Int J Legal Med (2018) 132:67–81
DOI 10.1007/s00414-017-1691-6

ORIGINAL ARTICLE

Comprehensive examination of conventional and innovative body fluid identification approaches and DNA profiling of laundered blood- and saliva-stained pieces of cloths

G. Kulstein[1] · P. Wiegand[1]

Received: 25 July 2017 / Accepted: 12 September 2017 / Published online: 29 September 2017

Abstract Body fluids like blood and saliva are commonly encountered during investigations of high volume crimes like homicides. The identification of the cellular origin and the composition of the trace can link suspects or victims to a certain crime scene and provide a probative value for criminal investigations. To erase all traces from the crime scene, perpetrators often wash away their traces. Characteristically, items that show exposed stains like blood are commonly cleaned or laundered to free them from potential visible leftovers. Mostly, investigators do not delegate the DNA analysis of laundered items. However, some studies have already revealed that items can still be used for DNA analysis even after they have been laundered. Nonetheless, a systematical evaluation of laundered blood and saliva traces that provides a comparison of different established and newly developed methods for body fluid identification (BFI) is still missing. Herein, we present the results of a comprehensive study of laundered blood- and saliva-stained pieces of cloths that were applied to a broad range of methods for BFI including conventional approaches as well as molecular mRNA profiling. The study included the evaluation of cellular origin as well as DNA profiling of blood- and saliva-stained (synthetic fiber and cotton) pieces of cloths, which have been washed at various washing temperatures for one or multiple times. Our experiments demonstrate that, while STR profiling seems to be sufficiently sensitive for the individualization of laundered items, there is a lack of approaches for BFI with the same sensitivity and specificity allowing to characterize the cellular origin of challenging, particularly laundered, blood and saliva samples.

Keywords Body fluid/tissue identification · Forensic RNA analysis · Messenger RNA · Blood · Saliva · STR profiling

Introduction

Evidence of blood is commonly encountered during investigations and is often involved in cases of homicide, aggravated assault or sexual assault. Like blood, saliva is highly associated with serious and major crime, especially sexual assault cases [1] and burglary. The identification of the cellular origin of the biological fluid often has probative value for criminal investigations. In some cases it can be crucial for the outcome of the whole trial. Currently, the main methods for body fluid and tissue identification (BFI) are based on chemical tests or immunological approaches [2, 3] and can be categorized as presumptive or confirmatory tests depending on whether their results are likely—but indefinite—or conclusive [4].

Blood, as well as saliva, is a highly complex mixture of cells, enzymes, proteins, and inorganic substances [2, 5]. Blood comprises a fluid portion, plasma, and a cellular portion that consists of erythrocytes (red blood cells), leucocytes (white blood cells), and thrombocytes (platelets). Most of the common presumptive tests target hemoglobin (hb), a protein that is enriched in the cytoplasm of the erythrocytes and is responsible for oxygen transportation from the respiratory organs to the remaining tissue and organs of the body. Heme has the ability to catalyze an oxidation-reduction reaction. Therefore, common presumptive tests, based on this catalytic

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00414-017-1691-6) contains supplementary material, which is available to authorized users.

✉ G. Kulstein
Galina.Kulstein@uniklinik-ulm.de

[1] Institute of Legal Medicine, University Hospital Ulm, Albert-Einstein-Allee 23, 89081 Ulm, Germany

reaction, often use hydrogen peroxide as oxidant. The most commonly applied reductants are phenolphthalein, leucomalachite green, tetramethylbenzidine (TMB), and luminol [5].

Saliva is mostly composed of water (> 99%) in the presence of buccal epithelia cells, enzymes, salts, mucin, and alpha-amylase (α-amylase), which is responsible for the breakdown of starch [2]. Because of its abundancy in human saliva, most of the presumptive tests target α-amylase. Confirmatory assays for blood and saliva are mainly based on immunochromatography and allow to differentiate between human and non-human body fluids. Despite their sensitivity, caution is required in the interpretation of presumptive and confirmatory tests since they show a range of drawbacks, e.g., false positive results with other oxidants like plant peroxidases [6] or false negative results due to the high-dose hook effect [7].

Because of the various limitations, new approaches for BFI have been investigated. Molecular mRNA analysis [8–10], microRNA (miRNA) approaches [11–16], and DNA methylation [17, 18] seem to be the most promising tools to overcome the disadvantages of current techniques. However, there is no universal technique that can be used or recommended for BFI to this day.

To fill the lack of a missing comparison of approaches for BFI, this work aims to compare established methods to recently developed innovative methods using challenging blood and saliva samples. Because perpetrators often try to wash away visible traces of the victim's blood off their clothes by machine washing or because victims try to wash away biological evidence in order to not be reminded of the assault they experienced, we investigated the potential of BFI methods with samples that have been laundered under varying conditions. The comprehensive analysis included, on the one side, commonly used enzymatic as well as immunochromatographic approaches and, on the other side, mRNA profiling. The comparison is based on different washing scenarios for blood as well as for saliva samples: the experimental design included distinct fabric types, multiple washing steps and considered the influence of different washing temperatures and storage periods prior to laundering. Besides RNA extraction, DNA from traces on fabrics was extracted and analyzed to test to which extent DNA of blood and saliva donors retains in different fabrics after laundering and if STR profiling will be promising.

Materials and methods

Sample collection and preparation

Blood and saliva samples were provided from healthy donors. All samples were obtained after informed consent and with approval of the Medical Ethics Committee at the University Hospital of Ulm, Germany. Blood has been drained by venipuncture. Native saliva was provided in 5 ml tubes after abstinence from food and drinking for 30 min. For the laundry experiments, cotton and synthetic pieces of cloths were prepared (size 13 × 18 cm). The same piece of cloth was spotted with 20 and 100 μl blood or with 20 and 100 μl of saliva and dried overnight (saliva and blood were not mixed on cloths). Distances between 20 and 100 μl volume traces ranged approximately from 5 to 10 cm. Altogether, six different experimental scenarios have been tested per body fluid (Fig. 1). Blood- and saliva-stained cloths were laundered in a LG Intellowasher WD-16110FD washing machine (that was not cleaned before washing) using a standardized washing program with a duration of 120 min and a spin cycle of 1000 rounds per minute (rpm) either at 40 or at 60 °C by a first washer (hereinafter termed "washer 1"). A liquid detergent (Denk mit, DM) without phosphatases was added. No fabric conditioner was used during the washes. Items with the same temperature and fabrics were washed together (without addition of other garments). After washing, the items were allowed to air-dry overnight and, dependent on the protocol, were either processed immediately or washed again under the same conditions. Laundered items were stored in envelopes until further processing. The identical samples were used for presumptive testing as well as for RNA processing. Cut-out areas for presumptive testing measured approximately 0.25 cm^2. In addition, cloths for LumiScene Ultra detection were prepared. Pieces of cloths that were used for LumiScene Ultra detection were neither subjected to other presumptive tests nor RNA/DNA profiling. Unwashed synthetic and cotton pieces of cloths spotted with the same amount of blood or saliva were included into the analysis and used as positive controls. Sporadically, controls of unstained areas (that were supposed to serve as transfer and negative controls, hereinafter only referred to as "transfer controls") were added to test for mutual nucleic acids transfer. Transfer controls were prepared by multiple cut-outs of distinct areas throughout the entire washed cloth piece. Positive and transfer controls were treated like body fluid stained traces and included in all subsequent analyses including presumptive testing. Except experimental designs D1 and D2, which were repeated twice, scenarios B, C, and E were repeated four times. To test whether the model of washing machine or the type of liquid detergent have an influence on the results, four replicates of experimental design A were additionally washed with a Miele washing machine Ed 111-W 5873 by a second researcher (hereinafter named "washer 2") with 1000 rpm at 60 °C for 120 min using laundry detergent powder from Persil (Henkel, Düsseldorf). One further wash was accomplished by the washing machine of washer 1 in combination with the washing powder of washer 2, Persil. Overall, this resulted in 24 samples for experimental design A (for 20 and 100 μl volume samples together).

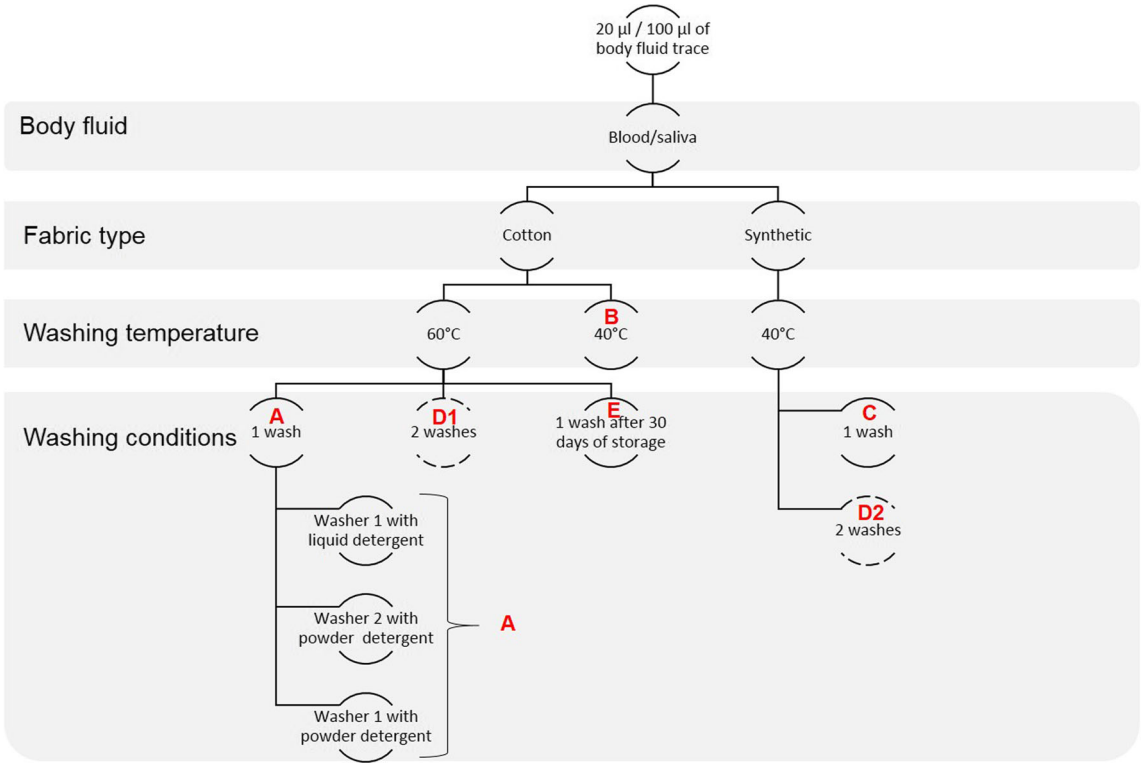

Fig. 1 Experimental scenarios and conditions of washed items. Each experimental design was conducted for blood- as well as for saliva-stained pieces of cloths. Design assignment is given in bold. Dashed lines of experimental design indicate experiments with two replicates. Undashed lines stand for experimental designs that have been repeated four times

Presumptive testing

Presumptive testing for blood

Hemastix Hemastix (Siemens) is a plastic reagent strip that reacts in the presence of hb by cleavage of the oxygen molecules of H_2O_2 and catalyzation of 3,3′,5,5′-tetramethylbenzidine that results in a change of color from colorless to greenish [19]. The test was accomplished by pressing and rubbing the moistened filter paper of the test stripe against the washed stains.

Fecal occult blood (FOB) test The FOB test (Diagnostic Systems, Holzheim) is an immunochromatographic quick test that selectively recognizes human hb through an antibody-antigene reaction. For testing, a portion of the washed sample was cut out and included into the collecting tube provided with extraction buffer. Four drops of the well-mixed solution were transferred via dosing cap onto the samples window of the test cassette. After 10 min of running time and incubation, the result was recorded.

RSID™-Blood The RSID™-Blood test (Galantos Genetics, Mainz) is an immunochromatographic assay that uses two monoclonal mouse antibodies for glycophorin A detection. Glycophorin A is a red blood cell specific protein that prevents cellular aggregation. For testing, a piece of the washed sample material was incubated for at least 60 min in the universal incubation buffer. Afterwards 100 µl of the lysate was pipetted into the sample window and the result was recorded after 10 min of incubation.

LumiScene Ultra LumiScene Ultra is a highly sensitive chemical blood search solution that helps to visualize latent bloodstains at crime scenes, where attempts were made to remove or wash away blood traces. LumiScene Ultra uses the peroxidase-like activity of hb to reduce its reductant. The chemiluminescense emission can be measured during a period of 15 s with a peak at 525 nm. LumiScene Ultra (Loci Forensic Products, Nieuw-Vennep) reagent was prepared according to the recommendation of the manufacturer.

Presumptive testing for saliva

Alternate light source (ALS) An alternate light source (ALS) is a simple and non-destructive method to visualize the natural fluorescence of different body fluids [20].

Bandwidths ranging from 365 to 445 (blue light) in combination with yellow or orange filter goggles are commonly used for the detection of body fluids like semen, saliva, or urine [21, 22]. The items in our study were examined before

and after washing using a crime-lite (foster + freeman) with blue light (wavelength 430–470 nm) in combination with orange googles.

Phadebas® Amylase The Phadebas® Amylase test (Magle Life Sciences, Lund) is designed to detect α-amylase, which is present in human saliva. Four hundred microliters of Phadebas® solution (that was prepared according to manufacturer's recommendations) and 100 μl of water were added to each sample. Mixtures were incubated for 30 min at 37 °C. To stop the reaction, 100 μl of 0.5 M NaOH was added and samples were centrifuged for 5 min at 13000 rounds per minute. The presence of α-amylase was indicated by a blue coloration of the supernatant of the sample.

RSID™-Saliva The RSID™-Saliva test (Galantos Genetics, Mainz) detects α-amylase by utilization of two monoclonal antibodies. For amylase detection, a portion of the washed stain was cut out and extracted for at least 60 min in the universal incubation buffer. Afterwards 100 μl of the lysate was pipetted into the sample window. The result was recorded after 10 min.

RNA and DNA extraction

Prior to RNA extraction, ambient RNases from all surfaces and devices used for RNA analysis were removed with RNaseZap (Merck, Darmstadt). Only RNase-free reagents and consumables were used.

Extraction was realized by two subsequent extraction procedures on an automated device. For each extraction half of the washed or unwashed stains were used. First DNA was extracted using the Maxwell® 16 RSC Blood DNA Kit (AS1400) on the Maxwell® 16 RSC platform (Promega, Mannheim) according to the manufacturer's recommendation. DNA was eluted in 60 μl of elution buffer. Subsequently, the other half of the stain was used to extract RNA following the manufacturer's instructions for RNA analysis with the Maxwell® 16 RSC miRNA Tissue Kit (AS1460, Promega, Mannheim), which extracts total RNA but enriches shorter RNAs like miRNAs. Elution volume was 50 μl of RNase-free water. RNA and DNA extracts were stored at − 20 °C after extraction.

Quantification

DNA quantification

DNA extracts were quantified with the PowerQuant® System (Promega, Mannheim) according to the manufacturer's recommendations using 5 μl of extract. The dilution series for stand curve assessment goes down to 0.0032 ng/μl, so the assay can detect very small amounts of human DNA. We are aware that accuracy of measurements decreases under this detection limit. Amplification was carried out on the 7500 Real-Time PCR System (Applied Biosystems, Darmstadt). Data were analyzed with the PowerQuant® Analysis Tool v1.0.0.0.

RNA quantification

Total RNA quantity was assessed with the QuantiFluor® RNA System applying the protocol for low RNA yields on the Quantus™ Fluorometer (Promega, Mannheim). Measurements were done according to the manufacturer's recommendation diluting 2 μl of RNA extract in 100 μl of 1xTE buffer and 98 μl of 1 to 1000 diluted QuantiFluor RNA dye. According to the manufacturer, the system is able to measure RNA ranging from 0.1 to 500 ng/μl.

Reverse transcription

Reverse transcription mRNA

cDNA synthesis was performed with the Tetro cDNA Synthesis Kit (Bioline, Luckenwalde) according to the manufacturer's instructions. Briefly, in the first step 12 μl of RNA extract was combined with 1 μl of random hexamers (40 μM), 1 μl dNTPs (10 mM), 1 μl RNase Inhibitor (10 U/μl), 4 μl 15xRT buffer and 1 μl MMLV Reverse Transcriptase (200 U/μl) to a final volume of 20 μl.

Reverse transcription was performed on a Biometra Thermocycler for 10 min at 25 °C and 30 min at 45 °C. Reaction was terminated by incubation at 85 °C for 5 min. After cDNA synthesis, samples were stored at − 20 °C. Reactions were prepared in duplicates. RT- reactions (RNA extracts including all reagents for cDNA synthesis but Reverse Transcriptase) were included to test for potential genomic DNA contamination.

Endpoint PCR and capillary electrophoresis

STR typing

STR typing was performed with the Investigator ESSplex SE QS (Qiagen, Hilden) according to the manufacturer's protocol on a Biometra thermocycler with 29 cycles. An ABI PRISM 3130 Genetic Analyzer was used for product separation and detection of amplicons. For the capillary electrophoresis 1 μl of PCR product was added to 12 μl Hi-Di™ formamide and 0.5 μl size standard BTO 550. Reaction plates were sealed and incubated for 5 min at 92 °C for denaturation. After denaturation, samples were cooled on ice for 5 min. Samples were injected for 1 s at 1.2 kV using a 4-capillary, 36 cm array with performance optimized polymer (POP) 7 as a separation matrix. The resulting data were analyzed with the GeneMapper

ID v3.2. Threshold for heterozygotes was set to 50 relative fluorescent units (rfu) and for homozygotes to 150 rfu.

mRNA profiling

Endpoint PCR was performed with 4 μl of cDNA synthesis product in a reaction volume of 25 μl according to Lindenbergh et al. [23]. In addition to the cDNA synthesis product each reaction contained 12.5 μl of 2× multiplex PCR master Mix and 5× of the Primer Mix Tissue-ID. Based on previous publications and with permission of the Netherlands Forensic Institute (NFI) the mRNA marker set of the NFI was adapted for BFI in our laboratory [23–25]. The assay includes 19 markers of which three are supposed to be detectable in blood samples: Hemoglobin subunit beta (HBB), Delta-aminolevulinate synthase 2 (ALAS2) and Cluster of Differentiation 93 (CD93). Two markers are used for saliva assignment: Histatin 3 (HTN3) and Statherin (STATH). Two housekeeping genes, Actin beta (ACTB) and 18S ribosomal RNA (18S rRNA), that are ubiquitously expressed in all body fluids and tissues, are additionally incorporated into the assay. Amplification was conducted on a Biometra thermocycler applying following conditions: 15 min at 95 °C, 33 cycles of 20 s at 94 °C, 30 s at 60 °C and 40 s at 72 °C, followed by an incubation of 45 min at 60 °C.

PCR products were separated and detected with an ABI PRISM 3130 using Pop 7 polymer. The run was accomplished with the multi-capillary Dye set for G5, the DS-33 Matrix Standard kit (Thermo Fisher Scientific, Darmstadt). GeneScan 500 LIZ dye size standard (Thermo Fisher Scientific, Darmstadt) was used as internal lane standard. Per reaction 0.1 μl of lane standard was mixed with 8.9 μl of formamide. Reaction plates were sealed and incubated for 5 min at 92 °C for denaturation. After denaturation samples were cooled on ice for 5 min. Samples were injected for 12 s at 1.2 kV. Duplicates of each sample were performed and analyzed with the GeneMapper ID v3.2. Threshold for peak calling was 50 rfu.

Data analysis

Statistical analysis was performed with GraphPad Prism 6 (Graphpad Software Inc., La Jolla, CA, USA).

Results

Presumptive testing and mRNA profiling

To make sure that all presumptive tests work properly, positive controls of blood and saliva were included and tested before washed samples were subjected to presumptive testing. All tests showed clear positive results for unwashed 20 and 100 μl volume samples. Because quantification with the Quantus Fluorometer is generally not human-specific and therefore not reliable, the highest input of 12 μl for RT reaction was chosen for each sample. To test for potential DNA contamination, RT- samples were included into the downstream analysis. Further, positive controls of blood-stained pieces of cloths as wells as transfer controls were examined in the subsequent analyses. All RT- controls were negative and showed no interpretable RNA profiles, indicating a successful DNase treatment during RNA extraction.

Blood

Unexpectedly, after laundering at 40 °C (Fig. 2b) and even at 60 °C (Fig. 2c) a visual examination of the cloths revealed that blood traces were still present on cotton. This was observed for washed cotton cloths of washer 1, but not for cotton cloths that were washed at 60 °C by washer 2 (Fig. 2d) or on synthetic fiber after laundering at 40 °C (Fig. 2f). As expected, after laundering immunochromatographic detection by either the FOB or the RSID™-Blood test showed negative results for all samples independent of the experimental design, type of fabric or sample volume (see Table 1). Yet, Hemastix results were positive for all samples, except for the cohort of experimental design A that was washed by washer 2. These four replicates did not react with Hemastix. Additionally, also 71% (5/7) of the transfer control samples exhibited positive Hemastix results. Specific LumiScence Ultra reaction was still visible on cloths after washing at 40 and 60 °C. Like the positive control, samples reacted instantly with the LumiScene Ultra and showed a blue luminescence that persisted for over 1 min. However, it was noticed that fluorescence intensity diminished with increasing washing temperature. Interestingly, LumiScene Ultra reaction on 40 °C washed cloths was not limited to blood-spotted areas, but spread throughout the entire cloth (Fig. 3).

RNA quantities were assessed with the QuantiFluor® RNA System. Controls of 20 and 100 μl blood on cotton and synthetic fiber were quantified before washing. The means of unwashed controls on cotton were 2100 pg/μl and 5000 pg/μl for 20 and 100 μl volume samples, respectively. RNA amounts of samples on synthetic fiber resulted in a mean of 2700 pg/μl for 20 μl volume samples and 7100 pg/μl for 100 μl of blood. Overall, RNA quantification after laundering was successful in 82% of the samples. The remaining samples (especially samples from experimental design A) showed RNA amounts below the detection limit (below blank).

The mRNA assay includes three markers that are used for blood detection: HBB, ALAS2 and CD93. In addition, both housekeeping genes (ACTB and 18S rRNA) were evaluated for each sample. Positive controls on both cotton and synthetic material (20 μl as well as 100 μl blood samples) showed the

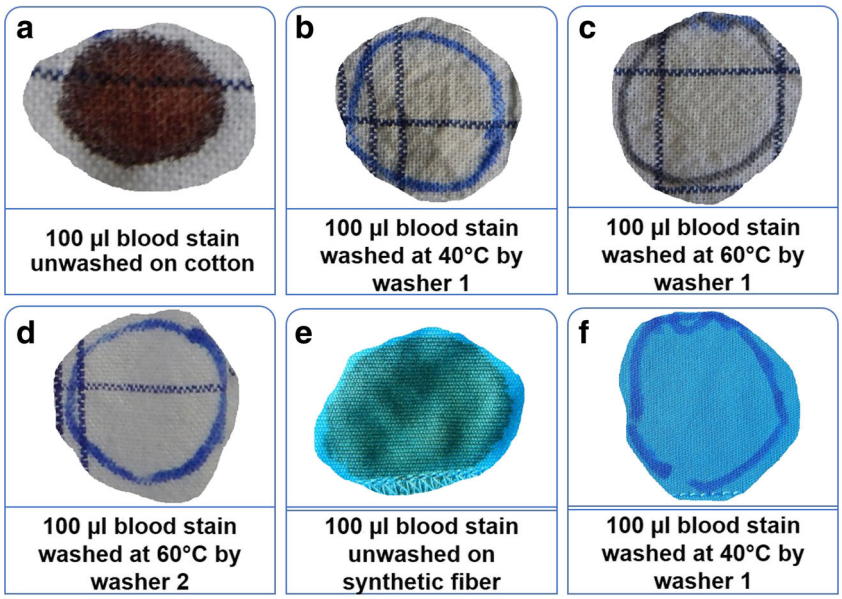

Fig. 2 Unwashed blood stains after trace generation with 100 µl of blood on cotton (**a**) and synthetic fiber (**e**). Blood residues after washing at 40 °C (**b** and **f**) and 60 °C by washer 1 (**c**) and washer 2 (**d**)

presence of all three blood-specific markers with high peaks (500–8500 relative fluorescence units (rfu)). After washing, blood-specific detection was successful in only 9% (4/56) of all analyzed blood samples. RNA profiles were generated mainly for 100 µl volume samples on cotton that have been washed at 40 °C (experimental design B; Table 3). Peaks were observed for HBB, ACTB and 18S rRNA (supplementary figure 1). Nonetheless, detectable signals decreased compared to control samples and ranged between 100 and 3000 rfu. For samples taken from double-washed cloths or from items that have been washed at 60 °C (experimental designs A, D1, D2 and E), no blood-specific targets were found. For the evaluation of transfer scenarios, transfer controls were subjected to RNA profiling, too. All transfer controls revealed no interpretable profiles. Therefore, we conclude that transfer scenarios of blood that happened during the laundering experiments were not measurable on RNA level.

Saliva

Prior to laundering, the localization and visualization of saliva traces was possible with an ALS at a wavelength of 430–470 nm and orange goggles. This observation was made for traces on synthetic fiber as well as on cotton and is in concordance to previously published statements [22]. However, the fluorescence was weak and hardly visible (Fig. 4, 1a-d). Traces on both fibers lost their fluorescence after laundering at 40 °C (Fig. 4, 2a-d). After laundering, presumptive testing with the Phadebas® Amylase test was graded positive for traces on cotton and synthetic pieces of cloths that have been washed at 40 °C as well as for cloths stored at room temperature for a period of 30 days before washing. Detection of saliva on items washed at 60 °C for one or two times was not possible anymore (Table 2). No positive results were observed for transfer controls. The immunochromatographic detection of α-amylase with the RSID™-Saliva test exhibited sporadically positive results for experimental design E only: α-amylase was detected in all 100 µl volume samples and in the moiety of 20 µl volume samples (Table 2).

Mean RNA yields of saliva control samples on cotton were 1400 pg/µl for 20 µl and 3900 pg/µl for 100 µl volume samples. The means of unwashed controls placed on synthetic fiber resulted in 2800 pg/µl and 3700 pg/µl for 20 and 100 µl volume samples, respectively. As expected, after washing RNA yields reduced in all experimental designs. Measurable RNA was recorded for 75% of all quantified

Table 1 Overview of presumptive test results of washed blood stains. Amounts of replicates of experimental designs B, C, and E were 4. Eight replicates (4 by washer 1 and 4 by washer 2) were conducted for experimental design A. Experimental design D was duplicated for cotton and synthetic fiber

Experimental design	A		B		C		D1 + D2		E	
Volume [µl]	20	100	20	100	20	100	20	100	20	100
Hemastix	4/8	4/8	4/4	4/4	4/4	4/4	2/2	2/2	4/4	4/4
FOB	0/8	0/8	0/4	0/4	0/4	0/4	0/2	0/2	0/4	0/4
RSID	0/8	0/8	0/4	0/4	0/4	0/4	0/2	0/2	0/4	0/4

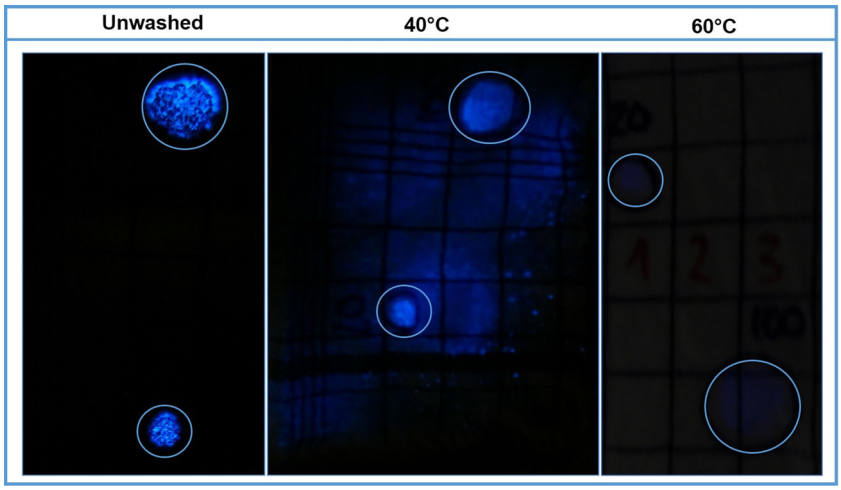

Fig. 3 Blood detection on cotton pieces of cloths before and after washing at 40 and 60 °C with LumiScene Ultra (areas of luminescence are outlined)

samples. Subsequent RNA profiling failed in all samples (including transfer controls) irrespective of washing temperature or fabric type for saliva-specific as well as for housekeeping markers.

DNA quantification and STR profiling

STR profiling was graded according to the German database guidelines meaning that profiles were evaluated as (i) reportable to the database, (ii) informative, but not reportable to the database and (iii) not informative. A profile is reportable when three of the five main loci (SE33, D21S11, vWA, TH01 and FGA) and altogether five out of eight loci (additionally D3S1358, D8S1179 and D18S51) can be designated. If this is not the case, the profile is still informative and can be used to assign or exclude a person of interest but is not applicable for an incorporation into the database. A profile is not informative at all when not enough alleles were typed and even an assignment or exclusion to a suspected person is not feasible anymore.

Blood stains

After co-extraction, DNA was quantified with the PowerQuant system. For blood, the unwashed cotton controls resulted for 20 μl stains in a mean DNA amount of 970 pg/μl and for 100 μl in 9850 pg/μl. Unwashed controls of synthetic cloths contained 1430 pg/μl and 8210 pg/μl for 20 and 100 μl stains, respectively. DNA quantification results of blood-stained cloths are summarized in Fig. 5.

DNA amounts in cotton decreased after washing at 40 and 60 °C with higher temperature leading to greater loss. This was observed for 20 μl as well as 100 μl volume samples (Fig. 5—A + B). Comparably, DNA amounts of 20 and 100 μl samples on synthetic fiber decreased after washing at 40 °C (Fig. 5—C). The comparison of cotton and synthetic fibers washed at 40 °C shows that cotton seems to retain DNA better than synthetic fibers since DNA amounts of cotton samples were higher for both sample volumes.

To test whether an increased amount of washing procedures will result in a decrease of DNA amounts, samples were washed twice with the application of the same washing

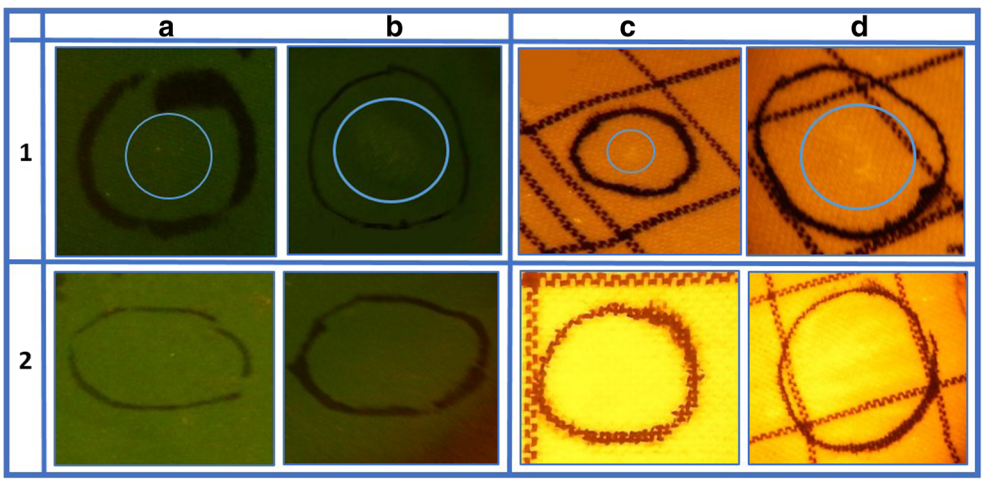

Fig. 4 Light source detection (areas are outlined) of saliva traces on synthetic and cotton fibers at 430–470 nm wavelength with orange googles of (A1 + C1) 20 μl saliva traces and (B1 + D1) 100 μl saliva traces. Loss of fluorescence after washing at 40 °C of (A2 + C2) 20 μl saliva traces and (B2 + D2) 100 μl saliva traces

Table 2 Overview of presumptive test results of washed saliva stains. Amounts of replicates of experimental designs B, C and E were 4. Eight replicates (4 by washer 1 and 4 by washer 2) were conducted for experimental design A. Experimental design D was duplicated for cotton and synthetic fiber. Amounts in brackets indicate weak positive test result

Experimental design	A		B		C		D1 + D2		E	
Volume [μl]	20	100	20	100	20	100	20	100	20	100
Phadebas®	0/8	1/8	(3/4)	(4/4)	(4/4)	(4/4)	0/2	0/2	(4/4)	(4/4)
RSID™	0/8	0/8	0/4	0/4	0/4	0/4	0/2	0/2	(2/4)	(4/4)

conditions. DNA amounts of both fabric types decreased after the double washing procedure (Fig. 5—D1 + D2).

Further, the results indicate that storage time has no significant influence on DNA amounts (Fig. 5—E). To test whether the type of washing machine or the type of washing detergent had an influence on DNA retention after washing, an additional wash was performed with a more modern washing machine and a branded powder detergent (by washer 2). The quantification results show that the combination of a newer washing machine and a non-liquid detergent leads to decreased DNA amounts for 20 and 100 μl volume samples (Fig. 6). To find out whether the washing machine or the washing detergent caused the reduction, washer 1 additionally conducted a wash with his washing machine in combination with the detergent of washer 2. The wash resulted in lower DNA yields compared to the prior wash of washer 1. However, DNA concentrations were still higher than those of washer 2.

Altogether, 91.1% of all samples yielded DNA profiles that were qualitatively good enough to be reported to the database (Table 3). Reportable profiles were analyzed independent of the experimental design, meaning that neither fabric type nor washing temperature seem to have an impact on STR profiling outcome. However, it was observed that STR profiles of 100 μl volume samples showed a better performance in all experimental designs.

Overall samples of washer 2 yielded lower DNA amounts and showed more allelic drop-outs than samples of washer 1. Nevertheless, all 100 μl volume traces washed by washer 2 resulted in full and reportable profiles, whereas 20 μl samples were informative but not reportable. Also, all blood-stained cotton samples that were washed at 60 °C of the second cohort of washer 1 showed reportable profiles (except one 20 μl volume sample that was informative, but not reportable). Because of the similar results, we decided to summarize all samples of washer 1 and washer 2 for further evaluation.

To test the possibility of transfer of blood from stained to unstained sections of the cloths, we also evaluated STR typing of transfer controls. Transfer controls were taken from washed cloths where no blood was applied before washing. During the experiment, seven control samples were conducted.

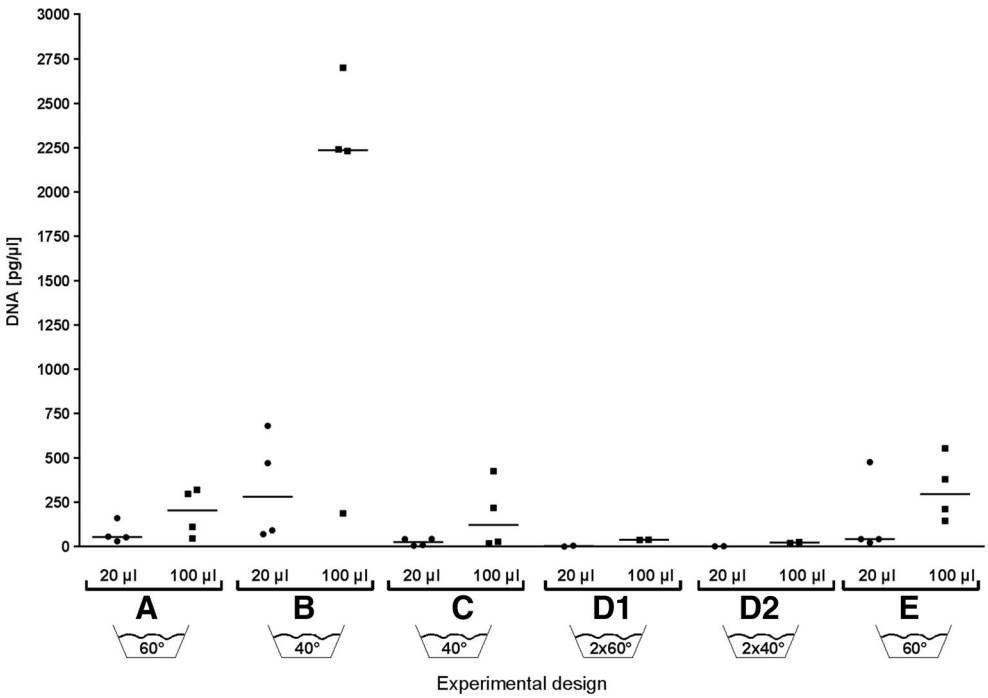

Fig. 5 Overview of DNA quantification results (pg/μl) of all experimental designs for 20 and 100 μl volume samples of blood. Line indicates median. Experimental design A: cotton washed at 60 °C, B: cotton washed at 40 °C, C: synthetic fiber washed at 40 °C, D1: cotton washed twice at 60 °C, D2: synthetic fiber washed twice at 40 °C, E: cotton washed at 60 °C after 30 days of storage

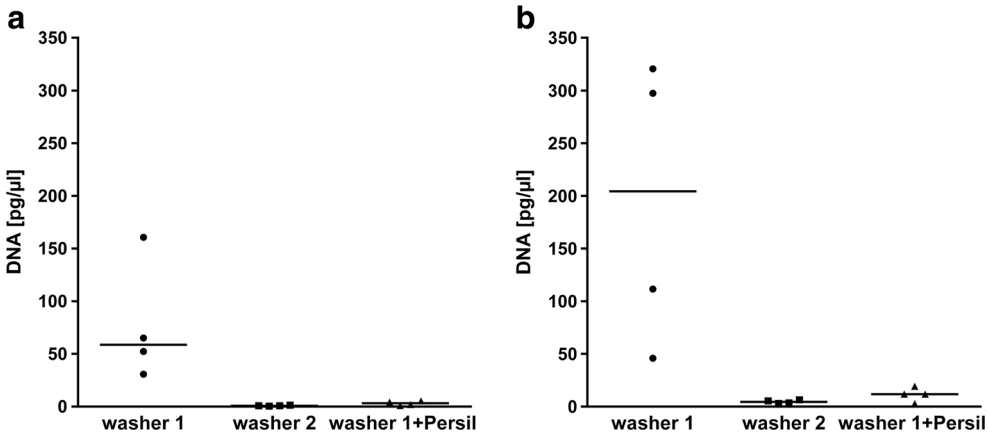

Fig. 6 Comparison of DNA amounts in pg/µl after washing of blood-stained cotton cloths at 60 °C by washer 1 and washer 2. **a** 20 µl and **b** 100 µl volume samples. Line at median

STR profiling of transfer samples resulted in 28.6% (2/7) reportable, 28.6% (2/7) informative, but not reportable and 42.9% (3/7) not informative profiles. Reportable profiles were mainly generated from controls that have been taken from cotton cloths. Most of the alleles matched the profiles of the blood donors of the experiments. Allelic drop-ins with unknown origin were also observed.

Saliva stains

Compared to blood, saliva samples yielded lower DNA amounts. Unwashed controls for 20 µl and 100 µl traces on cotton contained mean values of 430 pg/µl and 3460 pg/µl, respectively.

During DNA quantification of the positive controls, it was noticed that the DNA quantities extracted from the saliva samples of the two individuals (that donated saliva for the experiments) varied strongly. We hypothesize that the outliners that were observed during the study (especially in the scenarios B and E including 100 µl volume traces) resulted from those inter-individual DNA differences. Generally, it is known that saliva samples of different individuals show variable cell composition and amounts of cells, which results in distinct DNA amounts after extraction.

After laundering, cotton pieces of cloths that were washed at 40 °C showed reduced DNA amounts for 20 and 100 µl volume samples (Fig. 7—B). Washing at 60 °C resulted in even more reduced median amounts, at least for 20 µl volume samples (Fig. 7—A). Unwashed

Table 3 Overview of mRNA and STR profiling results for distinct experimental designs of blood-stained cloths. * Washes of washer 1 and washer 2 were summarized. Amounts in brackets indicate unsteady detection with one reaction resulting in a positive and one reaction in a negative result

Experimental design	Sample volume [µl]	RNA quantification range [pg/µl]	RNA analysis					DNA analysis			
			mRNA					DNA quantification median [pg/µl]	STR profiling		
			HBB	ALAS2	CD93	ACTB	18S rRNA		Reportable	Informative, not reportable	Not informative
A*	20	0–900	0/12	0/12	0/12	0/12	0/12	3	7/12	4/12	1/12
	100	0–1000	0/12	0/12	0/12	0/12	0/12	11.8	12/12	–	–
B	20	1100–1700	1/8	0/4	0/4	0/4	(4/4)	280	4/4	–	–
	100	4200–5200	3/8	0/4	0/4	2/4	4/4	2240	4/4	–	–
C	20	0–20	0/4	0/4	0/4	0/4	(2/4)	30	4/4	–	–
	100	100–500	(1/4)	0/4	0/4	0/4	(3/4)	120	4/4	–	–
D1	20	200–700	0/2	0/2	0/2	0/2	0/2	3	2/2	–	–
	100	600–900	0/2	0/2	0/2	0/2	(1/2)	40	2/2	–	–
D2	20	300–500	0/2	0/2	0/2	0/2	0/2	2	2/2	–	–
	100	300–400	0/2	0/2	0/2	0/2	(1/2)	20	2/2	–	–
E	20	1300–2200	0/4	0/4	0/4	0/4	0/4	40	4/4	–	–
	100	3800–6000	0/4	0/4	0/4	0/4	(4/4)	300	4/4	–	–

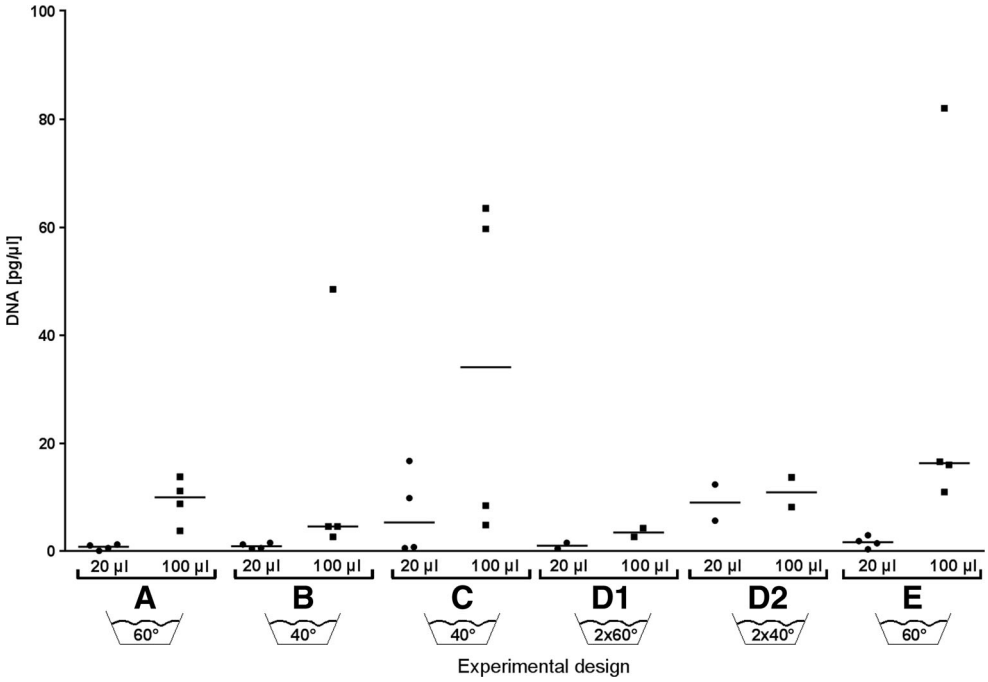

Fig. 7 Overview of DNA quantification results (pg/μl) of all experimental designs for 20 and 100 μl volume samples of saliva. Line indicates median. Experimental design A: cotton washed at 60 °C, B: cotton washed at 40 °C, C: synthetic fiber washed at 40 °C, D1: cotton washed twice at 60 °C, D2: synthetic fiber washed twice at 40 °C, E: cotton washed at 60 °C after 30 days of storage

controls of synthetic fiber resulted in mean DNA concentrations of 540 pg/μl for 20 μl stains and 3880 pg/μl for 100 μl stains. Manufacturers usually recommend laundering synthetic fibers at 40 °C, this is why we did not include any other washing temperatures into our experimental design. Washing of synthetic fibers at 40 °C resulted in decreased DNA yields for both, 20 and 100 μl samples. Compared to cotton traces that were washed at 40 °C and in contrast to the results of blood stained cloths, synthetic DNA retention rates were higher.

Double washing of cotton and synthetic cloths reduced the DNA amounts further for both volumes (Fig. 7—D1+D2).

The influence of the storage interval of 30 days on saliva traces in cotton showed that after laundering at 60 °C, DNA amounts increased for both volumes compared to samples that have been stored only overnight before washing (Fig. 7—E). The quantification results of the additional washing procedure by washer 2 indicated that DNA amounts of saliva samples decreased for 20 and 100 μl volume samples (Fig. 8a+b).

In total, reportable profiles were typed for 25 of 48 (52.1%) saliva traces. Eighty percent of the reportable profiles resulted from 100 μl volume samples. Informative profiles were generated by 20 μl as well as 100 μl volume samples. Altogether, 16 of 48 (33.3%) samples contained enough information for inclusion or exclusion of suspects. Only 14.6% yielded no informative profiles. Those profiles contained only data from 20 μl volume samples (Table 4). Differences were observed between samples that have been washed by washer 1 and washer 2. Informative profiles of 20 μl samples (of washer 1) were obtained in 75%, while 20 μl volume samples of washer 2 were only informative in 25%. Also, 100 μl volume samples of washer 2 exhibited qualitatively inferior STR profiles, where 75% resulted in informative, but not reportable profiles. In comparison, after washing by washer 1 all samples resulted in full, reportable profiles. Six transfer control samples were conducted during the experiments with saliva traces. Although up to 13 drop-in alleles were observed in one control, none of the samples resulted in reportable or informative profiles.

Discussion

The present study investigated the potential of conventional and novel methods for BFI of blood- and saliva-spotted pieces of cloths that have been laundered under varying conditions. Besides, the applicability of STR profiling of laundered samples was assessed. The examination comprised six different washing scenarios for two distinct fabrics (cotton and synthetic fiber). Small numbers of published empirical studies have already demonstrated that STR profiling of laundered blood- and saliva-stained items is feasible [26, 27] and investigated the possibilities of secondary transfer during laundering [28]. However, none of the studies have, to our knowledge, conducted a comprehensive comparison of BFI methods including RNA profiling of differentially laundered items so far.

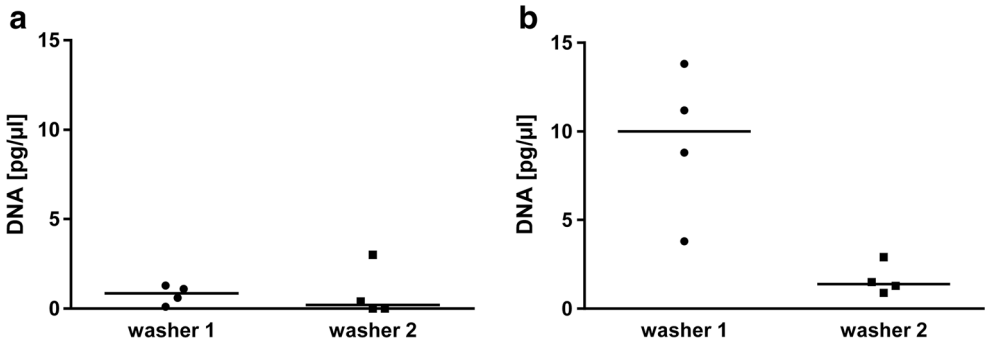

Fig. 8 Comparison of DNA amounts in pg/µl after washing of saliva-stained cotton cloths at 60 °C by washer 1 and washer 2. **a** 20 µl and **b** 100 µl volume samples. Line at median

Conventional BFI

Blood traces

Visualization and localization of crime scene related traces is a major challenge in forensic sciences. Generally, bloodstains occur frequently at crime scenes and can in principle be detected with the naked eye. Unfortunately, exposure to water or cleaning reagents dilute the blood until it cannot be detected by the naked eye anymore. In a recent publication, Edler et al. stated that it is possible to observe macroscopical residues of blood on cotton fibers after washing at different washing temperatures [27]. In our study, we were also able to detect blood stains macroscopically after laundering of cotton cloths at 40 °C and also at 60 °C by washer 1 (Fig. 2). Further, we agree with Edler et al. that fabric type and density have an effect on the retention of blood and that blood does not adhere well to smooth fabrics like polyester, because we could not observe blood residues on synthetic fiber. Contrary to our findings, Kamphausen et al. did not see any blood leftovers after washing 1.5 ml blood stains neither by hand nor by machine [28].

In cases where macroscopical examination fails to visualize blood on laundered items, chemiluminescence-based approaches like LumiScene Ultra can be used to detect latent blood traces. LumiScene Ultra is known to be one of the most sensitive methods for localization of latent blood traces. Referring to Radacher et al., LumiScene Ultra is even more sensitive than BlueStar® and LumiScene and should preferably be used for crime scene investigations [29]. In our study, application of LumiScene Ultra for blood detection resulted in a chemiluminescent reaction for 40 and 60 °C washed items; again only for cloths that have been washed by washer 1. This finding supports the results of Adair et al. [30] and Edler et al. [27] who also observed a chemiluminescence in the vast majority of their laundered samples. However, Edler et al.

Table 4 Overview of mRNA and STR profiling results for distinct experimental designs of saliva-stained cloths. * Washes of washer 1 and washer 2 were summarized

Experimental design	Sample volume [µl]	RNA quantification range [pg/µl]	RNA analysis				DNA analysis			
			mRNA				DNA quantification median [pg/µl]	STR profiling		
			STATH	HTN3	ACTB	18S rRNA		Reportable	Informative, not reportable	Not informative
A*	20	0–900	0/8	0/8	0/8	0/8	0.4	–	4/8	4/8
	100	0800–2000	0/8	0/8	0/8	0/8	3	5/8	3/8	–
B	20	0–60	0/4	0/4	0/4	0/4	1	–	4/4	–
	100	0–500	0/4	0/4	0/4	0/4	5	3/4	1/4	–
C	20	0	0/4	0/4	0/4	0/4	5	2/4	1/4	1/4
	100	0–300	0/4	0/4	0/4	0/4	34	4/4	–	–
D1	20	0	0/2	0/2	0/2	0/2	1	–	1/2	1/2
	100	670–710	0/2	0/2	0/2	0/2	4	2/2	–	–
D2	20	100–110	0/2	0/2	0/2	0/2	9	2/2	–	–
	100	100–700	0/2	0/2	0/2	0/2	11	2/2	–	–
E	20	40–100	0/4	0/4	0/4	0/4	2	1/4	2/4	1/4
	100	40–1000	0/4	0/4	0/4	0/4	16	4/4	–	–

noticed the most intense reaction at 60 °C compared to 95° and 30 °C [27]. In our study, the intensity of chemiluminescence decreased with increasing washing temperature.

During presumptive testing, striking differences have been observed between the enzymatic and chemical tests and immunochromatographic applications. The Hemastix test, which is one of the most common and simplest tests for hb detection, showed positive reactions in all samples that have been washed by washer 1. Compared to the Hemastix test, the FOB test did not react with laundered blood traces. The RSID™-Blood test showed positive reactions only sporadically. The reason for this might be that immunological assays are generally known to be less sensitive than enzymatic or chemical tests. The lowest limit of detection of the RSID™-Blood e.g. is much higher than the detection limit of Hemastix, ranging between 0.02 [31] and 0.05 μl blood [7]. This finding is concordant to Mushtaq et al. who compared four presumptive tests after hand-washing of blood-stained fabrics. Of the four tested approaches—Kastle-Meyer test, leucomalachite green, tetramethylbenzidine and Hemastix—the latter showed the highest sensitivity. Positive results were obtained for six different detergents on 12 investigated fabric types [32].

Despite its high sensitivity, the results of Hemastix and LumiScene Ultra should be treated with caution, because it is known that the presence of strong oxidants, such as chlorine-containing detergents or peroxidases e.g. from plants can cause false positive results [33–35].

Saliva traces

Since the Phadebas® test shows the highest sensitivity with a detection limit of 1000 nl [36], this method is immensely distributed in forensic case work. In our study, the Phadebas® test also exhibited the highest sensitivity compared to immunochromatographical approaches. Laundered saliva-stained cloths reacted in 50% of the samples with the Phadebas® reagent. Unfortunately, the Phadebas® test, which detects amylase, is not capable of differentiating between salivary amylases and amylases from other sources e.g. pancreatic or urinary amylases. Therefore, the application of Phadebas® can result in false positive reactions and should be interpreted with caution [34, 36].

Although the RSID™-Saliva test has a detection limit of 10 nl [36], detection failed in all laundered samples.

RNA profiling

In our study, HBB has been shown to be the most sensitive blood mRNA marker and was still detectable after laundering. HBB is a globin that makes up hemoglobin in red blood cells. Other studies already demonstrated that HBB is very sensitive and detected HBB in a minimum volume of 0.001 μl blood [24, 37–39]. The increased sensitivity compared to other blood specific mRNAs might result from the high abundance of erythrocytes in blood cells, which are around 700 times more abundant than leukocytes. Even though erythrocytes do not contain cellular DNA or mRNA themselves, their precursors, termed reticulocytes, contain mRNA and make up 1% of red blood cells [24].

Compared to the blood mRNA markers, none of the saliva specific mRNA candidates (STATH and HTN3) were detected in saliva stains after laundering. STATH encodes a calcium regulator in saliva [40] and HTN3 encodes a histidine rich protein involved in the non-immune host defense in the oral cavity [41, 42]. Generally, both markers show specific expression for saliva, are equally sensitive [24] and have a similar sensitivity to conventional BFI methods like the RSID™-Saliva test [43]. Failed detection of saliva specific mRNAs in laundered samples might occur because both markers are predominantly expressed in human parotid and sublingual-submandibular (salivary) glands [9]. Also, it is known that mRNAs that are abundant in fresh saliva are more prone to fast degradation by extracellular RNases [34]. Further, environmental studies showed that both markers exhibit an increased sensitivity to hydrolytic damage [44, 45]. In 2009, Zubakov et al. demonstrated that the detection limit of blood-specific mRNA markers in their study was lower than that of saliva-specific markers and suggested that cellular origin might be a considerable factor influencing RNA stability [46]. We agree with this observation, and suggest that blood is more resistant to laundering compared to saliva.

Even though laundering experiments have already been presented by van den Berge et al. [47], they were not able to detect RNA in blood and saliva stains of laundered items. This may be because they did not set up a systematical study covering different experimental scenarios.

The differences in mRNA detection of blood samples in both studies may also result from the distinct RNA data interpretation approaches. The NFI applies the "x = n/2" rule as described in [24] analyzing 4 replicates. In this experimental design—as BFI input was known—our interpretation is only based on the evaluation of two replicates. Samples were assigned to a certain body fluid as soon as at least one specific marker was observed.

Despite the concerns about the low stability of RNA, the long-term persistence of RNA molecules under certain circumstances and scenarios has been shown in various studies for environmentally challenged [45] or aged samples [23, 48]. With this study, we also showed that RNA is robust enough to survive laundry at 40 °C. However, because RNA profiling was limited in our study, the possibility of analyzing laundered samples with other approaches should be considered. Besides RNA profiling, BFI research also focuses on DNA methylation [17, 18], proteomic-based identification of body fluids [49, 50] and miRNA detection [11–13, 15]. Also, first results

of NGS-based solutions for mRNA detection are available [51, 52]. Applying high coverage analysis in combination with yielded targeting of so-called transcript stable regions (STaRs) may result in a more specific amplification of highly degraded transcripts [53]. Further, it is hypothesized that miRNAs might be more suitable for the analysis of challenging and degraded forensic samples due to their small (18 to 24 nucleotides) size and the stabilizing effect of Argonaute proteins [54, 55].

DNA yields and STR typing

Altogether, our findings are concordant with results of previous research studies, which showed that DNA profiling of laundered blood stains is possible [27, 28]. Like Kamphausen et al., we could show that results differed between distinct cell types [28]. Recovered DNA amounts from single-donor washed blood stains ranged from 0 to 2700 pg/μl. DNA concentrations of saliva-stained cloths after laundering were between 0 and 82 pg/μl. Ninety-eight percent of the blood stains resulted in reportable or informative DNA profiles matching the blood donors. In contrast, reportable and informative profiles of saliva stains were analyzed in 85% of all samples. Overall, saliva stains showed lower DNA amounts than blood stains, and consequently, a lower probability of typing full profiles (blood 91%; saliva 52%).

Some studies have already shown that transfer of blood particles from one item to another is possible, either by direct [56] or indirect transfer [57]. Moreover, Goray et al. showed that transfer scenarios occur more likely with wet blood stains [58]. In their examination, Kamphausen et al. proved that secondary transfer also occurs in a wet environment during laundering [28]. Receiving cloths contained full or partial profiles in almost 80% of the samples. Our findings support this observation. However, in our study, transfer controls employed in the blood experiments resulted in 57% reportable or informative profiles. The difference of the transfer rates may be explained by the limited sample amounts in our study. Because the study focuses on BFI, only transfer controls were rated as transfer scenarios. Concerning transfer of saliva scenarios, our findings are concordant with Kamphausen et al. [28]. Transfer of DNA was not observed in saliva laundering scenarios. Saliva samples are generally known to produce less complete profiles after transfer [50, 59].

To identify potential correlations of washing parameters and outcome of DNA analysis, multiple variables were adjusted during washing procedures: distinct washing machines, detergents and washing temperatures were included into the analysis. Like Andrews et al. [26], we observed higher DNA yields from cotton cloths washed at 40 °C compared to items washed at 60 °C for blood samples. Also, prolongation of storage time between deposition of traces and washing procedure seems to slightly improve DNA yields of blood as well as of saliva samples. This might be attributed to the effect, which has already been described for seminal fluid stains. The lag period can enhance the resistance of cellular material to the washing procedure, allowing the cells to be trapped deep in the fabric instead of being deposited on the surface [60]. This observation might be important since lag periods between offense and police investigation are possible and can range from hours to years.

Kamphausen et al. postulated that STR profiling of laundered samples is successful independent of the washing procedure (hand or machine) or the addition of detergent [28]. Here, we could show that despite the STR profiling success the washing detergent seems to have an influence on overall yields. DNA amounts reduced when a powder detergent was used for both washing machine types of washer 1 and 2. However, we agree with Edler et al. [27] that washing temperature does not seem to have significant influence on cleaning results.

Dependent on the brand, laundry detergents have different chemical compositions. Typically, detergents consist mainly of surfactants, water softener, bleaching agents, washing alkalis and enzymes like proteases, amylases, lipases or celluloses. In addition, some include fragrances, brighteners and stabilizers. In a comparison of 6 different detergents, Mushtaq et al. concluded that Ariel removes stains most effectively. However, their argumentation was based on results of presumptive testing, not on DNA analysis [32]. Like Persil, also Ariel is a powder detergent. Still, further experiments are required to ascertain whether powdery detergents remove stains more efficiently than liquid detergents do.

Conclusion

In the comparison of conventional and innovative methods for BFI of laundered blood and saliva traces, we could show for the first time that mRNA of the blood-specific marker HBB is robust enough to survive a laundering process of 2 h at 40 °C including a washing detergent. Compared to blood traces, RNA detection of saliva-specific markers failed for laundered saliva-stained cloths. Likewise, conventional tests showed limitations for both body fluids. Immunochromatographic assays failed to detect blood as well as saliva in all samples. Generally, enzymatic and chemical assays proved to be the most suitable applications for BFI in laundered sample. However, caution is needed in the interpretation, because both are known to have disadvantages like the lack of specificity and sample consumption. Compared to RNA profiling, those approaches are simple, cost-effective and can be performed very quickly. RNA profiling is more elaborate and cost-intensive. Interpretation of RNA profiles requires practice and long-term experience. Therefore, at this stage, we conclude

that innovative approaches for BFI cannot replace current methods for BFI, but rather supplement them. A combination of conventional and innovative methods for BFI may be a good alternative approach for the analysis of challenging case work samples. Experts could not only benefit from increasing probative value of synergized applications but also enhance their expertise in promising technologies. Even though the breakthrough solution for BFI is still not established yet, the sampling of laundered blood and saliva stains is still worthwhile, because the majority of laundered samples (98% of blood and 85% of saliva traces) can be used for individualization and incorporation of profiles into national databases.

Acknowledgements We kindly thank our blood and saliva donors for supplying samples. We also want to acknowledge Titia Sijen and Margreet van den Berge from the NFI for their support and helping hand with the establishment of mRNA analysis in our laboratory.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

1. Bond JW, Hammond C (2008) The value of DNA material recovered from crime scenes. J Forensic Sci 53:797–801. https://doi.org/10.1111/j.1556-4029.2008.00746.x
2. 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. https://doi.org/10.1016/j.forsciint.2009.02.013
3. Harbison SA, Fleming RI (2016) Forensic body fluid identification: state of the art. Res Reports Forensic Med Sci 6:11–23. https://doi.org/10.2147/RRFMS.S57994
4. Muro CK, Doty KC, de Souza FL, Lednev IK (2016) Forensic body fluid identification and differentiation by Raman spectroscopy. Forensic Chem 1:31–38. https://doi.org/10.1016/j.forc.2016.06.003
5. Li R (2015) Forensic serology. CRC Press, Boca Raton
6. Cox M (1991) A study of the sensitivity and specificity of four presumptive tests for blood. J Forensic Sci:1503–1511
7. Schweers BA, Old J, Boonlayangoor PW, Reich KA (2008) Developmental validation of a novel lateral flow strip test for rapid identification of human blood (rapid stain identification blood). Forensic Sci Int Genet 2:243–247. https://doi.org/10.1016/j.fsigen.2007.12.006
8. Bauer M, Patzelt D (2003) Protamine RNA as molecular marker for spermatozoa in semen stains. Int J Legal Med 117:175–179. https://doi.org/10.1007/s00414-002-0347-2
9. Juusola J, Ballantyne J (2005) Multiplex mRNA profiling for the identification of body fluids. Forensic Sci Int 152:1–12. https://doi.org/10.1016/j.forsciint.2005.02.020
10. 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 Sci Int Genet 3:80–88. https://doi.org/10.1016/j.fsigen.2008.11.003
11. Hanson EK, Lubenow H, Ballantyne J (2009) Identification of forensically relevant body fluids using a panel of differentially expressed microRNAs. Anal Biochem 387:303–314. https://doi.org/10.1016/j.ab.2009.01.037
12. Courts C, Madea B (2011) Specific microRNA signatures for the detection of saliva and blood in forensic bodyfluid identification. J Forensic Sci 56:1464–1470. https://doi.org/10.1111/j.1556-4029.2011.01894.x
13. Wang Z, Luo H, Pan X, Liao M, Hou Y (2012) A model for data analysis of microRNA expression in forensic body fluid identification. Forensic Sci Int Genet 6:419–423. https://doi.org/10.1016/j.fsigen.2011.08.008
14. 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 Sci Int Genet 7:116–123. https://doi.org/10.1016/j.fsigen.2012.07.006
15. Zubakov D, Boersma AWM, Choi Y, van Kuijk PF, Wiemer EA, Kayser M (2010) microRNA markers for forensic body fluid identification obtained from microarray screening and quantitative RT-PCR confirmation. Int J Legal Med 124:217–226. https://doi.org/10.1007/s00414-009-0402-3
16. Park JL, Park AM, Kwon OH, Lee HC, Kim JY, Seok HH, Lee WS, Lee SH, Kim YS, Woo KM, Kim SY (2014) Microarray screening and qRT-PCR evaluation of microRNA markers for forensic body fluid identification. Electrophoresis 35:362–3068. https://doi.org/10.1002/elps.201400075
17. Frumkin A, Wasserstrom A, Budowle B, Davidson A (2011) DNA methylation-based forensic tissue identification. Forensic Sci Int Genet 5:517–524. https://doi.org/10.1016/j.fsigen.2010.12.001
18. Lee HY, Jung SE, Lee EH, Yang WI, Shin KJ (2016) DNA methylation profiling for a confirmatory test for blood, saliva, semen, vaginal fluid and menstrual blood. Forensic Sci Int Genet 24:75–82. https://doi.org/10.1016/j.fsigen.2016.06.007
19. Poon H, Elliott J, Modler J, Frégeau C (2009) The use of Hemastix® and the subsequent lack of DNA recovery using the Promega DNA IQTM system. J Forensic Sci 54:1278–1286. https://doi.org/10.1111/j.1556-4029.2009.01173.x
20. Miranda GE, Prado FB, Delwing F, Darunge E Jr (2014) Analysis of the fluorescence of body fluids on different surfaces and times. Sci Justice 54:427–431. https://doi.org/10.1016/j.scijus.2014.10.002
21. Nelson DG, Santucci KA (2002) An alternate light source to detect semen. Acad Emerg Med 9:1045–1048
22. Vandenberg N, Orschoot RAH (2006) The use of polilight in the detection of seminal fluid, saliva, and bloodstains and comparison with conventional chemical-based screening tests. J Forensic Sci 51:361–370. https://doi.org/10.1111/j.1556-4029.2006.00065.x
23. 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 Sci Int Genet 6:565–577. https://doi.org/10.1016/j.fsigen.2012.01.009
24. Lindenbergh A, Maaskant P, Sijen T (2013) Implementation of RNA profiling in forensic casework. Forensic Sci Int Genet 7:159–166. https://doi.org/10.1016/j.fsigen.2012.09.003
25. 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 Sci Int Genet 20:19–129. https://doi.org/10.1016/j.fsigen.2015.10.011
26. Andrews C, Coquoz R (1994) PCR DNA typing of washed stains. In: Bär W, Fiori A, Rossi U (eds) Advances in forensic haemogenetics 5. Springer Verlag, Heidelberg, pp 343–345. https://doi.org/10.1007/978-3-642-78782-9_90
27. Edler C, Gehl A, Kohwagner J, Walther M, Krebs O, Augustin C, Klein A (2017) Blood trace evidence on washed textiles—a systematic approach. Int J Legal Med 131:1179–1189. https://doi.org/10.1007/s00414-017-1549-y

28. Kamphausen T, Fandel SB, Gutmann JS, Bajanowski T, Poetsch M (2015) Everything clean? Transfer of DNA traces between textiles in the washtub. Int J Legal Med 129:709–714. https://doi.org/10.1007/s00414-015-1203-5
29. Radacher M, Dunkelmann B, Höckner G, Neuhuber F, Pölzgutter E, Breksler E, Baderer D, Steinletzberger N (2011) Luminol im Vergleich mit Fluorescein und Blue Star, Blue Star Forensic Magnum im Vergleich mit Lumiscene. Kriminalistik 3:180–184
30. Adair TW, Shaw RL (2005) Enhancement of bloodstains on washed clothing using luminol and LVC reagents. I.A.B.P.A, News
31. Horjan I, Barbaric L, Mrsic G (2016) Applicability of three commercially available kits for forensic identification of blood stains. J Forensic Leg Med 38:101–105. https://doi.org/10.1016/j.jflm.2015.11.021
32. Mushtaq S, Rasool N, Firiyal S (2016) Detection of dry bloodstains on different fabrics after washing with commercially available detergents. Aust J Forensic Sci 48:87–94. https://doi.org/10.1080/00450618.2015.1029971
33. Tobe SS, Watson N, Daeid NN (2007) Evaluation of six presumptive tests for blood, their specificity, sensitivity, and effect on high molecular-weight DNA. J Forensic Sci 52:102–109. https://doi.org/10.1111/j.1556-4029.2006.00324.x
34. 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. Int J Legal Med 122:135–142. https://doi.org/10.1007/s00414-007-0182-6
35. An JH, Shin KJ, Yang WI, Lee HY (2012) Body fluid identification in forensics. BMB Rep 45:545–553
36. Pang BCM, Cheung BKK (2008) Applicability of two commercially available kits for forensic identification of saliva stains. J Forensic Sci 53:1117–1122. https://doi.org/10.1111/j.1556-4029.2008.00814.x
37. Haas C, Hanson E, Kratzer A, Bär W, Ballantyne J (2011) Selection of highly specific and sensitive mRNA biomarkers for the identification of blood. Forensic Sci Int Genet 5:449–458. https://doi.org/10.1016/j.fsigen.2010.09.006
38. Haas C, Hanson E, Anjos MJ, Banemann R, Berti A, Borges E (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. https://doi.org/10.1016/j.fsigen.2012.10.011
39. Kohlmeier F, Schneider PM (2012) Successful mRNA profiling of 23 years old blood stains. Forensic Sci Int Genet 6:274–276. https://doi.org/10.1016/j.fsigen.2011.04.007
40. Raj PA, Edgerton M, Levine MJ (1990) Salivary histatin 5: dependence of sequence, chain length, and helical conformation for candidacidal activity. J Biol Chem 265:3898–3905
41. Sabatini LM, Carlock LR, Johnson GW, Azen EA (1987) cDNA cloning and chromosomal localization (4q1 1-13) of a gene for statherin, a regulator of calcium in saliva. Am J Hum Genet 41:1048–1060
42. 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
43. Sakurada K, Ikegaya H, Fukushima H, Akutsu T, Watanabe K, Yoshino M (2009) Evaluation of mRNA-based approach for identification of saliva and semen. Legal Med 11:125–128. https://doi.org/10.1016/j.legalmed.2008.10.002
44. 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. https://doi.org/10.1111/j.1556-4029.2007.00652.x
45. Sirker M, Schneider PM, Gomes I (2016) A 17-month time course study of human RNA and DNA degradation in body fluids under dry and humid environmental conditions. Int J Legal Med 130:1431–1438. https://doi.org/10.1007/s00414-016-1373-9
46. 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. https://doi.org/10.1007/s00414-008-0249-z
47. van den Berge M, Ozcanhan G, Zijlstra SA, Lindenbergh A, Sijen T (2016) Prevalence of human cell material: DNA and RNA profiling of public and private objects and after activity scenarios. Forensic Sci Int Genet 21:81–89. doi:https://doi.org/10.1016/j.fsigen.2015.12.012
48. Nakanishi H, Hara M, Takahashi S, Takada A, Saito K (2014) Evaluation of forensic examination of extremely aged seminal stains. Legal Med 16:303–307. https://doi.org/10.1016/j.legalmed.2014.04.002
49. van Steendam K, de Ceuleneer M, Dhaenens M, van Hoofstat D, Deforce D (2013) Mass spectrometry-based proteomics as a tool to identify biological matrices in forensic sciences. Int J Legal Med 127:287–298. https://doi.org/10.1007/s00414-012-0747-x
50. Dammeier S, Nahnsen S, Veit J, Wehner F, Ueffing M, Kohlbach O (2016) Mass-spectrometry-based proteomics reveals organ-specific expression patterns to be used as forensic evidence. J Proteome Res 15:182–192. https://doi.org/10.1021/acs.jproteome.5b0070
51. Hanson E, Ingold S, Haas C, Ballantyne J (2015) Targeted multiplexed next generation RNA sequencing assay for tissue source determination of forensic samples. Forensic Sci Int Genet Supplement Series 5:e441–e443. https://doi.org/10.1016/j.fsigss.2015.09.175
52. Zubakov D, Kokmeijer I, Ralf A, Rajagopalan N, Calandro L, Wootton S, Langit R, Chang C, Lagace R, Kayser M (2015) Towards simultaneous individual and tissue identification: a proof-of-principle study on parallel sequencing of STRs, amelogenin, and mRNAs with the ion torrent PGM. Forensic Sci Int Genet 14:122–128. https://doi.org/10.1016/j.fsigen.2015.04.002
53. Lin MH, 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. https://doi.org/10.1016/j.fsigen.2015.09.012
54. Winter J, Diederichs S (2011) Argonaute proteins regulate microRNA stability: increased microRNA abundance by Argonaute proteins is due to microRNA stabilization. RNA Biol 8:1149–1157. https://doi.org/10.4161/rna.8.6.17665
55. Hutvagner G, Simard MJ (2008) Argonaute proteins: key players in RNA silencing. Nat Rev Mol Cell Biol 9:22–32. https://doi.org/10.1038/nrm2321
56. Goray M, Mitchell JR, van Oorschot RAH (2012) Evaluation of multiple transfer of DNA using mock case scenarios. Legal Med 14:40–46. https://doi.org/10.1016/j.legalmed.2011.09.006
57. Wiegand P, Heimbold C, Klein R, Immel U, Stiller D, Klintschar M (2010) Transfer of biological stains from different surfaces. Int J Legal Med 125:727–731. https://doi.org/10.1007/s00414-010-0424-x
58. Goray M, Eken E, Robert J, Mitchell RJ, van Oorschot RAH (2010) Secondary DNA transfer of biological substances under varying test conditions. Forensic Sci Int Genet 4:62–67. https://doi.org/10.1016/j.fsigen.2009.05.001
59. Phipps M, Petricevic S (2007) The tendency of individuals to transfer DNA to handled items. Forensic Sci Int 168:162–168. https://doi.org/10.1016/j.forsciint.2006.07.010
60. Brayley-Morris H, Sorrell A, Revoir AP, Meakin GE, Courts DS, Morgan RM (2015) Persistence of DNA from laundered semen stains: implications for child sex trafficking cases. Forensic Sci Int Genet 19:165–171. https://doi.org/10.1016/j.fsigen.2015.07.016