally be performed to verify the first investigative outcome. Each of above-mentioned human body fluids comprises a specific composition of different components (see Table 1), which are important for the respective cells to perform their specific functions. Conventional tests take advantage of these properties to identify a certain fluid type, e.g., the oxygen and carbon dioxide transport molecule hemoglobin (Schechter, 2008) as a specific component of blood, the prostate-specific antigen, a glycoprotein enzyme that cleaves semenogelins in the seminal coagulum (Balk *et al.*, 2003) as a specific component of semen, or amylase, an enzyme that catalyzes the hydrolysis of starch into sugars (Kaczmarek and Rosenmund, 1977) as a specific component of saliva.

Table 1 Biochemical composition of the three most important body fluids for forensic investigations
Each human body fluid is composed of a unique set of biochemical components, which allows the cells to perform their typical functions.

Blood	Semen	Saliva
Hemoglobin	Acid phosphatase	Amylase
Fibrinogen	Prostate-specific antigen	Lysozyme
Erythrocytes	Spermatozoa	Mucin
Albumin	Choline	Buccal epithelial cells
Glucose	Spermine	Thiocynate
Immunoglobulins	Semenogelin	Potassium
	Zinc	Bicarbonate
	Citric acid	Phosphorus
	Lactic acid	Glucose
	Fructose	Immunoglobulins
	Urea	
	Ascorbic acid	
	Immunoglobulins	

Table adapted from Virkler and Lednev 2009.

It is important to keep in mind that the identification of body fluids using conventional methods (described in sections 1.2.1 to 1.2.4) and DNA profiling techniques (described in section 1.1) are separate, independent tests. The determination of a secretion´s origin is performed prior to the DNA isolation process and utilized reagents can have a detrimental effect on subsequent profiling results. Sometimes, only a small amount of biological material can be recovered at a crime scene limiting the number of tests that can be performed (Virkler and Lednev, 2009). Furthermore, the interpretation of mixtures is also compromised, especially if one component is solely present in a small quantity, e.g., a minor portion of saliva mixed with a major portion of blood. In such a case, the specific activity of salivary amylase decreases resulting in a hampered detection capacity (Tsutsumi *et al.*, 1991). The association of a generated DNA profile to a certain body fluid is also not possible since the tested molecules, e.g., hemoglobin and DNA, do not necessarily need to originate from the same cell.

In the following sections 1.2.1 to 1.2.3 different conventional identification approaches for blood, saliva and semen are described, which are routinely performed in forensic casework. Other tests, such as tests for vaginal secretion, urine or sweat, are still problematic, mainly due to inconsistent constituents of the fluids, low sensitivity of tests and false positive results, or low occurrence of particular body fluids (Virkler, 2009). The determination of menstrual blood also remains challenging until today for which reason no reliable test is actually available. In section 1.2.4 another technique, the visualization of touched traces, is shortly described to complete the picture of examination methods commonly applied in real casework scenarios.

1.2.1 Identification of blood

Blood, composed of different cellular and plasmatic components, i.e., hemoglobin, fibrinogen, erythrocytes, leucocytes, albumin, glucose and immunoglobulins (Virkler, 2009), is by far the most common human body fluid that can be encountered at a crime scene setting and can usually be identified visually without the need for special tests. However, an unambiguous confirmation is often legally required for testimonies in court. If visible stains are available, investigators can collect sufficient material to

perform all different tests needed for a doubtless origin confirmation of the discovered liquid and to perform subsequent DNA profiling. However, abundant evidence material is not always available and cleaning attempts, or a challenging surface on which a stain was found may impede the detection of minor unnoticed bloodstains. It has been shown that the use of water or 10 % bleach, or the substrate on which a stain was deposited can have a major impact on the DNA profiling process (Gross *et al.*, 1999). Furthermore, the familiar rust-red color of the blood changes as the stain ages hampering its detection.

Nowadays, there are numerous presumptive and confirmatory tests available for the positive confirmation of blood. The most commonly used presumptive tests, which can be classified into chemiluminescent, chemical and immunological, are, i.e., the luminol test, the Kastle-Meyer test, the leucomalachite green (LMG) test, Hemastix®, Hemident™, Bluestar©, Hexagon OBTI test, and RSID™–blood test. Tests, which are confirmatory in nature, can be categorized as microscopic, crystal and immunological tests, spectroscopic and chromatographic methods (Virkler, 2009). In the following paragraphs, four different test types are described in more detail.

A simple and rapid method for the detection of blood that can be performed immediately at a crime scene, is their visualization deploying an alternate light source (ALS) (Vandenberg and van Oorschot, 2006). Lamps, which emit more than 700 nm in combination with infrared filters, can be used, e.g., to locate latent bloodstains on dark clothing (Grassberger and Schmid, 2013). This technique does not require any sample material making it highly suitable for a first assessment of both visual and non-visual traces. Nevertheless, a long-term exposure or an exposure to certain wavelengths should be avoided to prevent the degradation of DNA evidence (J. Andersen and Bramble, 1997; McNally *et al.*, 1989).

Luminol is a presumptive test reagent, which can also be used for the detection of minute quantities of latent bloodstains, especially, when large areas or dark surfaces need to be examined. As the use of an ALS, this reagent can only serve as a first evaluation of unknown stains and further presumptive and/or confirmatory tests need to be performed. Areas, which are subject for investigations, are sprayed with an alkaline solution of luminol and an activating oxidant, usually hydrogen peroxide

(Madea, 2015). The iron from hemoglobin located in red blood cells of vertebrates serves as catalyst and enhances the oxidation of luminol, which thereupon emits blue light visible in a darkened room (Barni *et al.*, 2007; Madea, 2015). The method is not human-specific but highly suitable for the inspection of fuscous, absorbent exhibits like carpets, doormats or pillowcases (Madea, 2015). A disadvantage of luminol is that it also reacts with bleach (sodium hypochlorite) and other cleaning regents leading to the occurrence of false positives test results (Barni, 2007). Chlorophyll and rust can also have falsifying effects on the test (Madea, 2015). Furthermore, the dilution of a small stain of blood can reduce the chance of obtaining valuable DNA profiling results. Although it has been shown that some luminol formulas can have a detrimental effect on following DNA analysis (Quinones *et al.*, 2006), alternative formulas exist which do not have a disadvantageous influence (Barni, 2007; Della Manna and Montpetit, 2000).

The LMG test, a catalytic test method, is based on the peroxidase-like activity of the hemoglobin´s heme group (Madea, 2015; Virkler, 2009). It can easily be utilized to obtain a first clue about the identity of the examined sample but it cannot determine whether it is of human or animal origin. Due to its simplicity, the test can be performed directly at a crime scene. Hemoglobin cleaves the oxygen molecules from H_2O_2 and catalyzes the reduced form of leucomalachite green (LMG) to the oxidized turquois product indicating a positive test result.

A more precise test is the Rapid Stain Detection of human blood (RSID™-blood, Galantos) test, which detects human glycophorin A present in the membrane of erythrocytes (Anstee, 1990). The immunochromatographic assay, a lateral flow strip test (see Fig. 4), uses two monoclonal antibodies, each recognizing a distinct glycophorin A epitope (Schweers *et al.*, 2008). Once the sample, which is prepared in a diluent buffer, is added to the test cassette, it diffuses towards the test line area. Along the way, the diluent redissolves the pre-dispersed monoclonal antibodies consisting of colloidal gold conjugated to anti-human glycophorin A. If present, glycophorin A will be bound by these antibodies. Complexes are captured by the attached anti-glycophorin A antibodies at the test line causing a color change from transparent to red (Schweers, 2008).

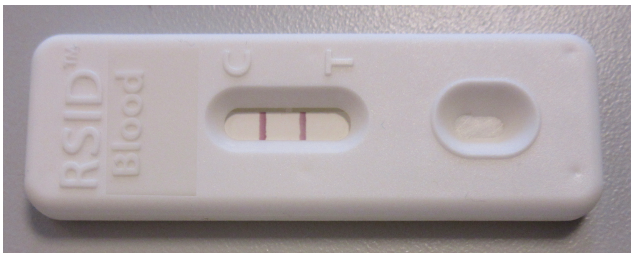


Fig. 4 Rapid Stain Detection of human blood (RSID™-blood, Galantos)
The picture shows a positive test result for blood; the left (control) line indicates that the test performed properly, the right (test) line indicates that human blood was detected (picture: Institute of Legal Medicine, Cologne).

The detection of blood is a very important endeavor since the occurrence of this particular body fluid can be linked to violent felonies and homicide, and its confirmation can strongly influence the direction of an investigation.

1.2.2 Identification of saliva

The detection of saliva can also be seen as important as the detection of blood, e.g., it can become relevant furnishing the proof of oral sex assaults. However, the identification of saliva has proven to be more challenging than the one of blood since this particular human body fluid appears colorless and transparent making it nearly invisible for the naked eye. Therefore, it is often necessary to perform tests to confirm the presence of this human body fluid. In the following paragraphs, different methods for the identification of saliva are described in more detail. Most of the tests, which are used in daily forensic routine, are of a presumptive nature (Myers and Adkins, 2008).

The most popular presumptive test for the identification of saliva is based on the activity of amylase (Virkler, 2009). The enzyme is responsible for the hydrolysis of starch into sugars as a first part of the chemical digestion process. Although amylase can also be found in other human secretions, e.g., vaginal secretions or semen, the quantity for its detection is not sufficient (Whitehead and Kipps, 1975) for which reason it is a suitable candidate for the indirect identification of salivary traces. The starch-iodine test is a basic approach widely used in the field of molecular biology. In the presence of iodine, starch, which can be bound in agar or be in solution, appears blue. If saliva, and hence amylase is applied, the enzyme breaks down the

polysaccharide helices hampering the integration of added iodine. In this case, the agar or the solution remains colorless. This test method is not suitable for mixtures containing blood since the result can turn out false negative (Tsutsumi, 1991).

The Phadebas® test principle was already introduced to the forensic community in the 70s (Willott, 1974). Since that time, the method has been steadily improved for forensic practice enabling the exact localization of saliva stains suitable for further DNA analysis procedures (Hedman *et al.*, 2008). By now, two test categories exist: the press test and the tube test[5]; both similar in their functionality. Principle of the press test is a color change, which can be observed when the coated paper is pressed against a stain of saliva. The immobilized substrate, a water-insoluble starch polymer carrying a blue dye, is hydrolyzed by salivary amylase forming water-soluble blue fragments, which diffuse through the filter paper pores. They can be observed on the paper surface indicating the enzyme´s presence and activity[6]. In comparison, the tube test is more sensitive than the press test. Amylase hydrolyzes starch microspheres, the dye is released into solution and can subsequently be read semi-quantitatively utilizing a spectrophotometer.

A more precise method is the RSID™-saliva test (Galantos). Main difference to above described methods is that two anti-salivary amylase monoclonal antibodies are used to detect the enzyme itself, rather than its activity (Old *et al.*, 2009). The principle is similar to the one of RSID™-blood (see section 1.2.1).

1.2.3 Identification of semen

Besides blood and saliva, semen is one of the most important and commonly encountered human body fluids. It can give valuable information about a course of events related to a possible act of crime, e.g., sexual assault, permitting the corroboration of claims or the exoneration of suspects. In principle, the male-specific secretion consists of two major components, which can be adducted for analysis

[5] http://www.phadebas.com/products/forensic-saliva-test-products (last access at 14th April 2018)

[6] http://www.phadebas.com/archive (last access at 14th April 2018)

procedures: seminal fluid, which again consists of several proteins and other chemical compounds, and spermatozoa. Since spermatozoa are exclusively produced in sexual organs of men and hence are present only in semen, tests are available which perform unambiguously and reliably for that particular body fluid. However, it needs to be considered that not every male person produces spermatozoa. Some diseases, birth defects or surgical interventions, such as vasectomy, can lead to deceptive, negative results. Furthermore, some identification approaches can generate false positive results due to the overlapping enzyme composition of some body fluids. Acid phosphatase (AP) is present in semen but can also be found in other fluids like vaginal secretion. In the following paragraphs, different test types are described in more detail.

One of the simplest and effective, however highly presumptive detection method for semen is the use of an ALS in order to pre-screen the crime scene or evidentiary items for further examination procedures (see Fig. 5A). Molecules, such as flavin or choline-conjugated proteins, fluoresce when irradiated with a light source of a certain wavelength (Olson, 2011); the excitation spectrum ranges from 350 to 500 nm (Stoilovic, 1991). Advantages of this method are its convenience, low incurring costs, and non-destructiveness, which is of particular importance if only low amounts of sample material is available. A disadvantage is that other molecules can fluoresce similar to the ones in semen wherefore an unambiguous identification of examined stains is not possible (Santucci *et al.*, 1999).

A chemical, more precise presumptive test for semen is the AP test, which is frequently applied in forensic practice (An, 2012; Virkler, 2009). The enzyme is secreted by the prostate gland and can be found in large quantities in the seminal fluid (Kaye, 1949). To indicate the presence of AP, a solution of monophenolic phosphoric acid, or its ester, and diazonium salt chromogen in an acetate buffer of pH 5 is given to the sample. The enzyme, if present, hydrolyses its substrate to phenol and phosphate ion. At the same time, the produced phenol is coupled with the diazonium salt, resulting in a dark purple color (Raju and Iyengar, 1964). In practice, sodium alpha naphtylphosphate (substrate) and Fast Blue B (salt) have proven to be suitable for forensic applications (Butler, 2011; Schleyer *et al.*, 1995). There is, however, a drawback in the method´s accuracy since the enzyme, and similar isoenzymes, are also produced

in other human organs (Bull *et al.*, 2002). A detection of semen is anyhow possible as the concentration of APs is comparably higher in semen than, e.g., in vaginal secretion, and the reagent does not react with all other APs, like the APs of red blood cells (Schleyer, 1995).

The prostate-specific antigen (PSA; also known as p30), a glycoprotein produced by the prostatic gland, can also be used as a marker for semen. After its secretion into the seminal fluid, including vasectomized or azoospermic individuals, it is responsible for the cleavage of semenogelins in the seminal coagulum, allowing the spermatozoa to move. One of the most commonly utilized tests is the OneStep ABAcard® PSA (Abacus Diagnostics). The principle behind this confirmatory method relies on an antigen-antibody reaction: a mobile monoclonal anti-human PSA antibody dye conjugate binds to PSA and migrates along the strip towards a control zone where immobilized anti-Ig antibodies reside. The formation of complexes causes a concentration of dye particles. As a result, a visible line appears indicating the presence of PSA (Hochmeister *et al.*, 1999; Kearsey *et al.*, 2001). However, caution is advised using this test due to its ambiguity. PSA is not exclusively expressed in prostatic glands. It could be demonstrated that PSA is also produced in specific tissue of the female urethra and could, consequently, be detected in female urine (Schmidt *et al.*, 2001).

The RSID™-semen test (Galantos), another immunochromatographic assay whose functionality is similar to the one of RSID™-blood and RSID™-saliva, detects human semenogelin; a protein that encases ejaculated spermatozoa. The test uses two types of anti-human semenogelin monoclonal antibodies: one is immobilized on the test line of the test cassette; the other one is conjugated to colloidal gold particles (Old *et al.*, 2012). Once the sample is deposited and the semenogelin antigen-antibody gold complexes arrive at the test line via lateral bulk flow through the test cassette, they are captured by the attached antibodies causing a color change from transparent to red at the test line position (Old, 2012; Sato *et al.*, 2004). Both the ABAcard® PSA test and RSID™-semen test have shown similar results regarding their specificity for semen (Boward and Wilson, 2013).

In contrast to the aforementioned tests, different staining methods for spermatozoa can be applied to unambiguously confirm the presence of spermatozoa. The most

commonly used techniques are Christmas tree, hematoxylin-eosin (see Fig. 5B), and alkaline fuchsin (Allery *et al.*, 2001). A mentioned above, the microscopic identification of semen is not possible and can lead to false negative results if the male responsible for the stain is azoospermic. In such a case, other tests need to be performed, e.g., the PSA test.

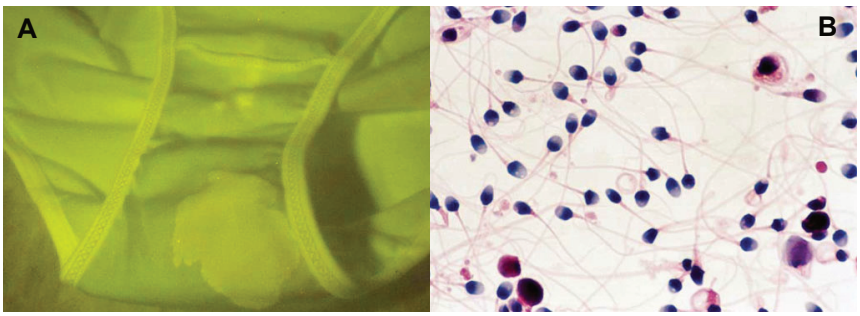


Fig. 5 Visualization of semen and spermatozoa

A: Detection of dried ejaculate using a Crime-lite® (Foster Freeman); a commercial alternate light source (ALS) device emitting wavelengths of 450 nm. An orange filter goggle was used to take the picture (picture: Institute of Legal Medicine, Cologne). B: Hematoxylin-eosin (HE) stained spermatozoa (picture taken from (Aksoy *et al.*, 2012)).

1.2.4 Visualization of touch traces

The visualization of touch traces can become important in cases where porous surfaces need to be examined, e.g., paper or similar materials. Since in most instances only some skin cells can be recovered, the forensic practitioner needs to know exactly where to swab the evidentiary item in order to collect as much biological material as possible for further analyses. This approach can save time and costs. For this purpose, ninhydrin spray is a frequently used reagent enabling the detection of latent fingerprints (Odén and Hofsten, 1954). Amino acids residing in sweat react with the chemical, 2,2-dihydroxyindane-1,3-dione, resulting in a deep purple color (see Fig. 6), which is also known under the name Ruhemann´s purple (Ruhemann, 1910).

Fig. 6 Ninhydrin-visualized fingerprint
Amino acids in sweat react with 2,2-dihydroxyindane-1,3-dione (ninhydrin) resulting in a deep purple color (Ruhemann´s purple). Picture taken from: https://smartphysik.de/Forensik/Ninhydrin.html[7].

1.3 Molecular genetic-based approaches for forensic body fluid and tissue identification

Currently used catalytic, enzymatic or immunologic tests for body fluid identification suffer from multiple noteworthy limitations (see section 1.2). Important to consider, especially regarding forensic investigations where solely limited amounts of evidence material can be secured, are the destruction or consumption of significant portions of available original sample material risking that subsequent DNA analysis procedures may be hampered or even impossible. Furthermore, the lack of specificity and low sensitivity of some conventional tests can be problematic. Aiming to overcome mentioned limitations, new techniques are urgently required to fulfill the challenging demands of forensic investigations.

In the following section 1.3.1 body fluid and tissue-specific ribonucleic acid (RNA) profiling approaches are described representing the major subject of this thesis. A further methodical approach currently drawing attention is the analysis of cell type-specific DNA methylation patterns. A brief overview on this topic is given in section 1.3.2.

[7] https://smartphysik.de/Forensik/Ninhydrin-10g-CAS-Nr-485-47-2-98::3.html (last access at 16th April 2018)

1.3.1 Body fluid and tissue-specific RNA profiling

The human body is composed of various cell types which require a specific set of proteins to fulfill their particular functions (Pontén *et al.*, 2009). In 1958, Francis Crick presented his assumptions and ideas about the synthesis of proteins (Crick, 1958), which is still known as the central dogma of molecular biology (Crick, 1970). In his work, the scientist develops the sequence hypothesis, which basically states that the specific sequence of the genetic material (DNA or RNA) displays the code for the amino acid sequence of proteins. Nowadays, it is well known that RNA, and in this special case messenger RNA (mRNA), represents the link between the genome and the proteome. The particularly expressed subset of genes, the transcriptome, thus reflects a cell´s protein composition and can, theoretically, be utilized as a tool for cell type inference; similar as it is possible to determine a cell type via its specific proteins, e.g., semen via PSA or saliva via amylase (see section 1.2). The development of molecular genetic-based techniques using mRNA for body fluid identification purposes has therefore been in focus of forensic research (Bauer and Patzelt, 2003; Courts and Madea, 2010; Fleming and Harbison, 2010; Haas *et al.*, 2009, 2011, 2012, 2013, 2014; Hanson *et al.*, 2009; Juusola and Ballantyne, 2005; Lindenbergh, Berge, *et al.*, 2013; Lindenbergh *et al.*, 2012; Silva *et al.*, 2015; van den Berge *et al.*, 2014).

One mRNA molecule can be translated several times whereby multiple proteins result. Their uncontrolled enrichment is prevented by different nucleases, which degrade mRNAs back into single nucleotides after a certain period of time (Eulalio *et al.*, 2007; Parker and Sheth, 2007). Both mRNA translation and degradation are hence closely linked processes (Braun and Young, 2014; Wilusz *et al.*, 2001), which allow fine adjustment of a cell´s protein level and reaction to environmental influences (Loscalzo, 2010; Marsit *et al.*, 2006). In living cells, two general pathways exist governing cellular mRNA levels. Both are realized by exonucleases, which initiate the degradation from opposite sides of the respective molecules (Schoenberg and Maquat, 2012). These processes are initiated i) by the shortening the poly-A-tail (deadenylation) by deadenylases (Schwede *et al.*, 2008) and ii) by hydrolyzing the 5´ cap (decapping) by decapping enzymes (She *et al.*, 2008). Subsequently, the mRNA body is degraded either from 5´ to 3´ or from 3´ to 5´ end (Houseley *et al.*, 2006; Parker and Song, 2004). In

addition, the decomposition of some mRNAs can as well be initiated by sequence-specific endonuclease or in response to microRNAs (miRNAs) or small interfering RNA (siRNAs) (Parker, 2004). Depending on the complementary of the miRNA binding sequence and involved proteins, the target mRNA molecule is either degraded or the mRNA translation is inhibited (Wienholds and Plasterk, 2005).

MiRNA has also crucial functions for the cell. Research efforts of the last 15 years revealed the importance of these small molecules in several processes of plant and animal development and also many others biological functions, such as cell proliferation, apoptosis and gene silencing (Bartel, 2004). In this context, many studies have engaged with the role of miRNAs and their dysregulated expression in human diseases, especially in cancer pathogenesis (Alvarez-Garcia and Miska, 2005; Esquela-Kerscher and Slack, 2006; Gregory and Shiekhattar, 2005; Liu *et al.*, 2012; Takahashi *et al.*, 2014; Volinia and Croce, 2013). Associated miRNAs are also called oncomiRs, reflecting the importance of this RNA class in the field of molecular genetics, pathology and medicine. More recently, miRNAs are also in the spotlight of forensic scientists due to their cell type-specific expression patterns (Courts, 2010; Hanson, 2009; Hanson and Ballantyne, 2013; Silva, 2015).

Compared to currently utilized catalytic, enzymatic or immunologic tests, mRNA and miRNA profiling methods seem to offer advantages in a number of ways. When analyzing forensically relevant biological material it is highly important to consider the size of stains (already stated in section 1.2). If the amount of sample material is too low, it is often not possible to perform more than one conventional test limiting the success of an analysis. In 2004, Alvarez *et al.* described an optimized method to isolate mRNA and DNA simultaneously from the same physiological body fluid stain, which renders the possibility to get information about the sample type performing RNA analysis and to individualize the same specimen performing DNA analysis (Alvarez *et al.*, 2004). Such a method allows working in a material saving manner, which is a prerequisite in the field of forensic genetics. A further advantage of using RNA profiling techniques compared to conventional methods is the possibility to develop multiplex assays, meaning that different markers for several body fluids or tissue types can be analyzed simultaneously in one experiment (Fleming, 2010; Haas, 2009; Juusola, 2005;

Lindenbergh, 2012). Suitable markers, however, firstly need to be identified, tested and finally validated for casework, as RNA is known to be readily degraded due to ambient ribonucleases (RNAses). Once implemented into forensic routine use, the accurate detection of a cell´s origin in a non-destructive manner will ensure that also small quantities of sample material can be investigated.

Two main approaches for RNA profiling exist, which are described in the following sections 1.3.1.1 and 1.3.1.2. In the publications presented in this thesis, endpoint PCR was used to detect body fluid-specific mRNAs and real-time quantitative PCR (qPCR) was applied to identify and validate relevant miRNAs for body fluid identification. Both methods are comprehensively compared in section 4.1.

1.3.1.1 Endpoint PCR for mRNA detection

Total RNA has to be extracted from different crime scene stains of unknown origin. For body fluid-specific detection, RNA molecules need to be reversely transcribed (RT) into complementary DNA (cDNA) molecules. In publication 1 (see section 3.1) the iScriptTM cDNA Synthesis kit (Bio-Rad) was used containing a blend of oligo(dT) and random hexamer primers[8]. Both primer types ensure that a variety of targets can be covered and provide the essential starting point for reverse transcription. Once the cDNA is generated, it serves as template for subsequent endpoint PCR, which can either be performed in singleplex assays (only one marker) or in multiplex assays (two or more markers). For each cell- or body fluid-specific transcript, a pair of PCR primers (one of these labelled with a fluorescent dye) is used to generate DNA fragments, which can be separated and detected by CE.

After separating the amplified fragments according to their specific length via CE, results are depicted as EPG comparable to those of STR profiles. If a peak of expected fragment length is observed, it means that the corresponding mRNA transcript was present in the casework sample, which in turn provides evidence for a particular sample type. The analysis of mRNA targets via endpoint PCR therefore, theoretically, results in a yes/no decision. However, since endpoint PCR is not a quantitative method, no

[8] http://www.bio-rad.com/webroot/web/pdf/lsr/literature/4106228.pdf (last access at 14th April 2018)

reliable information can be given about the amount of mRNA in a tested sample. The observed peak height only provides very rough guidance about the amount of RNA transcript. Although this approach appears to be comparatively simple, a certain expertise is needed to ensure data reliability.

To evaluate RNA profiles, an entirely different interpretation strategy is required compared to those of DNA profiling (Lindenbergh *et al.*, 2013). Several reasons, such as different expression levels for specific mRNAs in certain cell types or their regulation by biological, physiological or environmental factors cause an imbalance in peak heights hampering the analysis of RNA profiling results. Over-amplified peaks, bleed-through signals or amplification artefacts can be the consequence (Lindenbergh, 2013). Marker drop-outs or drop-ins may occur, either due to physiological conditions or due to the presence of non-specific transcripts in a cell (van den Berge, 2014). Quantifying the RNA before cDNA synthesis therefore does not necessarily allow the determination of an optimal reaction input since the relative amounts of the mRNA transcripts of interest are not equal in one cell (or cell type). Only the total amount of RNA in a sample, including RNA of non-human origin, can be measured. To overcome this issue, Lindenbergh *et al.*, 2013 suggested a scoring system of six different categories from "observed" to "not observed" for an unbiased assessment of RNA profiling results. Basis for their interpretation approach is an initial test series of different cDNA amounts subjected to a multiplex endpoint PCR containing two to three different primer pairs for each body fluid or cell type of interest. RNA profiles of "too low" or "too high" cDNA input (without signal or over-amplified) are discarded from the evaluation procedure. The final analysis is carried out using the cDNA amount giving the best range of peak heights and performing four identical PCR replicates. Signals of good peak morphology, which are above the detection threshold, are determined and a consensus is formed across the remaining informative RNA profiles by scoring the results from the four replicates and the two or three amplicons, giving rise to eight or 12 individual signals for each cell type. Intermediate categories, such as "observed and fits" or "sporadically observed, no reliable statement possible", help to explain body fluid mixtures and to reflect the reliability of obtained results.

1.3.1.2 Real-time quantitative PCR for miRNA detection

In comparison to endpoint PCR, real-time quantitative PCR (qPCR) (Heid *et al.*, 1996; Higuchi *et al.*, 1992, 1993) is a more complex method and the laboratory workflow requires more extensive planning. In general, two major types of chemistries are routinely used: SYBR green, an intercalating dye (Zipper *et al.*, 2004) which can detect the formation of any PCR product due to its specificity for double-stranded DNA molecules (Butler, 2011), and highly specific hydrolysis probes (Holland *et al.*, 1991). In the following, the qPCR approach using Taqman® chemistry is explained in more detail due to its relevance for the present thesis.

As described in section 1.3.1.1 the analysis process is also initiated with the extraction of RNA from crime scene stains followed by cDNA synthesis, which can either be performed in singleplex or in multiplex assays. The used RT primers are already specific for the markers of interest resulting in cDNA samples containing a multitude of particular cDNA molecules. In subsequent real-time PCR, specialized probes are used, each consisting of i) a target-specific sequence complementary to a corresponding cDNA sequence, ii) a fluorescent reporter dye at its 5´end and iii) a non-fluorescent quencher dye at its 3´end (Butler, 2011). During the reaction, the probe anneals specifically to its complementary single-stranded cDNA sequence. Due to the quencher, which is located in close proximity to the reporter, the fluorescence emission is inhibited by a physical mechanism called fluorescence resonance energy transfer (FRET) (Förster, 1948). During the amplification process the *Taq* DNA polymerase hydrolyzes the probes via its 5´-3´ exonuclease activity. By separating the quencher from the reporter, the inhibition effect is abrogated resulting in an increased fluorescence emission. The fluorescence intensity is directly proportional to the amount of PCR product and can be monitored in real-time by measuring the fluorescence emission after each PCR cycle (Butler, 2011). A proper computer software constructs an amplification plot using the data collected during the PCR (Arya *et al.*, 2005). The quantification measure is the so-called cycle of quantification (Cq). The Cq value is the cycle number at which the fluorescence signal of the detected PCR product starts to increase significantly (exponential) and corresponding amplification curve exceeds the

detection threshold; a defined fluorescence signal level (see Fig. 7). The value is inversely correlated to the logarithm of the initial amount of applied cDNA template.

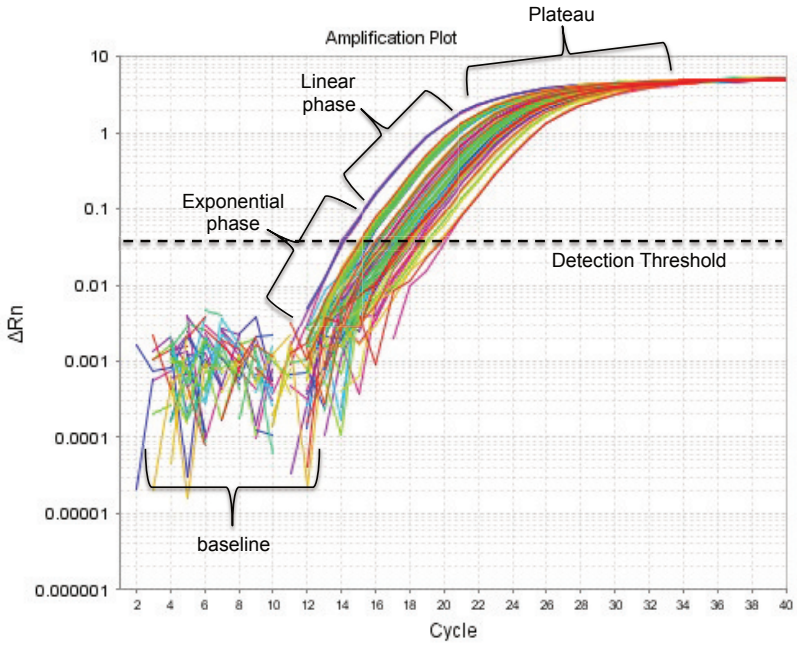


Fig. 7 Exemplary real-time PCR amplification plot

In the presented plot, the initial amplification of cDNA is too low to be detected and is displayed as noisy baseline (this phase is called lag phase). After PCR cycle 12 the specific amplification begins to be exponential, which causes a rapid and significant increase of fluorescence emission depicted as rising curve. The PCR efficiency is, ideally, at 100 % during that phase. A linear phase and a plateau phase, representing saturation due to the reduced availability of PCR components like free dNTPs or probes, follow the exponential phase. Decreasing PCR efficiency characterize both phases. The crossing point of the amplification curve with the detection threshold line is called Cq. This value can be used to calculate the initial amount of cDNA template. The lower the Cq, the more template was available (and vice versa). (ΔRn (normalized fluorescence) = Rnf (fluorescence emission of the product at each time point) – Rnb (fluorescence emission of the baseline)).

The quantification of nucleic acids can be carried out absolutely (e.g., to determine the DNA concentration of a sample) or relatively (e.g., to assess the amount of a certain transcript in a sample in relation to another transcript). For an absolute quantification, a standard curve is required, which is obtained by simultaneously amplifying a series of DNA samples of known concentrations and plotting the logarithm of those DNA concentrations against obtained Cqs. By comparing the Cq of a sample of unknown concentration with the generated linear standard curve, the inference about the starting amount of DNA can be made. To obtain relative quantification results, the expression of target genes, also known as genes of interest (GOIs), needs to be brought into relation with the expression of stably expressed genes (data are expressed as

fold-change of expression levels). Those reference genes, also called endogenous controls, are used to correct experimentally induced, non-biological variations between samples. For an accurate normalization, however, it is advised to use multiple or at least three validated reference genes (Vandesompele *et al.*, 2002). These need to be determined in a separate experiment (before main experiments are performed) since the application of inappropriate reference genes can have a critical impact on data interpretation. To ensure comparability between different qPCR runs it is also important to use inter-run-calibrators (IRCs), identical samples, which are additionally analyzed on each qPCR plate to eliminate technical ("run-to-run") variations (Hellemans *et al.*, 2007). The extensive normalization and calibration effort is the reason why an experiment requires more intense planning, especially when many samples are going to be examined. The evaluation of final qPCR results for body fluid determination necessitates further data editing and knowledge about data interpretation since values are not absolute and not as distinct as mRNA profiling results. This is going to be discussed in more detail in section 4.1.4.2.

1.3.2 Body fluid and tissue-specific DNA methylation profiling

Epigenetics refer to heritable alterations concerning cellular phenotypes or gene functions, which do not derive from genome sequence modifications but from superficial chromatin changes (Berger *et al.*, 2009) – in other words: a change in phenotype without changing the genotype. Such alterations are caused by various mechanisms, i.e., DNA methylation or histone modification (Vidaki *et al.*, 2013). Especially DNA methylation, a biochemical DNA modification in which a methyl group ($-CH_3$) is added to the 5´ position of the pyrimidine ring of cytosines in CpG dinucleotides (Suzuki and Bird, 2008), has a crucial impact on the regulation of gene expression and is therefore in focus of various molecular genetic disciplines such as cancer research (Daniel *et al.*, 2011; Momparler and Bovenzi, 2000) or the investigation of aging (Gonzalo, 2010; Johnson *et al.*, 2012). Also external factors like stress or smoking can influence the methylation status (Alegría-Torres *et al.*, 2011; K. W. K. Lee and Pausova, 2013; Teschendorff *et al.*, 2015). Differential methylation patterns

between different cell types have also been demonstrated (Shen *et al.*, 2007; Straussman *et al.*, 2009) and can be used in a forensic context for body fluid and tissue identification (J.-L. Park *et al.*, 2014; Sijen, 2015; Vidaki, 2013). On these grounds, many studies have already been engaged in the development of DNA methylation profiling techniques for this purpose (Forat *et al.*, 2016; Frumkin *et al.*, 2011; Holtkötter *et al.*, 2017; Jung *et al.*, 2016; H. Y. Lee *et al.*, 2012, 2015, 2016; S.-M. Park *et al.*, 2013). Recently, Lee *et al.* (H. Y. Lee, 2015, 2016) designed a multiplex methylation SNaPshot assay aiming to identify human body fluids (blood, saliva, semen, menstrual blood and vaginal secretion). Using only nine CpG sites it was possible to distinguish between above-mentioned body fluids. In a collaborative exercise, which was conducted by seven laboratories, first results about the applicability of methylation-based body fluid typing could be generated (Jung, 2016).

In comparison to conventional methods for the determination of body fluid samples of unknown origin, DNA methylation-based approaches offer various advantages: i) standard laboratory procedures can be used enabling automation consequently leading to lower error rates, ii) the consumption of evidence material is reduced due to the fact that tests can be performed using DNA samples which are already generated for the purpose of DNA profiling, and iii) it has been suggested that tests show less cross-reactivity (higher specificity) and increased sensitivity (Frumkin, 2011). Furthermore, iv) DNA is notorious for its stability compared to proteins or RNA molecules ensuring analysis success even of highly degraded or aged samples (Forat, 2016; Frumkin, 2011; Holtkötter, 2017). Similar to RNA profiling techniques, v) DNA methylation analyses offer the possibility to develop multiplex assays for the simultaneous identification of several cell types in one approach saving precious sample material (Frumkin, 2011). One of the most convincing advantages of DNA methylation profiling over conventional tests or RNA profiling approaches is, however, that vi) analyses can be performed even after the main investigation has already been completed and original sample material is no longer available (An, 2012; Holtkötter, 2017; H. Y. Lee, 2016); assuming that suitable DNA extracts still exist.

2 Motivation and aims

In a forensic casework context, it is significant to determine the source of a body fluid in order to choose the most suitable method for DNA extraction purposes, ensuring a high sample quality for subsequent STR profiling. Further, it is also important to know a secretion´s origin for the reconstruction of events that have taken place in the course of a crime. As an example, larger amounts of fresh blood are fairly easy to identify; smaller amounts of dried blood are, on the other hand, more challenging to detect due to the color change from dark red to brownish. Such stains require a positive determination to assess their evidentiary value in court.

One of the major drawbacks when working with forensically relevant stains is the limited availability of sufficient biological evidence material. Often only minute quantities can be secured at a crime scene, which can hinder a successful processing of samples. In such a case it would be highly desirable to decide beforehand which test to apply, since sample material may not be sufficient for the performance of several tests. The amount of sample material could be so small that even the application of a single presumptive test to clarify its origin would not be possible. An additional challenge is that the biological material of interest can be compromised by degradation, e.g., in case it was subjected to harsh environmental conditions such as humidity, UV-light, or the exposure to microorganisms.

Conventional tests for body fluid identification, which comprise technologically diverse methods, suffer from noteworthy limitations, e.g., the need for larger volumes of sample material, a reduced assay specificity/sensitivity, or the detrimental effect of some reagents on subsequent DNA profiling attempts (see section 1.2). On the contrary, RNA-based profiling approaches appear to offer various advantages over

conventional approaches, e.g., the possibility to co-analyze both RNA and DNA or the combined analysis of several specific markers for different cell types in a single assay (see section 1.3). For future laboratory routine use it would be highly desirable to have a methodical approach available, which determines relevant body fluids in a single assay using a robust and highly specific set of markers, and without compromising DNA profiling attempts.

Based on these considerations, the following objectives were defined:

- Assessment of the degradation process of mRNA under different environmental conditions (robustness of mRNA-based body fluid analysis)
- Assessment of extraction efficiencies of different methods (RNA extraction only vs. co-extraction of RNA and DNA)
- Evaluation of miRNA-based body fluid analysis as alternative method to mRNA analysis
- Methodical comparison of both approaches (multiplex endpoint PCR for the detection of mRNA vs. real-time quantitative PCR for the detection of miRNA)

3 Publications

3.1 17-month time course study of human DNA and RNA degradation in body fluids under dry and humid environmental conditions

Sirker, M., Schneider, P.M. and Gomes, I. (2016). A 17-month time course study of human RNA and DNA degradation in body fluids under dry and humid environmental conditions. International Journal of Legal Medicine, 1–8.

https://doi.org/10.1007/s00414-016-1373-9

Int J Legal Med
DOI 10.1007/s00414-016-1373-9

ORIGINAL ARTICLE

A 17-month time course study of human RNA and DNA degradation in body fluids under dry and humid environmental conditions

Miriam Sirker[1] · **Peter M. Schneider**[1] · **Iva Gomes**[1]

Received: 11 December 2015 / Accepted: 29 April 2016

Abstract Blood, saliva, and semen are some of the forensically most relevant biological stains commonly found at crime scenes, which can often be of small size or challenging due to advanced decay. In this context, it is of great importance to possess reliable knowledge about the effects of degradation under different environmental conditions and to use appropriate methods for retrieving maximal information from limited sample amount. In the last decade, RNA analysis has been demonstrated to be a reliable approach identifying the cell or tissue type of an evidentiary body fluid trace. Hence, messenger RNA (mRNA) profiling is going to be implemented into forensic casework to supplement the routinely performed short tandem repeat (STR) analysis, and therefore, the ability to co-isolate RNA and DNA from the same sample is a prerequisite. The objective of this work was to monitor and compare the degradation process of both nucleic acids for human blood, saliva, and semen stains at three different concentrations, exposed to dry and humid conditions during a 17-month time period. This study also addressed the question whether there are relevant differences in the efficiency of automated, magnetic bead-based single DNA or RNA extraction methods compared to a manually performed co-extraction method using silica columns. Our data show that mRNA, especially from blood and semen, can be recovered over the entire time period surveyed without compromising the success of DNA profiling; mRNA analysis indicates to be a robust and reliable technique to identify the biological source of aged stain material. The co-extraction method appears to provide mRNA and DNA of sufficient quantity and quality for all different forensic investigation procedures. Humidity and accompanied mold formation are detrimental to both nucleic acids.

Keywords Forensic science · Body fluid identification · mRNA profiling · STR profiling · Degradation · DNA/RNA co-extraction

Introduction

The identification of human body fluids combined with the analysis of DNA to generate individual-specific short tandem repeat (STR) profiles can help to clarify circumstances of a criminal act and establish a link between a crime scene, a victim, and a perpetrator, conversely, to exonerate innocent suspects from a falsely accused crime. However, conventional presumptive chemical, enzymatic, or immunological tests, which are routinely performed by forensic investigators to identify the biological origin of a variety of different body fluids [1], can comprise disadvantages regarding the destruction of precious stain material, in particular, when the analytical strategy includes several tests and subsequent DNA analysis. In addition, casework samples encountered at crime scenes or collected from victims can often be challenging due to limited quantity of material

Preliminary results on mRNA analyses from up to 1 year of storage were published in an extended abstract of the proceedings of the 25th ISFG congress in Melbourne 2013 (Forensic Sci Int Genet Suppl Ser 4 (2013) e164–e165)

Electronic supplementary material The online version of this article (doi:10.1007/s00414-016-1373-9) contains supplementary material, which is available to authorized users.

✉ Miriam Sirker
Miriam.Sirker@uk-koeln.de

[1] Institute of Legal Medicine, Faculty of Medicine, University of Cologne, Cologne, Germany

Published online: 16 May 2016

and/or comprised quality due to advanced decay, e.g., caused by UV light, humidity, or microorganisms. All of this has to be considered before starting the laboratory investigation in order to preserve precious evidence material. Therefore, the possibility to co-extract RNA and DNA from the same stain sample provides an approach fulfilling the pivotal requirements for efficient and comprehensive sample processing in routine forensic use [2–5]. This material-saving isolation technique allows to perform obligate STR profiling and, beyond that, multiplexed messenger RNA (mRNA) profiling to identify several body fluids in one assay [6–9]. Although RNA is notorious for its instability due to, e.g., ubiquitously present ribonucleases, implicating obvious issues for forensic approaches, the feasibility of identifying body fluids using mRNA-based approaches instead of sample-consuming protein-based methods has improved and many studies have focused on this forensic application [10–18]. Surprisingly, an unexpected high stability of a number of relevant RNA markers was observed, and their value for forensic casework has already been demonstrated [9, 19–24]. However, for applied forensic casework, the knowledge about the molecular decay, both for RNA and DNA, is crucial to assess the success of planned laboratory investigation, and thus, more detailed and systematic research on this issue is required.

In the present study, we intended to assess the degradation process of mRNA under different environmental conditions during a 17-month time period for cell-specific expressed genes: hemoglobin beta (HBB) [25] and beta-spectrin (SPTB) [26] for blood, statherin (STATH) [27] and histatin 3 (HTN3) [27, 28] for saliva, and protamine 1 and 2 (PRM1, PRM2) [29, 30] for semen. Blood, saliva, and semen represent the most frequently detected and, consequently, the most important body fluids found at crime scenes or recovered from victims. The chosen markers have been demonstrated and scrutinized to be reliable for forensic casework due to their specificity, sensitivity, stability, and performance. In our study, the sensitivity of these markers was tested with body fluid-specific duplex endpoint PCR assays for three different sample amounts. The amounts of 5, 0.5, and 0.05 μL were chosen to test the stability of each mRNA body fluid marker in relation to the initial amount deposited and the storage period under the given environmental conditions. We decided to use endpoint PCR as detection method rather than quantitative real-time PCR, as it has already been successfully used in casework due to its multiplexing capabilities [6–9]. Furthermore, two RNA extraction methods were compared in terms of their efficiency. The second objective of this work was to explore the parallel degradation of DNA in the same samples, which was either obtained by manual RNA/DNA co-extraction or by a separately performed automated DNA isolation.

Materials and methods

Sample preparation and RNA/DNA extraction

Body fluid samples (venous blood, saliva, and semen) were collected with informed consent from three different, apparently healthy volunteer participants. Blood (female donor) was collected by venipuncture (with anticoagulation treatment); saliva (female donor) was collected in a sterile 1.5-μL Eppendorf tube, and freshly ejaculated semen was collected in a sterile 50-mL plastic tube. For every extraction method going to be used (single RNA, single DNA, and co-extraction) and for two different storage conditions (dry and humid), 5-, 0.5-, and 0.05-μL-sized mock stains were prepared in triplicates, respectively, for all three samples types resulting in six filter paper cards with 27 specimens per extraction time point (Supplementary Fig. S1). To be able to dispense the low-level stains (0.5 and 0.05 μL), 1:10 and 1:100 dilutions of all sample types were prepared in advance using 0.9 % NaCl for a final pipetting volume of 5 μL of each sample and dilution. Dried specimens (1053 in total) were either deposited at room temperature (20–25 °C), exposed to day light and dry (no special humidity control) or humid conditions until extraction (RNA and/or DNA) in a designated location within a secure storage room also used to keep casework items. Humidity was achieved by storing the samples in a transparent plastic box using fabric soaked in distilled water (water was added regularly to ensure constant humidity close to 100 %; no direct contact between moisture and samples). After the indicated storage periods, DNase/RNase free tweezers and scissors were used to cut out individual stains.

RNA extraction

To remove ambient RNases, all surfaces and devices utilized during the extraction procedure were cleaned using RNaseZap® (Ambion). Furthermore, only RNase-free reagents, plastic consumables, and instruments were used. Total RNA was extracted approximately every 2 months with the column-based Allprep DNA/RNA/miRNA co-extraction kit and also with an automated silica-based RNA purification method using the EZ1 robot (both Qiagen). The extractions were conducted including a DNase digestion step. The final elution volumes were 30 μL (Co) or 50 μL (EZ1), respectively.

DNA extraction

DNA was extracted as described above simultaneously with RNA using the co-extraction kit, and further, an automated silica-based DNA purification was carried out using the M48 robot (Qiagen). The final elution volumes were 100 μL (Co) or 50 μL (M48), respectively.

Extractions were performed according to manufacturer's guidelines, and RNA and DNA samples were stored at −20 °C until further use.

cDNA synthesis and endpoint PCR for mRNA profiling

mRNA was reversely transcribed (RT) into complementary DNA (cDNA) using the iScript™ cDNA Synthesis kit (Bio-Rad) following manufacturer's protocol in a final reaction volume of 10 μL containing 5 μL of total RNA. cDNA was subsequently amplified in three different duplex mRNA assays with tissue-specific primers [6, 9, 30, 31] (see Supplementary Table S1): HBB (0.05 μM) and SPTB (1.05 μM) for blood, STATH and HTN3 (both 0.2 μM) for saliva, and PRM1 (0.2 μM) and PRM2 (0.12 μM) for semen. Forward primers were 5′-labeled with 6-FAM (HBB, SPTB, STATH, HTN3, PRM2) or TMR (PRM1). The final reaction volume was 12.5 μL containing 2 μL cDNA, 6.25 μL 2× Multiplex PCR Mastermix (Qiagen), 1.25 μL Q-solution (Qiagen), 2 μL nuclease-free water, and 1 μL primer mix, either for blood, saliva, or semen. The cycling conditions were as follows: initial denaturation at 95 °C for 15 min, followed by 36 cycles of 94 °C for 20 s, 55 °C for 30 s, 72 °C for 40 s, and final elongation at 72 °C for 5 min. Post PCR purification was performed using Sephadex® (GE Healthcare) to remove dye blobs and to increase low-peak signal intensities.

DNA quantification and amplification

Genomic DNA concentrations were determined using the Quantifiler® human DNA quantification kit and the ABI 7500 fast real-time PCR system (both Applied Biosystems) according to manufacturer's protocol. Subsequently, samples were amplified with the Investigator Decaplex SE kit (Qiagen). The final reaction volume was 12.5 μL containing 6.25 μL 2× Multiplex PCR Mastermix and 1.25 μL Decaplex primer mix (both Qiagen). According to quantification results, either maximum volume (5 μL) of template DNA was added to the PCR reaction (when the measured value was ≤100 pg/μL) or 0.5 ng as the optimal amount. The thermal cycling conditions were as follows: initial denaturation at 95 °C for 15 min, followed by 5 cycles of 94 °C for 30 s, 62 °C for 2 min, and 72 °C for 75 s, followed by 25 cycles of 94 °C for 30 s, 60 °C for 2 min, and 72 °C for 75 s. The final elongation was at 68 °C for 60 min (optimized Universal Multiplex Cycling Conditions, Qiagen).

Capillary electrophoresis and analysis of RNA and DNA profiles

RNA

Purified PCR products were separated and detected with an AB 3130 Genetic Analyzer and POP-4 polymer. A dye set which included FAM and TAMRA and the internal lane standard ILS600 (Promega) were used. Raw data were analyzed with GeneMapper® Software ID v3.2 (Applied Biosystems). For data compilation, mean values were calculated from relative fluorescence unit (RFU) peak heights obtained from the biological triplicates. As endpoint PCR is not a quantitative method, for ease of data interpretation, RFU intervals were defined and converted into a relative scale with five categories from 100 to 0 (>5000 RFU = 100, 5000-2500 RFU = 75, 2500-1000 RFU = 50, 1000-100 RFU = 25, <100 RFU = 0).

DNA

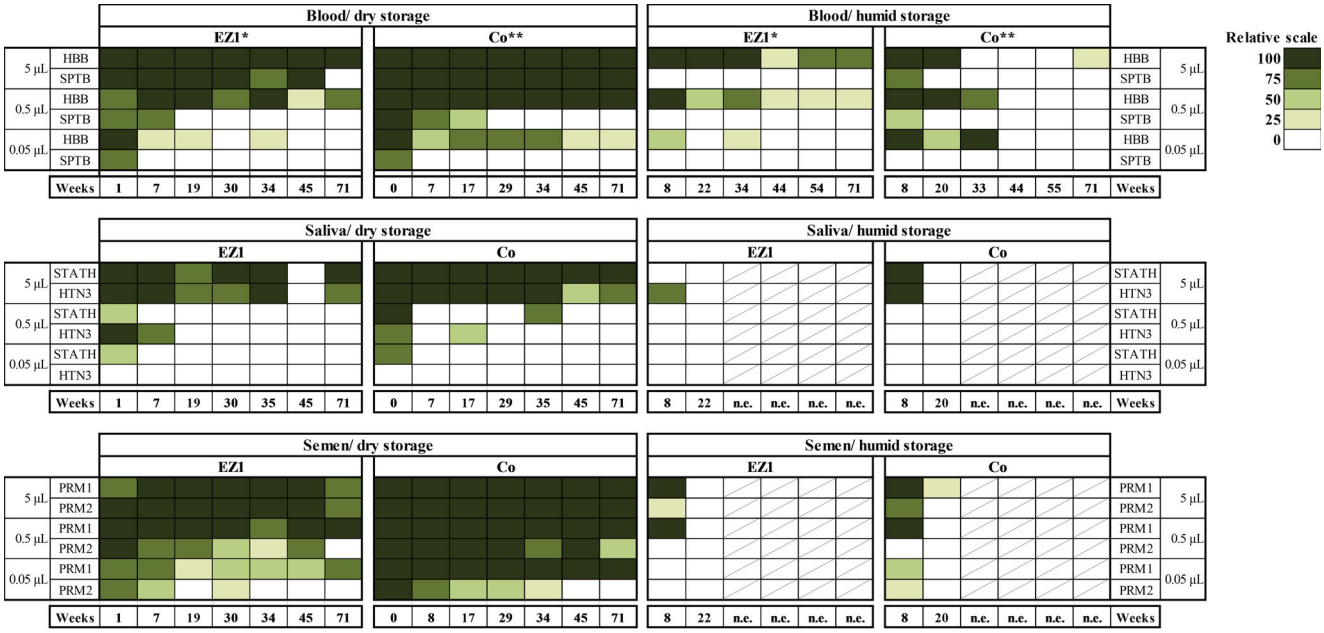
PCR products were directly separated without preceding purification using the AB 3130 instrument and software under standard conditions. An analytical threshold of 50 RFU was used. Genotypes were confirmed by comparison with reference profiles (unexposed controls), assessed by counting successfully amplified alleles, and the results were compared to the number of expected alleles. Calculated percentages of observed alleles were obtained by averaging the results of the biological triplicates.

Results and discussion

The effect of nucleic acid degradation caused by different environmental conditions (aridity or humidity at room temperature) was demonstrated using body fluid-specific duplex endpoint PCR assays for the detection of RNA and an STR profiling kit for the detection of DNA. Blood, saliva, and semen mock stains of different sample volume (5, 0.5, and 0.05 μL) were prepared and exposed for varying time periods up to 72 weeks, followed by different extraction methods (single RNA, single DNA, and DNA/RNA co-extraction). The time points for sample collection were not exactly the same for dry/humid samples as well as the three extraction methods due to a limitation in the number of samples to be processed simultaneously.

mRNA profiling

The complete mRNA profiling results are summarized in Fig. 1. Post PCR purification strongly improved the quality of the results in terms of a reduced appearance of dye blobs and increased peak heights so that even RNA from low-level body fluid samples (0.05 μL) could be detected. This is of particular importance for forensic stains, which are often of small size, like microscopically small spots of a certain body fluid, or in different states of degradation due to the impact of UV light, ubiquitously present RNases, or humidity, which often cause mold formation depending on the given environmental conditions. In general, blood and semen stains were



n.e., no extraction; *automated silica based RNA purification method; **Allprep DNA/RNA/miRNA co-extraction

Fig. 1 Heat map of mRNA profiling results: Defined RFU intervals were converted into a relative scale depicted by colors of decreasing intensity (>5000 RFU = 100, 5000–2500 RFU = 75; 2500–1000 RFU = 50; 1000–100 RFU = 25; <100 RFU = 0). No extraction was performed for the samples stored under humid conditions when no results had been obtained from samples extracted at the preceding time point

much more affected by increased mold formation due to humidity in comparison to saliva stains. Mold formation occurred randomly within the same type of body fluid and introduced a variation affecting some of the data that was beyond experimental control. Nevertheless, the mold-affected samples were included into the analysis, as such a process would also occur in a real scenario. It was observed that 5 µL mock stains stored in humidity were affected more frequently by mold formation than samples with 0.5 and 0.05 µL, most likely due to the fact that more biological substrate was present.

HBB and PRM1 seemed to be the most stable and reliable markers explored. Both markers could be detected up to 70/71 weeks of dry storage. Even in very small-sized samples (0.05 µL), the detection of cell-specific transcripts was possible, but, as expected, the amplification signals were less strong. Blood samples stored in humidity could also be detected with HBB for up to 71 weeks of storage (5 µL) or up to 33/34 weeks (0.05 µL), respectively. This was to be expected since it had been demonstrated previously that HBB was detectable in 23-year-old blood stains on various substrates [19]. However, in contrast to the previous study, which analyzed large bloodstains of more than 50 µL, it is encouraging to observe that long-term detection of blood is also possible in samples of very low volume. Semen samples could be detected with PRM1 for up to 20 weeks of humid storage (5 µL) or up to 8 weeks (0.05 µL), respectively. Semen marker PRM2 also provided strong amplification signals, comparable to those of PRM1, but it was less sensitive, especially regarding the low-level samples. Saliva markers STATH and HTN3 provided good results for the dry-stored 5 µL samples, but they did not show reliable results for the 0.5 and 0.05 µL stains. Both markers dropped out already at the beginning of this study. Under humid conditions, only very limited results could be obtained after 8 weeks for the 5 µL saliva samples. Apparently, the saliva markers exhibited an increased sensitivity to hydrolytic damage which was also shown in a previous study on RNA degradation in different body fluids by Setzer et al. [20].

The long-term persistence of RNA molecules under dry conditions may have several reasons. Fordyce et al. [32] discussed that the dehydration of a sample reduces the activity of RNases, which causes protection of the nucleic acid against its decay. Another general reason for the extended survival of RNA molecules may be their ability to resist spontaneous hydrolytic depurination due to stronger N-glycosidic bonds and depyrimidation processes and to form secondary and tertiary structures which prevent the phosphodiester bond hydrolysis [32]. Based on these considerations, the authors even state that in some cases, RNA can be more stable than DNA.

In a recent study based on massively parallel sequence analysis of transcript regions with high coverage from degraded body fluids, Lin et al. [33] have observed that PCR primers targeted to transcript stable regions (StaRs) are able to consistently and specifically amplify a wide range of RNA biomarkers in various body fluids of varying degradation levels. HTN3 was one of the tested markers, and the authors observed a fourfold increased sensitivity when StaR primers were used.

This implies that newly designed primers following this strategy could lead to greatly improved sensitivities in detecting forensic samples from degraded body fluids. Thus, not only the marker but also the most stable target transcript region may have a strong impact on the success rate of mRNA body fluid analysis.

Total DNA yield and STR profiling

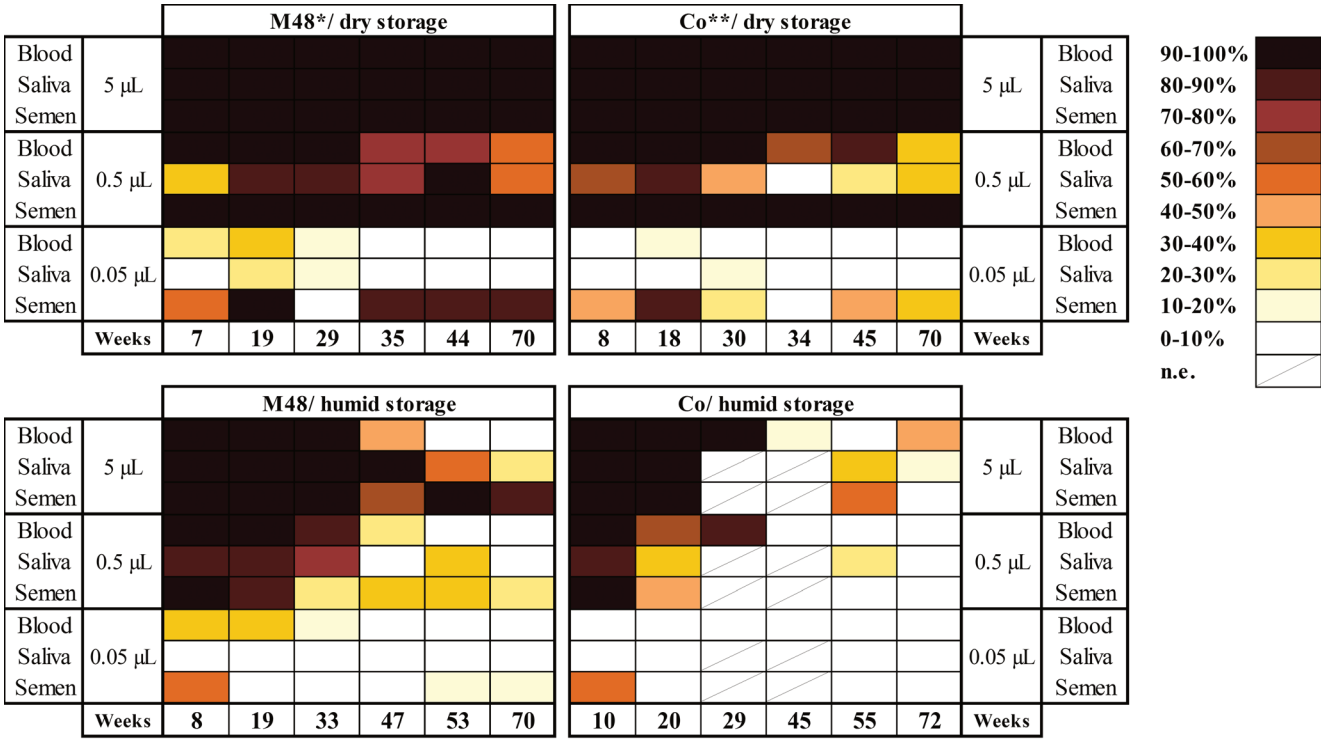
DNA quantification results for the 5 μL samples stored under dry environmental conditions are presented in Supplementary Fig. S2. Blood samples showed average total DNA yields of 8.47 ± 5.86 ng (extracted with the M48 robot) and 6.50 ± 2.88 ng (extracted with the Allprep kit) recovered from all samples over the whole time period of 72 weeks. In comparison, the total DNA yield recovered from saliva samples was slightly higher for both methods with 20.47 ± 7.55 and 18.70 ± 4.06 ng, respectively. Semen samples revealed the highest total DNA yields which were in average 212.92 ± 136.89 ng (M48) and 149.21 ± 91.78 ng (Allprep), a discrepancy of 70 % between both extraction procedures, which was solely observed in this case (5 μL and dry storage). In general, we could not assert an unambiguous difference between the two utilized methods. Quantification results of samples stored under humid environmental conditions are not presented. Due to randomly occurring mold growth, the total DNA yields measured were rather low and values showed more fluctuations between body fluid types depending on the degree of mold affection so that a direct comparison was not meaningful.

An initial STR analysis of unexposed controls (immediately extracted after preparation of specimens) was performed to generate reference profiles for the used body fluid mock stains (Supplementary Fig. S3). The optimal input volume for the PCR was determined according to quantification results to ensure that STR typing success is correlated only with the total DNA yield and not with the changing DNA concentrations after different extraction methods. The total numbers of amplified alleles, which represent full profiles based on 11 markers of the Investigator Decaplex SE kit, were as follows: 22 alleles for blood, 21 for saliva, and 22 for semen. The genotypes of the long-term stored samples were confirmed by comparison to the references, and the successfully amplified alleles were counted to determine the percentage of the obtained alleles per sample. The results are summarized in Fig. 2. We are aware of course that samples exhibiting less than 50-60 % of the alleles of a full profile are not quite informative in casework. However, all observed alleles down to a single allele per profile, which is represented by 4.5 and 4.7 % of the full reference profiles, respectively, were included to allow the full assessment of sample decay. In general, differences could be observed between the disparate body fluid types, between the 5, 0.5, and 0.05 μL sample volumes, respectively, and between the different storage conditions (dry and humid). Overall, blood samples extracted with the M48 robot showed better results than those obtained by co-extraction with the column-based method.

As expected, the effect of dry storage conditions on the success rates of STR analysis was comparatively low (see examples in Supplementary Fig. S4). It was possible to detect the different profiles of blood, saliva, and semen donors during the whole time period. The peak heights decreased when analyzing lower concentrated samples (0.5 and 0.05 μL compared to 5 μL), but it was still possible to detect single alleles. Overall, the results from semen were more complete than those from blood, and blood performed better than saliva. Differences between sample types could be explained by diverging cell densities of undiluted body fluids, which was already apparent from DNA quantification results (semen has a higher density of cells compared to blood and saliva). It can also be speculated that different cell membrane structures have variable protective effects to conserve the genetic material, and hence, it may be more difficult for, e.g., microorganisms or UV radiation to degrade the nucleic acids from sperm cells compared to buccal cells in saliva. In a study by Hall and Ballantyne [34], the UV-induced damage in bloodstains was analyzed. In this regard, the authors mention that protection against degradation caused by UV radiation could be provided by the tight connection of the DNA to proteins (e.g., histones) and the cellular milieu of the DNA. Protection could be provided due to radiation-absorbing molecules in the cytoplasm, e.g., proteins or RNA. Also, the genetic material is located in the nucleus of a cell, which represents an additional protective layer besides the cell membrane. In a later study by Hall et al. [34], the assumption was made that the dehydration of a biological specimen can provide additional protection by limiting the diffusibility of damaging agents. The authors also refer to a previously described conformation change of the DNA from a relaxed form B (physiological) to a compact form A [34, 35]. Form A distorts the steric relationship of the bases preventing dimer formation causing some types of defects. The abovementioned mechanisms could theoretically be responsible for the conservation of the genetic material against molecular damage, such as, e.g., base modification, single and/or double strand breaks, or damage by photoproducts [34].

Samples stored under humid conditions showed great differences regarding the percentages of successfully amplified alleles compared to samples stored under dry conditions. After 47 (M48) or 45 (co-extraction) weeks of storage, a sharp decline of the success rates of amplified alleles was observed for all body fluid types and all volumes (see example in Supplementary Fig. S4).

Although blood samples stored under dry conditions showed stable results over the whole time period of this study (full profiles for all 5 μL samples, partial profiles for 0.5 μL



n.e., no extraction; *automated silica based DNA purification method; **Allprep DNA/RNA/miRNA co-extraction

Fig. 2 Heat map of DNA profiling results: Percentages of successfully amplified alleles were converted into a scale depicted by colors of decreasing intensity (from 100 to 0 % in 10 % steps). For a number of samples stored under humid conditions and collected after 29 and 45 weeks, respectively, no extraction was performed due to the fact that no RNA had been recovered previously (see Fig. 1)

samples, and at least a few detected alleles for the 0.05 µL samples), blood samples stored under humid conditions showed comparably low percentages of successfully amplified alleles. In particular, after 47 weeks of storage, the success rate decreased drastically. As mentioned above, this may be explained by the increased mold formation, which preferentially affected the blood and semen stains as they represent good medium for microorganisms to colonize and proliferate. The degree of mold growth had a direct and strong correlation with the success of DNA amplification. Dissing et al. [36] showed that humidity in the air is the main reason for microbial activity since the availability of water is crucial for cells to perform biochemical processes. Their exoenzymes and general uncatalyzed hydrolytic reactions cause the degradation of DNA. In contrary, dehydration of a sample reduces the possibility for enzymatic reactions to occur, and the drying process of a sample also inactivates endogenic enzymes responsible for degradation.

Efficiency of extraction methods

RNA extraction

Our typing results demonstrate that mRNA can be recovered in sufficient quality and quantity by means of both methods. Nevertheless, the column-based co-extraction method seemed to be slightly more suitable to recover mRNA from samples stored under humid conditions. In general, the automated silica-based method (EZ1) also produced good results, but compared to the co-extraction method, it tended to provide lower signals, possibly due to a higher elution volume (50 µL for EZ1 vs. 30 µL for co-extraction). For this reason, lower concentrated samples often dropped out completely. An advantage of the co-extraction method is an adjustable elution volume, which can be very helpful to increase the chance of obtaining higher concentrated RNA extracts from small stains. This confirms previous observations by Akutsu et al. [37] who reported that the quality of extracted RNA is comparable using either a manual column-based method or the automated EZ1 technology. Although they found the column-based extracted RNA showing less sensitive RT-PCR results, we could not see limitations or significant differences performing end-point PCR analysis. A recent study comparing five different extraction methods for forensic RNA analysis performed by Grabmüller et al. [38] revealed that the Allprep kit was less efficient regarding RNA extraction compared to the other methods but gave the best results for DNA extraction. The authors also showed that the co-extracted DNA was of a high quality proving that the method is suitable for forensic applications.

DNA extraction

The results demonstrate that DNA can be simultaneously extracted with RNA without loss of material and compromising the potential to generate a DNA profile. Depending on the storage periods, it was also possible to generate partial profiles of very small amounts of dried body fluids. To increase the DNA recoverability of low-level samples, a subsequent precipitation might be useful to concentrate the purified DNA (or RNA) for subsequent applications but was not tested in the current study. Generally, it must be taken into account that the observed variation in the recoverability of nucleic acids may be caused by additional environmental, biological, and technical factors beyond our control, e.g., a variable density of cells among the physiological triplicates as well as randomly occurring mold formation and growths.

Concluding remarks

In this study, the time-dependent degradation of both RNA and DNA from small amounts of human body fluids stored under two different environmental conditions was compared, and different extraction methods were explored in terms of their efficiency. For the set of six genes tested in the present study, mRNA profiling has been demonstrated to be a sensitive method for positive identification of blood, saliva, and semen, representing the most common biological stains found at crime scenes. Reliable markers, especially for blood (HBB) and semen (PRM1), provide the possibility to detect stains of small size with the help of the manual co-extraction method as well as with the EZ1 robot. The automated extraction procedure provides the advantage of a minimized ribonuclease contamination risk and represents a fast and effective method to extract RNA. Nevertheless, the column-based Allprep DNA/RNA/miRNA co-extraction method seems to be more suitable, in particular to recover mRNA from humidity-degraded samples, although moisture and accompanying mold formation had a detrimental effect on the nucleic acid stability in particular after longer storage periods. Furthermore, STR analysis has shown that DNA is stable for a long time of dry storage, and full profiles can be generated for each type of body fluid. Both the automated extraction and the manual co-extraction provide DNA of good quality, and no great difference between results was observed. However, there are obvious challenges to amplify DNA from very small size stains, e.g., 0.05 μL. Furthermore, the impact of humidity and microorganisms always needs to be considered.

In conclusion, the co-extraction method provides the opportunity to analyze both mRNA and DNA from the same stain without loss of material or quality of the sample. The high stability of mRNA and DNA is promising for routine forensic use as it exhibits the potential to examine both the biological and individual source of stains exposed to different environmental conditions for long time periods.

Acknowledgments This work was financially supported from the European Union Seventh Framework Programme (FP7/2007-2013) under grant agreement no. 285487 (EUROFORGEN-NoE). We would like to thank our volunteers for donating the biological samples for this study and Ana Freire Aradas for helpful discussions.

References

1. Virkler K, Lednev IK (2009) Analysis of body fluids for forensic purposes: from laboratory testing to non-destructive rapid confirmatory identification at a crime scene. Forensic Sci Int 188:1–17. doi:10.1016/j.forsciint.2009.02.013
2. Alvarez M, Juusola J, Ballantyne J (2004) An mRNA and DNA co-isolation method for forensic casework samples. Anal Biochem 335:289–298. doi:10.1016/j.ab.2004.09.002
3. Bauer M, Patzelt D (2003) A method for simultaneous RNA and DNA isolation from dried blood and semen stains. Forensic Sci Int 136:76–78. doi:10.1016/S0379-0738(03)00219-6
4. Bowden A, Fleming R, Harbison S (2011) A method for DNA and RNA co-extraction for use on forensic samples using the Promega DNA IQ^{TM} system. Forensic Sci Int Genet 5:64–68. doi:10.1016/j.fsigen.2009.11.007
5. Watanabe K, Iwashima Y, Akutsu T et al (2014) Evaluation of a co-extraction method for real-time PCR-based body fluid identification and DNA typing. Leg Med 16:56–59. doi:10.1016/j.legalmed.2013.11.002
6. Juusola J, Ballantyne J (2005) Multiplex mRNA profiling for the identification of body fluids. Forensic Sci Int 152:1–12. doi:10.1016/j.forsciint.2005.02.020
7. Fleming RI, Harbison S (2010) The development of a mRNA multiplex RT-PCR assay for the definitive identification of body fluids. Forensic Sci Int Genet 4:244–256. doi:10.1016/j.fsigen.2009.10.006
8. Lindenbergh A, de Pagter M, Ramdayal G et al (2012) A multiplex (m)RNA-profiling system for the forensic identification of body fluids and contact traces. Forensic Sci Int Genet 6:565–577. doi:10.1016/j.fsigen.2012.01.009
9. Haas C, Klesser B, Maake C et al (2009) mRNA profiling for body fluid identification by reverse transcription endpoint PCR and realtime PCR. Forensic Sci Int Genet 3:80–88. doi:10.1016/j.fsigen.2008.11.003
10. Nussbaumer C, Gharehbaghi-Schnell E, Korschineck I (2006) Messenger RNA profiling: a novel method for body fluid identification by real-time PCR. Forensic Sci Int 157:181–186. doi:10.1016/j.forsciint.2005.10.009
11. Haas C, Hanson E, Kratzer A et al (2011) Selection of highly specific and sensitive mRNA biomarkers for the identification of blood. Forensic Sci Int Genet 5:449–458. doi:10.1016/j.fsigen.2010.09.006
12. Haas C, Hanson E, Bär W et al (2011) mRNA profiling for the identification of blood—results of a collaborative EDNAP exercise. Forensic Sci Int Genet 5:21–26. doi:10.1016/j.fsigen.2010.01.003
13. Haas C, Hanson E, Anjos MJ et al (2012) RNA/DNA co-analysis from blood stains—results of a second collaborative EDNAP exercise. Forensic Sci Int Genet 6:70–80. doi:10.1016/j.fsigen.2011.02.004
14. Haas C, Hanson E, Anjos MJ et al (2013) RNA/DNA co-analysis from human saliva and semen stains—results of a third collaborative EDNAP exercise. Forensic Sci Int Genet 7:230–239. doi:10.1016/j.fsigen.2012.10.011

15. Haas C, Hanson E, Anjos MJ et al (2014) RNA/DNA co-analysis from human menstrual blood and vaginal secretion stains: results of a fourth and fifth collaborative EDNAP exercise. Forensic Sci Int Genet 8:203–212. doi:10.1016/j.fsigen.2013.09.009
16. Visser M, Zubakov D, Ballantyne KN, Kayser M (2011) mRNA-based skin identification for forensic applications. Int J Legal Med 125:253–263. doi:10.1007/s00414-010-0545-2
17. Fang R, Manohar CF, Shulse C et al (2006) Real-time PCR assays for the detection of tissue and body fluid specific mRNAs. Int Congr Ser 1288:685–687. doi:10.1016/j.ics.2005.10.064
18. Hanson EK, Ballantyne J (2010) RNA profiling for the identification of the tissue origin of dried stains in forensic biology. Forensic Sci Rev 22:145–157
19. Kohlmeier F, Schneider PM (2012) Successful mRNA profiling of 23 years old blood stains. Forensic Sci Int Genet 6:274–276. doi:10.1016/j.fsigen.2011.04.007
20. Setzer M, Juusola J, Ballantyne J (2008) Recovery and stability of RNA in vaginal swabs and blood, semen, and saliva stains. J Forensic Sci 53:296–305. doi:10.1111/j.1556-4029.2007.00652.x
21. Bauer M, Polzin S, Patzelt D (2003) Quantification of RNA degradation by semi-quantitative duplex and competitive RT-PCR: a possible indicator of the age of bloodstains? Forensic Sci Int 138:94–103
22. Zubakov D, Hanekamp E, Kokshoorn M et al (2008) Stable RNA markers for identification of blood and saliva stains revealed from whole genome expression analysis of time-wise degraded samples. Int J Legal Med 122:135–142. doi:10.1007/s00414-007-0182-6
23. Zubakov D, Kokshoorn M, Kloosterman A, Kayser M (2009) New markers for old stains: stable mRNA markers for blood and saliva identification from up to 16-year-old stains. Int J Legal Med 123:71–74. doi:10.1007/s00414-008-0249-z
24. Nakanishi H, Hara M, Takahashi S et al (2014) Evaluation of forensic examination of extremely aged seminal stains. Leg Med Tokyo Jpn 16:303–307. doi:10.1016/j.legalmed.2014.04.002
25. Levings PP, Bungert J (2002) The human beta-globin locus control region. Eur J Biochem FEBS 269:1589–1599
26. Chu ZL, Wickrema A, Krantz SB, Winkelmann JC (1994) Erythroid-specific processing of human beta spectrin I pre-mRNA. Blood 84:1992–1999
27. Sabatini LM, Ota T, Azen EA (1993) Nucleotide sequence analysis of the human salivary protein genes HIS1 and HIS2, and evolution of the STATH/HIS gene family. Mol Biol Evol 10:497–511
28. Sabatini LM, Azen EA (1989) Histatins, a family of salivary histidine-rich proteins, are encoded by at least two loci (HIS1 and HIS2). Biochem Biophys Res Commun 160:495–502
29. Bauer M, Patzelt D (2003) Protamine mRNA as molecular marker for spermatozoa in semen stains. Int J Legal Med 117:175–179. doi:10.1007/s00414-002-0347-2
30. Steger K, Pauls K, Klonisch T et al (2000) Expression of protamine-1 and -2 mRNA during human spermiogenesis. Mol Hum Reprod 6:219–225
31. Juusola J, Ballantyne J (2003) Messenger RNA profiling: a prototype method to supplant conventional methods for body fluid identification. Forensic Sci Int 135:85–96
32. Fordyce SL, Kampmann M-L, van Doorn NL, Gilbert MTP (2013) Long-term RNA persistence in postmortem contexts. Investig Genet 4:7. doi:10.1186/2041-2223-4-7
33. Lin M-H, Albani PP, Fleming R (2016) Degraded RNA transcript stable regions (StaRs) as targets for enhanced forensic RNA body fluid identification. Forensic Sci Int Genet 20:61–70. doi:10.1016/j.fsigen.2015.09.012
34. Hall A, Sims LM, Ballantyne J (2014) Assessment of DNA damage induced by terrestrial UV irradiation of dried bloodstains: Forensic implications. Forensic Sci Int Genet 8:24–32. doi:10.1016/j.fsigen.2013.06.010
35. Hall A, Ballantyne J (2004) Characterization of UVC-induced DNA damage in bloodstains: forensic implications. Anal Bioanal Chem 380:72–83. doi:10.1007/s00216-004-2681-3
36. Dissing J, Søndervang A, Lund S (2010) Exploring the limits for the survival of DNA in blood stains. J Forensic Leg Med 17:392–396. doi:10.1016/j.jflm.2010.08.001
37. Akutsu T, Kitayama T, Watanabe K, Sakurada K (2015) Comparison of automated and manual purification of total RNA for mRNA-based identification of body fluids. Forensic Sci Int Genet 14:11–17. doi:10.1016/j.fsigen.2014.09.007
38. Grabmüller M, Madea B, Courts C (2015) Comparative evaluation of different extraction and quantification methods for forensic RNA analysis. Forensic Sci Int Genet 16:195–202. doi:10.1016/j.fsigen.2015.01.006

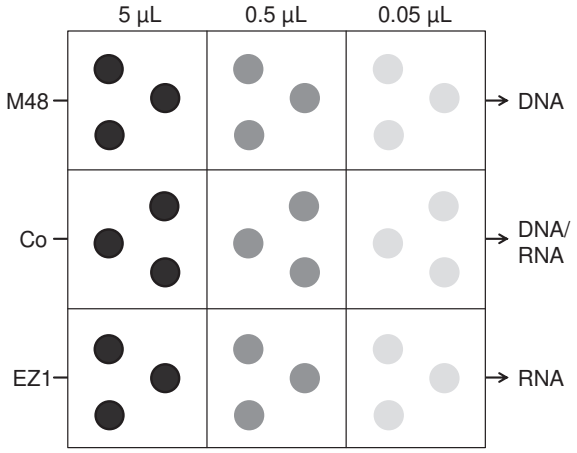


Fig. S1: Preparation scheme of body fluid mock stains

In total, 39 filter paper cards (21 for dry, including 3 for unexposed controls, and 18 for humid storage conditions) were prepared with 5, 0.5 and 0.05 µL stains (biological triplicates) of the particular body fluid (blood, saliva or semen). After certain storage periods (6 time points) samples were extracted with different methods (M48 robot for single DNA extraction, Allprep DNA/RNA/miRNA co-extraction for both nucleic acids and EZ1 robot for single RNA extraction).

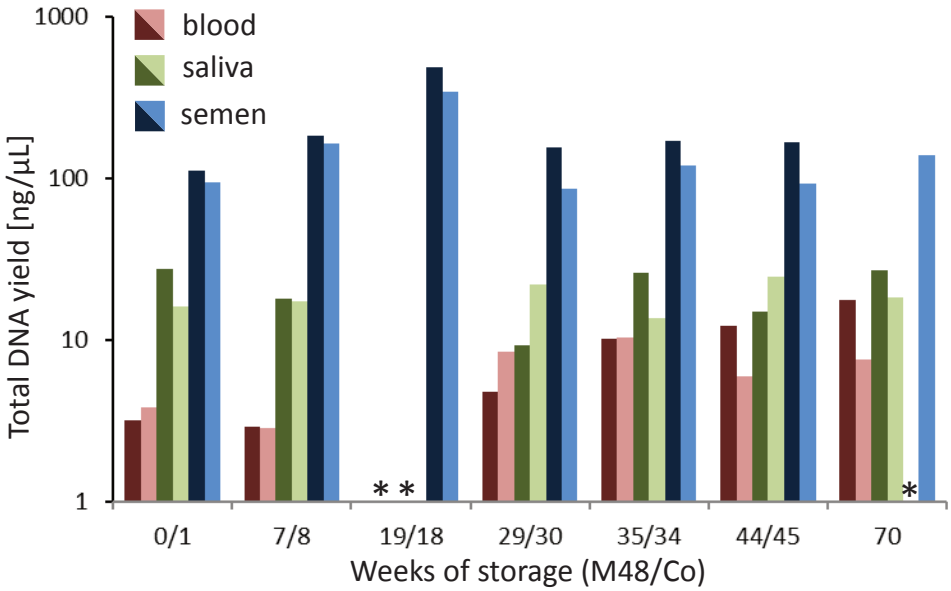


Fig. S2: Total DNA yields of dry stored 5 µL samples

All darker shaded bars of each color pair (1st, 3rd and 5th bar of each group) represent the results of M48 extracted samples, all lighter shaded bars (2nd, 4th and 6th) represent the results of RNA/DNA co-extracted samples. Asterisks indicate where no quantification results were obtained (for blood and saliva after 19 or 18 weeks of storage, respectively, (both methods) and for semen after 70 weeks of storage (M48 extracted). Extraction of samples was performed after 0, 7, 19, 29, 35, 44 and 70 weeks of storage with the M48 robot and after 1, 8, 18, 30, 34, 45 and 70 weeks using the Allprep co-extraction kit.

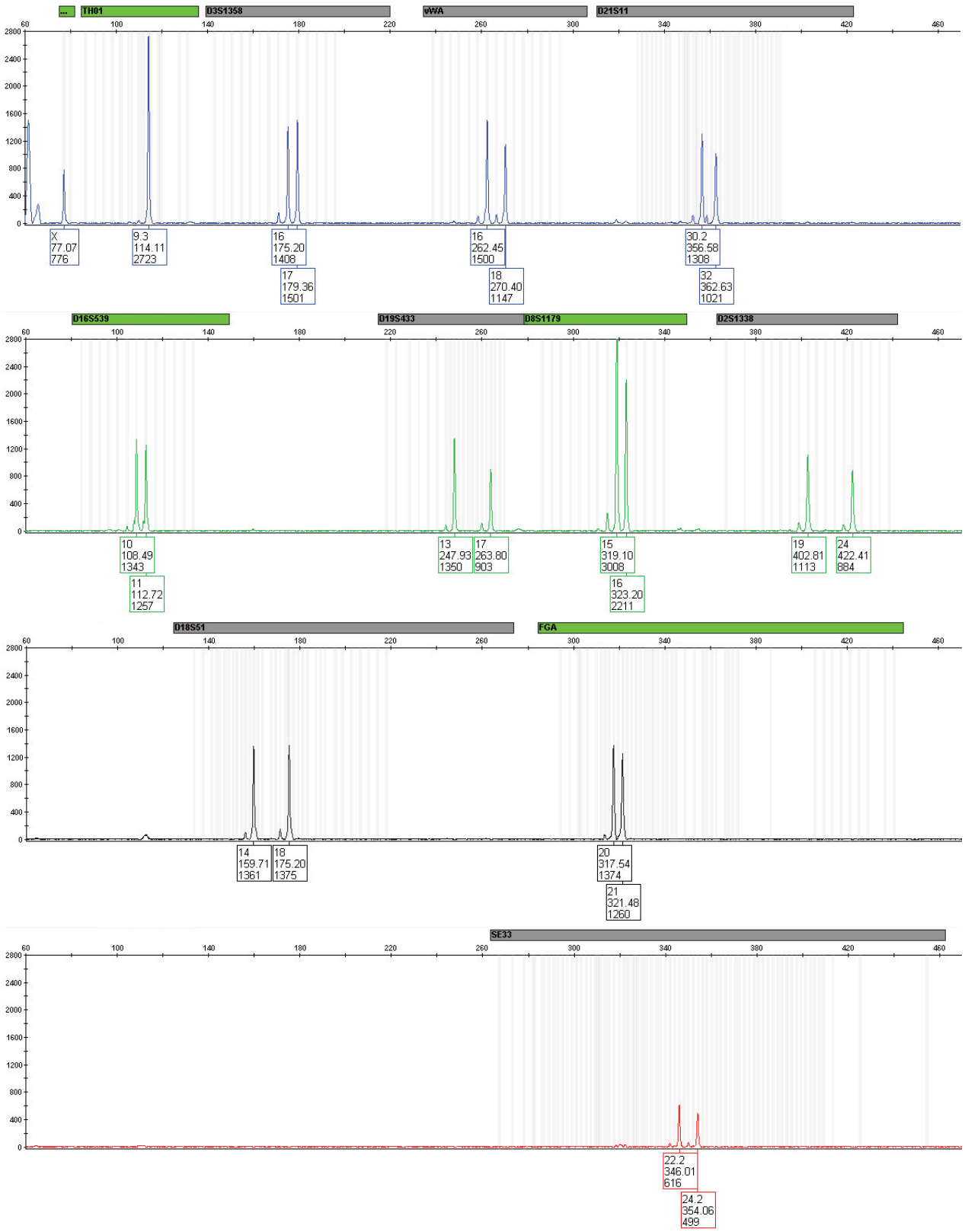


Fig. S3

Exemplary electropherogram from an unexposed blood specimen (reference STR profile). Sample was extracted with the M48 robot and DNA was analyzed with the Investigator Decaplex SE kit (Qiagen).

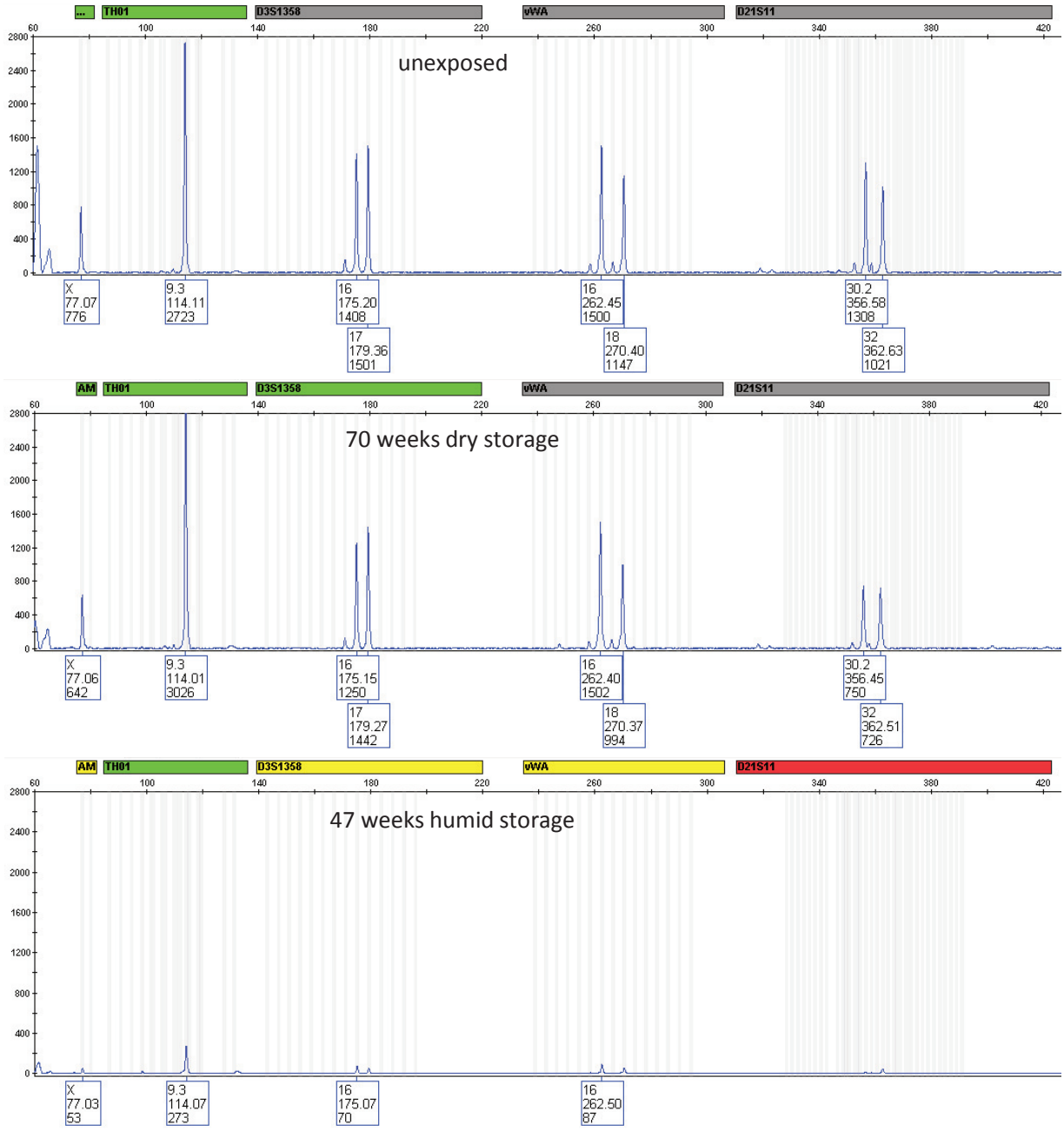


Fig. S4

Exemplary electropherograms from blood samples (blue channel) after dry and humid storage periods demonstrating the effects of degradation under humid conditions. Samples were extracted with the M48 robot and DNA was analyzed with the Investigator Decaplex SE kit (Qiagen).

Table S1 Endpoint PCR primer sequences for body fluid specific genes

Body Fluid	Gene	Primer Sequences (F and R)/ Dyes	Amplicon Size [bp]	Reference
Blood	HBB	F: 5´-6FAM -GCA CGT GGA TCC TGA GAA C R: 5´-ATG GGC CAG CAC ACA GAC	61	[9]
	SPTB	F: 5´-6FAM-AGG ATG GCT TGG CCT TTA AT R: 5´-ACT GCC AGC ACC TTC ATC TT	247	[6]
Saliva	STATH	F: 5´-6FAM-TTT GCC TTC ATC TTG GCT CT R: 5´-CCC ATA ACC GAA TCT TCC AA	93	[9]
	HTN3	F: 5´-6FAM-GCA AAG AGA CAT CAT GGG TA R: 5´-GCC AGT CAA ACC TCC ATA ATC	134	[6, 31]
Semen	PRM1	F: 5´-TMR-GCC AGG TAC AGA TGC TGT CGC AG R: 5´-TTA GTG TCT TCT ACA TCT CGG TCT	153	[30]
	PRM2	F: 5´-6FAM-GTG AGG AGC CTG AGC GAA CGC R: 5´-TTA GTG CCT TCT GCA TGT TCT CTT C	294	[30]

F, forward primer; R, reverse primer

3.2 Impact of using validated or standard reference genes for miRNA qPCR data normalization in cell type identification

Sirker, M., Liang, W., Zhang, L., Fimmers, R., Rothschild, M. A., Gomes, I., Schneider, P.M. and the EUROFORGEN-NoE Consortium (2015). Impact of using validated or standard reference genes for miRNA qPCR data normalization in cell type identification. Forensic Science International: Genetics Supplement Series, 5, e199–e201.

http://dx.doi.org/10.1016/j.fsigss.2015.09.080

Forensic Science International: Genetics Supplement Series 5 (2015) e199–e201

Contents lists available at ScienceDirect

Forensic Science International: Genetics Supplement Series

journal homepage: www.elsevier.com/locate/FSIGSS

Impact of using validated or standard reference genes for miRNA qPCR data normalization in cell type identification

M. Sirker[a,*], W. Liang[b], L. Zhang[b], R. Fimmers[c], M.A. Rothschild[a], I. Gomes[a], P.M. Schneider[a] and the EUROFORGEN-NoE Consortium

[a] Institute of Legal Medicine, Faculty of Medicine, University of Cologne, Cologne, Germany
[b] Institute for Forensic Medicine, Sichuan University, Chengdu, Sichuan, China
[c] Institute of Biometrics, Informatics and Epidemiology, University of Bonn, Bonn, Germany

ARTICLE INFO

Article history:
Received 28 August 2015
Accepted 16 September 2015
Available online 24 September 2015

Keywords:
Forensic science
miRNA profiling
Body fluid identification
Reference genes
GeNorm
Normalization

ABSTRACT

For the analysis of cell type-specific miRNA expression patterns qPCR is currently the method of choice owing to its high accuracy. However, to obtain reliable results, a proper normalization strategy is an absolute prerequisite, which is often underestimated. To demonstrate the importance of using a set of suitable reference genes, we tested two normalization strategies by comparing gene expressions of tissue-specific miRNA targets normalized against: (1) previously validated endogenous controls (miR92 and miR374) and (2) a commonly used miRNA reference (U6B).

1. Introduction

In recent years, miRNA profiling for the identification of human body fluids and tissues has strongly inspired the field of forensic molecular biology [1–3]. The so-called miRNome, the entirety of all miRNAs expressed at a given time point and under certain conditions, represents a unique cell signature which thereby enables the determination of its biological origin. Utilizing qPCR technology, which is characterized by high sensitivity and specificity, miRNA expression patterns can easily be evaluated. Nevertheless, to achieve accurate and reproducible data, non-biological variances in between examined sample sets and runs need to be corrected by using suitable and verified references which fit the experimental requirements for a specific setup. In the present study, we therefore determined the stability of 10 candidate references in a set of forensically relevant body fluids and skin cells by using the global mean normalization algorithm geNorm [4]. Identified genes were applied as normalizers for the analysis of cell type-specific target gene expression levels. Furthermore, the same data set was corrected independently with U6B, and the results of both approaches were compared to assess the impact of the chosen normalizers on the relative quantity of miRNA targets.

2. Material and methods

2.1. Samples

Blood, saliva, semen and skin samples were collected and stored dry on FTA cards or sterile cotton swabs. miRNA was extracted using the miRNeasy Mini kits (Qiagen) as quickly as possible after sample taking to prevent degradation. The quantity of RNA was determined using the NanoDrop 2000 UV/Vis spectrophotometer (Thermo Scientific).

2.2. Validation of endogenous controls

Expression of candidate references (miR26b, miR92, miR191, miR374, miR423, miR484, RNU24, RNU48, RNU44 and RNU47) was analyzed using the Fast SYBR® Green Master Mix with previously conducted cDNA synthesis of RNA samples using TaqMan® MicroRNA Reverse Transcription kit (both Life Technologies). PCR efficiencies were calculated using LinRegPCR (2014.5) [5] and the most stable expressed genes were determined using geNorm. For the heterogeneous group of samples examined, the mean gene stability value M was increased to 1 before analyzing the data [6].

* Corresponding author at: Institute of Legal Medicine, Faculty of Medicine, University of Cologne, Cologne, Germany, Melatengürtel 60/62, 50823 Cologne. Tel.: +49 221 478 89447; fax: +49 221 478 88370.
E-mail address: Miriam.Sirker@uk-koeln.de (M. Sirker).

http://dx.doi.org/10.1016/j.fsigss.2015.09.080
1875-1768/

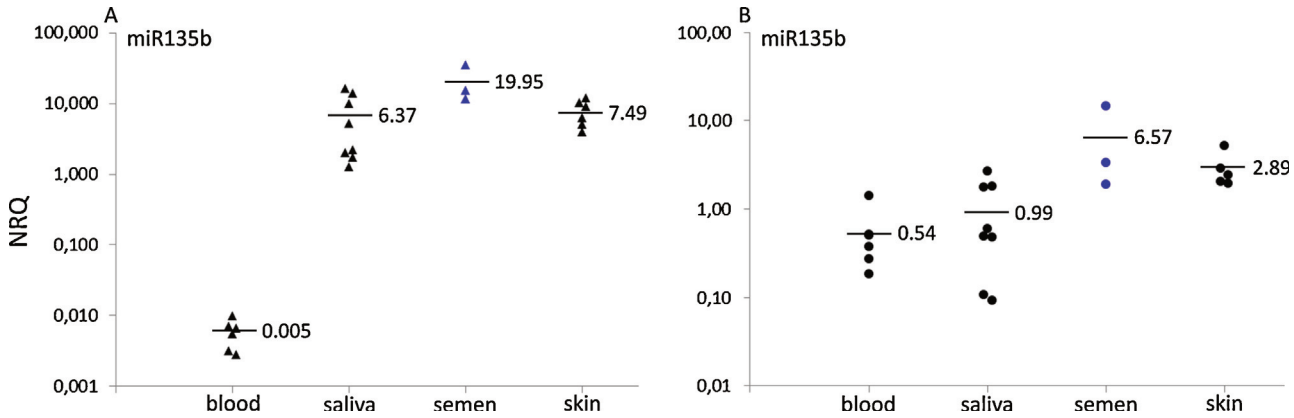


Fig. 1. Effect of normalization to qPCR results. LinRegPCR corrected data of semen-specific marker miR135b were (A) normalized against both validated reference genes miR92 and miR374 and (B) normalized against the commonly used standard endogenous control U6B. Numbers indicate the mean NRQ values, respectively.

2.3. cDNA synthesis and qPCR of target-specific genes

Multiplexed cDNA synthesis was performed using the TaqMan® MicroRNA Reverse Transcription kit for creating custom reverse transcription (RT) pools (Life Technologies). For this purpose, a RT primer pool was created consisting of individual RT primers for miR451 and miR16 (blood-specific), miR205 and miR658 (saliva-specific), miR10b and miR135b (semen-specific) and miR203 (skin-specific). All TaqMan® assays were run in triplicate.

2.4. Data analysis

LinRegPCR corrected qPCR data were normalized against the validated references (miR92 and miR374) and against the standard reference U6B using the software qbase^{plus} (Biogazelle). Finally, normalized relative quantities (NRQs) were compared.

3. Results and discussion

3.1. Validation and determination of the most suitable miRNA normalizers

Applying the geNorm algorithm, the following stability rank order was obtained: miR92 < miR191 < miR374 < miR484 < miR423 < RNU48. Due to missing data, candidates miR26b, RNU24, RNU44 and RNU47 were automatically excluded from analysis (no detectable expression of these targets across the tested sample set). After changing from SYBR® Green to TaqMan®, miR191 was excluded due to chemistry related differences. With M-values of 0.545 and 0.658, respectively, candidates miR92 and miR374 were determined to be the most stable expressed genes and thus used as normalizers for following experiments.

3.2. Impact assessment of using validated or non-validated normalizers on miRNA target expression

The choice of endogenous controls for normalization influenced the relative quantity of examined markers. Regarding the blood-specific marker miR16, it was clearly possible to differentiate blood (mean NRQ: 276.28) from the other body fluids and skin (mean NRQs < 1) using U6B as normalizer. In comparison, it was not possible to distinguish blood cells from the other cell types when miR92 and miR374 were applied as normalizers. All mean NRQs scattered in a range between 0.19 and 2.52. Results for miR451 also showed the possibility for an unambiguous identification of blood, applying both U6B and the previously validated references. Solely, the expression of blood compared to all other samples was much higher using U6B (mean NRQ of blood: 2384.53). Applying miR92 and miR374, blood samples showed a mean NRQ of 32.32.

Regarding both semen markers and skin marker miR203, an unambiguous identification of the cell type's origin was only possible if both validated endogenous controls were used as normalizers. Data of semen-specific marker miR135b generally showed higher expressions when using miR92 and miR374 to correct the relative quantities measured (Fig. 1). The NRQs for blood clustered around 0.005, while the values for the other cell types showed higher expressions (highest mean NRQ of 19.95 in semen samples). This indicates that miR135b is not specifically expressed in semen but rather in epithelial cells. Data for skin-specific marker miR203 revealed similar results showing high expression values in semen, saliva and skin samples. In comparison, NRQs of all sample types clustered in a narrow range when data were corrected with U6B. In these cases, a positive cell type identification was not possible. Both saliva markers (miR205 and miR658) did not give any results probably due to technical problems.

4. Conclusions

Applying either validated or standard reference genes to normalize qPCR data lead to different interpretations of results. There is an indication that for an accurate normalization it is important to use validated reference genes: with miR92 and miR374 it was possible to differentiate a certain body fluid/ skin using specific markers (4 out of 5), with U6B it was less efficient to identify a sample's origin (2 out of 5). A further study analyzing more samples per body fluid/ tissue type and more markers is currently carried out.

Conflict of interest

None.

Role of funding

M.S. has received financial support from the European Union Seventh Framework Programme (FP7/2007-2013) under grant agreement n° 285487 (EUROFORGEN-NoE).

Acknowledgments

We would like to thank our volunteers for donating the biological samples.

References

[1] E.K. Hanson, H. Lubenow, J. Ballantyne, Identification of forensically relevant body fluids using a panel of differentially expressed microRNAs, Anal. Biochem. 387 (2009) 303–314.
[2] D. Zubakov, A.W.M. Boersma, Y. Choi, et al., MicroRNA markers for forensic body fluid identification obtained from microarray screening and quantitative RT-PCR confirmation, Int. J. Legal Med. 124 (2010) 217–226.
[3] C. Courts, B. Madea, Specific micro-RNA signatures for the detection of saliva and blood in forensic body-fluid identification: micro-RNAs for forensic body fluid identification, J. Forensic Sci. 56 (2011) 1464–1470.
[4] J. Vandesompele, K. De Preter, F. Pattyn, et al., Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes, Genome Biol. 3 (2002) research0034.1–research0034.11.
[5] J.M. Ruijter, C. Ramakers, W.M.H. Hoogaars, et al., Amplification efficiency: linking baseline and bias in the analysis of quantitative PCR data, Nucleic Acids Res. 37 (2009) e45.
[6] J. Hellemans, G. Mortier, A. De Paepe, et al., qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data, Genome Biol. 8 (2007) R19–R19.

3.3 Evaluating the forensic application of 19 microRNAs as biomarkers in body fluid and tissue identification

Sirker, M., Fimmers, R., Schneider, P.M. and Gomes, I. (2017). Evaluating the forensic application of 19 target microRNAs as biomarkers in body fluid and tissue identification. Forensic Science International. Genetics, 27, 41–49.

https://doi.org/10.1016/j.fsigen.2016.11.012

Forensic Science International: Genetics 27 (2017) 41–49

Contents lists available at ScienceDirect

Forensic Science International: Genetics

journal homepage: www.elsevier.com/locate/fsig

Research paper

Evaluating the forensic application of 19 target microRNAs as biomarkers in body fluid and tissue identification

M. Sirker[a,*], R. Fimmers[b], P.M. Schneider[a], I. Gomes[a]

[a] Institute of Legal Medicine, Faculty of Medicine, University of Cologne, Cologne, Germany
[b] Institute of Biometrics, Informatics and Epidemiology, University of Bonn, Bonn, Germany

ARTICLE INFO

Article history:
Received 26 August 2016
Received in revised form 18 November 2016
Accepted 30 November 2016
Available online 2 December 2016

Keywords:
Forensic science
Body fluid identification
Skin
miRNA profiling
RT qPCR
Normalization

ABSTRACT

RNA-based body fluid and tissue identification has evolved as a promising and reliable new technique to classify type and source of biological evidence in crime cases. In particular, mRNA-based approaches are currently on the rise to replace conventional protein-based methods and are increasingly implemented into forensic casework. However, degradation of these nucleic acid molecules can cause issues on laboratory scale and need to be considered for a credible investigation. For this reason, the analysis of miRNAs using qPCR has been proposed to be a sensitive and specific approach to identify the origin of a biological trace taking advantage of their small size and resistance to degradation. Despite the straightforward workflow of this method, suitable endogenous controls are inevitable when performing real-time PCR to ensure accurate normalization of gene expression data in order to allow a meaningful interpretation. In this regard, we have validated reference genes for a set of forensically relevant body fluids and tissues (blood, saliva, semen, vaginal secretions, menstrual blood and skin) and tested 15 target genes aiming to identify abovementioned sample types. Our data showed that preselected endogenous controls (miR26b, miR92 and miR484) and miR144, initially selected as potential marker for the detection of menstrual blood, were the most stable expressed genes among our set of samples. Normalizing qPCR data with these four validated references revealed that only five miRNA markers are necessary to differentiate between the six different cell types selected in this study. Nevertheless, our observations in the present study indicate that miRNA analysis methods may not provide straightforward data interpretation strategies required for an implementation in forensic casework.

1. Introduction

Various kinds of biological traces of human origin can be encountered at a crime scene, which may serve as physical evidence, either to connect a suspect to the criminal offense or to exonerate an innocent person. Therefore, it is of great importance to isolate and analyze trace DNA to generate an individual-specific STR profile and, likewise important, to unambiguously confirm the biological origin of this DNA (e.g., did the recovered DNA stain originate from peripheral or menstrual blood?). The latter can help to clarify the circumstances of a criminal act. In this respect, several conventional presumptive tests are routinely performed, which are based on chemical, enzymatic or immunological reactions [1]. Due to disadvantages regarding the sensitivity and specificity of these tests, destruction or complete consumption of valuable stain material, and the disability to identify certain kinds of body fluids (e.g., vaginal secretions), alternative techniques are currently emerging to bridge abovementioned gaps. These methods include co-extraction of RNA and DNA enabling an efficient processing of limited sample amounts and allowing the preservation of DNA for parallel STR analysis [2–8]. Beyond that, the analysis of, e.g., messenger RNA (mRNA) permits a parallel analysis of different biological fluids in a multiplexed manner [9–11]. However, mRNA stability issues due to the ubiquitously presence of ribonucleases and further detrimental factors like humidity or UV light have been reported [12–14], and the search for alternative markers, which can withstand degradation to a certain extent, is ongoing.

MicroRNAs (miRNAs), a class of non-coding and differentially expressed RNA molecules which play crucial roles in regulating cellular processes at post-transcriptional level [15], have been proposed to enable the identification of cell types for forensic purposes [16–23]. Due to their small size of 18–22 nucleotides in length, these RNAs are known to be less susceptible to degradation

* Corresponding author at: Institute of Legal Medicine, Faculty of Medicine, University of Cologne, Melatengürtel 60/62, Cologne 50823, Germany.
E-mail address: Miriam.Sirker@uk-koeln.de (M. Sirker).

http://dx.doi.org/10.1016/j.fsigen.2016.11.012
1872-4973/

by environmental factors, which is an obvious advantage of these molecules to serve as useful biomarkers.

In the last decade, reverse transcription quantitative real-time PCR (RT-qPCR) has been widely used in miRNA expression analysis and seems to be suitable to determine a certain body fluid by its specific miRNA signature [16–20]. However, reliable detection and quantification of miRNA expression patterns using qPCR requires a proper normalization strategy including the use of validated endogenous controls. To accomplish accurate and reproducible data, appropriate reference genes, at least three as suggested by Vandesompele et al. [24] when a validation study cannot be performed, are necessary to normalize qPCR data as described in the MIQE guidelines [25].

As a first main objective of the present study we intended to validate i) appropriate reference genes for normalization of miRNA gene expression data in the context of human body fluid and tissue identification, and to identify ii) specific miRNA markers for the identification of forensically relevant body fluids (miR16 and miR451 for peripheral blood, miR203, miR205, and miR124* for saliva, miR943 and miR10b for semen, miR4286 and miR1280 for vaginal secretions, miR144, miR185 and miR142-3p for menstrual blood) and skin (miR3169, miR139 and miR494).

Although a set of potential promising markers is already described in relevant forensic literature, not many studies have followed up and confirmed these findings to investigate if an implementation of miRNA expression profiling in routine casework could provide trustworthy results and someday replace conventional methods. As a second objective we tried to assess the benefits of miRNA profiling based on our results and tried to compare these findings with pertinent literature.

2. Materials and methods

2.1. Biological samples

Peripheral blood samples were collected by puncturing the finger pulp with a sterile safety-lancet and spotting the blood directly onto a sterile cotton swab (both instruments from Sarstedt). Freshly ejaculated semen from non-vasectomized males was provided by the donors in sealed Falcon tubes. Volunteers who contributed with liquid saliva were asked to avoid drinking/eating, smoking or dental hygiene activities at least 30 min prior to sampling. Specimens were subsequently prepared by immersing sterile cotton swabs into the sample (semen/saliva) until they were fully soaked. Menstrual blood and semen-free vaginal secretion samples were collected by the female donors themselves using sterile cotton swabs. All body fluid specimens (eight different donors per sample type) were dried and stored at room temperature until extraction, which was conducted within 24 h after sample taking to avoid degradation. Skin samples of eight volunteers were collected by rubbing sterile cotton swabs over the face or palms of hands (epidermal cells). All participants of this study were in an apparently good state of health to prevent possible influences in miRNA expression levels due to pathologies or diseases and also gave informed consent for the use of their cell material as approved by the local ethics committee.

2.2. RNA extraction and quantification

To remove ambient RNases, all surfaces and devices utilized during the extraction procedure were thoroughly cleaned using RNase-Zap® (Ambion). Furthermore, only RNase-free reagents, plastic consumables and instruments were used. Samples were extracted using the miRNeasy Mini kit (Qiagen) according to manufacturer's protocols. A treatment with DNase-I was included in the protocol to remove potential genomic DNA traces. The quantity of RNA was assessed using the NanoDrop 2000 UV/Vis spectrophotometer (Thermo Scientific). Extracts were immediately stored at −20 °C until further use.

2.3. Selection of reference and target genes

A panel of four promising candidate reference genes for qPCR data normalization was initially selected, encompassing the markers miR26b, miR92, miR374 and miR484 (Supplementary Table S1). Marker miR92 and miR374 were previously determined to be the most stable expressed genes across a tested sample set that included the forensically relevant body fluids/tissues blood, semen, saliva and skin [26]. Although miR484 was not selected as an endogenous control in the abovementioned study, the marker, however, demonstrated stable gene expression across sample types. Hu et al. [27] also determined miR484 as the most stable expressed gene out of the tested candidates across different cancer specimens and healthy controls. For these reasons and the wish to include more potential stable reference genes miR484 was also included. Marker miR26b was chosen due to its good performance as a reference gene in another study [28].

The following 15 markers were selected as candidate target genes by literature research: miR16 and miR451 for peripheral blood, miR203, miR205, and miR124* for saliva, miR943 and miR10b for semen, miR4286 and miR1280 for vaginal secretions, miR144, miR185, and miR142-3p for menstrual blood, and miR3169, miR139, and miR494 for skin (Supplementary Table S1).

2.4. Complementary DNA synthesis and pre-amplification

Multiplexed cDNA synthesis and pre-amplification were performed using the TaqMan® MicroRNA Reverse Transcription and PreAmp Master Mix Kits (both Applied Biosystems) according to manufacturer's instructions for creating custom reverse transcription (RT) pools. The RT primer pool was created consisting of 19 individual RT primers for markers miR16, miR451, miR203, miR205, miR124*, miR943, miR10b, miR4286, miR1280, miR144, miR185, miR142-3p, miR3169, miR139, miR494, miR26b, miR92, miR374, and miR484, with 0.05× each in the final mix. RT reactions were performed in a volume of 15 μL containing 24 ng of total RNA. RT(−) controls were contemporaneously performed to assert possible contaminations with genomic DNA. The RT temperature profile consisted of an initial hold for 30 min at 16 °C, followed by 30 min at 42 °C and 5 min at 85 °C. The manufacturer suggests a pre-amplification step when the amount of total RNA is between 1 and 350 ng. To achieve higher sensitivity, 2.5 μL of the cDNA was used in a subsequent pre-amplification step which was performed in a total volume of 25 μL using a custom PreAmp primer pool consisting of the selected 19 TaqMan® MicroRNA assays, 0.2× each in a final volume of 500 μL. Thermal cycling conditions were as follows: initial hold for 10 min at 95 °C, followed by 2 min at 55 °C and 2 min at 72 °C, then 12 cycles of 95 °C, 15 s and 60 °C for 4 min were carried out. The final hold was at 99.9 °C for 10 min. PreAmp products were diluted 1:10 with 0.1× TE (pH 8.0) and immediately used to perform qPCR.

2.5. Real-time quantitative polymerase chain reaction

All TaqMan® assays were run in triplicates on an AB 7500 Fast Real-Time PCR instrument using TaqMan® Universal PCR Master Mix, No AmpErase® UNG 2x (Applied Biosystems) according to manufacturer's protocol. Reactions were performed in a volume of 15 μL containing 1.5 μL diluted PreAmp product. To eliminate possible 'run-to-run' variations, 3 different RNA samples (extracted from blood, saliva and semen) were included as inter run calibrators (IRCs) on all qPCR plates [29]. Thermal cycling

conditions were as follows: hold of 95 °C for 10 min, followed by 40 cycles of 15 s at 95 °C, and 1 min at 60 °C.

2.6. Determination of reference genes for normalization and qPCR data analysis

Prior to qPCR data analysis, the data set was corrected with the independent software for primary analysis of raw qPCR data, the LinRegPCR (version 2014.5) [30]. LinRegPCR calculates Cq and PCR efficiency values based on fluorescent amplification curves. The corrected values were then used for further analyses. The determination of the most suitable reference genes was carried out by using the global mean normalization algorithm geNorm [24]. geNorm is implemented in the reference gene validation software qbase^{plus} (Biogazelle, Ghent, Belgium) which calculates a gene stability measure (M-value) for each candidate. Before analyzing the data, the mean stability value M was manually changed to 1 and the coefficient of variation CV was set to 0.5, which are the recommended analyses settings for a heterogeneous group of samples as the ones examined in the present study [29]. As there are often only limited amounts of biological material available from forensic samples, the Cq exclusion criteria threshold was set from 35 to 38 cycles. Therefore, values of Cq >38 (non-specific amplification) were automatically excluded from the analysis. Subsequently, the LinRegPCR corrected qPCR data were calibrated using the three IRCs, and normalized against the validated references by the qbase^{plus} software. Calibrated normalized relative quantities (CNRQs) were exported and utilized to assess the potential of miRNA markers to identify a given cell type. CNRQ values of samples not showing amplification were arbitrarily set to 0.000001 to prevent that these would be excluded from further analysis. Box plots were generated using the open source software RStudio version 0.99.447.

2.7. ROC analyses for pairwise comparison of sample types

ROC analyses (receiver operator characteristic) were calculated for each miRNA marker using the CNRQ values, and for each possible pair of body fluids taking the area under the ROC curves (AUC). This analysis allowed us to assess the general capability of each marker to discriminate between the given pair of body fluids. AUC values of 0.5 indicate no discrimination power and the maximal possible value AUC = 1 stands for a perfect separation between the body fluid/tissue types. AUC = 1 also means that the empirical distributions found for the CNRQ values for a given marker in a pair of two different sample types did not overlap.

2.8. MIQE guidelines compliance

The experiments complied with the essential requirements reported in the MIQE guidelines [25] to achieve reliable and unambiguous qPCR results.

3. Results

All negative controls, which were routinely performed in all cDNA synthesis, pre-amplification and qPCR reactions to monitor possible contaminations, did not show unspecific amplification signals. RT(−) controls were also free of indications for residual genomic DNA.

We chose to perform a pre-amplification step prior to qPCR reactions due to the low starting amount of 24 ng RNA input in the cDNA synthesis. This is the recommended procedure in the manufacturer's protocol for creating custom RT (and pre-amplification) pools when a starting amount between 1–350 ng of total RNA is used. It has been shown that this additional step improves the overall assay sensitivity without influencing the relative expression levels [31–33].

3.1. Validation of reference genes

Variations, which are experimentally induced and of non-biological nature such as the differences of nucleic acid input between samples, need to be eliminated to allow for accurate interpretation of the expression changes. These variations can be removed by the use of reference genes. To validate the preselected candidate panel of reference genes, a total of eight samples were screened per each body fluid and for skin. After importing LinRegPCR corrected data into qbase^{plus}, technical triplicates were manually inspected for Cq outliers (the difference in Cq value between the replicate with the highest Cq value and the replicate with the lowest Cq value −ΔCq- should be smaller than 0.5 cycles [34]). After excluding the replicates that did not pass this criterion, the geNorm algorithm was applied. Defining all 19 genes as "reference targets", the following M-values were obtained for the candidate reference genes (the lower the M value, the more stable the expression): miR484: 1.782, miR92: 1.834 and miR26b: 2.16 (Fig. 1). Surprisingly, miR374 did not show a stable expression across the tested sample set and therefore no M-value could be obtained. Instead, miR16 (potential peripheral blood marker) and miR144 (potential menstrual blood marker) revealed high expression stabilities. Although the average expression stability, M-value, of miR16 (1989) queued between miR92 and miR26b, we chose miR144 (2.464) to serve as fourth normalizer keeping at least two potentially specific miRNAs for each cell type. The average expression stabilities of all other miRNAs were above 3.316 and thus too large to be considered. To monitor the influence of either using miR16 or miR144 as reference genes in combination with the other stable references miR484, miR92 and miR26b, we normalized the data set twice to both strategies and compared the expression patterns (CNRQ values) of miRNAs (Supplementary Fig. S1). In general, the distances of median values to each other were similar, solely the scaling changed slightly. Since the impact on the resulting CNRQ values was negligible, we kept miR144 as a reference gene rather than miR16 as substitution for miR374. Ideally, the M-values should be below M = 1 as described in Hellemans et al. [29]. We are aware of the fact that our data deviate from this recommended value. A probable explanation for the decreased stability could be the diverse set of biological samples of human body fluids and tissue types examined in our study that

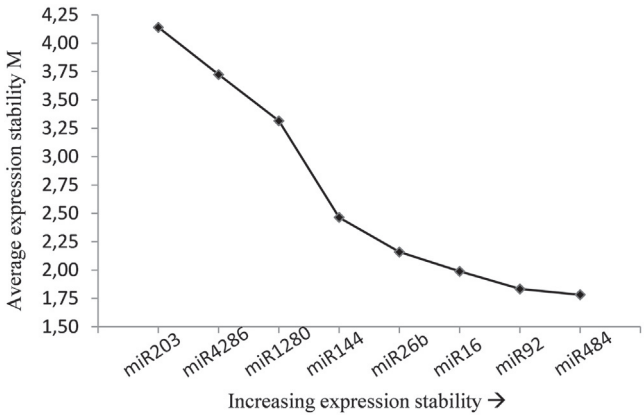


Fig. 1. geNorm expression stability plot. Average expression stability M of the remaining targets. Genes with the lowest M-value are the most stable expressed across the set of different body fluids and skin. geNorm of all 19 genes.

represent complex mixtures composed of completely different cell types that mimic the real forensic scenarios. Therefore, identifying highly stable reference genes across such a set of heterogeneous samples can be a complex task. Nevertheless, the sensitivity in the interpretation of qPCR data demands for the use and validation of reference genes instead of the use of no controls at all. For subsequent qPCR data analyses, all raw gene expression data were normalized against the four references miR26b, miR92, miR144, and miR484.

3.2. Evaluation of target genes for body fluid/tissue identification

Normalization of qPCR data of target genes was performed against the validated miR26b, miR92, miR144, and miR484 control genes. The expected specificities (based on initial marker selection) and the actual observed specificities of 14 potential cell type specific markers and one reference gene are summarized in Table 1. The corresponding CNRQ box plots are depicted in Figs. 2 and 3. Surprisingly, only six out of these markers showed expected specificities for the set of samples analyzed in this study, namely miR16, miR451, miR10b, miR1280, miR4286 and miR3169. Among these, only miR451 and miR10b showed clear separation capacities enabling a distinct and positive identification of peripheral blood and semen, respectively, in accordance with previous reports [7,16,18,35]. The remaining four genes did not show the expected specificities as increased overlapping expression levels could be observed across more than one body fluid/tissue type (Figs. 2 and 3).

Initially, miR16 and miR451 were selected as peripheral blood specific markers [7,16,18,19,35,36]. In general, both miRNAs revealed highest expression levels in peripheral and menstrual blood compared to saliva, semen, vaginal secretions and skin (non-blood samples). An unambiguous differentiation between peripheral blood and menstrual blood was, however, not possible using either miR16 or miR451. In fact, the expression of both genes was highest in menstrual blood. Due to the composition of these two body fluids an overlap in expression was indeed expected and had been described earlier [21]. Although miR16 was preselected as peripheral blood specific marker, it did not allow differentiating blood from other cell types (Fig. 2A). However, the median CNRQ of blood (3.32) and menstrual blood (4.51) were higher compared to the other sample types (saliva: 0.70, semen: 2.40, skin: 0.22 and vaginal secretion: 0.87). In fact, the median value of peripheral blood was comparable with semen, and therefore only a vague assumption can be made that miR16 could be suitable for the detection of blood. In addition, according to the obtained data in our study, it is also not the best candidate for blood due to overlapping expression levels with other body fluids (e.g., peripheral/menstrual blood, and semen or vaginal secretions) (Fig. 2A). Marker miR16 showed, in the previous reference gene validation study, an M-value of 1.989 in the geNorm analysis, therefore the target gene expression results were not surprising as a low M-value represents a stable expression across the examined sample set. On the other hand, miR451 was found to be a stronger and more reliable marker for identifying blood in general. A cross reaction of peripheral blood and menstrual blood, however, prohibits the differentiation of these two blood types based on this marker alone (Fig. 2B).

Markers miR124*, miR203, and miR205 [7,16,18,20,35] were selected as potential saliva-specific markers. All three markers did not show any specificity to saliva at all (Fig. 2C–E), however, miR203 exhibited a markedly increased median CNRQ value in vaginal secretions (Fig. 2E), and was considered as a marker to differentiate this sample type from peripheral blood, saliva, and semen.

MiR10b and miR943 were chosen as potential semen markers [16,17]. Marker miR10b permitted the unequivocal identification of semen samples. In comparison, all other sample types showed much lower expression levels (Fig. 2F). In contrast, miR943 was not semen specific at all, exhibiting elevated expression levels in saliva and skin, but not in both blood types and semen (Fig. 2G).

Candidate markers for vaginal secretion identification (miR1280 and miR4286) [35] showed similar expression patterns across the tested sample set with slightly increased expression in menstrual blood and vaginal secretion (Fig. 3H and I). Although the median value for vaginal secretion was actually the highest for both markers, the difference compared to the other sample types was not sufficient for a clear distinction.

Candidate genes for the identification of menstrual blood were miR142-3p and miR185 [35,37]. Both markers exhibited slightly elevated expression levels across almost all sample types without clear specificities (Fig. 3J and K).

Table 1
Expected and observed specificities of selected miRNA markers and their potential for body fluid/tissue identification.

Expected specificities			Observed specificities	
Described specificity of miRNA markers[a]	miRNA assay name[b]	Body fluid identification[c]	Body fluid identification[c]	Determined specificity of miRNA markers
Blood (venous)	miR16	(+)	(+)	(Menstrual blood)
	miR451	++	++	Peripheral blood/Menstrual blood[d]
Saliva	miR124*	–	+	Vaginal secretion/Menstrual blood[d]
	miR203	–	+	Vaginal secretion
	miR205	–	+	Vaginal secretion
Semen	miR10b	+++	+++	Semen
	miR943	–	+	Saliva/Skin[d]
Vaginal secretion	miR1280	(+)	(+)	Vaginal secretion/Menstrual blood[d]
	miR4286	(+)	(+)	Vaginal secretion/Menstrual blood[d]
Menstrual blood	miR142-3p	–	–	/
	miR185	–	–	/
Skin	miR139	–	–	/
	miR494	–	–	/
	miR3169	+	+	Skin
Candidate reference genes	miR374	/	+++	Semen

[a] Body fluids/tasks for which miRNA assays with potential body fluid/tissue specificity were initially selected by literature search.
[b] All assays are *homo sapiens* (hsa) specific.
[c] body fluid identification not possible; (+) weak association with body fluid; +moderate, ++ good and +++ unambiguous body fluid identification.
[d] Cross reaction.

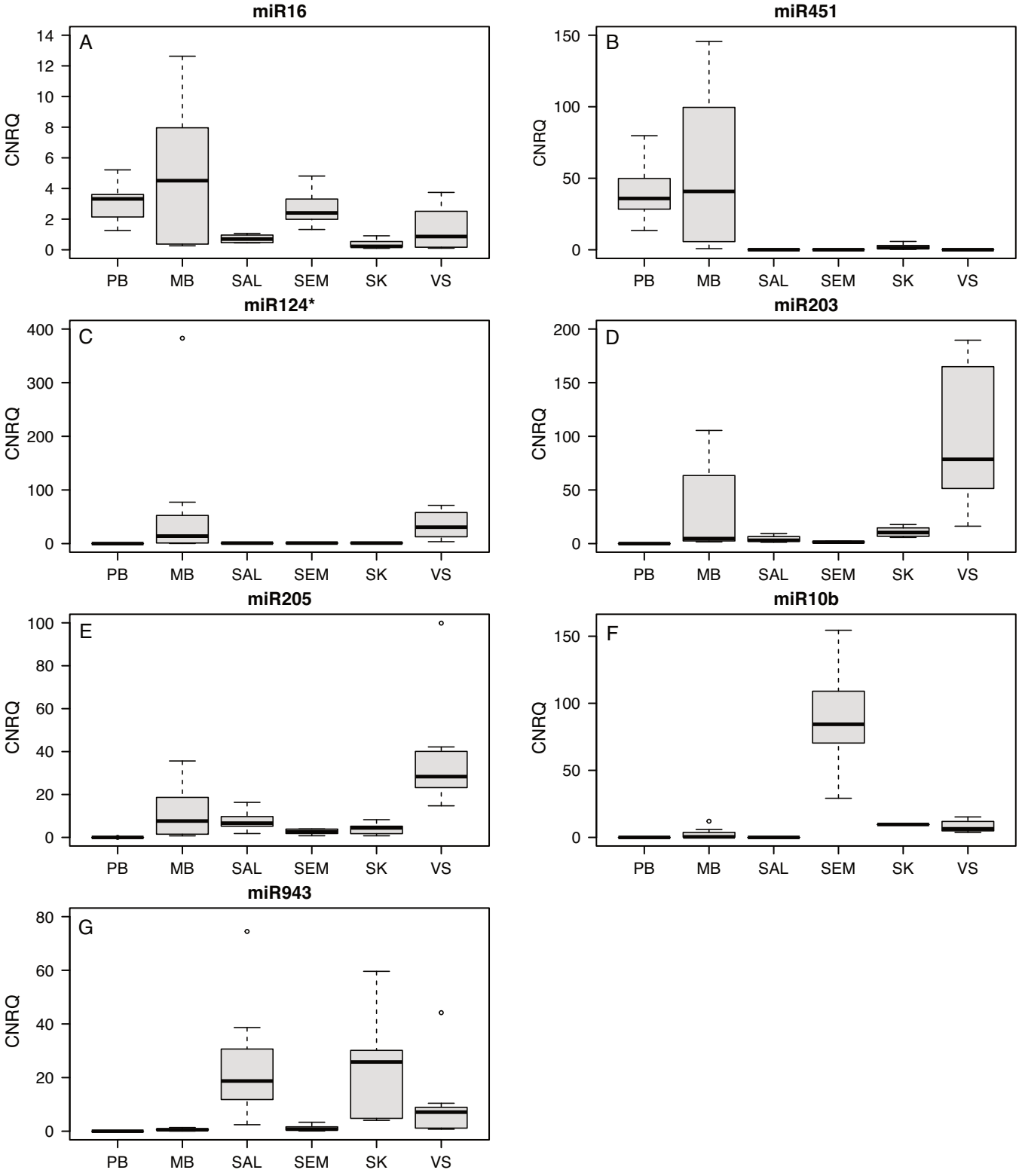


Fig. 2. Calibrated normalized relative quantities (CNRQs) of seven miRNA markers. Markers miR16 and miR451 were initially selected as peripheral blood specific, miR124*, miR203 and miR205 as saliva specific, and miR10b and miR943 as semen specific. Normalization was performed using validated endogenous controls miR26b, miR92, miR484, and miR144. Results are depicted in box plots presenting the median with interquartile range, lower (25%) and upper (75%) quartiles. Whiskers indicate the maximum/minimum values without outliers. Outliers are presented as circles. Peripheral blood (PB), menstrual blood (MB), saliva (SAL), semen (SEM), skin (SK) and vaginal secretions (VS).

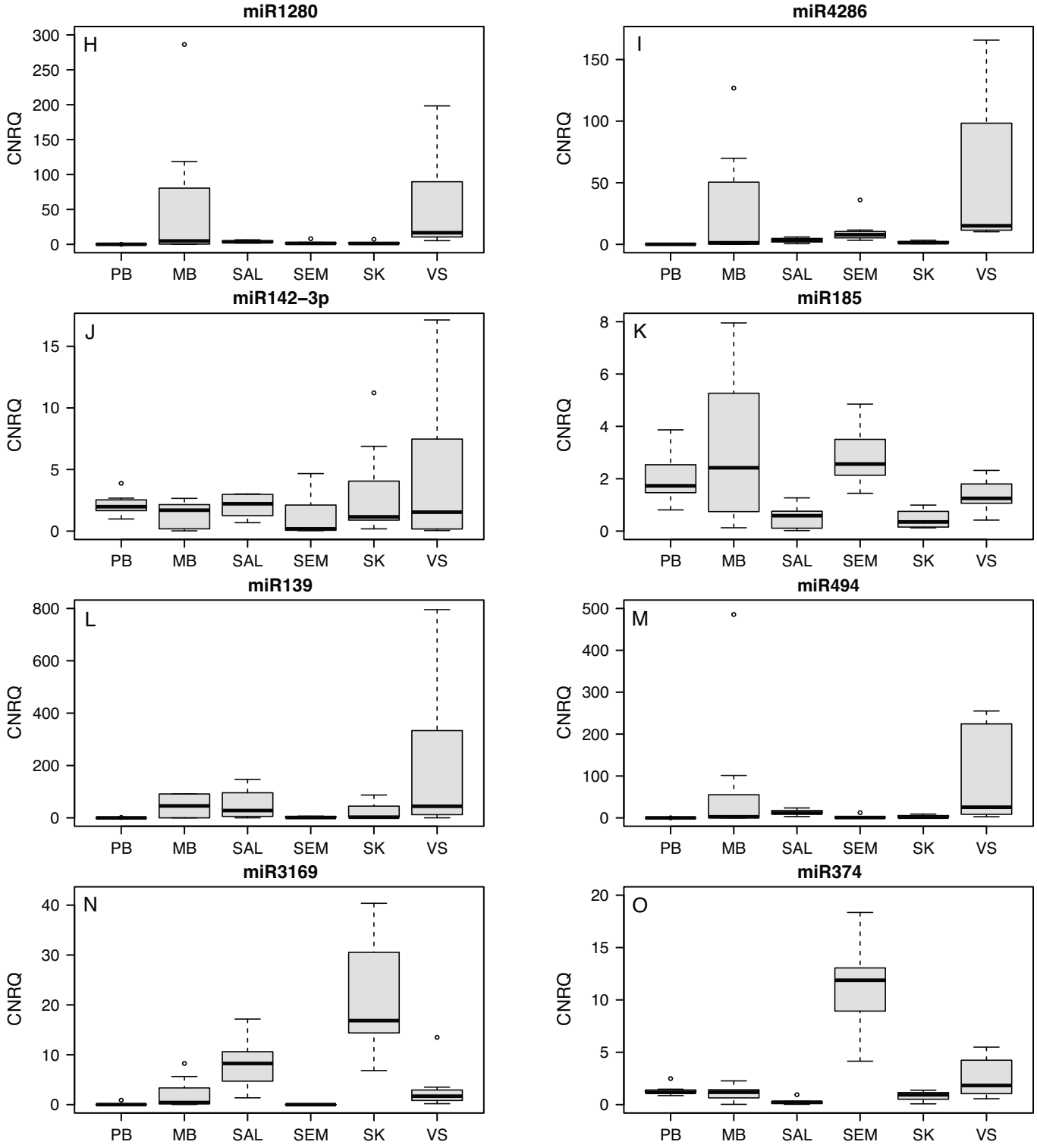


Fig. 3. Calibrated normalized relative quantities (CNRQs) of eight miRNA markers. Markers miR1280 and miR4286 were initially selected as vaginal secretion specific, miR142-3p, and miR185 as menstrual blood specific, and miR139, miR494, and miR3169 as skin specific. Normalization was performed using validated endogenous controls miR26b, miR92, miR484, and miR144. Results are depicted in box plots presenting the median with interquartile range, lower (25%) and upper (75%) quartiles. Whiskers indicate the maximum/minimum values without outliers. Outliers are presented as circles. Peripheral blood (PB), menstrual blood (MB), saliva (SAL), semen (SEM), skin (SK) and vaginal secretions (VS).

Table 2
Selection of markers for the separation of body fluids/tissues based on AUC values.

Body fluid/tissue pairs	miRNA markers for body fluid/tissue differentiation (AUC[a] = 1)				
	miR10b	miR203	miR374	miR451	miR943
PB/MB	x	x			x
PB/SAL		x		x	x
PB/SEM	x	x	x	x	x
PB/SK	x	x		x	x
PB/VS	x	x		x	x
MB/SAL				x	x
MB/SEM	x		x	x	
MB/SK					x
MB/VS				x	
SAL/SEM	x		x		
SAL/SK	x			x	
SAL/VS	x	x			
SEM/SK	x	x	x	x	x
SEM/VS	x	x			
SK/VS				x	

[a] AUC: area under ROC (receiver operating characteristics) curve; AUC = 1 correlates with a 100 % separation between two cell types; x: marker enabling a 100 % separation of the given body fluid/tissue pair.

To identify skin, miR139, miR494, and miR3169 were initially selected [35]. However, miR139 and miR494 (Fig. 3L and M) did not exhibit specificities for skin and, furthermore, were not suitable to separate any other body fluids (Table 2). In contrast, miR3169 showed clearly increased expression levels in skin and saliva, and furthermore, moderately increases in menstrual blood and vaginal secretion sample types (Fig. 3N). As expected, skin represents a challenging tissue since epithelial cells are present also in other body fluids, such as saliva and vaginal secretion, which complicates an unambiguous differentiation.

Finally, miR374, initially selected as candidate reference gene, showed a strikingly and surprisingly high expression in semen compared to all other sample types (Fig. 3O), so that this marker can be applied for specifically detecting this body fluid.

3.3. Body fluid/tissue differentiation using ROC analyses

To further assess the ability of the miRNA markers here selected to distinguish between different body fluids and skin, ROC analyses were performed (receiver operator characteristic) for each marker and each possible pair of sample types by calculating the area under the ROC curve (AUC). The resulting AUC values of the pairwise comparisons are compiled in Supplementary Table S2. As a first observation of these results we found several combinations for which we had an empirically perfect separation (AUC = 1), which is likely due to the fact that the number of analyzed samples per body fluid/tissue type was comparably low for such kind of analyses. We could identify at least one “perfect” marker with AUC = 1 for each pair of sample types. Therefore we did not investigate further AUC values below 1 to maintain a more conservative approach due to the low number of samples.

Summarizing all results obtained by calculating AUC values for each body fluid/tissue pair, we could identify a minimal set of five miRNAs enabling the positive identification of all six sample types analyzed in this study: miR10b, miR203, miR374, miR451, and miR943 (Table 2). The differentiation of peripheral blood and non-blood samples was straightforward using only marker miR451. However, to overcome the issue of separating menstrual blood from non-blood samples, it was necessary to use one more marker such as miR943 for the separation of menstrual blood and skin. To distinguish between the two blood types, three markers (miR10b, miR203, and miR943) showed sufficient separation capacities, although they were initially not selected to be menstrual blood specific (Table 1). Hence, it should be possible to distinguish peripheral blood from all other secretions or skin using two markers (minimal set of miR451 and miR943). In comparison, the differentiation between the non-blood samples was more challenging due to similarities in the composition of examined body fluid types, as, e.g., epithelial cells are found both in vaginal secretion and saliva. In those cases only two markers or one marker could be identified, respectively, e.g., miR451 and miR10b for the separation between skin and saliva or miR943 for skin and menstrual blood (as mentioned above). The identification of semen was straightforward, comparable to peripheral blood. Using miR451 it was possible to separate semen from peripheral and menstrual blood samples due to very low CNRQ values (<0.1). As listed in Table 2 also other markers showed sufficient pairwise differentiation capacities, e.g., miR10b or miR374. Distinguishing between semen and saliva, and semen and skin, the same two markers are found to be most informative (e.g., see Figs. 2 F and 3 O, vs. Fig. 2D).

4. Discussion

MicroRNA profiling has been investigated during the last few years in a number of studies to assess its potential as a robust technique for determining the body fluid/tissue type in forensic casework as well as its implementation [16–21]. In the present study, miR10b, miR203, miR374, miR451 and miR943 were identified using ROC analysis for the identification of five forensically relevant body fluids and skin specimen types (blood, saliva, semen, vaginal secretions, menstrual blood and skin). The five markers allowed an individual pairwise distinction of one body fluid/tissue type in comparison to one or more other sample types (Table 2). However, to overcome the challenge of technical variances due to the high heterogeneity of the examined sample set, a set of four candidate endogenous controls (miR26b, miR92, miR144 and miR484) was identified and validated for normalizing miRNA gene expression data (for the conditions and group of the sample types studied) before target gene analysis. Our approach was based on a strategy to search for and try to employ the most promising miRNA markers identified in previous studies for various body fluids and tissue types, and to compare them under standardized conditions using a defined set of samples. Our results

indicate that the specificity of a given marker depends on the overall comparison of the analyzed sample set. Consequently we found that a marker showing specificity, e.g., for saliva in comparison to blood (miR203 [18,20]) was much stronger expressed in vaginal secretion (Fig. 2D). In addition, miR943 selected to be specific for semen [17] was markedly expressed only in saliva and skin (Fig. 2G). It appears that a comparison of results across different experimental conditions is severely hampered by variations in the differences of detection methodologies (SYBR Green vs. Taqman assays); diversity in the selection of reference genes and normalization procedures [38]; and the use of inter run calibrators to reduce technical run-to-run variation [29].

It has been suggested that miRNA analysis has several advantages over the use of messenger RNA (mRNA), such as being less prone to degradation due to smaller molecule size and the application of the highly sensitive qPCR technique [22,23]. However, as demonstrated in this study, the unambiguous differentiation between peripheral blood and menstrual blood as well as the separation of skin and epithelial cell containing body fluids remains challenging, representing a major problem for forensic applications. For example, in sexual assault cases, when the determination of the blood type (peripheral blood vs. menstrual blood) is considered crucial for the outcome of an investigation it can be rather difficult using miRNA markers, as these are characterized by a more universal expression pattern. Compared to multiplex mRNA analysis using endpoint PCR [11,39,40], the more complex and sensitive laboratory workflow and data analysis using real-time qPCR for miRNA profiling is not so appealing. In addition, the extensive calibration and normalization procedures needed for qPCR analysis complicate the implementation into routine casework. Above all, it has to be pointed out that an apparent lack of exclusive marker specificity for one cell type and a simple interpretation method makes miRNA profiling difficult to apply for the identification of body fluid and tissue types in forensic genetics. This applies even more to the analysis and differentiation of mixed body fluids, which was neither attempted in the present study, nor in another recently published study [21]. Despite a large number of publications on miRNA analysis from recent years there are still no promising results that would allow applying this approach in real casework. The identification of an unknown crime scene sample or the components of a body fluid mixture will always require the inclusion of a set of reference samples for direct comparison to ensure correct result interpretation, as the data for the unknown samples can only be assessed in the context of a larger data set of defined samples. The inclusion of a sufficient number of reference genes in addition to the set of body fluid specific miRNA markers is also a crucial requirement. In contrast, a clear strategy has been suggested for the implementation of mRNA analysis in routine casework [41]. The analysis of mRNA molecules has also repeatedly been shown to be possible even a long time after trace deposition [14,42–47]. For that reason it can be assumed that mRNAs are not that prone to decay as often assumed. In principle, the same scrutiny as for miRNA markers, including adherence to the MIQE guidelines, should also be applied to mRNA analysis; however, methods like endpoint PCR represent a compromise in this regard providing a number of practical advantages over the described miRNA profiling technique: markers are more specific, corresponding facilities are already available in most forensic laboratories, the use of labelled PCR primers on cDNA followed by fragment analysis allows to establish multiplexes where all markers can be combined into a single assay to be used for small amounts of casework samples, and data evaluation is similar to well-known STR profile analysis.

In conclusion, there is still place for improvement of both mRNA and miRNA analyses for their implementation in body fluid/tissue identification. We strongly agree Sauer et al. [21] that body fluid identification based on miRNA profiling would highly benefit from inter-laboratory trials, ideally using a set of ubiquitously used markers, to achieve a level of standardization that would allow a better assessment of this experimental approach and its suitability for casework application.

Acknowledgements

This work was financially supported by the European Union Seventh Framework Programme (FP7/2007-2013) under grant agreement n° 285487 (EUROFORGEN-NoE). We would also like to thank our volunteers for the kind donation of the biological samples for this study.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fsigen.2016.11.012.

References

[1] K. Virkler, I.K. Lednev, Analysis of body fluids for forensic purposes: from laboratory testing to non-destructive rapid confirmatory identification at a crime scene, Forensic Sci. Int. 188 (2009) 1–17, doi:http://dx.doi.org/10.1016/j.forsciint.2009.02.013.
[2] M. Alvarez, J. Juusola, J. Ballantyne, An mRNA and DNA co-isolation method for forensic casework samples, Anal. Biochem. 335 (2004) 289–298, doi:http://dx.doi.org/10.1016/j.ab.2004.09.002.
[3] M. Bauer, D. Patzelt, A method for simultaneous RNA and DNA isolation from dried blood and semen stains, Forensic Sci. Int. 136 (2003) 76–78, doi:http://dx.doi.org/10.1016/S0379-0738(03)00219-6.
[4] A. Bowden, R. Fleming, S. Harbison, A method for DNA and RNA co-extraction for use on forensic samples using the Promega DNA IQ™ system, Forensic Sci. Int. Genet. 5 (2011) 64–68, doi:http://dx.doi.org/10.1016/j.fsigen.2009.11.007.
[5] K. Watanabe, Y. Iwashima, T. Akutsu, K. Sekiguchi, K. Sakurada, Evaluation of a co-extraction method for real-time PCR-based body fluid identification and DNA typing, Leg. Med. 16 (2014) 56–59, doi:http://dx.doi.org/10.1016/j.legalmed.2013.11.002.
[6] D. van der Meer, M.L. Uchimoto, G. Williams, Simultaneous analysis of microRNA and DNA for determining the body fluid origin of DNA profiles, J. Forensic Sci. 58 (2013) 967–971, doi:http://dx.doi.org/10.1111/1556-4029.12160.
[7] E.J. Omelia, M.L. Uchimoto, G. Williams, Quantitative PCR analysis of blood- and saliva-specific microRNA markers following solid-phase DNA extraction, Anal. Biochem. 435 (2013) 120–122, doi:http://dx.doi.org/10.1016/j.ab.2012.12.024.
[8] Y. Li, J. Zhang, W. Wei, Z. Wang, M. Prinz, Y. Hou, A strategy for co-analysis of microRNAs and DNA, Forensic Sci. Int. Genet. 12 (2014) 24–29, doi:http://dx.doi.org/10.1016/j.fsigen.2014.04.011.
[9] J. Juusola, J. Ballantyne, Multiplex mRNA profiling for the identification of body fluids, Forensic Sci. Int. 152 (2005) 1–12, doi:http://dx.doi.org/10.1016/j.forsciint.2005.02.020.
[10] R.I. Fleming, S. Harbison, The development of a mRNA multiplex RT-PCR assay for the definitive identification of body fluids, Forensic Sci. Int. Genet. 4 (2010) 244–256, doi:http://dx.doi.org/10.1016/j.fsigen.2009.10.006.
[11] C. Haas, B. Klesser, C. Maake, W. Bär, A. Kratzer, mRNA profiling for body fluid identification by reverse transcription endpoint PCR and realtime PCR, Forensic Sci. Int. Genet. 3 (2009) 80–88, doi:http://dx.doi.org/10.1016/j.fsigen.2008.11.003.
[12] A. Hall, J. Ballantyne, Characterization of UVC-induced DNA damage in bloodstains: forensic implications, Anal. Bioanal. Chem. 380 (2004) 72–83, doi:http://dx.doi.org/10.1007/s00216-004-2681-3.
[13] A. Hall, L.M. Sims, J. Ballantyne, Assessment of DNA damage induced by terrestrial UV irradiation of dried bloodstains: forensic implications, Forensic Sci. Int. Genet. 8 (2014) 24–32, doi:http://dx.doi.org/10.1016/j.fsigen.2013.06.010.
[14] M. Setzer, J. Juusola, J. Ballantyne, Recovery and stability of RNA in vaginal swabs and blood, semen, and saliva stains, J. Forensic Sci. 53 (2008) 296–305, doi:http://dx.doi.org/10.1111/j.1556-4029.2007.00652.x.
[15] D.P. Bartel, MicroRNAs: genomics, biogenesis, mechanism, and function, Cell 116 (2004) 281–297, doi:http://dx.doi.org/10.1016/S0092-8674(04)00045-5.
[16] E.K. Hanson, H. Lubenow, J. Ballantyne, Identification of forensically relevant body fluids using a panel of differentially expressed microRNAs, Anal. Biochem. 387 (2009) 303–314, doi:http://dx.doi.org/10.1016/j.ab.2009.01.037.
[17] D. Zubakov, A.W.M. Boersma, Y. Choi, P.F. van Kuijk, E.A.C. Wiemer, M. Kayser, MicroRNA markers for forensic body fluid identification obtained from

microarray screening and quantitative RT-PCR confirmation, Int. J. Leg. Med. 124 (2010) 217–226, doi:http://dx.doi.org/10.1007/s00414-009-0402-3.
[18] C. Courts, B. Madea, Specific micro-RNA signatures for the detection of saliva and blood in forensic body-fluid identification: micro-RNAs for forensic body-fluid identification, J. Forensic Sci. 56 (2011) 1464–1470, doi:http://dx.doi.org/10.1111/j.1556-4029.2011.01894.x.
[19] Z. Wang, J. Zhang, H. Luo, Y. Ye, J. Yan, Y. Hou, Screening and confirmation of microRNA markers for forensic body fluid identification, Forensic Sci. Int. Genet. 7 (2013) 116–123, doi:http://dx.doi.org/10.1016/j.fsigen.2012.07.006.
[20] Z. Wang, J. Zhang, W. Wei, D. Zhou, H. Luo, X. Chen, Y. Hou, Identification of saliva using MicroRNA biomarkers for forensic purpose, J. Forensic Sci. 60 (3) (2015) 702–706, doi:http://dx.doi.org/10.1111/1556-4029.12730 Epub 2015 Feb 17.
[21] E. Sauer, A.-K. Reinke, C. Courts, Differentiation of five body fluids from forensic samples by expression analysis of four microRNAs using quantitative PCR, Forensic Sci. Int. Genet. 22 (2016) 89–99, doi:http://dx.doi.org/10.1016/j.fsigen.2016.01.018.
[22] C. Courts, B. Madea, Micro-RNA —a potential for forensic science? Forensic Sci. Int. 203 (2010) 106–111, doi:http://dx.doi.org/10.1016/j.forsciint.2010.07.002.
[23] C. Silva, A.L. Teixeira, M.J. Carneiro de Sousa, R. Medeiros, Forensic miRNA: potential biomarker for body fluids? Forensic Sci. Int. Genet. 14 (2015) 1–10, doi:http://dx.doi.org/10.1016/j.fsigen.2014.09.002.
[24] J. Vandesompele, K. De Preter, F. Pattyn, B. Poppe, N. Van Roy, A. De Paepe, F. Speleman, Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes, Genome Biol. 3 (2002) (research0034.1-research0034.11).
[25] S.A. Bustin, V. Benes, J.A. Garson, J. Hellemans, J. Huggett, M. Kubista, R. Mueller, T. Nolan, M.W. Pfaffl, G.L. Shipley, J. Vandesompele, C.T. Wittwer, The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments, Clin. Chem. 55 (2009) 611–622, doi:http://dx.doi.org/10.1373/clinchem.2008.112797.
[26] M. Sirker, W. Liang, L. Zhang, R. Fimmers, M.A. Rothschild, I. Gomes, P.M. Schneider, Impact of using validated or standard reference genes for miRNA qPCR data normalization in cell type identification, Forensic Sci. Int. Genet. Suppl. Ser. 5 (2015) e199–e201, doi:http://dx.doi.org/10.1016/j.fsigss.2015.09.080.
[27] Z. Hu, J. Dong, L.-E. Wang, H. Ma, J. Liu, Y. Zhao, J. Tang, X. Chen, J. Dai, Q. Wei, C. Zhang, H. Shen, Serum microRNA profiling and breast cancer risk: the use of miR-484/191 as endogenous controls, Carcinogenesis 33 (2012) 828–834, doi:http://dx.doi.org/10.1093/carcin/bgs030.
[28] L. Wong, K. Lee, I. Russel, C. Chen, Endogenous controls for real-Time quantitation of miRNA using TaqMan MicroRNA assays, Appl. Biosyst. Appl. Note (2010) Publ. (2010) 127AP 11-01. Available online: https://www3.appliedbiosystems.com/cms/groups/mcb_marketing/documents/generaldocuments/cms_044972.pdf.
[29] J. Hellemans, G. Mortier, A. De Paepe, F. Speleman, J. Vandesompele, qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data, Genome Biol. 8 (2007), doi:http://dx.doi.org/10.1186/gb-2007-8-2-r19 (R19-R19).
[30] J.M. Ruijter, C. Ramakers, W.M.H. Hoogaars, Y. Karlen, O. Bakker, M.J.B. van den Hoff, A.F.M. Moorman, Amplification efficiency: linking baseline and bias in the analysis of quantitative PCR data, Nucleic Acids Res. 37 (2009), doi:http://dx.doi.org/10.1093/nar/gkp045 (e45-e45).
[31] Y. Chen, J.A.L. Gelfond, L.M. McManus, P.K. Shireman, Reproducibility of quantitative RT-PCR array in miRNA expression profiling and comparison with microarray analysis, BMC Genomics 10 (2009) 407, doi:http://dx.doi.org/10.1186/1471-2164-10-407.
[32] J. Le Carré, S. Lamon, B. Léger, Validation of a multiplex reverse transcription and pre-amplification method using TaqMan® MicroRNA assays, Front. Genet. 5 (2014), doi:http://dx.doi.org/10.3389/fgene.2014.00413.
[33] P. Mestdagh, T. Feys, N. Bernard, S. Guenther, C. Chen, F. Speleman, J. Vandesompele, High-throughput stem-loop RT-qPCR miRNA expression profiling using minute amounts of input RNA, Nucleic Acids Res. 36 (2008) e143, doi:http://dx.doi.org/10.1093/nar/gkn725.
[34] qbase plus manual rev 2014-11-10; https://www.biogazelle.com/sites/default/files/public_file/qbaseplus_manual.pdf, n.d.
[35] E.K. Hanson, K. Rekab, J. Ballantyne, Binary logistic regression models enable miRNA profiling to provide accurate identification of forensically relevant body fluids and tissues, Forensic Sci. Int. Genet. Suppl. Ser. 4 (2013) e127–e128, doi:http://dx.doi.org/10.1016/j.fsigss.2013.10.065.
[36] Z. Wang, H. Luo, X. Pan, M. Liao, Y. Hou, A model for data analysis of microRNA expression in forensic body fluid identification, Forensic Sci. Int. Genet. 6 (2012) 419–423, doi:http://dx.doi.org/10.1016/j.fsigen.2011.08.008.
[37] E.K. Hanson, M. Mirza, K. Rekab, J. Ballantyne, The identification of menstrual blood in forensic samples by logistic regression modeling of miRNA expression, Electrophoresis 35 (2014) 3087–3095, doi:http://dx.doi.org/10.1002/elps.201400171.
[38] E. Sauer, B. Madea, C. Courts, An evidence based strategy for normalization of quantitative PCR data from miRNA expression analysis in forensically relevant body fluids, Forensic Sci. Int. Genet. 11 (2014) 174–181, doi:http://dx.doi.org/10.1016/j.fsigen.2014.03.011.
[39] T. Sijen, Molecular approaches for forensic cell type identification: on mRNA, miRNA, DNA methylation and microbial markers, Forensic Sci. Int. Genet.. 18 (2015) 21–32, doi:http://dx.doi.org/10.1016/j.fsigen.2014.11.015.
[40] A. Lindenbergh, M. de Pagter, G. Ramdayal, M. Visser, D. Zubakov, M. Kayser, T. Sijen, A multiplex (m)RNA-profiling system for the forensic identification of body fluids and contact traces, Forensic Sci. Int. Genet. 6 (2012) 565–577, doi:http://dx.doi.org/10.1016/j.fsigen.2012.01.009.
[41] A. Lindenbergh, P. Maaskant, T. Sijen, Implementation of RNA profiling in forensic casework, Forensic Sci. Int. Genet. 7 (2013) 159–166, doi:http://dx.doi.org/10.1016/j.fsigen.2012.09.003.
[42] F. Kohlmeier, P.M. Schneider, Successful mRNA profiling of 23 years old blood stains, Forensic Sci. Int. Genet.. 6 (2012) 274–276, doi:http://dx.doi.org/10.1016/j.fsigen.2011.04.007.
[43] M. Bauer, S. Polzin, D. Patzelt, Quantification of RNA degradation by semi-quantitative duplex and competitive RT-PCR: a possible indicator of the age of bloodstains? Forensic Sci. Int. 138 (2003) 94–103.
[44] D. Zubakov, E. Hanekamp, M. Kokshoorn, W. van Ijcken, M. Kayser, Stable RNA markers for identification of blood and saliva stains revealed from whole genome expression analysis of time-wise degraded samples, Int. J. Leg. Med. 122 (2008) 135–142, doi:http://dx.doi.org/10.1007/s00414-007-0182-6.
[45] D. Zubakov, M. Kokshoorn, A. Kloosterman, M. Kayser, New markers for old stains: stable mRNA markers for blood and saliva identification from up to 16-year-old stains, Int. J. Leg. Med. 123 (2009) 71–74, doi:http://dx.doi.org/10.1007/s00414-008-0249-z.
[46] H. Nakanishi, M. Hara, S. Takahashi, A. Takada, K. Saito, Evaluation of forensic examination of extremely aged seminal stains, Leg. Med. Tokyo Jpn. 16 (2014) 303–307, doi:http://dx.doi.org/10.1016/j.legalmed.2014.04.002.
[47] M. Sirker, P.M. Schneider, I. Gomes, A 17-month time course study of human RNA and DNA degradation in body fluids under dry and humid environmental conditions, Int. J. Leg. Med. (2016) 1–8, doi:http://dx.doi.org/10.1007/s00414-016-1373-9.

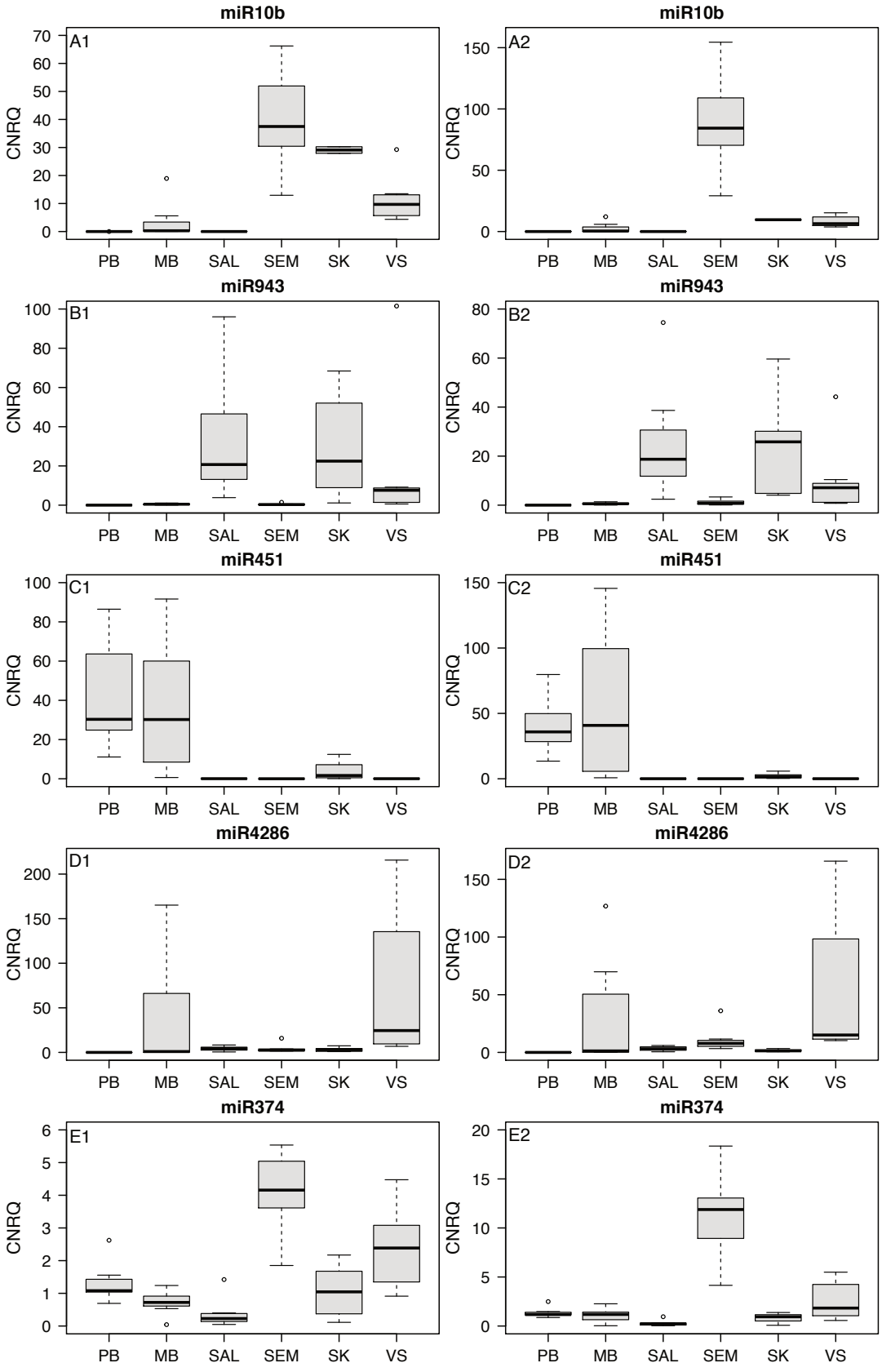


Fig. S1: Comparison of two different normalization strategies

Calibrated normalized relative quantities (CNRQs) of five exemplary selected miRNA markers. A1-E1: data normalized against miR26b, miR92, miR484, and miR16; A2-E2: data normalized against miR26b, miR92, miR484, and miR144. Results are depicted in box plots presenting the median with interquartile range, lower (25%) and upper (75%) quartiles. Whiskers indicate the maximum/ minimum values without outliers. Outliers are presented as circles. Peripheral blood (PB), menstrual blood (MB), saliva (SAL), semen (SEM), skin (SK) and vaginal secretions (VS).

Supplementary Table S1 Characteristics of 15 potential miRNA markers for body fluid/ tissue identification and four candidate reference genes for qPCR data normalization.

Body fluid/ Task	miRNA assay name[a]	TaqMan Assay ID	Reference
Peripheral blood	miR16	000391	[16,19,35,36]
	miR451	001105	[7,16,18,35]
Saliva	miR124*	002197	[35]
	miR203	000507	[18,20]
	miR205	000509	[7,16,18,35,20]
Semen	miR10b	002218	[16]
	miR943	002188	[17]
Vaginal secretion	miR1280	002835	[35]
	miR4286	241500_mat	[35]
Menstrual blood	miR142-3p	000464	[35]
	miR144	002676	[17,35,37]
	miR185[b]	002271	[17,35,37]
Skin	miR139	001096	[35]
	miR494	002365	[35]
	miR3169	243425_mat	[35]
Candidate reference genes	miR26b	000407	[28]
	miR92	000430	[28,26]
	miR374	000563	[28,26]
	miR484	001821	[27]

[a] all assays are *homo sapiens* (hsa) specific
[b] described as venous blood marker by Zubakov *et al*. [17]

Supplementary Table S2 AUC values calculated by pairwise comparison of all body fluids/ tissues for each marker.

Body fluid/ tissue pairs	miRNA assay name	AUC[a]	Body fluid/ tissue pairs	miRNA assay name	AUC[a]	Body fluid/ tissue pairs	miRNA assay name	AUC[a]
PB/MB	miR374	0.429	PB/SK	miR943	1	MB/SK	miR205	0.661
PB/MB	miR451	0.5	PB/SK	miR1280	1	MB/SK	miR185	0.813
PB/MB	miR16	0.516	PB/SK	miR3169	1	MB/SK	miR10b	0.857
PB/MB	miR185	0.563	PB/SK	miR4286	1	MB/SK	miR16	0.891
PB/MB	miR142-3p	0.688	PB/VS	miR142-3p	0.609	MB/SK	miR451	0.891
PB/MB	miR139	0.875	PB/VS	miR374	0.609	MB/SK	miR3169	0.979
PB/MB	miR3169	0.875	PB/VS	miR185	0.75	MB/SK	miR124*	1
PB/MB	miR10b	1	PB/VS	miR16	0.781	MB/SK	miR943	1
PB/MB	miR124*	1	PB/VS	miR3169	0.958	MB/VS	miR139	0.357
PB/MB	miR203	1	PB/VS	miR139	0.964	MB/VS	miR142-3p	0.453
PB/MB	miR205	1	PB/VS	miR10b	1	MB/VS	miR185	0.625
PB/MB	miR494	1	PB/VS	miR124*	1	MB/VS	miR124*	0.643
PB/MB	miR943	1	PB/VS	miR203	1	MB/VS	miR1280	0.688
PB/MB	miR1280	1	PB/VS	miR205	1	MB/VS	miR3169	0.703
PB/MB	miR4286	1	PB/VS	miR451	1	MB/VS	miR374	0.714
PB/SAL	miR142-3p	0.516	PB/VS	miR494	1	MB/VS	miR16	0.719
PB/SAL	miR185	0.964	PB/VS	miR943	1	MB/VS	miR4286	0.734
PB/SAL	miR139	0.969	PB/VS	miR1280	1	MB/VS	miR494	0.75
PB/SAL	miR374	0.984	PB/VS	miR4286	1	MB/VS	miR203	0.828
PB/SAL	miR16	1	MB/SAL	miR139	0.375	MB/VS	miR10b	0.839
PB/SAL	miR124*	1	MB/SAL	miR1280	0.5	MB/VS	miR943	0.886
PB/SAL	miR203	1	MB/SAL	miR205	0.547	MB/VS	miR205	0.891
PB/SAL	miR205	1	MB/SAL	miR4286	0.594	MB/VS	miR451	1
PB/SAL	miR451	1	MB/SAL	miR16	0.625	SAL/SEM	miR451	0.563
PB/SAL	miR494	1	MB/SAL	miR203	0.641	SAL/SEM	miR142-3p	0.734
PB/SAL	miR943	1	MB/SAL	miR142-3p	0.703	SAL/SEM	miR139	0.75
PB/SAL	miR1280	1	MB/SAL	miR494	0.719	SAL/SEM	miR1280	0.844
PB/SAL	miR3169	1	MB/SAL	miR185	0.821	SAL/SEM	miR203	0.875
PB/SAL	miR4286	1	MB/SAL	miR374	0.821	SAL/SEM	miR4286	0.891
PB/SAL	miR10b	1	MB/SAL	miR124*	0.825	SAL/SEM	miR205	0.906
PB/SEM	miR16	0.641	MB/SAL	miR3169	0.906	SAL/SEM	miR494	0.922
PB/SEM	miR185	0.734	MB/SAL	miR10b	1	SAL/SEM	miR943	0.984
PB/SEM	miR142-3p	0.75	MB/SAL	miR451	1	SAL/SEM	miR10b	1
PB/SEM	miR139	0.917	MB/SAL	miR943	1	SAL/SEM	miR124*	1
PB/SEM	miR10b	1	MB/SEM	miR139	0.5	SAL/SEM	miR16	1
PB/SEM	miR124*	1	MB/SEM	miR16	0.516	SAL/SEM	miR185	1
PB/SEM	miR203	1	MB/SEM	miR185	0.547	SAL/SEM	miR374	1
PB/SEM	miR205	1	MB/SEM	miR142-3p	0.578	SAL/SEM	miR3169	1
PB/SEM	miR374	1	MB/SEM	miR1280	0.578	SAL/SK	miR185	0.5
PB/SEM	miR451	1	MB/SEM	miR494	0.641	SAL/SK	miR943	0.531
PB/SEM	miR494	1	MB/SEM	miR4286	0.641	SAL/SK	miR139	0.583
PB/SEM	miR943	1	MB/SEM	miR943	0.65	SAL/SK	miR142-3p	0.625
PB/SEM	miR1280	1	MB/SEM	miR205	0.656	SAL/SK	miR4286	0.766
PB/SEM	miR3169	1	MB/SEM	miR203	0.969	SAL/SK	miR205	0.786
PB/SEM	miR4286	1	MB/SEM	miR124*	1	SAL/SK	miR374	0.821
PB/SK	miR142-3p	0.661	MB/SEM	miR10b	1	SAL/SK	miR1280	0.828
PB/SK	miR374	0.768	MB/SEM	miR374	1	SAL/SK	miR3169	0.833
PB/SK	miR185	0.969	MB/SEM	miR451	1	SAL/SK	miR16	0.844
PB/SK	miR10b	1	MB/SEM	miR3169	1	SAL/SK	miR203	0.906
PB/SK	miR124*	1	MB/SK	miR142-3p	0.464	SAL/SK	miR494	0.938
PB/SK	miR16	1	MB/SK	miR139	0.5	SAL/SK	miR10b	1
PB/SK	miR139	1	MB/SK	miR494	0.521	SAL/SK	miR124*	1
PB/SK	miR203	1	MB/SK	miR4286	0.547	SAL/SK	miR451	1
PB/SK	miR205	1	MB/SK	miR1280	0.594	SAL/VS	miR16	0.5
PB/SK	miR451	1	MB/SK	miR203	0.609	SAL/VS	miR451	0.563
PB/SK	miR494	1	MB/SK	miR374	0.612	SAL/VS	miR142-3p	0.578

[a] AUC: area under ROC (receiver operating characteristics) curve; AUC values of 0.5 indicate no discrimination power and the maximal possible value AUC = 1 stands for a perfect separation between the body fluid/ tissue types. AUC = 1 also means that the empirical distributions found for the CNRQ values for a given marker in a pair of two different sample types did not overlap.

B = peripheral blood; MB = menstrual blood; SAL = saliva; SEM = semen; SK = skin; VS = vaginal secretions

Supplementary Table S2 (continued)

Body fluid/ tissue pairs	miRNA assay name	AUC[a]
SAL/VS	miR139	0.607
SAL/VS	miR494	0.672
SAL/VS	miR3169	0.781
SAL/VS	miR943	0.821
SAL/VS	miR185	0.857
SAL/VS	miR1280	0.969
SAL/VS	miR205	0.984
SAL/VS	miR374	0.984
SAL/VS	miR10b	1
SAL/VS	miR124*	1
SAL/VS	miR203	1
SAL/VS	miR4286	1
SEM/SK	miR1280	0.484
SEM/SK	miR124*	0.5
SEM/SK	miR205	0.643
SEM/SK	miR142-3p	0.732
SEM/SK	miR139	0.778
SEM/SK	miR494	0.813
SEM/SK	miR4286	0.984
SEM/SK	miR10b	1
SEM/SK	miR16	1
SEM/SK	miR185	1
SEM/SK	miR203	1
SEM/SK	miR374	1
SEM/SK	miR451	1
SEM/SK	miR943	1
SEM/SK	miR3169	1
SEM/VS	miR451	0.469
SEM/VS	miR142-3p	0.672
SEM/VS	miR139	0.738
SEM/VS	miR16	0.781
SEM/VS	miR943	0.821
SEM/VS	miR185	0.875
SEM/VS	miR4286	0.891
SEM/VS	miR494	0.953
SEM/VS	miR374	0.969
SEM/VS	miR1280	0.984
SEM/VS	miR124*	1
SEM/VS	miR10b	1
SEM/VS	miR203	1
SEM/VS	miR205	1
SEM/VS	miR3169	1
SK/VS	miR142-3p	0.518
SK/VS	miR139	0.571
SK/VS	miR10b	0.625
SK/VS	miR16	0.672
SK/VS	miR943	0.714
SK/VS	miR374	0.804
SK/VS	miR185	0.938
SK/VS	miR494	0.938
SK/VS	miR203	0.969
SK/VS	miR3169	0.979
SK/VS	miR1280	0.984
SK/VS	miR124*	1
SK/VS	miR205	1
SK/VS	miR451	1
SK/VS	miR4286	1

[a] AUC: area under ROC (receiver operating characteristics) curve; AUC values of 0.5 indicate no discrimination power and the maximal possible value AUC = 1 stands for a perfect separation between the body fluid/ tissue types. AUC = 1 also means that the empirical distributions found for the CNRQ values for a given marker in a pair of two different sample types did not overlap.

PB = peripheral blood; MB = menstrual blood; SAL = saliva; SEM = semen; SK = skin; VS = vaginal secretions

4 Discussion

For a criminal investigation the determination of a body fluid stain of unknown composition can be crucial as it can significantly change the case´s outcome. Using conventional tests, it is not yet possible to identify particular body fluids with certainty, like vaginal secretions or menstrual blood, or to identify the components of mixed body fluids in a single approach, respectively. RNA-based profiling approaches, on the other hand, seem to represent reliable and sensitive detection methods fulfilling the essential demands of forensic casework. Since the analysis methods are based on PCR, multiple fluid types can be examined in a single multiplex assay saving time and precious sample material. The widely held opinion that RNA degrades shortly after its deposition could not be confirmed in this thesis as it has also been shown in other studies (Kohlmeier and P.M. Schneider, 2012; Nakanishi *et al.*, 2014; Setzer *et al.*, 2008; Zubakov *et al.*, 2008, 2009). The stability of those nucleic acids in forensically relevant body fluids thus enables the examination of aged stain material representing a fundamental prerequisite for forensic casework.

For future applications it would be desirable to have a method available, which could identify different kinds of body fluids in one single assay using a robust and highly specific set of markers, without compromising DNA profiling attempts. Therefore, the application of mRNA-based profiling approaches using endpoint PCR assays compared to miRNA-based profiling approaches using qPCR assays was investigated. Aim of the first part of the work was i) to assess the degradation process of mRNA (and DNA) under different environmental conditions during various time periods, ii) to test the sensitivity of selected markers using body fluid-specific endpoint PCR assays for three different sample amounts (5, 0.5 and 0.05 µL), and iii) to compare extraction methods regarding their efficiency. Aim of the second part of the work was i) to validate

reference genes for the normalization of miRNA gene expression data, ii) to identify specific miRNA markers for the determination of forensically relevant body fluids, and iii) to assess the benefits of miRNA profiling using qPCR.

In following sections, the different methodologies, which were applied in the present work, are critically analyzed in terms of their significance for forensic casework. Extraction methods, especially regarding their efficiency and final RNA/DNA yield, RNA quantification and the assessment of RNA quality, the different detection methods, and data analyses are in focus of the discussion. In comparison to the mRNA profiling approach outlined in "*17-month time course study of human DNA and RNA degradation in body fluids under dry and humid environmental conditions*" (publication 1, see section 3.1), which demonstrated to be straightforward both on laboratory scale and data analysis, the miRNA profiling approach applied in "*Evaluating the forensic application of 19 microRNAs as biomarkers in body fluid and tissue identification*" (publication 3, see section 3.3) showed serious drawbacks concerning the interpretation of results. Major point of criticism is the complex and sensitive laboratory workflow and the extensive calibration and normalization procedures required for the analysis of qPCR results. A previous validation of appropriate reference genes is essential for an accurate normalization, which could be shown in "*Impact of using validated or standard reference genes for miRNA qPCR data normalization in cell type identification*" (publication 2, see section 3.2). Regarding their advantages and disadvantages, the different approaches for body fluid identification using mRNA and miRNA are compared and discussed.

4.1 Comparison of mRNA and miRNA profiling approaches

In the following sections, methods that have been used in the publications of this thesis are discussed to assess their significance for forensic casework, focusing on extraction methods for DNA and RNA, final DNA yield and subsequent STR profiling success, RNA quantification, RNA detection methods (endpoint PCR vs. real-time quantitative PCR), and data analysis.

4.1.1 Extraction methods and final yield of DNA/RNA

Extraction methods can be de divided into two classes: methods that yield in solely one type of nucleic acid, either RNA or DNA, and methods that enable the simultaneous extraction of both nucleic acid types. In most cases it is sufficient to isolate only DNA for the purpose of generating an individual DNA profile. However, if RNA should also be analyzed for the purpose of identifying the cell or tissue type of a crime scene sample, the decision how to begin the laboratory investigation needs to be made right at the outset, especially if only a limited sample amount is available, which would be depleted after DNA extraction. In this regard, RNA can be extracted exclusively, or, both RNA and DNA can be obtained at the same time from the same forensic stain. Co-extracting RNA and DNA can therefore be advantageous because it renders the possibility of analyzing both the DNA for identification purposes, and the RNA to determine the biological origin of the generated DNA profile (as described in section 1.3.1).

In publication 1 (see section 3.1) different methods were applied to extract nucleic acids from forensic mock stains of different size (5, 0.5 and 0.05 µL), which were stored for certain time intervals up to 17 month, and compared in terms of their efficiency regarding the final DNA yield and endpoint PCR success for both mRNA and DNA analysis (the environmental influence of humidity is discussed separately in section 4.2). Solely RNA was obtained by automated silica-based purification using the EZ1 robot or utilizing the manual column-based Allprep DNA/RNA/miRNA co-extraction kit. DNA was extracted simultaneously with RNA using the co-extraction kit, and further, an automated silica-based DNA purification was carried out using the M48 robot. To measure the method´s efficiencies DNA extracts were quantified using the Quantifiler® human DNA quantification kit and subsequently amplified using the Investigator Decaplex SE kit. The yield of body fluid-specific mRNA was indirectly estimated after performing endpoint PCR and subsequent CE. By grouping the obtained peak heights of body fluid-specific signals into five categories based on relative values (see Table 2), the RNA profiling success could be evaluated (see Fig. 1 of publication 1). Of course, this estimate is not comparable to the results obtained from

qPCR as performed in publication 3 for quantitative miRNA assessment, but it helps to assess the robustness of the mRNA duplex assays.

Table 2 Conversion of peak heights into relative values for the ease of endpoint PCR data interpretation (publication 1)

RFU	Relative value	Meaning
> 5000	100	Very high signal in EPG (over-amplified peaks possible)
5000-2500	75	High signal in EPG
2500-1000	50	Moderate signal in EPG
1000-100	25	Low signal in EPG
<100	0	Signal too low; unspecificity possible

RFU = relative fluorescence unit; EPG = electropherogram

4.1.1.1 Final DNA yield of body fluid mock stains

In Table 3 the DNA quantification results of 5, 0.5 and 0.05 µL-sized stains stored under dry environmental conditions are presented. The two different DNA extraction methods are compared (extraction performed using the M48 robot and the Allprep co-extraction kit, respectively). As expected, semen showed the highest quantification results for both methods. This body fluid exhibits the highest cell density compared to the other human fluid types examined; on average, 80 to 100 million spermatozoa per mL (Faller and Schünke, 2008), which corresponds to 80000 to 100000 spermatozoa per µL. In comparison, 1 µL of blood contains an average of 4000 to 8000 leucocytes (Faller, 2008), which explains the lower quantification results (8.47 and 6.50 ng total DNA measured for 5 µL bloodstains compared to 212.92 and 149.21 ng total DNA measured for 5 µL semen stains, respectively). The number of nucleated cells present in 1 mL of saliva amounts to approximately 8 million[9], corresponding to 8000 cells in 1 µL. Assuming that the blood specimen contained a lower number of leucocytes (depending on the test person´s condition, which was declared as healthy), the higher total DNA yield assessed for 5 µL saliva stains can also be explained (20.47 and 18.70 ng total DNA measured for 5 µL saliva stains compared to 8.47 and 6.50 ng total DNA measured for 5 µL bloodstains, respectively). However, the values obtained for

[9] https://en.wikipedia.org/wiki/Saliva#Contents (last access at 15th April 2018)

the 0.5 µL samples do not support this assumption. Samples extracted with the M48 robot showed similar values for both saliva and blood, whereas samples extracted with the Allprep kit resulted in a higher total DNA yield for blood samples. The DNA concentrations measured for the saliva samples were on average below 5 pg/µL or gave no result (0.000 ng/µL). Consequently, a calculation of the average total DNA amount was not performed. The same applies to 0.05 µL samples, with the exception of semen samples extracted with the M48 robot.

Table 3 Quantification results of 5, 0.5 and 0.05 µL dry stored body fluid mock stains (publication 1)
Total DNA yields were calculated by averaging over quantification results obtained from different extraction time points. The different elution volumes were taken into account (50 µL for the M48 robot and 100 µL for the Allprep co-extraction kit).

Body fluid	Stain size [µL]	M48 robot	Allprep co-extraction kit
Semen	5	212.92 ± 136.89 ng	149.21± 91.78 ng
	0.5	18.82 ± 7.16 ng	6,21 ± 2.23 ng
	0.05	1.57 ± 0.76 ng	/
Saliva	5	20.47 ± 7.55 ng	18.70 ± 4.06 ng
	0.5	1.33 ± 1.18 ng	/
	0.05	/	/
Blood	5	8.47 ± 5.86 ng	6.50 ± 2.88 ng
	0.5	1.42 ± 1.14 ng	2.50 ± 2.63 ng
	0.05	/	/

Regarding the total DNA yield, distinct disparities between extraction methods were solely observed for semen samples. For blood and saliva samples no clear difference was detected. Variations in yield may occur due to variable densities of cells amongst the different specimens caused by sedimentation of the cells in their liquid medium during mock stain preparation, pipetting disparities or further technical factors. Although samples were frequently mixed, a cell-sedimentation cannot be entirely excluded. Results for samples stored under humid environmental conditions are not presented. A comparison would not have been meaningful as measured values differed significantly (between samples and methods) due to randomly occurring mold growth and the degree of mold affection, which was beyond experimental control.

4.1.1.2 DNA profiling

To allow for a further evaluation of the extraction efficiencies, samples were investigated with regard to their DNA profiling success. By performing an initial STR analysis of unexposed controls, which were extracted immediately after mock stain preparation, full reference profiles of the three sample donors were obtained. The detected alleles were counted representing 100 % for each donor. The same procedure was followed with the exposed samples in order to eventually determine the percentage of successfully amplified alleles. Although a DNA profile containing only half of the expected number of alleles (or less) may not be informative for forensic casework, results of those partial profiles are shown in order to illustrate the degradation process (see Fig. 2 of publication 1).

As described, the success rate of generating informative DNA profiles from 5 µL mock stains was mostly unaffected by dry storage conditions for both extraction methods. Looking at the results for 0.5 µL mock stains, the calculated percentages of successfully amplified alleles reflect the quantification results discussed above. For semen samples it was possible to generate full profiles during the whole investigative time period, whereas blood and saliva showed a variable range of profiles (0 to 100 % successfully amplified alleles). As expected, 0.05 µL mock stains showed the most incomplete profiles due to decreased peak heights and associated allelic drop-outs. In general, no degradation effect could be observed over time; solely for 0.5 µL blood samples: it was possible to generate full profiles after 29 and 30 weeks of storage, respectively; after 35 and 34 weeks, respectively, partial profiles with 90 to 30 % successfully amplified alleles could be generated. In summary, semen samples performed best, followed by blood samples and finally by saliva samples. This ranking order could be observed within each stain size group. A decrease in peak heights was expected for smaller sized stains and could also be monitored. A degradation effect was barely noticed. Samples extracted with either the M48 robot or the co-extraction kit performed comparably.

The effect of humidity to the STR analysis success rate was, in contrast, clearly noticeable. Results obtained during the first 33 weeks of humid storage were similar to those of dry storage, especially for the M48 extracted samples. After 47 weeks of

storage, a decline in success rates of amplified alleles could be observed for all three sample types and for all different mock stain sizes. Detailed results can be found in Fig. 2 of publication 1. A particular difference between the two extraction methods used could not be ascertained. However, after 47 weeks of storage the M48 extracted samples performed slightly better. More information regarding the performance of both extraction methods can be found in section 4.1.1.4 where efficiencies are compared for samples stored under dry and humid conditions in terms of DNA and RNA recoverability, respectively.

As mentioned in section 4.1.1.1, randomly occurring mold growth hindered a meaningful comparison of quantification results for samples exposed to humid environmental conditions. The colonization of certain microorganisms and accompanied DNA degradation is caused by the interaction of various factors. Human blood, for example, contains different salts, proteins, carbohydrates and micronutrients besides its cellular components (Faller, 2008), representing a good growth medium. The nutritive value of this body fluid is therefore a fundamental factor playing a key role for microbial settlement and proliferation shortly after a sample is deposited outside of the (human) body. The substrate composition on which the sample has been applied also has a strong influence on the microbial species, which will settle. Since the specimens in publication 1 (blood, semen and saliva) were spotted on sterile filter paper cards, mentioned impact is not discussed here. As further notable factor, the availability of water, present in the form of air humidity in publication 1 (see section 3.1), is necessary for biochemical reactions to occur; not only for the endogenic enzyme activity but also for the enzyme activity of accumulating microorganisms (Dissing *et al.*, 2010). Over time, the cells naturally start to decompose whereby nutrient-rich cytoplasm is liberated (Alaeddini *et al.*, 2010; Hofreiter *et al.*, 2001) which is, in turn, used by the microorganisms for their further growth and proliferation. The more microorganisms are present, the more they can contribute to the enzymatic DNA disruption, which begins as soon as the cell is no longer provided with energy. The simultaneous decay of cellular structures, like the nuclear envelope, increases the vulnerability for DNA degradation due to the lack of physical protection. Intracellular and microbial nucleases are jointly responsible for the degradation of DNA, whereas the STR analysis success

rate gets worse over time (see Fig. 2 of publication 1). Besides enzyme-catalyzed processes, which are, moreover, dependent on the ambient pH level and temperature, uncatalyzed reactions are also responsible for the fragmentation of nucleic acid molecules, such as hydrolytic reactions, oxidative reactions, or radiation (Alaeddini, 2010; Hofreiter, 2001; Lindahl, 1993). Conversely to the afore discussed influence of water/humidity on DNA degradation, rapid desiccation can help to preserve the DNA (Hofreiter, 2001), explaining the STR profiling success of samples stored under dry conditions. Different studies have demonstrated that DNA can successfully be recovered from ancient biological material, e.g., obtained from museum collections (Mitchell, 2015; Rowe *et al.*, 2011), and analyzed even after years of controlled exposure (Hofreiter, 2001). However, other factors, such as mentioned oxidation or background radiation, also contribute to the degradation process wherefore a long-term DNA preservation is, however, limited (Hofreiter, 2001; Lindahl, 1993).

4.1.1.3 RNA profiling

As mentioned in section 4.1.1, the yield of body fluid-specific mRNAs was indirectly estimated. To begin with, endpoint PCR and subsequent CE were performed. To allow for a better comparison, the obtained peak heights were grouped into five categories based on relative values (see Table 2), and those categories were then used to assess the robustness of the mRNA duplex assays.

In terms of detectability over time, blood-specific HBB and semen-specific PRM1 were the most reliable markers explored. In 5 µL-sized mock stains, both could be detected up to 70/71 weeks of dry storage (with both extraction methods). As expected, the smaller volume samples showed weaker signals in the EPGs; however, they could also be detected (e.g., HBB showed a signal strength between 1000 and 100 RFU in 0.05 µL co-extracted bloodstains) after 71 weeks of storage. In fact, PRM1 could even be detected in 0.05 µL co-extracted semen stains for the entire time period, as well as HBB in 0.5 µL co-extracted bloodstains. Both showed signal intensities above 5000 RFU. The second blood-specific marker, SPTB, also performed reliably for 5 µL-sized stains. In contrast to HBB, SPTB showed signal drop-outs after 19 and 29 weeks in 0.5 µL stains and after 7 weeks in 0.05 µL stains, respectively. The second

semen-specific marker, PRM2, provided amplification signals comparably strong to those of PRM1, but it was overall less sensitive. Especially the volume-reduced samples were more difficult to detect. Both saliva-specific markers, STATH and HTN3, provided good results regarding the 5 μL stains stored under dry conditions. In contrast to the other markers tested, they dropped out though in 0.5 and 0.05 μL samples after a short time of dry storage (see Fig 1 of publication 1).

Summarized, semen markers performed best, followed by blood markers and finally by saliva markers; similar to the ranking order observed for the DNA profiling results. A decrease in peak heights, and hence also a decrease in the relative values, was monitored for smaller sized stains. A degradation effect was barely noticed in dry stored samples, also similar to DNA profiling results. In comparison to those results, however, a greater variation among the differently extracted samples could be observed. This was particularly noticeable for semen, but also for blood samples. While PRM1 exceptionally provided reliable results for the 0.05 μL semen stains extracted with the Allprep kit (RFUs > 5000), a noticeable signal intensity drop could be asserted in corresponding stains extracted with the EZ1 robot. The maximum peak heights were below 5000 RFU, more precisely between 5000 and 100 RFU. For PRM2, the peak height decrease was already noticeable for the 0.5 μL-sized semen stains extracted with the EZ1 robot, whereas co-extracted 0.5 μL stains could be detected reliably (with exception for week 34 and 71, where solely signals below 5000 RFU were recorded). Detailed results can be found in Fig. 1 of publication 1.

The effect of humidity to the detectability of the different body fluid-specific markers was considerable. Both saliva-specific markers (STATH and HTN3) and both semen-specific markers (PRM1 and PRM2) dropped out after short time of storage. After 20/22 weeks it was already not possible anymore to obtain signals for all three sample volumes tested. The only exception was PRM1, which showed low signal intensities for 5 μL stains. Blood samples performed unexpectedly well compared to semen samples under humid conditions. Marker HBB was detectable up to 71 weeks (5 and 0.5 μL) with signal intensities ranging from 5000 to 100 RFU for the EZ1 extracted samples. On the contrary, HBB showed amplification results until week 33 and dropped out entirely afterwards for the co-extracted samples. Also unexpected was

the earlier drop-out of HBB in 5 µL samples compared to the lower sample volumes: results from 5 µL samples could be obtained until week 20; after 33 weeks of storage HBB dropped out using the smaller volume samples. The re-appearance of HBB amplification signals after 71 weeks could be explained by an unequal mold growth across the prepared specimens, which was beyond experimental control. As observed for the dry storage conditions, SPTB showed a weaker sensitivity. The marker was solely detectable after 8 weeks of humid storage. The environmental influence of humidity on the stability of RNA is discussed in more detail in section 4.2.

4.1.1.4 Efficiencies of extraction methods

As aforementioned, co-extraction renders the possibility of analyzing both DNA and RNA of very small biological stains and is therefore, theoretically, the methodical approach of choice for challenging crime scene samples. Summarizing obtained profiling results regarding the efficiency of different extraction methods (manual column-based Allprep DNA/RNA/miRNA co-extraction kit compared to automated silica-based purification approaches using the M48 robot for DNA or the EZ1 robot for RNA), it was noticeable that overall manually co-extracted samples performed slightly better than samples isolated with the robots (see Table 4).

Table 4 Efficiencies of extraction methods

Extraction methods, the manual column-based Allprep DNA/RNA/miRNA co-extraction kit and automated silica-based purification robots (M84 for the extraction of DNA and EZ1 for the extraction of RNA), are compared and their efficiency is assessed based on endpoint PCR success for both mRNA and DNA analysis. Categories + (satisfactory), ++ (good) and +++ (very good) were defined based on the overall profiling success and the possibility to obtain informative results.

Nucleic acid type	Storage condition	Allprep co-extraction kit	M48*/ EZ1**
DNA	Dry	++	++*
DNA	Humid	++	+++*
RNA	Dry	+++	++**
RNA	Humid	++	+**

DNA could be extracted simultaneously with RNA from dry stored body fluid specimens without the loss of material or compromising the DNA profiling success.

Obtained results were comparable to those of dry stored samples extracted with the M48 robot. The only difference was found for samples stored under humid conditions, which performed better when extracted with the M48 robot. For example, nearly complete DNA profiles (80-90 % of expected alleles) could be obtained analyzing the 5 µL semen samples after 70 weeks of storage, whereas corresponding samples extracted with the Allprep kit did not show amplification results (see Fig. 2 of publication 1). Regarding the efficiency of both RNA extraction methods it could be shown that the co-extracted samples performed more reliably than samples extracted with the EZ1 robot (dry and humid storage conditions). Especially blood and semen specimens performed very well, meaning that the obtained signal strengths were above 5000 RFU even after 71 weeks of dry storage. As expected, the humid stored body fluid samples showed reduced peak heights for all markers investigated (over time and with decreasing sample volumes). At the beginning of the study, however, co-extracted samples could be detected more reliably (e.g., the blood-specific marker HBB could be detected in 0.05 µL blood samples after 33 weeks of humid storage (>5000 RFU); corresponding samples extracted with the EZ1 robot only gave signal intensities between 1000 and 100 RFU). The convincing efficiency of the column-based Allprep DNA/RNA/miRNA co-extraction kit was solely reduced concerning the STR analysis success of humid stored samples. To increase the recoverability of DNA, a precipitation or a reduction of the elution volume might be useful in order to concentrate the purified nucleic acids. Since the elution volume can be easily adjusted when using the Allprep co-extraction columns, it would be the most obvious and simple attempt to enrich the DNA. Although the manufacturer suggests a minimal elution volume of 100 µL, a reduced volume of elution buffer, e.g. from 100 to 75 µL, applied to the column membrane should also resolve a sufficient number of molecules. Unfortunately, there was no time within this study to test this approach.

As shown in a study by Grabmüller *et al.*, in which five different commonly applied mRNA/miRNA extraction methods were compared, the Allprep DNA/RNA/miRNA co-extraction kit performed best regarding DNA yield and profiling success (Grabmüller *et al.*, 2015). Since it is a prerequisite for forensic casework routine to be able to perform STR analysis, it is encouraging to see that no limitations were

ascertainable concerning the DNA profiling performance. This result by Grabmüller *et al.* is consistent with the conclusions based on the herein presented study (publication 1, see section 3.1). Total DNA yields obtained from co-extracted samples were comparable to those of samples extracted with the M48 robot (with the exception of semen samples; see section 4.1.1.1), which is a positive outcome since the robot should be specialized providing high quantity DNA extracts. The other four methods tested by Grabmüller *et al.* are not designed for co-extraction but the authors tried to obtain DNA, i.e., from the organic phases left over after phase separation steps or the flow through waste. It could be shown that only one method, the NucleoSpin® miRNA kit (Macherey-Nagel), produced full STR profiles from the remaining swab material extracted with the PrepFiler® Forensic DNA Extraction kit (Thermo Fisher Scientific). The total DNA yield was, however, below the Allprep kit results. Regarding the overall efficiency (DNA and RNA extraction) the authors presented a ranking including the following categories: RNA yield, RNA quality, RNA expression analysis, DNA yield and DNA profiling. Surprisingly, the Allprep kit scored among the worst. The comparably bad result is due to low RNA yield and quality, the RNA expression analysis and arising costs per sample. Since all other methods are exclusively designed for mRNA/miRNA extraction it is, presumably, not surprising that these approaches yielded better scores in the study ranking. In this case, the presented findings, however, do not correspond with those of publication 1 (see section 3.1). Here, the co-extraction method performed throughout slightly better than the automated silica-based purification approach using the EZ1 robot (see Table 4), which has a specialized RNA extraction program like the M48 robot for DNA. The better efficiency of the co-extraction method is therefore somewhat unexpected.

Summarized, the Allprep DNA/RNA/miRNA co-extraction kit has advantages and disadvantages regarding the total nucleic acid yield and profiling success of extracted samples, both for DNA and RNA. The overall performance is, however, satisfactory: it was possible to successfully create full STR profiles and to identify their biological sources; even of stains with small volumes and for long storage time periods.

4.1.2 RNA quantification

The RNA quantities of the differently stored body fluid samples (publication 1, see section 3.1) were not determined prior to cDNA synthesis. The reverse transcription of mRNA into cDNA was performed in a final reaction volume of 10 µL containing 5 µL of total RNA. An approach using a maximum input volume without prior quantification has also been described in other studies (Lindenbergh, 2012, 2013; van den Berge, 2014; van den Berge *et al.*, 2016). Emanating from real casework samples, which are, in most cases, of limited quantity, we intended to work in a material saving manner, and, on these grounds, to add a maximum volume of RNA into the reaction. This was done equally for all samples to ensure the comparability of final data. According to the manufacturer of the iScript™ cDNA Synthesis kit used in publication 1, the RNA capacity in the cDNA reaction is 1 µg total RNA in 20 µL. Since a reduced reaction volume of 10 µL was used, 500 ng total RNA is the maximum amount to be added to the cDNA synthesis reaction. Such high amounts are rarely available when analyzing forensic stains. For confirmation purposes, the total RNA extracts obtained from 5 µL sized stains of all three sample types (blood, semen and saliva) were quantified using the NanoDrop 2000 UV/Vis spectrophotometer (Thermo Fisher Scientific). All measured quantities were, without exception, below 100 ng/µL, ensuring the optimum synthesis efficiency when applying 5 µL of RNA into the reaction. As described in section 4.1.1, the final RNA yield was estimated indirectly by performing endpoint PCR, subsequent CE, and conversion of obtained peak heights into relative values (see Table 2). As expected, the 5 µL body fluid samples revealed the highest values compared to samples of tenfold reduced volumes (0.5 and 0.05 µL). Furthermore, semen and blood samples showed the strongest signals in corresponding EPGs, which imply that those sample types were more concentrated than saliva samples (also see section 4.1.1).

Before performing qPCR experiments, and in contrary to above discussed experimental approach, it is strongly recommended to determine the RNA concentrations of all samples to be analyzed to ensure an equal RNA input for reliable data comparability (Bustin *et al.*, 2009). The RNA quantities of the samples used in publications 2 and 3 (see sections 3.2 and 3.3) were therefore measured using the

NanoDrop instrument. This device provides information about both the concentration and purity of a tested sample by measuring the UV absorbance ratio at 260 and 280 nm. The presence of DNA or residual phenol in a sample would alter the ratio and consequently indicate the decline in RNA purity (Bustin, 2009). Since this quantification method is, however, not specific to RNA molecules, the fluorescence-based Qubit assay, which uses a fluorescent RNA-binding dye, was additionally applied to confirm the NanoDrop measurements (data not shown). As the results of both approaches were comparable we decided to continue working with the NanoDrop; mainly due to its cost-efficiency and rapid feasibility. For a certain sample type, divergent RNA concentrations were measured caused by non-technical factors. Blood was collected, e.g., by puncturing the finger pulp of study participants. It was found that individuals bled to varying degrees and that the cotton swabs were therefore soaked with different amounts of blood. Furthermore, the RNA content in an equal amount of biological material varies naturally between individuals due to the different extent of specific gene expression influenced by personal physiological conditions or environmental factors. Other sample types also underlie mentioned influences resulting in RNA extracts varying in their concentration. In publication 3 (see section 3.3), blood showed the lowest quantification results, followed by skin, menstrual blood, semen, saliva and vaginal secretion (see Fig. 8). Due to the disparities in sample collection, a direct comparison and evaluation of those results is, however, not meaningful.

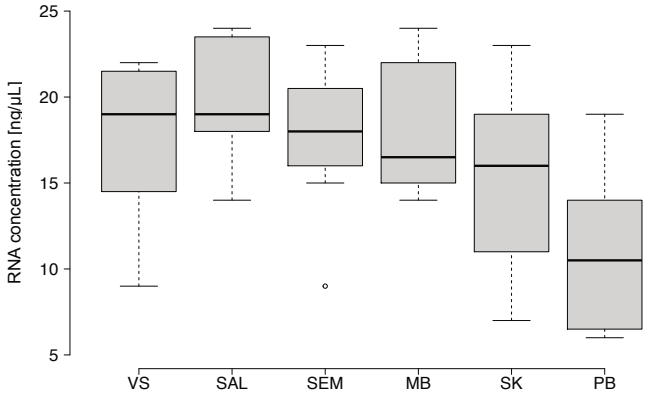


Fig. 8 RNA concentrations of different body fluids and skin samples (publication 3)

The different body fluid and skin samples (n = 8, respectively) were quantified using the NanoDrop 2000 UV/Vis spectrophotometer (Thermo Fisher Scientific). Results are depicted in box plots presenting the median with interquartile range, lower (25%) and upper (75%) quartiles. Whiskers indicate the maximum/minimum values without outliers. Outliers are presented as circles. Abbreviations are as follows: vaginal secretions (VS), saliva (SAL), semen (SEM), menstrual blood (MB), skin (SK) and peripheral blood (PB).

4.1.3 Endpoint PCR vs. real-time quantitative PCR

In this section, the laboratory efforts of both methods, endpoint PCR for the detection of mRNA targets and qPCR for the detection of miRNA targets, are compared and discussed in terms of their applicability for forensic casework.

Endpoint PCR: As described in section 1.3.1.1, sample processing for endpoint PCR detection starts with extracting the nucleic acids and subjecting the RNA to a cDNA synthesis reaction. A prior RNA quantification is not necessarily required. In order to obtain comparable results, the same amount of RNA should consistently be applied into reactions as shown in publication 1 and other studies (Lindenbergh, 2012, 2013; van den Berge, 2014, 2016). However, depending on the objective of the analysis (e.g., the detection of sample decay or the identification of sample types), the experimental procedure has to be continued differently. Either as carried out in publication 1 (a standard volume of cDNA was added into the endpoint PCR reaction), or as presented in the studies of Lindenbergh *et al.* (a series of different cDNA amounts is initially subjected to the reaction in order to determine which of these amounts result in the most informative EPGs). Both experimental strategies are easy to implement in short time representing a moderate effort (also see Table 5). Once the cDNA has been generated, it can be amplified either in singleplex or multiplex assays. Multiplex approaches are, however, advantageous due to the fact that multiple markers can be analyzed simultaneously, which also saves precious cDNA for subsequent analyses. The execution of an endpoint PCR for the detection of mRNA targets is straightforward and can be carried out in the vast majority of forensic laboratories, as the methodology is similar to that of the standard PCR for DNA profiling purposes. The PCR is followed by CE. Although it is not mandatory, a prior purification of the PCR products can have a positive effect on the CE results and is recommended to remove unspecific dye blobs and to increase the intensities of low peaks in the EPGs. Both purification and CE are basic laboratory procedures and, as the endpoint PCR described above, easy to be carried out in a standard forensic laboratory. The evaluation of results is the most complex step in relation to the entire work process. A continuing discussion can be found in section 4.1.4.

Table 5 Comparison of endpoint PCR and qPCR regarding their laboratory efforts

Both methods, endpoint PCR for the detection of mRNAs and qPCR for the detection of miRNAs, are compared in terms of their laboratory efforts. The expenditure of time, the implementation potential in a standard forensic laboratory and required expertise have been taken into account to define the categories + (moderate/regular), ++ (increased) and +++ (extensive/complex).

Analytical steps	Endpoint PCR for mRNA detection	qPCR for miRNA detection
RNA extraction	+	+
RNA quantification	(+)	+
cDNA synthesis	+	+
(Pre-Amplification)	/	+
PCR	+	++
Post PCR purification and CE	+	/
Data processing/ evauation	+ to ++	+++
∑ Time for experimental procedure [h]	+	++ to +++

/: not required

Real-time quantitative PCR: To detect cell type-specific miRNA targets and to assess their relative expression, qPCR was utilized in publication 3 (and 2). The effort of extracting the RNA and synthesizing cDNA is comparable to that described for mRNA detection using endpoint PCR. The main difference lies in the use of specific cDNA synthesis primers, which convert exactly those RNA sequences into cDNA that are to be detected afterwards by highly specific Taqman® probes. The creation of a custom primer pool is an additional effort representing a further assay specification. cDNA synthesis is followed by an optional pre-amplification step, which is recommended in the manufacturer´s protocol for creating custom RT (and pre-amplification) pools. In case of a low starting amount of RNA (1-350 ng) pre-amplifying the targets of interest can improve the overall assay sensitivity. Nevertheless, each supplementary processing step represents a risk of shifting the gene expression ratio of analyzed targets and thus differences are possibly more difficult to display. It could, however, be shown that the relative expression levels remain unaffected (Chen *et al.*, 2009; Le Carré *et al.*, 2014; Mestdagh *et al.*, 2008). The effort for this step is comparatively remote and is similar to a regular PCR. All subsequent expenditures of laboratory work (real-time PCR reaction setup and data processing/evaluation) deviate from the procedures described for mRNA target detection via endpoint PCR. First of all, the pre-amplification product needs to be diluted (in case the optional pre-amplification step has been performed

before). After that, the 96-well qPCR plate(s) have to be prepared with great care and accuracy: samples are applied in triplicates (to compensate for pipetting inaccuracies afterwards) for each miRNA marker separately and IRCs are required in addition to the test samples allowing for detection and elimination of technical variations between different qPCR runs. Those are usually samples that have to be analyzed on every plate of a qPCR experiment or study and enable a software-based calculation of a correction (or calibration) factor. This renders the possibility to proceed as if all samples were analyzed in the same run (Hellemans, 2007). Furthermore, appropriate reference genes need to be selected in advance (in a separate validation study), which are crucial for the normalization of gene expression data (Bustin, 2009). This process corrects technical sample-specific variations, e.g., disparities in the total amount of added cDNA, which would falsify the relative quantification results. Vandesompele *et al.* suggest to utilize at least three references to ensure an accurate normalization (Vandesompele, 2002). With a large number of samples and a large number of genes to be tested, it is necessary to choose an appropriate experimental setup (arrangement of samples on the qPCR plate(s)). Hellemanns *et al.* describe the *sample maximization approach* as preferable if a larger number of samples are to be examined. Different genes should then be analyzed in different runs if not enough free wells are available (Hellemans, 2007). The processing and evaluation of data is, compared to the analysis of mRNA profiling results, the most complex part of the entire analytical process and requires expertise in the use of suitable analysis software and statistics. Especially challenging is the proper interpretation of obtained data, since values are relative and many factors, such as gene expression discrepancies between tested individuals, the choice of reference genes (see publication 2, section 3.2) or the erroneous handling of the analysis software, can significantly influence the final analysis outcome. A detailed discussion concerning the evaluation of RNA profiling results can be found in section 4.1.4.2.

4.1.4 Evaluation of RNA profiling results

Both alternative RNA profiling approaches described in this thesis (see sections 3.1, 3.2 and 3.3), endpoint PCR for the detection of mRNA targets and qPCR for the

detection of miRNA targets, differ particularly in their evaluation procedure, as mentioned in sections 1.3.1.1, 1.3.1.2 and 4.1.3. While endpoint PCR results appear to be readily analyzed (comparable to STR profiling data), qPCR data are far more complex and require a further comprehensive processing. In the following sections 4.1.4.1 and 4.1.4.2 both evaluation procedures are described in more detail. The extensive normalization process of qPCR data is discussed separately in sections 4.1.4.2.1 and 4.1.4.2.2.

4.1.4.1 Endpoint PCR

The evaluation of endpoint PCR results starts with EPGs similar to those of STR profiles, although their assessment is obviously quite different from STR genotyping. If a peak of good morphology is visible in its designated position, a corresponding mRNA transcript was hence present in the casework sample. However, the peaks do not necessarily have to have an unambiguous shape. Looking at the endpoint PCR results of publication 1 (see section 3.1), split peaks of decreased size and wider shape could be detected for 5 µL samples extracted after the first dry storage periods (data not shown). This effect can also be observed when high amounts of DNA are amplified for STR profiling purposes. It is caused by an incomplete 3´A nucleotide addition during PCR (Magnuson *et al.*, 1996). Those peaks can even appear smaller in size than peaks of samples with a lower DNA content. For this reason, all peak heights above 5000 RFU were converted into the highest relative value of 100 representing a distinct signal. After translating obtained peak heights into relative values (0, 25, 50, 75 and 100, see section 4.1.1, Table 2) and assessing whether the peaks are real or possible amplification artefacts, the main part of the analysis procedure is completed. The relative values can only be used as a vague guidance for the amount of mRNA transcript in a sample, since endpoint PCR is not a quantitative method. However, this simplified evaluation concept only applies to the analysis approach presented in publication 1 where body fluid-specific duplex PCR assays (two markers for each target) were used for the detection of corresponding body fluids (e.g., a blood duplex PCR assay for the detection of blood). Other studies, such as the one by Lindenbergh *et al.* (2013) or by van den Berge *et al.* (2014), follow more complex evaluation approaches: with the help

of a multiplex PCR, body fluids of unknown origin can be discerned by generating multiple specific signals in the EPG. These are evaluated for the most reliable results based on all informative RNA profiles that were obtained following a serial dilution input approach to determine the most suitable amount of cDNA (Lindenbergh, 2013) and subsequent scoring the obtained profiling results yielding in eight or 12 individual signals for each cell type (also see section 1.3.1.1). Even if this procedure appears to be more complex and certainly depends on expertise, it can be carried out relatively fast and does not require any further analysis software since it encompasses "solely" the evaluation of generated EPGs and scoring of body fluid-specific signals. It compensates for the fact the mRNA is not equally expressed in the target cells or body fluids of interest, as expression levels may be influenced both by endogenous and exogenous factors.

4.1.4.2 Real-time quantitative PCR

As mentioned in section 4.1.3, the evaluation of qPCR data is, compared to that of endpoint PCR data, more complex and requires expertise for a proper performance of described analytical steps plus the handling of analysis software(s). The methodical procedure is known to be the most accurate and specific for gene expression studies, which is why it is often referred to as the *gold standard*, but at the same time the method is prone to external interferences due to its sensitivity and the experimental set-up. A proper normalization strategy for qPCR data is therefore strongly required in order to minimize technically induced variations between the samples to be compared. Those differences can be introduced at a number of stages throughout the experimental process (Dheda *et al.*, 2005), e.g., via sample input, RNA extraction or reverse transcription, wherefore the choice of normalization strategy is not that trivial since the methods have their respective advantages and drawbacks.

The accuracy of qPCR results is dependent on a proper normalization. Several strategies of variable effectiveness are available for that purpose (Huggett *et al.*, 2005). For example, normalization can be carried out using the applied sample size/volume, which, however, has no influence on fluctuating efficiencies of the individual methodical steps of the experimental procedure. A more effective and reliable way to

normalize data is the use of reference control genes. Control gene transcripts originate from the same sample as the target gene transcripts and pass through the same experimental workflow. For that reason, both transcript types underlie the same conditions, e.g., they are extracted and reversely transcribed into cDNA with the same efficiency. Theoretically, final qPCR data can therefore be compared more reliably.

To ensure the suitability of a selected reference, it needs to fulfill a mandatory requirement: a constant expression across all sample types to be tested (D'haene *et al.*, 2012; Dheda, 2005; Vandesompele, 2002). If the reference gene expression, however, varies between different sample types, the assay noise increases. Subtle expression differences of the target gene in different sample types can thus be overlooked or biologically meaningful changes cannot be distinguished from technical variations, leading to misinterpretations of study findings. To avoid erroneous results, it is thus strongly recommended to experimentally validate reference genes for qPCR data normalization (Bustin, 2009; Dheda, 2005).

In 2009, it could be shown that a group of reference genes results in a better data normalization than typically applied calibration strategies using only a single reference gene (Mestdagh *et al.*, 2009). This method, introduced as the *global mean normalization method* using a mean miRNA expression value, exceeded other normalization strategies regarding its performance to reduce technical variations and accentuate true biological changes. It is, however, only applicable if a large number of miRNAs per sample, e.g., the entire miRNome, is to be tested. In experiments where a larger sample set and only a limited set of target genes are to be analyzed, it is sufficient to include a reduced number of reference genes. The geometric mean of carefully selected miRNAs/small RNA controls resembling the mean performs equally well compared to the mean miRNA expression value itself (Mestdagh, 2009). Previous studies have already shown that several references perform better than individual references (Huggett, 2005; Vandesompele, 2002).

4.1.4.2.1 Validation of reference genes for data normalization

Reference genes for data normalization differ between studies and their experimental objective. A common practice is the utilization of a single standard reference to control

for error between the tested samples. In general, small non-coding RNAs are used comprising both small nuclear RNAs, like U6B, and small nucleolar RNAs, such as U24 or U26 (Mestdagh, 2009). This approach, however, has been called into question as it was becoming increasingly apparent that the expression of some of those genes can vary substantially in certain biological sample material leading to false results (Dheda, 2005). To overcome that issue it is highly recommended to perform a pilot validation study in order to identify the most stable expressed genes in a representative sample set (Bustin, 2009; D'haene, 2012; Mestdagh, 2009; Vandesompele, 2002).

A validation study usually starts with a selection of candidate references, e.g., from microarray analysis studies (Nelson *et al.*, 2004; Thomson *et al.*, 2004). If it is not possible to carry out such a large-scale pilot screening, it is also legitimate to identify and compile a suitable set of potential candidates through a comprehensive literature search. Selected candidate references are processed in parallel to the genes of interest, meaning cDNA synthesis, pre-amplification and qPCR (see publication 3, section 3.3). This ensures that changes in the expression level between samples are not caused by technical factors: all samples are equally processed and subjected to the same experimental conditions as well as to the same methodical efficiencies. For the final determination of the most suitable reference gene(s) out of selected candidates, software applications such as geNorm (Vandesompele, 2002), NormFinder (C. L. Andersen *et al.*, 2004) or BestKeeper (Pfaffl *et al.*, 2004) are available.

A convenient solution is provided by the reference gene validation software qbase^{plus} (Biogazelle): it uses the incorporated global mean normalization algorithm geNorm in a modified version, which calculates a gene stability measure (M-value) for each candidate on the basis of non-normalized expression levels (Vandesompele, 2002). The M-value is based on the assumption that the expression ratio of two ideal reference genes is the same across all samples. The variation of the expression ratios of two real reference genes reflects the fact that the expression of at least one gene is, however, not constant. An increasing ratio variation is therefore associated with decreasing expression stability. To determine the M-value, the pairwise variation for every reference gene with all other candidates is calculated as the standard deviation of the logarithmically transformed expression ratios. The progressive exclusion of the candidates with the highest M-value

finally leads to a combination of the two most stable expressed reference genes (Vandesompele, 2002). Results are graphically depicted according to the expression stability of reference genes across the set of different sample types tested (see Fig. 1 of publication 3). The lower the values, the more stable expressed are the genes (Hellemans, 2007).

In publication 2 (see section 3.2) it could be shown that the choice of reference gene, either standard or validated, can have a strong impact on qPCR data normalization and interpretation. Results for blood and semen-specific markers are presented in Fig. 9; results for skin-specific markers are depicted in Fig. 10. Both saliva markers, miR205 and miR658, did not give any results probably due to technical issues. The direct comparison of normalized relative quantities (NRQ) values was no definitive analysis strategy, like the scoring system suggested by Lindenbergh *et al.* (2013) for the interpretation of mRNA profiling data, but merely served to demonstrate the influence of certain reference genes to data interpretation. It was ascertainable that marker miR16 was the only one giving imprecise results when data were normalized using validated references. Obtained NRQ values scattered closely together. In comparison, it was possible to differentiate blood (mean NRQ: 276.28) from the other cell types investigated (mean NRQs <1) when data were normalized using the standard reference gene U6B. All other markers gave more precise results when data were normalized applying validated references (see Fig. 9 and Fig. 10).

Marker miR203, initially selected as skin marker, showed greater potential to serve as future blood-specific marker (see Fig. 10). Although miR203 had highest expression in skin (mean NRQ: 29.59), saliva and semen scattered closely (mean NRQs: 18.45 and 19.34); possibly due to the similarity of samples containing a certain proportion of epithelial cells. Blood values on the other hand clustered around a mean NRQ of 0.003. The same could also be observed for marker miR135b (see Fig. 9). It was solely possible to differentiate one sample type from the others when validated references were used for data normalization. Applying U6B, NRQs were similar even showing overlaps so that it was not possible to differentiate between the different sample types examined.

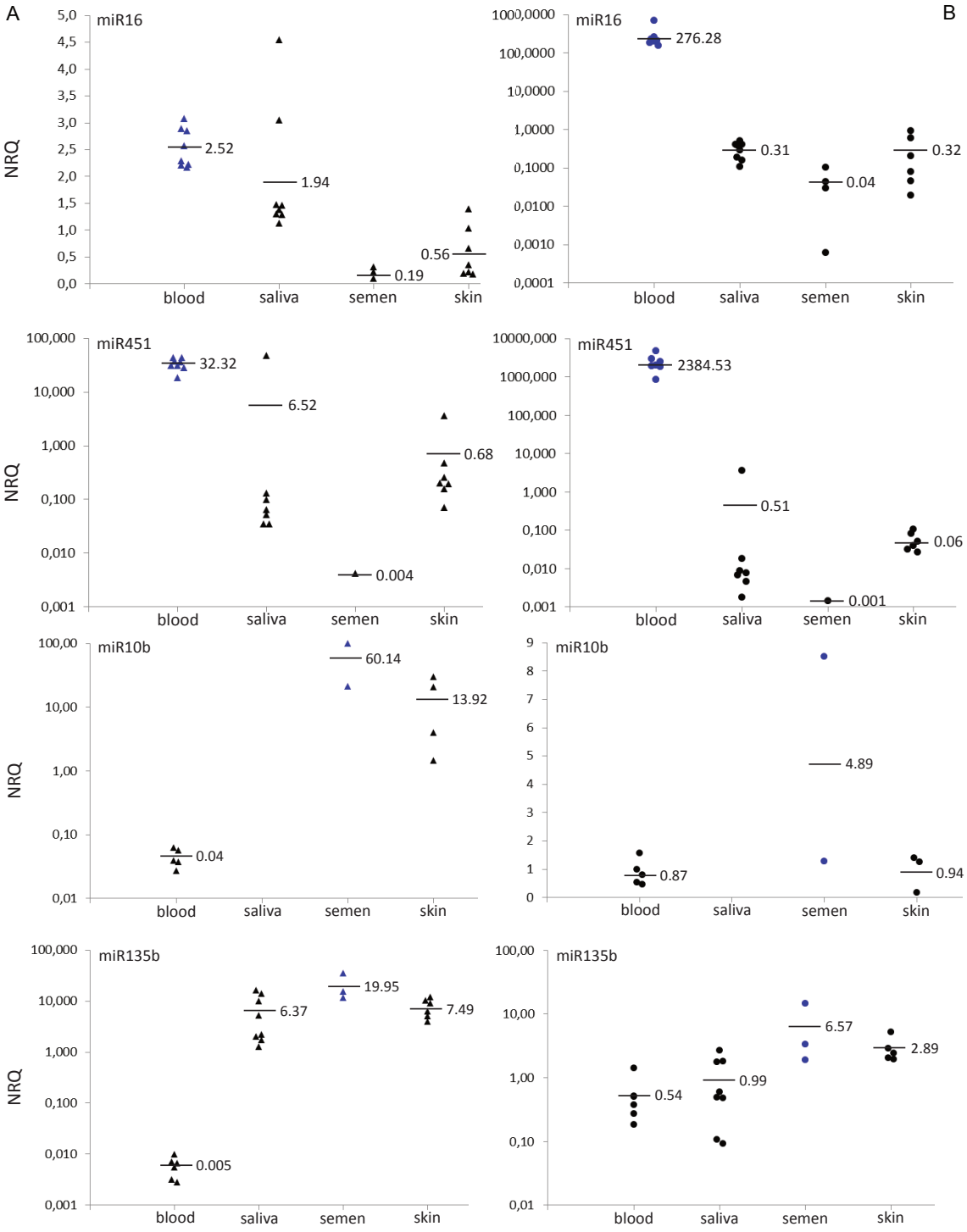


Fig. 9 Comparison of the effect of normalization to qPCR results of blood (miR16 and miR451) and semen markers (miR10b and miR135b)

Data were normalized (A; left site) against validated reference genes (miR92 and miR374) and (B; right site) against the commonly used standard endogenous control (U6B). Normalized relative quantities (NRQs) are plotted against particular body fluids (blood, saliva, semen) and skin. Numbers indicate the mean NRQs, respectively.

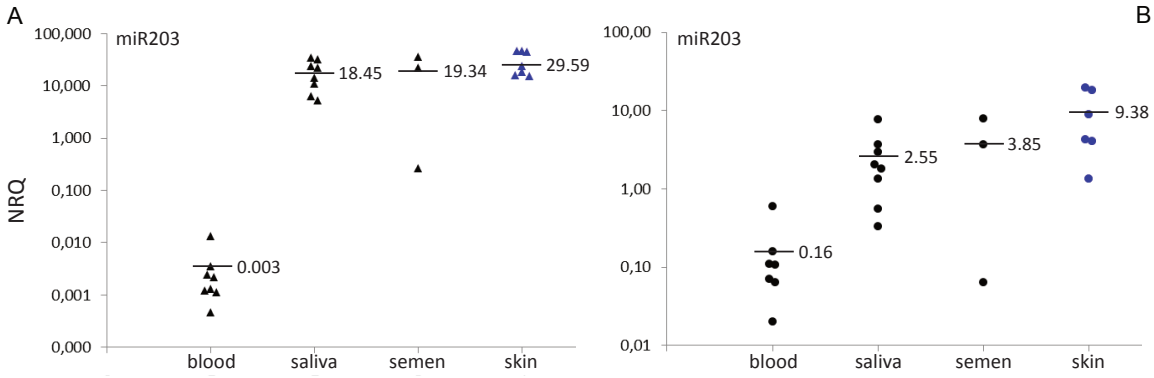


Fig. 10 Comparison of the effect of normalization to qPCR results of skin marker miR203

Data were normalized (A; left site) against validated reference genes (miR92 and miR374) and (B; right site) against the commonly used standard endogenous control (U6B). Normalized relative quantities (NRQs) are plotted against particular body fluids (blood, saliva, semen) and skin. Numbers indicate the mean NRQs, respectively.

Reagent costs can limit the number of reference genes to be evaluated, wherefore a careful compilation of the initial candidate set is required. Of course, if once a valid set of references is determined, it is not necessary to repeat the described procedure. It can be regarded as a nonrecurring effort, which, however, should be carried out with great care to guarantee the reliability of qPCR results.

4.1.4.2.2 Normalization of miRNA data

A proper normalization strategy is essential for an accurate interpretation of gene expression results as described in section 4.1.4.2.1. To accomplish this, a PCR efficiency correction needs to be carried out before the actual calculation of NRQs since it is preferable to work with the true efficiency of a gene assay instead of a hypothetical one. This step is recommended in the qbase^{plus} manual[10] and can be performed, e.g., using independent software like LinRegPCR (Ruijter *et al.*, 2009). Instead of calculating the efficiency from the slope of a serial dilution of a target gene, LinRegPCR averages the sample-specific efficiencies to obtain a target gene-specific efficiency using fluorescent amplification curves. Corrected qPCR data can be exported

[10] https://www.qbaseplus.com/sites/default/files/public_file/qbaseplus_manual.pdf (last access at 14th April 2018)

in a file format, which can directly be imported into the analysis software (qbase^{plus}). Cq values can subsequently be subjected to a quality control including the inspection of technical replicas. By default, the replicate variability threshold is set to 0.5, meaning that the difference in Cq value between the replicate with the highest Cq value and the replicate with the lowest Cq value must be smaller than 0.5 cycles[11]. Outliers, which may bias further calculations, can manually be excluded. As soon as all recommended quality controls have been carried out, reference genes that have previously been identified by applying the geNorm algorithm can be defined as *reference targets*; the remaining target genes are defined as *targets of interest*. The consecutive data normalization is performed according to the calculation workflow schematically depicted in Fig. 11. The fourth step is solely necessary if a target gene is measured in different qPCR runs, leading to a further technical variation, which needs to be corrected for reliable data interpretation. By measuring the Cq or NRQ difference between the IRCs (identical samples for the same target) of two qPCR runs, it is possible to calculate a correction or calibration factor (CF) to remove the run-to--run variation (Hellemans, 2007). The calibrated normalized relative quantities (CNRQs) can then be exported from the software for further statistical evaluation.

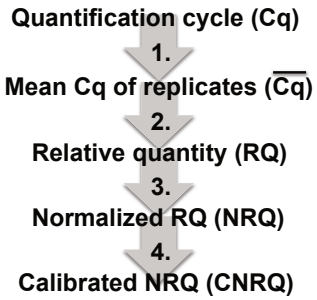


Fig. 11 Scheme of the qbase^{plus} calculation workflow

The software qbase^{plus} initially calculates the arithmetic mean of the Cq values of technical replicas, respectively (step 1). Subsequently, those data are converted into relative quantities (RQs) by subtracting the individual mean values from the highest Cq value (step 2). The normalization is carried out dividing the RQs by the normalization factor (NF) of these reference genes (step 3). NFs are the geometric means of the RQs of at least three reference genes in each sample examined. Finally, the calibration of NRQ data against their mean value (calibration factor, CF) (step 4) enables the analysis of samples from different qPCR runs. Picture adapted from Hellemans *et al.* 2007.

[11] https://www.qbaseplus.com/sites/default/files/public_file/qbaseplus_manual.pdf (last access at 14th April 2018)

Although qPCR is the most accurate method for detecting subtle expression differences of target genes in different tissue types due to its high specificity, sensitivity and accuracy, it nevertheless has its drawbacks. One of the most serious disadvantages is certainly, as already mentioned, the enormous effort of data processing, which has only been briefly outlined above. The utilization of software tremendously simplifies RQ calculation and data normalization though, as none of the mathematical steps need to be executed by the experimenter himself. However, qbase^{plus} contains a variety of configuration options requiring expertise for the appropriate selection of analysis settings. Otherwise the calculations cannot be performed correctly representing an obvious source of error. In addition, the user interface is not intuitive enough for untrained use impeding the quick acquisition and confident handling of the software. When planning a qPCR experiment, it is furthermore important to consider the availability of sufficient sample material as well as the utilization of IRCs if one gene is tested in more than one qPCR runs. This would obviously be the case if miRNA profiling would be applied for the analysis of real casework samples. It should therefore be ensured that the same IRC sample material is available for all future miRNA profiling experiments.

4.2 Stability of RNA in forensically relevant stain material

In a living cell, the half-life periods of RNA molecules underlie different regulatory mechanisms to prevent an accumulation of molecules, which are no longer required, and to allow fine adjustment and reactivity to environmental influences (see section 1.3.1). In forensically relevant stain material these mechanisms collapse since internal biochemical processes terminate with ending energy supply (Osellame *et al.*, 2012). Other factors, like the activity of nucleases or the extracellular osmolarity, can then have an influence on what happens to the cells and their ribonucleic acids in the further course of the process. For a long time mRNA was assumed to be readily degraded due to ambient RNases (Osellame *et al.*, 2012); difficult to imagine that those fragile mRNAs might be suitable markers. Contrary to this expectation, it could be

demonstrated that specific blood and saliva mRNA markers show stable expression patterns even up to 180 days after trace deposition (Bauer, 2007). Other research groups could confirm the unexpected stability of different body fluid-specific mRNAs (Zubakov, 2009). A long-term persistence of 23 years as described by Kohlmeier and Schneider (2012), may be explained by sample dehydration which reduces the activity of RNases (Kohlmeier, 2012; Nakanishi, 2014; Setzer, 2008; Zubakov, 2009). Fordyce *et al.* also discussed the ability of RNA molecules to resist spontaneous hydrolytic depurination as a reason for their extended viability. In some instances, RNA may even be more stable than DNA (Alaeddini, 2010; Fordyce, Kampmann, *et al.*, 2013). In a further study by Fordyce *et al.*, ancient RNA derived from 725 year-old desiccated maize seeds has successfully been analyzed (Fordyce, Ávila-Arcos, *et al.*, 2013). However, in order for RNA to be preserved for decades, or even centuries, a protective barrier is essential. Cells lacking such a protective coating experience hydrolytic damage when exposed to humidity, which leads to significantly decreased marker stability. In addition, humidity stimulates the spreading of certain environmental microorganisms, which use the biological material as substrate for their growth (see also section 4.1.1.2). A further detrimental factor for some types of body fluids, e.g., for saliva or vaginal secretions, is its endogenous flora of microorganisms (Setzer, 2008). Analogously to DNA degradation processes, physical or chemical factors also need to be considered, such as pH, the presence of metal cations, UV-light, or the presence of oxygen (Fordyce, 2013).

In publication 1 (see section 3.1) the RNA stability was indirectly measured by its recovery and detectability after performing PCR and CE (see Fig. 1 of publication 1). It could be shown that even after 17 months of storage, body fluid-specific mRNAs were still intact and positive results could be obtained (blood and semen markers showed the best results; see section 4.1.1.3). It could also be shown that humidity had a detrimental effect on the recoverability of certain markers examined. In conclusion, the high stability and robustness of mRNAs were confirmed, which is promising for forensic routine use. Often, crime scenes remain unnoticed for a long time and, additionally, they may be affected by different environmental factors. In those cases, it is still possible to recover sufficient biological material for body fluid identification purposes.

Knowledge about the mechanisms of RNA degradation and the best approach to ensure a potential analytical success will aid improving the way of securing evidence and interpreting RNA results.

4.3 Methods using epigenetics for body fluid identification – an alternative approach to RNA profiling techniques?

Although DNA methylation profiling seems to be the method of choice according to advantages described in section 1.3.2, the method still suffers from a relatively high demand of original sample material required for a complete bisulfite conversion (Sijen, 2015). In studies published in the past few years, various DNA amounts have been applied and different processing approaches were followed (see Table 6). To date, no uniform strategy for body fluid identification purposes is yet pursued making it difficult to implement DNA methylation profiling in forensic routine work. However, it appears that an approach using bisulfite conversion, PCR and single-base extension (SBE) is the most frequently used and, simultaneously, the most reasonable since the DNA integrity is maintained; in comparison to an approach where target sequences are digested by methylation-sensitive restriction enzymes. An incomplete digestion or differences in enzyme activities could hamper the analysis by distorting the methylation ratios and consequently compromise the interpretation of results (Vidaki, 2013). For that reason, this methodological approach seems to be more difficult to implement. Contrary to this, bisulfite treatment does not destroy the DNA structure, but merely alters it (Vidaki, 2013).

Recently, NGS technology is being used more and more frequently. Such new methods can significantly increase the sensitivity, but they are still far too expensive for routine use. It could, however, be shown that successful DNA methylation profiles were obtained with as low as 50 pg of starting material applying a bisulfite pyrosequencing-based assay (Vidaki *et al.*, 2016). Forat *et al.* determined a detection limit between 100 and 200 pg for the different assays tested in their study (Forat, 2016).

Table 6 Comparison of recently published DNA methylation studies for body fluid identification

Different recently published studies for body fluid identification using DNA methylation are compared in terms of their laboratory strategy and the amount of DNA used.

Study	Laboratory strategy	DNA amount
Frumkin *et al.*, 2011	Endonuclease digestion (HhaI), PCR and CE	1 ng
Lee *et al.* 2012	Bisulfite conversion, PCR, cloning and SBE	>100 ng
Forat *et al.*, 2016	Bisulfite conversion, PCR and: bisulfite sequencing, msSNuPE* or NGS	2 ng (25 pg to 5 ng**)
Lee *et al.*, 2016/ Jung *et al.* 2016	Bisulfite conversion, PCR and SBE	100 ng
Vidaki *et al.*, 2016	Bisulfite conversion, PCR and pyrosequencing	100 ng to 1 ng (to 10 pg**)
Holtkötter *et al.*, 2017	Bisulfite conversion, PCR and SBE	200 to 500 ng

*msSNuPE = Methylation-Sensitive Single Nucleotide Primer Extension Assay (=SBE); ** amounts of DNA tested for assay sensitivity

Although the sensitivity increases due to the use of new sequencing methods and thereby DNA methylation profiling becomes more and more interesting for forensic applications, certain aspects cannot be ignored. A serious drawback to be viewed critically is that this approach is often based on differential methylation levels. In comparison to mRNA analysis where a yes/no answer can be generated (a marker is either present or absent), the results obtained from DNA methylation profiling are more vague (a CpG site is more or less methylated). Differences between individuals are influenced by age, pathophysiological conditions or environmental factors making it even more difficult to analyze and interpret results. Body fluid mixture interpretation may also be hindered as methylation levels can get diluted (Sijen, 2015). If, e.g., a fictitious marker shows a medium high degree of methylation for blood while the degree of methylation is lower for saliva, it is, theoretically, possible to discriminate between the two body fluids; in a 1:1 mixture of blood and saliva the total methylation degree will drop since the two individual values interfere with each other (T. Sijen, Netherlands Forensic Institute (NFI), personal communication).

Analyzing mRNA it could be shown that positive and unambiguous identification results could be obtained even when starting material of 0.05 µL was examined (see publication 1, section 3.1); especially blood and semen performed quite reliably. For stains of this size, an average DNA concentration of less than 5 pg/µL or 0.000 ng/µL

was measured for corresponding co-extracted samples. Lindenbergh *et al.* also obtained full RNA profiles from 0.05 µL sized body fluid samples. It could also be demonstrated that RNA is almost as stable as DNA and can therefore be analyzed even months or years after sample deposition (Kohlmeier, 2012; Nakanishi, 2014; Setzer, 2008; Zubakov, 2008, 2009 and publication 1).

Both DNA methylation and RNA profiling methods have comparable advantages over conventional methods and can thus be seen as alternative, supportive approaches to each other, which can be applied depending on the special requirements of the case. Epigenetic-based methods are an alternative when sufficient sample material exists or when only DNA extracts have been retained after the main investigation has already been completed. When only limited amounts of a body fluid or body fluid mixtures could be secured at a crime scene, RNA-based profiling methods are preferable; also because this method has so far been applied to investigate a broader spectrum of different cell types. DNA methylation profiling, to date, renders the possibility to determine commonly encountered body fluids, like blood, semen, saliva, vaginal mucosa, menstrual secretion and skin, whereas RNA profiling additionally enables the identification of nasal blood, nasal secretion, sweat and urine (Sijen, 2015).

4.4 Significance of RNA analysis for forensic casework and future perspectives

In the last decade, alternatives to the previously described conventional tests for body fluid identification (see section 1.2) have been increasingly discussed and explored by the forensic community since many of the conventional methods exhibit serious drawbacks regarding their laboratory workflow, specificity, and sensitivity (see Table 7). Precious evidence material is diluted by the use of certain reagents, for example luminol for the visualization of low volume sized stains (see section 1.2.1), which may impair subsequent DNA amplification reactions. Some of the other methods are not specific enough to provide evidence for an unambiguous cell type inference, such as the amylase test for the detection of saliva (see section 1.2.2); the enzyme is also expressed

in breast milk and sweat (Virkler, 2009). In contrast, mRNA-based approaches allow for a convenient and specific identification of body fluids as shown in publication 1 (see section 3.1). In particular, the potential to examine multiple markers for different cell types in a single multiplex assay (Fleming, 2010; Juusola, 2005; Lindenbergh, 2012) is highly advantageous and could easily replace the large number of serological and biochemical tests currently applied in forensic routine.

Table 7 Advantages and drawbacks of RNA profiling techniques compared to conventional methods

	RNA profiling	Conventional Methods
Advantages	Lower amounts of sample material required	Lower costs for single tests
	Co-analysis of RNA and DNA possible	Time saving if only one test is performed
	Highly sensitive and specific markers	
	Multiplexed analysis possible; therefore good cost-benefit factor and time saving	
	No detrimental effect on subsequent DNA analysis	
Drawbacks	More analytical steps required; therefore more prone for errors	Higher amounts of sample material needed, as each marker has to be tested individually
	Extracted RNA sensitive to RNase degradation	Cross-reactivity possible; therefore reduced specificity
		Reagents can interfere with DNA analysis

In forensic context, many samples encountered at a crime scene or collected from individuals, e.g., from victims of sexual assault, can represent mixtures of various cell types contributed by one ore more individuals. The degree of involvement of one contributor to the mixture can also vary considerably. The most common sample types are blood-blood mixtures, vaginal mucosa-penile skin, or vaginal mucosa-semen (Sijen, 2015). With conventional methods it is impossible to distinguish different cell types, especially if one donor contributed many times stronger to the mixture than another. A collaborative exercise supported by the EUROFORGEN-NoE project explored the usefulness of forensic cell type identification by mRNA profiling (van den Berge, 2014). A set of different specimens, single-source and body fluid mixtures, was prepared and sent to the participating laboratories. They were asked to analyze the samples using a 20-marker multiplex allowing for an identification of blood, saliva, semen, skin,

menstrual secretions and vaginal mucosa, and to evaluate the results applying an "$x = n/2$" scoring system which is built on replicate RNA analyses and the ratio "observed/theoretically possible" peaks for each cell type (Lindenbergh, 2013) (see also section 1.3.1.1). It could be shown that different single-source cell types can successfully be determined. Mixtures, on the other hand, were more difficult to interpret, especially those that consisted of body fluids in which markers are co-expressed, e.g., blood in menstrual secretion (van den Berge, 2014). Results demonstrate that the method still needs some improvement but clearly it is one of the most promising approaches, not least because it could be shown that markers are unexpectedly stable (Kohlmeier, 2012; Nakanishi, 2014; Setzer, 2008; Zubakov, 2008, 2009 and publication 1). A concrete guideline for the evaluation of mRNA data, as it is already routinely used for casework at the NFI, could help starting to implement mRNA profiling methods in German laboratories; at least for uncompromised human secretions/tissues. Further collaborative exercises are required to assess the usefulness of described scoring system for compromised sample material, to further train users and expand their expertise.

Despite disadvantages of RNA profiling methods, e.g. compared to DNA methylation profiling techniques described in section 1.3.2, the analysis of RNA, especially of mRNA, represents a convincing alternative, especially to conventional approaches. In particular cases it is also preferable to DNA methylation analyses. Co-extracting DNA and RNA enables both STR profiling attempts and the determination of body fluid stains of unknown origin; even of minute amounts of biological material as could be shown in publication 1 (see section 3.1). However, when investigating such low volume samples, the difficulty of associating a DNA profile with its cellular origin arises (as described in section 1.1.2). Harteveld *et al.* proposed an empirical evaluation approach to assess whether peak height data of DNA and RNA profiles can be utilized to implicate donor and cell type for a set of designed mixtures containing two cell types of different donors (Harteveld *et al.*, 2013) (see Fig. 12). The conclusion is based on different amounts of contributed body fluids. However, this method appears to be too prone for interferences so that an application in forensic routine seems unrealistic. Peak heights in the RNA EPG may vary due to technical

factors or the expression levels of investigated markers can fluctuate between individuals due to various endogenous or exogenous factors causing peak height variations. An association of donor and cell type is therefore not that reliable and cannot be recommended. Nevertheless, the idea behind the methodological approach is significant for future developments, namely combining the information about a sample´s cellular source with an individual.

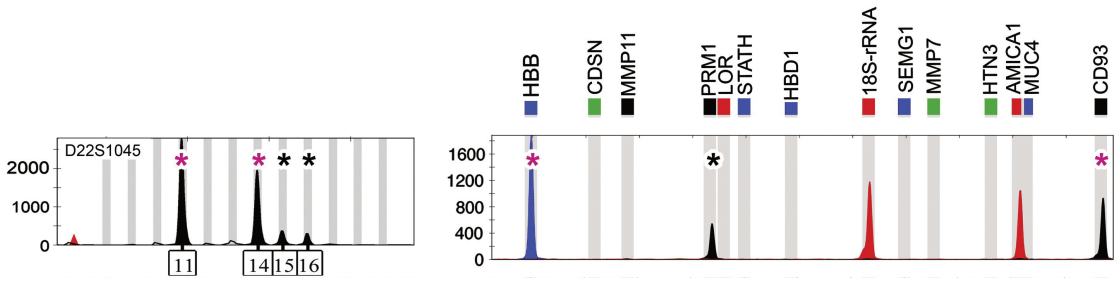


Fig. 12 DNA profile section and RNA profile of a semen-blood mixture

Exemplary image section of a DNA profile (locus D22S1045; left site) and the corresponding RNA profile (right site) of a 1:5 semen-blood mixture. The pink asterisks indicate the components of donor A, who has the genotype 11/14 in D22S1045, and black asterisks indicate components of donor B, who has the genotype 15/16. HBB and CD39 are blood-specific markers, PRM1 is a semen-specific marker. 18S-rRNA was included into the multiplex as housekeeping marker. AMICA1 indicates blood. Figure taken and adapted from Harteveld *et al.*, 2013.

In another study performed by Hanson *et al.*, the successful use of a targeted multiplex RNA NGS assay for tissue source identification was demonstrated (Hanson *et al.*, 2015). The authors could determine blood, semen, saliva, vaginal secretions, menstrual blood and skin in single source samples as well as mixed samples with a limited number of contributors. More recently, within the EUROFORGEN-NoE project, Ingold *et al.* developed a method based on the approach introduced by Hanson *et al.* (2015), enabling the association of tissue-specific mRNA transcripts to the donor of a stain by targeted massive parallel sequencing (MPS) (Ingold *et al.*, 2017, 2018). Within those RNA stretches of interest, coding region SNPs (cSNPs) could be identified which render the possibility to discriminate between different individuals. It is thus possible to get both information about the donor of the stain and the fluid type (Ingold, 2017, 2018). For routine use, however, this approach seems to be quite extensive and far from being practically implemented at the present. Besides the need to have a sufficient number of informative cSNPs at hand, suitable to distinguish

between the persons of interest in a given case, not every forensic laboratory has the expensive equipment that is required for such an investigation.

A known and general problem is the susceptibility of ribonucleic acids to degradation. In a study by Lin *et al.* it could be observed that PCR primers targeted to transcript stable regions (StaRs) are able to consistently and specifically amplify a wide range of mRNA markers in diverse body fluid types of varying degradation levels (Lin *et al.*, 2016). For saliva-specific marker HTN3 the authors stated a fourfold increased sensitivity when StaR primers were used. In order to increase the sensitivity of previously identified and tested markers, and to retain methodical approaches that have already been successfully applied in routine work, it would be reasonable to improve assay performance with "simple means", e.g., by compiling a set of StaR primers, optimizing and combining those assay amendments with suggested evaluation protocols.

Unfortunately, there are still not yet specific markers for all body fluids, such as markers that can unambiguously identify vaginal secretions or menstrual blood. These secretions represent complex blends of similar composition. That is why other methods might be interesting in order to overcome this weakness, such as profiling the human microbial community, e.g., lactobacilli in vaginal secretion (Harbison and Fleming, 2016; Sijen, 2015). However, disadvantage of the examination of microbial species is that important body fluids, such as blood or semen, are eventually not covered by available markers (due to the respective secretions nature), which is why other marker types appear more promising. It is not only important to find proper markers for each body fluid or tissue type, but it is also important that a method allows a combined analysis of all markers in one approach for being suitable for forensic casework.

The use of miRNAs as suitable markers for cell type identification purposes has been proposed by several research groups (Courts, 2010; Courts and Madea, 2011; Hanson, 2009; Sauer *et al.*, 2016; Silva, 2015; Wang *et al.*, 2013, 2015; Zubakov *et al.*, 2010) and was also evaluated in publication 3 (see section 3.3). Despite several disadvantages regarding data processing (see section 4.1.4.2), the methodical approach using qPCR is the most accurate for the detection of minute expression differences between target genes. Nevertheless, there are several points arguing against the routine

use of miRNA profiling, e.g., the increased co-expression of various miRNAs. A single miRNA may have multiple mRNA targets and any given target may have multiple miRNAs. A key limitation therefore is their specificity (Harbison, 2016).

No consensus has yet been reached in the analysis and evaluation of miRNA data; especially regarding the normalization procedure (described in section 4.1.4.2.2). Only a few candidate reference miRNAs have yet been reported hindering a standardized processing of qPCR data, and, furthermore, there are not yet suitable markers available for all kinds of fluids or tissues, such as nasal blood, sweat and urine (Sijen, 2015). This demonstrates the urgent need for inter-laboratory exercises in order to identify and compile a consistent set of target and reference genes, and to develop a meaningful data analysis strategy. In comparison, the analysis of mRNA eventually appears to be more straightforward for an implementation in forensic casework.

Besides all mentioned advantages and disadvantages of using RNA to identify human body fluids and tissues, there are further important aspects to be considered. One of the most important challenges of working with RNA, in general, is the correct storage and handling of the samples; starting with sample securing by responsible and trained police officers. Although it could be shown that RNA can survive intact longer than expected when deposited outside the human body, ones extracted the molecules are more fragile than DNA, e.g., due to their single stranded nature (Lodish *et al.*, 2001). However, as long as there is awareness of the sensitivity of the molecules and appropriate precautions are taken, such as the dry storage of samples and working on ice, no problems should arise during the processing of RNAs.

The identification of forensically relevant body fluids and tissues is an exciting and dynamic discipline in the field of forensic molecular biology. Advancement in technology takes place relatively fast, especially with regard to the use of new sequencing methodologies. There are already many promising approaches that deliver reliable results, such as mRNA profiling and the scoring system suggested by Lindenbergh *et al.* (2013). Uniform utilization of a method and adherence to guidelines for the evaluation of RNA profiling results are essential for an implementation in forensic routine. As there are already concrete proposals in this respect, drafted by the NFI, an application in forensic casework is conceivable. The information obtained can

be helpful answering complex questions during case interpretation, e.g., about the reconstruction of a crime course of events.

5 Summary

Forensic genetics addresses questions in criminal investigations that can be answered using molecular genetic-based techniques. The individualization of biological material is one of the primary objectives in order to associate trace evidence encountered at a crime scene to a suspect or to exclude a person from being a trace donor. Other important responsibilities in forensic routine are the identification of human remains, e.g., of decomposed bodies or mass disaster victims (disaster victim identification, DVI), and kinship or paternity testing (for private/judicial parentage reports or in immigration cases).

The basic molecular genetic tool for abovementioned forensic applications is the analysis of short tandem repeats (STRs), which are located in non-coding regions of the DNA and amplified by polymerase chain reaction (PCR). By now, STRs are the most widely used marker type: they underwent an almost 20 years lasting process of standardization, resulting in highly accurate typing system used to solve crimes within and between countries. In order to take further advantage of STR profiles obtained from criminal cases for investigative work, national DNA databases, like the *DNA-Analyse Datei* (DAD) maintained by the German Federal Criminal Police Office (Bundeskriminalamt, BKA), were launched in the late 1990s. Their main intention is the storage of DNA profiles of suspects and convicted offenders, and crime scene traces for comparison purposes attempting to identify donors of biological trace evidence.

With regard to the investigation of criminal offences, the identification of a certain forensically relevant body fluid or tissue type may serve as additional key evidence in legal proceedings. The information obtained can be essential reconstructing the crime

course of events, which can support a prosecution or exonerate a suspect. Caution is, nevertheless, required due to the uncertainty of association of a body fluid with a DNA profile, which has drastically increased due to the sensitivity of today´s amplification methods. They allow obtaining information from just a few cells, meaning that the detection of a DNA profile has to be interpreted with great caution, as its source can be ambiguous. For that reason, it is fundamental to determine the biological origin of the DNA evidence.

Several technologically diverse detection methods, presumptive and confirmatory in nature, have been developed over the years, such as tests based on enzymatic or immunochemical reactions. With those routinely utilized conventional methods it is currently possible to identify blood, saliva, semen and urine. However, tests hold several disadvantages regarding their application for forensically relevant questions. One major drawback is the requirement of larger quantities of original sample material, which is often not available in the forensic context. Furthermore, evidence material can be diluted by the use of certain reagents (e.g., luminol for the visualization of minute bloodstains) or degraded by extended exposure to certain wavelengths (e.g., using an alternate light source (ALS)), which both may interfere with subsequent STR profiling attempts. Other methods are not specific enough to provide evidence for an unambiguous cell type inference, such as the amylase test for saliva detection. In order to be able to provide the best possible results for forensic casework, it would therefore be highly desirable to have a (single) method available that could overcome aforementioned technically inherent limitations.

In recent years, the potential of RNA (both messenger RNA (mRNA) and microRNA (miRNA)) or DNA methylation patterns between different cell types for body fluid identification purposes has been in focus of forensic research. Comparing the different methodical approaches, which are based on molecular genetic techniques, respectively, advantages and disadvantages regarding their laboratory procedures and suitability for forensic issues can be found. For example, DNA methylation profiling demands a comparably high amount of original sample material but can, however, be useful in cases where only DNA extracts have been retained after the main investigation has already been completed. A serious drawback is that this approach is

often based on differential methylation levels, which may be influenced by various internal (e.g., a person´s age) or external (environmental) factors. RNA profiling, on the other hand, offers considerable advantages that are crucial for the processing of forensic trace material. Co-extracting DNA and RNA theoretically enable both STR profiling attempts and the determination of body fluid stains out of one sample. The capability to examine multiple, specific markers for different cell types in a single multiplex assay is highly advantageous and could replace the great number of currently applied conventional tests. A single assay approach furthermore reduces the risk of contamination compared to an approach where multiple assays need to be prepared. For those reasons, the analysis of specific RNA markers seems to be a very promising tool for body fluid and tissue identification. For an implementation into forensic casework the method, however, needs to fulfill important criteria, such as sensitivity, specificity or stability of examined markers. For a long time, RNA was assumed to be readily degraded due to ambient ribonucleases (RNases) wherefore an evaluation of their susceptibility for degradation is as crucial as the standardization of data evaluation.

In the first part of this thesis (publication 1, see section 3.1), different mRNA markers for the determination of the three forensically most relevant body fluids blood, saliva and semen were examined, assessing the degradation process of the nucleic acids, both RNA and DNA, over a period of 17 months under dry and humid storage conditions (robustness of mRNA-based body fluid analysis). A comparison was drawn between the efficiencies of different extraction methods (RNA extraction only vs. co-extraction of RNA and DNA) and, furthermore, different quantities of original sample material (5, 0.5 and 0.05 µL), which have been subjected to nucleic acid extraction, were investigated. It could be shown that mRNA, contrary to the common assumption of its proneness for degradation, is generally stable when stored under dry conditions and can still be successfully detected after more than one year of deposition outside the human body. However, mold-formation poses a major problem for the recoverability of mRNA since body fluids, especially blood, represent a good growth medium for microorganisms. Within a short time, affected samples can no longer be used for a successful mRNA analysis. In this regard, a general "issue" is the availability of water, which is required for all biochemical reactions to occur; for endogenic enzyme

activity as well as for the enzyme activity of accumulating microorganisms. Our typing results demonstrated that mRNA can, nevertheless, be recovered successfully, in sufficient quality and quantity, from samples stored under dry conditions by means of both methods. The automated extraction using the EZ1 robot provides the advantage of a minimized ribonuclease contamination risk; the column-based Allprep DNA/RNA/miRNA co-extraction method, however, seems to be more suitable, in particular to recover mRNA from humidity degraded samples since the elution volume can be adjusted manually. As expected, it was more difficult to obtain results from 0.05 µL stains compared to 5 µL stains. Blood marker HBB and semen marker PRM1, however, performed quite reliably, even for minute sample quantities. The results furthermore demonstrated that DNA can be simultaneously extracted with RNA without loss of biological material or compromising the potential to generate a DNA profile, which is an absolute prerequisite for the forensic routine use of this type of extraction method.

In the second part of this thesis, miRNA-based body fluid analysis was evaluated as alternative method to mRNA analysis. It was suggested that miRNA expression profiling would hold advantages over mRNA profiling, such as being less prone to degradation due to a smaller molecule size and the application of the highly sensitive qPCR technique. It was, however, found that a suitable data normalization strategy is inevitable, as it has a decisive impact on the evaluation of results and their interpretation may vary significantly depending on the applied references (publication 2, see section 3.2). In this regard, different reference genes were tested, showing that the use of a single standard reference gene, such as U6B, did not allow an unambiguous differentiation between the body fluid types tested. In comparison, using a previously validated set of several reference genes, a clearer distinction could be achieved. Subsequently, appropriate reference genes were included to be validated and target genes for body fluid and tissue identification should be determined under standardized conditions using a defined set of samples (publication 3, see section 3.3). Although potential markers have already been described previously, it is not yet clear if miRNA expression profiling provides trustworthy results for real routine casework. Five markers (miR10b, miR203, miR374, miR451 and miR943) could be identified for the

inference of five body fluids and skin cells. They allowed an individual pairwise distinction of one body fluid/tissue type in comparison to one or more other body fluid/tissue types. It was observed that the specificity of a given marker depends on the overall comparison of the analyzed sample set. The obtained results were, however, not as clear-cut compared to the yes/no decision obtained by mRNA profiling using endpoint PCR. Data underlie much greater technically induced fluctuations, which need to be normalized applying an appropriate strategy. Findings of publication 3 revealed a number of drawbacks regarding the processing of qPCR data, such as the selection of suitable reference genes, the inclusion of numerous control samples up to the evaluation of results. The complex experimental and data processing procedures therefore do not appear appealing for routine use.

In conclusion, a change from conventional methods to molecular genetic-based techniques would be absolutely desirable due to the technical advantages of those approaches compared to the drawbacks of conventional tests. The analysis of mRNA seems to be more promising for an implementation in forensic routine than the analysis of miRNA. Unfortunately, there is still not yet a sufficient number of specific markers for all body fluids available, such as markers that can unambiguously identify vaginal secretions or menstrual blood, which both have a strong forensic relevance. Moreover, no generally agreed level of standardization regarding the evaluation of data has yet been achieved and the examination of body fluid mixtures remains challenging, demonstrating the necessity for future inter-laboratory exercises. A further important step to make is the association of tissue-specific mRNA transcripts to the donor of a stain. The definite origin of a DNA profile needs to be assured since it could significantly change the outcome of an investigation.

6 Zusammenfassung

Die forensische Genetik befasst sich mit Fragen strafrechtlicher Ermittlungsverfahren, die mit Hilfe molekulargenetischer Methoden untersucht werden können. Die Individualisierung von biologischem Material ist eines der Hauptanliegen mit dem Ziel, die an einem Tatort vorgefundenen Spuren einem Verdächtigen zuzuordnen bzw. eine Person als Spurenleger auszuschließen. Die Identifizierung menschlicher Überreste, z.B. von fäulnisveränderten Leichen oder Opfern von Massenkatastrophen (*disaster victim identification*, DVI) sowie Verwandtschaftsanalysen oder Vaterschaftstests (für private/gerichtliche Abstammungsgutachten oder für die Beantragung von Einreisevisa bei Familienzusammenführungen) stellen weitere wichtige Aufgabenbereiche der forensischen Routine dar.

Das wichtigste molekulargenetische Werkzeug für die oben genannten forensischen Einsatzbereiche ist die Analyse von *short tandem repeats* (STRs), die sich in den nicht codierenden Bereichen der DNA befinden und durch Polymerase-Kettenreaktion (PCR) amplifiziert werden. Mittlerweile sind STR-Systeme der am weitesten verbreitete Markertyp: sie durchliefen einen fast 20 Jahre andauernden Standardisierungsprozess, der in einem hochpräzisen Typisierungssystem resultierte, welches zur Aufklärung von Straftaten innerhalb eines Landes bzw. zwischen den Ländern eingesetzt wird. Um die aus Kriminalfällen gewonnenen STR-Profile für die polizeiliche Ermittlungsarbeit nutzen zu können, wurden Ende der 90er Jahre nationale Datenbanken, wie die DNA-Analyse-Datei (DAD) des Bundeskriminalamtes (BKA), ins Leben gerufen. Ihr Hauptanliegen ist die Speicherung von DNA-Profilen verdächtiger Personen, verurteilter Straftäter sowie von Tatortspuren zu Vergleichszwecken, mit dem Ziel Spurenverursacher zu identifizieren.

Im Bezug auf die Untersuchung von Straftaten kann die Identifizierung einer bestimmten forensisch relevanten Körperflüssigkeit oder Gewebeart als zusätzlicher Schlüsselbeweis in Gerichtsverfahren dienen. Die gewonnenen Informationen können für die Rekonstruktion eines Tathergangs von wesentlicher Bedeutung sein, wodurch eine Anklage unterstützt oder eine tatverdächtige Person entlastet werden kann. Dennoch ist Vorsicht geboten, da die Unsicherheit der Assoziation einer Körperflüssigkeit mit einem DNA-Profil durch die Sensitivität heutiger Amplifikationsmethoden drastisch gestiegen ist. Sie erlauben es, Informationen aus ein paar wenigen Zellen zu erhalten, was bedeutet, dass der Nachweis eines DNA-Profils mit Vorsicht zu interpretieren ist, da seine zelluläre Herkunft ungewiss sein kann. Aus diesem Grund ist es wichtig, den biologischen Ursprung von DNA-Spuren zu bestimmen.

Im Laufe der Jahre wurden diverse technologisch unterschiedliche Nachweisverfahren entwickelt (präsumptiv und konfirmatorisch), die auf enzymatischen oder immunochemischen Reaktionen basieren. Mit diesen routinemäßig verwendeten, konventionellen Methoden ist es derzeit möglich, Blut, Speichel, Sperma und Urin zu identifizieren. Solche Tests weisen jedoch eine Reihe von Nachteilen hinsichtlich ihrer Anwendung bei forensisch relevanten Fragestellungen auf. Ein wesentlicher Nachteil besteht darin, dass größere Mengen an Probenmaterial benötigt werden, welche im forensischen Kontext oftmals nicht zur Verfügung stehen. Außerdem kann das Beweismaterial durch die Verwendung bestimmter Reagenzien verdünnt (z.B. durch Luminol zur Visualisierung latenter Blutflecken), oder durch eine längerfristige Exposition gegenüber bestimmter Wellenlängen degradiert werden (z.B. durch die Verwendung einer alternativen Lichtquelle (ALS)). Beides kann eine nachfolgende STR-Typisierung beeinträchtigen. Andere Methoden sind nicht spezifisch genug, um einen eindeutigen Zelltypnachweis zu erbringen, wie z.B. der Amylasetest für die Bestimmung von Speichel. Um bestmögliche Ergebnisse für die forensische Fallarbeit liefern zu können, wäre es daher sehr wünschenswert, eine (einzige) Methode zur Verfügung zu haben, welche die oben genannten, technisch bedingten Einschränkungen überwinden könnte.

In jüngster Zeit steht das Potenzial von RNA (sowohl von mRNA als auch von miRNA) bzw. das Potenzial von DNA-Methylierungsmustern zwischen verschiedenen Zelltypen für die Identifizierung von Körperflüssigkeiten im Mittelpunkt der forensischen Forschung. Vergleicht man die verschiedenen methodischen Ansätze, die jeweils auf molekulargenetischen Techniken basieren, so lassen sich jeweils Vor- und Nachteile hinsichtlich ihrer Abläufe im Labor sowie ihrer Eignung für forensische Fragestellungen feststellen. Beispielsweise erfordert die Untersuchung von DNA-Methylierungsmustern eine vergleichsweise hohe Menge an Probenmaterial, sie kann aber hilfreich sein, wenn nach Abschluss der Hauptuntersuchung nur noch DNA-Extrakte zur Verfügung stehen. Ein gravierender Nachteil ist, dass dieser Ansatz oft auf differenziellen Methylierungsniveaus beruht, die von verschiedenen internen (z.B. dem Alter einer Person) oder externen (Umwelt-) Faktoren beeinflusst werden können. Die Analyse von RNA bietet hingegen erhebliche Vorteile, die für die Bearbeitung von forensischem Spurenmaterial unerlässlich sind. Die simultane Extraktion von DNA und RNA ermöglicht, theoretisch, sowohl eine STR-Typisierung als auch die Bestimmung von Körperflüssigkeiten aus einer einzigen Probe heraus. Die Möglichkeit, mehrere spezifische Marker für verschiedene Zelltypen in einem einzigen Multiplex-Assay zu untersuchen, ist äußerst vorteilhaft und könnte die große Zahl der derzeit verwendeten konventionellen Tests ersetzen. Ein Ansatz, der auf einem einzigen Untersuchungsverfahren beruht, reduziert zudem das Kontaminationsrisiko im Vergleich zu einem Ansatz, bei dem verschiedene Analysen vorbereitet werden müssen. Aus diesen Gründen scheint die Analyse spezifischer RNA-Marker vielversprechend für die Identifizierung von Körperflüssigkeit und Gewebe zu sein. Für eine Implementierung in forensische Fallarbeit muss die Methode jedoch wichtige Kriterien, wie die Sensitivität, Spezifität oder Stabilität der untersuchten Marker, erfüllen. Lange Zeit wurde angenommen, dass RNA aufgrund von Ribonukleasen (RNasen) in der Umgebung leicht abgebaut werden kann, weshalb eine Bewertung ihrer Degradationsanfälligkeit ebenso entscheidend ist wie die Standardisierung der Datenbewertung.

Im ersten Teil der vorliegenden Dissertation (Publikation 1, siehe Abschnitt 3.1) wurden verschiedene mRNA-Marker für die Bestimmung der drei forensisch

relevantesten Körperflüssigkeiten Blut, Speichel und Sperma untersucht, wobei der Abbauprozess der Nukleinsäuren, sowohl der RNA als auch der DNA, über einen Zeitraum von 17 Monaten unter verschiedenen Lagerbedingungen beurteilt wurde (Robustheit der mRNA-basierten Körperflüssigkeitsanalyse). Es wurde ein Vergleich zwischen den Effizienzen verschiedener Extraktionsmethoden (RNA-Extraktion vs. Co-Extraktion von RNA und DNA) gezogen. Darüber hinaus wurden unterschiedliche Mengen an Probenmaterial (5, 0,5 und 0,05 µL), die einer Nukleinsäure-Extraktion unterzogen wurden, getestet. Es konnte gezeigt werden, dass mRNA, entgegen der allgemeinen Annahme ihrer Degradationsanfälligkeit, bei trockener Lagerung generell stabil ist und auch nach mehr als einem Jahr der Lagerung außerhalb des menschlichen Körpers noch erfolgreich nachgewiesen werden kann. Die Bildung von Schimmelpilzen stellt jedoch ein großes Problem für die Gewinnung der mRNA dar, da Körperflüssigkeiten, insbesondere Blut, ein gutes Wachstumsmedium für Mikroorganismen darstellen. Innerhalb kurzer Zeit können betroffene Proben nicht mehr für eine erfolgreiche mRNA-Analyse verwendet werden. Ein generelles "Problem" ist die Verfügbarkeit von Wasser, welches für alle biochemischen Reaktionen benötigt wird, sowohl für die endogene Enzymaktivität als auch für die Enzymaktivität akkumulierender Mikroorganismen. Unsere Typisierungsergebnisse zeigten, dass mRNA dennoch mit beiden Methoden in ausreichend hoher Qualität und Quantität aus trocken gelagerten Proben gewonnen werden kann. Die automatisierte Extraktion mit dem EZ1-Roboter bietet den Vorteil eines minimierten Ribonuklease-Kontaminationsrisikos; die säulenbasierte Allprep DNA/RNA/miRNA Co-Extraktionsmethode scheint insgesamt jedoch besser geeignet zu sein, insbesondere, um mRNA aus feuchtigkeitsdegradierten Proben zu gewinnen, da das Elutionsvolumen manuell angepasst werden kann. Wie erwartet war es schwieriger, Ergebnisse aus den 0,05 µL großen Spuren zu erhalten als aus denen von 5 µL Menge. Der Blut-Marker HBB sowie der Sperma-Marker PRM1 lieferten jedoch auch bei kleinsten Probenmengen recht zuverlässige Ergebnisse. Die Ergebnisse zeigten zudem, dass DNA und RNA gleichzeitig extrahiert werden können, ohne dass es zum Verlust biologischen Materials kommt, oder die Möglichkeit ein DNA-Profil zu erzeugen beeinträchtigt wird, was eine absolute Grundvoraussetzung für die forensische Routineanwendung dieser Art von Extraktionsmethode ist.

Im zweiten Teil der vorliegenden Arbeit wurde die miRNA-basierte Körperflüssigkeitsanalyse als alternative Methode zur mRNA-Analyse evaluiert. Dabei wurde geprüft, ob die miRNA-Expressionsanalyse Vorteile gegenüber der mRNA-Analyse bietet, wie z.B. eine geringere Degradationsanfälligkeit aufgrund der kleineren Molekülgröße sowie die Anwendung der hochempfindlichen qPCR-Technik. Es wurde jedoch festgestellt, dass eine geeignete Strategie zur Datennormalisierung unabdingbar ist, da diese einen entscheidenden Einfluss auf die Bewertung der Ergebnisse hat bzw. deren Interpretation in Abhängigkeit von den verwendeten Referenzen stark variieren kann (Publikation 2, siehe Abschnitt 3.2). In diesem Zusammenhang wurden verschiedene Referenzgene getestet, wobei sich zeigte, dass die Verwendung eines einzigen Standard-Referenzgens, wie z.B. U6B, keine eindeutige Unterscheidung zwischen den getesteten Körperflüssigkeitstypen zulässt. Im Vergleich dazu konnte mit einem zuvor validierten Set mehrerer Referenzgene eine eindeutigere Differenzierung erzielt werden. Anschließend wurden geeignete Referenzgene zur Validierung herangezogen und Zielgene für die Identifizierung von Körperflüssigkeit und Gewebe unter standardisierten Bedingungen anhand eines definierten Probensatzes bestimmt (Publikation 3, siehe Abschnitt 3.3). Obwohl potenzielle Marker bereits zuvor beschrieben wurden, konnte nicht sicher geklärt werden, ob die miRNA-Expressionsanalyse verlässliche Ergebnisse für echte Routinefälle liefert. Fünf Marker (miR10b, miR203, miR374, miR451 und miR943) konnten für den Rückschluss auf fünf Körperflüssigkeiten und Hautzellen identifiziert werden. Sie erlaubten eine individuelle paarweise Unterscheidung eines Körperflüssigkeits-/Gewebetyps im Vergleich zu einem oder mehreren anderen Körperflüssigkeits-/Gewebetypen. Es wurde beobachtet, dass die Spezifität eines bestimmten Markers vom Gesamtvergleich des analysierten Probensatzes abhängt. Die Ergebnisse waren jedoch nicht ganz so eindeutig im Vergleich zu der Ja/Nein Entscheidung, die bei der mRNA-Analyse mittels Endpunkt-PCR erzielt wird. Die Daten unterliegen wesentlich größeren, technisch bedingten Schwankungen, die mit einer geeigneten Strategie normalisiert werden müssen. Die Ergebnisse der Publikation 3 offenbarten eine Reihe von Nachteilen bei der Verarbeitung von qPCR-Daten, wie die Auswahl geeigneter Referenzgene, die Einbeziehung zahlreicher Kontrollproben bis hin zur Auswertung

der Ergebnisse. Die komplexen Versuchs- und Datenverarbeitungsverfahren erscheinen daher für den Routineeinsatz nicht überzeugend.

Zusammenfassend lässt sich sagen, dass ein Wechsel von konventionellen Methoden hin zu molekulargenetischen Techniken aufgrund der technischen Vorteile dieser Ansätze im Vergleich zu den Nachteilen herkömmlicher Tests äußerst wünschenswert wäre. Die Analyse von mRNA scheint für eine Implementierung in die forensische Routine vielversprechender zu sein, als die Analyse von miRNA. Leider gibt es noch nicht für alle Körperflüssigkeiten eine ausreichende Zahl spezifischer Marker, wie z.B. solche für die Identifizierung von Vaginalsekret oder Menstruationsblut, die beide eine starke forensische Relevanz aufweisen. Darüber hinaus wurde noch kein allgemein anerkannter Grad an Standardisierung bezüglich der Datenauswertung erreicht und die Untersuchung von Mischungen verschiedener Köperflüssigkeiten verbleibt schwierig, was die Notwendigkeit für künftige Ringversuche zeigt. Ein weiterer wichtiger Schritt ist die Assoziation gewebespezifischer mRNA-Transkripte mit einem Spurenleger. Die eindeutige Herkunft eines DNA-Profils muss sichergestellt sein, da sie das Ergebnis einer Untersuchung erheblich verändern kann.

7 References

Aksoy, E., Aktan, T. M., Duman, S., Cuce, G. (2012). Assessment of Spermatozoa Morphology under Light Microscopy with Different Histologic Stains and Comparison of Morphometric Measurements. *International Journal of Morphology*, **30** (4), 1544–1550.

Alaeddini, R., Walsh, S. J., Abbas, A. (2010). Forensic implications of genetic analyses from degraded DNA--a review. *Forensic Science International. Genetics*, **4** (3), 148–157.

Alegría-Torres, J. A., Baccarelli, A., Bollati, V. (2011). Epigenetics and lifestyle. *Epigenomics*, **3** (3), 267–277.

Ali, S., Müller, C. R., Epplen, J. T. (1986). DNA finger printing by oligonucleotide probes specific for simple repeats. *Human Genetics*, **74** (3), 239–243.

Allery, J. P., Telmon, N., Mieusset, R., Blanc, A., Rougé, D. (2001). Cytological detection of spermatozoa: comparison of three staining methods. *Journal of Forensic Sciences*, **46** (2), 349–351.

Alvarez, M., Juusola, J., Ballantyne, J. (2004). An mRNA and DNA co-isolation method for forensic casework samples. *Analytical Biochemistry*, **335** (2), 289–298.

Alvarez-Garcia, I., Miska, E. A. (2005). MicroRNA functions in animal development and human disease. *Development*, **132** (21), 4653–4662.

An, J. H., Shin, K.-J., Yang, W. I., Lee, H. Y. (2012). Body fluid identification in forensics. *BMB Reports*, **45** (10), 545–553.

Andersen, C. L., Jensen, J. L., Ørntoft, T. F. (2004). Normalization of Real-Time Quantitative Reverse Transcription-PCR Data: A Model-Based Variance Estimation Approach to Identify Genes Suited for Normalization, Applied to Bladder and Colon Cancer Data Sets. *Cancer Research*, **64** (15), 5245–5250.

Andersen, J., Bramble, S. (1997). The effects of fingermark enhancement light sources on subsequent PCR-STR DNA analysis of fresh bloodstains. *Journal of Forensic Sciences*, **42** (2), 303–306.

Anderson, S., Bankier, A.T., Barrell, B.G., de Bruijn, M.H.L., Coulson, A.R., Drouin, J., Eperon, I.C., Nierlich, D.P., Roe, B.A., Sanger, F., Schreier, P.H., Smith, A.J.H., Staden, R., Young, I.G. (1981). Sequence and organization of the human mitochondrial genome. *Nature*, **290** (5806), 457–465.

Anstee, D. J. (1990). Blood group-active surface molecules of the human red blood cell. *Vox Sanguinis*, **58** (1), 1–20.

Arber, W., Linn, S. (1969). DNA modification and restriction. *Annual Review of Biochemistry*, **38**, 467–500.

Arya, M., Shergill, I. S., Williamson, M., Gommersall, L., Arya, N., Patel, H. R. (2005). Basic principles of real-time quantitative PCR. *Expert Review of Molecular Diagnostics*, **5** (2), 209–219.

Bader, P., Niethammer, D., Willasch, A., Kreyenberg, H., Klingebiel, T. (2004). How and when should we monitor chimerism after allogeneic stem cell transplantation? *Bone Marrow Transplantation*, **35** (2), 107–119.

Balk, S. P., Ko, Y.-J., Bubley, G. J. (2003). Biology of Prostate-Specific Antigen. *Journal of Clinical Oncology*, **21** (2), 383–391.

Ballantyne, K.N., Ralf, A., Aboukhalid, R., Achakzai, N.M., Anjos, M.J., Ayub, Q., Balažic, J., Ballantyne, J., Ballard, D.J., Berger, B., Bobillo, C., Bouabdellah, M., Burri, H., Capal, T., Caratti, S., Cárdenas, J., Cartault, F., Carvalho, E.F., Carvalho, M., Cheng, B., Coble, M.D., Comas, D., Corach, D., D'Amato, M.E., Davison, S., de Knijff, P., De Ungria, M.C.A., Decorte, R., Dobosz, T., Dupuy, B.M., Elmrghni, S., Gliwiński, M., Gomes, S.C., Grol, L., Haas, C., Hanson, E., Henke, J., Henke, L., Herrera-Rodríguez, F., Hill, C.R., Holmlund, G., Honda, K., Immel, U.-D., Inokuchi, S., Jobling, M.A., Kaddura, M., Kim, J.S., Kim, S.H., Kim, W., King, T.E., Klausriegler, E., Kling, D., Kovačević, L., Kovatsi, L., Krajewski, P., Kravchenko, S., Larmuseau, M.H.D., Lee, E.Y., Lessig, R., Livshits, L.A., Marjanović, D., Minarik, M., Mizuno, N., Moreira, H., Morling, N., Mukherjee, M., Munier, P., Nagaraju, J., Neuhuber, F., Nie, S., Nilasitsataporn, P., Nishi, T., Oh, H.H., Olofsson, J., Onofri, V., Palo, J.U., Pamjav, H., Parson, W., Petlach, M., Phillips, C., Ploski, R., Prasad, S.P.R., Primorac, D., Purnomo, G.A., Purps, J., Rangel-Villalobos, H., Rębała, K., Rerkamnuaychoke, B., Gonzalez, D.R., Robino, C., Roewer, L., Rosa, A., Sajantila, A., Sala, A., Salvador, J.M., Sanz, P., Schmitt, C., Sharma, A.K., Silva, D.A., Shin, K.-J., Sijen, T., Sirker, M.,

Siváková, D., Škaro, V., Solano-Matamoros, C., Souto, L., Stenzl, V., Sudoyo, H., Syndercombe-Court, D., Tagliabracci, A., Taylor, D., Tillmar, A., Tsybovsky, I.S., Tyler-Smith, C., van der Gaag, K.J., Vanek, D., Völgyi, A., Ward, D., Willemse, P., Yap, E.P., Yong, R.Y., Pajnič, I.Z., Kayser, M. (2014). Toward Male Individualization with Rapidly Mutating Y-Chromosomal Short Tandem Repeats. *Human Mutation*, **35** (8), 1021–1032.

Balogh, M. K., Burger, J., Bender, K., Schneider, P.M., Alt, K. W. (2003). STR genotyping and mtDNA sequencing of latent fingerprint on paper. *Forensic Science International*, **137** (2–3), 188–195.

Bär, W., Brinkmann, B., Budowle, B., Carracedo, A., Gill, P., Lincoln, P., Mayr, W., Olaisen, B. (1997). DNA recommendations. Further report of the DNA Commission of the ISFH regarding the use of short tandem repeat systems. International Society for Forensic Haemogenetics. *International Journal of Legal Medicine*, **110** (4), 175–176.

Barni, F., Lewis, S. W., Berti, A., Miskelly, G. M., Lago, G. (2007). Forensic application of the luminol reaction as a presumptive test for latent blood detection. *Talanta*, **72** (3), 896–913.

Bartel, D. P. (2004). MicroRNAs: Genomics, Biogenesis, Mechanism, and Function. *Cell*, **116** (2), 281–297.

Bauer, M. (2007). RNA in forensic science. *Forensic Science International: Genetics*, **1** (1), 69–74.

Bauer, M., Patzelt, D. (2003). Protamine mRNA as molecular marker for spermatozoa in semen stains. *International Journal of Legal Medicine*, **117** (3), 175–179.

Berger, S. L., Kouzarides, T., Shiekhattar, R., Shilatifard, A. (2009). An operational definition of epigenetics. *Genes & Development*, **23** (7), 781–783.

Boward, E. S., Wilson, S. L. (2013). A comparison of ABAcard(®) p30 and RSID™-Semen test kits for forensic semen identification. *Journal of Forensic and Legal Medicine*, **20** (8), 1126–1130.

Braun, K. A., Young, E. T. (2014). Coupling mRNA Synthesis and Decay. *Molecular and Cellular Biology*, **34** (22), 4078–4087.

Budowle, B., Allard, M. W., Wilson, M. R., Chakraborty, R. (2003). Forensics and mitochondrial DNA: applications, debates, and foundations. *Annual Review of Genomics and Human Genetics*, **4**, 119–141.

Budowle, B., Bieber, F. R., Eisenberg, A. J. (2005). Forensic aspects of mass disasters: strategic considerations for DNA-based human identification. *Legal Medicine (Tokyo, Japan)*, **7** (4), 230–243.

Bull, H., Murray, P. G., Thomas, D., Fraser, A. M., Nelson, P. N. (2002). Acid phosphatases. *Molecular Pathology*, **55** (2), 65–72.

Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L., Vandesompele, J., Wittwer, C.T. (2009). The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments. *Clinical Chemistry*, **55** (4), 611–622.

Butler, J. (2006). Genetics and genomics of core short tandem repeat loci used in human identity testing. *Journal of Forensic Sciences*, **51** (2), 253–265.

Butler, J. (2009). *Fundamentals of Forensic DNA Typing*. Elsevier Academic Press.

Butler, J. (2011). *Advanced Topics in Forensic DNA Typing: Methodology*. Elsevier Academic Press.

Butler, J., Levin, B. C. (1998). Forensic applications of mitochondrial DNA. *Trends in Biotechnology*, **16** (4), 158–162.

Carracedo, A., Lareu, M. V. (1998). Development of New STRs for Forensic Casework: Criteria for Selection, Sequencing & Population Data and Forensic Validation. *Proceeding of the Ninth International Symposium on Human Identification*, 89–107.

Chen, Y., Gelfond, J. A. L., McManus, L. M., Shireman, P. K. (2009). Reproducibility of quantitative RT-PCR array in miRNA expression profiling and comparison with microarray analysis. *BMC Genomics*, **10**, 407.

Coble, M.D., Loreille, O.M., Wadhams, M.J., Edson, S.M., Maynard, K., Meyer, C.E., Niederstätter, H., Berger, C., Berger, B., Falsetti, A.B., Gill, P., Parson, W., Finelli, L.N. (2009). Mystery solved: the identification of the two missing Romanov children using DNA analysis. *PloS One*, **4** (3), E4838.

Consortium, T. 1000 G. P. (2015). A global reference for human genetic variation. *Nature*, **526** (7571), 68.

Courts, C., Madea, B. (2010). Micro-RNA – A potential for forensic science? *Forensic Science International*, **203** (1–3), 106–111.

Courts, C., Madea, B. (2011). Specific Micro-RNA Signatures for the Detection of Saliva and Blood in Forensic Body-fluid Identification: Micro-RNAs For Forensic Body-fluid Identification. *Journal of Forensic Sciences*, **56** (6), 1464–1470.

Crick, F. (1958). On protein synthesis. *Symposia of the Society for Experimental Biology*, **12**, 138–163.

Crick, F. (1970). Central dogma of molecular biology. *Nature*, **227** (5258), 561–563.

Daniel, F. I., Cherubini, K., Yurgel, L. S., de Figueiredo, M. A. Z., Salum, F. G. (2011). The role of epigenetic transcription repression and DNA methyltransferases in cancer. *Cancer*, **117** (4), 677–687.

Dausset, J. (1958). Iso-leuko-antibodies. *Acta Haematologica*, **20** (1–4), 156–166.

Della Manna, A., Montpetit, S. (2000). A novel approach to obtaining reliable PCR results from luminol treated bloodstains. *Journal of Forensic Sciences*, **45** (4), 886–890.

D'haene, B., Mestdagh, P., Hellemans, J., Vandesompele, J. (2012) miRNA Expression Profiling: From Reference Genes to Global Mean Normalization, in: Fan, J.-B. (ed.) *Next-Generation MicroRNA Expression Profiling Technology*. Humana Press,822, pp. 261–272.

Dheda, K., Huggett, J.F., Chang, J.S., Kim, L.U., Bustin, S.A., Johnson, M.A., Rook, G.A.W., Zumla, A. (2005). The implications of using an inappropriate reference gene for real-time reverse transcription PCR data normalization. *Analytical Biochemistry*, **344** (1), 141–143.

Dissing, J., Søndervang, A., Lund, S. (2010). Exploring the limits for the survival of DNA in blood stains. *Journal of Forensic and Legal Medicine*, **17** (7), 392–396.

Dungern, E., Hirschfeld, L. (1910). Ueber Vererbung gruppenspezifischer Strukturen des Blutes. *Z Immunforsch*, **6**, 284–292.

Dussoix, D., Arber, W. (1965). Host specificity of DNA produced by Escherichia coli. IV. Host specificity of infectious DNA from bacteriophage lambda. *Journal of Molecular Biology*, **11**, 238–246.

Eduardoff, M., Gross, T.E., Santos, C., Puente, M. de la, Ballard, D., Strobl, C., Børsting, C., Morling, N., Fusco, L., Hussing, C., Egyed, B., Souto, L., Uacyisrael, J., Court, D.S., Carracedo, Á., Lareu, M.V., Schneider, P.M., Parson, W., Phillips, C., Parson, W., Phillips, C. (2016). Inter-laboratory evaluation of the EUROFORGEN Global

ancestry-informative SNP panel by massively parallel sequencing using the Ion PGM™. *Forensic Science International: Genetics*, **23**, 178–189.

Edwards, A., Civitello, A., Hammond, H. A., Caskey, C. T. (1991). DNA typing and genetic mapping with trimeric and tetrameric tandem repeats. *American Journal of Human Genetics*, **49** (4), 746–756.

Ellegren, H. (2004). Microsatellites: simple sequences with complex evolution. *Nature Reviews. Genetics*, **5** (6), 435–445.

Esquela-Kerscher, A., Slack, F. J. (2006). Oncomirs — microRNAs with a role in cancer. *Nature Reviews Cancer*, **6** (4), 259.

Eulalio, A., Behm-Ansmant, I., Izaurralde, E. (2007). P bodies: at the crossroads of post-transcriptional pathways. *Nature Reviews. Molecular Cell Biology*, **8** (1), 9–22.

Faller, A., Schünke, M. (2008). *Der Körper des Menschen: Einführung in Bau und Funktion*. 15., komplett überarbeitete Auflage. Stuttgart; New York: Thieme.

Fleming, R. I., Harbison, S. (2010). The development of a mRNA multiplex RT-PCR assay for the definitive identification of body fluids. *Forensic Science International. Genetics*, **4** (4), 244–256.

Fonneløp, A. E., Johannessen, H., Egeland, T., Gill, P. (2016). Contamination during criminal investigation: Detecting police contamination and secondary DNA transfer from evidence bags. *Forensic Science International. Genetics*, **23**, 121–129.

Forat, S., Huettel, B., Reinhardt, R., Fimmers, R., Haidl, G., Denschlag, D., Olek, K. (2016). Methylation Markers for the Identification of Body Fluids and Tissues from Forensic Trace Evidence. *PLoS ONE*, **11** (2).

Fordyce, S.L., Ávila-Arcos, M.C., Rasmussen, M., Cappellini, E., Romero-Navarro, J.A., Wales, N., Alquezar-Planas, D.E., Penfield, S., Brown, T.A., Vielle-Calzada, J.-P., Montiel, R., Jørgensen, T., Odegaard, N., Jacobs, M., Arriaza, B., Higham, T.F.G., Ramsey, C.B., Willerslev, E., Gilbert, M.T.P. (2013). Deep sequencing of RNA from ancient maize kernels. *PloS One*, **8** (1), E50961.

Fordyce, S. L., Kampmann, M.-L., Doorn, N. L. van, Gilbert, M. T. P. (2013). Long-term RNA persistence in postmortem contexts. *Investigative Genetics*, **4** (1), 7.

Förster, T. (1948). Zwischenmolekulare Energiewanderung und Fluoreszenz. *Annalen Der Physik*, **437** (1–2), 55–75.

Frazer, K.A., Ballinger, D.G., Cox, D.R., Hinds, D.A., Stuve, L.L., Gibbs, R.A., Belmont, J.W., Boudreau, A., Hardenbol, P., Leal, S.M., Pasternak, S., Wheeler, D.A., Willis, T.D., Yu, F., Yang, H., Zeng, C., Gao, Y., Hu, H., Hu, W., Li, C., Lin, W., Liu, S., Pan, H., Tang, X., Wang, J., Wang, W., Yu, J., Zhang, B., Zhang, Q., Zhao, Hongbin, Zhao, Hui, Zhou, J., Gabriel, S.B., Barry, R., Blumenstiel, B., Camargo, A., Defelice, M., Faggart, M., Goyette, M., Gupta, S., Moore, J., Nguyen, H., Onofrio, R.C., Parkin, M., Roy, J., Stahl, E., Winchester, E., Ziaugra, L., Altshuler, D., Shen, Yan, Yao, Z., Huang, W., Chu, X., He, Y., Jin, L., Liu, Y., Shen, Yayun, Sun, W., Wang, Haifeng, Wang, Yi, Wang, Ying, Xiong, X., Xu, L., Waye, M.M.Y., Tsui, S.K.W., Xue, H., Wong, J.T.-F., Galver, L.M., Fan, J.-B., Gunderson, K., Murray, S.S., Oliphant, A.R., Chee, M.S., Montpetit, A., Chagnon, F., Ferretti, V., Leboeuf, M., Olivier, J.-F., Phillips, M.S., Roumy, S., Sallée, C., Verner, A., Hudson, T.J., Kwok, P.-Y., Cai, D., Koboldt, D.C., Miller, R.D., Pawlikowska, L., Taillon-Miller, P., Xiao, M., Tsui, L.-C., Mak, W., Song, Y.Q., Tam, P.K.H., Nakamura, Y., Kawaguchi, T., Kitamoto, T., Morizono, T., Nagashima, A., Ohnishi, Y., Sekine, A., Tanaka, T., Tsunoda, T., Deloukas, P., Bird, C.P., Delgado, M., Dermitzakis, E.T., Gwilliam, R., Hunt, S., Morrison, J., Powell, D., Stranger, B.E., Whittaker, P., Bentley, D.R., Daly, M.J., Bakker, P.I.W. de, Barrett, J., Chretien, Y.R., Maller, J., McCarroll, S., Patterson, N., Pe'er, I., Price, A., Purcell, S., Richter, D.J., Sabeti, P., Saxena, R., Schaffner, S.F., Sham, P.C., Varilly, P., Stein, L.D., Krishnan, L., Smith, A.V., Tello-Ruiz, M.K., Thorisson, G.A., Chakravarti, A., Chen, P.E., Cutler, D.J., Kashuk, C.S., Lin, S., Abecasis, G.R., Guan, W., Li, Y., Munro, H.M., Qin, Z.S., Thomas, D.J., McVean, G., Auton, A., Bottolo, L., Cardin, N., Eyheramendy, S., Freeman, C., Marchini, J., Myers, S., Spencer, C., Stephens, M., Donnelly, P., Cardon, L.R., Clarke, G., Evans, D.M., Morris, A.P., Weir, B.S., Johnson, T., Mullikin, J.C., Sherry, S.T., Feolo, M., Skol, A., Zhang, H., Matsuda, I., Fukushima, Y., Macer, D.R., Suda, E., Rotimi, C.N., Adebamowo, C.A., Ajayi, I., Aniagwu, T., Marshall, P.A., Nkwodimmah, C., Royal, C.D.M., Leppert, M.F., Dixon, M., Peiffer, A., Qiu, R., Kent, A., Kato, K., Niikawa, N., Adewole, I.F., Knoppers, B.M., Foster, M.W., Clayton, E.W., Watkin, J., Muzny, D., Nazareth, L., Sodergren, E., Weinstock, G.M., Yakub, I., Birren, B.W., Wilson, R.K., Fulton, L.L., Rogers, J., Burton, J., Carter, N.P., Clee, C.M., Griffiths, M., Jones, M.C., McLay, K., Plumb, R.W., Ross, M.T., Sims, S.K., Willey, D.L., Chen, Z., Han, H., Kang, L., Godbout, M., Wallenburg, J.C., L'Archevêque, P., Bellemare, G., Saeki, K., Wang, Hongguang, An, D., Fu, H., Li, Q., Wang, Z., Wang, R., Holden, A.L., Brooks, L.D., McEwen, J.E., Guyer, M.S., Wang, V.O., Peterson, J.L., Shi, M., Spiegel, J., Sung, L.M., Zacharia, L.F., Collins, F.S., Kennedy, K., Jamieson, R., Stewart, J. (2007). A second generation human haplotype map of over 3.1 million SNPs. *Nature*, **449** (7164), 851–861.

Frumkin, D., Wasserstrom, A., Budowle, B., Davidson, A. (2011). DNA methylation-based forensic tissue identification. *Forensic Science International: Genetics*, **5** (5), 517–524.

Geserick, G., Wirth, I. (2012). Genetic Kinship Investigation from Blood Groups to DNA Markers. *Transfusion Medicine and Hemotherapy*, **39** (3), 163–175.

Gill, P. (2014). *Misleading DNA Evidence: Reasons for Miscarriages of Justice*. Elsevier Academic Press.

Gill, P., Brinkmann, B., d'Aloja, E., Andersen, J., Bar, W., Carracedo, A., Dupuy, B., Eriksen, B., Jangblad, M., Johnsson, V., Kloosterman, A.D., Lincoln, P., Morling, N., Rand, S., Sabatier, M., Scheithauer, R., Schneider, P.M., Vide, M.C. (1997). Considerations from the European DNA profiling group (EDNAP) concerning STR nomenclature. *Forensic Science International*, **87** (3), 185–192.

Gill, P., Fereday, L., Morling, N., Schneider, P.M. (2006). The evolution of DNA databases--recommendations for new European STR loci. *Forensic Science International*, **156** (2–3), 242–244.

Gill, P., Haned, H., Bleka, O., Hansson, O., Dørum, G., Egeland, T. (2015). Genotyping and interpretation of STR-DNA: Low-template, mixtures and database matches-Twenty years of research and development. *Forensic Science International. Genetics*, **18**, 100–117.

Gill, P., Ivanov, P.L., Kimpton, C., Piercy, R., Benson, N., Tully, G., Evett, I., Hagelberg, E., Sullivan, K. (1994). Identification of the remains of the Romanov family by DNA analysis. *Nature Genetics*, **6** (2), 130–135.

Gill, P., Jeffreys, A. J., Werrett, D. J. (1985). Forensic application of DNA 'fingerprints'. *Nature*, **318** (6046), 577–579.

Gill, P., Whitaker, J., Flaxman, C., Brown, N., Buckleton, J. (2000). An investigation of the rigor of interpretation rules for STRs derived from less than 100 pg of DNA. *Forensic Science International*, **112** (1), 17–40.

Gonzalo, S. (2010). Epigenetic alterations in aging. *Journal of Applied Physiology*, **109** (2), 586–597.

Grabmüller, M., Madea, B., Courts, C. (2015). Comparative evaluation of different extraction and quantification methods for forensic RNA analysis. *Forensic Science International: Genetics*, **16**, 195–202.

Grassberger, M., Schmid, H. (2013). *Todesermittlung: Befundaufnahme und Spurensicherung*. 2 Bearb. u. erg. Stuttgart: Wissenschaftliche Verlagsgesellschaft.

Gregory, R. I., Shiekhattar, R. (2005). MicroRNA Biogenesis and Cancer. *Cancer Research*, **65** (9), 3509–3512.

Gross, A. M., Harris, K. A., Kaldun, G. L. (1999). The effect of luminol on presumptive tests and DNA analysis using the polymerase chain reaction. *Journal of Forensic Sciences*, **44** (4), 837–840.

Haas, C., Hanson, E., Anjos, M.J., Ballantyne, K.N., Banemann, R., Bhoelai, B., Borges, E., Carvalho, M., Courts, C., De Cock, G., Drobnic, K., Dötsch, M., Fleming, R., Franchi, C., Gomes, I., Hadzic, G., Harbison, S.A., Harteveld, J., Hjort, B., Hollard, C., Hoff-Olsen, P., Hüls, C., Keyser, C., Maroñas, O., McCallum, N., Moore, D., Morling, N., Niederstätter, H., Noël, F., Parson, W., Phillips, C., Popielarz, C., Roeder, A.D., Salvaderi, L., Sauer, E., Schneider, P.M., Shanthan, G., Court, D.S., Turanská, M., van Oorschot, R. a. H., Vennemann, M., Vidaki, A., Zatkalíková, L., Ballantyne, J. (2014). RNA/DNA co-analysis from human menstrual blood and vaginal secretion stains: results of a fourth and fifth collaborative EDNAP exercise. *Forensic Science International. Genetics*, **8** (1), 203–212.

Haas, C., Hanson, E., Anjos, M.J., Banemann, R., Berti, A., Borges, E., Carracedo, A., Carvalho, M., Courts, C., De Cock, G., Dötsch, M., Flynn, S., Gomes, I., Hollard, C., Hjort, B., Hoff-Olsen, P., Hríbiková, K., Lindenbergh, A., Ludes, B., Maroñas, O., McCallum, N., Moore, D., Morling, N., Niederstätter, H., Noel, F., Parson, W., Popielarz, C., Rapone, C., Roeder, A.D., Ruiz, Y., Sauer, E., Schneider, P.M., Sijen, T., Court, D.S., Sviežená, B., Turanská, M., Vidaki, A., Zatkalíková, L., Ballantyne, J. (2013). RNA/DNA co-analysis from human saliva and semen stains--results of a third collaborative EDNAP exercise. *Forensic Science International. Genetics*, **7** (2), 230–239.

Haas, C., Hanson, E., Anjos, M.J., Bär, W., Banemann, R., Berti, A., Borges, E., Bouakaze, C., Carracedo, A., Carvalho, M., Castella, V., Choma, A., De Cock, G., Dötsch, M., Hoff-Olsen, P., Johansen, P., Kohlmeier, F., Lindenbergh, P.A., Ludes, B., Maroñas, O., Moore, D., Morerod, M.-L., Morling, N., Niederstätter, H., Noel, F., Parson, W., Patel, G., Popielarz, C., Salata, E., Schneider, P.M., Sijen, T., Sviežena, B., Turanská, M., Zatkalíková, L., Ballantyne, J. (2012). RNA/DNA co-analysis from blood stains--results of a second collaborative EDNAP exercise. *Forensic Science International. Genetics*, **6** (1), 70–80.

Haas, C., Hanson, E., Bär, W., Banemann, R., Bento, A.M., Berti, A., Borges, E., Bouakaze, C., Carracedo, A., Carvalho, M., Choma, A., Dötsch, M., Duriancíková, M., Hoff-Olsen, P., Hohoff, C., Johansen, P., Lindenbergh, P.A., Loddenkötter, B., Ludes, B., Maroñas, O., Morling, N., Niederstätter, H., Parson, W., Patel, G., Popielarz, C., Salata, E., Schneider, P.M., Sijen, T., Sviezená, B., Zatkalíková, L., Ballantyne, J. (2011). mRNA profiling for the identification of blood--results of a collaborative EDNAP exercise. *Forensic Science International. Genetics*, **5** (1), 21–26.

Haas, C., Klesser, B., Maake, C., Bär, W., Kratzer, A. (2009). mRNA profiling for body fluid identification by reverse transcription endpoint PCR and realtime PCR. *Forensic Science International. Genetics*, **3** (2), 80–88.

Hanson, E. K., Ballantyne, J. (2013). Circulating microRNA for the identification of forensically relevant body fluids. *Methods in Molecular Biology (Clifton, N.J.)*, **1024**, 221–234.

Hanson, E. K., Ingold, S., Haas, C., Ballantyne, J. (2015). Targeted multiplexed next generation RNA sequencing assay for tissue source determination of forensic samples. *Forensic Science International: Genetics Supplement Series*, **5**, E441–e443.

Hanson, E. K., Lubenow, H., Ballantyne, J. (2009). Identification of forensically relevant body fluids using a panel of differentially expressed microRNAs. *Analytical Biochemistry*, **387** (2), 303–314.

Harbison, S. A., Fleming, R. I. (2016, February 10). Forensic body fluid identification: state of the art. *Research and Reports in Forensic Medical Science*.

Harteveld, J., Lindenbergh, A., Sijen, T. (2013). RNA cell typing and DNA profiling of mixed samples: Can cell types and donors be associated? *Science & Justice*, **53** (3), 261–269.

Hedman, J., Gustavsson, K., Ansell, R. (2008). Using the new Phadebas® Forensic Press test to find crime scene saliva stains suitable for DNA analysis. *Forensic Science International: Genetics Supplement Series*, **1** (1), 430–432.

Heid, C. A., Stevens, J., Livak, K. J., Williams, P. M. (1996). Real time quantitative PCR. *Genome Research*, **6** (10), 986–994.

Hellemans, J., Mortier, G., De Paepe, A., Speleman, F., Vandesompele, J. (2007). qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. *Genome Biology*, **8** (2), R19–R19.

Higuchi, R., Dollinger, G., Walsh, P. S., Griffith, R. (1992). Simultaneous amplification and detection of specific DNA sequences. *Bio/Technology (Nature Publishing Company)*, **10** (4), 413–417.

Higuchi, R., Fockler, C., Dollinger, G., Watson, R. (1993). Kinetic PCR analysis: real-time monitoring of DNA amplification reactions. *Bio/Technology (Nature Publishing Company)*, **11** (9), 1026–1030.

Hochmeister, M.N., Budowle, B., Rudin, O., Gehrig, C., Borer, U., Thali, M., Dirnhofer, R. (1999). Evaluation of prostate-specific antigen (PSA) membrane test assays for the forensic identification of seminal fluid. *Journal of Forensic Sciences*, **44** (5), 1057–1060.

Hofreiter, M., Serre, D., Poinar, H. N., Kuch, M., Pääbo, S. (2001). Ancient DNA. *Nature Reviews. Genetics*, **2** (5), 353–359.

Holland, P. M., Abramson, R. D., Watson, R., Gelfand, D. H. (1991). Detection of specific polymerase chain reaction product by utilizing the 5'----3' exonuclease activity of Thermus aquaticus DNA polymerase. *Proceedings of the National Academy of Sciences of the United States of America*, **88** (16), 7276–7280.

Holtkötter, H., Beyer, V., Schwender, K., Glaub, A., Johann, K.S., Schürenkamp, M., Sibbing, U., Banken, S., Wiegand, P., Pfeiffer, H., Dennany, L., Vennemann, M., EUROFORGEN-NoE Consortium, Vennemann, M. (2017). Independent validation of body fluid-specific CpG markers and construction of a robust multiplex assay. *Forensic Science International. Genetics*, **29**, 261–268.

Hopkinson, D. A., Spencer, N., Harris, H. (1963). Red cell acid phosphatase variants: a new human polymorphism. *Nature*, **199**, 969–971.

Houseley, J., LaCava, J., Tollervey, D. (2006). RNA-quality control by the exosome. *Nature Reviews Molecular Cell Biology*, **7** (7), 529–539.

Huggett, J., Dheda, K., Bustin, S., Zumla, A. (2005). Real-time RT-PCR normalisation; strategies and considerations. *Genes and Immunity*, **6** (4), 279–284.

Hutchison, C. A., Newbold, J. E., Potter, S. S., Edgell, M. H. (1974). Maternal inheritance of mammalian mitochondrial DNA. *Nature*, **251** (5475), 536–538.

Ingold, S., Dørum, G., Hanson, E., Berti, A., Branicki, W., Brito, P., Elsmore, P., Gettings, K.B., Giangasparo, F., Gross, T.E., Hansen, S., Hanssen, E.N., Kampmann, M.-L., Kayser, M., Laurent, F.-X., Morling, N., Mosquera-Miguel, A., Parson, W., Phillips, C., Porto, M.J., Pośpiech, E., Roeder, A.D., Schneider, P.M., Schulze Johann, K., Steffen, C.R., Syndercombe-Court, D., Trautmann, M., van den Berge, M., van der Gaag, K.J., Vannier, J., Verdoliva, V., Vidaki, A., Xavier, C., Ballantyne, J., Haas, C. (2018). Body fluid identification using a targeted mRNA massively parallel sequencing approach - results of a EUROFORGEN/EDNAP collaborative exercise. *Forensic Science International. Genetics*, **34**, 105–115.

Ingold, S., Haas, C., Dørum, G., Hanson, E., Ballantyne, J. (2017). Association of a body fluid with a DNA profile by targeted RNA/DNA deep sequencing. *Forensic Science International: Genetics Supplement Series*, **6**, E112–e113.

Jeffreys, A. J., Wilson, V., Thein, S. L. (1985a). Hypervariable 'minisatellite' regions in human DNA. *Nature*, **314** (6006), 67–73.

Jeffreys, A. J., Wilson, V., Thein, S. L. (1985b). Individual-specific 'fingerprints' of human DNA. *Nature*, **316** (6023), 76–79.

Jobling, M. A., Gill, P. (2004). Encoded evidence: DNA in forensic analysis. *Nature Reviews Genetics*, **5** (10), 739–751.

Johnson, A. A., Akman, K., Calimport, S. R. G., Wuttke, D., Stolzing, A., de Magalhães, J. P. (2012). The Role of DNA Methylation in Aging, Rejuvenation, and Age-Related Disease. *Rejuvenation Research*, **15** (5), 483–494.

Jung, S.-E., Cho, S., Antunes, J., Gomes, I., Uchimoto, M.L., Oh, Y.N., Di Giacomo, L., Schneider, P.M., Park, M.S., van der Meer, D., Williams, G., McCord, B., Ahn, H.-J., Choi, D.H., Lee, Y.H., Lee, S.D., Lee, H.Y. (2016). A collaborative exercise on DNA methylation based body fluid typing. *Electrophoresis*, **37** (21), 2759–2766.

Juusola, J., Ballantyne, J. (2005). Multiplex mRNA profiling for the identification of body fluids. *Forensic Science International*, **152** (1), 1–12.

Kaczmarek, M. J., Rosenmund, H. (1977). The action of human pancreatic and salivary isoamylases on starch and glycogen. *Clinica Chimica Acta; International Journal of Clinical Chemistry*, **79** (1), 69–73.

Kan, Y. W., Dozy, A. M. (1978). Polymorphism of DNA sequence adjacent to human beta-globin structural gene: relationship to sickle mutation. *Proceedings of the National Academy of Sciences of the United States of America*, **75** (11), 5631–5635.

Kaye, S. (1949). Acid phosphatase test for identification of seminal stains. *The Journal of Laboratory and Clinical Medicine*, **34** (5), 728–732.

Kearsey, J., Louie, H., Poon, H. (2001). Validation Study of the "Onestep ABAcard® PSA Test" Kit for RCMP Casework. *Canadian Society of Forensic Science Journal*, **34** (2), 63–72.

Khan, F., Agarwal, A., Agrawal, S. (2004). Significance of chimerism in hematopoietic stem cell transplantation: new variations on an old theme. *Bone Marrow Transplantation*, **34** (1), 1–12.

Kimpton, C. P., Gill, P., Walton, A., Urquhart, A., Millican, E. S., Adams, M. (1993). Automated DNA profiling employing multiplex amplification of short tandem repeat loci. *Genome Research*, **3** (1), 13–22.

Kirk, P. (1953). *Crime investigation: physical evidence and the police laboratory*. Interscience Publishers, Inc.: New York.

Kohlmeier, F., Schneider, P.M. (2012). Successful mRNA profiling of 23 years old blood stains. *Forensic Science International: Genetics*, **6** (2), 274–276.

Landsteiner, K. (1901). Ueber Agglutinationserscheinungen normalen menschlichen Blutes. *Wiener Klinische Wochenschrift*, **14**, 1132–1134.

Le Carré, J., Lamon, S., Léger, B. (2014). Validation of a multiplex reverse transcription and pre-amplification method using TaqMan® MicroRNA assays. *Frontiers in Genetics*, **5**.

Lee, H.Y., An, J.H., Jung, S.-E., Oh, Y.N., Lee, E.Y., Choi, A., Yang, W.I., Shin, K.-J. (2015). Genome-wide methylation profiling and a multiplex construction for the identification of body fluids using epigenetic markers. *Forensic Science International. Genetics*, **17**, 17–24.

Lee, H. Y., Jung, S.-E., Lee, E. H., Yang, W. I., Shin, K.-J. (2016). DNA methylation profiling for a confirmatory test for blood, saliva, semen, vaginal fluid and menstrual blood. *Forensic Science International: Genetics*, **24**, 75–82.

Lee, H. Y., Park, M. J., Choi, A., An, J. H., Yang, W. I., Shin, K.-J. (2012). Potential forensic application of DNA methylation profiling to body fluid identification. *International Journal of Legal Medicine*, **126** (1), 55–62.

Lee, K. W. K., Pausova, Z. (2013). Cigarette smoking and DNA methylation. *Frontiers in Genetics*, **4**.

Lessig, R., Edelmann, J., Aspinall, L., Krumm, P., Bastisch, I., Wiegand, P., Hohoff, C., Steinlechner, M., Roewer, L. (2011). German standards for forensic molecular genetics investigations in cases of mass disaster victim identification (DVI). *Forensic Science International: Genetics*, **5** (3), 247–248.

Lin, M.-H., Albani, P. P., Fleming, R. (2016). Degraded RNA transcript stable regions (StaRs) as targets for enhanced forensic RNA body fluid identification. *Forensic Science International. Genetics*, **20**, 61–70.

Lindahl, T. (1993). Instability and decay of the primary structure of DNA. *Nature*, **362** (6422), 709–715.

Lindenbergh, A., Berge, M. van den, Oostra, R.-J., Cleypool, C., Bruggink, A., Kloosterman, A., Sijen, T. (2013). Development of a mRNA profiling multiplex for the inference of organ tissues. *International Journal of Legal Medicine*, **127** (5), 891–900.

Lindenbergh, A., de Pagter, M., Ramdayal, G., Visser, M., Zubakov, D., Kayser, M., Sijen, T. (2012). A multiplex (m)RNA-profiling system for the forensic identification of body fluids and contact traces. *Forensic Science International. Genetics*, **6** (5), 565–577.

Lindenbergh, A., Maaskant, P., Sijen, T. (2013). Implementation of RNA profiling in forensic casework. *Forensic Science International. Genetics*, **7** (1), 159–166.

Litt, M., Luty, J. A. (1989). A hypervariable microsatellite revealed by in vitro amplification of a dinucleotide repeat within the cardiac muscle actin gene. *American Journal of Human Genetics*, **44** (3), 397–401.

Liu, N., Chen, N.-Y., Cui, R.-X., Li, W.-F., Li, Y., Wei, R.-R., Zhang, M.-Y., Sun, Y., Huang, B.-J., Chen, M., He, Q.-M., Jiang, N., Chen, L., Cho, W.C.S., Yun, J.-P., Zeng, J., Liu, L.-Z., Li, L., Guo, Y., Wang, H.-Y., Ma, J. (2012). Prognostic value of a microRNA signature in nasopharyngeal carcinoma: a microRNA expression analysis. *The Lancet. Oncology*, **13** (6), 633–641.

Lodish, H., Berk, A., Zipursky, S. L., Matsudaira, P., Baltimore, D., Darnell, J. E. (2001). *Molekulare Zellbiologie*. 4th ed. Spektrum Akademischer Verlag.

Loscalzo, J. (2010). The cellular response to hypoxia: tuning the system with microRNAs. *The Journal of Clinical Investigation*, **120** (11), 3815–3817.

Madea, B. (2015). *Rechtsmedizin - Befunderhebung, Rekonstruktion, Begutachtung*. 3rd ed. Springer.

Magnuson, V.L., Ally, D.S., Nylund, S.J., Karanjawala, Z.E., Rayman, J.B., Knapp, J.I., Lowe, A.L., Ghosh, S., Collins, F.S. (1996). Substrate nucleotide-determined non-templated addition of adenine by Taq DNA polymerase: implications for PCR-based genotyping and cloning. *BioTechniques*, **21** (4), 700–709.

Marsit, C. J., Eddy, K., Kelsey, K. T. (2006). MicroRNA responses to cellular stress. *Cancer Research*, **66** (22), 10843–10848.

Martin, P. D., Schmitter, H., Schneider, P.M. (2001). A brief history of the formation of DNA databases in forensic science within Europe. *Forensic Science International*, **119** (2), 225–231.

McNally, L., Shaler, R. C., Baird, M., Balazs, I., De Forest, P., Kobilinsky, L. (1989). Evaluation of deoxyribonucleic acid (DNA) isolated from human bloodstains exposed to ultraviolet light, heat, humidity, and soil contamination. *Journal of Forensic Sciences*, **34** (5), 1059–1069.

Meselson, M., Yuan, R. (1968). DNA restriction enzyme from E. coli. *Nature*, **217** (5134), 1110–1114.

Mestdagh, P., Feys, T., Bernard, N., Guenther, S., Chen, C., Speleman, F., Vandesompele, J. (2008). High-throughput stem-loop RT-qPCR miRNA expression profiling using minute amounts of input RNA. *Nucleic Acids Research*, **36** (21), E143.

Mestdagh, P., Van Vlierberghe, P., De Weer, A., Muth, D., Westermann, F., Speleman, F., Vandesompele, J. (2009). A novel and universal method for microRNA RT-qPCR data normalization. *Genome Biology*, **10** (6), R64–R64.

Mitchell, A. (2015). Collecting in collections: a PCR strategy and primer set for DNA barcoding of decades-old dried museum specimens. *Molecular Ecology Resources*, **15** (5), 1102–1111.

Momparler, R. L., Bovenzi, V. (2000). DNA methylation and cancer. *Journal of Cellular Physiology*, **183** (2), 145–154.

Mullis, K. B., Faloona, F. A. (1987). Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction. *Methods in Enzymology*, **155**, 335–350.

Myers, J. R., Adkins, W. K. (2008). Comparison of modern techniques for saliva screening. *Journal of Forensic Sciences*, **53** (4), 862–867.

Nakanishi, H., Hara, M., Takahashi, S., Takada, A., Saito, K. (2014). Evaluation of forensic examination of extremely aged seminal stains. *Legal Medicine (Tokyo, Japan)*, **16** (5), 303–307.

Nelson, P. T., Baldwin, D. A., Scearce, L. M., Oberholtzer, J. C., Tobias, J. W., Mourelatos, Z. (2004). Microarray-based, high-throughput gene expression profiling of microRNAs. *Nature Methods*, **1** (2), 155.

Odén, S., Hofsten, B. V. (1954). Detection of Fingerprints by the Ninhydrin Reaction. *Nature*, **173** (4401), 449–450.

Old, J., Schweers, B. A., Boonlayangoor, P. W., Fischer, B., Miller, K. W. P., Reich, K. (2012). Developmental validation of RSID™-Semen: a lateral flow immunochromatographic strip test for the forensic detection of human semen. *Journal of Forensic Sciences*, **57** (2), 489–499.

Old, J., Schweers, B. A., Boonlayangoor, P. W., Reich, K. A. (2009). Developmental validation of RSID-saliva: a lateral flow immunochromatographic strip test for the forensic detection of saliva. *Journal of Forensic Sciences*, **54** (4), 866–873.

Olson, S. R. (2011, October 19). Forensic Tests for Semen: What you should know. *Forensic Science in North Carolina*. Available from: https://ncforensics.wordpress.com/2011/10/19/forensic-tests-for-semen-what-you-should-know/

Osellame, L. D., Blacker, T. S., Duchen, M. R. (2012). Cellular and molecular mechanisms of mitochondrial function. *Best Practice & Research. Clinical Endocrinology & Metabolism*, **26** (6), 711–723.

Park, J.-L., Kwon, O.-H., Kim, J.H., Yoo, H.-S., Lee, H.-C., Woo, K.-M., Kim, S.-Y., Lee, S.-H., Kim, Y.S. (2014). Identification of body fluid-specific DNA methylation markers for use in forensic science. *Forensic Science International: Genetics*, **13**, 147–153.

Park, S.-M., Park, S.-Y., Kim, J.-H., Kang, T.-W., Park, J.-L., Woo, K.-M., Kim, J.-S., Lee, H.-C., Kim, S.-Y., Lee, S.-H. (2013). Genome-wide mRNA profiling and multiplex quantitative RT-PCR for forensic body fluid identification. *Forensic Science International: Genetics*, **7** (1), 143–150.

Parker, R., Sheth, U. (2007). P bodies and the control of mRNA translation and degradation. *Molecular Cell*, **25** (5), 635–646.

Parker, R., Song, H. (2004). The enzymes and control of eukaryotic mRNA turnover. *Nature Structural & Molecular Biology*, **11** (2), 121–127.

Parson, W. (2009). Bedeutung der mtDNA-Analyse für forensische Fragestellungen. *Rechtsmedizin*, **19** (3), 183–194.

Pereira, R., Phillips, C., Alves, C., Amorim, A., Carracedo, A., Gusmão, L. (2009a). A new multiplex for human identification using insertion/deletion polymorphisms. *Electrophoresis*, **30** (21), 3682–3690.

Pereira, R., Phillips, C., Alves, C., Amorim, A., Carracedo, Á., Gusmão, L. (2009b). Insertion/deletion polymorphisms: A multiplex assay and forensic applications. *Forensic Science International: Genetics Supplement Series*, **2** (1), 513–515.

Pfaffl, M. W., Tichopad, A., Prgomet, C., Neuvians, T. P. (2004). Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper--Excel-based tool using pair-wise correlations. *Biotechnology Letters*, **26** (6), 509–515.

Phillips, C. (2012). Application of Autosomal SNPs and Indels in Forensic Analysis. *Forensic Science Review*, **24** (1), 43–62.

Pontén, F., Gry, M., Fagerberg, L., Lundberg, E., Asplund, A., Berglund, L., Oksvold, P., Björling, E., Hober, S., Kampf, C., Navani, S., Nilsson, P., Ottosson, J., Persson, A., Wernérus, H., Wester, K., Uhlén, M. (2009). A global view of protein expression in human cells, tissues, and organs. *Molecular Systems Biology*, **5**, 337.

Prinz, M., Carracedo, A., Mayr, W.R., Morling, N., Parsons, T.J., Sajantila, A., Scheithauer, R., Schmitter, H., Schneider, P.M. (2007). DNA Commission of the International Society for Forensic Genetics (ISFG): recommendations regarding the role of forensic genetics for disaster victim identification (DVI). *Forensic Science International. Genetics*, **1** (1), 3–12.

Purps, J., Siegert, S., Willuweit, S., Nagy, M., Alves, C., Salazar, R., Angustia, S.M., Santos, L.H., Anslinger, K., Bayer, B., Ayub, Q., Wei, W., Xue, Y., Tyler-Smith, C., Bafalluy, M.B., Martínez-Jarreta, B., Egyed, B., Balitzki, B., Tschumi, S., Ballard, D., Court, D.S., Barrantes, X., Bäßler, G., Wiest, T., Berger, B., Niederstätter, H., Parson, W., Davis, C., Budowle, B., Burri, H., Borer, U., Koller, C., Carvalho, E.F., Domingues, P.M., Chamoun, W.T., Coble, M.D., Hill, C.R., Corach, D., Caputo, M., D'Amato, M.E., Davison, S., Decorte, R., Larmuseau, M.H., Ottoni, C., Rickards, O., Lu, D., Jiang, C., Dobosz, T., Jonkisz, A., Frank, W.E., Furac, I., Gehrig, C., Castella, V., Grskovic, B., Haas, C., Wobst, J., Hadzic, G., Drobnic, K., Honda, K., Hou, Y., Zhou, D., Li, Y., Hu, S., Chen, S., Immel, U.-D., Lessig, R., Jakovski, Z., Ilievska, T., Klann, A.E., García, C.C., de Knijff, P., Kraaijenbrink, T., Kondili, A., Miniati, P., Vouropoulou, M., Kovacevic, L., Marjanovic, D., Lindner, I., Mansour, I., Al-Azem, M., Andari, A.E., Marino, M., Furfuro, S., Locarno, L., Martín, P., Luque, G.M., Alonso, A., Miranda, L.S., Moreira, H., Mizuno, N., Iwashima, Y., Neto, R.S.M., Nogueira, T.L., Silva, R., Nastainczyk-Wulf, M., Edelmann, J., Kohl, M., Nie, S., Wang, X., Cheng, B., Núñez, C., Pancorbo, M.M. de, Olofsson, J.K., Morling, N., Onofri, V., Tagliabracci, A., Pamjav, H., Volgyi, A., Barany, G., Pawlowski, R., Maciejewska, A., Pelotti, S., Pepinski, W., Abreu-Glowacka, M., Phillips, C., Cárdenas, J., Rey-Gonzalez, D., Salas, A., Brisighelli, F., Capelli, C., Toscanini, U., Piccinini, A.,

Piglionica, M., Baldassarra, S.L., Ploski, R., Konarzewska, M., Jastrzebska, E., Robino, C., Sajantila, A., Palo, J.U., Guevara, E., Salvador, J., Ungria, M.C.D., Rodriguez, J.J.R., Schmidt, U., Schlauderer, N., Saukko, P., Schneider, P.M., Sirker, M., Shin, K.-J., Oh, Y.N., Skitsa, I., Ampati, A., Smith, T.-G., Calvit, L.S. de, Stenzl, V., Capal, T., Tillmar, A., Nilsson, H., Turrina, S., De Leo, D., Verzeletti, A., Cortellini, V., Wetton, J.H., Gwynne, G.M., Jobling, M.A., Whittle, M.R., Sumita, D.R., Wolańska-Nowak, P., Yong, R.Y., Krawczak, M., Nothnagel, M., Roewer, L. (2014). A global analysis of Y-chromosomal haplotype diversity for 23 STR loci. *Forensic Science International. Genetics*, **12** (100), 12–23.

Quinones, I., Sheppard, D., Harbison, S. A., Elliot, D. (2006). Comparative analysis of luminol formulations. *Can. Soc. Forensic Sci. J.*, **40**, 53–63.

Raju, P., Iyengar, N. (1964). Acid Phosphatase Reaction as a Specific Test for the Identification of Seminal Stains. *Journal of Criminal Law and Criminology*, **55** (4), 522.

Raymond, J. J., van Oorschot, R. A. H., Gunn, P. R., Walsh, S. J., Roux, C. (2009). Trace evidence characteristics of DNA: A preliminary investigation of the persistence of DNA at crime scenes. *Forensic Science International. Genetics*, **4** (1), 26–33.

Roewer, L., Arnemann, J., Spurr, N. K., Grzeschik, K. H., Epplen, J. T. (1992). Simple repeat sequences on the human Y chromosome are equally polymorphic as their autosomal counterparts. *Human Genetics*, **89** (4), 389–394.

Rowe, K.C., Singhal, S., Macmanes, M.D., Ayroles, J.F., Morelli, T.L., Rubidge, E.M., Bi, K., Moritz, C.C. (2011). Museum genomics: low-cost and high-accuracy genetic data from historical specimens. *Molecular Ecology Resources*, **11** (6), 1082–1092.

Ruhemann, S. (1910). Triketohydrindene hydrate. *Journal of the Chemical Society, Transactions*, (97), 2025–2031.

Ruijter, J.M., Ramakers, C., Hoogaars, W.M.H., Karlen, Y., Bakker, O., van den Hoff, M.J.B., Moorman, A.F.M. (2009). Amplification efficiency: linking baseline and bias in the analysis of quantitative PCR data. *Nucleic Acids Research*, **37** (6), E45–e45.

Saiki, R. K., Bugawan, T. L., Horn, G. T., Mullis, K. B., Erlich, H. A. (1986). Analysis of enzymatically amplified beta-globin and HLA-DQ alpha DNA with allele-specific oligonucleotide probes. *Nature*, **324** (6093), 163–166.

Sanchez, J.J., Phillips, C., Børsting, C., Balogh, K., Bogus, M., Fondevila, M., Harrison, C.D., Musgrave-Brown, E., Salas, A., Syndercombe-Court, D., Schneider, P.M., Carracedo,

A., Morling, N. (2006). A multiplex assay with 52 single nucleotide polymorphisms for human identification. *Electrophoresis*, **27** (9), 1713–1724.

Sanger, F., Nicklen, S., Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Sciences of the United States of America*, **74** (12), 5463–5467.

Santucci, K. A., Nelson, D. G., McQuillen, K. K., Duffy, S. J., Linakis, J. G. (1999). Wood's lamp utility in the identification of semen. *Pediatrics*, **104** (6), 1342–1344.

Sato, I., Kojima, K., Yamasaki, T., Yoshida, K., Yoshiike, M., Takano, S., Mukai, T., Iwamoto, T. (2004). Rapid detection of semenogelin by one-step immunochromatographic assay for semen identification. *Journal of Immunological Methods*, **287** (1–2), 137–145.

Sauer, E., Reinke, A.-K., Courts, C. (2016). Differentiation of five body fluids from forensic samples by expression analysis of four microRNAs using quantitative PCR. *Forensic Science International. Genetics*, **22**, 89–99.

Schechter, A. N. (2008). Hemoglobin research and the origins of molecular medicine. *Blood*, **112** (10), 3927–3938.

Schiff, F. (1924). Wie häufig läßt sich die Blutgruppendiagnose in Paternitätsfragen heranziehen? *Aerztl Sachverst Ztg*, **30**, 231–233.

Schiff, F., Adelsberger, L. (1924). Die Blutgruppendiagnose als forensische Methode. *Aerztl Sachverst Ztg*, **30**, 101–103.

Schleyer, F., Oepen, I., Henke, J. (1995). *Humanbiologische Spuren: Sicherung, Nachweis und Analyse in Kriminaltechnik und forensischer Medizin*. Vol. 32. Kriminalistik-Verlag.

Schmidt, S., Franke, M., Lehmann, J., Loch, T., Stöckle, M., Weichert-Jacobsen, K. (2001). Prostate-specific antigen in female urine: a prospective study involving 217 women. *Urology*, **57** (4), 717–720.

Schneider, H., Sommerer, T., Rand, S., Wiegand, P. (2011). Hot flakes in cold cases. *International Journal of Legal Medicine*, **125** (4), 543–548.

Schneider, P.M. (1997). Basic issues in forensic DNA typing. *Forensic Science International*, **88** (1), 17–22.

Schneider, P.M., Martin, P. D. (2001). Criminal DNA databases: the European situation. *Forensic Science International*, **119** (2), 232–238.

Schoenberg, D. R., Maquat, L. E. (2012). Regulation of cytoplasmic mRNA decay. *Nature Reviews Genetics*, **13** (4), 246–259.

Schulz, M. M., Reichert, W. (2002). Archived or directly swabbed latent fingerprints as a DNA source for STR typing. *Forensic Science International*, **127** (1), 128–130.

Schwede, A., Ellis, L., Luther, J., Carrington, M., Stoecklin, G., Clayton, C. (2008). A role for Caf1 in mRNA deadenylation and decay in trypanosomes and human cells. *Nucleic Acids Research*, **36** (10), 3374–3388.

Schweers, B. A., Old, J., Boonlayangoor, P. W., Reich, K. A. (2008). Developmental validation of a novel lateral flow strip test for rapid identification of human blood (Rapid Stain Identification™-Blood). *Forensic Science International: Genetics*, **2** (3), 243–247.

Setzer, M., Juusola, J., Ballantyne, J. (2008). Recovery and stability of RNA in vaginal swabs and blood, semen, and saliva stains. *Journal of Forensic Sciences*, **53** (2), 296–305.

She, M., Decker, C.J., Svergun, D.I., Round, A., Chen, N., Muhlrad, D., Parker, R., Song, H. (2008). Structural basis of dcp2 recognition and activation by dcp1. *Molecular Cell*, **29** (3), 337–349.

Shen, L., Kondo, Y., Guo, Y., Zhang, J., Zhang, L., Ahmed, S., Shu, J., Chen, X., Waterland, R.A., Issa, J.-P.J. (2007). Genome-wide profiling of DNA methylation reveals a class of normally methylated CpG island promoters. *PLoS Genetics*, **3** (10), 2023–2036.

Sijen, T. (2015). Molecular approaches for forensic cell type identification: On mRNA, miRNA, DNA methylation and microbial markers. *Forensic Science International. Genetics*, **18**, 21–32.

Silva, S. S., Lopes, C., Teixeira, A. L., Carneiro de Sousa, M. J., Medeiros, R. (2015). Forensic miRNA: potential biomarker for body fluids? *Forensic Science International. Genetics*, **14**, 1–10.

Southern, E. M. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. *Journal of Molecular Biology*, **98** (3), 503–517.

Stoilovic, M. (1991). Detection of semen and blood stains using polilight as a light source. *Forensic Science International*, **51** (2), 289–296.

Strassmann, G. (1927). Die forensische Bedeutung der Blutgruppenfrage. *Z Med Beamte*, **40**, 327–336.

Straussman, R., Nejman, D., Roberts, D., Steinfeld, I., Blum, B., Benvenisty, N., Simon, I., Yakhini, Z., Cedar, H. (2009). Developmental programming of CpG island methylation profiles in the human genome. *Nature Structural & Molecular Biology*, **16** (5), 564–571.

Suzuki, M. M., Bird, A. (2008). DNA methylation landscapes: provocative insights from epigenomics. *Nature Reviews Genetics*, **9** (6), 465–476.

Takahashi, R., Miyazaki, H., Ochiya, T. (2014). The role of microRNAs in the regulation of cancer stem cells. *Epigenomics and Epigenetics*, **4**, 295.

Tautz, D. (1993). Notes on the definition and nomenclature of tandemly repetitive DNA sequences. *EXS*, **67**, 21–28.

Teschendorff, A.E., Yang, Z., Wong, A., Pipinikas, C.P., Jiao, Y., Jones, A., Anjum, S., Hardy, R., Salvesen, H.B., Thirlwell, C., Janes, S.M., Kuh, D., Widschwendter, M. (2015). Correlation of Smoking-Associated DNA Methylation Changes in Buccal Cells With DNA Methylation Changes in Epithelial Cancer. *JAMA Oncology*, **1** (4), 476–485.

Thomson, J. M., Parker, J., Perou, C. M., Hammond, S. M. (2004). A custom microarray platform for analysis of microRNA gene expression. *Nature Methods*, **1** (1), 47.

Tsutsumi, H., Higashide, K., Mizuno, Y., Tamaki, K., Katsumata, Y. (1991). Identification of saliva stains by determination of the specific activity of amylase. *Forensic Science International*, **50** (1), 37–42.

Tully, G., Sullivan, K. M., Gill, P. (1993). Analysis of 6 VNTR loci by 'multiplex' PCR and automated fluorescent detection. *Human Genetics*, **92** (6), 554–562.

Urquhart, A., Kimpton, C. P., Downes, T. J., Gill, P. (1994). Variation in short tandem repeat sequences--a survey of twelve microsatellite loci for use as forensic identification markers. *International Journal of Legal Medicine*, **107** (1), 13–20.

van den Berge, M., Bhoelai, B., Harteveld, J., Matai, A., Sijen, T. (2016). Advancing forensic RNA typing: On non-target secretions, a nasal mucosa marker, a differential co-extraction protocol and the sensitivity of DNA and RNA profiling. *Forensic Science International: Genetics*, **20**, 119–129.

van den Berge, M., Carracedo, A., Gomes, I., Graham, E. a. M., Haas, C., Hjort, B., Hoff-Olsen, P., Maroñas, O., Mevåg, B., Morling, N., Niederstätter, H., Parson, W., Schneider, P.M., Court, D.S., Vidaki, A., Sijen, T. (2014). A collaborative European exercise on mRNA-based body fluid/skin typing and interpretation of DNA and RNA results. *Forensic Science International. Genetics*, **10**, 40–48.

van Oorschot, R. A. H., Jones, M. K. (1997). DNA fingerprints from fingerprints. *Nature*, **387** (6635), 767–767.

Van Rood, J. J., Van Leeuwen, A. (1963). Leukocyte grouping. A method and its application. *The Journal of Clinical Investigation*, **42**, 1382–1390.

Vandenberg, N., van Oorschot, R. A. H. (2006). The use of Polilight in the detection of seminal fluid, saliva, and bloodstains and comparison with conventional chemical-based screening tests. *Journal of Forensic Sciences*, **51** (2), 361–370.

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., Speleman, F. (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biology*, **3** (7), Research0034.1-Research0034.11.

Vidaki, A., Daniel, B., Court, D. S. (2013). Forensic DNA methylation profiling--potential opportunities and challenges. *Forensic Science International. Genetics*, **7** (5), 499–507.

Vidaki, A., Giangasparo, F., Syndercombe Court, D. (2016). Discovery of potential DNA methylation markers for forensic tissue identification using bisulphite pyrosequencing. *ELECTROPHORESIS*, **37** (21), 2767–2779.

Virkler, K., Lednev, I. K. (2009). Analysis of body fluids for forensic purposes: From laboratory testing to non-destructive rapid confirmatory identification at a crime scene. *Forensic Science International*, **188** (1–3), 1–17.

Volinia, S., Croce, C. M. (2013). Prognostic microRNA/mRNA signature from the integrated analysis of patients with invasive breast cancer. *Proceedings of the National Academy of Sciences of the United States of America*, **110** (18), 7413–7417.

Walsh, P. S., Fildes, N. J., Reynolds, R. (1996). Sequence analysis and characterization of stutter products at the tetranucleotide repeat locus vWA. *Nucleic Acids Research*, **24** (14), 2807–2812.

Wang, Z., Zhang, J., Luo, H., Ye, Y., Yan, J., Hou, Y. (2013). Screening and confirmation of microRNA markers for forensic body fluid identification. *Forensic Science International: Genetics*, **7** (1), 116–123.

Wang, Z., Zhang, J., Wei, W., Zhou, D., Luo, H., Chen, X., Hou, Y. (2015). Identification of Saliva Using MicroRNA Biomarkers for Forensic Purpose. *Journal of Forensic Sciences*, N/A-N/A.

Weber, J. L., May, P. E. (1989). Abundant class of human DNA polymorphisms which can be typed using the polymerase chain reaction. *American Journal of Human Genetics*, **44** (3), 388–396.

Wheeler, D.A., Srinivasan, M., Egholm, M., Shen, Y., Chen, L., McGuire, A., He, W., Chen, Y.-J., Makhijani, V., Roth, G.T., Gomes, X., Tartaro, K., Niazi, F., Turcotte, C.L., Irzyk, G.P., Lupski, J.R., Chinault, C., Song, X., Liu, Y., Yuan, Y., Nazareth, L., Qin, X., Muzny, D.M., Margulies, M., Weinstock, G.M., Gibbs, R.A., Rothberg, J.M. (2008). The complete genome of an individual by massively parallel DNA sequencing. *Nature*, **452** (7189), 872–876.

Whitehead, P. H., Kipps, A. E. (1975). A test paper for detecting saliva stains. *Journal - Forensic Science Society*, **15** (1), 39–42.

Wienholds, E., Plasterk, R. H. A. (2005). MicroRNA function in animal development. *FEBS Letters*, **579** (26), 5911–5922.

Willott, G. M. (1974). An improved test for the detection of salivary amylase in stains. *Journal - Forensic Science Society*, **14** (4), 341–344.

Wilusz, C. J., Wang, W., Peltz, S. W. (2001). Curbing the nonsense: the activation and regulation of mRNA surveillance. *Genes & Development*, **15** (21), 2781–2785.

Wong, Z., Wilson, V., Jeffreys, A. J., Thein, S. L. (1986). Cloning a selected fragment from a human DNA 'fingerprint': isolation of an extremely polymorphic minisatellite. *Nucleic Acids Research*, **14** (11), 4605–4616.

Zipper, H., Brunner, H., Bernhagen, J., Vitzthum, F. (2004). Investigations on DNA intercalation and surface binding by SYBR Green I, its structure determination and methodological implications. *Nucleic Acids Research*, **32** (12), E103.

Zubakov, D., Boersma, A. W. M., Choi, Y., van Kuijk, P. F., Wiemer, E. A. C., Kayser, M. (2010). MicroRNA markers for forensic body fluid identification obtained from

microarray screening and quantitative RT-PCR confirmation. *International Journal of Legal Medicine*, **124** (3), 217–226.

Zubakov, D., Hanekamp, E., Kokshoorn, M., van Ijcken, W., Kayser, M. (2008). Stable RNA markers for identification of blood and saliva stains revealed from whole genome expression analysis of time-wise degraded samples. *International Journal of Legal Medicine*, **122** (2), 135–142.

Zubakov, D., Kokshoorn, M., Kloosterman, A., Kayser, M. (2009). New markers for old stains: stable mRNA markers for blood and saliva identification from up to 16-year-old stains. *International Journal of Legal Medicine*, **123** (1), 71–74.

8 Lebenslauf

Mein Lebenslauf wird aus Gründen des Datenschutzes in der elektronischen Fassung meiner Arbeit nicht veröffentlicht.