- Vom Schuss zur Spur -

Molekulargenetische Analysen von Rückschleuderspuren nach Schüssen auf biologische Ziele

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Inhaltsverzeichnis

Publikationen

Die der vorliegenden kumulativen Dissertationsschrift zugrunde liegenden Arbeiten befassen sich mit der unterschiedlichen Analysierbarkeit der verschiedenen Nukleinsäuren und deren vielseitigen Anwendung im Bereich der forensischen Molekulargenetik.
Die Publikationen sind am Institut für Rechtsmedizin des Universitätsklinikums Bonn entstanden und wurden bereits in diversen Fachzeitschriften veröffentlicht:

1. 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 (*doi*: 10.1016/j.fsigen.2015.01.006).

2. Grabmüller M, Schyma C, Euteneuer J, Madea B, Courts C (2015) "Simultaneous analysis of nuclear and mitochondrial DNA, mRNA and miRNA from backspatter from inside parts of firearms generated by shots at "triple contrast" doped ballistic models." Forensic Science, Medicine and Pathology 11(3): 365-375 (*doi*: 10.1007/s12024-015-9695-3).

3. Grabmüller M, Cachée P, Madea B, Courts C (2016) "How far does it get? — The effect of shooting distance and type of firearm on the simultaneous analysis of DNA and RNA from backspatter recovered from inside and outside surfaces of firearms." Forensic Science International 258: 11-18 (*doi*: 10.1016/j.forsciint.2015.10.030).

4. Grabmüller M, Schyma C, Madea B, Eichhorst T, Courts C (2017) "RNA/DNA co-analysis on aged bloodstains from adhesive tapes used for gunshot residue collection from hands." Forensic Science, Medicine, and Pathology 13(2): 161-169 (*doi*: 10.1007/s12024-017-9864-7).

5. Grabmüller M, Courts C, Madea B, Eichhorst T, Schyma C (2017) "RNA/DNA co-analysis from bloodstains on aged polyvinyl-alcohol gloves prepared for securing evidence from the hands of victims of fatal gunshot injuries." International Journal of Legal Medicine 132(1): 53-66 (*doi*: 10.1007/s00414-017-1687-2).

6. Grabmüller M, Madea B, Courts C "RNA degradation in up to 8 year old blood traces as a measure of time elapsed since deposition." (Das Manuskript befindet sich derzeit im Revisionsprozess beim „International Journal of Legal Medicine").

Die notwendigen Lizenzen zum Nachdruck der Publikationen in dieser Dissertation wurden von den entsprechenden Verlagen erteilt.

Des Weiteren wurden die einzelnen Publikationen auf nationalen wie auch auf internationalen Kongressen als Präsentationen bzw. Poster vorgestellt und unter anderem als „Extended Abstract“ veröffentlicht:

1. 93. Jahrestagung der Deutschen Gesellschaft für Rechtsmedizin (DGRM), September 2014, Greifswald. Präsentation: „Vergleichende Analyse von RNA-Extraktionsmethoden für die forensische Spurenartidentifikation”.

2. 24. Frühjahrstagung der Deutschen Gesellschaft für Rechtsmedizin (DGRM, Region Nord), Mai 2015, Köln. Präsentation: „Analyse von mtDNA und RNA in Rückschleuderspuren aus dem Waffeninneren nach Beschuss „Triple Contrast“ - dotierter ballistischer Modelle“.

3. 26. Weltkongress der Internationalen Gesellschaft für Forensische Genetik (ISFG), August/September 2015, Krakau. Poster: M. Grabmüller, P. Cachée, C Courts. “On the effect of shooting distance, ballistic model construction, doping and weapon type on the simultaneous analysis of DNA and RNA from backspatter recovered from inside surfaces of firearms.” Forensic Science International: Genetics Supplement Series 5 (2015) e644-e646.

4. 36. Spurenworkshop der Deutschen Gesellschaft für Rechtsmedizin (DGRM), Februar 2016, Essen. Präsentation: „RNA/DNA Ko-Analyse an gealterten Spurensicherungsfolien von Händen von Tatverdächtigen oder Opfern tödlicher Schussverletzungen“.

5. 25. Frühjahrstagung der Deutschen Gesellschaft für Rechtsmedizin (DGRM, Region Nord), Mai 2016, Rostock. Präsentation: „RNA/DNA Ko-Analyse an gealterten Spurensicherungsfolien von Händen von Tatverdächtigen oder Opfern tödlicher Schussverletzungen“.

6. 95. Jahrestagung der Deutschen Gesellschaft für Rechtsmedizin (DGRM), August 2016, Heidelberg, Präsentation: „Eine RNA/DNA Ko-Analyse aus Backspatter, asserviert von den Händen von Opfern tödlicher Schussverletzungen mittels der Polyvinylalkoholmethode”.

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Zusammenfassung

Pro Jahr werden in Deutschland mehrere Tausend Straftaten mit Schusswaffengebrauch registriert. Die Aufklärung des meist komplexen Szenarios durch eine objektive und allumfassende Rekonstruktion des entsprechenden Tathergangs ist von großem forensischem, wie auch juristischem Interesse. Die Analyse des Spurenbildes nach einem Schuss auf ein biologisches Ziel muss daher neben morphologischen und wundballistischen Befunden auch die Untersuchung von Rückschleuderspuren einschließen. Voraussetzung für eine erfolgreiche Tatrekonstruktion sind entsprechende Analysen, die sowohl Schütze und Opfer als auch die verwendete Feuerwaffe einbeziehen können.

Rückschleuderspuren, die bei einem Schuss auf ein biologisches Ziel durch wundballistische Effekte aus der Eintrittswunde retrograd zur Schussrichtung hin zur Feuerwaffe zurückgeschleudert werden, können in und an der Schusswaffe sowie am Schützen und in dessen Umgebung nachgewiesen werden. Jedoch bestimmt eine Vielzahl von verschiedenen Parametern die Entstehung und das Auftreten der Rückschleuderspuren am biologischen Ziel. Um die Variablen zur Entstehung und Verteilung der Rückschleuderspur nachvollziehen zu können, erfordert es aufwendige wissenschaftliche Studien, da nicht die Möglichkeit gegeben ist die Entstehung der Rückschleuderspur in einem einzigen Schussvorgang allumfassend zu untersuchen.

Die in dieser Dissertationsschrift zusammengefassten Studien repräsentieren eine forensisch-interdisziplinäre Analyse, die wesentliche Einzelprozesse eines (Nah-)Schussgeschehens von der Entstehung bis zur Konsolidierung einer biologischen Spur umfasst. Diese komplexe Analyse beinhaltet die Untersuchung eines geeigneten RNA-Isolationsverfahrens, die Gewinnung und Prüfung der Haltbar- und Beständigkeit verschiedener Nukleinsäuren aus dem Feuerwaffenlauf, die Untersuchung der Abhängigkeit des Spurenaufkommens von der Schussentfernung, die Analyse verschiedener Spurensicherungsmethoden von

Schussrückständen auf Händen tatbeteiligter Personen, sowie der Korrelation des RNA-Abbaus mit der vergangenen Zeit seit Spurenlegung.

Die folgenden Ergebnisse konnten im Einzelnen erarbeitet werden:

1. Keines der untersuchten RNA-Isolationsverfahren genügte allen Anforderungen einer umfassenden Ko-Extraktion von RNA und DNA. Jede dieser Methoden weist spezifische Vor- und Nachteile auf. Als Empfehlung sollten zunächst vor Beginn einer Studie die verfügbaren Methoden geprüft werden, um deren Eigenschaften explizit auf die Bedürfnisse der vorliegenden Versuchsanordnung anzupassen.

2. Die mitochondriale DNA-Analyse des „Triple Contrast"-dotierten biologischen Materials, gesammelt von Innenflächen von Schusswaffen, kann als eine alternative Methode gegenüber der Standard-STR-Genotypisierung angesehen werden, wenn lediglich unzureichende und/oder stark degradierte nukleäre DNA aus Rückschleuderspuren gewonnen werden kann.
   Die Ergebnisse unterstreichen die Nützlichkeit der „Triple Contrast"-Methode und demonstrieren darüber hinaus die Kompatibilität mit forensischer Körperflüssigkeitsidentifikation, basierend auf sparsamer Ko-Extraktion und quantitativer Analyse von körperflüssigkeitsspezifischer mRNA und miRNA.

3. Eine robuste Korrelation zwischen RNA/DNA-Ausbeute, Schussentfernung und/oder Feuerwaffentyp konnte nicht festgestellt werden. Somit kann die Menge an vorhandenen Nukleinsäuren in Rückschleuderspuren aus dem Inneren einer Schusswaffe nicht zuverlässig dazu verwendet werden, um die Schussentfernung bei einem Tathergang zu rekonstruieren.
   Dennoch konnte die Möglichkeit der erfolgreichen Gewinnung von Nukleinsäuren sowie der Trefferzonenlokalisierung anhand spezifisch exprimierter RNA aus Hirngewebe und/oder Blut bei Schussdistanzen von bis zu 15 bzw. 30 cm demonstriert werden. Diese Ergebnisse belegen, dass Rückschleuderspuren sowohl außen als auch im Inneren von Feuerwaffenoberflächen auftreten und nicht nur bei absoluten Nahschüssen als wertvolle Quelle forensischen Beweismaterials angesehen werden sollten.

4. Die von den untersuchten Spurensicherungsfolien extrahierten Nukleinsäuren wiesen im Vergleich zur PVAL-Methode zwar für eine erfolgreiche DNA-Profilierung unzureichende DNA-Quantität und -Qualität auf, gestatteten aber die Detektion blutspezifischer miRNA. Deutlich zeigte sich, dass aus der wasserlöslichen PVAL-Matrix auch nach knapp 20 Jahren Lagerung erfolgreich STR-Profile generiert werden konnten und der Nachweis von blut- und hirnspezifischer miRNA-Expression gelang.
   Die Asservierung von Spurenmaterial mittels Spurensicherungsfolien von Händen tatbeteiligter Personen bei Schusswaffendelikten zeigt langfristig nachteilige Auswirkungen auf das biologische Spurenmaterial, wohingegen die Probennahme mittels PVAL deutliche Vorteile aufweist.

5. Keiner der untersuchten mRNA-Marker wies einen stetigen und zeitlich bedingten RNA-Degradationsprozess auf, der für eine robuste Korrelation mit der seit der Deponierung des Spurenmaterials vergangenen Zeit erforderlich wäre, um letztlich eine zuverlässige Berechnung des Alters der Spur, basierend auf einer Regressionsanalyse, zu ermöglichen. Unterschiede konnten lediglich zwischen Proben die bis zu 90 Tage gealtert und solchen, die bis zu acht Jahre gealtert waren, festgestellt werden. Darüber hinaus konnte diese Methode bei realen Fallbeispielen erfolgreich angewendet werden, um deren Alter korrekt einer dieser beiden Alterskategorien zuzuordnen.
   Des Weiteren wurde ein methodisches Rahmenwerk für die quantitative Bewertung des Potentials von RNA-Kandidaten für die forensische Spurenaltersschätzung erarbeitet, das in zukünftigen Forschungsarbeiten eingesetzt werden kann.

1 Allgemeine Einleitung

Die von Edmond Locard [1] formulierte „Locard'sche Regel", die auch als „Locard'sches Austauschprinzip" bekannt ist, ist eines der wichtigsten Prinzipien der modernen Forensik. Es besagt, dass „jeder Kontakt eine Spur hinterlässt", welche das Resultat eines wechselseitigen Austausches von physischen Materialien zweier an einer Tat beteiligter Komponenten (Person, Ort, Objekt) ist [1]. Somit resultiert selbst ein flüchtiger Kontakt in einem Materialtransfer zwischen Spurenverursacher und Spurenträger. Oft ist es schwierig zu bestimmen, ob die an einem Tatort gefundenen Beweise während des Verbrechens zurückgelassen oder bereits bei früheren Interaktionen tatbeteiligter Personen hinterlassen wurden. Wenn diese in ihrer Vorgeschichte persönlichen Kontakt hatten, ist es nicht ungewöhnlich, dass Spuren eines Verdächtigen auf das Opfer gelangen oder vice versa [2]. Um bei der Verfolgung von Straftaten zu ermitteln, wie und von wem eine Spur an einen Tatort gelangt, ist jede beteiligte Ermittlungsbehörde an eine der drei hierarchisch angeordneten Interpretationsebenen gebunden [3, 4]. Zunächst muss von Sachverständigen diverser Disziplinen auf den ersten beiden Ebenen ermittelt und geklärt werden, welchen Ursprung die am Tatort gefundene Spur hat („Quellenebene") bzw. der zweiten „Handlungsebene", wie das vorhandene Spurenbild entstanden ist. Auf der dritten und letzten Ebene, der „Schuld-Ebene" obliegt die Beantwortung der Schuldfrage durch die Aufklärung des Tatmotivs dem zuständigen Gericht [3, 4]. Die wichtigsten an einem Tatort gefundenen biologischen Spuren von forensischer Relevanz sind meist sehr kleine Antragungen abgestreifter Hautzellen, Haare, Körperflüssigkeiten und/oder Gewebsfragmenten an bzw. auf Personen oder Objekten in unmittelbarer Umgebung, die einen Rückschluss auf die an einer Tat beteiligten Personen und/oder den Handlungshergang erlauben [5]. Auf der „Quellenebene" kann aus einer an einem Tatort gefundenen Spur mittels nicht-kodierender Short Tandem Repeat (STR)-Systeme, ein individualspezifisches Desoxyribonukleinsäure (DNA)-Profil erstellt werden. Die Art der Spur bzw. die in einer Mischspur enthaltenen Spurenarten können mitunter wichtige Informationen zur Rekonstruktion eines

Tatgeschehens liefern [6]. Bei Kapital-, Sexual- oder sonstigen Delikten ist die Beantwortung der Alternativfrage bedeutsam, vor allem wenn sich identische DNA-Profile aus einer Spur detektieren lassen, diese aber unterschiedlichen körperlichen Ursprungs sind, was gravierende Auswirkungen auf die Rekonstruktion des Tathergangs bzw. Handlungsablaufes haben kann [5].

Neben der forensischen Individualisierung einer Spur wurde die RNA-basierte Bestimmung von Körperflüssigkeiten in den letzten Jahren zu einem bedeutsamen Forschungsgebiet. Während Spuren, die beispielsweise Organgewebe enthalten, seltener als Körperflüssigkeiten angetroffen werden, können sie dennoch relevante, zur Tatrekonstruktion beitragende Informationen liefern. Somit stieg das Interesse auch bei Schusswaffendelikten, den körperlichen Ursprung einer Spur zu detektieren. Allerdings ist neben der Identifizierung der Spurenart bzw. der Gewebsherkunft oder die Klärung des Spurenalters etc. nicht mittels der Standard-DNA-Untersuchung zu erreichen. Durch Analyse der Ribonukleinsäure (RNA) können o.g. Fragestellungen beantwortet werden. Weiterhin konnte bereits gezeigt werden, dass die Untersuchung von RNA kompatibel und parallel zur DNA-Analyse durchführbar ist [7–13]. Damit eröffnet sich die Möglichkeit, sogar bei Minimalspuren und ohne zusätzlichen Probenverbrauch nicht nur das DNA-Profil des Spurenlegers (Individualisierung) zu erstellen, sondern auch die Spurenart zu bestimmen (Kontextualisierung).

1.1 Todesfälle durch Schusswaffen

Todesfälle durch Schusswaffen sind im rechtsmedizinischen Untersuchungsgut keine alltäglich vorkommenden Ereignisse, deren Aufklärung und Rekonstruktion dennoch stets im Fokus von wissenschaftlichem sowie öffentlichem Interesse steht [14]. Schusstodesfälle können auf drei unterschiedliche Weisen geschehen: durch ein Tötungsdelikt (Mord, Totschlag), durch Suizid oder durch einen Unfall [15].

Im Jahr 2016 betrug die Anzahl der sich in Deutschland in legalem Besitz befindlichen Feuerwaffen ca. 5,3 Millionen. Davon verteilen sich ca. 2,4 Millionen auf Jäger, Sportschützen, Sammler oder Sicherheitsunternehmen [16]. Die Anzahl der sich in Umlauf befindlichen illegalen Schusswaffen liegt nach Schätzungen des Bundesministeriums des Inneren (BMI) mit ca. 20 Millionen deutlich darüber [16]. Laut Bundeskriminalamt (BKA) wurden im selben Jahr

insgesamt 9967 Straftaten unter Verwendung von Schusswaffen begangen [17], was einen Anstieg von 967 Fällen (10,7 %) zum Vorjahr 2015 darstellt [18]. Hierbei wird zwischen „mit Schusswaffe gedroht" und „mit Schusswaffe geschossen" unterschieden. 5542 (2015: 4711) Fälle wurden registriert, bei denen es zu einer Schussabgabe auf Personen oder Objekten kam. Am häufigsten wurden Schüsse während einer kriminellen Handlung in Nordrhein-Westfalen in 921 Fällen (2015: 814), in Bayern in 860 Fällen (2015: 776) sowie in Niedersachen in 734 Fällen (2015: 708) abgegeben [17, 18].

Von den insgesamt 5542 begangenen Straftaten im Jahr 2016 wurden 158 Straftaten gegen das Leben (darunter Mord und Totschlag in Versuch und Vollendung) registriert, 28 mehr als im Vorjahr, bei denen Schusswaffen zum Einsatz kamen [17, 18]. Ob die tödlichen Schüsse von legalen oder illegalen Feuerwaffen abgegeben wurden, wird hierbei nicht unterschieden. Im Vergleich zu den Tötungsdelikten verübten im Jahr 2015 insgesamt 10078 Personen eine vorsätzliche Selbstbeschädigung, darunter 272 Suizide (2,7 %), vornehmlich von Männern (95 %), die mit einer Handfeuerwaffe sowie Gewehr, Schrotflinte oder schweren Feuerwaffe begangen wurden [19]. Darüber hinaus fehlen in Deutschland Informationen und retrospektive Studien bezüglich Inzidenz, epidemiologischer, demografischer und geografischer Verteilung. Während die Mortalitätsraten nach Feuerwaffengebrauch in Europa bzw. Ozeanien sowie Asien im internationalen Vergleich deutlich niedrig liegen [20], zählt die Sterblichkeit durch Schusswaffengebrauch neben tödlichen Verkehrsunfällen zu den häufigeren Todesursachen in den Vereinigten Staaten [21]. In einer retrospektiven Studie von 2016 untersuchten Grinshteyn & Hemenway [22] die Inzidenz von Todesfällen nach Schusswaffengebrauch des Jahres 2010. Hierbei lag die Gesamtbevölkerung der Vereinigten Staaten bei 309 Millionen Menschen. Die Gesamtbevölkerung der anderen 22 untersuchten Länder bei 664 Millionen. Dennoch entfielen 82 % aller Todesfälle durch Feuerwaffen auf die Vereinigten Staaten. Zudem lag die Rate von Mord bzw. Suizid durch Schusswaffengebrauch 25-mal bzw. acht-mal höher [22]. Im Jahr 2012 waren tödliche Verletzungen durch Feuerwaffen die Haupttodesursache bei männlichen Afroamerikanern im Alter zwischen 15 und 34 Jahren [21]. Weiterhin legten Oyetunji et al. [23] unter Einbeziehung der „National Trauma Data Bank" eine epidemiologische Auswertung von 577 pädiatrischen Schusstodesfällen aus einer zwei-Jahres Periode (2007-2008) vor. Die meisten Todesfälle durch Schusswaffen ereigneten sich demnach mit 33,6 % in häuslicher Umgebung bei denen 288 Kinder afroamerikanischer Herkunft und 102 Kinder weißen Hauttyps durch

Schusswaffen starben, davon 72,8 % vorsätzlich, 12,7 % selbstverschuldet und 8,2 % durch einen Unfall [23].

Aus diesen Zahlen wird ersichtlich, dass Untersuchungen im Zusammenhang mit Feuerwaffendelikten ein bedeutender Rang für die forensischen Wissenschaften zukommt. Wichtig ist hierbei, das meist komplexe Szenario objektiv und umfassend zu rekonstruieren um den Tathergang bewerten zu können. Die einzelnen Analysen können sowohl die Untersuchung des Opfers, des Schützen als auch die Feuerwaffe einschließen.

1.2 Forensische Ballistik

1.2.1 Forensische Untersuchungen bei Schusswaffendelikten

Die Untersuchung von Schusswaffendelikten nimmt in der forensischen Disziplin eine Sonderstellung ein. Verletzungen durch Schusswaffenprojektile gelten als Sonderform des stumpfen Traumas [5] und werden durch das mit hoher Geschwindigkeit (250 m/s – 1000 m/s) durch den Waffenlauf getriebene Projektil und dessen schädigender Einwirkung auf den Körper verursacht [5]. Im Wesentlichen wird das Ausmaß einer Schussverletzung durch physikalische Parameter des Geschosses, die Eigenschaften des penetrierten Gewebes sowie anatomische Bedingungen beeinflusst.

Die Aufklärung von Schusswaffendelikten sollte laut Gross & Kunz [24] auf den polizeilichen und rechtsmedizinischen Ermittlungen am Tatort sowie der forensisch-autoptischen Untersuchung des Verstorbenen beruhen. Die Untersuchung des Tatorts, das Auffinden und Sichern von Hülsen und Projektilen sowie biologischen Spuren ist Aufgabe der Sachverständigen des Erkennungsdienstes („Handlungsebene“). Eine systematische und detaillierte Erfassung des Leichenfundortes [25], die Lage des Verstorbenen und der Schusswaffe [25–27] unter Betrachtung der Position der Arme, Blut- und/oder Gewebsantragungen auf den Händen [27] sowie der Bekleidung sollten dokumentiert werden. Dies ist u.a. von Bedeutung, da stets die Frage zu stellen ist, ob es sich beim Leichenfundort um den Tatort handelt. Weiterhin werden hierzu kriminaltechnische Untersuchungen, die die Zuordnung des Geschosses zur Feuerwaffe sowie die Untersuchung der Hülse erlauben, durchgeführt. Die Erhebung wundballistischer Befunde an der Leiche, wie Einschuss- und

Ausschusszeichen, Schussentfernungs-, Schussrichtungs- und Schusshandbestimmung gehört zur Expertise des Rechtsmediziners. Neben seiner primären Aufgabe, der Unterscheidung zwischen einem Suizid vom einem Fremdverschulden, soll er Untersuchungen über die Wirkung des Projektils im biologischen Ziel sowie die Erkennung und Identifizierung von (nicht)-biologischen Rückständen nach Schussabgabe vornehmen. Dies kann durch Betrachtung der Einschussstelle, insbesondere der Morphologie geschehen, um zwischen einem absoluten Nahschuss, relativen Nahschuss sowie Fernschuss zu unterscheiden. Die Ausprägung der Einschussstelle hängt nicht nur von der Schussentfernung, sondern in erheblichem Maße vom verwendeten Waffentyp sowie von der eingesetzten Munition ab.

Bei der Schussabgabe wird ein Projektil nach Verbrennung des Treibsatzes aufgrund des entstehenden Drucks aus dem Waffenlauf getrieben. Dabei entstehen heiße Gase, die die Bestandteile der Vortriebsladung sowie Komponenten des Zündsatzes, dem Projektil, der Patronenhülse und der Schusswaffe selbst verdampfen und rekondensieren lassen [28–30]. Verschiedene Schusswaffen- und Patronenarten befördern die Bestandteile unterschiedlich weit und finden sich auf dem Schützen, insbesondere dessen Bekleidung und/oder Händen sowie in unmittelbarer Umgebung der Einschussstelle des Opfers wieder. Diese nicht biologischen Schussrückstände (engl. „gunshot residue“, GSR), auch Schmauch genannt, bestehen aus anorganischen metallischen Schmelzprodukten wie Blei, Barium und Antimon und organischen Kohlenstoffanteilen (Ruß) [31–34] mit einer Größe von 1 bis 150 µm [35], die meist mit bloßem Auge nicht sichtbar sind. Anhand der Verteilung und Dichte von Schmauchspuren lassen sich Rückschlüsse ziehen, ob die Person den Schuss abgegeben oder nur in Kontakt mit einer in der Umgebung befindlichen Schmauchwolke gekommen ist (Kontamination) [36]. Zur Asservierung der Schussrückstände wurden verschiedene Spurensicherungsmethoden etabliert und in kumulierende und topographische Verfahren unterteilt. Zu den kumulierenden Verfahren gehört die Sicherung mittels kleiner mit Klebefolie beschichteter Stempel („Tape-Lift“-Verfahren), das Vakuum-Lifting bei Bekleidung oder das Abtupfen der Hand mit Watte. Nachteile dieser Verfahren bestehen in unspezifischen sowie falsch-positiven Ergebnissen und erlauben lediglich einen qualitativen Nachweis der GSR-Bestandteile einer bestimmten Stelle. Wohingegen mittels topographischer Verfahren, wie Spurensicherungsfolien oder der Polyvinylalkohol (PVAL)-Methode [34] mit anschließender Rasterelektronenmikroskopie-/Röntgenmikro (REM-/EDX)-Analyse, eine realitätsgetreue Abbildung des Spurenbildes auf der Hand darstellbar ist. Mit diesen Verfahren ist es möglich, eine Aussage bezüglich einer potenziellen Schussabgabe zu treffen,

da allein der Nachweis von GSR auf der Hand einer Person noch keine Schussabgabe durch diese beweist [25, 37, 38]. In der forensischen Fallarbeit hat sich der Schmauchnachweis mittels Spurensicherungsfolien und anschließender REM-/EDX-Analyse durchgesetzt. Durchgeführte Studien zeigen, dass sich mittels Spurensicherungsfolien [39–41] und PVAL [42] biologisches Material von unterschiedlichen Oberflächen sichern und daraus DNA-Profile generieren lassen.

Finden sich Anzeichen eines absoluten Nahschusses, sollten eingehende Untersuchungen der waffenseitigen und schussbedingten Antragungen mitunter an der Schusshand, der Feuerwaffe und den tatbeteiligten Personen auf biologische Spuren durchgeführt werden. Die erstmals Ende des 19. Jahrhundert von Hans Gross erwähnte Analyse von Blutspurenmustern [43] rückte die bis dato noch unbekannte Bedeutung dieser Analyse als wichtigen Aspekt der forensischen Tatortrekonstruktion in ein neues Licht. Im Jahr 1924 untersuchte Werkgartner [44] systematisch in einer experimentellen Studie Spurenbilder, die durch Verletzungen von Mündungsabdrücken bei der Beibringung von Kontaktschüssen entstanden waren. Er konnte beobachten, dass aus der Eintrittswunde rückgeschleudertes biologisches Material wie Blut und/oder Gewebe auf die Finger der Schusshand gelangt war. Brüning [45] konnte in mehreren Fällen von Schusswaffengebrauch Fett und Gewebsteile im Laufinneren nachweisen. 1931 erläuterte Weimann [46] die forensische Bedeutung von Spuren, die aus rückgeschleudertem (biologischem) Material vornehmlich auf und in der Feuerwaffe resultieren. Drei Jahre später untersuchten Brüning und Wiethold [47] systematisch Spuren von biologischem Material auf Außen- und Innenflächen von Schusswaffen, die bei suizidalen Schüssen eingesetzt wurden.

Nach absoluten und relativen Nahschüssen auf ein biologisches Ziel können durch wundballistische Effekte biologische Materialien, u.a. Blut, Gewebepartikel sowie Knochenfragmente aus der Eintrittswunde retrograd zur Schussrichtung zurückgeschleudert werden. Dieses Phänomen wurde zunächst als „Rückschleuderspuren“ [44, 48] bekannt und später als „Backspatter“ bezeichnet [49, 50]. Neben den aus einer perforierenden Ausschusswunde in Richtung der Flugbahn des Projektils herausgeschleuderten sogenannten „Forwardspatter“, kommt Backspatterspuren eine bedeutende forensische Relevanz zur Tatrekonstruktion zu, da sie Erkenntnisse über beteiligte Personen, mögliche Erkenntnisse zur Trefferlokalisation und Korrelation mit wundballistischen Effekten ermöglichen. Das Vorkommen und die Menge an Backspatter hängt von verschiedenen Faktoren wie der Schussentfernung [46, 48, 50, 51], dem Kaliber der Waffe [52], des Waffentyps [53] sowie der

anatomischen Lage der Eintrittswunde [14] ab. Ein absoluter Nahschuss gegen den Schädel mittels einer großkalibrigen Feuerwaffe kann im Verhältnis mehr Backspatter produzieren, als ein Fernschuss mit kleinkalibriger Schusswaffe gegen den Rumpf. Rückschleuderspuren sind opferspezifisch und belegen durch ihr Vorhandensein räumliche Nähe [14]. Sie lassen sich auf der Außenseite sowie auf inneren Oberflächen der Feuerwaffe wie dem Lauf [11, 54–59], auf dem Schützen u.a. der Schusshand [37, 38, 52, 60–62] sowie Personen und Objekten in der nahen Umgebung [51] finden, wo durch Transfer des biologischen Materials des Opfers diese bestehen bleiben und für nachfolgende forensische Analysen gesichert werden können (Abbildung 1).

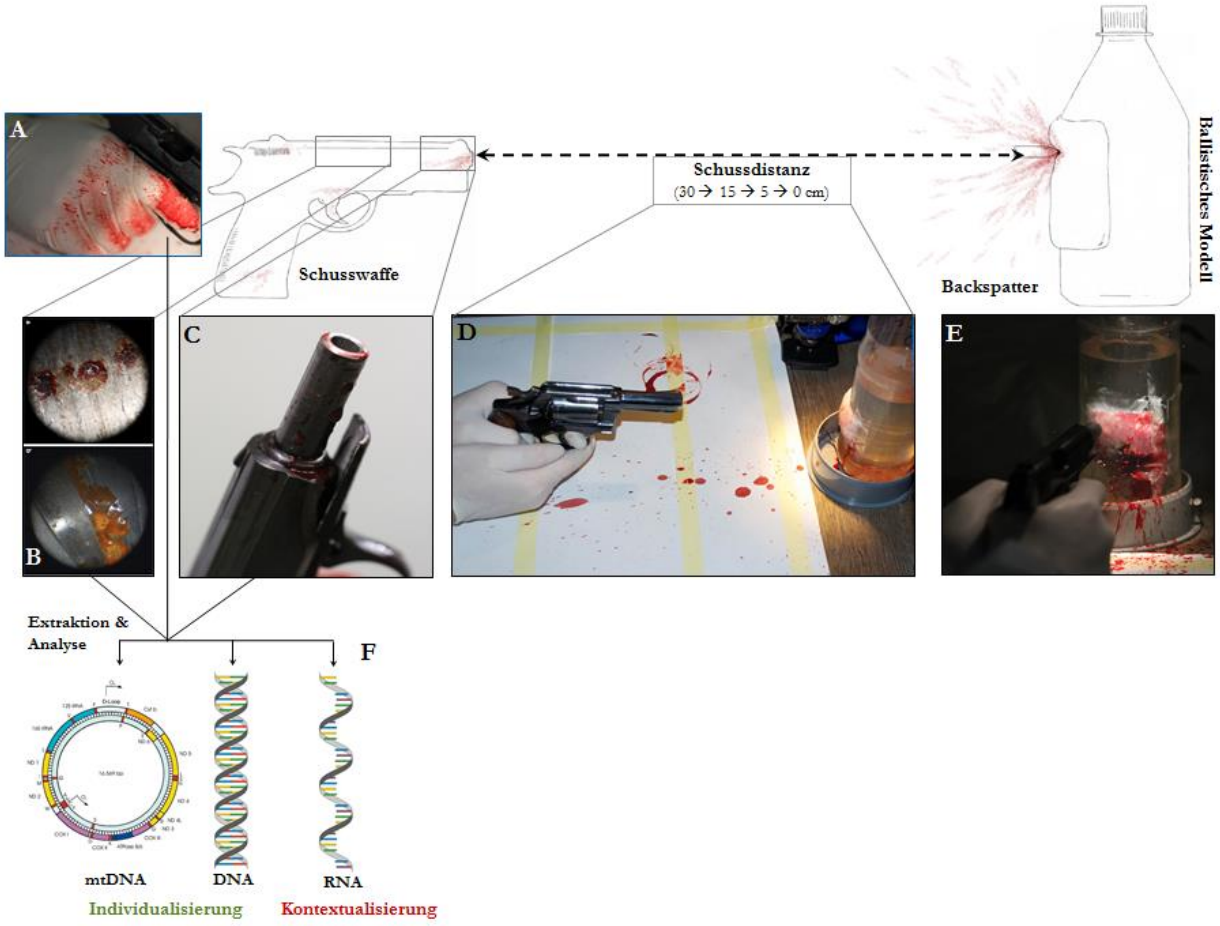

Abb. 1: Entstehung, Verteilung und Analyse von Rückschleuderspuren
A: Rückschleuderspuren an der Schusshand (absoluter Nahschuss, halbautomatische Pistole Kaliber 7.65 Browning (eigene Aufnahme)); **B: endoskopische Innenaufnahme des Feuerwaffenlaufs** (oben: Gewebespur, unten: Korrosion und Rückschleuderspuren (Bilder aus [58])); **C: Makroskopisch sichtbare Backspatter auf inneren und äußeren Oberflächen** (absoluter Nahschuss, halbautomatische Pistole Kaliber 7.65 Browning (eigene Aufnahme)); **D: Makro- und Mikrorückschleuderspuren** (Schuss aus 15 cm Entfernung, Revolver Kaliber .38 Spezial (eigene Aufnahme)); **E: Austreten von Backspatter nach Aufprall des Projektils auf das ballistische Modell** (Schuss aus 15 cm Entfernung, halbautomatische Pistole Kaliber 9 x 19 mm (eigene Aufnahme)); **F: Simultane Extraktion und Analyse von mtDNA und nDNA zur Individualisierung sowie mRNA und miRNA zur Kontextualisierung von Rückschleuderspuren** (Bilder aus [5] und [63])

Obwohl sich in der Literatur viele Veröffentlichungen zum Thema Wundballistik finden, ist jedoch die Erzeugung, Verteilung und Konsolidierung von Rückschleuderspuren nicht vollständig geklärt und systematische Forschung zu den Aspekten von Präsenz, Menge und spezifischen Mustern von Backspatter fehlt. Dennoch werden zur Entstehung, Dynamik und Verteilung drei physikalische Mechanismen diskutiert [64] wobei die Rückschleuderspuren von einer Vielzahl von ballistischen und anatomischen Parametern abhängig sind. Für die Beschleunigung von Blut- und/oder Gewebeteilchen notwendige kinetische Energie kann erstens durch den subkutanen Gaseffekt, zweitens durch sogenanntes „tail splashing" oder drittens die temporäre Kavitation erzeugt werden. In den meisten Fällen bedingt die Kombination aller drei Faktoren die Entstehung eines Backspatters.

1. Bei absoluten Nahschüssen entsteht beim *subkutanen Gaseffekt* bedingt durch die rasche Ausdehnung heißer Mündungsgase die unterhalb der elastischen Haut, insbesondere zwischen der Haut und dem darunterliegenden Knochen eingeschlossen sind, ein taschenartiger Raum, bei dessen Kollaps die Beschleunigungskraft biologisches Material in Kombination mit dem resultierenden Rückstrom des austretenden Gases aus der Eintrittswunde herausschleudert [44, 46, 48, 51, 64, 65].

2. Unmittelbar nach Eindringen eines Projektils in das Gewebe findet entlang der lateralen Oberfläche des Geschosses retrograd in Richtung der Eintrittswunde, eine konische Rückwärtsströmung von Flüssigkeit und/oder Gewebeteilchen statt („*tail splashing*") [50, 64–67].

3. Eine *temporäre* intrakranielle *Kavitation* ist das Ergebnis eines Einschusses und des damit in Zusammenhang stehenden radialen, ein nach allen Seiten Verdrängen von Gewebe und der darauffolgende Kollaps der temporären Wundhöhle. Durch den entstehenden Überdruck, der im Weichteilgewebe nach dem Eindringen eines Geschosses gebildet wird, werden Gewebeteilchen oder Flüssigkeit rückwärts herausgeschleudert [46, 64, 65, 68]. Mit Flüssigkeit gefüllte, anatomische Hohlraumstrukturen wie der Schädel, das Herz oder das Auge bieten beste Voraussetzungen für die Bildung einer starken temporären Kavitation, den Rückstoß von Gewebe und/oder Flüssigkeit und somit die Entstehung von Backspatter [64].

Um wundballistische Prozesse sowie die Entstehung von Rückschleuderspuren eines Schussgeschehens systematisch zu untersuchen, sollten experimentelle Schüsse auf ballistische Modelle erfolgen. In der Vergangenheit beschränkten sich solche experimentellen Studien meist auf die Verwendung lebender Tiere [62, 64, 65, 69, 70] oder Tierkadaver [65],

welche heutzutage aufgrund tierethischer Bedenken sowie des damit verbundenen Zeitaufwandes kaum mehr durchgeführt werden. Um diese Einschränkungen zu umgehen, haben einige Forschungsgruppen verschiedene ballistische, vornehmlich „Kopf"-Modelle etabliert [54, 56, 71–77], um die Prozesse des Schussgeschehens besser simulieren, untersuchen und nachvollziehen zu können.

Es ist offensichtlich, dass die Interpretation der Ereignisse eine umfassende Auswertung sämtlicher Befunde und Spuren unter Einbeziehung der Ermittlungsergebnisse erfordert und neben den Erkenntnissen des kriminaltechnischen Dienstes und der Rechtsmediziner auch die molekulargenetische Analyse von biologischem Material einbezogen werden sollte.

In dieser Dissertationsschrift werden vornehmlich die zentralen Aspekte der Detektion von Rückschleuderspuren aus dem Waffeninneren sowie auf der Schusshand tatbeteiligter Personen untersucht.

1.2.2 Relevanz der Nukleinsäure-Analytik in der forensischen Schusswaffenuntersuchung

Im Zusammenhang mit Schusswaffendelikten richten sich die routinemäßig durchgeführten forensischen Nukleinsäure-Analysen vornehmlich auf die Identifizierung der schussabgebenden Person. Hierzu kann durch STR-Typisierung der sich beispielsweise am Griff, am Abzug oder auf Patronen [78, 79] befindlichen DNA-haltigen (Haut-)Zellen bewiesen werden, dass die tatverdächtige Person in Verbindung zur Feuerwaffe steht. Neben dem Schützen kann mitunter die Person, die durch eine Schusswaffe verletzt oder getötet wurde, in oder an der Schusswaffe Spuren hinterlassen. Diese Rückschleuderspuren, vor allem auf inneren Oberflächen, können wertvolle forensische Beweise für eine Verbindung zwischen Feuerwaffe und Opfer sein, wenn beispielsweise der Schütze nach einem Delikt versucht, diese zu reinigen. Dennoch wurde der Aspekt, dass innere Oberflächen einer Feuerwaffe potenzielle Spurenträger für die Nukleinsäure-Analytik sein können, bis ins das Jahr 2011 vernachlässigt. Hierzu haben Courts et al. [54] erstmalig an ballistischen Modellen gezeigt, dass Rückschleuderspuren die bei absoluten Nahschüssen mit unterschiedlichen Waffentypen erzeugt wurden, ausreichend biologisches Material im Laufinneren einer Waffe hinterlassen können um ein vollständiges, zur Identifikation des

Opfers ausreichendes DNA-Profil zu erstellen. Selbst dann noch, wenn ein weiterer „Reinigungsschuss" durch dieselbe Feuerwaffe abgegeben wurde [54, 55, 57]. Erweitert und bestätigt wurde diese Untersuchungsmethode im Rahmen einer retrospektiven Studie von Suizid- und Tötungsdeliktfällen [55], bei der erste Empfehlungen zur Asservierung des Spurenmaterials aus dem Inneren von Schusswaffenläufen gegeben wurden. Des Weiteren konnten die Erkenntnisse bei der Aufklärung eines familiären Mehrfach-Mordes angewendet und dabei festgestellt werden, dass typisierbare DNA nicht nur im Laufinneren einer Feuerwaffe, sondern auch auf anderen inneren Oberflächen gefunden werden konnte [57]. Diese Ergebnisse belegen mithin, dass innere Oberflächen von Schusswaffen, insbesondere der Lauf, als wichtige und wertvolle Quelle forensischen Beweismaterials anzusehen sind. Sie legen nahe, dass die DNA trotz ungünstiger Bedingungen (z.B. Lagerungsdauer, Nachschuss) im Laufinnern überdauern und damit eine zeitliche Stabilität aufweisen kann. Allerdings beschränkten sich diese Studien lediglich auf die Untersuchung genomischer DNA (gDNA). Über die Erhaltung und Analysierbarkeit mitochondrialer DNA (mtDNA) aus dem Inneren von Schusswaffen lagen bis dato keine Erkenntnisse vor. Wenn die STR-basierte Genotypisierung aufgrund der Degradierung nukleärer DNA (nDNA) infolge der extremen Bedingungen während der Schussabgabe versagt, bietet die mtDNA-Analyse möglicherweise eine nützliche Alternative.

Neben der Zuordnung der tatbeteiligten Personen zu einem Schussgeschehen ist die Erkenntnis der Trefferlokalisation von hohem forensischem Interesse. Um einen Tathergang vollständig zu rekonstruieren, kann die Identifizierung der Spurenart/Gewebsherkunft mittels RNA-Analytik die klassischen kriminaltechnischen Untersuchungen komplementieren. Die Identifikation der Spurenart bzw. Gewebsherkunft ist bei Beteiligung mehrere Feuerwaffen und Täter von entscheidender Bedeutung, da das perforierende Geschoss und/oder eine spezifische Schusswunde des Opfers der tatbeteiligten Feuerwaffe zugeordnet werden kann [78].

Die Möglichkeit der Individualisierung der erhaltenen Blut- und Gewebespuren durch Nukleinsäure-Analyse galt es in der Folge zu überprüfen, da hierüber eine exakte Zuordnung zur Person und darüber hinaus eine mögliche Trefferlokalisation zweifelsfrei erfolgen kann.

1.3 Forensisch-molekulargenetische Methoden

1.3.1 DNA-Analytik

Nachdem Jeffreys et al. [80] im Jahr 1984 individualspezifische DNA-Muster, den sogenannten „genetischen Fingerabdruck", entdeckten, läutete dies eine neue Ära für die forensische Spurenanalytik und Abstammungsbegutachtung ein [81]. Ein Jahr später konnte in England die erste Straftat unter Einbeziehung eines DNA-Profils aufgeklärt werden. Ein weiterer Meilenstein wurde in den 90 er-Jahren von Mullis et al. [82] mit der Einführung der Polymerase-Kettenreaktion (engl. „polymerase chain reaction", PCR) gesetzt. Vor Einführung der PCR wurden große Mengen (ca. 1-2 µg) gut erhaltener hochmolekularer DNA, beispielsweise aus Blutspuren, für aufwändige Restriktionsfragmentlängen-Polymorphismus (RFLP)-Analysen benötigt [83]. Nun bestand für die forensischen Wissenschaftler die Möglichkeit mittels der PCR, in kürzerer Zeit geringere Mengen (z.B. 100 pg) an biologischem Spurenmaterial zu untersuchen und aussagekräftig zu beurteilen. Somit lässt sich durch Vergleich mittels forensischer DNA-Analyse generierter DNA-Profile eine tatrelevante biologische Spur einer Person zuordnen bzw. eine Person als Spurenleger ausschließen oder ein unbekannter Leichnam identifizieren.

Ein DNA-Profil wird durch Analyse von Short Tandem Repeat-Systeme erstellt und basiert auf dem Prinzip des Längenpolymorphismus' [84, 85]. Dies sind kurze, tandemartig hintereinander angeordnete sich wiederholende Nukleotid-Motive in nicht-kodierenden Bereichen des Genoms [86], welche sich in einer Population in der Anzahl der Wiederholungseinheiten pro DNA-System, den Allelen unterscheiden. Dadurch kann bei Kombination mehrerer unabhängiger STR-Systeme die Unterscheidung von Individuen ermöglicht werden. Forensisch relevant sind hauptsächlich DNA-Systeme mit Tetranukleotid-Repeats, also aus vier Nukleotiden bestehenden Wiederholungsmotiven, wie beispielsweise $(AATG)_n$ [86]. Zwischen Populationen verschiedener Ethnien unterscheidet sich die Häufigkeit des Vorkommens einzelner Allele (Allelfrequenzen) z.T. erheblich, wohingegen innerhalb einer Population die Allele einer Häufigkeitsverteilung unterliegen.

In der forensischen DNA-Analytik ist die kapillarelektrophoretische Analyse von angereicherten STRs die Goldstandard-Methode zur Erstellung von DNA-Profilen. In einer Multiplex-PCR-Reaktion werden simultan alle zu untersuchenden STR-Systeme in einer Reaktion amplifiziert [87–90]. Um die PCR-Fragmente gleicher Länge in der

nachfolgenden kapillarelektrophoretischen Auftrennung voneinander unterscheiden zu können, bedarf es aufeinander abgestimmte lokusspezifische Primerpaare [91, 92], wobei einer der beiden Primer mit einem Fluorophor in einer von mehreren verschiedenen Farben markiert ist [92, 93]. Bei der anschließenden fluoreszenzbasierten Fragmentlängenanalyse (Kapillarelektrophorese, CE) können Längenpolymorphismen in DNA-Fragmenten analysiert werden. Die CE beruht auf dem Prinzip der konventionellen Elektrophorese, einer trägerfreien Trennmethode, die die Allele entsprechend ihrer Größe über eine Kapillare auftrennt. Laserlicht trifft die Fragmente und damit die Fluorophore, die an das jeweilige Fragment gebunden sind. Diese werden in der jeweiligen Farbe angeregt und von einem Detektor registriert. Mit Hilfe einer speziellen Software wird aus den Rohdaten und Abgleich mit einer spezifischen Allel-Leiter sowie eines internen Längenstandards ein Elektropherogramm erstellt [85, 88, 94].

Dennoch kann die Analyse von STR-Systemen an ihre Grenzen stoßen, beispielsweise bei stark degradiertem Probenmaterial. Alternative Möglichkeiten wie die Analyse der mitochondrialen DNA bestehen, bei denen PCR-Produkte generiert werden, die für eine anschließende Sequenzierung eingesetzt werden können.

1.3.1.1 Mitochondriale DNA

Die mitochondriale DNA-Sequenzierung stellt in der Forensik eine technologische Nische dar [95] und bietet dennoch neben der klassischen STR-Analyse bei stark degradiertem und altem Untersuchungsmaterial sowie bei Proben, die keine ausreichende Menge an nukleärer DNA enthalten (z.B. Haarschaft, Knochen oder Zähne) [96–102], einen alternativen Analyseweg zur Klärung des Spurenlegers.

Das ringförmig geschlossene, 16569 Basenpaar (Bp) große, extranukleäre mtDNA-Genom kodiert 37 Gene und befindet sich im Inneren eines Mitochondriums [103]. Mitochondrien sind nur in Eukaryoten vorkommende Organellen mit Doppelmembran, die wesentliche zelluläre Funktionen ausüben. Im Vergleich zur nDNA liegt das mtDNA-Genom pro Zelle in hundert bis tausend Kopien vor [104–106], wodurch mit hoher Wahrscheinlichkeit auch unter ungünstigen Bedingungen noch intakte mtDNA-Kopien zu finden sind [84, 104, 105, 107, 108]. Zur Beantwortung forensischer Fragestellungen ist momentan ein knapp Zehntel langer Abschnitt des mtDNA-Genoms relevant. Der „displacement loop“

(D-Loop) auch Kontrollregion genannte Bereich ist eine 1,2 Kilobasen (kb) lange Sequenz der mtDNA, die überwiegend regulatorische Funktion besitzt [109]. Bei der Analyse werden spezifische Abschnitte amplifiziert und mittels Sanger-Sequenzierung Sequenzpolymorphismen, insbesondere Single-Nukleotid-Polymorphismen (SNPs) der zwei hypervariablen Regionen (HVR 1 & HVR 2) des D-Loops nachgewiesen [84]. Somit ist nicht wie bei den STR-Systemen die Länge, sondern die exakte Basensequenz von Bedeutung.

Da die mtDNA aus der Oozyte stammt, ist diese somit bei allen verwandten Personen einer mütterlichen Linie identisch. Diese spezielle Art der Vererbung (maternaler Erbgang) bedingt, dass keine Differenzierung zwischen mütterlich verwandten Personen stattfinden kann [110–112]. Im Rahmen forensischer Individualisierungsuntersuchungen werden die Sequenzierungsergebnisse mit einer erstmals von Anderson et al. [103] vorgestellten und nach Anpassungen und Korrekturen inzwischen als „revised Cambridge Reference Sequence" (rCRS) bezeichneten Konsensussequenz abgeglichen [113]. Die Unterscheide in der Basenabfolge einer sequenzierten Probe zur rCRS werden notiert und stellen den mitochondrialen Haplotypen einer Person dar. Für die biostatistische Bewertung der mtDNA-Ergebnisse wird die relative Häufigkeit der Haplotypen innerhalb einer Population mit Hilfe von Datenbanken (z.B. EMPOP) abgeschätzt und nicht wie bei den STR-Systemen, deren Allelfrequenzen multipliziert werden [114].

Da der D-Loop weniger polymorph ist, schränkt dieser Faktor seine Aussagekraft im Vergleich zu den hochvariablen STR-Systemen ein, da zufällig ausgewählte Individuen dieselben Haplotypen aufweisen können. Damit ist selbst bei vollständig übereinstimmender mtDNA zwischen Spur und Vergleichsperson eine eindeutige Zuordnung eines Spurenlegers nicht gewährleistet. Allerdings kann eine Person bei abweichenden mtDNA-Haplotypen sicher als Spurenverursacher ausgeschlossen werden. Die Anwesenheit von verschiedenen mitochondrialen DNA-Molekülen innerhalb eines Individuums wird als Heteroplasmie bezeichnet [115–117]. Hierbei können sowohl Sequenz- als auch Längen-Heteroplasmien auftreten, die den Sequenzvergleich zwischen möglichen Spurenlegern und einer Spur erschweren.

Das grundlegende Prinzip des Sequenzierungsverfahrens beruht auf der Kettenabbruchsynthese nach Sanger [118]. In vier identischen Ansätzen werden neben den üblichen Desoxynukleosidtriphosphaten (dNTPs) je eine der vier Basen als Didesoxynukleosidtriphosphat (ddNTP) zugegeben. Eine Kettenverlängerung kann nur am 3'-OH-Ende eines Nukleotids erfolgen. Die ddNTPs besitzen hierbei keine

3'-Hydroxylgruppe, die für die Anknüpfung weiterer Nukleotide notwendig ist. Aufgrund dessen kommt es im neusynthetisierten Strang an dieser Stelle zu einem Kettenabbruch bei der pro Ansatz DNA-Fragmente unterschiedlicher Länge entstehen. Erweitert und vereinfacht wurde diese Methode durch Einsatz unterschiedlicher Fluoreszenzfarbstoff markierten Didesoxynukleotide. Somit können alle vier ddNTPs in einem Reaktionsgefäß analysiert werden. Mittels Kapillarelektrophorese können die entstandenen Produkte aufgetrennt und detektiert werden. Die Basenabfolge des sequenzierten Bereiches wird im Elektropherogramm ausgegeben.

Die in einer der hier aufgeführten Studien angewandte „Mito-Mini"-Methode zur mtDNA-Analyse nach Eichmann und Parson [119] beruht auf der Amplifizierung der gesamten mitochondrialen Kontrollregion, aufgeteilt in zwei Multiplex-PCRs. Hierbei wurden jeweils fünf sich nicht überlappende Bereiche, pro Multiplex-PCR ko-amplifiziert. Die Überlappungen treten somit nur zwischen den Amplifikaten der beiden unterschiedlichen Multiplex-Reaktionen auf.

1.3.2 RNA-Analytik

In der forensischen Literatur wurde im Jahr 1984 erstmals die postmortale Synthese von RNA von Oehmichen & Zilles [120] erwähnt. Phang et al. [121] untersuchten 10 Jahre später die Genexpression in postmortalem Gewebe durch Nachweis von RNA mittels Reverser Transkriptions-PCR (RT-PCR). Mit fortschreitenden methodischen Entwicklungen im RNA-Analyseprozess, der die RNA-Extraktion, die reverse Transkription sowie die quantitative bzw. „Echtzeit"-PCR (engl. „real-time quantitative" PCR, qPCR) [122] umfasst, stieg das Interesse, RNA-analytische Methoden auch im forensischen Kontext anzuwenden [123]. Die Quantifizierung der komplementären DNA (engl. „complementary DNA", cDNA) mittels qPCR erlaubt eine exakte Bestimmung der Ausgangsmenge eines RNA-Moleküls und somit eine Aussage bezüglich des differentiellen Expressionsstatus' des Gens, das durch diese RNA repräsentiert wird.

Die geringere Stabilität und höhere Anfälligkeit für Degradation von RNA stellte die RNA-basierte Genexpressionsanalytik als forensische Routineanwendung zunächst in Frage. Spuren können durch Feuchtigkeit, UV-Exposition, hohe Temperaturen, etc. angegriffen werden, was deren Qualität (gemessen als: engl. „RNA integrity number", RIN) [124] und

dadurch die Reproduzierbarkeit von Ergebnissen mindern kann. Wider Erwarten [125, 126] zeigte sich, dass RNA-Moleküle weniger degradationsanfällig und stabiler sind als zuvor angenommen [8, 127–130]. Zudem gelang es, aus altem und degradiertem Spurenmaterial wie Blut [131–133] oder Knochen [134] eine RNA-Analyse durchzuführen. Dennoch wird davon ausgegangen, dass einzelsträngige RNA-Transkripte ab gewisser Länge anfälliger für Degradation durch physikalische und chemische Einflüsse, insbesondere ubiquitär vorkommende RNasen sind, als DNA-Moleküle vergleichbarer Länge.

Das forensische Potenzial und die vielseitigen Einsatzmöglichkeiten für Transkriptom- und Genexpressionsanalysen in der forensischen messenger RNA (mRNA)-Analytik belegen inzwischen zahlreiche Studien [127, 130, 135–143]. Im Jahr 2009 demonstrierten Hanson et al. [144] erstmalig, dass die differentielle microRNA (miRNA)-Expression zur forensisch-genetischen Identifikation von Spurenarten genutzt werden kann. Weniger relevant ist hierbei die Klärung miRNA-vermittelter Prozesse wie die der Zelldifferenzierung, Proliferation und Apoptose, vielmehr liegt der Augenmerk auf dem Vergleich differentieller Expressionsniveaus einzelner miRNAs zwischen den jeweiligen Spurenarten. Neben der Identifikation von Körperflüssigkeiten [9, 10, 133, 144–151] und Organgeweben [11, 152, 153], die in der vorliegenden Dissertationsschrift bei Untersuchungen von Schusswaffendelikten Anwendung fanden, bestehen weitere Einsatzmöglichkeiten der forensischen RNA-Analyse vor allem in Schätzungen zum Wundalter und Heilungsprozessen [154–160], Bestimmungen des Post-Mortem Intervalls (PMI) [161–169] oder des Alters einer biologischen Spur [170–176]. Des Weiteren ist die Bestimmung der Spurenablagezeit [177, 178] wie auch das Alter des Spurenlegers möglich [179, 180]. Realisierbar ist die postmortale Feststellung einer Schwangerschaft [181] sowie die Todesursache [182–192] auf molekulargenetischer Ebene abzugrenzen. Toxikogenetische Untersuchungen [193–202] können darüber hinaus die Aufklärung beispielsweise von Intoxikationen unterstützen.

Die Beweisinformationen, die aus biologischen Spuren erhalten werden, können für die ermittelnden Behörden einen Beitrag zur Rekonstruktion des Tatgeschehens leisten. Zusammenfassend kann durch die bisherigen Erkenntnisse als belegt gelten, dass RNA-Analytik im Bedarfsfall im forensischen Routinebetrieb einzusetzen ist.

1.3.2.1 Messenger RNA

Die Messenger RNA auch Boten-RNA genannt, ist das während der Transkription gebildete RNA-Transkript was die im Zellkern kodierten genetischen Information aus diesem ins Zytoplasma zu den Ribosomen, dem Ort der Proteinbiosynthese, transportiert. Sie bildet somit eine Art dynamische Zwischenstufe, die als komplementäre Abschrift eines Gens der DNA Informationen für die Synthese einer Polypeptidkette bzw. eines Proteins enthält. Die Gesamtheit aller mRNA-Moleküle einer Zelle zu einem bestimmten Zeitpunkt wird als „Transkriptom" bezeichnet, was für jede Zell- bzw. Gewebeart spezifisch ist [203], somit ist es möglich, das differenzielle Expressionsniveau verschiedener mRNAs zu messen und diese einer bestimmten Spurenart zuzuordnen. Wohingegen im Vergleich alle DNA-Moleküle in (fast) allen Zellen, bis auf wenige Abweichungen, identisch sind. Heute ist die Identifizierung von Spurenarten, insbesondere der Körperflüssigkeitsbestimmung eine etablierte und validierte Standardmethode in forensischen Laboratorien verschiedener Länder [204, 205]. Weiterführende Studien leisten einen Beitrag, um einzelne Aspekte dieser Methode zu verbessern und neue RNA-Signaturen für weitere biologische Spurenarten zu etablieren und zu validieren [206–208].

Während der Umschreibung von DNA in mRNA wird bei Eukaryoten zunächst ein unreifes Vorläufermolekül der mRNA, die sogenannte „prä-mRNA", durch die DNA-abhängige RNA-Polymerase synthetisiert [209]. Diese durchläuft bereits während der laufenden Transkription spezifische und komplexe Prozessierungsschritte, bevor die nachfolgende Translation beginnen kann. Hierzu wird im Zellkern die prä-mRNA mittels dreier Reaktionen in die reife Form der mRNA überführt. Beim sogenannten „Spleißen" (engl. „splice" = verbinden) werden die nicht-kodierenden DNA-Sequenzen (Introns) aus der prä-mRNA entfernt und die kodierenden DNA-Sequenzen (Exons) miteinander verbunden [210]. Hierbei kann es zum „alternativen Spleißen", zu Umlagerungen, Umorientierungen oder Entfernen von Exons kommen, so dass durch verschiedene Kombination der Exone für ein einzelnes Gen mehrere unterschiedliche reife mRNAs entstehen können, wodurch sich die Zahl der möglichen Proteine erhöht. Alternatives Spleißen ist die Grundlage für die Vielfalt der Proteinvarianten des Proteoms [211]. Katalysiert wird dieser Prozess von einem Ribonukleoproteinkomplex, dem „Spleißosom". Als weitere ko-transkriptionelle Modifikationen erhält die prä-mRNA beim „Capping" am 5'-Ende einen am Stickstoff-Atom methylierten Guanylrest [212] sowie bei der „Polyadenlyierung" eine Poly(A)-Sequenz aus 250–300 Adeninresten [213] an das 3'-Ende angefügt [214, 215]. Während das „Capping"

einen schnelleren und gezielten Export der reifen mRNA aus dem Zellkern ins Cytoplasma gewährleistet [216], ist die Funktion der angefügten Poly-Adenin-Sequenz noch nicht abschließend geklärt [214]. Sicher aber führt der Prozess der „Polyadenylierung“ zu einer Erhöhung der Stabilität der mRNA durch Schutz vor Degradation sowie eine Erhöhung der Translatierbarkeit [217–220].

1.3.2.2 MicroRNA

MicroRNAs bilden eine evolutionär konservierte Klasse [221–223] von kurzen, nicht-kodierenden Ribonukleinsäure-Molekülen mit einer Länge von 18 bis 24 Nukleotiden, die durch spezifische komplementäre Basenpaarung mit dem 3‘-untranslatierten Bereich (3‘-UTR) der Ziel-messengerRNA die Proteinbiosynthese regulieren [224–228]. MiRNAs agieren in einem komplexen funktionalen Netzwerk, innerhalb dessen eine miRNA bis zu Hunderte verschiedener Zielgene kontrollieren kann. Die Expression eines einzelnen Gens kann somit durch mehrere verschiedene miRNAs gleichzeitig reguliert werden [229–232]. Die erste miRNA wurde 1993 im Fadenwurm (*Caenorhabditis elegans*) entdeckt, wo die miRNA *lin-4* in einer Entwicklungsphase des Wurmes die Proteinmenge des *lin-14* herunterreguliert [233]. Als im Jahr 2000 *let-7* als weitere miRNA im Fadenwurm gefunden und in nahezu allen Metazoen nachgewiesen werden konnte [234], wurde die Bezeichnung „microRNA“ für die neue Molekülklasse im Jahr 2001 eingeführt [221–223, 225, 235, 236]. Bioinformatische Schätzungen und Analysen postulieren, dass 30-60 % des menschlichen Genoms bzw. über 60 % proteinkodierender Gene [237] durch miRNAs reguliert werden [229, 237, 238] und bis zu 1000 verschiedene miRNAs im Genom enthalten sind [226, 239, 240], wobei die Funktion vieler miRNAs bislang unbekannt ist [241]. Das miRNom, die Gesamtheit aller zu einem bestimmten Zeitpunkt in einer Zelle vorhandenen miRNAs, enthält daher vielfältige biologische Informationen [242] und Veränderungen der Expression hunderter mRNAs können potentiell durch lediglich eine oder wenige miRNAs gesteuert werden [229, 237, 243, 244]. In der Datenbank „miRBase“, sind zum Zeitpunkt der Niederschrift dieser Dissertation 1982 humane miRNA-Vorläufergene, die in 2693 reife miRNAs prozessiert werden, annotiert und katalogisiert [245–248].

Mit Ausnahme des Y-Chromosoms sind miRNA-kodierende Gene über das gesamte Genom verteilt, wobei deren Strukturierung und Aktivität sehr unterschiedlich sein kann.

Hierbei kann eine miRNA von einem eigenen Gen kodiert (monocistronisch) oder in einem Intron eines protein-kodierenden Gens lokalisiert sein (intronisch), mit welchem sie gemeinsam transkribiert wird (als sog. „MiRtron“) oder mehrere miRNAs befinden sich in einem Gencluster, aus dem sie von einem gemeinsamen Promotor transkribiert werden (polycistronisch) [221, 222, 249–251]. Hierbei kann des Weiteren zwischen intergenischen, intronischen und exonischen miRNA-Genen unterschieden werden [221–223, 249, 250]. MiRNAs weisen eine gewebsspezifische Expression auf [144, 145, 252, 253].

Die miRNA-vermittelte Regulation spielt bei zahlreichen zellulären Prozessen und Signalwegen [254–256] wie beispielsweise der Neurogenese [257, 258], der Hämatopoese [259], der Stressantwort und Differenzierung [260–262], der Entwicklung der Skelett- und Herzmuskulatur [263–265], der Immunantwort [266–268], dem Fettstoffwechsel [269], der Zellteilung und der Apoptose [270–273] eine essentielle Rolle.

Bei Säugern umfasst die Prozessierung der Vorläufermoleküle zu reifen miRNAs mehrere Schritte. Die Vorläufermoleküle werden von miR-Genen transkribiert und die RNA-Polymerase II erzeugt Vorstufen der miRNAs, auch „primary-miRNA“ (pri-miRNA) genannt, die lange Haarnadel-Strukturen enthalten [274–277]. Noch im Nukleus werden diese pri-miRNAs durch einen „Mikroprozessor“-Komplex bearbeitet. Dieser Komplex besteht aus einer RNase III (*Drosha*) und dem Doppelstrang-RNA-Bindedomäne-Protein (dsRBD) *DGCR8* [278]. Er erzeugt aus den pri-miRNAs 60 bis 110 Bp lange Haarnadel-Strukturen mit einem 2 - 3 Nukleotid langen 3‘-Überhang, die sogenannten „pre-miRNAs“ [279–283]. Anschließend werden die pre-miRNAs durch Exportin-5, welches deren 3‘-Überhang erkennt, in einem Ran-Guanosintriphosphat (Ran-GTP)-abhängigen Prozess aus dem Zellkern in das Zytoplasma transportiert [283–287]. Im Zytoplasma werden die pre-miRNAs durch *Dicer* (Ribonuklease Typ III) und das Doppelstrang-RNA-bindende Protein TRBP (engl. „trans-activation response [TAR]-RNA-binding protein“) in reife, doppelsträngige miRNAs von ca. 22 Bp Länge und nur teilweise gepaarten Basen geschnitten [288–291]. Lediglich ein Strang der reifen ds-miRNA, der „Guide“-Strang, wird aufgrund der thermodynamischen Stabilität des 5‘-Endes von einer katalytisch aktiven RNase (Argonaut-Protein, Ago2) [292, 293] in den trimeren RNA-induzierten Silencing Komplex (RISC) eingebaut [294–296]. Dieser trimere Komplex, bestehend aus *Dicer*, dem dsRBD-Protein TRBP und Ago2, wird von der miRNA durch komplementäre Basenpaarung zu ihrer Ziel-mRNA geleitet [295, 296], wobei die am 5‘-Ende der miRNA gelegenen Nukleotide an Position 2 bis 7 der sogenannten „Seed“-Region bedeutsam sind [229, 297, 298].

Der verbleibende zweite reife miRNA-Strang wird degradiert. Der tatsächliche Mechanismus der durch miRNA vermittelten Genregulation hängt vom Grad der Komplementarität zwischen der miRNA und ihrer Ziel-mRNA ab. Insbesondere von RNA-Bindeproteinen der Argonaut-Familie [290]. Eine nur teilweise bestehende Komplementarität der Bindesequenz kann zu einer Destabilisierung der mRNA durch eine Deadenylierung führen [299, 300] aber auch eine Inhibition der Translation der mRNA bewirken [301–303]. MicroRNAs besitzen unterschiedliche Expressionsmuster, die eine Vielzahl von Zielgenen regulieren. Besteht vollständige Komplementarität, so wird die mRNA enzymatisch gespalten [304–307] und nachfolgend in speziellen zytoplasmatischen Zentren, den „Verarbeitungskörpern" (P-Körpern), abgebaut (Abbildung 2) [308–311]. Die Anwesenheit der Argonaut-Proteine lässt dabei vermuten, dass durch eine RISC-vermittelte Translationshemmung mRNAs in die P-Körper transportiert werden [312]. Unter bestimmten Umständen kann neben der negativen posttranskriptionellen miRNA-Regulation eine Hochregulation der Genexpression auftreten [241, 313].

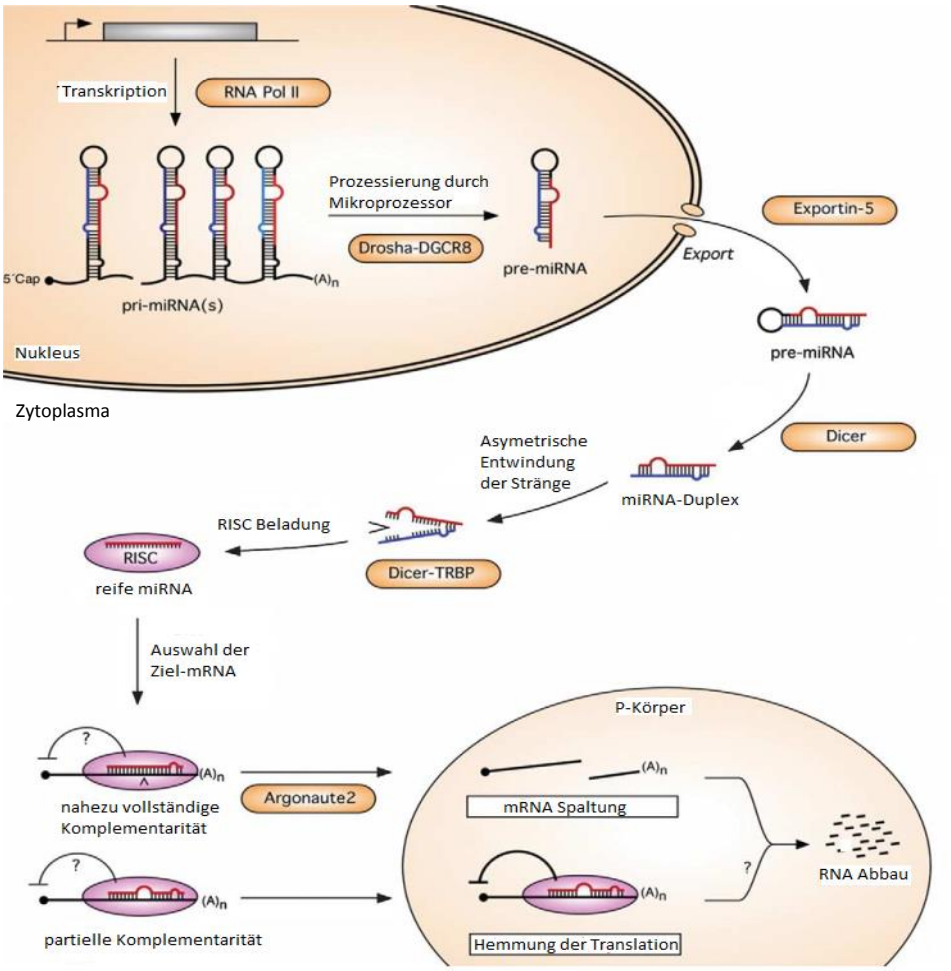

Abb. 2: Schematische Darstellung der miRNA-Biogenese (modifiziert nach [314])

Zur Bestimmung des mRNA- sowie miRNA-Expressionsniveaus, in nach Schüssen auf ballistische Modelle wie auch biologische Ziele gewonnenen Proben, wurde die für RNA-Quantifizierung als Goldstandard angesehene Methode der Reversen Transkription mit anschließender quantitativer Echtzeit-PCR unter Verwendung von TaqMan®-Hydrolysesonden eingesetzt [315–317]. Das Prinzip beruht auf der konventionellen Polymerase-Kettenreaktion und ermöglicht in Verbindung mit Fluoreszenzmessungen die Quantifizierung der gesuchten Sequenz.

Für die auf PCR basierende Analyse ist es notwendig, die RNA in komplementäre DNA umzuschreiben. Das dafür verwendete Enzym „Reverse Transkriptase" wurde ursprünglich aus Retroviren gewonnen, deren Erbinformation im Viruspartikel als RNA vorliegt. Um doppelsträngige DNA aus ihrer einzelsträngigen genomischen RNA herzustellen, verwenden Retroviren das viruseigene Enzym um im Anschluss ihre Gene mittels der „Integrase" in die DNA ihrer Wirtsorganismen zu integrieren [318, 319]. Die „Reverse Transkriptase" ist in der Lage, einen zur RNA komplementären DNA-Strang zu synthetisieren, indem es an das 3'-Ende eines an die RNA gebundenen Primers weitere Desoxynukleotide polymerisiert.

Für eine mRNA-spezifische reverse Transkription können Oligo-dT-Primer eingesetzt werden, da diese komplementär zur Poly-A-Sequenz eukaryotischer mRNA sind. Bei der Analyse von miRNA können spezifische Primer, beispielsweise sogenannte „Stem-Loop" Primer verwendet werden, die eine kurze einzelsträngige, zum 3'-Ende des betreffenden Gentranskripts (reife miRNA), komplementäre Sequenz besitzen und eine Haarnadelstruktur (engl. „stem loop") bilden [320]. Diese Primer sind aufgrund ihrer Stem-Loop Struktur als spezifisch und sensitiv für die miRNA-Analyse anzusehen. Es besteht die Annahme, dass für eine effektive RT-Reaktion die Basenpaarung der kurzen Primer innerhalb der Haarnadelstruktur die thermische Stabilität erhöht [320, 321].

Um die RNA-Expressionsniveaus vergleichen zu können, werden die RT-Produkte mittels qPCR quantifiziert. Bei einer Variante dieser Technik wird die 5'-3'-Exonukleaseaktiviät der dabei eingesetzten *Taq*-DNA-Polymerase ausgenutzt. Hierbei werden ein miRNA-spezifischer Vorwärtsprimer und ein universeller, gegen die in der Haarnadelstruktur der Stem-Loop Primer befindliche Sequenz komplementärer Rückwärtsprimer eingesetzt. Zusätzlich hybridisiert ein Oligonukleotid, eine sogenannte „Sonde", mit der Sequenz zwischen den beiden spezifischen Primern. Eine Variante dieser Sonden, die als Hydrolyse-Sonden bezeichnet werden, tragen an ihrem 5'-Ende einen Reporterfluoreszenzfarbstoff sowie

am 3'-Ende einen sogenannten „Quencher". Bei einer intakten Hydrolyse-Sonde wird durch die räumliche Nähe des Quenchers zum Fluorophor die Reporterfluoreszenzemission nahezu vollständig absorbiert (Fluoreszenz-Resonanzenergietransfer, FRET). Ist die gesuchte Zielsequenz vorhanden, lagert sich die Sonde zwischen den Bindestellen für die Amplifikationsprimer an und wird durch die 5'-3'-Exonukleaseaktivität der *Taq*-DNA-Polymerase während der Neustrangsynthese in kleine Fragmente geschnitten („hydrolysiert"). Dadurch kommt es zur Zerstörung der Sonde, wodurch der Reporterfluorophor vom Quencher getrennt wird, welcher in Folge dessen das Fluoreszenzsignal nicht mehr absorbieren kann. Die Zunahme der messbaren Reporterfluoreszenz wird nach jedem Zyklus detektiert und ist proportional zum Zuwachs der Menge an Sonden-Zielsequenzen im Reaktionsgefäß. Anhand der erhaltenen Fluoreszenzsignale kann die Quantifizierung für jede Probe-Assay-Kombination in der exponentiellen Phase am Ende jedes PCR-Zyklus' vorgenommen werden. Eine exakte Quantifizierung ist nur in dieser exponentiellen Phase möglich, da während dieses Zeitraums ein linearer Zusammenhang zwischen dem Anstieg der Fluoreszenzintensität und der Zunahme der Menge des gesuchten DNA-Moleküls besteht. Von der *Taq*-DNA-Polymerase werden nur gebundene Sonden fragmentiert, nicht an die Zielsequenz hybridisierte Sonden bleiben intakt und senden kein Fluoreszenzsignal aus. Als C_q-Wert (engl. „cycle of quantification") ist jener PCR-Zyklus definiert, bei dem das Fluoreszenzsignal erstmals einen definierten Schwellenwert erreicht. Je niedriger der erhaltene C_q-Wert, desto höher die Ausgangsmenge des sich im Reaktionsansatz befindlichen Zielmoleküls und desto früher wurde der Schwellenwert erreicht oder vice versa. Ein niedriger C_q-Wert repräsentiert somit ein hohes Expressionsniveau. Von den erhaltenen C_q-Werten kann rechnerisch auf die exakte Ausgangsmenge des Zielmoleküls rückgeschlossen werden.

Um die Experimente der in dieser Dissertationsschrift aufgeführten Studien unabhängig und reproduzierbar durchzuführen, fanden die von Bustin et al. 2009 [322] formulierten Mindestanforderungen für die Dokumentation von qPCR-Experimenten, die MIQE-Richtlinien (engl. „minimum information for publication of qualitative real-time PCR experiments"), Anwendung [321, 323, 324]. Es ist erforderlich, eine strenge Standardisierung des Versuchsablaufs einzuhalten, sowie eine sachgerechte Normalisierung der erhaltenen Expressionsdaten vorzunehmen, um einen korrekten, unverfälschten Vergleich der relativen Expression von Ziel-miRNAs in mehreren Proben zu gewährleisten und dabei die Einflüsse nicht-biologischer Störvariablen zu eliminieren [242, 325].

2 Ziele

In der forensischen Wissenschaft kommt den Untersuchungen von Straftaten im Zusammenhang mit Schusswaffengebrauch ein bedeutender Rang zu. Für eine in ihrer Gesamtheit evidenzbasierte sowie detaillierte Charakterisierung entstandener Spurenbilder, als Voraussetzung für die objektive Rekonstruktion von Tathergängen, müssen vielseitige Aspekte zur Bewertung eines meist komplexen Szenarios berücksichtigt werden. Die Untersuchung und Interpretation des Backspatters, der u.a. bei absoluten sowie relativen Nahschüssen entsteht, können unabhängige Erkenntnisse hinsichtlich des Opfers, des Schützen, der Feuerwaffe sowie eine eindeutige Verbindung der Waffe mit Opfer(n) und Schütze liefern. Hierzu zählen die DNA-basierte Individualisierung spurengebender Personen und die RNA-vermittelte Kontextualisierung der Spur etwa zu ihrem körperlichen bzw. geweblichen Ursprung. Über die Gewinnung und Analysierbarkeit von RNA aus dem Waffeninneren unter den Aspekten der Haltbarkeit, der Schussentfernung sowie des Spurenalters gibt es jedoch bis dato keine systematischen Erkenntnisse.

Die Ziele, der in dieser Dissertationsschrift zusammengefassten Studien, bestanden daher in der transdisziplinären Untersuchung und Bewertung einzelner Aspekte und Abläufe rund um die Entstehung und Konsolidierung forensisch relevanter Spuren nach Schüssen mit einer Feuerwaffe auf ein biologisches Ziel.

Neben wissenschaftlich validierten Erkenntnissen sollten darüber hinaus praxisfähige Empfehlungen für den polizeilichen Routinebetrieb, insbesondere den kriminaltechnischen Erkennungsdienst zur Spurensicherung am Tatort sowie für forensisch-wissenschaftliches Laborpersonal zur erfolgreichen Sicherung, optimalen Lagerung und Analyse des Beweismaterials bei Feuerwaffendelikten erarbeitet werden.

Folgende Fragestellungen sollen bearbeitet werden:

1. Existiert eine auf forensische Bedürfnisse optimal abgestimmte Methode zur Ko-Extraktion von RNA und DNA, die vor allem bei Minimalspuren ausreichende Ergebnisse ermöglicht? Hierzu wurden für fünf kommerziell erhältliche RNA-Extraktionskits vergleichende Untersuchungen an Minimalproben von forensisch relevanten Körperflüssigkeiten durchgeführt.

2. Sind aus mittels der zuvor entwickelten „Triple Contrast"-Methode dotierten ballistischen Modellen nach Schüssen gewonnene biologische Spuren in forensisch-molekulargenetischen Folgeuntersuchungen auswertbar?
   Zu diesem Zweck wurde der Einsatznutzen der „Triple Contrast"-Methode evaluiert, indem ihre Kompatibilität mit der forensischen Analyse nukleärer und mitochondrialer DNA geprüft wurde. Anschließend wurde die Anwendbarkeit simultan extrahierter mRNA und miRNA aus Backspatter von inneren Oberflächen verschiedener Feuerwaffentypen nach experimentellen Schüssen auf ballistische Modelle geprüft.

3. Welchen Einfluss weißt die Schussentfernung auf die Menge und Analysierbarkeit von Nukleinsäuren aus Backspatterspuren im Inneren von Schusswaffen auf und besteht die Möglichkeit der Spurenartidentifikation bzw. Trefferzonenlokalisierung?
   Systematisch wurde hierzu das Spurenaufkommen auf den Innenflächen verschiedener Typen von Feuerwaffen nach Schüssen auf ballistische Modelle aus unterschiedlichen Entfernungen untersucht.

4. Sind verschiedene topographische Spurensicherungsmethoden (Spurensicherungsfolien oder PVAL-Methode), gleichermaßen geeignet Schussrückstände zu charakterisieren sowie einen diskriminierenden Informationsgewinn aus rückgeschleudertem biologischem Material an der Schusshand zu ermöglichen?
   Dafür wurde systematisch die Anwendbarkeit der Nukleinsäure-Analytik an bis zu 20 Jahre altem Spurenmaterial von den Händen tatbeteiligter Personen aus Schusswaffendelikten untersucht, welches mittels o.g. Methoden gesichert wurde.

5. Können geeignete RNA-Marker zur Bestimmung der seit Spurenlegung vergangenen Zeit mittels der Quantifizierung differentieller RNA-Degradation etabliert werden?
   Über eine zeitabhängige Messreihe sowie anhand von Proben aus Realfällen wurde die Degradation verschiedene RNA-Marker quantifiziert und auf Korrelation zum Probenalter geprüft.

Die sich aus den einzelnen Fragestellungen ergänzenden und/oder bestätigenden experimentellen Ergebnisse können einen synergistisches Effekt begründen, der letztlich durch die Integration der Analysen-Ergebnisse, nach einem Schusswaffengebrauch, einen höheren Beweiswert generieren kann, als in Summe jede der Einzeluntersuchungen erzielt.

3 Vergleich verschiedener Extraktions- und Quantifizierungsmethoden für die forensische RNA-Analyse

3.1 Einleitung

Bei der Begehung eines Gewaltverbrechens wird biologisches Material zwischen dem Opfer und dem Täter übertragen sowie am Tatort hinterlassen [1]. Solches im Rahmen von Ermittlungen vorfindliches Spurenmaterial kann heterogene Mischungen verschiedener Körperflüssigkeiten wie Blut, Speichel, Sperma, Vaginalsekret und Menstruationsblut umfassen. Routinemäßig wird die DNA, aufgrund ihrer Robustheit und hoher Differenzierbarkeit zwischen Individuen, aus diesem Spurenmaterial isoliert. Daher ist die DNA-basierte Identifikation von Personen sowie die Zuordnung von Tatverdächtigen, Opfern und Tatorten von Bedeutung, erlaubt jedoch nicht die Bestimmung des vorhandenen biologischen Materials.

Ein RNA-Isolationsverfahren, welches den Ansprüchen an die forensisch-molekulargenetische Routinearbeit, deren vornehmliches Interesse sich in der Regel auf die Analyse von DNA richtet, genügen soll, muss mithin die zusätzliche und simultane Extraktion von DNA aus derselben Spur ermöglichen, um aus dem vorhandenen Material die Fragen nach der Urheberschaft (Individualisierung) und nach der Spurenart (Kontextualisierung) beantworten zu können. Einige Studien befassen sich bereits mit forensischer RNA/DNA Ko-Extraktion [7–13] und deren Zweckmäßigkeit und Anwendbarkeit von standardmäßig ausgestatteten forensischen Routinelabors wurde bereits in mehrfach durchgeführten internationalen Ringversuchen belegt [326–331].

Für eine Untersuchung im forensischen Kontext, in dem häufig umweltexponiertes, gealtertes und/oder degradiertes Probenmaterial auftritt, wurde die RNA lange als zu instabil und degradationsanfällig angesehen [125, 126]. Mehrere Studien im letzten Jahrzehnt haben diese Ansicht im Bereich der forensischen Molekulargenetik jedoch relativiert [8, 127–130].

Doch während für die DNA-Analytik spezifische, auf forensische Bedürfnisse und Bedingungen angepasste kommerziell erhältliche Extraktions-Kits zur Verfügung stehen, existieren bis dato und trotz des steigenden Bedarfs angesichts der verschiedenen Anwendungsmöglichkeiten keine speziell für RNA-Moleküle optimierte Isolationsverfahren, die die Eigenschaften und Probleme von forensischem Probenmaterial (extrem geringe Mengen, Umweltexposition, UV-Bestrahlung, feuchte Lagerung, Kontamination mit Inhibitoren etc.) berücksichtigen und keine Studie, die vorhandene Verfahren einem systematischen Vergleich unterzieht. Für den Praktiker ist die Wahl des geeigneten RNA-Isolationsverfahren von wesentlicher Bedeutung. Die Ergebnisse nachfolgender Analysen hängen maßgeblich von der Ausbeute und Qualität der isolierten RNA-Moleküle ab. Dies gilt, insbesondere wenn wie im forensischen Kontext mit Spuren geringer Menge und/oder reduzierter Qualität gearbeitet wird. Hierbei sollte ein Isolationsverfahren verfügbar sein, das aus geringen Mengen Spurenmaterial beide Fragestellungen nach Ursprung und Spurenart beantworten kann. In Anbetracht der nachfolgenden Studien, bei denen forensisches Probenmaterial u.a. aus Realfällen einbezogen, sowie Schüsse auf experimentelle ballistische Modelle abgegeben wurden und hierbei mit weitaus geringeren Nukleinsäure-Ausbeuten als bei Probenmaterial beispielsweise der Diagnostik gerechnet werden musste, sollte zunächst die für diese spezielle Fragestellung bestgeeignete Extraktionsmethode gefunden werden, die die Ko-Extraktion von RNA und DNA in ausreichender Quantität und Qualität gestattet.

In der vorliegenden Originalpublikation werden die Ergebnisse der ersten vergleichenden Evaluation von fünf verschiedenen kommerziell verfügbaren Extraktionsverfahren präsentiert ((1) mirVana™ miRNA Isolation Kit, (2) Trizol® Reagent, (3) NucleoSpin® miRNA Kit, (4) AllPrep DNA/RNA Mini Kit und (5) RNeasy® Mini Kit). Die Evaluation umfasste die Messung der Quantität und Qualität isolierter mRNA bzw. miRNA aus forensisch relevanten Spurenmaterialien wie getrockneten und gealterten Blut-, Speichel-, Mundschleimhaut- und Spermaproben, die Überprüfung von deren Eignung für differenzielle Expressionsmessungen sowie die Überprüfung ihrer Kompatibilität der Ko-Extraktion von RNA/DNA die für STR-Genotypisierung.

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Comparative evaluation of different extraction and quantification methods for forensic RNA analysis

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ABSTRACT

Since about 2005, there is increasing interest in forensic RNA analysis whose versatility may very favorably complement traditional DNA profiling in forensic casework. There is, however, no method available specifically dedicated for extraction of RNA from forensically relevant sample material.

In this study we compared five commercially available and commonly used RNA extraction kits and methods (mirVana™ miRNA Isolation Kit Ambion; Trizol® Reagent, Invitrogen; NucleoSpin® miRNA Kit Macherey-Nagel; AllPrep DNA/RNA Mini Kit and RNeasy® Mini Kit both Qiagen) to assess their relative effectiveness of yielding RNA of good quality and their compatibility with co-extraction of DNA amenable to STR profiling.

We set up samples of small amounts of dried blood, liquid saliva, semen and buccal mucosa that were aged for different time intervals for co-extraction of RNA and DNA. RNA quality was assessed by determination of 'RNA integrity number' (RIN) and quantitative PCR based expression analysis. DNA quality was assessed via monitoring STR typing success rates.

By comparison, the different methods exhibited considerable differences between RNA and DNA yields, RNA quality values and expression levels, and STR profiling success, with the AllPrep DNA/RNA Mini Kit and the NucleoSpin® miRNA Kit excelling at DNA co-extraction and RNA results, respectively.

Overall, there was no 'best' method to satisfy all demands of comprehensible co-analysis of RNA and DNA and it appears that each method has specific merits and flaws. We recommend to cautiously choose from available methods and align its characteristics with the needs of the experimental setting at hand.

1. Introduction

RNA was first mentioned in forensic literature by Oehmichen et al. in 1984 reporting on post mortal biosynthesis of DNA and RNA [1]. Ten years later, Phang et al. described the reverse transcription (RT) and PCR analysis of RNA from post mortem tissues [2]. Since the development of quantitative or 'real time' PCR [3] in 1996 enabling an exact determination of mRNA quantities, an upsurge of interest in RNA and transcriptomic analyses ensued that also entrained the forensic science community.

Today, numerous studies document the versatility of transcriptome and gene expression analyses in the processing of remarkably diverse forensic problems the most prominent being body fluid identification (BFI) based on differentially expressed mRNA [4–8] and micro-RNA (miRNA) [9–13]. Indeed, several international trials of RNA based forensic BFI have already been conducted with encouraging results [14–18] and a practicable procedure for the implementation of RNA in routine casework has been proposed [19].

Other forensic problems that were tackled utilizing RNA analysis comprised the estimation of wound age [20–22] and post mortem interval [23–25], the discrimination of potential causes of death [26,27], toxico-genetic analyses [28,29], the forensic entomologic assessment of fly development [30,31], and the post mortal detection of pregnancy [32].

Despite the rising interest in different applications of RNA analysis and whereas several kits for DNA extraction specifically adapted to forensic needs and conditions are commercially available (e.g., PrepFiler® Forensic DNA Extraction Kit by Life Technologies, QIAamp™ DNA Investigator Kit by Qiagen, and NucleoSpin® DNA Trace by Macherey-Nagel), there is as yet and to the best of our knowledge no RNA extraction method especially dedicated to forensic samples and circumstances. Thus, a plethora of different methodologies to extract and quantify RNA is represented in the literature concerning the application of forensic RNA analysis.

In the present work we present the results of the first comparative assessment of five different commonly applied

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methods of mRNA/miRNA extraction from forensically relevant materials i.e., dried and aged samples of blood, saliva, buccal mucosa and semen.

2. Material and methods

2.1. Sample collection and preparation

For this study, three different body fluids (EDTA-free blood drawn by venipuncture, liquid saliva and buccal mucosa) were obtained from five healthy Caucasian individuals (four females, one male, ranging from 24 to 36 years of age). In addition, samples of freshly ejaculated semen were provided by five men (ranging from 25 to 45 years of age) and collected in sealed plastic tubes that were stored at −80 °C until use. All volunteers provided informed consent and the study protocol was reviewed and approved by the ethics committee of the Hospital of the University of Bonn. For all volunteers DNA profiles had been generated in advance for comparison.

All body fluids per individual were collected within a 1 h interval and transferred to DNA-free forensic swabs (Sarstedt AG & Co., Nümbrecht, Germany) within 30 s. 3 μl aliquots of blood, liquid saliva and fresh semen were pipetted onto cotton swabs. Samples of buccal mucosa were obtained with forensic swabs applying the common sampling procedure. All samples were dried and used on the same day or after storage (dark, room temperature) for five or fourteen days.

2.2. RNA extraction

The following five commercial RNA extraction kits were used on EDTA-free blood, liquid saliva, fresh semen and buccal mucosa samples, according to the manufacturer's instructions with slight modifications (Fig. 1, upper half).

(1) mirVana™ miRNA Isolation Kit (Ambion, Austin, TX, USA).
(2) Trizol® Reagent (Invitrogen, Darmstadt, Germany).
(3) NucleoSpin® miRNA Kit (Macherey-Nagel, Düren, Germany).
(4) AllPrep DNA/RNA Mini Kit (Qiagen, Hilden, Germany).
(5) RNeasy® Mini Kit (Qiagen).

Before RNA extraction was performed all surfaces and devices utilized in the process were thoroughly cleansed using RNase-Zap® (Ambion) and Roti®-Nukleinsäurefrei (Carl Roth, Karlsruhe, Germany) to remove all traces of ambient RNases and nucleid acid contaminations. Only RNase-free reagents and plastic consumables were used.

Prior to all extractions, swabs (whole cotton tip or approximately 2 cm^2) were cut into small pieces with scissors and NucleoSpin® Forensic Filters (Macherey-Nagel) were used to separate lysate from discardable solid material by centrifugation at maximum speed (21,000 × g) for 3 min after incubation with lysis buffer.

Total RNA containing small RNA was extracted from all kinds of samples using (1) the mirVana™ miRNA Isolation Kit.

The samples were incubated with 350 μl Lysis Buffer at 56 °C for 1 h. The RNA was eluted in 30 μl of nuclease-free water pre-heated to 95 °C.

Using (2) the Trizol® Reagent total RNA was extracted from all kinds of samples and total RNA was eluted in 50 μl of nuclease-free water.

Total RNA including small RNAs was extracted from all kinds of samples using (3) the NucleoSpin® miRNA Kit. Total RNA was eluted in 30 μl of nuclease-free water pre-heated to 90 °C.

The (4) AllPrep DNA/RNA Mini and (5) RNeasy® Mini Kits were used to extract total RNA from all kinds of samples. 350 μl Buffer RLT Plus containing 3.5 μl 14.3 M 2-mercaptoethanol was added to each sample then incubated at 56 °C for 3 h. Total RNA was eluted in 30 μl of nuclease-free water pre-heated to 95 °C.

All RNA extracts were stored at −80 °C until further processing. Potential contaminant genomic DNA was removed by subsequent DNase I digestion provided by TURBO DNA-free™ Kit (Ambion), for all extraction methods, except (3), following the manufacturer's protocol.

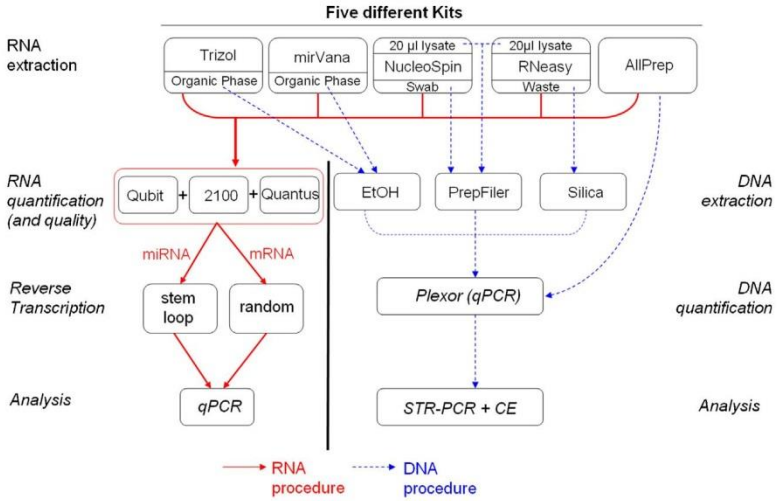

Fig. 1. Schematic representation of RNA and DNA procedures in this study.
2100: Agilent 2100 Bioanalyzer using the RNA 6000 Nano and Pico kits; organic phase: the organic phase containing the DNA remains after phase separation and removal of the aqueous phase; waste: the first remaining flow through from the RNeasy® spin column; swab: the sample swab remaining in the NucleoSpin® Forensic Filter after application of lysis buffer and centrifugation; stem loop: miRNA specific RT protocol using TaqMan® stem loop primers; random: non-specific reverse transcription using random priming; EtOH: extraction by ethanolic precipitation; Silica: using QIAquick Spin Columns; STR-PCR + CE: STR multiplex PCR using the PowerPlex® 17 ESX kit and subsequent capillary electrophoresis (CE); qPCR: quantitative PCR to measure mRNA/miRNA expression level; solid arrow: RNA procedure; dashed arrow: DNA procedure. Note: RNA input amounts for Reverse Transcription were based solely on Quantus measurements.

2.3. RNA quantification and integrity assessment

Total RNA quantity was measured by four methods:

(I) Quant-iT™ RNA Assay Kit on a Qubit® fluorometer (both Invitrogen),

(IIa) the RNA 6000 Nano Kit and (IIb) the RNA 6000 Pico Kit both on a Agilent 2100 Bioanalyzer (all three Agilent, Böblingen, Germany) and

(III) QuantiFluor® RNA Dye on a Quantus™ Fluorometer (both Promega, Mannheim, Germany).

For all kits, 1 μl of RNA extract was used and all measurements were performed according to the manufacturers' recommendations.

RNA quality, represented by the RNA integrity number (RIN) [33] was assessed using the 2100 Bioanalyzer and both the RNA 6000 Nano and RNA 6000 Pico Kit compliant to provided protocols.

2.4. Reverse transcription and qPCR

Reverse transcription (RT) of RNA to generate complementary DNA (cDNA) was performed using the High Capacity Reverse Transcription Kit with random hexamer primers for mRNA and the TaqMan® MicroRNA Reverse Transcription Kit with target-specific stem-loop primers for miRNAs (both Life Technologies, Darmstadt, Germany) according to manufacturer's instructions and in a total volume of 20 μl and 15 μl, respectively. Each mRNA RT-reaction contained 20 ng of total RNA (as determined by Quantus™ method (III)), 1 × RT Buffer, 100 mM dNTPs, 1 × Random Primers and 50 U MultiScribe™ Reverse Transcriptase.

Each miRNA RT reaction contained 10 ng of total RNA (as determined by Quantus™ method (III)), 1 × RT Primers, 50 U MultiScribe™ Reverse Transcriptase, 1 mM dNTPs, 3.8 U RNase Inhibitor, and 1 × RT buffer.

All RT-reactions were performed on a T3 Thermocycler (Biometra, Göttingen, Germany) with the following temperature profile: for RT of mRNA: 25 °C for 10 min, 37 °C for 120 min, and 85 °C for 5 min. For RT of miRNA: 16 °C for 30 min, 42 °C for 30 min, and 85 °C for 5 min.

To assess potential contamination with genomic DNA, RNA extraction negative controls (i.e., empty swab), RT(−)-controls (i.e., RT reaction without reverse transcriptase) and H_2O controls (i.e., RT reaction with water instead of RNA template) were set up. RT reaction products were stored at −20 °C.

For mRNA, qPCR was performed using a target-specific TaqMan® Assay for β-Actin which is commonly used as a reference gene (Supplementary Table 1) and the TaqMan® Universal PCR Master Mix No AmpErase® UNG (both Life Technologies), according to manufacturer's protocol. 4 μl of the corresponding RT reaction product were used in each qPCR with 1 × TaqMan® Universal PCR Master Mix and 1 × specific TaqMan® Assay adding up to a total reaction volume of 20 μl.

For miRNA, qPCR reactions were performed using three target-specific TaqMan® Assays commonly used as reference genes (Supplementary Table 1) and the TaqMan® Universal PCR Master Mix II, No AmpErase® UNG (both Life Technologies) as per manufacturer's protocol: Each 20 μl reaction contained 1.3 μl of the appropriate RT reaction product, 1 × TaqMan® Universal PCR Master Mix and 1 × specific TaqMan® Assay.

All sample/assay combinations were run in duplicates, both for mRNA and miRNA. QPCR cycling conditions: 95 °C for 10 min, followed by 40 cycles with 95 °C for 15 s and 60 °C for 1 min performed on an ABI Prism 7500 (Life Technologies).

Data collection took place during the 60 °C step by the SDS software version 1.2.3 (Life Technologies). Cycle of quantification (C_q) measurements were automatically determined by the SDS software and were exported for additional analysis. A C_q value of <35 was considered to indicate successful PCR and thus implied RNA quality suitable for expression analysis.

To facilitate reliable and unequivocal interpretation of the qPCR results reported herein, all information that is rated 'essential' according to the MIQE guidelines [37] is reported, where applicable.

All RNA procedures following extraction (quantification, reverse transcription and analysis) are summarized in Fig. 1 (lower half, left side).

2.5. DNA co-extraction, quantification and STR profiling

Co-extraction of DNA was performed for all sample types utilizing different methods depending on the precedent RNA extraction method.

Firstly, DNA was extracted for selected samples from the organic phases left over after the phase separation steps in (1) and (2) according to the Trizol® Reagent DNA isolation procedure based on ethanolic precipitation. Secondly, DNA was extracted from the flow through waste generated in the first centrifugation step from (5) using the silica column based QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's instructions.

Thirdly, DNA was extracted from the swab material left over after lysis from (3) using the PrepFiler® Forensic DNA Extraction Kit (Life Technologies).

Fourthly, DNA was extracted from 20 μl of lysate that had been diverted from (3) and (5) after incubation, using the PrepFiler® Forensic DNA Extraction Kit according to manufacturer's prescription.

Lastly, DNA was extracted from (4) using the "DNA route" of the AllPrep DNA/RNA Mini Kit compliant with the manufacturer's instruction.

DNA concentration and potential PCR inhibition were quantified by qPCR using Plexor® HY System (Promega) according to manufacturer's protocol on an ABI Prism 7500 (Life Technologies).

STR-Multiplex-PCR was performed using the Powerplex® ESX 17 Kit (Promega) according to the provider's instruction. PCR products were detected on a 310 Genetic Analyzer (Life Technologies). Analysis of raw data and DNA profile generation was done with GeneMapper v3.2 software (Life Technologies). The detection threshold for a positive result was defined as 50 RFUs.

All DNA procedures (extraction, quantification and analysis) are summarized in Fig. 1 (lower half, right side).

2.6. Data analysis and statistics

Data analysis, interpretation, and statistical calculations were performed using MS EXCEL and SPSS software v.22 (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Comparison of methods for RNA quantification and quality measurement

Table 1 gives a comparison of average RNA quantification results by quantification method over all samples included in the study.

A large variance of measurements of RNA yield and quality as represented by the RIN was observed both between the different methods of extraction and quantification (Table 2, upper part). The highest total RNA concentrations were measured using the RNA 6000 Nano Kit (IIa) and the Quantus™ Fluorometer (III) after extraction with the RNeasy® Mini Kit (5) followed by Trizol®

Table 1
Overall comparison of RNA quantification methods.

	Qubit	Quantus	6000 Nano	6000 Pico
Mean*	6.40	4.10	7.50	0.80
SD*	16.85	10.6	14.62	4.93
Median*	0.00	0.40	3.50	0.00
RIN	–	–	0.3	1.3

RIN, RNA integrity number (mean); –, does not apply.
* Values in [ng/μl].

Reagent (2) and the mirVana™ miRNA Isolation Kit (1). In contrast, the measurements with the Qubit® fluorometer (I) exhibited the lowest overall RNA concentrations with negative results in up to 68% of samples. RIN values determined by the RNA 6000 Pico Kit were highest (maximum of 2.5) in extracts from the NucleoSpin® miRNA Kit extracts (3) and could be determined in 93% of samples, followed by extracts from the RNeasy® Mini Kit (5) (maximum 1.0, 55% of samples) and extracts from the Trizol® Reagent method (2) (maximum 1.1; 43% of samples). The lowest RIN values resulted from the AllPrep DNA/RNA Mini Kit extracts (4) where in 75% of samples RIN could not be determined.

RNA yields and RIN values also exhibited considerable variation between the quantification methods by sample types (blood, buccal mucosa, liquid saliva and semen) (Table 2, middle part). All quantification methods reported highest RNA yields and RIN values from samples of buccal mucosa (RNA 6000 Nano Kit: 10.48 ng/μl; Qubit®: 9.48 ng/μl; Quantus™: 5.70 ng/μl; RIN-Pico: 2.4 RIN-Nano: 1.0). Samples of liquid saliva (3 μl) exhibited RNA yields between 3.07 ng/μl (RNA 6000 Nano Kit) and 1.40 ng/μl (Qubit® measurement). In blood samples (3 μl), RNA was detectable only by the Quantus™ Fluorometer (0.13 ng/μl) and the RNA 6000 Nano Kit (2.43 ng/μl). The Qubit® fluorometer failed to detect RNA in 74 of 75 cases for blood, 65 of 75 samples for semen and 38 of 75 samples for liquid saliva. The lowest total RNA amount was recovered from semen samples (RNA 6000 Nano Kit: 3.30 ng/μl; Quantus™: 0.28 ng/μl; Qubit®: 0.00 ng/μl).

The age of samples also influenced RNA quantity and quality in that higher age was correlated with decreasing RNA yields whereas RIN values did not vary with sample age (Table 2, lower part).

Overall, the Qubit® fluorometer produced the highest number of negative results. In 181 out of 300 tested samples (60%), which were mostly blood and semen, the Qubit® fluorometer failed to detect RNA. In contrast, the Quantus™ Fluorometer obtained positive results over all extraction methods and body fluids in over 98% of samples.

The determination of RIN using the RNA 6000 Nano Kit failed in 263 out of 300 samples (88%), predominantly in blood, liquid saliva and semen.

Note: due to the noisy fluorescence baseline of the RNA 6000 Nano Kit (IIa) and because the Quantus™ Fluorometer method (III) exhibited the least negative results and the lowest standard deviations we chose this method (III) for all downstream measurements to determine input RNA amounts for reverse transcription as well as for the ranking of a method's RNA yield.

Also, because of considerable variances between the quantification methods and to reduce bias due to a non-normal distribution of values with gross outliers median was applied instead of mean for comparison.

3.2. RNA yield and integrity by extraction method and sample type

For buccal mucosa, the RNeasy® Mini Kit indicates the highest RNA yield (20.00 ng/μl) and integrity values (RIN: 2.3). For blood, the NucleoSpin® miRNA Kit produced the highest integrity values whereas the RNeasy® Mini Kit scored the highest total RNA yields (Supplementary Table 2).

When comparing extraction methods overall, the mirVana™ miRNA Isolation Kit generated the highest RNA yield followed by

Table 2
Overall comparison of RNA quantification and integrity by RNA extraction methods, body fluids and sample age.

	Method	Qubit	Quantus	6000 Nano	6000 Pico	RIN-N	RIN-P
Mean* (median*); ±SD* n[a]	AllPrep	4.55 (0.00); ±15.05 41	2.55 (0.20); ±7.92 2	4.10 (1.87); ±7.24 0	0.12 (0.02); ±0.34 0	0.2 (0.0); ±0.7 57	0.5 (0.0); ±0.9 45
	mirVana	4.56 (0.00); ±11.88 40	3.78 (0.56); ±8.65 0	5.44 (3.67); ±7.34 0	0.10 (0.01);±0.34 0	0.2 (0.0); ±0.8 54	1.0 (0.0); ±1.3 40
	Trizol	8.36 (0.00); ±18.00 32	3.93 (0.27); ±9.56 2	8.90 (3.67); ±13.32 0	0.20 (0.03); ±0.48 0	0.4 (0.0); ±1.0 54	1.3 (1.1); ±1.2 34
	NucleoSpin	6.36 (0.65); ±16.70 29	3.84 (0.43); ±10.98 0	8.69 (3.41); ±20.69 0	3.41 (0.32); ±10.64 0	0.2 (0.0); ±0.7 51	2.2 (2.5); ±1.0 4
	RNeasy	8.29 (0.00); ±21.29 40	6.38 (0.53); ±14.57 0	10.32 (5.02); ±18.47 1	0.34 (0.04); ±0.95 0	0.6 (0.0); ±1.1 43	1.3 (1.0); ±1.4 27
	Body fluid	**Qubit**	**Quantus**	**6000 Nano**	**6000 Pico**	**RIN-N**	**RIN-P**
Mean* (median*); ±SD* n[b]	Blood	0.02 (0.00); ±0.17 74	0.15 (0.13); ±0.14 4	2.76 (2.43); ±0.14 1	0.06 (0.02); ±0.10 0	0.0 (0.0); ±0.1 74	0.9 (0.0); ±1.2 44
	Buccal mucosa	23.51 (9.48); ±27.26 5	14.82 (5.70); ±17.21 0	19.09 (10.48); ±25.33 0	2.98 (0.42); ±9.57 0	1.1 (1.0); ±1.4 48	2.1 (2.4); ±1.2 12
	Saliva	1.83 (1.40); ±2.52 37	0.88 (0.46); ±1.18 0	3.76 (3.07); ±3.89 0	0.22 (0.05); ±0.47 0	0.1 (0.0); ±0.5 71	1.1 (0.0); ±1.2 41
	Semen	0.34 (0.00); ±1.00 65	0.52 (0.28); ±0.69 0	4.34 (3.30); ±4.10 0	0.07 (0.02); ±0.15 0	0.1 (0.0); ±0.4 70	1.1 (1.0); ±1.2 38
	Time interval	**Qubit**	**Quantus**	**6000 Nano**	**6000 Pico**	**RIN-N**	**RIN-P**
Mean* (median*); ±SD* n[c]	Day 0	7.96 (0.00); ±18.61 63	4.37 (0.28); ±11.28 1	8.79 (3.41); ±18.22 0	1.39 (0.03); ±7.47 0	0.3 (0.0); ±0.7 82	1.1 (1.0); ±1.2 49
	Day 5	6.57 (0.00); ±18.62 57	4.36 (0.46); ±12.05 1	8.06 (3.06); ±15.75 1	0.85 (0.04); ±4.05 0	0.4 (0.0); ±1.1 82	1.5 (1.7); ±1.3 39
	Day 14	4.74 (0.00); ±12.60 63	3.54 (0.37); ±8.16 2	5.62 (3.34); ±7.65 0	0.26 (0.03); ±0.65 0	0.3 (0.0); ±0.8 87	1.3 (1.2); ±1.4 44

RIN-N, RIN by Nano-Kit; RIN-P, RIN by Pico-Kit; SD, standard deviation; *n*: no RNA detected out of [a]: 60, [b]: 7 5 and [c]:100 samples.
* Values in [ng/μl]

the RNeasy® Mini Kit and the NucleoSpin® miRNA Kit. In contrast, the AllPrep DNA/RNA Mini Kit obtained the lowest RNA yield and RIN values. All methods were less effective in extracting RNA from samples of blood and semen and these body fluids produced the lowest RNA yields and RIN values (Supplementary Table 2).

3.3. DNA-extraction and yield

Of the five DNA co-extraction methods, the AllPrep DNA/RNA Mini Kit (3) produced highest DNA yields from samples of blood, buccal mucosa, liquid saliva and semen with an average DNA yield of 1.27 ng/μl and sufficient DNA for STR profiling in 96% of samples (Table 3).

The extraction from the flow through waste taken from the RNeasy® Mini Kit (5) resulted in an overall DNA yield of 1.55 ng/μl and sufficient DNA for STR profiling in 58% of samples. In contrast, extraction from the 20 μl lysate diverted from (5) produced a lower overall DNA concentration of 0.38 ng/μl but a higher rate of samples (83%) with sufficient DNA for STR profiling.

Extraction from both the leftover swab material and the 20 μl lysate diverted from the NucleoSpin® miRNA Kit (3) put out overall sufficient DNA concentrations and identical rates of samples (~67%) with sufficient DNA for STR profiling.

The lowest DNA concentrations were detected after extraction from the organic phases left back by the Trizol® Reagent method (2) and mirVana™ miRNA Isolation Kit (1) (DNA concentration: 0.009 ng/μl and 0.004 ng/μl, respectively; 17% and 25% of samples with sufficient DNA, respectively).

In comparison over the various body fluids by extraction method, samples of buccal mucosa extracted with the AllPrep DNA/RNA Mini Kit (4) and from the flow through waste from the RNeasy® Mini Kit (5) exhibited the highest DNA yield. In contrast, samples of blood and buccal mucosa produced lowest DNA yields especially when using the Trizol® Reagent (2) and mirVana™ miRNA Isolation Kit (1) co-extraction methods.

3.4. Quantification and STR profiling of co-extracted DNA

STR profiles were generated for selected samples representing all body fluids that were aged 14 days. Three distinct result categories were defined:

"++", full profile, indicating that 17 of 17 possible STR systems were identified correctly;

"+", identifiable, indicating that at least 8 and up to 16 of 17 possible STR systems were identified correctly, which was considered sufficient for forensic identification, and

"–", not identifiable, indicating that less than 8 of 17 possible STR systems were identified correctly. Table 3 summarizes the quantification and typing results.

Full STR-profiles were obtained from extracts generated by the AllPrep DNA/RNA Mini Kit and from the remaining swab material from the NucleoSpin® miRNA Kit extracted with the PrepFiler® Forensic DNA Extraction Kit. In contrast, the DNA co-extraction procedure from the Trizol® Reagent based on ethanolic precipitation did not produce any identifiable profiles neither from extracts from the Trizol® Reagent nor from the mirVana™ miRNA Isolation Kit.

3.5. Expression analysis of mRNA and miRNA

To assess general suitability for relevant downstream analyses of mRNA and miRNA extracted by the different methods, expression levels of β-Actin mRNA as well as miR-191, miR-93 and RNU6B were determined by qPCR in selected samples aged 0, 5, and 14 days and representing all body fluids from two different individuals each. Again, three distinct result categories were defined:

"++", both samples scored C_q-values <35;

"+", one samples scored a C_q-value <35;

"–", no sample scored a C_q-value <35.

Table 4 and Supplementary Table 3 summarize the results.

Please note that the sole purpose of the qPCR experiments included in this study was to prove the general suitability of the RNA extracted using the different methods for downstream qPCR. The results do not and are not intended to present any differential, body-fluid specific expression values of the assessed mRNA and miRNAs.

mRNA-Expression: C_q values varied considerably by sample type. Samples of blood and buccal mucosa exhibited low C_q of β-Actin (representing high start amounts of RNA) for all extraction methods. In contrast, samples of saliva and semen showed low β-Actin expression especially for RNA extracted by the AllPrep DNA/RNA Mini Kit and Trizol® Reagent method (only + or – results).

By comparison, samples from the NucleoSpin® miRNA Kit exhibited stable C_q values (++ results) over all sample types and ages and no "–" results. The RNeasy® Mini Kit came in second. In addition, higher sample age was correlated with higher C_q values.

miRNA-Expression: miRNA C_q values also varied between samples. Again, samples of blood and buccal mucosa exhibited low C_q values (representing high start amounts of RNA) overall. For liquid saliva, positive values (+ or ++) could only be obtained from

Table 3
Co-extraction of DNA and profiling suitability.

RNA extraction method	AllPrep	RNeasy		NucleoSpin		Trizol	mirVana
DNA extraction method		Waste	Lysate	Swab	Lysate	EtOH	EtOH
DNA yield							
Blood	0.15	0.0001	0.145	0.014	0.043	0.000065	0.00
Buccal mucosa	14.13	7.19	0.18	1.44	3.052	0.039	0.085
Liquid saliva	0.57	–	0.132	–	0.057	–	–
Semen	4.93	–	2.984	–	0.106	–	–
DNA yield overall	1.27	1.55	0.38	0.041	0.12	0.009	0.004
Sufficiency	95.83	58.33	83.33	66.67	66.67	16.67	25.00
DNA profiling							
Full Profile (++)[%]	100	0	–	50	–	0	0
Identifiable (+) [%]	0	50	–	50	–	0	0
Non-identifiable (–) [%]	0	50	–	0	–	100	100

DNA yield (median), [ng/μl]; sufficiency, % of samples yielding sufficient DNA (100 pg) for STR multiplex; PCR Full Profile (++), 17/17; Identifiable (+), 8–16/17; Non-identifiable (–), 0–7/17; –, not done.

Table 4
Comparison of mRNA and miRNA by extraction kits and body fluids (day 0).

	mRNA	AllPrep	RNeasy	NucleoSpin	Trizol	mirVana
Blood	β-Actin	++	++	++	+	–
Buccal mucosa	β-Actin	+	+	++	+	++
Saliva	β-Actin	+	+	++	–	–
Semen	β-Actin	–	+	++	–	–
Blood	RNU6B	–	–	++	–	+
Buccal mucosa	miR-191	++	++	++	++	++
Saliva	miR-93	–	–	+	++	–
Semen	RNU6B	–	–	–	–	–

++, both samples scored a C_q-value <35; +, one sample scored a C_q-value <35; –, no sample scored a C_q-value <35.

samples at 0 days of age extracted by the Trizol® Reagent method and NucleoSpin® miRNA Kit. Overall, high miRNA C_q values were observed for semen samples (only "–" results). In contrast to mRNA-expression, sample age had no influence on C_q values.

All negative controls showed negative results for all extracted samples.

3.6. Ranking of RNA extraction methods

Table 5 presents a ranking of RNA extraction methods. The ranking includes seven equally weighed categories. For the categories 'RNA yield', 'RNA quality', 'RNA expression analysis', 'DNA yield', and 'DNA profiling', highest overall values were considered best, for the category 'costs', the lowest price (in €) per sample was considered best. The category 'handling' represents an average of duration (the shorter the better), contamination risk (number of tube changes, the less the better), 'difficulty of RNA extraction' and 'difficulty of DNA extraction' (both rated from 0 to 2, the lower the better) (Supplementary Table 4).

Overall, the NucleoSpin® miRNA Kit extraction method achieved the highest ranking. The mirVana™ miRNA Isolation Kit produced highest total RNA concentrations, followed by the RNeasy® Mini Kit. For RNA quality and expression analysis, the NucleoSpin® miRNA Kit scored highest. The lowest RNA quantity, integrity and expression values were obtained with the AllPrep DNA/RNA Mini Kit. In contrast, the AllPrep DNA/RNA Mini Kit achieved the best results for DNA co-extraction and STR profiling. The NucleoSpin® miRNA Kit had the best handling while Trizol® Reagent and NucleoSpin® miRNA Kit had the lowest price. The AllPrep DNA/RNA Mini Kit is the most expensive.

4. Discussion

The aim of the study was to compare several different methods of RNA extraction and quantification commonly used in forensic RNA research (Supplementary Table 5) and to assess their relative effectiveness for extracting and quantifying total RNA from forensically relevant materials. To realistically mimic forensic trace material we set up swabbed buccal mucosa, small amounts of dried blood, liquid saliva and semen specimens that were aged for various time intervals.

Table 5
Overall ranking of RNA extraction methods.

	AllPrep	RNeasy	NucleoSpin	Trizol	mirVana
RNA yield	5	2	3	4	1
RNA quality	5	3	1	2	4
RNA expression analysis	5	3	1	3	2
DNA yield	1	2	2	4	4
DNA profiling	1	2	2	4	4
Handling	2	2	1	4	5
Costs (price per sample)	5	3	2	1	4
Overall	4	2	1	3	4

Ranks are from 1 (best) to 5 (worst).

The four RNA quantification methods assessed herein produced largely different results for RNA concentration and integrity (RIN). As total RNA amounts cannot be determined via qPCR, which is considered gold standard for forensic DNA quantification [74], it was sobering to observe such incongruity between several established methods of RNA quantification. Ultimately, we chose the Quantus™ Fluorometer method as basis for our comparison of RNA yields between extraction methods because it produced the fewest negative results and lowest standard deviations. Nevertheless, we strongly recommend sticking to one quantification method throughout any comprehensive set of experiments, as this will at least provide relative accuracy between measurements whereas results will apparently not be concordant between different methods of quantification.

The different extraction methods also varied strongly in RNA yield and integrity values. Overall, the RNeasy® Mini Kit and the NucleoSpin® miRNA Kit produced highest RNA yields and integrity results, especially from samples of blood and buccal mucosa and blood and semen, respectively. The NucleoSpin® miRNA Kit is the only method in our panel with an integrated DNase digestion step, which may account for higher RNA quality. This kit is designed for the isolation of large RNA and small RNAs in separate fractions from a broad range of sample materials. However, the protocol requires many instances of tube openings, so the risk of cross contamination may be increased.

The mirVana™ miRNA Isolation Kit and the Trizol® Reagent exhibited sufficient RNA yields and integrity values but both methods rely on phenolic extraction [75] and thus have a difficult handling requiring good practice in phase separation for the recovery of the aqueous phase with avoiding disturbance of interphase or organic phase, which may influence efficiency and consistency of RNA recovery.

Notably, the AllPrep DNA/RNA Mini Kit being the only dedicated DNA/RNA co-extraction kit in our panel and whose extraction principle is similar to the RNeasy® Mini Kit showed the weakest performance in RNA extraction efficiency and integrity values. In addition, both methods are focused on extraction of RNA molecules >200 bp so that large portions of small RNAs may be lost. This is reflected in the poor miRNA expression results of the AllPrep DNA/RNA Mini Kit and the RNeasy® Mini Kit as discussed below.

To be acceptable for forensic genetic casework routine that nearly always comprises DNA profiling, any RNA extraction procedure consuming precious sample material must permit co-extraction of DNA suitable for STR profiling. Consequently, several studies address forensic RNA/DNA co-extraction [71,76] and RNA/DNA co-extraction from forensic samples has already been assessed in various international trials [15–17]. Although a recent study presented simultaneous extraction of DNA and miRNA without physical phase separation [61] we decided to part extraction routes for co-isolation to generate sufficient amounts of RNA. This approach allowed isolation of both kinds of nucleic acids without a loss of total RNA eluate.

In comparison, the AllPrep DNA/RNA Mini Kit achieved best results for DNA quantification and STR profiling which was to be expected as this method is specifically designed for RNA/DNA co-extraction. However, given this kit's weak RNA performance mentioned above its focus was clearly put on DNA extraction. Satisfactory DNA results (quantity and STR profiling) were also obtained with PrepFiler® DNA extracts from the lysates diverted from RNA extraction by the NucleoSpin® miRNA Kit and the RNeasy® Mini Kit. As in any case RNA yield and quality were not compromised by removing small amounts of lysate while being superior to AllPrep DNA/RNA Mini Kit RNA results, these

procedures are recommended when focusing on RNA extraction. In contrast, co-extraction of DNA suitable for STR profiling failed for the Trizol® Reagent method and mirVana™ miRNA Isolation Kit when the remaining organic phase was tapped. However, in a previous study diverting lysate from the mirVana™ miRNA isolation procedure and extracting DNA using PrepFiler® Forensic DNA Extraction Kit was shown to produce DNA fit for STR profiling [67] and could be applied alternatively.

Overall, three out of five tested RNA extraction methods isolate ample amounts of RNA from limited size samples and enable co-extraction of DNA sufficient and suitable for forensic identification.

β-Actin expression was detectable in almost all tested samples, proving that all extraction methods included in this study produced mRNA suitable for expression analysis. However, the considerably different expression values for β-Actin between sample types and extraction methods underscore once more that this so called 'housekeeping gene' and, for that matter, any other gene should not uncritically be chosen as an endogenous reference for qPCR data normalization. Instead, any normalization strategy and particularly when intended for forensic purposes should a priori be shown to be reliable [66].

In addition to mRNA, feasibility and practicability of forensic miRNA analysis based on qPCR is being assayed since 2009 by several groups [9,11,13,77]. Due to its superior stability, analysis of miRNA recommends itself to forensic investigation. In this study, miRNA expression was detectable in all sample types except semen extracts from all methods. The negative results from semen samples may be attributable to low RNA yields combined with intrinsically low expression of RNU6B in this body fluid.

Within the limitations of the present study, our findings suggest the use of the NucleoSpin® miRNA Kit if downstream qPCR analysis is to be performed as this kit exhibited the fewest negative results.

Parsimonious use of scarce sample material is crucial to forensic casework and this and other studies show, that the same sample may provide for multiple analysis of different nucleic acids and that 'waste' is not necessarily void of analyzable material. Omelia et al. reported successful miRNA expression analysis from blood and saliva samples that were previously extracted using standard DNA extraction [57] and Pena-Llopis et al. recently demonstrated simultaneous isolation of high-quality DNA, RNA miRNA and proteins from tissues for genomic applications using a self devised method combining the mirVana™ miRNA Isolation Kit and the AllPrep DNA/RNA Mini Kit [78].

Future work thus could include more and also 'homebrew' RNA extraction methods and should expand on sample types and conditions and number of individuals. At present, with no perfect method for RNA/DNA co-extraction from forensic samples available, one has to choose wisely and should be ready to compromise.

5. Conclusion

An RNA extraction method optimally adapted to forensic needs should be capable of extracting from various different kinds of compromised samples sufficient amounts of not too degraded total RNA without losing small RNA and facilitate co-extraction of DNA amenable for STR profiling. Unfortunately, as this study clearly shows, this applies to none of the methods investigated herein and to the best of our knowledge, such a method does not exist at present. This underscores the necessity to carefully choose from available methods for RNA extraction before experiments with forensic scope are set up and to fit its strengths and weaknesses to the given experimental settings' needs. Our results may also serve as an incentive for suppliers of forensic research consumables to acknowledge the rising interest in forensic RNA analysis and devise and validate a kit specifically dedicated for RNA/DNA co-extraction from forensic type samples.

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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.2015.01.006.

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SUPPLEMENTARY TABLE 1:
Specifications of the RNA Assays

Official Gene Symbol	NCBI-Alias	mRNA/miRNA used in	NCBI-/miRBase-mature sequence accession	TaqMan Assay ID	Target Sequence (amplicon length in base pairs)	References
ACTB	*BRWS; PS1TP5BP1*	B, BM, SA, SE	-	Hs01060665_g1	sequence not disclosed by manufacturer	-
hsa-miR-93-3p	-	BM	MIMAT0004509	002139	ACUGCUGAGCUAGCACUUCCCG (22)	[9]
hsa-miR-191-5p	-	SA	MIMAT0000440	002299	CAACGGAAUCCCAAAAGCAGCUG (23)	[9]
RNU6-2	*U6, RNU6B*	B, SE	NR_002752	001093	CGCAAGGATGACACGCAAATTCGT GAAGCGTTCCATATTTTT (42)	[11,13,34,35]

NCBI: National Center for Biotechnology Information; *miRBase:* microRNA database [36]; *B*: Blood; *BM*: Buccal mucosa; *SA*: Saliva: *SE*: Semen; -: does not apply

SUPPLEMENTARY TABLE 2:

Comparison of RNA yield and quality by extraction method and sample type

	AllPrep	RNeasy	NucleoSpin	Trizol	mirVana
Blood					
Mean*	0.08	0.26	0.18	0.06	0.16
SD*	0.82	0.24	0.09	0.06	0.03
Median*	0.06	0.22	0.15	0.06	0.17
RIN	0.0	0.0	1.0	0.0	0.0
Buccal mucosa					
Mean*	3.32	23.65	14.13	14.21	12.74
SD*	14.03	21.58	18.86	15.23	13.96
Median*	3.25	20.00	4.05	5.70	4.00
RIN	0.0	2.3	1.1	2.2	1.1
Saliva					
Mean*	0.57	0.72	0.65	1.06	1.39
SD*	0.54	0.83	0.43	1.11	2.12
Median*	0.28	0.46	0.55	0.36	0.25
RIN	0.0	0.0	0.0	0.0	0.0
Semen					
Mean*	0.14	0.87	0.39	0.40	0.81
SD*	0.12	1.19	0.25	0.47	0.61
Median*	0.12	0.47	0.34	0.25	0.80
RIN	0.0	0.0	0.5	0.0	0.0
Overall					
Mean*	2.55	6.38	3.84	3.93	3.78
SD*	7.92	14.57	10.98	9.56	8.56
Median*	0.20	0.53	0.43	0.27	0.56
RIN	0.0	0.0	1.0	0.0	0.0

*values in [ng/µl]; *SD:* standard deviation; *RIN:* number of integrity (median)

SUPPLEMENTARY TABLE 3:

Comparison of mRNA and miRNA by extraction kits and body fluids (day 5 and 14)

	mRNA	**AllPrep**	**RNeasy**	**NucleoSpin**	**Trizol**	**mirVana**
Day 5						
Blood	β-Actin	++	++	++	++	+
Buccal mucosa	β-Actin	+	++	++	+	+
Saliva	β-Actin	-	++	++	++	+
Semen	β-Actin	+	+	++	+	+
Day 14						
Blood	β-Actin	-	+	++	-	++
Buccal mucosa	β-Actin	+	+	-	+	-
Saliva	β-Actin	+	-	+	-	+
Semen	β-Actin	-	+	++	-	-
	miRNA	**AllPrep**	**RNeasy**	**NucleoSpin**	**Trizol**	**mirVana**
Day 5						
Blood	RNU6B	-	-	++	++	+
Buccal mucosa	miR-191	++	++	++	++	++
Saliva	miR-93	-	-	-	-	-
Semen	RNU6B	-	-	-	-	-
Day 14						
Blood	RNU6B	+	-	++	++	++
Buccal mucosa	miR-191	-	-	++	++	++
Saliva	miR-93	-	-	-	-	-
Semen	RNU6B	-	-	-	-	-

(++) both samples scored a Cq-value < 35; (+) one sample scored a Cq-value < 35;
(-) no sample scored a Cq-value < 35

SUPPLEMENTARY TABLE 4:

Breakdown of 'handling' category

	AllPrep	RNeasy	NucleoSpin	Trizol	mirVana
Duration [min]	220	210	80	120	135
Rank	**5**	**4**	**1**	**2**	**3**
Contamination risk	11	11	16	8	12
Rank	**3**	**3**	**5**	**1**	**2**
Difficulty	2	2	2	0	1
Rank	**1**	**1**	**1**	**5**	**4**
Difficulty of DNA co-extraction	2	1	1	0	0
Rank	**1**	**2**	**2**	**4**	**4**
Rank overall	2	2	1	4	5

Ranks are from 1 (best) to 5 (worst)

SUPPLEMENTARY TABLE 5:

Overview of different methods of RNA extraction and quantification commonly used in forensic RNA research

	Extraction of				
Method used	**mRNA**	**miRNA**	**DNA**	**Objective**	**Reference**
PGI	x	-	x	BFI	[38]
PGI	x	-	-	BFI	[5]
PGI	x	-	x	BFI	[39]
PGI	x	-	-	BFI	[6]
PGI	x	-	-	BFI	[7]
PGI	x	-	-	BFI	[40]
PGI, (4), (5)	-	x	x	BFI	[9]
PGI	x	-	x	BFI	[41]
PGI	x	-	-	BFI	[42]
PGI, RNeasy Micro Kit	x	-	x	BFI	[43]
PGI	x	-	-	BFI	[44]
PC	-	x	-	BFI	[45]
(2)	x	x	-	reference genes (tissue)	[46]
(2)	-	x	-	BFI	[47]
(2)	x	-	-	age estimation (blood)	[48]
(2)	x	-	-	age estimation (hair)	[49]
(2)	x	-	-	PMI (organs; mice)	[25]
(2)	x	x	-	PMI (liver; mice)	[50]
(2), miRNeasy Mini Kit	-	x	-	EC miRNA (blood, plasma)	[51]
(5)	x	-	-	BFI	[52]
(5)	x	x	-	BFI	[53]
(5)	-	x	-	BFI	[11]
(5)	-	x	-	BFI	[54]
(5)	-	x	-	BFI	[55]
(5)	x	-	-	BFI	[56]
(5)	-	x	x	BFI	[57]
(5)	-	x	-	BFI	[58]
RNeasy Plus Mini Kit	x	-	-	BFI	[59]

miRNeasy Mini Kit	-	x	-	BFI	[60]
(5), Qiamp DNA Mini Kit, PC	-	x	x	BFI	[61]
(2), (5), RNeasy MiniElute Cleanup Kit	-	x	-	age estimation (blood)	[62]
(1), Qiamp DNA Mini	x	-	x	BFI	[63]
(1)	-	x	-	BFI	[12]
(1)	-	x	-	SIDS	[64]
(1)	-	x	-	time of death (vitreous humor)	[65]
(1)	-	x	-	BFI	[66]
(1)	-	x	-	BFI	[13]
(1)	x	x	x	backspatter (blood, brain)	[67]
(1), (3), (4), RiboPure™-Blood Kit	-	x	x	BFI	[68]
(1), Qiamp DNA Mini Kit	x	-	x	BFI	[69]
(4)	x	-	x	BFI	[70]
(4)	x	-	x	BFI	[15]
(4)	x	-	x	BFI	[71]
(4), (5)	x	-	x	BFI	[72]
(4), (5), RNeasy Micro Kit	x	-	x	BFI	[73]

PGI: Guanidinium thiocyanate–phenol–chloroform; *PC:* Phenol-chlorophorm; *(1):* mirVana™ miRNA Isolation Kit; *(2):* Trizol® Reagent; *(3):* NucleoSpin® miRNA Kit; *(4):* AllPrep DNA/RNA Mini Kit; *(5):* RNeasy® Mini Kit; *BFI*: Body-fluid identification; *PMI*: Post-mortem interval; *EC miRNA*: Extracellular circulating miRNA; *SIDS*: Sudden infant death syndrome

3.3 Zusammenfassung

Die Auswahl der in dieser Studie untersuchten RNA-Extraktionsverfahren basierte auf den bis dato in der einschlägigen Literatur beschriebenen Isolationsverfahren, welche im forensischen Kontext u.a. bei der Körperflüssigkeitsbestimmung [9–12, 130, 132, 133, 144, 146–148, 332–340], der Bestimmung des PMI [163, 167, 341] oder des Spurenalters [173–175] eingesetzt wurden. Die fünf kommerziellen RNA-Extraktionskits: (1) mirVana™ miRNA Isolation Kit, (2) Trizol® Reagent, (3) NucleoSpin® miRNA Kit, (4) AllPrep DNA/RNA Mini Kit und (5) RNeasy® Mini Kit wurden auf ihre Leistungsfähigkeit bei drei forensisch realistischen Körperflüssigkeiten wie EDTA-freiem Blut, frischem Samen, flüssigen Speichel und Mundschleimhaut-Proben, mittels vier verschiedener Messmethoden zur Erfassung von Quantität und Qualität und anschließender Messung der Expression von mRNA und miRNA sowie deren Eignung zur Erstellung von STR-Profilen hin überprüft.

Die vier untersuchten RNA-Quantifizierungsverfahren erbrachten überwiegend differierende Ergebnisse für RNA-Menge und -Qualität. Deutliche Divergenzen wurden zwischen den etablierten Methoden (Qubit® Fluorometer vs. Quantus™ Fluorometer vs. 2100 Bioanalyzer) bei der Quantifizierung der RNA beobachtet, da die Gesamt-RNA-Konzentration nicht mittels eines qPCR-Verfahrens bestimmt werden kann, wie es für die forensische DNA-Quantifizierung [342] als Standardmethode verwendet wird. Hierbei stellte sich die Messung mittels des Quantus™ Fluorometers als solide Grundlage für die nachfolgenden Versuche heraus. Der Vergleich der RNA-Ausbeuten zwischen den Extraktionsmethoden erbrachte die meisten positiven Ergebnisse bei geringster Standardabweichung.

Beim Vergleich der verschiedenen Extraktionsmethoden variierten die RNA-Ausbeuten und Qualitätswerte (gemessen als RIN) stark. Insgesamt erzeugten das RNeasy® Mini Kit und das NucleoSpin® miRNA Kit die höchsten RNA-Konzentrationen und Qualitätswerte, vornehmlich aus Blut- und Mundschleimhautproben bzw. Blut- und Sperma-Proben. Das NucleoSpin® miRNA Kit war in der vergleichenden Studie das einzige Isolationsverfahren, welches einen integrierten DNase-Verdauschritt enthält, was möglicherweise für die höhere RNA-Qualität verantwortlich ist. Das mirVana™ miRNA Isolation Kit und das Trizol® Reagent wiesen ausreichende RNA-Ausbeuten und Qualitätswerte auf, jedoch beruhen beide Methoden auf phenolischer Extraktion [343]. Für deren effiziente Anwendung eine ausreichende praktische Erfahrung in der händisch

durchzuführenden Phasentrennung für die Gewinnung der wässrigen Phase unter Vermeidung von Störungen der Inter- oder organischen Phase erforderlich ist. Das AllPrep DNA/RNA Mini Kit ist das einzig dedizierte DNA/RNA-Ko-Extraktions-Kit, dessen Isolationsprinzip ähnlich dem des RNeasy® Mini Kits ist. Dennoch erbrachte dieses Verfahren die schwächsten RNA-Extraktionsausbeuten und Qualitätswerte. Darüber hinaus sind beide Methoden für die Isolation von RNA-Molekülen > 200 Nukleotide optimiert, so dass eine Vielzahl kleiner RNA-Moleküle (z.B. miRNAs) verloren gehen kann. Dies spiegelt sich in den ungenügenden Expressionsergebnissen der isolierten miRNAs mittels des AllPrep DNA/RNA Mini Kits und des RNeasy® Mini Kits wider.

Das „Abzweigen" des RNA-Lysates für die nachfolgende DNA-Extraktion ermöglichte die Isolierung beider Nukleinsäure-Typen ohne Verlust des Gesamt-RNA-Eluates am Ende des Isolationsverfahrens. Diese und andere Studien zeigen, dass die kombinierte Analyse verschiedener Nukleinsäuren und der bei der RNA-Extraktion entstandene RNA-„Abfall" der Waschschritte nicht notwendigerweise frei von analysierbarem Material sind. Omelia et al. [9] präsentierten eine erfolgreiche miRNA-Expressionsanalyse von Blut und Speichelproben, bei denen die RNA-Moleküle zuvor mittels einer Standard-DNA-Methode extrahiert wurden. Weiterhin demonstrierten Pena-Llopis et al. [344] eine simultane Isolierung von DNA, RNA und Proteinen aus Geweben für genomische Anwendungen mit einer selbst entwickelten Methode, die eine Kombination der mirVana™ miRNA Isolation und des AllPrep DNA/RNA Mini Kits darstellte.

Wie erwartet erbrachte das AllPrep DNA/RNA Mini Kit die besten Ergebnisse für die DNA-Quantifizierung und STR-Genotypisierung, da diese Methode speziell für die DNA/RNA-Ko-Extraktion entwickelt wurde. Aufgrund dessen der Schwerpunkt dieser Methode eindeutig auf der Isolation der DNA-Moleküle liegt, bedingte dies wohl die geringe RNA-Effizienz. Zufriedenstellende DNA-Ergebnisse (hinsichtlich von Quantität und STR-Profilerstellung) erbrachten die „abgezweigten" RNA-Lysate des NucleoSpin® miRNA Kits und des RNeasy® Mini Kits, die mittels des PrepFiler® DNA Extraktions Kits isoliert worden waren. Da die RNA-Ausbeute und -Qualität durch das Abzweigen geringer Lysat-Mengen nicht beeinträchtigt wurde, scheinen diese Verfahren dem AllPrep DNA/RNA Mini Kit überlegen zu sein. Im Gegensatz dazu scheiterte die Ko-Extraktion von DNA aus der entnommenen Probe der verbliebenen organischen Phase mittels des Trizol® Reagenzes und des mirVana™ miRNA Isolation Kits.

Die RNA-basierte Expression von *β-Aktin* wurde in nahezu allen getesteten Proben gemessen, was beweist, dass alle Extraktionsmethoden, die in dieser Studie untersucht wurden, mRNA-Moleküle isolieren, die für eine Expressionsanalyse geeignet sind. Jedoch unterstreichen die deutlich unterschiedlichen Expressionswerte von *β-Aktin* zwischen den Probenarten und Extraktionsmethoden abermals, dass dieses häufig als „Haushalts-Gen" bezeichnete Gen nicht unkritisch als endogene Referenz für die qPCR-Datennormalisierung gewählt werden sollte. Stattdessen sollte für jede Versuchsreihe eine zuvor empirisch validierte Normalisierungsstrategie eingesetzt werden, die geeignete und robuste Referenz-Marker für die Identifikation der zu untersuchenden Körperflüssigkeit umfasst [345]. Neben der mRNA [127–129, 138, 332, 335, 346–348] wird die Machbarkeit und Anwendung der forensischen miRNA-Analyse seit 2009 von mehreren Gruppen untersucht [144–146, 148, 149]. In der vorliegenden Studie konnte die miRNA-Expression in allen Probentypen, mit Ausnahme von Samen und in mit allen Methoden hergestellten Extrakten nachgewiesen werden. Die negativen Ergebnisse der Samenproben sind wahrscheinlich auf niedrige RNA-Ausbeuten in Kombination mit einer intrinsisch niedrigen Expression von *RNU6B* in dieser Körperflüssigkeit zurückzuführen.

Eine RNA-Extraktionsmethode, die optimal auf die Eigenschaften forensischen Spurenmaterials angepasst ist, sollte es ermöglichen, aus verschiedenen Arten von Minimalspuren eine ausreichende Menge an intakten RNA-Molekülen zu isolieren, kurze RNA-Fragmente (miRNA) nicht zu verlieren, sowie eine Ko-Extraktion von DNA zu erlauben, um diese für die nachfolgende Erstellung von STR-Profilen zugänglich zu machen. Zusammenfassend zeigte diese Studie, dass keine der untersuchten RNA-Isolationsverfahren all diese Kriterien voll erfüllt. Insgesamt konnten aber drei der fünf untersuchten RNA-Isolationsmethoden ausreichende Mengen an RNA aus forensisch relevanten Spurenarten extrahieren und gestatteten zudem die Ko-Extraktion von DNA, die für eine forensische Identifizierung geeignet ist. Dies unterstreicht die Notwendigkeit sorgfältig aus den verfügbaren Methoden für die RNA-Extraktion auszuwählen und die Stärken und Schwächen des Verfahrens den Bedürfnissen der jeweiligen Versuchsanordnung anzupassen, bevor Experimente im forensischen Kontext durchgeführt werden sollten.

Aufbauend auf diesen Erkenntnissen wurden in den Folgearbeiten die RNA-Moleküle mittels des NukleoSpin® miRNA-Extraktionskits isoliert und mittels des Quantus™ Fluorometer quantifiziert. Die Eignung dieser Verfahren sollte in realistischen und experimentell erzeugten forensischem Probenmaterial durch die nachfolgenden Arbeiten weiter evaluiert werden.

4 Ballistische Modelle

4.1 Einleitung

Für eine evidenzbasierte Rekonstruktion und juristische Bewertung von Straftaten nach Feuerwaffengebrauch ist es von immenser Bedeutung das vorhandene Spurenmaterial sachgemäß zu analysieren, um letztlich den Tathergang in einen Kontext mit dem vorfindlichen Spurenbild bringen zu können. Ein wichtiges Ziel der forensisch-ballistischen Analyse ist hierbei zu klären, ob die Schusswunde durch einen Unfall, durch eigene oder fremde Hand entstanden ist.

Allerdings können kriminelle Handlungen, bei denen Schüsse auf ein biologisches Ziel abgefeuert wurden, nicht geplant oder kontrolliert werden. Für die systematische molekulargenetische Analyse von Backspatter sind daher experimentelle Schüsse auf standardisierte ballistische Modelle neben der morphologischen Untersuchung u.a. von wundballistischen Phänomenen, unentbehrlich. Angesichts des logistischen und finanziellen Aufwands, welcher mit der Durchführung experimenteller Schussversuche verbunden ist, aber auch im Sinne einer integrativen Erfassung der Zusammenhänge der einzelnen Aspekte des Spurenbilds, ist es wünschenswert, all diese Aspekte an demselben Modell gleichzeitig jeweils pro Schuss erfassen zu können. Hierzu haben Schyma et al. [56] das „Triple Contrast"-Modell entwickelt, welches mit Blut, Acyrlfarbe und Röntgenkontrastmittel dotiert ist und zusätzlich zur molekulargenetischen Analyse des Backspatters eine optische und bildgebende Darstellung des Wundkanals gestattet.

Neben der standardmäßig durchgeführten DNA-Analyse gewinnt die forensische Analyse von RNA zunehmend an Bedeutung [349]. Ihre Aussagequalität ist komplementär zur Analyse von DNA, indem sie der DNA-basierten Individualisierung die Möglichkeit zur (teilweisen) Rekonstruktion des Tatgeschehens mittels Kontextualisierung über das an einem Tatort gesicherte biologische Material beiordnet. Heute belegen zahlreiche Studien die Praktikabilität

der Analyse von mRNA und miRNA zur Körperflüssigkeits- und Organidentifikation auf der Basis des Nachweises von differentiell exprimierter mRNA [127–129, 138, 346–348] und miRNA [11, 133, 144–148, 152]. Es konnte gezeigt werden, dass RNA simultan mit DNA aus kleinsten Mengen forensischen Probenmaterials [350] und sogar Backspatter von inneren Oberflächen von Schusswaffen gewonnen werden konnte [11, 54–57]. Allerdings enthalten diese Proben aus der forensischen Fallarbeit häufig gealtertes und/oder degradiertes Spurenmaterial mit reduzierter Quantität und Qualität, wodurch eine Standard-STR-Typisierung von nDNA verhindert werden kann. In diesen Fällen ist eine Analyse der hypervariablen Regionen des Displacement Loops der mitochondrialen DNA als valide, zuverlässige und sensitive Alternative zur nDNA-Typisierung empfehlenswert [119, 351].

Ein weiterer, in vorherigen Studien zu Backspatter nicht systematisch untersuchter Aspekt ist der Einflusses der Schussdistanz auf die molekulargenetische Analyse von Backspatter aus dem Inneren von Schusswaffen [38, 64, 69]. Bislang wurde angenommen, dass Rückschleuderspuren nur nach absoluten Nahschüssen gesichert werden können. Die Abschätzung der Schussdistanz beruht unter anderem auf der Untersuchung und Interpretation des am Tatort vorgefundenen Spurenmaterials in Bezug zum Schussereignis. Neben der Schussdistanz können weitere Variablen wie Schusswinkel, Kaliber, Hindernisse in der Flugbahn des Projektils (z.B. Fenster, Bekleidung, etc.), Munitions- und Schusswaffenart sowie die Trefferzone, die zu beobachtende Backspatter-Muster beeinflussen.

Schussdistanzen werden grundsätzlich in vier Kategorien eingeteilt: 1. „absoluter Nahschuss“: die Mündung der Feuerwaffe ist in direktem Kontakt mit dem Ziel, 2. „näherer relativer Nahschuss“: die Mündung der Schusswaffe ist nur wenige Zentimeter vom Ziel entfernt, 3. „weiterer relativer Nahschuss“: die Feuerwaffe wird von einer Distanz abgefeuert, bei der keine Schusszeichen der Kategorien 1 (Schmauchhöhle) und 2 (Schmauchhof) entstehen, die aber dennoch kurz genug ist, sodass Backspatter und andere Schussrückstände den Schützen und dessen unmittelbare Umgebung erreichen können und 4. „Fernschuss“: ein Schuss aus einer so großen Entfernung, dass typische Nahschusszeichen fehlen [352].

Die in der ersten Originalpublikation beschriebene Arbeit, sollte die Anwendbarkeit der „Triple Contrast“-Methode nach Schyma et al. [56] durch die Bewertung ihrer Kompatibilität mittels der parallelen und integrativen Analyse von mtDNA und die gleichzeitige Untersuchung ko-extrahierter mRNA und miRNA aus Backspatter, welche aus dem Inneren von Schusswaffen nach experimentellen Schüssen auf ballistische Modelle gesammelt wurden, erweitern.

Das Ziel der zweiten Originalpublikation erstmalig beschriebenen Arbeit bestand in der systematischen Untersuchung forensisch auswertbarer Nukleinsäuren, welche aus Rückschleuderspuren von äußeren und inneren Oberflächen verschiedener Schusswaffen mit unterschiedlichen Kalibern nach experimentellen Schüssen aus verschiedenen Schussdistanzen gesichert werden konnten, wobei das Augenmerk besonders auf dem Einfluss der Schussdistanz lag.

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ORIGINAL ARTICLE

Simultaneous analysis of nuclear and mitochondrial DNA, mRNA and miRNA from backspatter from inside parts of firearms generated by shots at "triple contrast" doped ballistic models

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Abstract When a firearm projectile hits a biological target a spray of biological material (e.g., blood and tissue fragments) can be propelled from the entrance wound back towards the firearm. This phenomenon has become known as "backspatter" and if caused by contact shots or shots from short distances traces of backspatter may reach, consolidate on, and be recovered from, the inside surfaces of the firearm. Thus, a comprehensive investigation of firearm-related crimes must not only comprise of wound ballistic assessment but also backspatter analysis, and may even take into account potential correlations between these emergences. The aim of the present study was to evaluate and expand the applicability of the "triple contrast" method by probing its compatibility with forensic analysis of nuclear and mitochondrial DNA and the simultaneous investigation of co-extracted mRNA and miRNA from backspatter collected from internal components of different types of firearms after experimental shootings. We demonstrate that "triple contrast" stained biological samples collected from the inside surfaces of firearms are amenable to forensic co-analysis of DNA and RNA and permit sequence analysis of the entire mtDNA displacement-loop, even for "low template" DNA amounts that preclude standard short tandem repeat DNA analysis. Our findings underscore the "triple contrast" method's usefulness as a research tool in experimental forensic ballistics.

Keywords Backspatter · Molecular ballistics · Forensic RNA/miRNA analysis · mtDNA · "Triple contrast" method

Introduction

The analysis of bloodstain patterns, first mentioned in the nineteenth century [1] is an important aspect of forensic crime scene reconstruction. Caused by shots against biological targets a spray of biological material (e.g., blood and tissue) may be ejected a wound in the direction of the projectile's trajectory, but some material is propelled from the entrance wound back towards the firearm. This phenomenon has become known as the "backspatter effect" and when contact shots or shots from short distances are applied backspattered biological material may be found on the inside surfaces of the firearm (e.g., the barrel inside), on the shooter (e.g., on his/her hands), and the shooter's surroundings, where it can persist and be recovered for forensic analysis. As early as 1931, Weimann [2] demonstrated the forensic importance of traces and stains resulting from backspatter. Three years later, Brüning and Wiethold [3] first investigated and systematically evaluated traces of biological material on both outside and inside surfaces of firearms used in suicidal shootings, which was subsequently verified by other groups [4–6]. The persistence of DNA and RNA amenable for forensic analysis on internal parts of firearms has only recently been demonstrated [7–9].

However, criminal acts involving shooting firearms at biological targets cannot be planned or controlled;

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therefore experimental shootings using standardized ballistic models are indispensable for the systematic analysis of backspatter and the investigation of wound ballistic phenomena.

To facilitate efficient analysis with maximum utilization of evidence generated during expensive and cumbersome experimental shootings, Schyma et al. [10] developed the "triple contrast" method for the simultaneous analysis of backspatter encompassing short tandem repeat (STR) profiling of nuclear DNA (nDNA) and wound ballistic effects.

However, samples in forensic casework are frequently comprised of aged and/or degraded trace material of a reduced quantity and quality that may preclude standard STR profiling of nDNA. In these cases, the analysis of hyper-variable regions in the displacement loop (D-loop) of mitochondrial DNA (mtDNA) as a valid, reliable and sensitive alternative to nDNA typing is advisable, and robust methods for the analysis of the complete D-loop, even from highly degraded material, have already been described [11, 12].

Knowing the composition of biological material from a crime scene, e.g., whether a stain contains blood or semen, is complementary to DNA based identification and can be essential in reconstructing the course of events in criminal investigations. Today, numerous studies document the practicability of mRNA and microRNA (miRNA) analyses for body fluid identification (BFI) based on the detection of differentially expressed mRNA and miRNA [13]. Also, RNA can be co-extracted with DNA from small amounts of forensic samples [14] and even from backspatter recovered from the inner surfaces of firearms that had been fired at human targets [9].

The aim of the present study was to expand the applicability of the "triple contrast" method by evaluating its compatibility with forensic analysis of mtDNA and the simultaneous investigation of co-extracted mRNA and miRNA from backspatter collected from inside parts of firearms after experimental shootings.

Materials and methods

Blood collection and ethics

To generate ballistic samples we used EDTA-free blood drawn by venipuncture from three different healthy Caucasian individuals (one male, two females) with their informed consent. These persons had no physical contact whatsoever with any firearm and model construction used in this study. For comparison, DNA profiles had been generated from pristine volunteers' blood samples in advance. The study protocol was reviewed and approved by the ethics committee of the Hospital of the University of Bonn.

Ballistic models and sample collection

For this study ten different firearms were used: six revolvers (two .38 Special, three .357 Magnum and one 9 × 19 mm) and four semi-automatic pistols (all 9 × 19 mm). Caliber .38 Special (Magtech Ammunition Company Inc., Lino Lakes, USA) lead round-nose ammunition (projectile weight: 10.2 g) and 9 × 19 mm (Fiocchi Munizioni S.p.A, Lecco, Italy) full metal jacketed bullets (projectile weight: 10.2 g) were fired.

Ballistic models were constructed as described elsewhere [10]. Briefly, a triple mixture of 1 ml blood, 2 ml acrylic paint (CPM, Erkrath, Germany) and 2 ml Micropaque® (Guerbert, Brussels, Belgium), a barium sulphate-based radiocontrast agent, was sealed into thin 5 × 5 cm foil bags. Each bag was attached to a 0.5 l polyethylene bottle filled with 10 % gelatine "Ballistic III" (Gelita, Ebersbach, Germany) which was then covered with a 2–3 mm thick silicon layer and stored at 4 °C for 48 h until use.

Shots were fired with the muzzle touching the ballistic model. After each firing and before cleaning of the weapon, samples were collected using a modified double swab technique [15] by thoroughly wiping the inner surface of the proximal and distal part of the barrel with DNA-free forensic swabs (Sarstedt AG & Co, Nümbrecht, Germany) half moistened with sterile, desalted water, half dry.

Subsequently, the external and internal surfaces of the weapon were cleaned meticulously to prevent contamination.

DNA/RNA co-extraction, DNA quantification and STR profiling

DNA was extracted from collected samples using the PrepFiler® Forensic DNA Extraction Kit (Life Technologies, Darmstadt, Germany) according to the manufacturer's instructions. 40–35 µl of the eluate was diverted for RNA analysis (see below). All DNA extracts were stored at −80 °C until further processing.

DNA concentration and potential PCR inhibition were quantified by quantitative PCR (qPCR) using the Plexor® HY System (Promega, Mannheim, Germany) according to the manufacturer's protocol on an ABI Prism 7500 Sequence Detection System (Life Technologies).

STR-Multiplex-PCR was performed using the Powerplex® ESX 17 Kit (Promega) according to the provider's protocol for selected samples with input amounts between 4 and 500 pg of DNA. PCR products were detected on ABI 310 Genetic Analyzer (Life Technologies). Analysis of raw

data and DNA profile generation was done with the GeneMapper v3.2.1 software (Life Technologies). The analytical threshold for a positive result was set at 50 RFU.

Analysis of mitochondrial DNA

Due to the enhanced risk of contamination in mtDNA analysis, all procedures were performed in a working area dedicated to "low template" DNA. Also, prior to each work phase, all surfaces, machines, and devices utilized in the process were thoroughly cleansed using Roti®-Nukleinsäurefrei (Carl Roth, Karlsruhe, Germany) and Softa-Man® (B. Braun, Melsungen, Germany) to remove all traces of ambient nucleic acid contaminations.

The complete control region of the mtDNA was amplified according to the "Mito-Mini" method described by Eichmann and Parson [11]. Briefly, ten overlapping mini-amplicons are generated in two multiplex PCR assays which can then be sequenced.

Amplification was performed on a PTC-220 DNA Engine DYAD™ (MJ Research™, Waltham, USA) with the cycling conditions described in Eichmann and Parson: an initial denaturation step at 95 °C for 2 min followed by 39 cycles at 95 °C for 15 s, 57 °C for 10 s, and 72 °C for 10 s. Prior to sequencing, PCR products from all samples were examined via microfluid-gelelectrophoresis using the DNA 7500 Kit on an Agilent 2100 Bioanalyzer (both Agilent, Böblingen, Germany).

PCR products were then purified using ExoSAP-IT® (Affymetrix, Santa Clara, USA). For sequencing, the BigDye® Terminator v1.1 Cycle Sequencing Kit (Life Technologies) on a PTC-220 DNA Engine DYAD™ (MJ Research) was used as per manufacturer's prescription and with a primer concentration of 0.5 μM for all sequencing reactions. The sequencing reaction products were cleared of residual dye terminators using Centri-Sep™ spin columns (Life Technologies) according to the manufacturer's prescription. The sequence fragments were then separated via capillary electrophoresis on an ABI 310 Genetic Analyzer using POP-6 Polymer (both Life Technologies) and analysis of raw data was performed using Sequencing Analysis software v3.7 and SeqScape® software v2.1 (both Life Technologies).

Selection of blood specific genes of interest and reference genes for the normalization of miRNA- and mRNA-expression data

For the detection of blood and based on previous work [16–19] the following blood specific mRNA and miRNA were chosen: β-hemoglobin (*HBB*) *and miR-16* (Table 1). These assays do not amplify genomich DNA (gDNA), as they are specific for miRNA and complementary DNA (cDNA) (exon junction spanning), respectively.

Table 1 Specifications of the RNA Assays

Official Gene Symbol	NCBI-Alias	NCBI-/miRBase-mature sequence accession	TaqMan® Assay ID	Small RNA sequence (amplicon length in base pairs)	References
HBB	*CD113t-C, beta-globin*	NM_000518.4	Hs00747223_g1	–	
RPL37A	*L37A*	NM_000998.4	Hs01102345_m1	–	
hsa-miR-16-5p	*miRNA16-1*	MIMAT0000069	000391	UAGCAGCACGUAAAUAUUGGCG (22)	[48, 51]
SNORA66	*RNU66, U66*	NR_002444	001002	GUAACUGUGGUGAUGGAAAUGUGUUAGCCUCAGACA CUACUGAGGUGGUUCUUUCUAUCCUAGUACAGUC (70)	[17, 20]

HBB, ß-hemoglobin; *RPL37A*, ribosomal protein L37a; NCBI, National Center for Biotechnology Information; miRBase, microRNA database [21]; –, does not apply

Also, a panel of nine candidate reference genes for normalization of miRNA expression from blood comprising miRNAs and small nucleolar RNAs (snoRNA) was compiled based on the work of Sauer et al. [17]. The resulting panel of normalization candidates consisted of *miR-93*, *miR-191*, *RNU6-2*, *SNORA66*, *SNORA74A*, *SNORD24*, *SNORD38B*, *SNORD43*, and *SNORD49A*.

Assessment of the nine selected candidate reference genes was performed using the GenEx software v6 (multiD Analyses, Göteborg, Sweden), which integrates the geNorm [22] and NormFinder [23] algorithms to evaluate relative gene expression stability. In addition the MS Excel-based "BestKeeper" [24] algorithm was applied, which compares expression levels by pair-wise correlation analysis. Based on this analysis we chose *SNORA66* as best suited reference gene (Table 1).

For the selection of candidate reference genes for mRNA expression data normalization we applied the online accessible "Genevestigator" v4-35-1 (Nebion AG, Zurich, Switzerland), a multi-organ microarray data browsing algorithm [25]. To facilitate reference gene selection, "Genevestigator" offers the "RefGenes" application [26]. Using this tool, we chose ribosomal protein L37a (*RPL37A*) as the reference gene exhibiting minimal expression variance and similar expression intensity across a large set of relevant gene expression arrays for our pair of genes (gene of interest:reference gene) (Online Resource 1).

RNA quantification and integrity assessment

Prior to all RNA processing, all surfaces, machines, and devices utilized in the processes were thoroughly cleansed using RNase-Zap® (Ambion, Austin, TX, USA) and Roti®-Nukleinsäurefrei (Carl Roth) to remove all traces of ambient RNases and nucleic acid contaminations. Only RNase-free reagents and plastic consumables were used and RNA was kept on ice during processing or was stored at −80 °C until further proceedings.

Total quantity and quality of RNA in the eluate diverted from DNA extraction as described above were measured using QuantiFluor® RNA Dye on a Quantus™ Fluorometer (both Promega) and the RNA 6000 Pico Kit on an Agilent 2100 Bioanalyzer (both Agilent, Böblingen, Germany), respectively. For both methods, 1 µl of DNA extraction eluate was used and all measurements were performed according to the manufacturers' recommendations. RNA quality is represented by the "RNA integrity number" (RIN) as described elsewhere [27]. Note, that RNA was not extracted separately using any dedicated extraction kit but was taken directly from the eluate from the DNA extraction procedure.

Based on quantification results, all individual samples were diluted to a final concentration of 2 ng/µl with RNAse free water. If RNA yield could not be detected fluorometrically, the maximum input volumes for the reverse transcription reactions were used instead.

Reverse transcription (RT) and qPCR

cDNA was synthesized from mRNA and miRNA using the High Capacity Reverse Transcription Kit with random hexamer primers and the TaqMan® MicroRNA Reverse Transcription Kit with target-specific stem-loop primers (both Life Technologies) according to manufacturer's protocols and in total volumes of 20 µl and 15 µl, respectively. Each mRNA RT reaction consisted of 20 ng of total RNA, 1× RT Buffer, 100 mM dNTPs, 1× Random Primers, and 50U MultiScribe™ Reverse Transcriptase. Each miRNA RT reaction comprised 10 ng of total RNA, 1× RT Primers, 50U MultiScribe™ Reverse Transcriptase, 1 mM dNTPs, 3.8U RNase Inhibitor, and 1× RT Buffer.

All RT reactions were performed on a T3 Thermocycler (Biometra, Göttingen, Germany) with the following cycling conditions for mRNA: 25 °C for 10 min, 37 °C for 120 min, and 85 °C for 5 min. For RT of miRNA: 16 °C for 30 min, 42 °C for 30 min, and 85 °C for 5 min.

To detect potential cross-reactivity with genomic DNA, H_2O controls (i.e., RT reactions with water instead of RNA template) and RT(−) controls (i.e., RT reaction without reverse transcriptase) were set up.

RNA was kept on ice during all steps and RT reaction products were stored at −20 °C until further proceedings. For mRNA, qPCR was performed using target-specific TaqMan® Assays for the gene of interest and the reference gene (Table 1) and the TaqMan® Universal PCR Master Mix, No AmpErase® UNG (all Life Technologies), according to manufacturer's prescription. 4 µl of the corresponding RT reaction product were used in each qPCR with 1× TaqMan® Universal PCR Master Mix and 1× specific TaqMan®Assay adding up to a total reaction volume of 20 µl.

For miRNA, qPCR reactions were performed using two target-specific TaqMan® Assays for the gene of interest and the reference gene (Table 1) and the TaqMan® Universal PCR Master Mix II, No AmpErase® UNG (both Life Technologies) as per manufacturer's protocol: Each 20 µl reaction contained 1.3 µl of the respective RT reaction product, 1× TaqMan® Universal PCR Master Mix and 1× specific TaqMan®Assay.

All reaction components were kept on ice during processing.

All sample/assay combinations were run in technical duplicates conducted in MicroAmp® Optical 96-Well Reaction Plates (Life Technologies), both for mRNA and miRNA, with the following qPCR cycling conditions: 95 °C for 10 min, followed by 40 cycles with 95 °C for

15 s and 60 °C for 1 min. All qPCR were performed on an ABI Prism 7500 Sequence Detection System (Life Technologies).

For all quantification reactions the internal PCR control from the Quantifiler® Human DNA Quantification Kit (Life Technologies) was used as an inter plate calibrator. Data was collected during the 60 °C step by the SDS software v2.0.6 (Life Technologies). Raw fluorescence was recorded by the SDS software and then exported for further analysis.

Data analysis, normalization, statistics, and MIQE compliance

To calculate quantification cycle (C_q)-values and amplification efficiencies from SDS spread sheet exported R_n-values from mRNA and miRNA qPCR reactions, the LinRegPCR analysis program v2015.1 [28] was applied. For calculation of C_q-values a common threshold was set to −0.7 log (fluorescence). C_q-values deviating more than one cycle from the duplicate median were excluded from further processing. Normalization of computed C_q-values of genes of interest was performed using the GenEx software v6 (multiD Analyses). A C_q-value of < 35 was considered to indicate successful PCR implying a specific signal and thus RNA quality suitable for expression analysis.

Data analysis, interpretation, and statistical calculations (*t* tests in this study) were performed using MS EXCEL and SPSS software v.22 (SPSS Inc., Chicago, IL, USA). p values < 0.05 were interpreted as significant.

To facilitate reliable and unequivocal interpretation of the qPCR results reported herein, all information that is rated "essential" according to the MIQE guidelines [29] is reported, where applicable.

Results

DNA quantification and STR profiling of triplex mixtures

After experimental shots, biological material was collected from the anterior and posterior half of the barrel and analyzed separately. Positive DNA quantification results were obtained for all collected samples, however a large variance of DNA yield was observed between samples from different barrel parts as well as weapon types and blood donors. The highest DNA concentration (4.10 ng/µl) was obtained for a sample recovered from the anterior barrel half of a revolver 0.357 Magnum whereas the lowest DNA amount (0.0002 ng/µl) resulted from a sample collected from the posterior barrel half of a revolver 0.38 Special.

In general and as was to be expected, samples recovered from the front half of a barrel contained significantly more DNA (median 0.25 ng/µl, ±1.29 SD) than samples obtained from the rear half (median 0.01 ng/µl, ±0.19 SD; $p = 0.001$). Notably though, DNA yields varied between the weapon types (pistol vs. revolver) with samples collected from pistols exhibiting lower median DNA concentrations (0.14 ng/µl, ±1.06 SD) than those collected from revolvers (0.24 ng/µl, ±0.96 SD).

To assess the success rate of STR typing from "triplex mixture" samples as limited by DNA amount, DNA profiles were generated for selected samples with varying DNA yields and rated according to the following result categories:

"++", full STR profiles (17 of 17 possible STR systems), "+", identifiable profiles (at least 8–16 of 17 possible STR systems), and "−", not identifiable profiles (<8 of 17 possible STR systems).

"++" was achieved for samples with an STR-PCR input amount of >100 pg of DNA, "+" was achieved with an STR–PCR input amount of >40 pg up to ≤100 pg (except #16, Table 2, which produced a "−" despite of an input DNA amount of 80 pg), and "−" was obtained if the STR–PCR input amount of DNA was <40 pg.

The majority of DNA profiles consisted of eight or more successfully amplified and correctly typed STR loci (82 %). In one case (#13, Table 2) a mixed STR profile was obtained, indicating residual contamination from previous shootings. PCR inhibition was not observed in any case.

Analysis of mtDNA

Analysis of mitochondrial DNA was performed for selected samples with a DNA yield ≤0.007 ng/µl and these results are summarized in Table 2. For all tested samples, at least 8 of 10 fragments were successfully amplified as demonstrated via microfluid–gelelectrophoresis (an example is shown in Online Resource 2). To analyze mtDNA haplotypes all amplified fragments, representing the entire D-loop region of mtDNA, were sequenced for selected samples that exhibited a DNA amount of less than 100 pg and for which only partial or no STR profiles at all had been obtained (Table 2). In 73 % of samples and down to a DNA amount of 4 pg a complete alignment of all (10/10) amplicon sequences to the CRS reference sequence [30] was obtained. For the remaining samples at least 8 of 10 sequenced amplicons could be aligned. In general, sequence quality differed between multiplexes 1 and 2. Multiplex 2 amplicons on average exhibited lower sequence quality than multiplex 1 amplicons though

Table 2 STR typing and mtDNA sequencing results of selected samples across a range of DNA amounts

No.	Type	Caliber	Barrel part	DNA yield[a]	max. PCR input[b]	STR typing	Sequencing	Alignments	HVI	HVII
1	P	9 mm × 19	a	2.51	500	++	–	–	–	–
2	R	357 Magnum	a	2.35	500	++	–	–	–	–
3	P	9 mm × 19	p	0.91	500	++	–	–	–	–
4	R	38 special	p	0.49	500	++	–	–	–	–
5	R	9 mm × 19	a	0.37	500	++	–	–	–	–
6	P	9 mm × 19	p	0.20	500	++	–	–	–	–
7	R	357 Magnum	p	0.16	500	++	–	–	–	–
8	R	38 special	p	0.10	500	++	–	–	–	–
9	P	9 mm × 19	a	0.04	500	++	–	–	–	–
10	R	357 Magnum	a	0.04	500	++	–	–	–	–
11	R	38 special	p	0.007	100	+	Yes	10/10	Yes	Yes
12	R	357 Magnum	a	0.006	100	+	Yes	10/10	Yes	Yes
13	R	357 Magnum	p	0.006	100	+*	Yes	10/10	Yes	Yes
14	R	9 mm × 19	a	0.006	100	+	Yes	10/10	Yes	Yes
15	R	357 Magnum	p	0.005	80	+	–	–	–	–
16	P	9 mm × 19	p	0.004	80	–	Yes	9/10	Yes	No
17	R	38 special	a	0.004	70	+	Yes	8/10	Yes	No
18	R	357 Magnum	p	0.002	40	+	Yes	10/10	Yes	Yes
19	P	9 mm × 19	p	0.001	20	–	Yes	8/10	No	No
20	R	357 Magnum	a	0.001	20	–	Yes	10/10	Yes	Yes
21	R	9 mm × 19	p	0.0004	8	–	Yes	10/10	Yes	Yes
22	R	357 Magnum	p	0.0002	4	–	Yes	10/10	Yes	Yes

No., number; P, pistol; R, revolver; a, anterior barrel part; p, posterior barrel part; [a]Values in [ng/µl]; [b]pg of DNA in 17.5 µl; STR typing, PCR full profile (++): 17/17; Identifiable (+), 8–16/17; Non-identifiable (–), 0–7/17; *mixed STR profile; Alignments, successfully aligned amplicons; HVI/HVII, successful analysis of complete HVI/HVII; –, not done

overall a high sequence quality of completely aligned sequences was observed. Only in one case (#19, Table 2) was a complete alignment of HVI and HVII not possible. All haplotypes obtained correctly matched those of the pristine blood positive controls. In the case indicating contamination (#13, Table 2) in its STR profile, described above, a corresponding contamination was noticed in the mtDNA sequence (Fig. 1).

Quantity and integrity of co-extracted RNA

Total RNA (processed from eluates diverted from DNA extraction) exhibited considerable variation in quantity and quality (RIN) among all samples and quantification results and RIN values are summarized comprehensively in Online Resource 3.

Overall, the highest RNA yield (6.30 ng/µl) was obtained for a sample recovered from the anterior barrel half of a revolver 0.357 Magnum but an RNA amount above the limit of detection could only be determined in 49 % of samples. In 75 % of samples collected from pistol type weapons no RNA could be detected. In contrast, RNA was detectable in 67 % of samples recovered from revolvers and also exhibited a slightly higher average RNA concentration (0.56 ng/µl, ±1.27 SD) than those taken from pistols (0.55 ng/µl, ±1.37 SD). RNA integrity in terms of RIN was low on average, as expected, and did not vary significantly between weapon types (revolvers: average RIN: 1.7, ±1.0 SD; pistols: average RIN: 1.4, ±1.2 SD).

Comparing the different barrel halves, samples collected from the anterior half exhibited higher average RNA quantity but lower RNA quality (1.05 ng/µl, ±1.71 SD; RIN: 1.3, ±1.2 SD) than those that were taken from the posterior half (0.07 ng/µl, ±0.12 SD; RIN: 1.8, ±1.1 SD).

Expression analysis of mRNA and miRNA

To assess the general suitability for forensically relevant downstream analyses of RNA isolated from "triple mixture" samples, expression levels of blood specific mRNA (*HBB*) as well as miRNA (*miR-16*), were determined by

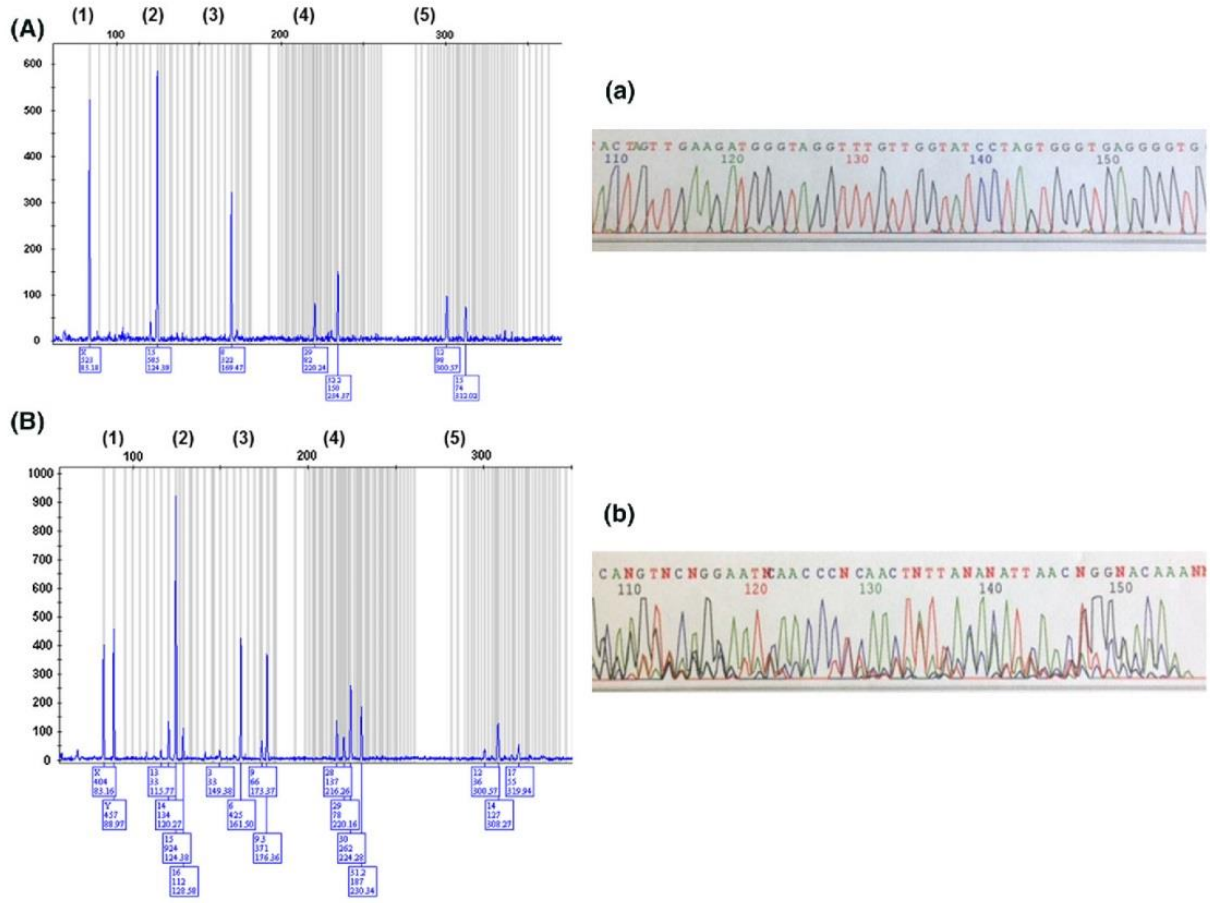

Fig. 1 Representative electropherograms of successful STR profiling and mtDNA sequencing. **A** Exemplary STR profile of a sample obtained from the posterior barrel half of a revolver cal. 357 Magnum with a template DNA amount of 40 pg (donor A). **a** Exemplary sequencing electropherogram of the same sample described using primer F160947. **B** Exemplary STR profile of a sample obtained from the posterior barrel half of a revolver cal. 357 Magnum with a template DNA amount of 100 pg (donor B), showing evidence of contamination **b** exemplary sequencing electropherogram of the same sample described using primer F160947, showing evidence of contamination. STR loci: (1) amelogenin; (2) D3S1358; (3) TH01; (4) D21S11; (5) D18S51

qPCR in selected samples (Table 3). The C_q-values calculated and normalized employing the LinReg and GeneEx softwares, respectively, varied between the tested RNAs.

Overall, normalized expression of *HBB* was blood specific ($C_{q,n} < 35$) in all selected samples. For five samples (#3, #4, #5, #7, and #12) the C_q-values for *SNORA66* were >35, these samples were excluded from further analysis. Lower C_q-values (representing high start amounts of RNA) were exhibited by miRNA *miR-16* (25.59, ±1.55 SD) than mRNA *HBB* (28.41, ±2.78 SD). The reference gene *RPLA37A* exhibited lower C_q-values (30.43, ±1.31 SD) than *SNORA66* (32.31, ±0.75 SD).

All negative controls showed no or unspecific results ($C_q > 35$) for all tested samples.

Discussion

In the present study we are first to describe the simultaneous extraction and forensic analysis of nuclear and mitochondrial DNA, mRNA and miRNA from traces of backspatter collected from the inside surfaces of firearms used in experimental shootings. Additionally, we demonstrate the compatibility of these analyses with multiple stained samples according to the "triple contrast" method [10].

Since its first description in 1925 by Brüning [31], backspatter pattern analysis has become an important tool for both forensic research and routine crime scene reconstruction, and extensive literature on the topic is available [3, 32].

Table 3 RNA yield, integrity and normalized expression levels of *HBB* and *miR-16* from selected samples

No.	Type	Caliber	Barrel part	[a]RNA yield	RIN	Normalized expression	
						HBB	*miR-16*
1	R	357 Magnum	a	6.30	–	0.36	−8.14
2	P	9 mm × 19	a	5.95	2.1	−1.46	−6.71
3	R	38 special	a	4.20	2.2	0.94	–
4	P	9 mm × 19	a	2.75	–	1.27	–
5	R	357 Magnum	a	2.60	–	1.56	–
6	P	9 mm × 19	a	2.15	2.3	−4.57	−7.68
7	P	9 mm × 19	a	0.96	–	−4.25	–
8	R	9 mm × 19	a	0.87	2.0	−3.81	−7.00
9	R	38 special	a	0.61	2.5	−1.80	−5.25
10	R	357 Magnum	a	0.60	–	−2.91	−5.84
11	R	357 Magnum	a	0.55	2.4	−4.31	−6.19
12	R	357 Magnum	a	0.51	2.4	−2.59	–
13	R	9 mm × 19	p	0.38	2.3	−4.24	−7.04
14	R	38 special	a	0.25	–	−2.34	−6.34
15	R	38 special	p	–	5.5	−1.80	−5.20
16	P	9 mm × 19	a	–	2.5	−4.11	−6.65
17	P	9 mm × 19	a	–	2.4	−0.64	−5.20
18	P	9 mm × 19	p	–	2.4	−5.75	−4.63
19	P	9 mm × 19	a	–	2.3	−5.32	−5.70
20	R	357 Magnum	p	–	2.1	−1.72	−5.65

No., number; P, pistol; R, revolver; a, anterior barrel part; p, posterior barrel part; [a]Values in [ng/µl]; RIN, number of integrity; HBB, ß-hemoglobin; miR-16, microRNA-16; Normalized expression, ΔC_q-value: C_q-value$_{(\text{gene of interest})} - C_q$-value$_{(\text{reference gene})}$; –, not detected

To make use of the information encoded in backspatter patterns encountered in cases of firearm-related crimes the conditions and circumstances for the generation and distribution of backspatter has to be understood. Therefore, systematic investigation of backspatter in controlled experimental settings employing standardized ballistic models [9, 33, 34] is a valuable research approach which is less challenging to realize and to replicate and thus more reproducible than shots at living animals [35–37]. Recently Kunz et al. [38] used spongy material in their models to address the distribution of bloodstains spattered back onto the weapon and the shooter's hand. The ballistic model used in this study was devised and tested by Lux et al. [9].

In forensic casework, STR genotyping is considered the gold standard for DNA-based identification because of its robustness and high power of discrimination. Courts et al. [34] previously demonstrated the persistence of biological traces in gun barrels after experimental shootings. Their results indicated that DNA isolated from backspatter on internal surfaces of firearms could yield full STR profiles eligible for forensic identification. The nDNA concentrations reported herein varied between 0.0002 ng/µl and 4.10 ng/µl which is consistent with the results of Courts et al. and a study by Schyma et al. [7] who described the persistence of biological traces in gun barrels after fatal contact shots. Both studies indicated that an STR profile eligible for forensic identification required DNA amounts >0.001 ng/µl. Additionally, Lux et al. quantified DNA from backspatter on the inside parts of seven firearms used for suicidal headshots, resulting in concentrations between 0.0015 ng/µl up to 0.0733 ng/µl [9].

These findings are confirmed by our own results in that comparable ranges of DNA yields were obtained and all samples with DNA amounts >0.001 ng/µl exhibited identifiable STR profiles.

In general and as was to be expected, samples recovered from the front half of firearm barrels contained about one order of magnitude more DNA than samples obtained from the rear half. This significant difference between DNA amounts implies a decline of backspatter with increasing distance from the muzzle. The intra-barrel difference was less pronounced in revolvers than in pistols.

STR analysis can be compromised if only highly degraded and/or minuscule amounts of nuclear DNA are available. While only one large molecule of linear nDNA is present per cell, mtDNA in contrast, is comparably short

and circular and thus not only exhibits enhanced resistance against degradation but is also present in hundreds, and up to a thousand, copies per cell. Consequently, mtDNA though being less discriminative has long since been employed in forensic casework in cases where STR profiling was unsuccessful due to limited or degraded nDNA [39], although it is mostly used only for the exclusion of non-perpetrators given its lack of specificity as compared to STR profiling. However, and to the best of our knowledge, there is as yet no account in the current forensic literature of the assessment of mtDNA isolated from backspatter on the inside surfaces of firearms. We therefore applied the "Mito-Mini" method specifically designed by Eichmann and Parson [11] for "highly degraded samples" to analyze the complete D-loop of the mtDNA in selected samples with nDNA yields insufficient for STR profiling, and found that down to an nDNA amount of 4 pg, representing less than the nDNA content of a single cell, a complete alignment of all ten amplicon sequences was possible. These results demonstrate that robust protocols for mtDNA analysis, such as "Mito-Mini" or "Mini-Midi-Mito" [40], can serve as alternative approaches for samples with degraded or very low amounts of nDNA, typically encountered in backspatter, that preclude standard short tandem repeat DNA analysis. Disadvantages of the "Mito-Mini" method and forensic mtDNA analysis in general are their high susceptibility to contamination, cumbersome and labor intensive procedures, relatively high costs, and reduced power of discrimination as compared to STR profiling. Therefore, mtDNA analysis is often regarded as a means of last resort to tackle otherwise unaccessible genetic evidence. As such however, mtDNA analysis clearly has its worth and can, as shown here, suitably be employed in the investigation of traces of micro backspatter.

In 1994, Phang et al. first introduced reverse transcription PCR based mRNA-based profiling as a potential tool to forensic genetics [41] which was later taken on by other groups [42–45]. International blind trial exercises of RNA based body fluid identification (BFI) [16, 46], dedicated workshops at forensic genetic conferences, and the implementation of RNA analysis in forensic routine casework in some countries [47], have helped to raise consciousness to the vast potential of forensic RNA analysis. Since 2009, to sidestep the inherent instability of mRNA, the first reports of forensic micro-RNA analysis were presented [18, 48, 49], proving their potential as forensically relevant markers [50]. Due to their short size (18–24 bp) and superior stability miRNA are substantially less prone to degradation than mRNA, which is a crucial asset to forensic investigation and forensic miRNA analysis and their usefulness is now constantly being explored further.

In this study, the presence of blood in the "triple contrast" stained sample material was demonstrated via the detection of expression of blood specific mRNA and miRNA, proving the compatibility of "triple contrast" doping of ballistic models with RNA analysis from backspatter. Whereas normalized blood specific expression of *HBB* mRNA was detectable in all tested samples, normalization of miRNA expression data failed in some samples that exhibited too low expression of the endogenous control (*SNORA66*) probably due to insufficient RNA yield.

Conclusion

We have demonstrated that "triple contrast" stained biological material from backspatter collected from the inside surfaces of gun barrels can successfully be subjected to the full bandwidth of forensic nucleic acid analyses, encompassing nuclear and mitochondrial DNA, mRNA, and miRNA.

We show that mtDNA analysis qualifies as an alternative approach to standard STR profiling in molecular ballistics if only small amounts of, and/or highly degraded nDNA, can be retrieved from traces of backspatter. Additionally, we demonstrate the compatibility of "triple contrast" staining of sample material in experimental shootings with forensic body fluid identification based on parsimonious co-extraction and quantitative analysis of blood specific mRNA and miRNA.

Thus, our findings considerably expand the versatility of the "triple contrast" method by broadening its applicability in experimental shootings to the simultaneous analysis of wound ballistic phenomena and all forensically relevant nucleic acid species. This allows for a comprehensive and "economical" realization of experimental shootings, underscoring the method's usefulness as a research tool in experimental forensic ballistics.

Key points

1. The "triple contrast" method of assessing backspatter can be expanded to encompass forensic co-analysis of DNA and RNA and permit sequence analysis of the entire mtDNA displacement-loop, even for "low template" DNA amounts that preclude standard short tandem repeat DNA analysis, demonstrating its versatility and economy.
2. Samples of backspatter collected from the inside surfaces of firearms are amenable to the full bandwidth of forensic nucleic acid analyses.

3. Analysis of mitochondrial DNA can be used as an alternative approach if standard STR profiling fails because of low template amounts and/or degradation of the sample.

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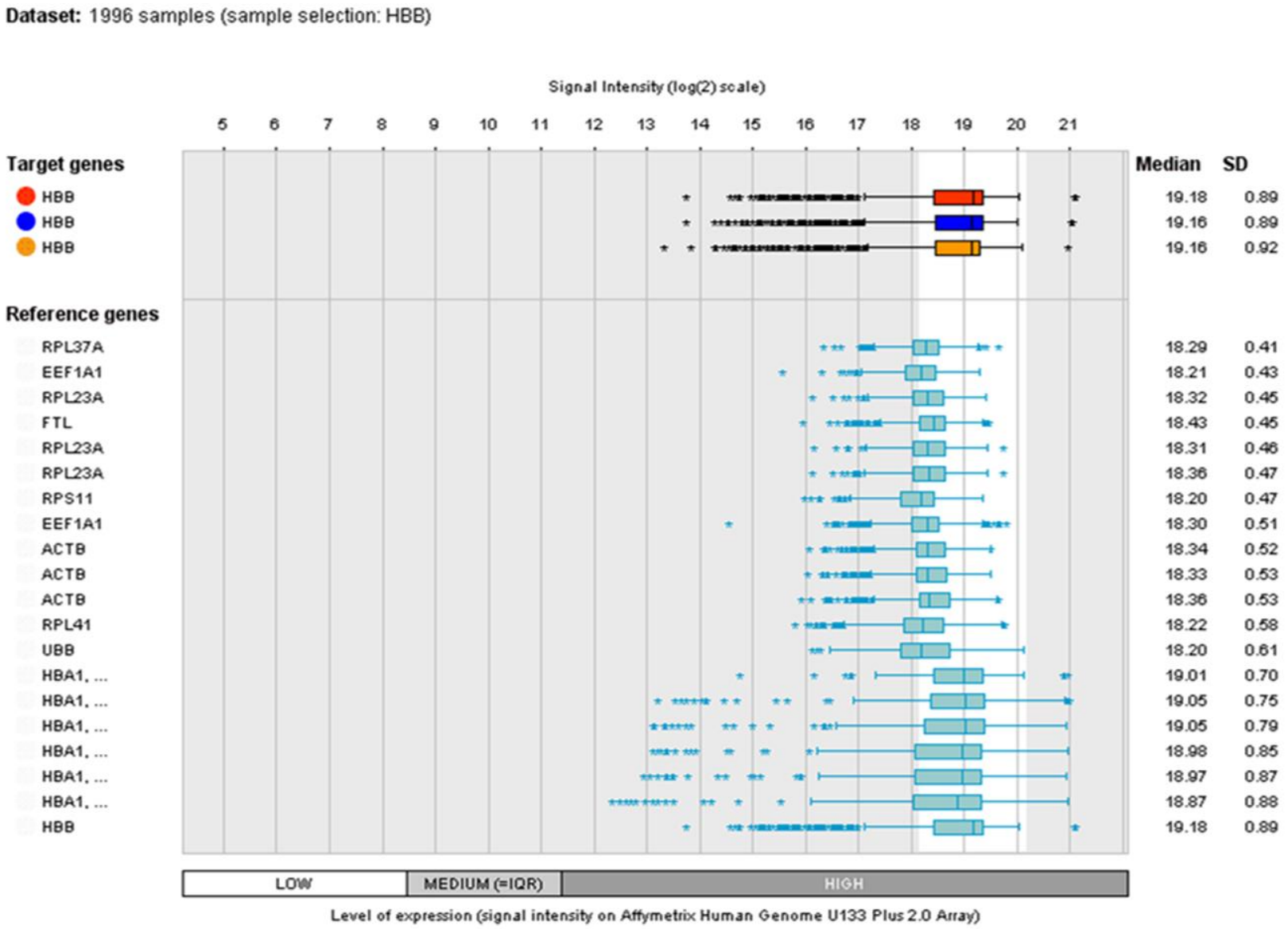
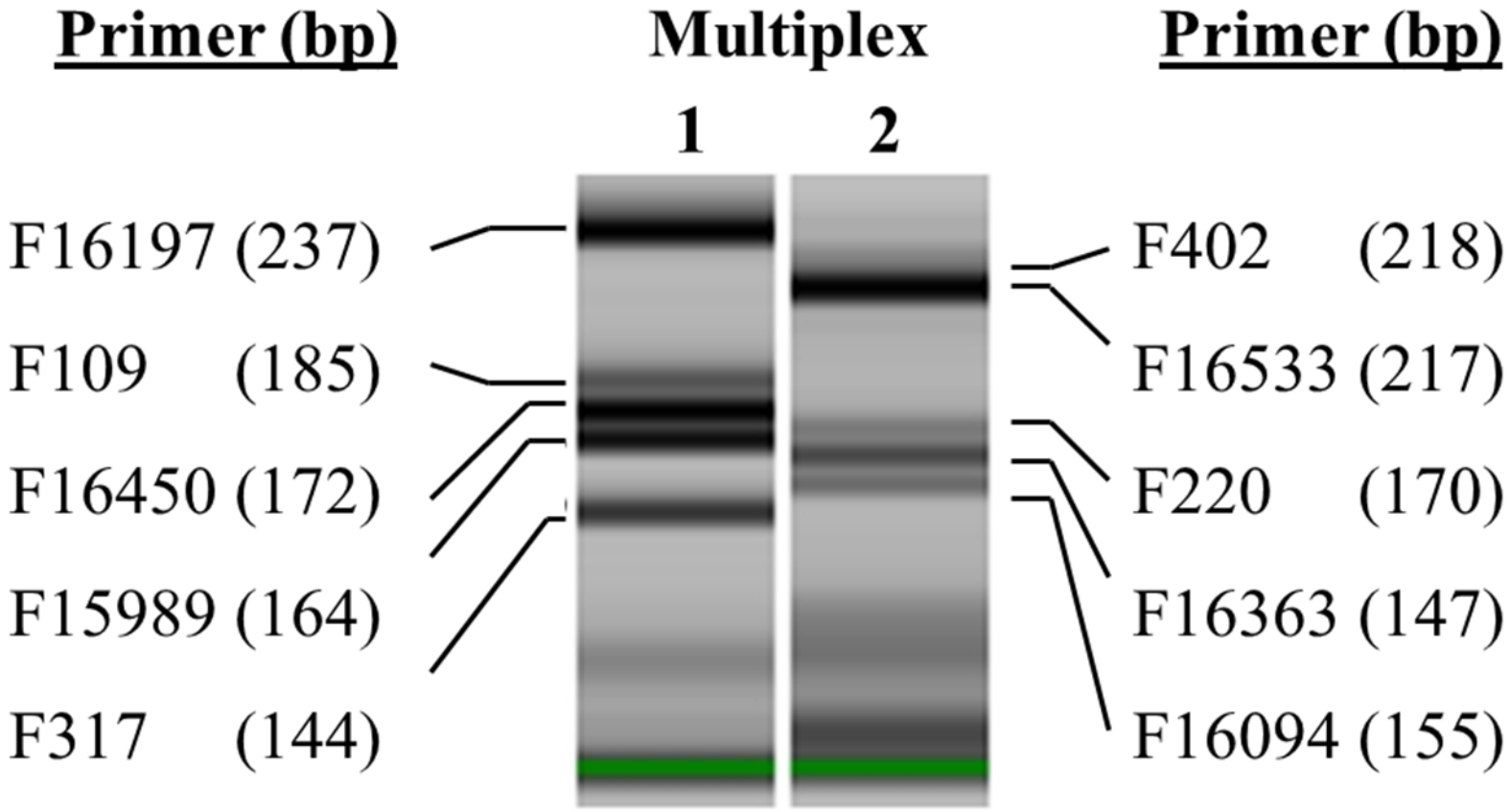


Online Resource 1 Selection of a reference gene for mRNA expression data normalization using the 'RefGenes' online resource



Online Resource 2 Imaging of PCR products resulting from two multiplex PCRs using "Mito-Mini" primer sets in "low template" samples. PCR products were separated via microfluid-gelelectrophoresis on an Agilent 2100 Bioanalyzer; Primer: primer name; bp: base pairs

ONLINE RESOURCE 3

Overview of all samples and analyses

No.	Donor	Type	Calibre	Barrel part	[a]DNA yield	[b]max. PCR input	STR typing	'Mito-Mini' Multiplex	Sequencing	Alignments	[a]RNA yield	RIN	mRNA expression		miRNA expression	
													HBB	*RPL37A*	*miR-16*	*SNORA 66*
1	A	R	38 special	a	0.004255	70	13/17 (+)	yes	yes	8/10	0.25	-	27.65	29.99	26.74	33.08
				p	0.006807	100	14/17 (+)	yes	yes	10/10	0.24	-				
	B			a	0.02017	350	---	---	---	---	-	1.1				
				p	0.02062	350	---	---	---	---	-	5.5	29.13	30.93	27.78	32.98
	C			a	0.05832	>500	---	yes	---	---	0.13	-				
				p	0.1067	>500	17/17 (++)	---	---	---	-	1.9				
2	A	R	357 Magnum	a	0.001381	20	0/17 (-)	yes	yes	10/10	-	-				
				p	0.0002153	4	0/17 (-)	yes	yes	10/10	0.14	1.2				
	B			a	0.04213	>500	17/17 (++)	---	---	---	0.26	1.5				
				p	0.01869	325	---	yes	---	---	-	2.1	29.02	30.74	26.00	31.65
	C			a	0.6957	>500	---	---	---	---	0.60	-	28.79	31.70	26.32	32.16
				p	0.01023	175	---	yes	---	---	-	1.9				
3	A	R	38 special	a	0.4941	>500	---	yes	---	---	0.52	-				
				p	0.009647	165	---	yes	---	---	0.21	1.0				
	B			a	0.06884	>500	---	---	---	---	0.61	2.5	29.55	31.35	26.78	32.03
				p	0.02864	>500	---	yes	---	---	0.07	2.6				
	C			a	3.709	>500	---	---	---	---	4.20	2.2	30.37	29.43	-	-
				p	0.4919	>500	17/17 (++)	yes	---	---	0.45	1.9				
4	A	R	357 Magnum	a	0.4614	>500	---	---	---	---	0.55	2.4	25.34	29.65	25.02	31.21
				p	0.005034	80	16/17 (+)	---	---	---	-	1.0				
	B			a	0.006055	100	14/17 (+)	yes	yes	10/10	0.03	2.5				
				p	0.006053	100	16/17 (+)	yes	yes	10/10	-	1.0				
	C			a	2.348	>500	17/17 (++)	---	---	---	2.60	-	30.99	29.43	-	-
				p	0.03343	>500	---	---	---	---	0.16	-				

	A			a	0.01668	290	---	yes	---	---	-	-				
				p	0.002247	40	9/17 (+)	yes	yes	10/10	-	2.4				
5	B	R	357 Magnum	a	0.03778	>500	---	---	---	---	0.51	2.4	29.96	32.55	-	-
				p	0.02329	400	---	---	---	---	0.28	2.3				
	C			a	4.101	>500	---	yes	---	---	6.30	-	30.01	29.65	25.04	33.18
				p	0.1693	>500	17/17 (++)	---	---	---	-	1.5				
	A			a	0.3754	>500	17/17 (++)	---	---	---	0.87	2.0	26.28	30.09	24.59	31.59
				p	0.02439	420	---	yes	---	---	0.38	2.3	25.85	30.09	24.10	31.14
6	B	R	9 mm x 19	a	0.006565	100	16/17 (+)	yes	yes	10/10	0.42	2.3				
				p	0.0004816	8	0/17 (-)	yes	yes	10/10	-	-				
	C			a	0.05627	>500	---	---	---	---	0.37	-				
				p	0.01363	230	---	yes	---	---	-	1.1				
	A			a	3.303	>500	---	yes	---	---	2.15	2.3	24.28	28.85	24.59	32.27
				p	0.9175	>500	17/17 (++)	yes	---	---	0.02	2.4				
7	B	P	9 mm x 19	a	0.2735	>500	---	---	---	---	-	2.3				
				p	0.06293	>500	---	yes	---	---	-	2.1				
	C			a	0.2245	>500	---	---	---	---	-	2.4	31.80	32.44	27.32	32.52
				p	0.035	>500	---	---	---	---	-	2.4				
	A			a	0.3637	>500	---	yes	---	---	-	2.3	24.70	30.02	25.98	31.68
				p	0.04921	>500	---	---	---	---	-	1.2				
8	B	P	9 mm x 19	a	2.514	>500	17/17 (++)	yes	---	---	5.95	2.1	28.94	30.40	26.74	33.45
				p	0.2534	>500	---	---	---	---	-	2.3				
	C			a	0.08187	>500	---	yes	---	---	-	2.5				
				p	0.004552	80	0/17 (-)	yes	yes	9/10	-	2.5				
	A			a	1.949	>500	---	---	---	---	1.45	-				
				p	0.02146	375	---	yes	---	---	-	1.0				
9	B	P	9 mm x 19	a	0.04668	>500	17/17 (++)	---	---	---	-	-				
				p	0.009123	155	---	yes	---	---	-	2.4	26.44	32.19	27.85	32.48
	C			a	3.311	>500	---	---	---	---	2.75	-	30.72	29.45	-	-
				p	0.001188	20	0/17 (-)	yes	yes	8/10	-	1.0				

10	A	P	9 mm x 19	a	0.4963	>500	---	yes	---	---	-	2.4	27.98	32.09	26.56	33.21
				p	0.01928	330	---	yes	---	---	-	2.1				
	B			a	0.02928	>500	---	---	---	---	-	2.5				
				p	0.1993	>500	17/17 (++)	yes	---	---	-	1.7				
	C			a	1.817	>500	---	---	---	---	0.96	-	28.20	32.45	-	-
				p	0.007829	135	---	yes	---	---	-	-				

A: blood from donor A; *B:* blood from donor B; *C*: blood from donor C; *P:* pistol; *R:* revolver; *a:* anterior barrel part; *p:* posterior barrel part; [a]values in [ng/µl]; [b]*max. PCR input:* pg of DNA in 17.5 µl; *STR typing:* PCR full profile (++): 17/17; Identifiable (+): 8-16/17; Non-identifiable (-): 0-7/17; *RIN:* number of integrity; *HBB*: ß-hemoglobin; *RPL37A*: ribosomal protein L37a; *miR-16*: microRNA-16; ---: not done; -: not detected

Online Resource 3 in
"Simultaneous analysis of nuclear and mitochondrial DNA, mRNA and miRNA from backspatter on inside parts of firearms generated by shots at "triple-contrast" doped ballistic models"

Forensic Science, Medicine and Pathology

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How far does it get?—The effect of shooting distance and type of firearm on the simultaneous analysis of DNA and RNA from backspatter recovered from inside and outside surfaces of firearms

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ABSTRACT

When a firearm projectile hits a biological target a spray of biological material (e.g. blood and tissue) is ejected from the entrance wound and propelled back into the direction of the firearm. This phenomenon has been termed 'backspatter' and if backspattered biological material reaches the firearm on its backward trajectory it may persist on and be recovered from the firearm's inside surfaces. Molecular genetic analysis of backspatter generated by contact shots and shots from very short distances has already been demonstrated to critically contribute to victim identification and the reconstruction of firearm-related crimes.

It is not known, however, up to what shooting distance can backspatter be found on firearms' inside surfaces and what influence the weapon's type and caliber has on backspatter attributes (e.g. reach, amount and distribution).

Therefore, the present pilot study investigated the effect of serval combinations of shooting distances and types of firearms and ammunitions on the analyzability of co-extracted DNA and micro-RNA in samples of backspatter collected from interior and exterior surfaces of the firearms after experimental shootings employing standardized ballistic models.

We demonstrate the limiting effect of shooting distance and the type of firearm on the yield of nucleic acids recovered from backspatter and the success rates of forensic DNA profiling and RNA based body-fluid and organ tissue identification in experimental shootings.

1. Introduction

Investigations at crime scenes after criminal acts involving gunshot injuries occurred often encompass the analysis of traces of blood and so-called 'backspatter'. Backspatter is produced by contact shots or shots from short distances at biological targets: A spray of biological material (e.g. blood and tissue fragments) is ejected from the entrance wound into the direction of the firearm. Traces of backspatter may be found on inside surfaces of the firearm (e.g. the barrel inside), on the shooter (e.g. on his/her hands, even his/her back and the shooter's surroundings, where it can persist and be recovered from for forensic analysis. Such traces may be a valuable source of biological material in forensic crime scene investigation and in reconstructing the course of events. The interpretation and analysis of backspatter has already demonstrated its potential for DNA based victim identification in a real case of triple homicide [1] and for hit zone implication via RNA mediated trace contextualization [2].

Another very important aspect of the evidence based reconstruction and legal appraisal of firearm related crimes is the distance from which a shot has been fired. Shooting distances are generally divided into four categories: (1) a 'contact shot' is stated when the firearm's muzzle is held directly against a target. In 'near contact shots' (2) the muzzle is only a few centimeters away from the target. An 'intermediate shot' (3) is stated when the weapon is fired at a distance that preclude signs of (1) and (2) but close enough that backspatter and/or other traces e.g. gunshot residues (GSR) may still reach the shooter and his/her immediate surroundings. Lastly, a 'distant shot' (4) is fired from so far a distance, that typical firearm related traces are not observed [3].

The estimation of the shooting distance is based inter alia on the examination and interpretation of traces at the crime scene that are related to the shooting event. Apart from shooting distance there are several other variables that may influence observable backspatter patterns, such as shot angle, caliber, obstacles in the

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Fig. 1. Overview of used firearms and ammunition for ballistic experiments. (A) a Smith & Wesson revolver caliber 0.38 Special (B) a FÉGARMY Arms Factory semi-automatic pistol caliber 7.65 mm Browning (C) an Astra semi-automatic pistol caliber 9 × 19 mm (D) a semi-jacket soft point bullet (158 gr) fired with revolver caliber 0.38 Special (E) a full metal jacket bullet (124 gr) fired with the two semi-automatic pistols.

projectile trajectory (e.g. windows, clothing), bullet and weapon type, and the target hit zone.

Judging from a thorough literature survey and to the best of our knowledge, previous studies on backspatter [4–6] have not included a systematic investigation of the effect of shooting distance on the molecular biological analysis of traces of backspatter recovered from inside surfaces of firearms and it is currently assumed, that backspatter is recoverable only after close range or contact shots.

Therefore, in this pilot study our aim was to systematically investigate whether backspatter containing forensically analyzable DNA and RNA can be recovered from inside surfaces of firearms after experimental shootings with different distances using different types of weapons and ammunition.

2. Material and methods

2.1. Samples from autopsy cases and ethics

We collected samples of femoral vein blood and brain tissue during three medico legal autopsies (one female, two males) at the Institute of Legal Medicine in Bonn. The samples were used to dope ballistic models as described below. For comparison, reference DNA profiles from pristine autopsy samples were generated as described below.

The study protocol was reviewed and approved by the ethics committee of the Hospital of the University of Bonn.

2.2. Firearms and ballistic models

Firearms and ammunition used for the experimental shootings are shown in Fig. 1 and listed in Table 1. Ballistic models were constructed as a slightly modified version of the ballistic model described elsewhere [7]. Briefly, 750 ml of 10% ballistic gelatine type 'Ballistic III' (Gelita, Ebersbach, Germany) were prepared following Fackler's instructions [8], filled into 1.5 l polyethylene bottles, and stored at 4 °C for 36 h. Subsequently, small 5 × 5 cm plastic foil bags filled with a mixture of 3 ml blood and 300 mg of ground brain tissue of the same source was sealed and attached to each bottle. Finally, the bag was fixed and covered with a 2–3 mm transparent silicon layer (Sista, Henkel AG & Co. KGaA, Düsseldorf, Germany) and stored at 4 °C for 16 h until use (Supplementary Fig. 1).

Table 1
Weapon types, ammunition and sampling locations per firearm.

Weapon	Manufacturer	Caliber	Ammunition	Bullet weight (gr)	Manufacturer	Sampling locations	Orientation
R	Smith & Wesson (USA)	0.38 Special	SJSP	158.0	Fiocchi Munizioni S.p.A (Italy)	All empty chambers Cylinder Anterior barrel half Posterior barrel half Muzzle Trigger guard	Exterior Exterior Interior Interior Exterior Exterior
P	Astra (Spain)	9 × 19 mm	FMJ	124.0	Sellier & Bellot (Czech Republic)	Slide, inner surface External barrel Anterior barrel half	Exterior Exterior Interior
P	FEG (Hungary)	7.65 mm Browning	FMJ	124.0	Sellier & Bellot (Czech Republic)	Posterior barrel half Muzzle Trigger guard	Interior Exterior Exterior

R: revolver; P: pistol; FEG: FÉGARMY Arms Factory; SJSP: semi-jacket soft point; FMJ: full metal jacket; gr: grain (1 gr = 0.065 g).

2.3. Experimental setup

The experimental setup consists of a table, the ballistic model (as described above), an action camcorder GoPro Hero 3 (GoPro Inc., San Mateo, CA, USA) and a table lamp. Pictures from resulting backspatter and ballistic models were documented with a Canon® EOS 60D (Canon Austria GmbH, Wien, Austria). On the table Whatman™ Filter Paper (GE Healthcare Life Sciences, Buckinghamshire, UK) was fixed and the different shooting distances (as mentioned below) had been marked on it. The ballisitc model was set up and fixed on a table in front of a bullet trap (Supplementary Fig. 2). The shooter was dressed with a Secutex® Pro 5/6 disposable hooded coverall (zetDress®, ZVG Zellstoff-Vertriebs-GmbH & Co. KG, Troisdorf, Germany), a Foliodress® face mask (Paul Hartmann AG, Heidenheim, Germany), two layers of Micro-Touch® sterile examination gloves (Ansell, Tamworth, UK), and protective goggles. The shots were fired two-handed with the hands resting on the table.

2.4. Experimental shootings and sampling procedure

The shooting distances were set at 30 cm, 15 cm, 5 cm and 0 cm, representing a contact shot with the muzzle touching the ballistic model. Shots were fired in order of descending distance (30 cm → 15 cm → 5 cm → 0 cm). After each firing, samples were collected using a modified double swab technique [9]: DNA-free forensic swabs (Sarstedt AG & Co, Nümbrecht, Germany) half moistened with sterile, desalted water, half dry were used to thoroughly wipe the respective sampling locations first with the moistened and then with the dry swab half. Sampling locations as per firearm are described in Table 1. After each shot, the external and internal surfaces of the weapon were cleaned meticulously to prevent contamination: Cleaning was done first mechanically, using a barrel brush and barrel cleaners of woollen felt that had been soaked with Ballistol, a purpose-made ballistic oil (F.W. Klever GmbH, Aham, Germany), to soundly rub and wipe the barrel from the inside and then chemically, using Roti®-Nukleinsäurefrei (Carl Roth, Karlsruhe, Germany).

2.5. RNA extraction, quantification and integrity assessment

Prior to all RNA processing, all surfaces, instruments and devices employed in the processes were thoroughly cleansed using RNase-Zap® (Ambion, Austin, TX, USA) and Roti®-Nukleinsäurefrei to remove all traces of ambient RNases and nucleic acid contaminations. Only RNase-free reagents and plastic consumables were used.

Prior to each extraction with the NucleoSpin® miRNA Kit (Macherey-Nagel, Düren, Germany) which had been shown to be best suited for forensic DNA/RNA co-extraction [10] and according to the manufacturer's instructions using the small RNA protocol, swabs (whole cotton tip or approximately 2 cm^2) were cut into small pieces with scissors. The swab fragments were then incubated with 350 µl lysis buffer and NucleoSpin® Forensic Filters (Macherey–Nagel) were used to separate RNA containing lysates from discardable solid material by centrifugation at 21000 × g for 3 min. Small RNA was eluted in 40 µl of nuclease-free water pre-heated to 90 °C. All RNA extracts were then stored at −80 °C until further processing.

Quantity and quality of small RNA were measured using QuantiFluor® RNA Dye with the Quantus™ Fluorometer (both Promega, Mannheim, Germany) and the RNA 6000 Pico Kit with an Agilent 2100 Bioanalyzer (both Agilent, Böblingen, Germany), respectively. For both methods, 1 µl of RNA extract was used and all measurements were performed according to the manufacturers' recommendations. RNA quality is represented by the 'RNA integrity number' (RIN) [11]. Based on quantification results, all individual samples were diluted to a final concentration of 2 ng/µl with RNAse free water. If RNA yield could not be detected fluorometrically, the maximum input volume allowed for the reverse transcription reaction was used instead.

2.6. DNA co-extraction, quantification and STR profiling

Co-extraction of DNA was performed for each sample by diverting 20 µl of its 350 µl RNA lysate to the PrepFiler® Forensic DNA Extraction Kit routine (Life Technologies, Darmstadt, Germany) processing it according to manufacturer's prescription. DNA was eluted in 50 µl of PrepFiler™ Elution Buffer. All DNA extracts were then stored at −20 °C until further processing.

DNA concentration and presence of PCR inhibitors were measured by quantitative PCR (qPCR) using the Plexor® HY System (Promega, Mannheim, Germany) according to the manufacturer's protocol on an ABI Prism 7500 Sequence Detection System (Life Technologies).

DNA profiling was performed by STR-Multiplex-PCR using the Powerplex® ESX 17 Kit (Promega) according to the provider's protocol for selected samples from interior and exterior surfaces with detectable DNA, representing each shooting distance for each weapon. PCR products were analyzed by capillary electrophoresis of PCR products on an ABI 310 Genetic Analyzer (Life Technologies). GeneMapper v3.2.1 software (Life Technologies) was used for data analysis and DNA profile compilation. The analytical threshold for a positive signal was set at 50 RFU.

2.7. Selection of blood and brain specific miRNA and reference genes for qPCR data normalization

Based on previous work [2,12,13] the following blood and brain specific microRNAs (miRNA) were chosen for the detection of blood and brain tissue: miR-16 (blood) and miR-124a (brain tissue) (Table 2).

To establish a reliable and reproducible strategy of data normalization, panels of fourteen candidate reference genes comprising miRNAs and small nucleolar RNAs (snoRNA) for the normalization of miRNA expression data from blood and brain tissue, were compiled based on the reports of Sauer et al. [12,18] (Supplementary Table 1, [16,19]).

Assessment of the fourteen selected candidate reference genes was performed using the GenEx software v6 (multiD Analyses, Göteborg, Sweden), which integrates the geNorm [20] and NormFinder [21] algorithms as well as the MS Excel-based 'BestKeeper' [22] to evaluate relative gene expression stability. Based on this analysis we chose miR-191 as the best suited

Table 2
Specifications of the RNA Assays.

Official gene symbol	NCBI-Alias	NCBI-/miRBase-Mature Sequence Accession	TaqMan assay ID	Small RNA Sequence (amplicon length in base pairs)	References
hsa-miR-16-5p	*miRNA16-1*	MIMAT0000069	000391	UAGCAGCACGUAAAUAUUGGCG (22)	[13,14]
mmu-miR-124a	*hsa-miRNA124-3p*	MIMAT0000422	001182	UAAGGCACGCGGUGAAUGCC (20)	[15]
hsa-miR-191-5p	–	MIMAT0000440	002299	CAACGGAAUCCCAAAAGCAGCUG (23)	[16]

NCBI: national center for biotechnology information; miRBase: microRNA database [17]; –: does not apply.

reference gene for both, blood and brain tissue specific miRNA expression data normalization (Table 2).

2.8. Reverse transcription (RT) and qPCR

Complementary DNA (cDNA) was synthesized from miRNA using the TaqMan® MicroRNA Reverse Transcription Kit employing target-specific stem-loop primers (both Life Technologies) in a total volume of 15 μl. Each miRNA RT reaction comprised 10 ng of total RNA, 1× RT Primers, 50 U MultiScribe™ Reverse Transcriptase, 1 mM dNTPs, 3.8 U RNase Inhibitor, and 1× RT Buffer.

All RT reactions were performed on a T3 Thermocycler (Biometra, Göttingen, Germany) with the following cycling conditions: 16 °C for 30 min, 42 °C for 30 min, and 85 °C for 5 min.

To detect potential genomic DNA and reagent contamination, RT(-) controls (i.e. RT reactions without reverse transcriptase) and H_2O controls (i.e. RT reactions with water instead of RNA template) were set up, respectively. RNA was kept on ice during all steps and RT reaction products were stored at −20 °C until further proceedings.

QPCR was performed using target-specific TaqMan® Assays (Life Technologies) (Table 2) for the selected blood and brain specific miRNAs and reference gene: Each 20 μl reaction contained 1.3 μl of the respective RT reaction product, 1× TaqMan® Universal PCR Master Mix, No AmpErase® UNG (Life Technologies) and 1× TaqMan®Assay. All reaction components were kept on ice during processing.

All sample/assay combinations were run in technical triplicates. All qPCR reactions were conducted in MicroAmp® Optical 96-Well Reaction Plates on an ABI Prism 7500 Sequence Detection System (both Life Technologies) with the following qPCR cycling conditions: 95 °C for 10 min, followed by 40 cycles with 95 °C for 15 s and 60 °C for 1 min.

To normalize inter-run variation between qPCR reaction plates the internal PCR control from the Quantifiler® Human DNA Quantification Kit (Life Technologies) was used as an inter-plate calibrator. Raw fluorescence data was collected during the 60 °C step by the SDS software v2.0.6 (Life Technologies) and then exported for further analysis.

2.9. Data analysis, normalization, statistics, and MIQE compliance

To calculate quantification cycle (C_q)-values and amplification efficiencies from raw data spread sheets exported from SDS software the LinRegPCR analysis program v2015.1 [23] was applied. For calculation of C_q-values a common threshold was set to −0.7 log (fluorescence). C_q-values deviating more than one unit from the triplicate median were excluded from further processing. Normalization of computed C_q-values of target specific miRNA was performed using the GenEx software v6 (multiD Analyses). A normalized C_q-value of <35 was considered to indicate successful PCR implying a target specific signal and thus RNA quantity and quality suitable for expression analysis.

Data analysis, interpretation, and statistical calculations were performed using MS EXCEL and SPSS software v.22 (SPSS Inc., Chicago, IL, USA).

To facilitate reliable and unequivocal interpretation of the qPCR results reported herein, all information that is rated 'essential' according to the MIQE guidelines [24] is reported, where applicable.

3. Results

3.1. DNA quantification

After shots had been fired at ballistic models from specified distances (from 30 cm down to 0 cm), backspatter containing biological material was collected from various sampling locations as described in Table 1. The samples were not pooled but analyzed separately. DNA was quantifiable in 83 and 54% of samples collected from exterior and interior surfaces, respectively. However, a wide range of DNA concentrations was observed between samples from interior and exterior surface parts as well as between samples from different weapon types.

3.1.1. Exterior vs. interior surfaces

When comparing samples collected from interior and exterior surfaces, samples from exterior surfaces always yielded higher DNA concentrations for all shooting distances and all weapons.

3.1.2. Front vs. rear barrel half

Also, in general and as was to be expected, samples recovered from the front half of a firearm's barrel inside contained one order of magnitude more DNA than samples obtained from the rear half. DNA concentrations varied considerably between the barrel halfs depending on the weapon type (pistols vs. revolver), however. On average, samples collected from pistol 7.65 mm Browning exhibited higher DNA concentrations in the barrel's front half (1 pg/μl, ±2 SD) than those collected from pistol 9 × 19 mm (0.8 pg/μl, ±0.8 SD) and revolver 0.38 Special (0.3 pg/μl, ±0.2 SD).

3.1.3. Comparing shooting distances

Comparing the samples from different shooting distances over the different weapon types as well as the barrel parts whence they were collected, a non-systematic distribution of DNA yields was observed (Table 3). Overall, the highest DNA concentrations were observed in samples from contact shots for pistol 7.65 mm Browning (200 pg/μl ±390 SD) and pistol 9 × 19 mm (60 pg/μl ±100 SD), however, with the revolver 0.38 Special the highest DNA concentration was observed at 5 cm shooting distance (10 pg/μl ±20 SD). No DNA was detected in samples collected from the internal barrel halves generated with the pistol 7.65 mm Browning from shooting distances >0 cm. Notably however, an indirect correlation of shooting distance and DNA yield was not observed.

3.2. STR profiling of co-extracted DNA

Only 33% of all samples contained more than 100 pg DNA in 17.5 μl (multiplex PCR reaction volume) which was considered sufficient for successful STR profiling. STR typing success rate was then assessed for selected samples with PCR input amounts of 2 pg–4.7 ng DNA. These samples encompassed swabbings of the weapons' internal ($n = 8$) and external ($n = 9$) surfaces. DNA profiles were rated according to the following result categories:

'++', full STR profiles (17 of 17 possible STR systems), '+', identifiable profiles (at least 8–16 of 17 possible STR systems) and '−', not identifiable profiles (less than 8 of 17 possible STR systems).

'++' was achieved for all tested samples with an STR PCR input amount of >51 pg of DNA, '+' and '−' were obtained with PCR input amounts of ≥21 pg up to ≤51 pg, and <16 pg of DNA, respectively.

In 37.5 and 50% of tested samples from interior and exterior surfaces, respectively, DNA profiles were rated '++' or '+'. In all other cases STR profiling was unsuccessful ('−'). Table 3 summarizes these results. Notably, in no case were identifiable profiles obtained from samples of shots fired from a distance of 30 cm whereas full STR profiles had been obtained from samples collected from interior and exterior surfaces of pistol 9 × 19 mm fired from distances of 5 and 15 cm, respectively.

Table 3
Overview of DNA and RNA results of selected samples.

Weapon	Distance [cm]	Location	Orientation	DNA results			RNA results			
				DNA concentration[c]	DNA input amount[d]	STR profiling	RNA[e] concentration	RIN	Correct detection	
									Blood	Brain
Revolver 0.38 Special	0	ec	e	18.6	325	++	2.05	–	*	*
		cp	e	0.3	5		0.98	2.3		
		a	i	0.4	7	–	1.45	2.3		
		p	i	–	–		1.85	2.4	*	*
	5	ec	e	43.0	753		5.05	2.5	*	*
		cp	e	–	–		1.10	1.5		
		a	i	0.4	7	–	1.25	1.5		
		p	i	0.2	4		1.70	1.5	*	–
	15	ec	e	0.5	9	–	1.60	1.1	*	*
		cp	e	–	–		1.10	1.3		
		a	i	–	–		0.87	1.3		
		p	i	–	–		1.04	1.3	–	–
	30	ec	e	–	–		1.50	1.8		
		cp	e	0.3	5	–	0.83	2.0	*	–
		a	i	0.2	4	–	0.69	1.9		
		p	i	–	–		1.45	1.8	–	–
Pistol 7.65 mm Browning	0	sa	e	26.4	461		0.76	2.4		
		eb	e	785.4	13745		20.00	2.4	*	*
		a	i	3.9	68	++	0.36	2.5	*	*
		p	i	0.2	4		–	1.7		
	5	sa	e	–	–		0.22	2.2		
		eb	e	2.9	51	++	0.42	–	*	–
		a	i	–	–		0.12	1.9	*	–
		p	i	–	–		0.09	1.3		
	15	sa	e	0.2	4	–	0.36	1.4	*	–
		eb	e	0.1	2		0.33	–		
		a	i	–	–		–	2.6	–	–
		p	i	–	–		–	2.5		
	30	sa	e	0.4	7	–	0.65	2.6	*	–
		eb	e	0.2	4		0.99	–	–	–
		a	i	–	–		0.18	2.2		
		p	i	–	–		–	1.6		
Pistol 9 mm × 19	0	sa	e	44.1	772		2.00	2.4	*	*
		eb	e	201.8	3532		1.50	2.3		
		a	i	1.2	21	+	–	1.5	*	*
		p	i	0.1	2		–	1.2		
	5	sa	e	2.5	44		–	2.4		
		eb	e	21.9	383	++	–	2.4	*	*
		a	i	1.7	30	+	–	2.2	*	*
		p	i	0.1	2		–	1.6		
	15	sa	e	1.3	23		–	2.2	*	*
		eb	e	2702	4729	++	11.00	2.4	*	*
		a	i	0.1	2	–	–	–		
		p	i	–	–		–	–		
	30	sa	e	0.9	16	–	0.28	–	*	–
		eb	e	0.7	12		–	1.3		
		a	i	0.1	2	–	–	1.0		
		p	i	0.1	2		–	2.2	*	–

ec: empty chamber; cp: chamber and projectile; a: anterior barrel part; p: posterior barrel part; sa: slide anterior; eb: external barrel.
[c] Values in [pg/μl].
[d] Amount of DNA (pg) in 17.5 μl (maximum input volume for multiplex STR-PCR reaction); STR profiling: PCR full profile (++): 17/17; Identifiable (+): 8–16/17; Non-identifiable (–): 0–7/17.
[e] Values in [ng/μl]; RIN: RNA integrity.
* Correct blood or brain detected; –: not detected.

3.3. RNA quantification, integrity and expression analysis

Overall and analogous to DNA, small RNA exhibited considerable variation in terms of quantity but only minor variation in terms of quality (RIN) between the different sampling locations and weapon types.

In 50 and 17% of samples from interior and exterior surfaces the RNA amount was below the limit of detection. Also, to assess the influence of shooting distance on the forensic analysis of RNA extracted from backspatter, expression levels of blood and brain specific miRNA *miR-16* and *miR-124a*, respectively, were determined by qPCR in selected samples representing all tested shooting distances and weapon types having been normalized as described above. Overall, blood specific normalized expression of *miR-16* ($C_{q,n} < 35$) was detected in 83% of all selected samples. Table 3 summarizes all RNA results.

3.3.1. Exterior vs. interior surfaces

When comparing samples collected from interior and exterior surfaces, samples from exterior surfaces always yielded higher RNA concentrations for all shooting distances and all weapons.

3.3.2. Front vs. rear barrel half

Comparing the internal barrel parts (anterior vs. posterior half), samples generated with the pistol 7.65 mm Browning produced higher RNA concentrations when collected from the anterior barrel part (0.17 ng/μl, ±0.15 SD) than from the posterior half (0.02 ng/μl, ±0.05 SD) whereas, notably, the reverse was observed with samples from the revolver 0.38 Special (anterior barrel half: 1.07 ng/μl, ±0.35 SD; posterior barrel half: 1.51 ng/μl, ±0.35 SD). No RNA could be detected for samples from the pistol 9 × 19 mm from any shooting distance.

3.3.3. Comparing weapons and shooting distances

RNA was detectable in all samples collected from the revolver 0.38 Special, whereas in 25% and 56% of samples collected from the pistol 7.65 mm Browning and pistol 9 × 19 mm, respectively, no RNA could be detected. Overall, the samples recovered from revolver 0.38 Special exhibited a similar average RNA concentration (1.53 ng/μl, ±1.01 SD) like those collected from pistol 7.65 mm Browning (1.53 ng/μl, ±4.93 SD). The lowest average RNA yield was obtained in samples from the pistol 9 × 19 mm (0.92 ng/μl, ±2.75 SD).

A correct detection of blood specific miRNA *miR-16* was obtained for all samples generated with pistol 9 × 19 mm, whereas detection of blood failed in 25% of samples generated with both revolver 0.38 Special and pistol 7.65 mm Browning. Also, presence of blood was correctly called in samples generated from all shooting distances up to 30 cm.

Notably however, detection of brain proved less sensitive as brain specific normalized expression levels of *miR-124a* ($C_{q,n} < 35$) was obtained only in 50% of selected samples and only for shooting distances up to 15 cm for revolver 0.38 Special and pistol 9 × 19 mm or contact shots (=0 cm) for the pistol 7.65 mm Browning.

3.3.4. Integrity

As expected of forensic samples of this type, RNA integrity as represented by the RIN was low in average but did not vary considerably over all samples or between the weapon types nor sampling locations (Table 3). Also, there was no correlation whatsoever between shooting distance and RNA integrity.

All negative controls were negative for all tested samples.

4. Discussion

The shooting distance is a crucial influence variable in the generation of trace patterns consisting of gunshot residues, forward and backspatter in experimental shootings and firearm-related crimes as well. If shooting distance is to be inferred from the trace patterns observed at the crime scene but also on crime-related persons (victim, shooter) and objects (firearm), however, its influence on these gun-shot derived forensically relevant emergences has to be systematically analyzed. And while there is some groundwork on the presence of backspatter on firearms and the shooter's hand after contact or close-range shots [6,25,26] investigations of the influence of the shooting distance on the amount and distribution of backspatter within firearms as yet received no attention in the literature. Herein, the purpose of the pilot study was to investigate in how far shooting distance correlates with the success rates of forensic analysis of DNA and miRNA recovered from traces of backspatter that were collected from inside and outside surfaces of firearms after experimental shots at standardized ballistic models employing different types of firearms and ammunitions.

Several previous studies on the persistence of biological traces within firearms now suggest that thorough and systematic investigation of backspatter should be acknowledged as an important tool for crime scene reconstruction including victim identification and ought to be routinely performed in the investigation of cases of firearm-related crimes [1,2,27,28]. To understand the conditions and variables influencing the generation and distribution of such traces the staging of a controlled experimental setting based on standardized ballistic models is advisable (as for instance in [6,28] while experiments employing live animals (e.g. [4,5]) which are highly complex but of only limited informative value as well as ethically questionable should be discouraged from. The experimental setting used in this study was devised [2] and proved its value as described in previous work [7].

STR genotyping is considered gold standard in forensic DNA-based identification. So one aim of our study was to evaluate the influence that shooting distance, type of firearm, caliber and ammunition have on amount, distribution and STR typing success rate of backspatter containing DNA. We found that, while DNA concentration were low on average, weapon type and ammunition strongly influenced the amount of DNA that could be garnered from samples collected from various outer and inner surfaces of the weapons. Samples recovered from the revolver produced DNA amounts sufficient for full STR profiles with shooting distances of up to 5 cm whereas full STR profiles were obtained with samples from both pistols fired from distances of up to 15 cm. However, no STR profiles could be produced from any samples from shooting distances >15 cm, implying a potential detection threshold due to a decline or 'thinning' of backspatter with increasing distance. However, while the DNA yields obtained from samples generated by contact shots are consistent with the results reported by Courts et al. [28] and Schyma et al. [27] who described the persistence of biological traces in gun barrels after experimental and fatal contact shots, respectively, we did not observe a distinct correlation between DNA yield and longer shooting distances. This is explainable by a departure in practice from a perfectly even distribution over an ideal spherical front of backspatter that in theory emerges from the entry wound in the target and a conic section of which enters the firearm's muzzle. Such departure (see Supplementary Fig. 3A) and the ensuing erratic and 'realistic' distribution of backspatter (that may even be found on the shooter's back as shown in Supplementary Fig. 3B) described herein may be caused by differences in caliber, ammunition and projectile velocity as well as minor shot-to-shot variances of the shooting angle, firearm's recoil and of the hand-made ballistic models that cannot be controlled for. This effect on backspatter formation could potentially be visualized in subsequent studies by recording the shooting procedure with a high speed camera with high resolution.

Another reason for limited DNA recovery could be the sampling procedure. Several samples were collected after each shot, however to assess the differential distribution of backspatter between several locations within the firearms (e.g. anterior and posterior half of the barrel's inside, inner sledge, bullet chambers etc.) samples were not pooled but processed separately. As this artificial compartmentalization of available material may have produced individual samples with DNA amounts below the limit of detection it is conceivable that pooling all available samples would increase STR typing success rate which seems advisable for casework application. This might even facilitate successful STR typing from shooting distances >15 cm.

Phang et al. [29] first introduced RT-PCR based mRNA profiling as an alternative approach for the identification of tissues and body fluids in forensic genetics. Some years later, with the rise of qPCR, mRNA based methods started spreading in forensic molecular biology [30–32] and since 2009 the potential of the analysis of differential expression of miRNA for forensic body fluid identification (BFI) is being explored [13,33,34]. Due to their short size (18–24 bp) miRNA are more stable and substantially less prone to

degradation than mRNA, which is a crucial asset to the investigation of forensic type samples.

In this study, we demonstrated the identification of blood and brain tissue via analysis of differentially expressed miRNA in blood and brain tissue containing samples obtained from minimal traces of backspatter. However, RNA yields varied considerably among samples and did not correlate with DNA yields. A reason for this observation might be that there are individual specific differences between the cellular DNA:miRNA ratios as well as between the RNA contents per mg of tissue or blood. Moreover, total RNA quantification is of only limited use to estimate a sample's miRNA content and might also be affected by degradation of large RNA molecules. Nevertheless, the analysis of blood and brain specific miRNA expression levels presented herein allows for a robust detection of blood and brain tissue even in samples from shooting distances of up to 30 cm, thereby exceeding the sensitivity of DNA analysis.

This difference may be explainable by a higher sensitivity of the miRNA based assays employing up to 35 cycles of amplification as well as that miRNA might be less affected by fragmentation caused by the physico-chemical strain associated with gunfire and thus be more available to analysis than DNA.

Also, there was a difference between detection rates of blood and brain tissue with brain detection being less sensitive. This might be due to a less sensitive miRNA assay but also due to a non-homogenous distribution of brain tissue in the blood filled plastic bag in the ballistic model. Apart from that our results are consistent with the findings of Lux et al. [2] who in a pilot study proved the principle that the detection of brain specifically expressed miRNA in backspatter from firearm-related injuries may crucially support the inference that a head shot has occurred. In addition to that, our results suggest that this method may be applied to shots from distances up to 15 cm and potentially even more if all collected samples are pooled and as it allows for RNA/DNA co-extraction can be done in parallel to standard forensic DNA profiling.

5. Conclusion

Herein, we are first to investigate the effect of serval combinations of shooting distances and types of firearms and ammunitions on the recovery rates and forensic analyzability of nucleic acids isolated from backspatter from inside and outside surfaces of firearms.

In summary, we demonstrate that recovery of analyzable DNA and RNA from blood and/or brain tissue was successful with shooting distances of up to 15 and 30 cm, respectively. These results suggest, that backspatter on outside but also inside surfaces of firearms should be regarded as a valuable source of forensic evidence not only in contact shots. However, no robust correlation between DNA/RNA yield and shooting distance and/or weapon type was observed so that the amount of nucleic acids in backspatter collected from within a firearm cannot be used to reliably infer shooting distance in crime scene reconstruction. To enhance success rate of this method in routine casework, the pooling of all samples recovered from inside surfaces of firearms used in firearm-related crimes is deemed advisable.

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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.forsciint.2015.10.030.

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Supplementary Table 1

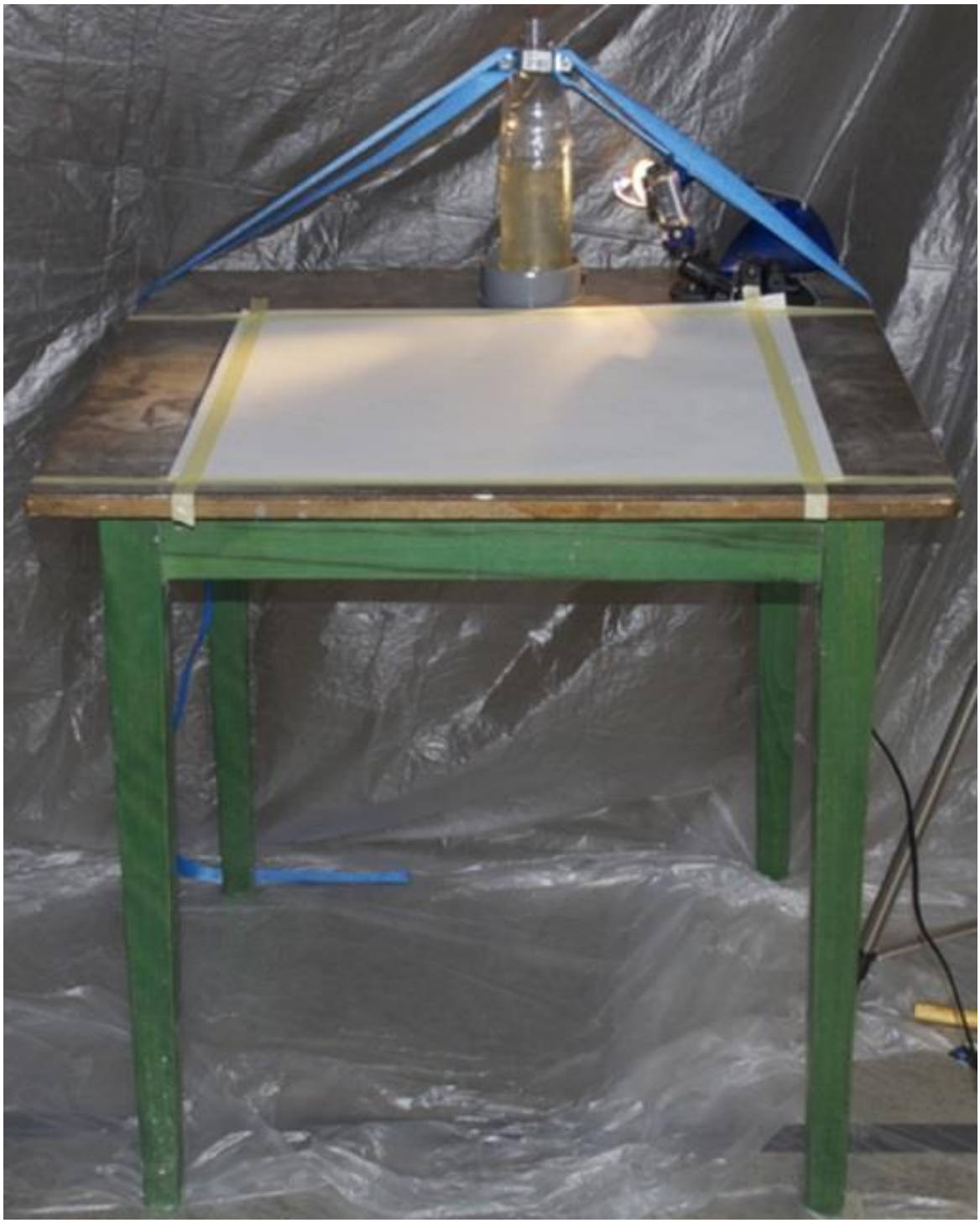
Specifications of the candidate reference genes

Source	Official Gene Symbol	NCBI-Alias	NCBI-/miRBase-mature sequence accession	TaqMan Assay ID	Small RNA sequence (amplicon length in base pairs)	References
blood/brain	hsa-miR-93-5p	---	MIMAT0000093	001090	CAAAGUGCUGUUCGUGCAGGUAG (23)	[16]
blood/brain	*hsa-miR-191-5p*	---	MIMAT0000440	002299	CAACGGAAUCCCAAAAGCAGCUG (23)	[16]
blood/brain	*RNU6-2*	*U6, RNU6B*	NR_002752	001093	CGCAAGGAUGACACGCAAAUUCGUGAAGCGUUCCAUAUUUUU (42)	[13,14,19]
brain	*RNY3*	*HY3, Y3*	AC005251	001214	CCAGTCACAGATTTCTTTGTTCCTTCTCCACTCCCACTGCATCACTTAACTAGCCTT (57)	[19]
blood	*SNORA66*	*RNU66, U66*	NR_002444	001002	GUAACUGUGGUGAUGGAAAUGUGUUAGCCUCAGACACUACUGAGGUGGUUCUUUCUAUCCUAGUACAGUC (70)	[19]
blood	*SNORA74A*	*U19, RNU19*	X94290	001003	UUGCACCUCUGAGAGUGGAAUGACUCCUGUGGAGUUGAUCCUAGUCUGGGUGCAAACAAUU (61)	[19]
brain	*SNORD18A*	*U18A*	AB061820	001204	CAGTAGTGATGAAATTCCACTTCATTGGTCCGTGTTTCTGAACCACATGATTTTCTCGGATGTTCTGATG (70)	[19]
blood/brain	*SNORD24*	*U24, RNU24*	NR_002447	001001	AUUUGCUAUCUGAGAGAUGGUGAUGACAUUUUAAACCACCAAGAUCGCUGAUGCA (55)	[14,19,33]
blood	*SNORD38B*	*U38B, RNU38B*	NR_001457	001004	CCAGUUCUGCUACUGACAGUAAGUGAAGAUAAAGUGUGUCUGAGGAGA (48)	[19]
blood	*SNORD43*	*U43, RNU43*	NR_002439	001095	GAACUUAUUGACGGGCGGACAGAAACUGUGUGCUGAUUGUCACGUUCUGAUU (52)	[19]
brain	*SNORD44*	*U44; RNU44*	NR_002750	001094	CCUGGAUGAUGAUAGCAAAUGCUGACUGAACAUGAAGGUCUUAAUUAGCUCUAACUGACU (60)	[14,19,33]
brain	*SNORD47*	*U47, RNU47*	X96647	001223	TAATGATTCTGCCAAATGAAATATAATGATATCACTGTAAAACCGTTCCATTTTGATTCTGAGGT (65)	[19]

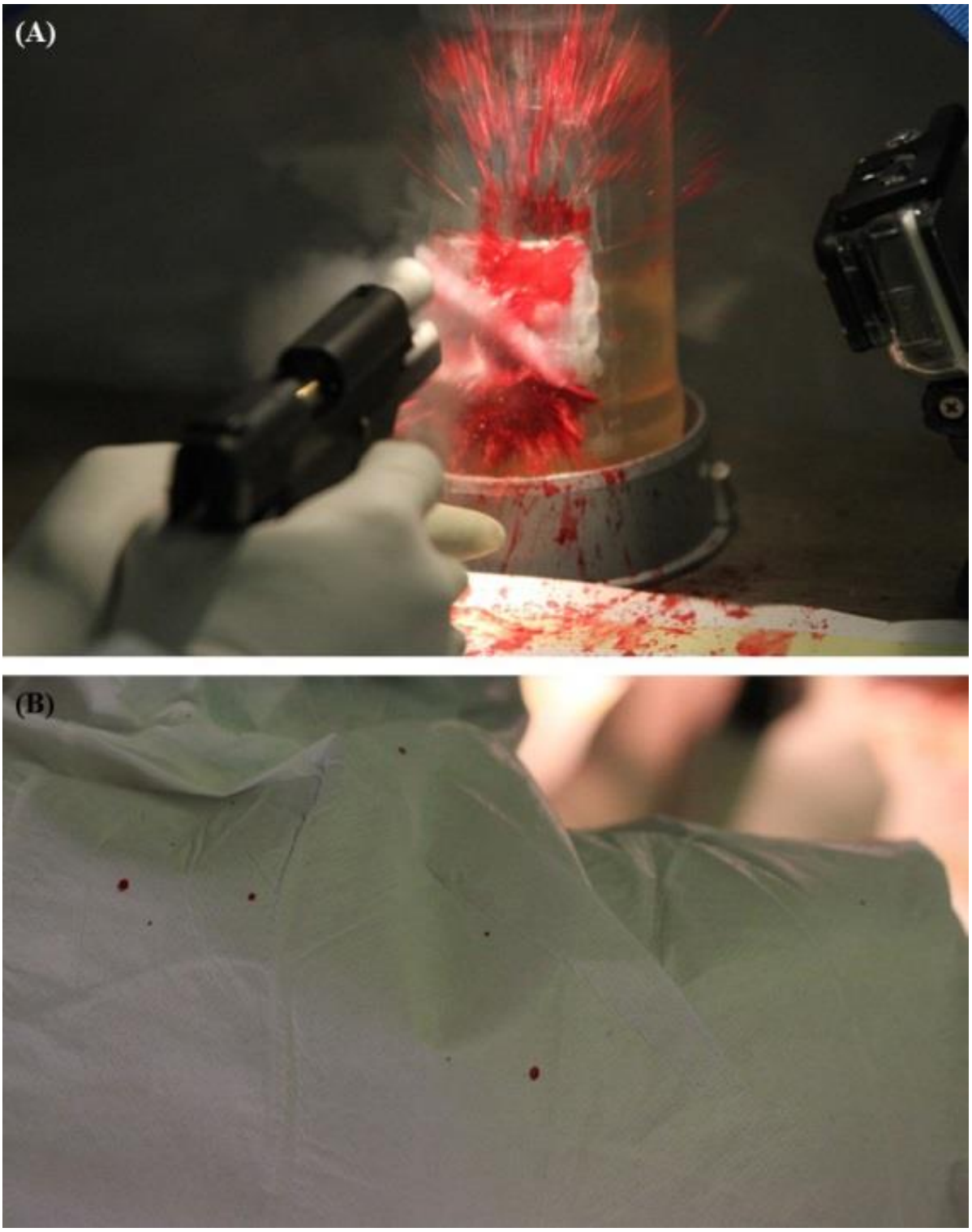
brain	*SNORD48*	*U48, RNU48*	NR_002745	001006	GAUGACCCCAGGUAACUCUGAGUGUGUCGCUGAUGCCAUCACCGCAGCGCUCUGACC (57)	[14,19,33]
blood/brain	*SNORD49A*	*U49, U49A, RNU49*	NR_002744	001005	CACUAAUAGGAAGUGCCGUCAGAAGCGAUAACUGACGAAGACUACUCCUGUCUGAUU (57)	[19]

NCBI: National Center for Biotechnology Information; *miRBase:* microRNA database [17]; ---: does not apply

Supplementary Figure 1: Ballistic model used for experimental shootings



Supplementary Figure 2: Installation for fixing and adjusting the ballistic models and to measure shooting distances



Supplementary Figure 3: Backspatter produced by a shot with pistol 9 x 19 mm
(A) The ballistic model being hit by a projectile (moment of impact) fired from a distance of 5 cm. **(B)** Splashes of backspattered blood were found on the back and right back shoulder of the shooter produced by a shot from a distance of 5 cm.

4.4 Zusammenfassung

Die systematische Untersuchung von Rückschleuderspuren in kontrollierten Schussexperimenten unter Verwendung standardisierter ballistischer Modelle [11, 54, 56, 59, 73, 76, 77] ist ein relevanter Forschungsansatz, der logistisch unkomplizierter und kosteneffizienter zu realisieren und zu reproduzieren ist als beispielsweise die Durchführung experimenteller Schüsse auf lebende Tiere [62, 64, 65, 69, 70], die aufgrund der Komplexität der Planung, Durchführung und angesichts der erheblichen anatomischen Unterschiede zwischen Tier und Mensch, insbesondere des Schädels, nur vermindert aussagekräftig und überdies ethisch fragwürdig sind.

Die hier vorgestellten Studien untersuchten erstmals systematisch die Eignung, Analysierbarkeit und Kompatibilität verschiedener Nukleinsäuren aus Rückschleuderspuren, die von inneren und äußeren Oberflächen von Feuerwaffen nach Abgabe von Schüssen auf verschiedenartig dotierte ballistische Modelle gesichert wurden.

4.4.1 Triple Contrast

Um die nach Feuerwaffengebrauch auf biologische Ziele in der Verteilung und Zusammensetzung von Backspatter enthaltene Information interpretieren zu können, müssen die Bedingungen und die Umstände für die Entstehung und Verteilung dieser Rückschleuderspuren geklärt und verstanden werden.

In diesem Schussexperiment wurden, auf die nach Schyma et al. [56] konstruierten ballistischen „Triple Contrast"-Modelle Schüsse mit 10 verschiedenen Feuerwaffen abgegeben, darunter sechs Revolver sowie vier halb-automatische Pistolen. Es wurden stets absolute Nahschüsse abgegeben und die Probennahme aus dem Waffeninneren erfolgte mittels der modifizierten Double-Swab-Technik [353] unter Verwendung eines je zur Hälfte befeuchteten und trockenen Abriebtupfers. Aus den gesammelten Proben des inneren Laufes wurden simultan DNA und RNA extrahiert. Eine Multiplex-STR-PCR wurde zur Erstellung forensischer DNA-Profile der genomischen DNA eingesetzt. Die Analyse der mtDNA erfolgte mittels des „Mito-Mini"-Verfahrens [119], bei dem der gesamte D-Loop einschließlich aller hoch-variablen Regionen der mtDNA (HVR I-III) amplifiziert und sequenziert wurde.

Um blutspezifisch exprimierte RNAs zu detektieren, wurden die Expressionsdaten des Gens *HBB* sowie der *hsa-miR-16-5p* mit den jeweiligen Referenzgenen *RPL37A* bzw. *SNORA66* normalisiert. Empirisch wurde die Eignung der hier eingesetzten Referenzgene aus einer Gruppe von Kandidatengenen, die auf Grundlage einschlägiger Literatur [345] sowie öffentlich verfügbaren Arraydaten [354] zusammengestellt worden waren, durch Verwendung gängiger Algorithmen (geNorm [355], NormFinder [356], BestKeeper [357] und Genevestigator [358]) unter dem Kriterium maximaler Stabilität geprüft.

Abermals bestätigte sich, dass innere Oberflächen von Schusswaffen als Quelle für forensisches Beweismaterial relevant sind, indem von dort die Sicherung von Backspatterspuren erfolgen kann, die geeignet für die Analyse verschiedener Nukleinsäurespezies (gDNA, mtDNA, mRNA und miRNA) sind [11, 54–57]. Positive DNA-Quantifizierungsergebnisse wurden für alle gesammelten Proben erhalten, jedoch konnte eine große Varianz der DNA-Ausbeuten zwischen Proben von verschiedenen Lauf-Teilen sowie aus den verschiedenen Waffentypen und den Probanden beobachtet werden. Im Allgemeinen und wie zu erwarten, enthielten Proben, die aus der vorderen Hälfte des Laufes gewonnen wurden, signifikant mehr DNA als Proben aus der hinteren Laufhälfte. Insbesondere enthielten die Proben, die aus Pistolen gesammelt wurden, niedrigere DNA-Konzentrationen als diejenigen aus Revolvern. Zur Analyse der mtDNA-Haplotypen wurden von ausgewählten Proben, die eine DNA-Menge von weniger als 100 pg aufwiesen und bei denen nur unvollständige oder keine STR-Profile erhalten wurden, alle amplifizierten Fragmente, die die gesamte D-Loop-Region der mtDNA repräsentieren, sequenziert. Es konnte gezeigt werden, dass mitochondriale DNA reproduzierbar gewonnen und analysiert werden konnte und damit die Analyse der mtDNA als alternativer und ergänzender Ansatz für die Standard STR-Profilierung in der molekulargenetischen Analyse von Backspatter nutzbar ist, wenn nur unzureichende und/oder stark degradierte genomische DNA aus Rückschleuderspuren gewonnen werden konnte. Trotz stark variierender und teils nicht messbarer RNA-Ausbeuten gelang es in allen untersuchten Proben, eine blutspezifische Expression der mRNA *HBB* nachzuweisen, während die Normalisierung der miRNA-Expressionsdaten in einigen Proben, aufgrund zu geringer Messwerte der endogenen Kontrolle (*SNORA66*), wahrscheinlich verursacht durch zu geringe Ausbeute und/oder zu starke Degradation, scheiterte und somit kein gültiges Messergebnis erzielt werden konnte.

Im Gesamten erweitern und belegen die Ergebnisse die Vielseitigkeit und die Kompatibilität der „Triple Contrast"-Methode, indem sie in experimentellen Schussversuchen die simultane Analyse von wundballistischen Phänomenen und die gesamte Bandbrandreite forensisch-molekulargenetisch relevanter Nukleinsäuresanalysen ermöglicht. Es wurde die Möglichkeit aufgezeigt, dass mRNA und miRNA simultan zur DNA [9, 10] aus Backspatter von inneren Oberflächen von Schusswaffen isoliert und analysieret werden kann, um die Individualisierung des Spurenmaterials um den Aspekt der Kontextualisierung zu erweitern. Dies ermöglicht, dass die gewonnenen Erkenntnisse sich einander in ihren Aussagequalitäten integrieren, bestätigen und/oder ergänzen können. Des Weiteren gestattet diese Methode eine „wirtschaftliche" Realisierung komplexer Spurenbilder erzeugender experimenteller Schussversuche, was die Nützlichkeit des Verfahrens als Forschungsinstrument in der experimentellen forensischen Ballistik unterstreicht.

4.4.2 Schussdistanz

Die Schussdistanz beeinflusst maßgeblich die Entstehung und Auffindbarkeit forensischen Spurenmaterials, wie Schmauch-, Forward- und Backspatterspuren, die sowohl bei experimentellen Schüssen, als auch bei Schusswaffendelikten erzeugt werden können.

In dieser experimentellen Schussreihe wurde erstmals systematisch der Einfluss der Schussentfernung mit unterschiedlichen Feuerwaffentypen und -kalibern auf Menge und Verteilung von Backspatter und darin enthaltener DNA und miRNA untersucht. Die Analyse umfasste ein modifiziertes ballistisches Modell nach Schyma et al. [56], welches nicht mit Acrylfarbe und Radiokontrastmittel, allerdings neben Blut mit Hirngewebe dotiert wurde. Die Schussdistanzen betrugen 30 cm, 15 cm, 5 cm und 0 cm (absoluter Nahschuss), aus denen Schüsse mit einem Revolver und zwei Pistolen auf je ein ballistisches Modell abgegeben wurden. Die Probennahme erfolgte jeweils von verschiedenen inneren und äußeren Oberflächen der Schusswaffen mittels einer modifizierten Double-Swab-Technik [353], mit nur einem je zur Hälfte befeuchteten und trockenen Abriebtupfer. Die RNA/DNA Ko-Extraktion erfolgte aus den gesammelten Proben. Zur standardmäßigen DNA-Typisierung der genomischen DNA erfolgte eine Multiplex-STR-PCR. Um blut- bzw. hirnspezifisch exprimierte RNAs zu detektieren, wurden die blutspezifische miRNA *hsa-mi*R-*16-5p* und die hirnspezifische miRNA *hsa-mi*R-*124a-3p* mit dem Referenzgen

hsa-miR-191-5p normalisiert. Die Auswahl des hier eingesetzten Referenzgens wurde empirisch aus einer Gruppe von Kandidatengenen, die auf Grundlage einschlägiger Literatur [345, 359–361] zusammengestellt worden waren und unter dem Kriterium maximaler Stabilität auf seine Eignung durch Verwendung gängiger Algorithmen (geNorm [355], NormFinder [356], BestKeeper [357] und Genevestigator [358]), geprüft.

Es konnte demonstriert werden, dass der Feuerwaffentyp und das -kaliber einen erheblichen Einfluss auf die Entstehung und die Reichweite von Rückschleuderspuren haben und nachweislich die Gewinnung von analysierbarer DNA und RNA aus Blut und/oder Hirngewebe erfolgreich war. Lediglich bei den Pistolen ließen sich für eine Identifikation ausreichende DNA-Profile bei Entfernungen von bis zu 5 cm (Pistole 7.65 mm Browning) bzw. 15 cm (Pistole 9 mm x 19) erstellen. Ein Grund für diese begrenzte DNA-Rückgewinnung könnte das Verfahren zur Probenentnahme sein. Nach jedem Schuss wurden mehrere Proben gesammelt. Um jedoch die unterschiedliche Verteilung von Backspatter zwischen mehreren Stellen innerhalb der Feuerwaffen (z.B. vordere und hintere Hälfte des Laufinneren, Innenschlitten, Geschosskammern etc.) zu bewerten, wurden die Proben nicht zusammengefasst, sondern separat bearbeitet. Während diese künstliche Kompartimentierung des gesamt verfügbaren Spurenmaterials möglicherweise einzelne Proben mit DNA-Mengen unterhalb der Nachweisgrenze produziert haben könnte, ist es vorstellbar, dass das Zusammenführen aller verfügbaren Proben die STR-Typisierungs-Erfolgsrate erhöht, ein Vorgehen, das für die Fallarbeit ratsam erscheint. Dies könnte sogar eine erfolgreiche STR-Profilerstellung aus Schussdistanzen > 15 cm ermöglichen.

Im Gegensatz dazu gelang der korrekte RNA-basierte Nachweis von Blut bzw. Gehirn bei allen Schusswaffentypen bis zur Entfernung von 30 cm bzw. 15 cm. Jedoch konnte keine robuste Korrelation zwischen RNA-/DNA-Ausbeute und Schussdistanz und/oder Waffentyp festgestellt werden, so dass die in bzw. an einer Feuerwaffe gesammelte Menge an Nukleinsäuren im Backspatter nicht zuverlässig als Indikator genutzt werden kann, um die Schussdistanz bei der Tatortrekonstruktion zweifelsfrei abzuleiten. Dennoch erlaubt die hier vorgestellte Analyse der spezifischen Expressionsmessung von blut- und hirnspezifischer miRNA einen robusten Nachweis dieser Körperflüssigkeit und des Organgewebes, auch bei Proben aus Schussdistanzen von bis zu 30 cm und übertrifft damit die Sensitivität der DNA-Analyse. Dieser Unterschied kann möglicherweise durch eine höhere Sensitivität der miRNA-basierten Assays mit bis zu 35 Amplifikationszyklen erklärt werden sowie dadurch,

dass miRNA weniger durch Fragmentierung beeinflusst wird, die durch die mit dem Schussgeschehen verbundenen physikochemischen Belastungen verursacht wird, als die DNA.

Im Gesamten folgt daraus, dass das Vorhandensein von Backspatter auf inneren und äußeren Oberflächen von Feuerwaffen als eine wertvolle Quelle forensischen Beweismaterials angesehen werden sollte. Dabei ist dies kein, wie zuvor vermutet, ausschließlich bei absoluten Nahschüssen auftretendes Phänomen, sondern die RNA-basierte Spurenartidentifikation kann sogar bei Schussdistanzen von bis zu 30 cm gelingen. Um weiterführende Erkenntnisse im Rahmen der routinemäßigen Spurensicherung zu erhalten, sollten grundsätzlich, vor allem aber bei Schussentfernungen jenseits der absoluten Nahschussweite, die inneren Oberflächen von Schusswaffen auf das Vorhandensein von Rückschleuderspuren geprüft werden. Als ratsam wird erachtet, die von der Schusswaffe gesammelten Proben vor der molekulargenetischen Analyse zusammenzuführen, um die Ausbeute zu maximieren und damit die Chance für eine erfolgreiche Untersuchung zu verbessern sowie die Möglichkeit der meist nicht routinemäßig durchgeführten Analyse der RNA zur Kontextualisierung der Spurenart hinsichtlich der Trefferzonenzuordnung zu erwägen.

5 Spurensicherungsmethoden an der Schusshand

5.1 Einleitung

Im Zusammenhang mit Schusswaffendelikten ist für die forensische Fallarbeit neben der Untersuchung der Schussdistanz [46, 48, 50, 51, 362–364], der Schussrichtung sowie der Einschusslokalisation die Bestimmung der Schusshand [37, 38, 52, 60–62] von elementarer Bedeutung.

Das Augenmerk liegt neben vorfindlichen chemischen Schussrückständen (GSR), die durch das Abbrennen von Zünd- und Treibladung entstanden sind, auch auf biologischen Spuren (u.a. Backspatter), welche aus der Eintrittswunde in Richtung der Schusswaffe zurückgeschleudert wurden. Das Verteilungsmuster und die Verteilungsdichte der Schussrückstände lassen Rückschlüsse auf die Spurentstehung zu [365, 366]. Die größte Relevanz solcher Schussrückstände kommt dabei mitunter dem Backspatter zu [33]. Das zunehmende Interesse der forensischen RNA-Analyse, deren Vielseitigkeit und Komplementarität die traditionelle DNA-Profilerstellung aus Spurenmaterial in der forensischen Fallarbeit erweitert, unterstreicht das Potential der Rückschleuderspuren als Quelle nicht nur für DNA sondern auch für RNA zu dienen. Diese Spuren können mittels topographischer Spurensicherungsmethoden wie flächendeckende Spurensicherungsfolien [39, 40] oder handschuhartige Polyvinylalkoholabzügen (PVAL) [34, 42] lagegerecht an der Schusshand asserviert und integrativ ausgewertet werden.

Das Vorhandensein von Backspatter und/oder GSR auf einer Hand oder einer anderen Oberfläche kann auf eine enge räumliche Nähe der Schusshand zur Einschussstelle im Moment der Schussabgabe hinweisen. Allerdings beweist die bloße Anwesenheit von schusswaffenbezogenen Spuren auf der Hand eines Individuums nicht zwangsläufig,

dass diese Person die Schusswaffe abgefeuert hat [25, 37, 38]. Dies kann zu einer falsch positiven oder falsch negativen Identifikation der Person führen, die eine Feuerwaffe abgefeuert hat. Erst das Schmauchverteilungsmuster an der Hand kann weiteren Aufschluss bringen. Daher ist es bei der Rekonstruktion des Tathergangs von wesentlicher Bedeutung, forensische und molekulargenetische Analysen an Spurenmaterial durchzuführen, das von Tatverdächtigen und/oder Opfern gesammelt wurden.

Spurensicherungsfolien verschiedener Formen und aus verschiedenen Materialien [39–41, 367, 368] werden bei der Spurensicherung am Tatort von den kriminaltechnischen Erkennungsdiensten verwendet, um Spurenmaterial das an Oberflächen von Beweismaterial haftet, zu asservieren. Solche Folien sind an der Schusshand simpel zu applizieren und können Haare, Epithelzellen, Schussrückstände oder Backspatter von ausgewählten Bereichen an den Händen oder Fingern von Verdächtigen oder Opfern aufnehmen. Anhand solchermaßen gesicherten Materials konnte bereits simultan der Nachweis von Schussrückständen sowie DNA erbracht werden [367, 369]. Ein Nachteil dieser Methode ist, dass das Schmauchverteilungsbild nicht exakt sichtbar gemacht werden kann [370].

Die 1993 von Merkel und Mailänder [34] vorgestellte topographische PVAL-Methode ist ein weiteres wesentliches Verfahren zur Sicherung von GSR oder biologischem Material von der Hand eines Schützen. Hierbei wird ein wasserlösliches, flüssiges Polymer verwendet, um die Hände von Tatverdächtigen oder Opfern für die in-situ-Konservierung der Rückschleuderspuren zu bedecken. Diese repräsentative Konservierung ermöglicht die Fertigung eines exakten Abbildes des vorhandenen Spurenmaterials an den Händen. Die Eignung dieser Technik für die Erkennung und Charakterisierung von GSR [32, 34, 365, 366] und ihre Kompatibilität mit der Singleplex-STR-Typisierung von DNA aus Backspatter [42] konnte bereits gezeigt werden.

Die vorliegenden Originalpublikationen untersuchten und bewerteten die Anwendbarkeit der forensischen Analyse von ko-extrahierter RNA/DNA an bis zu 20 Jahre altem biologischem Spurenmaterial, von Händen involvierter Personen von Schusswaffendelikten, welches mittels Spurensicherungsfolien bzw. der PVAL-Methode gesichert wurde.

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ORIGINAL ARTICLE

RNA/DNA co-analysis on aged bloodstains from adhesive tapes used for gunshot residue collection from hands

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Abstract In cases of firearm related fatalities a systematic investigation at the scene of death is indispensable to differentiate between self-inflicted and homicidal gunshot injuries. A common method to preserve gunshot residues (GSR) is their collection using adhesive tapes. However, the biological material gathered at the same time by the tapes would be of special interest if backspatter, ejected from the entrance wound against the direction of fire, could be detected. In the present study we examined the success rate of co-analysis of RNA and DNA recovered from biological traces sampled with adhesive tapes. The material originated from eight cases of fatal gunshots, taken from the hands of suspects or victims, examined 5 to 19 years ago for GSR. For all types of adhesive tapes tested, quantity and quality of the co-extracted nucleic acids was insufficient for successful DNA profiling, but was sufficient for the detection of blood-specific micro RNA (miRNA). In summary, sampling trace evidence from the hands of persons involved in fatal gunshots with adhesive tapes has a long-term detrimental effect on biological traces.

Keywords Adhesive tapes · Backspatter · Gunshot residues · Blood specific expression · RNA/DNA co-analysis

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Introduction

In recent years, interest in forensic RNA analysis, its versatility and complementarity to traditional DNA profiling of trace evidence in forensic routine casework, has increased. Thus, the identification of a suitable method for collecting non-biological (e.g. gunshot residues), as well as biological materials (e.g. traces of blood and tissue), from crime scenes and related items during forensic investigations, is an important prerequisite for obtaining adequate analysis results.

Widely applied techniques of (non-)biological evidence collection at crime scenes comprise swabbing, cutting out, or taping the area of interest. Adhesive tapes of various shapes and materials and tape-lifts (for direct scanning electron microscope analysis) are devices commonly used by police departments in crime scene investigations and can represent an important source of forensic evidence. Such tapes are straightforward to apply and can be used to collect samples including GSR, shed epithelial cells or backspatter from selected areas (e.g. from the hands or fingers of suspects or victims of crimes involving fatal gunshot injuries), which may be a source of DNA and/or RNA.

GSR are composed of burnt and unburnt particles containing vaporized and condensed materials (e.g. lead, barium, antimony and zinc) and arise from combustion products from primer, propellant, projectile and/or the weapon itself with particle sizes usually ranging from 0.5 to 100 μm [1].

Backspattered biological material and/or GSRs found on a shooter's hand represent important evidence for self-inflicted gunshot injury, however, the sheer presence of firearm-related backspatter and/or GSRs on any individual's hand does not necessarily prove that this person did discharge a firearm. Therefore, in reconstructing the course of events, it is very important to perform forensic and molecular genetic analysis on evidence materials collected from the suspect or victim.

Previous studies demonstrated successful DNA profiling and GSR analyses from different types of adhesive tapes used in forensic routine casework [2–4].

The object of the present study was to evaluate the applicability of RNA/DNA co-extraction and assess success rates of DNA-profiling and RNA-based blood identification of biological material collected from adhesive tape samples that were collected in forensic casework and stored for up to 19 years.

Material and methods

Samples and ethics

Adhesive tape samples originated from eight cases of fatal gunshots between April 1997 and October 2010, comprising six cases of suicide, one homicide and one accident. The samples were collected from specific areas of the hands of victims and suspects.

Three different types of adhesive tapes where used to collect evidence: Filmolux® 80 (Neschen AG, Bückeburg, Germany), Krimfo (Krimfo GmbH Kriminal- & Fototechnik, Norderstedt, Germany) and Scotch™ 800 (3 M Deutschland GmbH, Neuss, Germany).

The study protocol was reviewed and approved by the ethics committee of the Hospital of the University of Bonn.

Forensic analysis of GSR

Preservation of evidence and detection of gunshot residues

Filmolux® 80 tapes arrived after trace securing glued on silicone paper, Krimfo or Scotch™ 800 tapes were glued on neutral foils. To examine GSR, the adhesive tapes were opened under laboratory conditions, avoiding contamination. For chemographic detection of GSR adhesive tapes were treated with 2% tartaric acid and saturated sodium rhodizonate solution. After drying all tapes were fixed onto a new neutral foil and sealed. Purple, red or orange colored particles were detected using a stereo microscope Stemi 2000 C (Carl Zeiss Microscopy, Oberkochen, Germany) at 20 to 40 fold magnification. Samples of these particles were examined by non-destructive energy dispersive X-ray fluorescence analysis (Eagle μ-Probe II, EDAX Inc., Mahwah, U.S.A.) to verify characteristic GSR element composition. After the criminal investigations were officially closed by the prosecution the samples were archived in a dry and dark room and tapes that had been glued on neutral foils had not been opened until molecular genetic analyses were performed.

Molecular genetic analysis

To prevent possible bias molecular genetic investigations were blinded. No information about the cases, involved persons, weapons, etc. were disclosed to investigators performing the analyses.

Sampling procedure

First, adhesive tapes were examined macroscopically, and areas containing visible stains suspected to contain blood were cut out with a scalpel and separated from the neutral foil. For the sake of maximal sample retention pre-tests for the identification of blood were not performed. For each adhesive tape both separate and pooled samples (pooling together several cuttings) were generated and examined separately.

All samples collected from adhesive tapes for RNA and DNA analysis were treated before as described in the section 'Forensic analysis of GSR'.

RNA extraction, quantification and integrity assessment

Prior to all RNA processing, all surfaces, instruments and devices employed in the processes were thoroughly cleansed using RNase-Zap® (Ambion, Austin, TX, U.S.A.) and Roti®-Nukleinsäurefrei (Carl Roth, Karlsruhe, Germany) to remove all traces of ambient RNases and nucleic acid contaminations. Only RNase-free reagents and plastic consumables were used.

Nucleic acids were extracted using the NucleoSpin® miRNA Kit (Macherey-Nagel, Düren, Germany) according to the provided small RNA protocol with minor changes which had been shown to be well suited for co-extracting DNA and RNA from forensic type samples [5]. Briefly, cuttings were incubated for 10 min with 350 μl lysis buffer at room temperature and NucleoSpin® Forensic Filters (Macherey-Nagel) were then used to separate the liquid nucleic acid containing lysate from discardable solid material by centrifugation at 21000 x g for 3 min. The lysate was then processed according the protocol and small RNA was finally eluted in 40 μl of nuclease-free water pre-heated to 95 °C. All RNA extracts were stored at −80 °C until further processing.

Quantity and quality of small RNA were measured using QuantiFluor® RNA Dye with the Quantus™ Fluorometer (both Promega, Mannheim, Germany) and the RNA 6000 Pico Kit with an Agilent 2100 Bioanalyzer (both Agilent, Böblingen, Germany), respectively. For both methods, 1 μl of RNA extract was used and all measurements were performed according to the manufacturers' prescriptions. RNA quality is represented by the 'RNA integrity number' (RIN) [6]. Based on quantification results, all individual samples were diluted to a final concentration of 2 ng/μl with RNAse-free water. If RNA yield was too low to be detected fluorometrically, the maximum input volume for reverse transcription reactions was used instead.

DNA co- extraction, quantification and STR profiling

Co-extraction of DNA was performed for each sample by diverting 20 μl of its DNA containing RNA lysate to the DNA extraction procedure of the PrepFiler® Forensic DNA Extraction Kit (Life Technologies, Darmstadt, Germany), processing it according to manufacturer's instructions. All DNA extracts were then stored at −20 °C until further processing.

DNA concentration and presence of PCR inhibitors were quantified by quantitative PCR (qPCR) using the PowerQuant™ System (Promega, Mannheim, Germany) as recommended by the manufacturer on an ABI Prism 7500 Sequence Detection System (Life Technologies).

DNA profiling was performed by STR-Multiplex-PCR using the Powerplex® ESX 17 System (Promega) according to the provider's prescriptions for selected samples with input amounts between 4 and 54 pg of DNA. PCR products were detected on an ABI 310 Genetic Analyzer (Life Technologies). GeneMapper v3.2.1 software (Life Technologies) was used for raw data analysis and DNA profile compilation. The analytical threshold for a positive signal was set at 50 RFU.

Selection of blood specific miRNA and reference genes for qPCR data normalization

Based on previous work [7, 8] the blood specific *miR-451a* was chosen for the detection of blood (Table 1).

MiR-191 was chosen as best suited reference gene to serve as an endogenous control for the normalization of blood specific miRNA expression data (Table 1). This choice was based on the work of Sauer et al. [12] who had established a reliable and reproducible strategy for qPCR data normalization from forensically relevant body fluids in forensic type samples.

Reverse transcription (RT) and qPCR

Complementary DNA (cDNA) was synthesized from miRNA using the TaqMan® MicroRNA Reverse Transcription Kit employing target-specific stem-loop primers (both Life Technologies) in a total volume of 15 μl. Each miRNA RT reaction comprised 10 ng of total RNA, 1 x RT Primers, 50 U MultiScribe™ Reverse Transcriptase, 1 mM dNTPs, 3.8 U RNase Inhibitor, and 1 x RT buffer.

All RT reactions were performed on a T3 Thermocycler (Biometra, Göttingen, Germany) with the following cycling conditions: 16 °C for 30 min, 42 °C for 30 min, and 85 °C for 5 min.

To detect potential contaminations with genomic DNA or of our reagents, RNA extraction negative controls, RT(−) controls (i.e. RT reactions without reverse transcriptase), and H_2O controls (i.e. RT reactions with water instead of RNA template), were set up respectively. RNA was kept on ice during processing and RT reaction products were stored at −20 °C until further proceedings.

QPCR was performed using target-specific TaqMan® Assays (Life Technologies) (Table 1) for the selected blood specific miRNA and the reference gene: Each 20 μl reaction contained 1.3 μl of the respective RT reaction product, 1 x TaqMan® Universal PCR Master Mix, No AmpErase® UNG (Life Technologies) and 1 x TaqMan®Assay. All reaction components were kept on ice during processing.

All sample/assay combinations were run in technical triplicates. All qPCR reactions were conducted in MicroAmp® Optical 96-Well Reaction Plates on an ABI Prism 7500 Sequence Detection System (both Life Technologies) with the following qPCR cycling conditions: 95 °C for 10 min, followed by 40 cycles with 95 °C for 15 s and 60 °C for 1 min.

To normalize inter-run variation between qPCR reaction plates the internal positive control (IPC) from the PowerQuant™ System (Promega) was used as an inter-plate calibrator. Raw fluorescence data was collected during the 60 °C step by the SDS software v2.0.6 (Life Technologies) and then exported for further analysis.

Data analysis, normalization, statistics, and MIQE compliance

To calculate quantification cycle (C_q)-values and amplification efficiencies from raw data from SDS spread sheet exported R_n-values the LinRegPCR analysis program v2015.1 [13] was applied. For calculation of C_q-values a common threshold was set to −0.7 log (fluorescence). Sample replicates exhibiting C_q-values

Table 1 Specifications of the RNA assays

Official gene symbol	NCBI–/ miRBase-mature sequence accession	TaqMan assay ID	Small RNA sequence (amplicon length in base pairs)	References
hsa-miR-451a	MIMAT0001631	001141	AAACCGUUACCAUUACUGAGUU (22)	[9]
hsa-miR-191-5p	MIMAT0000440	002299	CAACGGAAUCCCAAAAGCAGCUG (23)	[10]

NCBI National Center for Biotechnology Information, *miRBase* microRNA database [11]

deviating more than one from the mean of the triplicates were excluded from further processing. Normalization of computed C_q-values of target specific miRNA was performed using the GenEx software v6 (multiD Analyses AB, Goteborg, Sweden). A normalized C_q-value <35 was considered to indicate successful PCR implying a target specific signal and thus RNA quantity and quality suitable for expression analysis.

Data analysis, interpretation, and statistical calculations were performed using MS EXCEL and SPSS software v.22 (SPSS Inc., Chicago, IL, U.S.A.).

To facilitate reliable and unequivocal interpretation of the qPCR results reported herein, all information that is rated 'essential' according to the MIQE guidelines [14] is reported, where applicable.

Results

Forensic analysis on gunshot residues

Non-biological material (e.g. gunshot residues) was sampled with adhesive tapes from different areas of the hands of suspects or victims after fatal gunshot injuries. In all cases the predominant primer element was lead. Therefore a microchemical treatment with 2% tartaric acid and saturated sodium rhodizonate solution was performed, to colorize GSR particles which could be detected by stereo microscopic examination (e.g. Fig. 1).

In three of eight cases (#1, #5 and #6) GSR was detectable (Table 2). In cases #2, #3 and #4 no GSR could be detected on the hands of the suspects. After reconstructing the circumstances and courses of events the suspects, respectively, they could be ruled out as shooters. In cases #7 and #8 (suicides), although the victims fired the guns themselves, no GSR could be detected stereo microscopically.

Molecular genetic analysis

Quantification and STR profiling of co-extracted DNA

Sampling biological material from hands of suspects or victims of fatal gunshot injuries, adhesive tapes were used as method for collection and preservation of evidence. Secured biological material (e.g. backspatter and/or epithelial cells) was then recovered from various locations of the adhesive tapes (e.g. Fig. 2 and Table 2). Samples were either analyzed separately or pooled (combining several cuttings) and then analyzed.

DNA was quantifiable in 33 and 20% of samples analyzed separately and pooled, respectively. In cases #1, #2, #3 and #5, no DNA was detectable. In all other cases, DNA concentrations ranged between 3.1 pg/μl (case #6; from the left palm of the hand) and 0.2 pg/μl (case #4; from the left back of the hand). Notably, DNA yields were low in average but no inhibition was observed.

None of the selected samples contained more than 100 pg DNA in 17.5 μl (maximum input volume for STR multiplex PCR) which had repeatedly shown to be sufficient for successful STR profiling in our laboratory (data not shown). The selected samples contained between 4 and 54 pg DNA in 17.5 μl. However, to assess the success rate of STR typing from samples collected from different adhesive tapes, DNA profiles were generated with PCR input amounts of 7–54 pg

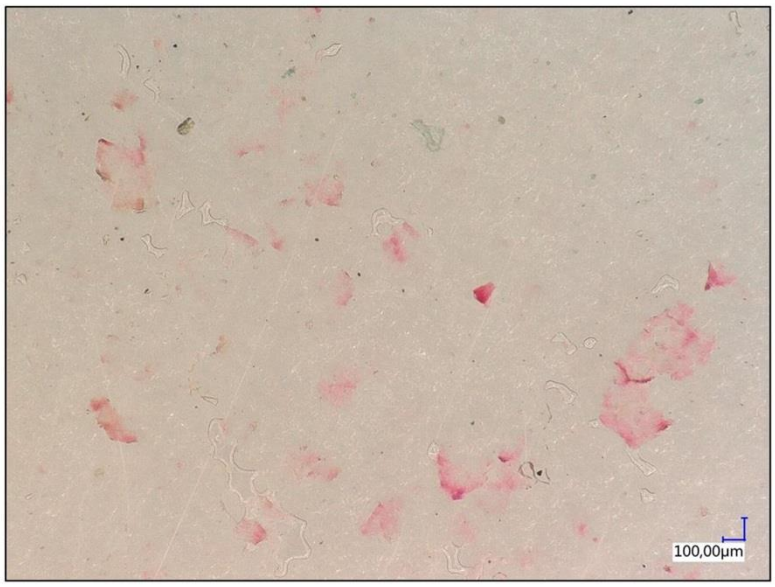

Fig. 1 Rhodizonate positive staining in an adhesive tape about 15 years after GSR collection. The purple color indicated lead which was confirmed by X-ray fluorescence analysis

Table 2 Overview of DNA and RNA results of analyzed adhesive tape samples

Case	No.	Manufacturer	Age of tapes (years)	Sex	GSR	Weapon	Shooter	Context	DNA results				RNA results	
									[a]DNA concentration	[b]DNA input amount	[c]Sufficient for STR profiling	STR profiling	RNA concentration	Correct detection
														blood
#1	1-L-BH	Krimfo	19	m	+	pistol	yes	homicide	–	–	–		–	–
	1-L-PH								–	–	–		–	–
	1-L-T/F												–	
#2	2-L-BH	Scotch™ 800	13	f	–	revolver	no	suicide	–	–	–		–	–
	2-R-BH								–	–	–		–	–
#3	3-L-BH	Scotch™ 800	10	m	–	shotgun	no	suicide					–	
	3-L-PH								–	–	–		–	–
	3-R-BH								–	–	–		–	–
	3-R-PH												–	
#4	4-L-BH	Scotch™ 800	10	m	–	revolver	no	suicide	0.2	4	no		–	–
	4-L-PH								0.4	7	no	–	–	
	4-R-BH								–	–	–		–	–
	4-R-PH								–	–	–		–	
#5	5-L-T	Filmolux® 80	18	m	+	revolver	yes	suicide	–	–	–		–	*
	5-L-FF												–	
	5-L-T/F								–	–	–		–	*
	5-R-T								–	–	–		–	*
	5-R-FF												–	
	5-R-T/F								–	–	–		–	–
#6	6-L-T	Filmolux® 80	5	f	+	revolver	no	accident					–	
	6-L-FF												–	
	6-L-MF												–	
	6-L-RF								0.6	11	no		–	
	6-L-LF												–	*
	6-L-PH								3.1	54	no	+	–	*
	6-R-T												–	
	6-R-FF												–	
	6-R-MF								–	–	–		–	
	6-R-RF												–	
	6-R-LF												–	–
	6-R-PH								0.7	12	no		–	*
#7	7-L-T	Filmolux® 80	7	f	–	blank pistol	yes	suicide					–	
	7-L-FF												–	
	7-L-MF								–	–	–		–	–
	7-L-PH i												–	
	7-L-PH o								2.3	40	no	–	–	*
	7-R-T												–	
	7-R-FF												–	
	7-R-MF								–	–	–		–	–
	7-R-RF												–	
	7-R-LF												–	
	7-R-PH i.												–	

Table 2 (continued)

Case	No.	Manufacturer	Age of tapes (years)	Sex	GSR	Weapon	Shooter	Context	DNA results				RNA results	
									[a]DNA concentration	[b]DNA input amount	[c]Sufficient for STR profiling	STR profiling	RNA concentration	Correct detection
	7-R-PH o.								–	–	–		–	*
#8	8-L	Filmolux® 80	5	m	–	blank pistol	yes	suicide	0.9	16	no	–	–	*
	8-R								0.4	7	no		–	*
Pooled samples	1-L-P								–	–	–		–	–
	2-L-P								–	–	–		–	–
	2-R-P								–	–	–		–	–
	3-L-P								–	–	–		–	–
	3-R-P								–	–	–		–	–
	4-L-P								–	–	–		–	–
	4-R-P								–	–	–		–	–
	5-L-P								–	–	–		–	–
	5-R-P								–	–	–		–	*
	6-L-P								2.3	40	no		–	*
	6-R-P								–	–	–		–	*
	7-L-P								–	–	–		–	*
	7-R-P								–	–	–		–	*
	8-L-P								1.4	25	no	–	–	*
	8-R-P								0.7	12	no		–	*

L Left hand, *R* Right hand, *BH* Back of the hand, *PH* Palm of the hand, *T/F* Thumb/forefinger, *T* Thumb, *F* Forefinger, *MF* Middle finger, *RF* Ring finger, *LF* Little finger, *i.* Inside, *o.* Outside, *P* Pooled samples, *m* Male, *f* Female, +: GSR positive; −: GSR negative

[a] values in [pg/μl]; [b] amount of DNA (pg) in 17.5 μl (maximum input volume for multiplex STR-PCR reaction); [c] *sufficient for STR profiling*: more >100 pg DNA amount

STR profiling: PCR full profile (++): 17/17; Identifiable (+): 8–16/17; Non-identifiable (−): 0–7/17; *: correct blood detected; −: not detected

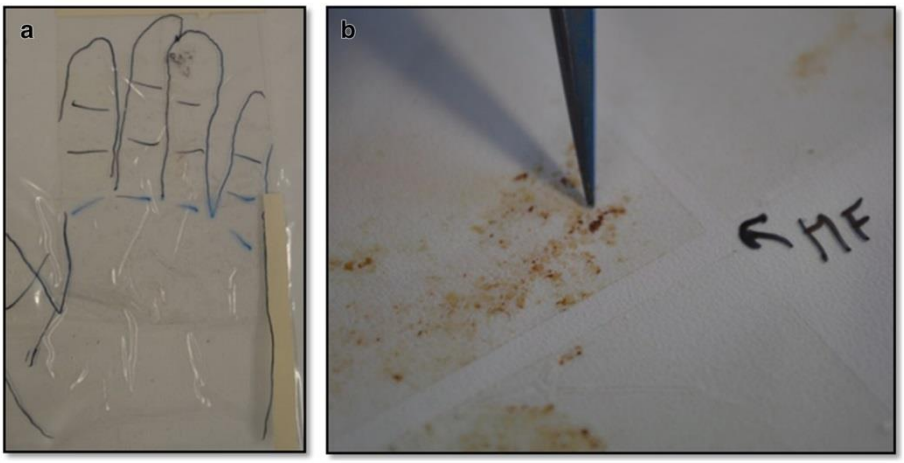

Fig. 2 Different types of adhesive tapes. **a** Whole adhesive tape from the right hand of a shooter (case #1). **b** Suspicious backspattered material sampled from the middle finger (MF) (case #6)

DNA and rated according to the following result categories: '++', full STR profiles (17 of 17 possible STR systems), '+', profiles suitable for identification (at least 8–16 of 17 possible STR systems), and '-', profiles unsuitable for identification (less than 8 of 17 possible STR systems).

Only one tested sample (input amount of 54 pg) recovered from a left palm was rated '+' for the DNA profile. In all other cases (75%) with input amounts of DNA < 40 pg STR profiling was unsuccessful ('-'). A full STR profile ('++') was not obtained for any tested sample. Table 2 summarizes these results.

Furthermore, no difference in DNA yield and STR typing success rate was observed between samples analyzed separately or after pooling.

Quantity and integrity of RNA

Overall, quantification of small RNA was not possible due to a lack of fluorometrically detectable RNA. There was, however, minor variation between different cases in terms of RNA quality (RIN) (data not shown), as expected for forensic samples of this type. Quantification results are summarized comprehensively in Table 2.

Expression analysis of miRNA

To assess the general suitability of samples recovered from different adhesive tape types for forensically relevant downstream analyses of RNA and the influence of the type of the tape on isolated biological material, expression levels of blood specific miRNA (*miR-451a*) was determined by qPCR in selected samples, representing different tested sampling locations and adhesive tape types (Table 2).

Overall, normalized expression of *miR-451a* that was considered to be indicative of blood ($C_{q,n} < 35$) was detected in 46% (cases #5, #6, #7 and #8) of all selected samples. There was no notable difference in successful detection of blood between samples analyzed separately or after pooling. Although small RNA was not detectable fluorometrically, detection via qPCR was successful.

All negative controls showed no or unspecific results ($C_q > 35$) for all tested samples.

Accordance of molecular genetic analysis and forensic chemical analysis

The unambiguous reconstruction of firearms related crimes requires a comprehensive search of traces, preservation of any available evidence, and integrated analysis to take into account all ascertained facts and circumstances. Not detecting GSR on a suspects hand does not necessarily indicate that he or she was not the shooter and vice versa. Therefore, when taking into account the circumstances of a crime complementary molecular genetic analysis of backspatter may support confirmation or exoneration of a suspect.

In cases # 2, #3 and #4 adhesive tape samples were taken from the hands of suspects found unconscious next to the deceased person. Forensic chemical analysis did not detect GSR on adhesive tape samples taken from the hands of the suspects. The results of an additional molecular genetic analysis of backspatter then confirmed the findings of the forensic chemical analysis independently: no blood specific miRNA (*miR-451a*) was detected on different locations on hands of suspects sampled with the adhesive tapes.

Although no GSR could be detected stereo microscopically in cases #7 and #8, the analysis of backspatter confirmed that the suicide victims had fired the gun.

Discussion

The present study was based on adhesive tapes which were used to collect gunshot residues between 5 and 19 years before

our current molecular genetic investigation. At that time GSR analysis had been performed [15]. We have now investigated the simultaneous extraction and forensic analysis of nuclear DNA and miRNA from aged traces of backspatter and shed epithelial cells that had been collected with adhesive tapes from different parts of the hands of victims, suspects or perpetrators of fatal gunshots. When investigating firearm-related crimes it is important to make use of the information contained in the composition and distribution of gunshot residues and/or backspatter to understand and reconstruct the conditions and circumstances of the events that led to their respective generation.

For the assessment of the value of a particular GSR evidence in linking a suspect to a crime it is important to distinguish whether the suspect did fire a gun in a specific situation, or whether he or she was not involved in the shooting: The detection of GSR on a person's hand or any other surface can occur if a firearm has been discharged in proximity of, or after contact with, a surface that is contaminated with GSR. This then can prompt a false positive or false negative interpretation of that person having fired a gun. In cases #7 and #8, although the suicide victims had fired the guns, no GSRs were detectable after forensic chemical analysis. Therefore, it is important to take into account all ascertained facts to reconstruct the circumstances of gun related crimes, including forensic chemical and molecular genetic analyses.

In forensic routine casework, STR profiling is considered the gold standard for DNA-based identification because of its robustness and high power of discrimination. In recent studies different types of adhesive tape had been used to collect shed epithelial cells, fingerprints [2], and other biological material, which was then subjected to DNA analysis to assess the success rate of STR-profiling [3, 4, 16, 17].

Gunnarsson et al. [17] analyzed over 3300 'mini-tape' samples and concluded that this sampling method would generate a significantly lower amount of DNA compared to two other methods they examined. More than 500 samples exhibited a DNA concentration lower than 6 pg/μl. These findings are in concordance with our results in comparable ranges of DNA concentrations, with DNA yields between 0.2–3.1 pg/μl.

Zech et al. [4] examined 150 biological samples from adhesive tapes collected in the investigation of authentic cases and obtained DNA profiles from more than half of the samples. The STR results from samples analyzed by Gunnarsson et al. [17] exhibited more mixed profiles than single profiles. Overall and in contrast, our samples yielded low DNA concentrations that were insufficient for successful STR profiling, and not even pooling several cuttings from the same tape sample generated sufficient amounts of DNA. A probable reason for such poor DNA recovery is the minimal size of traces of backspatter that were excised from the tape samples (Fig. 2, b). In comparison, other authors [3, 4, 17] have used whole adhesive tapes or swabbings of large parts [4] of the adhesive tapes for their analyses. Furthermore, degradation caused by aging (5–19 years), environmental exposure, and other conditions detrimental to DNA integrity, may have reduced recovery of typeable DNA.

One of these conditions, already mentioned by Zech et al. [4], could be the amount of glue contained in the adhesive tapes. Too much glue per square millimeter of tape may inhibit recovery of biological trace sample material and thus the process of genotyping, whereas too little of the adhesive substance results in lower levels of adhesion, so that less biological material will be collected. In addition, strength of adhesion will also be reduced when collecting moist blood stains as applying the non-absorbent tape will smear the sample, reducing the efficacy in the collection of other biological material [15].

We examined adhesive tapes from three different manufacturers and observed a difference of DNA concentrations between the respective products. Samples collected with the Filmolux® 80 adhesive tapes generated a positive DNA result in 42% of cases as compared to the Scotch™ 800 and Krimfo tapes (20%). In 2010, Barash et al. [3] examined four different types of adhesive tape and concluded that the use of three-layered adhesive tapes exhibits the highest efficiency in the collection of crime stains. Therefore, the type of adhesive tape appears to be another important factor in obtaining adequate DNA results.

Beside standard DNA analysis another important aspect in the reconstruction of the circumstances of a crime is the analysis of the pattern and composition of a stain, e.g. backspatter, by RNA-based identification of body-fluids and organ tissues. RNA was first mentioned in the forensic literature in 1984 by Oehmichen et al. [18] and in 1994 Phang et al. [19] introduced reverse transcription PCR based analysis of post-mortem RNA as a potential tool for forensic genetics. With the advent of quantitative PCR [20] interest in mRNA based methods started spreading in forensic molecular biology [21] and the potential of the analysis of differential expression of miRNA for forensic body fluid identification (BFI) has been explored since 2003 [22].

Due to their short size (18–24 bp) miRNA are more stable and substantially less prone to degradation than mRNA, which is a crucial asset to the investigation of forensic type samples. Thus, in this study, the presence of blood on the hands of victims or perpetrators of fatal gunshots was assessed via the identification of blood specific miRNAs in traces of backspatter. Although no RNA concentration could be determined fluorometrically a fluorescence-based detection of blood using qPCR was successful. Normalized blood specific expression of *miR-451a* was successfully detected in the tested samples originating from hands of suicide victims, whereas miRNA expression was negative in samples originating from the suspects. Apart from that, our results are consistent with the findings of Lux et al. [23] who, in a pilot study, proved the principle that the detection of brain specifically expressed

miRNA and mRNA in backspatter from firearm-related injuries may crucially support the inference that a head shot has occurred. Therefore, the investigation of gunshot residues and DNA should also include RNA based identification of body-fluids and organ tissues as an important and supporting factor in the interpretation and contextualization of biological traces to support the reconstruction of the circumstances of crimes.

Conclusion

Sampling minimal biological traces (like backspatter) collected from case work items with adhesive tapes we found that this non-destructive collection method is prone to unsuccessful DNA profiling when only minimal traces of material is available. However, analysis of RNA yields better success rates and besides the detection of gunshot residues and standard DNA analysis may be a supporting and exonerating supplement in reconstructing the circumstances of crimes.

Key points

1. The analysis of aged traces of biological material from adhesive tape samples can be expanded to encompass forensic co-analysis of DNA and RNA.
2. Samples of biological material collected with different types of adhesive tapes are amenable to the bandwidth of forensic nucleic acid analyses.
3. Collecting minimal biological traces from casework items with adhesive tapes this could entail a loss of traces.

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Compliance with ethical standards The study protocol was reviewed and approved by the ethics committee of the Hospital of the University of Bonn.

Conflict of interest All authors declare they have no conflict of interest.

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ORIGINAL ARTICLE

RNA/DNA co-analysis from bloodstains on aged polyvinyl-alcohol gloves prepared for securing evidence from the hands of victims of fatal gunshot injuries

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Abstract In contrast to cumulative techniques (e.g., tape-lift) for qualitative gunshot residues (GSR) analysis, topographic methods are commonly applied to preserve the integrity of evidence from a shooter's or victim's hand in cases of gun-related crimes. Topographic sampling techniques employing adhesive foils, latex, or the polyvinyl alcohol (PVAL) method enable unambiguous sampling of biological and non-biological trace material while preserving its spatial distribution and relation to each other. The PVAL method in particular allows for a topographically veridic and quantitative conservation of traces of GSR and biological stains that are embedded in the PVAL glove, because it completely removes these traces from the hand. The present study investigated the success rates of STR profiling and the detection of blood and brain-specific gene expression from minimal traces of blood splatter as well as parallel to the positive detection of gunshot residues embedded in 17 PVAL gloves taken from the hands of deceased persons in the context of homicide cases in the period between 1996 and 2003. The water-soluble PVAL matrix is shown to be fully compatible with successful STR profiling and the detection of blood- and brain-specific miRNA expression, even after up to 20 years of storage, demonstrating that this sampling technique offers advantages compared to other more simplistic sampling methods like taping.

Keywords Polyvinyl alcohol method · PVAL · Gunshot residues · Bloodstains · RNA/DNA co-analysis · Blood- and brain-specific expression

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00414-017-1687-2) contains supplementary material, which is available to authorized users.

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Introduction

It is widely accepted that in cases of firearm-related fatalities, a systematic investigation at the scene of death is indispensable to differentiate between self-inflicted [1–3], intentionally or accidentally caused gunshot injuries. In such cases, it is important that results of the analysis of available residual non-biologic material (e.g., gunshot residues (GSR)) caused by firearm discharge, and/or biological material ejected from the entrance wound into the direction of the firearm, i.e., "backspatter," are evaluated integratively. Notably, traces of backspatter have been demonstrated to be retrievable from inside surfaces of firearms [4–11], from the shooter's hand [12, 13] (also GSR) and the shooter's surroundings and may be of considerable value in forensic crime scene investigations and in reconstructing the course of events. Biological material located on the shooter's hand is an important cue indicating self-inflicted gunshot injury; however, the presence of firearm-related bloodstains and GSR on an individual's hand does not necessarily prove that this person did indeed discharge a firearm.

Topographic sampling utilizing adhesive foils or tapes, latex, or the polyvinyl alcohol (PVAL) technique, first

introduced in 1993 by Merkel and Mailänder [14], represents an essential procedure for sampling GSR or biological material from a shooter's hand. For the PVAL technique, a water soluble liquid polymer is used to cover the hands of suspects or victims for in situ preservation of residual evidence which allows for a representative conservation of trace materials by recording the positional information of the particles and backspatter on the hands. This method is suitable for the detection and characterization of GSR [14–17], and while its compatibility with singleplex STR typing has already been demonstrated [18], it remains to be seen whether it is appropriate for multiplex STR profiling and simultaneous investigation of co-extracted miRNA from backspatter retained from PVAL gloves.

The interest in RNA analysis steadily increased during the last decade in the forensic field. In 1984, Oehmichen et al. [19] were first to mention RNA in the forensic literature in their description of post-mortem RNA synthesis. Ten years later, Phang et al. [20] introduced gene expression analysis via reverse transcription PCR to forensic molecular biology. Following methodological advances in RNA analysis and quantification, forensic laboratories began to adopt RNA based techniques and more and novel applications employing mRNA and miRNA for forensic body fluid identification and organ tissue identification were being explored [21–25]. MicroRNAs (miRNA) are more stable and substantially less prone to degradation than mRNA because of their short size (18–24 bp) and exhibit less recognition sites for nucleases which are crucial advantages in the investigation of forensic type samples.

Therefore, the aim of the present study was to systematically investigate the feasibility of forensic analysis of miRNA and DNA that have been isolated from bloodstains and biological material embedded in PVAL gloves of the hands of persons involved in cases of homicide by gunshot.

Material and methods

Samples and ethics

Samples from polyvinyl gloves from the left and right hand of nine deceased persons of fatal gunshot injuries comprising seven homicides, one homicide-suicide, and one accident where collected between 1996 and 2003. The gloves were prepared as described elsewhere [14, 17]. Altogether, 17 PVAL gloves were examined, representing eight cases with gloves prepared from both hands and one case with a glove prepared only from the right hand.

The study protocol was reviewed and approved by the ethics committee of the Hospital of the University of Bonn.

Forensic analysis of GSR

Preservation of evidence and detection of gunshot residues

To examine GSR, PVAL gloves (Fig. 1) were treated with 2% tartaric acid and saturated sodium rhodizonate solution. After drying, purple-, red-, or orange-colored particles were detected using a stereo microscope Stemi 2000 C (Carl Zeiss Microscopy, Oberkochen, Germany) at 20- to 40-fold magnification. Samples of these particles were examined by non-destructive energy dispersive X-ray fluorescence analysis (Eagle μ-Probe II, EDAX Inc., Mahwah, USA) to verify GSR characteristic elemental composition. After closure of the criminal investigation by the prosecutor, the samples were archived in a dry and dark environment. No further examination had been done until the molecular genetic analyses were performed.

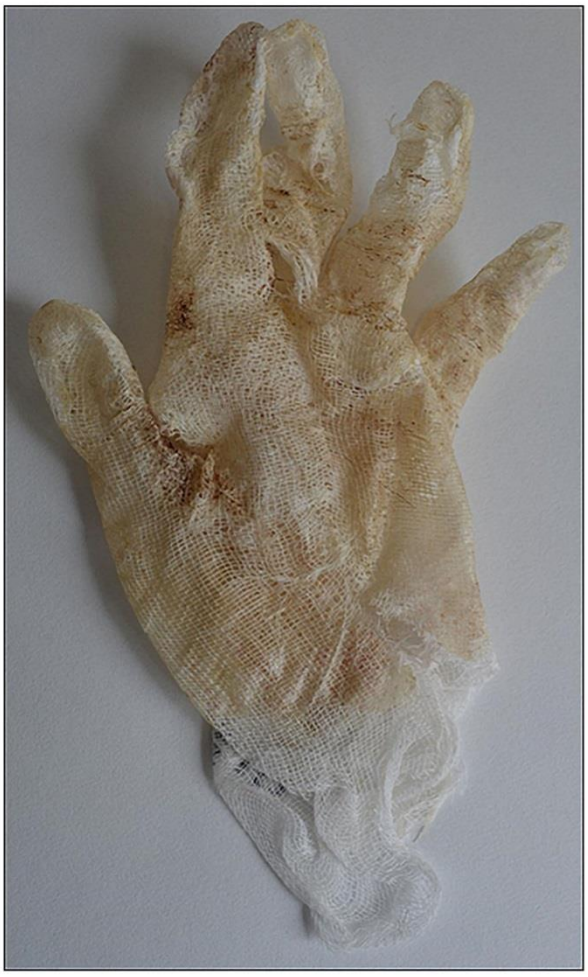

Fig. 1 PVAL glove from the right hand of a homicide victim (case #1) with blood-stained area between thumb and forefinger (palm shown, glove turned inside out)

Molecular genetic analysis

Sampling procedure

To prevent a possible bias the investigations were performed by experimentators blinded to case histories, involved persons, weapons, and shooters.

First, for the RNA/DNA co-analysis and DNA analysis, gloves were examined macroscopically, and areas containing visible stains suspected to contain blood were cut out into small pieces (approximately 1 cm^2) using sterile scissors (Fig. 2). Selected areas from each glove that were free of any visible trace of blood or other material were also cut out (N1) and, together with samples of excess gauze that had not been treated with PVAL (N2), served as negative controls for the RNA/DNA co-analysis. For each glove, both separate and pooled samples (pooling together several cuttings) were generated and examined separately. For the sake of maximal sample retention, presumptive blood tests were performed after selecting the samples for RNA/DNA co-extraction and DNA analysis. From each glove, one visible minimal trace suspected to contain blood was cut out. The identification of blood was performed using the Hexagon OBTI® test (Gesellschaft fuer Biochemica und Diagnostic mbH, Wiesbaden, Germany) which detects human hemoglobin.

RNA extraction, quantification, and integrity assessment

Prior to all RNA processing, all surfaces, instruments, and devices employed in the processes were thoroughly cleansed using RNase-Zap® (Ambion, Austin, TX, USA) and Roti®-Nukleinsäurefrei (Carl Roth, Karlsruhe, Germany) to remove all traces of ambient RNases and nucleic acid contaminations. Only RNase-free reagents and plastic consumables were used.

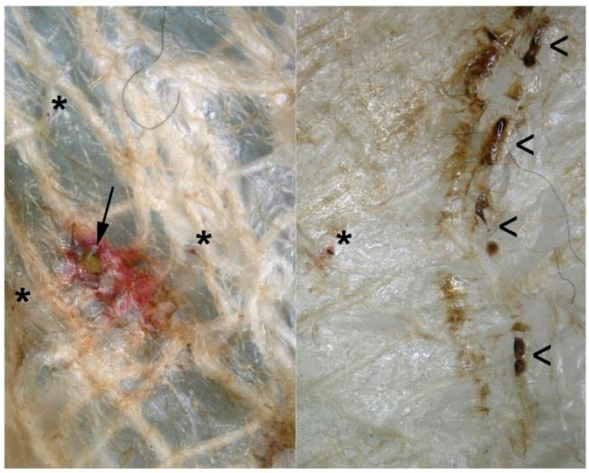

Fig. 2 Dorsum of the right glove of case #9. GSR particles < 10 μm (*). Powder particle (arrow) embedded in purple red rhodizonate positive area (lead confirmed). Minimal spatters (<) of biological material (blood positive presumptive test) used for the nucleic acid analysis.

Nucleic acids were extracted using the NucleoSpin® miRNA Kit (Macherey-Nagel, Düren, Germany) according to the provided small RNA protocol with minor changes which had previously been shown to be well suited for co-extracting DNA and RNA from forensic type samples [26]. Briefly, cuttings were incubated for 10 min with 350 μl lysis buffer at room temperature, and NucleoSpin® Forensic Filters (Macherey-Nagel) were then used to separate the liquid nucleic acid containing lysate from discardable solid material by centrifugation at 21000×*g* for 3 min. The lysate was then processed according to the protocol, and small RNA was finally eluted in 40 μl of nuclease-free water pre-heated to 95 °C. All RNA extracts were stored at − 80 °C until further processing.

Quantity and quality of RNA were measured using QuantiFluor® RNA Dye with the Quantus™ Fluorometer (both Promega, Mannheim, Germany) and the RNA 6000 Pico Kit with an Agilent 2100 Bioanalyzer (both Agilent, Böblingen, Germany), respectively. For both methods, 1 μl of RNA extract was used, and all measurements were performed according to the manufacturers' prescriptions. RNA quality is represented by the "RNA integrity number" (RIN) [27]. Based on quantification results, all individual samples were diluted to a final concentration of 2 ng/μl with RNase-free water. If RNA yield was too low to be detected fluorometrically, the maximum input volume for reverse transcription reactions was used instead.

DNA (co-) extraction, quantification, and STR profiling

Co-extraction of DNA was performed for each sample by diverting 20 μl of its DNA containing RNA lysate to the DNA extraction procedure of the PrepFiler® Forensic DNA Extraction Kit (Life Technologies, Darmstadt, Germany) processing it according to manufacturer's instructions.

DNA extraction of pooled samples was performed using the PrepFiler® Forensic DNA Extraction Kit (Life Technologies) according to manufacturer's prescription. All DNA extracts were then stored at − 20 °C until further processing.

DNA concentration, degradation, and the presence of PCR inhibitors were measured by quantitative PCR (qPCR) using the PowerQuant™ System (Promega, Mannheim, Germany) as recommended by the manufacturer on an ABI Prism 7500 Sequence Detection System (Life Technologies).

DNA profiling was performed for selected samples by STR multiplex PCR using the PowerPlex® ESI/ESX 17 System (Promega) according to the provider's prescriptions. Input amounts of DNA were used: separate samples between 11 pg and 16 ng, pooled samples between 7 and 500 pg

(RNA/DNA co-extraction) and pooled samples from DNA extraction between 53 pg and 10 ng.

PCR products were detected on an ABI 310 Genetic Analyzer (Life Technologies). GeneMapper v3.2.1 software (Life Technologies) was used for raw data analysis and DNA profile compilation. The analytical threshold for a positive signal was set at 50 RFU.

Selection of blood- and brain-specific miRNA and reference genes for qPCR data normalization

Based on previous work [28–31], the following blood- and brain-specific miRNAs were chosen for the detection of blood and brain tissue: *miR-451a* (blood) and *miR-124a* (brain tissue) (Table 1).

MiR-191 was chosen as best suited reference gene to serve as an endogenous control for the normalization of blood- and brain-specific miRNA expression data (Table 1). This choice was based on a selection procedure employing three well-established computational algorithms, geNorm [33], NormFinder [34], both implemented in the GenEx v6 software (multiD Analyses AB, Goteborg, Sweden) and the Excel-based BestKeeper [35]. Sauer et al. [36, 37] established a reliable and reproducible strategy for qPCR data normalization from forensically relevant body fluids and organ tissues in forensic type samples. They provided us with their raw expression data of nine carefully preselected candidate reference genes in dried blood stains and ten selected candidate reference genes for brain tissue from which the most stably expressed gene was determined to be in both cases *miR-191* (data not shown) (reference genes: see Supplementary Table 1) [38–40].

Reverse transcription and qPCR

Complementary DNA (cDNA) was synthesized from miRNA using the TaqMan® MicroRNA Reverse Transcription Kit employing target-specific stem-loop primers (both Life Technologies) in a total volume of 15 μl. Each miRNA reverse transcription (RT) reaction comprised 10 ng of total RNA, 1× RT Primers, 50 U MultiScribe™ Reverse Transcriptase, 1 mM dNTPs, 3.8 U RNase Inhibitor, and 1× RT buffer.

All RT reactions were performed on a T3 Thermocycler (Biometra, Göttingen, Germany) with the following cycling conditions: 16 °C for 30 min, 42 °C for 30 min, and 85 °C for 5 min.

To detect potential contaminations with genomic DNA or of our reagents, RNA extraction negative controls, RT(−) controls (i.e., RT reactions without reverse transcriptase) and H_2O controls (i.e., RT reactions with water instead of RNA template) were set up, respectively. RNA was kept on ice during processing, and RT reaction products were stored at − 20 °C until further proceedings.

QPCR was performed using target-specific TaqMan® Assays (Life Technologies) (Table 1) for the selected blood- and brain-specific miRNA and the reference gene. Each 20 μl reaction contained 1.3 μl of the respective RT reaction product, 1 x TaqMan® Universal PCR Master Mix, No AmpErase® UNG (Life Technologies) and 1 x TaqMan®Assay. All reaction components were kept on ice during processing.

All sample/assay combinations were run in technical triplicates. All qPCR reactions were conducted in MicroAmp® Optical 96-Well Reaction Plates on an ABI Prism 7500 Sequence Detection System (both Life Technologies) with the following qPCR cycling conditions: 95 °C for 10 min, followed by 40 cycles with 95 °C for 15 s and 60 °C for 1 min.

To normalize inter-run variation between qPCR reaction plates, the internal positive control (IPC) from the PowerQuant™ System (Promega) was used as an inter-plate calibrator. Raw fluorescence data was collected during the 60 °C step by the SDS software v2.0.6 (Life Technologies) and then exported for further analysis.

Data analysis, normalization, statistics, and MIQE compliance

To calculate quantification cycle (C_q)-values and amplification efficiencies from raw data from SDS spread sheet exported R_n-values, the LinRegPCR analysis program v2015.1 [41] was applied. Efficiencies outside 5% of the group median were excluded from mean efficiency calculation. For the calculation of C_q-values, a common threshold value was set to − 0.7 log (fluorescence). Sample

Table 1 Specifications of the RNA Assays

Official gene symbol	NCBI−/miRBase-mature sequence accession	TaqMan assay ID	Small RNA sequence (amplicon length in base pairs)	References
hsa-miR-451a	MIMAT0001631	001141	AAACCGUUACCAUUACUGAGUU (22)	[28, 29]
hsa-miR-124a	MIMAT0000422	001182	UAAGGCACGCGGUGAAUGCC (20)	[30]
hsa-miR-191-5p	MIMAT0000440	002299	CAACGGAAUCCCAAAAGCAGCUG (23)	[31]

NCBI National Center for Biotechnology Information, *miRBase* microRNA database [32]

replicates exhibiting C_q-values deviating more than one unit from the mean of the triplicates were excluded from further processing. Normalization of computed C_q-values of target-specific miRNA was performed using the GenEx software v6 (multiD Analyses AB). Pre-processing of qPCR encompassed the following steps in the given order: efficiency correction, averaging of technical qPCR replicates, and normalization with reference genes resulting in ΔC_q-values. A normalized C_q-value ($C_{q,n}$) < 35 was considered to indicate successful PCR implying a target-specific signal and thus RNA quantity and quality suitable for expression analysis.

Data analysis, interpretation, and statistical calculations were performed using MS EXCEL and SPSS software v.22 (SPSS Inc., Chicago, IL, USA).

To facilitate reliable and unequivocal interpretation of the qPCR results reported herein, all information that is rated "essential" according to the MIQE guidelines [42] is reported, where applicable.

Results

GSR analysis performed 1996–2003

In sampling (non-)biological material from the hands of seven homicide victims, one accident and one suicide case, the polyvinyl alcohol method was used as the method for the preservation of evidence.

Although the examined PVAL gloves were especially obtained from persons killed by third in the half of the cases, GSR particles had been detected. However, no GSR pattern had been found that indicated firing or handling a gun. Only in case #9 where the suspect first shot his wife and commit suicide afterwards by contact shot to the head, the GSR pattern on the PVAL of the right hand was found compatible with shooting (Fig. 2).

Presumptive blood test

The interpretation of a trace pattern is often of great importance for the reconstruction of an event. This also includes knowledge of the type of examined trace material. In the present study, it was all the more important, because the PVAL had been stored for many years so that the color contrasts of the stains had faded.

In case of 17 examined gloves, only five gloves (29%) exhibited a positive signal for the presumptive blood test, including two gloves (case #1, left hand and case #3, right hand) which showed a weak positive signal (Supplementary Fig. 1).

Quantification and STR profiling of (co-)extracted DNA

Secured biological material was collected from various locations of the PVAL gloves (Figs. 1 and 2 and Table 2). Samples were either analyzed separately or pooled (combining several cuttings) and then analyzed.

DNA was quantifiable in 76 and 94% of samples analyzed separately and pooled after RNA/DNA co-extraction, respectively. Only in case #8 was no DNA detectable from the right hand.

Pooled samples which had been supplied to DNA extraction exhibit for all stains quantifiable DNA concentrations. However, a wide range of DNA concentrations was observed between samples from different PVAL gloves. The highest DNA concentration (0.9 ng/μl) was obtained in a sample recovered from the left glove of case #4 whereas the lowest DNA amount (0.2 pg/μl) was measured in four samples collected from the right PVAL glove of case #2 and left and right glove of case #9. Notably though, DNA yields were low in average with no detectable inhibitions but different extents of degradation (data not shown).

After RNA/DNA co-extraction, only 16% of samples contained more than 100 pg DNA in 17.5 μl (maximum input volume for multiplex PCR reaction) which had reliably proved sufficient for successful STR profiling in our hands.

By contrast, after DNA extraction, one sample (case #8, right hand) contained less than 100 pg DNA in 17.5 μl multiplex PCR reaction volume.

Then, to assess the success rate of STR typing with samples collected from PVAL gloves, DNA profiles were generated from selected samples using PCR input amounts between 7 pg and 16 ng of DNA and were rated according to the following result categories:

"++," full STR profiles (17 of 17 possible STR systems), "+," identifiable profiles (at least 8–16 of 17 possible STR systems), and "−," not identifiable profiles (less than 8 of 17 possible STR systems).

RNA/DNA co-extraction

"++" was observed in three cases for STR PCR input amounts between 500 pg and 1.6 ng of DNA. In 58% of tested samples from left and right gloves, DNA profiles were rated "+" with STR PCR input amounts of > 11 pg and up to 1.0 ng (except for #6 R-2, which produced a "−" with an input DNA amount of 32 pg). STR profiling was unsuccessful ("−") for 30% if the STR PCR input amount of DNA was between 7 and 26 pg.

DNA extraction

Samples with STR PCR input amounts between 1.0 and 10 ng of DNA generated full STR profiles ("++") in more than half of the cases. DNA profiles were rated "+" in 41% of samples

Table 2 Overview of DNA and RNA results of selected PVAL samples

									DNA results			RNA results			
Case	No.	Age of gloves (years)	Sex	GSR	Weapon	Context	Entry wound (localization)	Presumptive blood test	DNA concentration [pg/µl]	[a]DNA input amount	[b]STR profiling	RNA concentration [ng/µl]	RIN	Stain identification	
														Blood	Brain
#1	1-L-1	19	f	+	Pistol	Homicide	Neck	+	1.3	23	+	--	2.5	*	
	1-L-2											--	1.2	--	
	1-L-3								10.2	179	+	1.85	2.5	*	
	1-L-N1											--	1.5	–	
	1-L-N2											--	1.5	--	
	1-R-1							–	2.7	47	+	--	2.6	*	
	1-R-2								--	--	/	--	2.0	*	
	1-R-3											--	1.0	--	
	1-R-N1											--	1.0	--	
	1-R-N2											--	1.1	--	
#2	2-R-1	19	m	–	Revolver	Homicide	Body	–	0.2	4	/	--	1.9	*	
	2-R-2								0.3	5	/	--	1.7	*	
	2-R-3											--	1.0	--	
	2-R-N1											--	1.0	--	
	2-R-N2											--	1.3	--	
#3	3-L-1	16	m	–	Pistol	Homicide	Neck	–	0.5	9	/	--	2.4	*	
	3-L-2											--	1.0	--	
	3-L-3								1.2	21	-	0.18	2.5	*	
	3-L-N1											--	1.0	--	
	3-L-N2											--	1.0	--	
	3-R-1							+	16.8	294	+	9.85	2.4	*	
	3-R-2								4.6	81	+	6.15	2.3	*	
	3-R-3											--	2.3	*	
	3-R-N1											--	1.0	--	
	3-R-N2											--	1.0	--	
#4	4-L-1	17	m	+	Pistol	Accident	Head	–	933.3	16,327	++	95.85	2.4	*	--
	4-L-2								--	--		3.05	2.6	*	*
	4-L-3											--	2.4	*	*
	4-L-N1											--	1.0	--	--
	4-L-N2											--	1.0	--	--
	4-R-1							–	57.0	998	+	6.65	2.5	*	*

Table 2 (continued)

Case	No.	Age of gloves (years)	Sex	GSR	Weapon	Context	Entry wound (localization)	Presumptive blood test	DNA results			RNA results			
									DNA concentration [pg/μl]	[a]DNA input amount	[b]STR profiling	RNA concentration [ng/μl]	RIN	Stain identification	
	4-R-2								48.2	884	+	1.50	2.4	*	--
	4-R-3											--	1.1	--	--
	4-R-4											--	2.4	*	*
	4-R-N1											--	1.3	--	--
	4-R-N2											--	1.0	--	--
#5	5-L-1	15	f	+	Revolver	Homicide	Neck	–				--	1.7	*	
	5-L-2								0.3	5	/	5.10	2.4	*	
	5-L-3								--	--	/	--	2.3	*	
	5-L-N1											--	1.0	--	
	5-L-N2											--	1.0	--	
	5-R-1							–	0.6	11	–	2.65	2.3	*	
	5-R-2											--	1.4	--	
	5-R-3								0.9	16	–	11.00	2.2	*	
	5-R-N1											--	1.0	--	
	5-R-N2											--	1.2	--	
#6	6-L-1	18	m	+	Revolver	Homicide	Neck	–	0.9	16	–	--	2.4	*	
	6-L-2											--	1.0	--	
	6-L-3								0.5	9	/	--	2.2	*	
	6-L-N1											--	1.0	--	
	6-L-N2											--	1.2	--	
	6-R-1							+				--	2.2	*	
	6-R-2								1.8	32	–	--	2.6	*	
	6-R-3								--	--	/	0.12	2.6	*	
	6-R-N1											--	---	--	
	6-R-N2											--	1.0	--	
#7	7-L-1	16	f	–	Pistol	Homicide	Neck	+	0.5	9	/	--	2.2	*	
	7-L-2								3.8	67	+	--	2.5	*	
	7-L-3											--	1.1	--	
	7-L-N1											--	1.0	--	
	7-R-1							–	0.7	12	–	--	1.1	--	
	7-R-2								0.3	5	/	--	1.2	--	
	7-R-3											--	1.0	--	

Table 2 (continued)

Case	No.	Age of gloves (years)	Sex	GSR	Weapon	Context	Entry wound (localization)	Presumptive blood test	DNA results			RNA results			
									DNA concentration [pg/μl]	[a]DNA input amount	[b]STR profiling	RNA concentration [ng/μl]	RIN	Stain identification	
	7-R-N1											--	1.2	--	
#8	8-L-1	12	f	–	Revolver	Homicide	Head	-				--	2.4	*	*
	8-L-2								--	--	/	--	2.0	*	*
	8-L-3								--	--	/	--	2.0	*	--
	8-L-N1											--	1.0	--	--
	8-L-N2											--	1.3	--	--
	8-R-1							–				--	1.0	--	--
	8-R-2								--	--	/	--	1.0	--	--
	8-R-3								--	--	/	--	1.0	--	--
	8-R-N1											--	1.0	--	--
	8-R-N2											--	1.0	--	--
#9	9-L-1	20	m	+	Revolver	Suicide	Head	+	0.2	4	/	0.77	2.2	*	*
	9-L-2								0.3	5	/	0.23	1.9	*	*
	9-L-3								0.2	4	/	0.36	2.0	*	*
	9-L-N1											--	1.1	--	--
	9-L-N2											--	1.0	--	--
	9-R-1							–	0.3	5	/	0.23	1.8	*	*
	9-R-2								0.2	4	/	0.22	1.7	*	*
	9-R-3								2.1	37	+	0.22	1.7	*	*
	9-R-N1											--	1.2	--	--
	9-R-N2											--	1.0	--	--
Pooled samples (RNA/DNA co-extraction)	1-L-P								25.6	448	++	3.65	2.3	*	
	1-R-P								0.6	11	+	--	2.5	*	
	2-R-P								0.6	11	–	--	2.6	*	
	3-L-P								8.5	149	+	8.50	2.3	*	
	3-R-P								28.3	495	++	19.50	2.3	*	
	4-L-P								1.9	33	+	--	2.6	*	*
	4-R-P								0.7	12	+	0.11	2.5	*	*
	5-L-P								2.6	46	+	9.70	2.3	*	
	5-R-P								2.3	40	+	8.90	2.3	*	
	6-L-P								1.5	26	–	0.33	2.5	*	
	6-R-P								2.7	47	+	1.20	2.6	*	

Table 2 (continued)

Case	No.	Age of gloves (years)	Sex	GSR	Weapon	Context	Entry wound (localization)	Presumptive blood test	DNA results			RNA results			
									DNA concentration [pg/μl]	[a]DNA input amount	[b]STR profiling	RNA concentration [ng/μl]	RIN	Stain identification	
	7-L-P								3.0	53	+	--	2.5	*	
	7-R-P								0.4	7	–	--	1.0	--	
	8-L-P								0.8	14	+	--	2.3	*	*
	8-R-P								--	--	--	--	1.3	--	--
	9-L-P								2.0	35	+	0.56	2.4	*	*
	9-R-P								0.8	14	–	0.22	2.2	*	*
Pooled samples (DNA extraction)	D1-L-P								590.5	10,334	++	/	/	/	/
	D1-R-P								456.5	7989	++	/	/	/	/
	D2-R-P								61.9	1083	++	/	/	/	/
	D3-L-P								10.9	191	+	/	/	/	/
	D3-R-P								18.0	315	+	/	/	/	/
	D4-L-P								87.5	1531	++	/	/	/	/
	D4-R-P								405.2	7091	++	/	/	/	/
	D5-L-P								27.5	481	+	/	/	/	/
	D5-R-P								58.8	1029	++	/	/	/	/
	D6-L-P								102.1	1787	+	/	/	/	/
	D6-R-P								12.5	219	+	/	/	/	/
	D7-L-P								593.3	10,383	++	/	/	/	/
	D7-R-P								134.8	2359	++	/	/	/	/
	D8-L-P								148.2	2594	+	/	/	/	/
	D8-R-P								3.0	53	–	/	/	/	/
	D9-L-P								29.5	516	+	/	/	/	/
	D9-R-P								425.5	7446	++	/	/	/	/

L, left hand; *R*, right hand; *N1*, piece of glove with no visible contamination; *N2*, piece of mull without PVAL; *P*, pooled samples; *D*, DNA; *m*, male; *f*, female; +, GSR/presumptive blood positive; –, GSR/presumptive blood negative; *RIN*, RNA integrity number

[a] *DNA input amount:* pg in 17.5 μl (maximum input volume for multiplex STR PCR reaction)-DNA amount > 100 pg which is sufficient for STR profiling

[b] *STR profiling*: PCR full profile (++): 17/17. Identifiable (+): 8-16/17. Non-identifiable (–): 0-7/17;

Stain identification: * correct blood/brain detected; – not detected; / not analyzed

generated with STR PCR input amounts > 190 pg and up to 500 pg. Only in one case (#8 right hand) was STR profiling unsuccessful "–" with the STR PCR input amount of DNA being 53 pg.

In case #9 where the PVAL glove was sampled from the shooter's hand, all examined traces exhibited the DNA profile of the shooter and not from the victim. Table 2 summarizes these results.

Quantity and integrity of RNA

Overall and analogous to DNA, small RNA exhibited considerable variation in terms of quantity but only minor variation in terms of, generally low, quality (RIN) as expected for forensic samples of this type between the different cases. The highest RNA yield (95.85 ng/μl) was obtained for a sample recovered from the left glove of case #4. Only in 28% of samples (21% of separately analyzed samples and 59% of pooled samples, respectively) a RNA concentration could be determined.

For the assessment of RNA quality, RIN values were established. Overall, regardless of the age of the samples, RIN values ranged between 1.0 and 2.6. Pooled samples exhibited RIN values between 2.2 and 2.6 (except cases #7 and #8; both right hands). In the subsequent qPCR-based blood- and brain-specific miRNA expression analysis, all samples with an RNA quality > 1.7 produced a significantly positive signal for blood and brain tissue, and vice versa ($p = 0.000$). Although cases #4 (both hands) and #8 (right hand) exhibited RIN values of 2.4 and 2.0, respectively, no brain-specific expression could be determined.

Quantification and quality results are summarized comprehensively in Table 2.

Expression analysis of miRNA

A downstream qPCR analysis was used to assess the general suitability of forensic RNA extracted from selected samples of backspatter that was recovered from PVAL gloves to produce blood- (*miR-451a*) and brain- (*miR-124a*) specific miRNA expression levels.

Although small RNA was not detectable fluorometrically in some samples, fluorescence-based detection of blood- and brain-specific miRNA expression via qPCR was successful. Normalized expression of *miR-451a* that was considered to be indicative of blood ($C_{q,n} < 35$) was detected in 77% of all samples: 88% for pooled samples and 73% for separately analyzed samples, respectively. Only in cases #7 and #8 (both right gloves) was the detection of blood-specific miRNA expression not successful (Table 2).

Additionally, for three cases (#4, #8, and #9) in which headshots had been delivered, brain-specific miRNA was examined. Overall, brain-specific miRNA could be detected on at least one sample of each hand, expect case #8 (right hand pooled sample).

In contrast, all selected areas from each glove free of any visible traces of blood or other material (Table 2: N1, N2) were negative for blood- and brain-specific miRNA expression. Furthermore, all negative controls (extraction negative, RT(−) and H_2O) showed no or unspecific results ($C_q > 35$) for all tested samples.

Discussion

In firearm-related fatalities, the differentiation between homicide, suicide, and accident can sometimes be exceedingly difficult, and a final assessment of the events can only be reached after a detailed analysis of all aspects and circumstances of the incident. The reconstruction is not only to be based on the analysis of the death scene but requires a complex and integrative analysis of all findings in the given case including non-biological material (e.g., gunshot residues), biological material (e.g., blood stains and backspatter), results of medico-legal autopsy, and forensic chemical analysis. Therefore, the aim of the present study was to investigate the simultaneous extraction and forensic analysis of miRNA and nuclear DNA from aged traces of bloodstains that had been collected with the PVAL method from hands of victims of fatal gunshot injuries after GSR analysis had been done more than 12 years ago.

For the assessment of the value of a particular piece of GSR evidence in linking a person to a crime, it is important to distinguish whether or not that person did indeed fire a gun in a specific situation. The topographic PVAL technique in contrast to other methods reproduced a true image of the original findings on the hand [18]. The position and spatial relations between all (non-)biological traces can precisely be reconstructed at any time, as they are accurately transferred form the hand to the glove. However, the detection of GSR on a person's hand or any other surface can also occur if a firearm has been discharged in proximity or after contact with a surface that is contaminated with GSR. This then can lead to false interpretation of the circumstances of events.

Although the PVAL gloves were sampled from eight persons who were killed by third, in four cases, GSR were detectable after forensic chemical and physical analysis. The origin of GSR on the hand/s of some victims might be explained by close range shots fired to the back of the victims' head or neck region so that particles of the discharge could reach their hands. However, only in case #9 where the husband had killed his wife, GSR showed a typical pattern indicating a shooter's hands. Therefore, a detailed reconstruction of the circumstances of firearm-related death has to go beyond the detection of GSR and should include analysis of biological material.

Hence, before commencing molecular genetic analysis, it should first be assessed what type of trace material is present.

Presumptive preliminary tests are intended to enable the identification of a stain and are conducted after visual examination. Conventional methods for blood identification include immunological, chemical, and enzymatic tests. These tests vary greatly in terms of sensitivity and specificity and comparably large amount of sample material are required to perform these tests which are problematic, since the amount of evidential biological material is usually very limited in forensic casework and especially in backspatter and bloodstain analysis. The Hexagon OBTI® test is known as a rapid and highly specific and sensitive one-step immunochromatographic assay for the detection of human hemoglobin and was used for qualitative detection of human blood on the PVAL gloves. Hochmeister et al. [43] found that an immunochromatographic 1-step test is robust and suitable for forensic analyses, being human specific. However, in our examination, perhaps due to the minute amount or time-wise degradation of trace material, the presumptive blood test gave out a positive result only in less than a third of the samples. In contrast, microRNA-based body fluid identification (BFI) has been shown to be very sensitive and robust against degradation and can be performed in parallel to DNA analysis without consuming any additional trace material [19]. RNA/DNA co-extraction and subsequent miRNA-based BFI can outperform conventional tests, providing a direct link to the crime scene stain. Combining these analyses can then answer of which individual the trace originates (individualization) and what kind of trace material it is (contextualization).

STR profiling is considered "gold standard" for DNA-based identification because of its robustness and high power of discrimination and the analysis of DNA, comprising quantification and the generation of STR profiles, from authentic material from homicide cases sampled using the PVAL method produced reliable results for ~ 20 year old trace material. Our findings confirm, what had already been pointed out by Schyma et al. [18]. They investigated 13 PCR systems including seven singleplex STR systems (HumTH01, HumVWA31, HumFES, HumF13B, HumFGA, D1S80, and CD4), five structure polymorphisms (LDLR, GYPA, D7S8, HBGG, and GC), and one VNTR system (HLA-DQα). Only five of these seven STR systems are still in use in contemporary multiplex STR kits, and only three (TH01, VWA, and FGA) are comprised by widely used highly robust next generation STR multiplex kits which had been developed for DNA analysis of small amounts of potentially degraded trace material in forensic laboratories. In 1999, a European Standard Set (ESS) of STR loci introduced [44], and there is a considerable difference concerning the power of discrimination between contemporary STR kits (comprising > 16 STR loci) and the kits available at that time (6 STR loci). Furthermore, we compared RNA/DNA co-extraction with the extraction of DNA alone. As expected, we obtained better results in STR profiling when using DNA extraction where a lower loss of DNA yield was observed as compared to RNA/DNA co-extraction. Thus, depending on the exact question concerning the circumstances of death, it should be assessed which analysis should be carried out. In such cases, it is advisable if be possible to sample several similar minimal traces located directly next to each other. From one sample, only DNA is then extracted from the other(s) DNA and RNA can be co-extracted. If in doubt e.g., if only one minimal trace is available, RNA analysis may be put on hold; alternatively, if the identity of the person in question had already been clarified or can be clarified without the need of DNA profiling, RNA can be extracted for downstream analysis instead. In summary, sampling biological material employing the PVAL technique with subsequent STR profiling using STR kits can produce reliable DNA profiles suitable for forensic identification with no observable inhibition and compared to other techniques applicable to recover traces from persons involved in firearm-related death e.g., adhesive tape the PVAL method produced superior results [45].

Besides standard DNA analysis, another important aspect in the reconstruction of the circumstances of a crime is the analysis of the pattern and composition of a stain, e.g., bloodstains, by RNA-based identification of body fluids and organ tissues. In this study, the presence of blood and in case of head shots especially brain tissue on hands of the deceased, sampled with the PVAL technique was assessed via the identification of blood- and brain-specific miRNA for the first time.

In general, the assessment of RNA integrity is an important step in RNA expression analysis if small fold-changes are to be detected. In case of forensic experiments, however, where the detection of much coarser differences (e.g., expression vs. no expression) is sufficient studies suggest that low RNA integrity values do not adversely affect RT-qPCR profiling results [47, 48], and no difficulties were encountered using similar types of specimens mimicking forensic evidence in identifying small nucleolar RNAs (snoRNAs) as reference genes [36]; hence, the recommendation of a minimum RIN of 7 for samples to be proceeded was disregarded [49, 50]. In our study, RNA integrity was measured using the 2100 Bioanalyzer which employs microfluidic electrophoretic separation of RNA by molecular weight and fluorometric detection generating an electropherogram [27]. Based on these measurements, the software then calculates the RIN as described in the "Material and methods" section. RNA samples with RIN values of 1.0 were considered as completely degraded, whereas RIN values of 10 indicate intact RNA molecules [27]. Minimal amounts of trace material aged up to 20 years had been investigated, and we measured RIN values between 1.0 and 2.6 which can probably be attributed to the presence of low template or old samples where RNA degradation occurs. We found that only samples with RIN values ≥ 1.7 produced significantly positive blood- or brain-specific expression. Therefore, it seems inappropriate to completely exclude a sample from molecular analysis because of insufficient or

poor RNA quality if a RIN of 7 is regarded as minimum. As expected, a rise in miRNA concentration was associated with lower RNA quality caused by the formation of small RNA fragments by degradation of longer RNAs which were falsely interpreted as miRNA, as the Bioanalyzer method only considers fragment length.

Despite the partly unsuccessful fluorometric determination of RNA concentrations in samples from the PVAL gloves normalized blood- and brain-specific expression of *miR-451a* and *miR-124a*, respectively, was successfully and correctly detected via qPCR in the tested samples. Brain-specific miRNA was detected on one hand of a victim, and on the PVAL glove in the suicide case #9. In both cases, this finding was related to a contact shot to the head with brain injury. Our results are in line with the findings of Lux et al. [51] who in a pilot study proved the principle that the detection of brain specifically expressed RNA in samples of backspatter from firearm-related injuries may crucially support the inference that a head shot has occurred. Brain-specific miRNA was detected on at least one hand of the victims, hit in the head, and on the shooter's hand. A recent study investigated various miRNAs to differentiate between several different tissues and skin [52]. Furthermore, they investigated samples of backspatter recovered from inside surfaces of gun barrels after experimental shootings and correctly determined and differentiated brain, heart, and skeletal muscle tissue in 13 of 18 backspatter samples. Hence, our investigations confirmed these previous results. Furthermore, no correlation between DNA and RNA yield and age of sample or weapon type was observed. The pooling of all samples recovered from deceased in firearm-related crimes is deemed advisable and can enhance success rate of this method in routine casework.

In case #9, we have demonstrated that sampled biological material from backspatter collected from the shooter's hand can successfully be subjected to the full bandwidth of chemical, physical, immunochromatographic and forensic nucleic acid analyses, encompassing nuclear DNA and miRNA-based body fluid, and organ tissue identification. We showed that after the person had committed suicide by a contact shot to the head blood- and brain-specific backspatter could be found on his hands. STR typing confirmed that the recovered traces of body fluid and organ tissue originated from him. We demonstrated that the correct detection of blood and brain tissue from the hands of deceased is feasible in up to 20 years old authentic trace material. The method is combining GSR and nucleic acid analysis for backspatter identification. The water-soluble PVAL matrix showed distinct advantages for successful extraction of nucleic acids in comparison with adhesive foils [45]. In case of adhesive foils, no successful STR profiling was obtained, because the glue reliably degrades DNA within a very short time. In contrast, the water-soluble PVAL matrix contains no solvents and facilitates better STR results. Another important difference between the adhesive foils and PVAL gloves is that too large a density of glue on the tape may interfere with the recovery of biological trace sample material and thus the process of genotyping, whereas too little of the adhesive substance results in lowered adhesiveness and hence reduced recovery of biological material. In addition, adhesiveness will also be reduced when collecting moist blood stains as applying the non-absorbent tape will smear the sample, reducing the efficacy of the collection of other biological material [16, 45]. Finally, the water-soluble matrix of PVAL is able to absorb a much greater quantity of the biological trace material present on the hands than adhesive foils can collect [16]. Consequently, the yield of nucleic acids will be greater, increasing the opportunity to identify the victim and the tissue injured.

Conclusion

In summary, we demonstrated that DNA and blood- and brain-specific miRNA from biological traces embedded in PVAL gloves recovered from case-related surfaces can successfully be recovered and analyzed even if only minimal amounts of material aged up to 20 years are available. However, to enhance the success rate of this method in routine casework, the pooling of all samples recovered from PVAL gloves used in firearm-related crimes appears to be advisable and generally advantageous. By demonstrating its high success rates, our results underscore the method's usefulness and recommend it above other non-destructive collection methods.

While we are confident to have shown that the PVAL method bears great potential for an application in forensic routine casework, ongoing research in this field is still necessary in our view to constantly expand the methodological repertoire, optimize, and adapt it to new methods of investigation such as forensic RNA analysis.

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Compliance with ethical standards The study protocol was reviewed and approved by the ethics committee of the Hospital of the University of Bonn.

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

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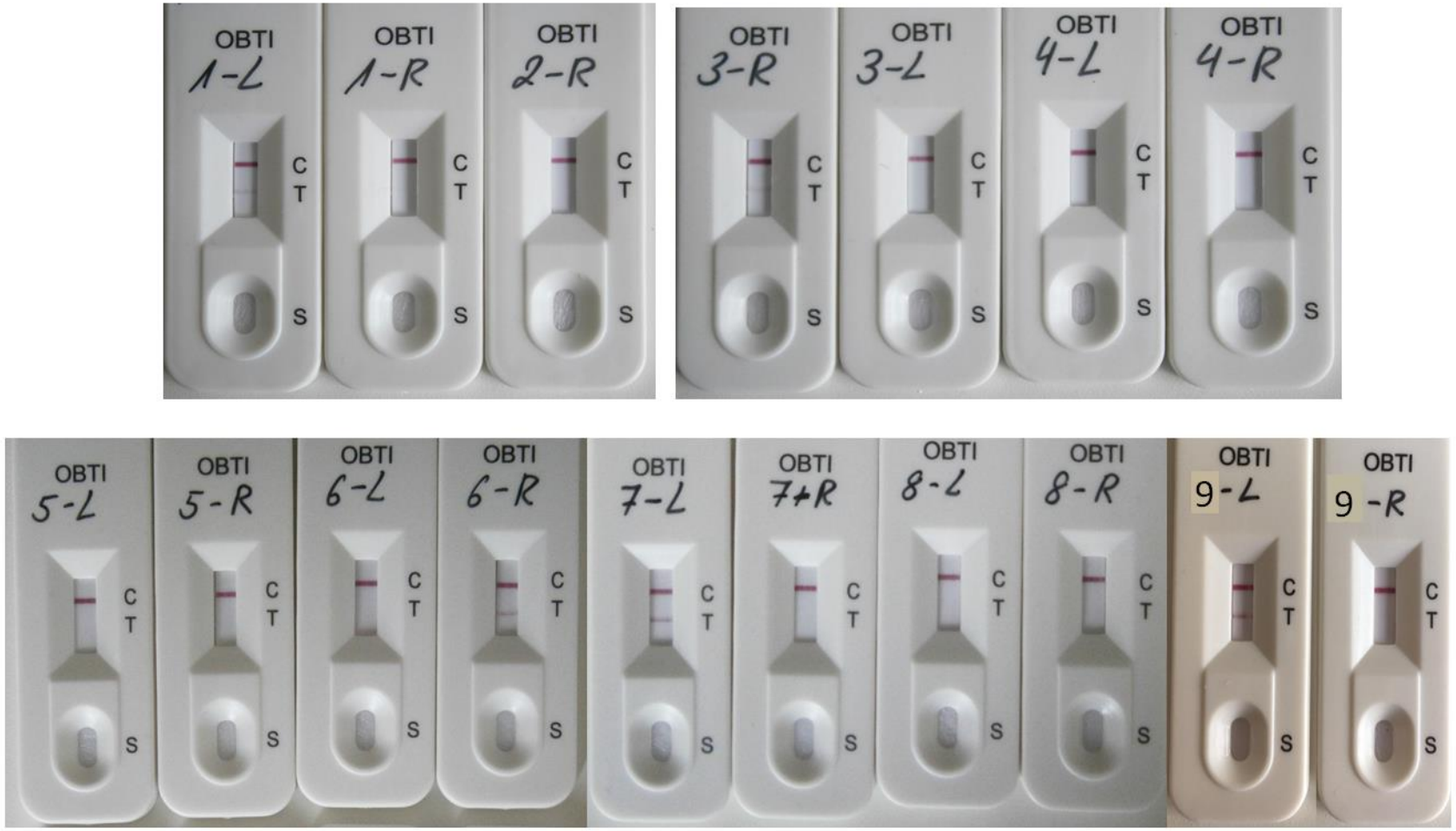

Supplementary Figure 1: Results of the preliminary blood test.
Positive results: 1-L, 3-R, 6-R, 7-L, 9-L

Supplementary Table 1

Specifications of the preselected candidate reference genes

Official Gene Symbol	NCBI-Alias	NCBI-/ miRBase-mature sequence accession	TaqMan Assay ID	Small RNA sequence (amplicon length in base pairs)	Reference gene for	References
hsa-miR-93-5p	-	MIMAT0000093	001090	CAAAGUGCUGUUCGUGCAGGUAG (23)	BL, BR	[31]
hsa-miR-191-5p	-	MIMAT0000440	002299	CAACGGAAUCCCAAAAGCAGCUG (23)	BL, BR	[31]
RNU6-2	*U6, RNU6B*	NR_002752	001093	CGCAAGGAUGACACGCAAAUUCGUGAAGCGUUCCAUAUUUUU (42)	BL, BR	[28, 29, 38, 39]
RNY3	*HY3, Y3*	AC005251	001214	CCAGUCACAGAUUUCUUUGUUCCUUCUCCACUCCCACUGCAUCACUUAACUAGCCUU (57)	BR	[38]
SNORA66	*U66, RNU66*	NR_002444	001002	GUAACUGUGGUGAUGGAAAUGUGUUAGCCUCAGACACUACUGAGGUGGUUCUUUCUAUCCUAGUACAGUC (70)	BL	[38]
SNORA74A	*U19, RNU19*	X94290	001003	UUGCACCUCUGAGAGUGGAAUGACUCCUGUGGAGUUGAUCCUAGUCUGGGUGCAAACAAUU (61)	BL	[38]
SNORD18A	*U18A*	AB061820	001204	CAGUAGUGAUGAAAUUCCACUUCAUUGGUCCGUGUUUCUGAACCACAUGAUUUUCUCGGAUGUUCUGAUG (70)	BR	[38]
SNORD24	*U24, RNU24*	NR_002447	001001	AUUUGCUAUCUGAGAGAUGGUGAUGACAUUUUAAACCACCAAGAUCGCUGAUGCA (55)	BL, BR	[38, 40]
SNORD38B	*U38B, RNU38B*	NR_001457	001004	CCAGUUCUGCUACUGACAGUAAGUGAAGAUAAAGUGUGUCUGAGGAGA (48)	BL	[38]
SNORD43	*U43, RNU43*	NR_002439	001095	GAACUUAUUGACGGGCGGACAGAAACUGUGUGCUGAUUGUCACGUUCUGAUU (52)	BL	[38]

SNORD44	*U44, RNU44*	NR_002750	001094	CCUGGAUGAUGAUAGCAAAUGCUGACUGAACAUGAAG GUCUUAAUUAGCUCUAACUGACU (60)	BR	[38-40]
SNORD47	*U47, RNU47*	X96647	001223	UAAUGAUUCUGCCAAAUGAAAUAUAAUGAUAUCACUG UAAAACCGUUCCAUUUUGAUUCUGAGGU (65)	BR	[38]
SNORD48	*U48, RNU48*	NR_002745	001006	GAUGACCCCAGGUAACUCUGAGUGUGUCGCUGAUGCC AUCACCGCAGCGCUCUGACC (57)	BR	[38, 40]
SNORD49A	*U49, U49A, RNU49*	NR_002744	001005	CACUAAUAGGAAGUGCCGUCAGAAGCGAUAACUGACG AAGACUACUCCUGUCUGAUU (57)	BL, BR	[38]

NCBI: National Center for Biotechnology Information; *miRBase:* microRNA database [32]; *BL*: blood; *BR*: brain

5.4 Zusammenfassung

Bei der Untersuchung von Delikten mit Schusswaffengebrauch ist es unerlässlich, die in der Zusammensetzung und Verteilung von Schussrückständen und/oder Rückschleuderspuren enthaltenen Informationen zu nutzen, um die Bedingungen und Umstände der Ereignisse, die jeweils zur Entstehung derselben geführt haben, verstehen und rekonstruieren zu können.

Bei den hier an Realfällen durchgeführten Analysen wurde erstmals die parallele Extraktion und forensische Analyse blut- und hirnspezifischer miRNA und nukleärer DNA aus gealterten Blutspuren überprüft, die vor den aktuellen Untersuchungen zum Sammeln von Schussrückständen verwendet und bis zur molekulargenetischen Analyse nicht weiter untersucht wurden [366]. Dieses (nicht-) biologische Spurenmaterial wurde mittels zweier verschiedener topographischer Spurensicherungsmethoden, wie den Spurensicherungsfolien bzw. der PVAL-Methode, von Händen von Opfern und Tatverdächtigen tödlicher Schussverletzungen gewonnen. Die Analyse umfasste eine RNA/DNA Ko-Extraktion aus ausgewählten Minimalspuren. Zur standardmäßigen DNA-Typisierung der genomischen DNA wurde eine Multiplex-STR-PCR durchgeführt. Um das Expressionsniveau der RNAs zu detektieren, wurden die Expressionswerte für die blutspezifische miRNA *hsa-miR-451a* und die hirnspezifische miRNA *hsa-miR-124a-3p* mit den Expressionswerten des Referenzgens *hsa-miR-191-5p* normalisiert. Empirisch belegt wurde dessen Eignung, indem es aus einer Gruppe von Kandidatengenen, bereitgestellt von Sauer et al. [345, 359], unter Verwendung der gängigen Algorithmen geNorm [355], NormFinder [356] und BestKeeper [357] unter dem Kriterium maximaler Stabilität als bestgeeignetes Referenzgen ausgewählt wurde.

Für die Beurteilung vorhandener Schussrückstände ist es wichtig zu unterscheiden, ob die verdächtige Person eine Waffe abgefeuert hat oder nicht. Hierzu wurde zunächst der Nachweis von Schussrückständen über eine mikrochemische Behandlung mit 2 %-iger Weinsäure und gesättigter Natriumrhodizonat-Lösung geführt, wodurch die GSR-Partikel angefärbt und anschließend durch stereomikroskopische Untersuchung nachgewiesen werden können. Von den asservierten Spurensicherungsfolien der Suizidenten waren, obwohl die Personen die Schusswaffe selbst abgefeuert hatten, nach einer forensisch-chemischen Analyse stereomikroskopisch keine Schussrückstände nachweisbar. Die topographische PVAL-Technik reproduziert im Gegensatz zu anderen Methoden ein wirklichkeitsgetreues Abbild des vorhandenen Spurenbildes an der Hand [42]. Die Position und die räumlichen

Beziehungen zwischen allen (nicht-) biologischen Spuren kann dadurch jederzeit präzise rekonstruiert werden, da sie exakt von der Hand auf den Handschuh übertragen werden. Obwohl in der Hälfte der Fälle die untersuchten PVAL-Handschuhe von Personen stammten, die durch Dritte getötet wurden, konnten GSR-Partikel nachgewiesen werden. Jedoch wurde kein GSR-Muster gefunden, das auf das Abfeuern oder die Handhabung einer Schusswaffe hinwies. Das Vorhandensein der Schussrückstände auf der Hand einiger Opfer könnte durch absolute Nahschüsse erklärt werden, die in den Nacken oder den Kopfbereich der Opfer abgegeben wurden, so dass die Schussrückstände deren Hände erreichen konnten. Nur in einem Fall, bei dem der Verdächtige zunächst seine Frau erschossen und sich anschließend durch einen aufgesetzten Kopfschuss suizidiert hatte, konnte ein mit einer Schussabgabe vereinbares GSR-Muster auf der rechten Hand nachgewiesen werden.

Vor Beginn der molekulargenetischen Analyse sollte zunächst beurteilt werden, welche Art von Spurenmaterial vorliegt. Präsumtive Vortests sollen die Erkennung der Art einer Spur ermöglichen. Herkömmliche Methoden zur Blutidentifikation umfassen immunologische, chemische und enzymatische Tests [371]. Diese Tests unterscheiden sich stark in Bezug auf Empfindlichkeit und Spezifität und eine vergleichsweise große Menge an Probenmaterial ist für eine erfolgreiche Durchführung erforderlich. Dies ist problematisch, da die Menge an vorhandenem biologischem Material in der forensischen Fallarbeit und insbesondere in der Backspatter- und Blutspurenanalyse normalerweise sehr begrenzt ist. Der Hexagon OBTI®-Test wurde zum qualitativen Nachweis humanen Hämoglobins (Blut) an den PVAL-Handschuhen eingesetzt. Die Untersuchung ergab - möglicherweise aufgrund der geringen Menge oder der zeitlich bedingten Degradation des Spurenmaterials - jedoch nur in weniger als einem Drittel der Proben ein positives Ergebnis. Im Gegensatz dazu konnte gezeigt werden, dass die miRNA-basierte Körperflüssigkeitsidentifikation sehr sensitiv und robust gegen Degradation ist sowie parallel zur DNA-Analyse durchgeführt werden kann, ohne zusätzliches Spurenmaterial zu verbrauchen. Die RNA/DNA Ko-Extraktion mit darauffolgender miRNA-basierter Körperflüssigkeitsidentifikation kann die herkömmlichen Tests im Ergebnis übertreffen und ersetzen sowie eine direkte Verbindung zum Spurenmaterial herstellen. Die Kombination dieser Analysen kann Aufschluss darüber geben, von welchem Individuum die Spur stammt (Individualisierung) und welche Art von Spurenmaterial vorliegt (Kontextualisierung).

In der forensischen Routinearbeit wird die STR-Typisierung aufgrund ihrer Robustheit und hohen Individualisierungspotenzials als Goldstandard für die DNA-basierte Identifizierung angesehen. Insgesamt erbrachten die Proben der Spurensicherungsfolien niedrige DNA-Konzentrationen, die für eine erfolgreiche STR-Genotypisierung nicht ausreichten. Sogar das Zusammenführen mehrerer Proben derselben Spurensicherungsfolie konnte keine ausreichenden DNA-Mengen erzeugen. Gründe für eine solche unzureichende Rückgewinnung typisierbarer DNA können die minimale Größe dieser Spuren, die Degradierung durch Alterung (5-19 Jahre), Umwelteinflüsse und andere die DNA-Qualität beeinträchtigende Bedingungen sein. Die wasserlösliche PVAL-Matrix zeigte hingegen deutliche Vorteile für eine erfolgreiche Extraktion von Nukleinsäuren im Vergleich zu den Spurensicherungsfolien [372]. Die PVAL-Methode erbrachte verlässliche Ergebnisse für das bis zu 20 Jahre alte Spurenmaterial. Alle vereinigten Proben wiesen quantifizierbare DNA-Konzentrationen auf. Darüber hinaus wurden die Effizienzen der RNA/DNA Ko-Extraktion und der DNA-Extraktion verglichen. Wie erwartet, wurden bei der STR-Genotypisierung bessere Ergebnisse erzielt, wenn die DNA-Extraktion durchgeführt wurde, hierbei konnte ein geringerer Verlust an DNA-Ausbeute im Vergleich zur RNA/DNA Ko-Extraktion beobachtet werden. In Abhängigkeit von der genauen Fragestellung zur Klärung der Todesumstände sollte daher abgewogen werden, welche Extraktionsart durchgeführt werden soll. In solchen Fällen empfiehlt es sich, mehrere gleichartige, direkt nebeneinander liegende Spuren zu erfassen. Von einer Probe wird anschließend nur DNA, von der/den anderen RNA/DNA ko-extrahiert. Im Zweifelsfall, wenn nur eine Minimalspur verfügbar ist und die Fragestellung der Individualisierung Vorrang hat, kann auf eine RNA-Analyse verzichtet werden. Alternativ, wenn die Identität der fraglichen Person bereits geklärt wurde oder geklärt werden kann, ohne dass dafür ein DNA-Profil erforderlich ist, kann stattdessen die RNA zur Körperflüssigkeits- bzw. Organbestimmung isoliert werden.

Zusammenfassend kann die Probennahme von biologischem Material unter Verwendung der PVAL-Technik mit anschließender STR-Typisierung zuverlässige DNA-Profile zur forensischen Identifizierung, ohne beobachtbare Inhibition, erzeugen. Ein weiterer wichtiger Unterschied zwischen Spurensicherungsfolien und PVAL-Handschuhen besteht darin, dass eine zu große Klebstoffdichte auf dem Klebeband die Rückgewinnung von biologischem Spurenmaterial und damit den Prozess der Genotypisierung beeinträchtigen kann, während zu wenig Klebstoff zu einer verminderten Haftfestigkeit und damit zu verminderter Haftfähigkeit führt [40]. Darüber hinaus wird die Haftfähigkeit verringert, wenn feuchte Blutspuren

gesammelt werden, da das Auftragen der nichtabsorbierenden Spurensicherungsfolie die Probe verschmiert, wodurch die Wirksamkeit der Sammlung anderer biologischer Materialien verringert wird [366]. Schließlich ist die wasserlösliche Matrix von PVAL in der Lage, eine viel größere Menge des auf den Händen vorhandenen biologischen Spurenmaterials zu absorbieren, als sich auf Spurensicherungsfolien ansammeln können [366]. Folglich ist bei Verwendung der PVAL-Methode eine höhere Ausbeute an Nukleinsäuren zu erwarten, was die Wahrscheinlichkeit erhöht, das Opfer und das verletzte Gewebe zu identifizieren.

Die Untersuchung von Schussrückständen und DNA sollte mitunter die RNA-basierte Identifizierung von Körperflüssigkeiten und Organgeweben als wichtigen und unterstützenden Faktor bei der Interpretation und Kontextualisierung biologischer Spuren zur Rekonstruktion der Umstände von Verbrechen umfassen. Obwohl bei den untersuchten Proben der Spurensicherungsfolien, die von Händen von Suizidopfern stammten, keine RNA-Konzentration fluorometrisch bestimmt werden konnte, gelang der fluoreszenzbasierte Nachweis blutspezifischer Expression von *miR-451a* in den getesteten Proben, wohingegen die miRNA-Expression in Proben, die von den Verdächtigen stammten, nicht nachweisbar war. Trotz der teilweise erfolglosen fluorometrischen Bestimmung der RNA-Konzentrationen in Proben der PVAL-Handschuhe, wurde in den getesteten Proben eine blut- bzw. hirnspezifische Expression von *miR-451a* bzw. *miR-124a* erfolgreich und korrekt nachgewiesen. Hirnspezifische miRNA wurde einerseits an einem Opfer und andererseits am PVAL-Handschuh des Suizidfalls nachgewiesen. In beiden Fällen handelte es sich beim Tatgeschehen um einen absoluten Nahschuss mit Kopfverletzung. In einem Fall konnte gezeigt werden, dass das entnommene biologisches Material aus Rückschleuderspuren, das von der Hand des Schützen gesammelt wurde, erfolgreich der vollen Bandbreite chemischer, physikalischer, immunchromatographischer und forensischer Nukleinsäureanalytik unterzogen werden konnte, einschließlich nukleärer DNA und miRNA-basierter Körperflüssigkeits- und Organgewebsidentifizierung. Nachdem die Person durch einen absoluten Nahschuss am Kopf Suizid begangen hatte, konnte in Spuren von Backspatter auf deren Händen blut- und hirnspezifische RNA-Expression gemessen werden. Die STR-Typisierung bestätigte, dass die gefundenen Spuren der Körperflüssigkeit und des Organgewebes von derselben Person stammten. Somit gelangen der Nachweis und die korrekte Identifizierung von Blut und Hirngewebe an den Händen von Verstorbenen in bis zu 20 Jahre altem authentischem Spurenmaterial.

Zusammenfassend zeigen die beiden Studien, dass die Sicherung minimaler biologischer Spuren (wie Backspatter), die mittels Spurensicherungsfolien an Händen von Opfern und Tatverdächtigen gesammelt wurden, bedingt eine erfolgreiche DNA-Profilerstellung erlaubt, wenn eine geringe Menge von Spurenmaterial verfügbar ist. Die Analyse von RNA führt jedoch zu besseren Erfolgsraten und kann neben der Detektion von Schmauchspuren und der Standard-DNA-Analyse eine unterstützende, kontexterweiternde Ergänzung bei der Rekonstruktion der Umstände von Straftaten sein. Es konnte gezeigt werden, dass DNA sowie blut- und hirnspezifische miRNA aus biologischen Spuren von PVAL-Handschuhen von fallbezogenen Oberflächen gewonnen und analysiert werden können, wenngleich nur minimale Mengen von bis zu 20 Jahre gealtertem Material zur Verfügung stehen.

Um jedoch die Erfolgsquote dieser Methode in der Routinearbeit zu erhöhen erscheint das Zusammenführen aller Proben, die aus PVAL-Handschuhen gewonnen wurden, ratsam und allgemein als vorteilhaft. Durch die Demonstration ihrer hohen Erfolgsrate unterstreicht diese Methode nach Delikten mit Schusswaffengebrauch, die Nützlichkeit und ihr großes Potenzial für eine Anwendung in der forensischen Routinearbeit sowie ihre Überlegenheit gegenüber anderen Spurensicherungsmethoden.

6 Messung der differentiellen RNA-Degradation zur Altersschätzung von Blutspuren

6.1 Einleitung

Neben der Urheberschaft oder der Spurenart ist auch die Kenntnis des Zeitpunktes, zu dem eine Straftat begangen worden ist, von hohem forensischem Interesse. Meist kann eine solche Zeitbestimmung jedoch nur indirekt durch die Ermittlung des Zeitpunktes, zu dem eine tatrelevante Spur entstanden ist, erreicht werden. Es ist jedoch nicht immer klar, ob sich die Nachweise auf den betreffenden Vorfall oder ein anderes damit nicht in Zusammenhang stehendes Ereignis beziehen. Eine Möglichkeit, den Zeitpunkt eines Schusswaffendelikts indirekt zu ermitteln, besteht darin, das Alter der dabei freigesetzten Blutspuren zu bestimmen, wodurch eine Verbindung zwischen dieser Spur und dem Zeitpunkt, zu dem die Tat begangen wurde, bereitgestellt wird.

Eine genaue Abschätzung des Alters von biologischem Spurenmaterial ist jedoch noch immer ein schwieriges und bisher nicht hinreichend gelöstes Problem in der forensischen Wissenschaft und wird durch die Tatsache verkompliziert, dass Proben, wie sie in der forensischen Routinearbeit auftreten, häufig gealtertes und/oder degradiertes Spurenmaterial in geringen Mengen und von reduzierter Qualität enthalten. Daher hat sich die Forschung in den letzten zehn Jahren zunehmend der forensischen RNA-Analyse zugewandt, die sich u.a. mit der Bestimmung des biologischen Alters einer Spur befasst. Verschiedene Forschungsansätze wurden entwickelt, um das Alter von Blutspuren zu bestimmen. Zunächst lag der Fokus der Forschung auf verschiedenen, nicht molekulargenetischen Methoden. Enzymatische [373, 374], spektroskopische [375–377], entomologische [378] und chromatographische Methoden wie Flüssigchromatographie (HPLC) [379–381]

sowie Elektronenspinresonanzspektroskopie (EPR) [382] wurden etabliert. Wie sich zeigte, konnten diese Methoden das Alter einer Blutspur nicht genau bestimmen und erfordern zudem eine aufwendige Vorbereitung sowie eine komplexe Ausstattung des Labors. Bis heute wurden verschiedene molekulargenetische Studien durchgeführt, um das Alter einer Blutspur oder einer anderen biologischen Spurenart zu bestimmen [170–172, 174–176]. Es wird angenommen, dass RNA schneller degradiert [125, 126] als DNA, und mehrere forensische Studien berichten über die differentielle Degradation abundanter RNA-Spezies in verschiedenen Probenarten [171–173]. Daher kann eine systematische Analyse des RNA-Abbaus ein praktikabler Ansatz sein, um das Alter biologischen Spurenmaterials zu bestimmen. Obwohl unterschiedliche forensische Methoden vorgestellt wurden, bleiben dennoch viele Fragen bezüglich des Verhaltens von RNA in geringen Mengen und/oder degradiertem Spurenmaterial bestehen.

Im vorliegenden Manuskript wird eine robuste Methodik vorgestellt, mittels derer mRNA-Marker auf ihr Potenzial hin untersucht werden, eine Altersschätzung für forensisch realistische Proben aus getrocknetem Blut zu ermöglichen. Dafür wurde exemplarisch die mRNA-Degradation von *ACTB*, *ALOX5AP*, *COX16*, *HBB_g1* und *SCGB1C1* in Proben aus frischem und gealtertem Blut gemessen, um eine mögliche Korrelation des RNA-Molekül-Abbaus mit dem Alter der Probe zu identifizieren. Die Anwendbarkeit der RNA-basierten Altersbestimmung wurde neben der Standardreihe anhand realer Fallbeispiele bewertet.

RNA degradation in up to 8 year old blood traces as a measure of time elapsed since deposition

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ABSTRACT

An accurate estimate of the age of a bloodstain can reveal the time that has elapsed since it was deposited, thereby establishing a link between the bloodstain and the point of time when a crime was committed. Standard forensic DNA analysis only allows for the individualization of biological trace material from a crime scene but provides no information as to when the trace had been deposited. Therefore, in the last decade considerably more research has been conducted applying forensic RNA analysis to determine the age of a biological stain. However, although various possible forensic applications have been suggested many questions still remain concerning the behavior of RNA in limited amounts of degrading material.

The aim of the present study was to identify suitable mRNA markers for time estimation in forensically realistic samples of dried blood aged up to 90 days and up to eight years utilizing quantitative PCR based RNA quantification. MRNA quantities were measured in fresh and aged blood to identify a potential correlation of RNA molecule degradation with sample age. Furthermore, the applicability of our method was assessed with real casework samples.

Our results show time-wise differences between the measured integrity values of the investigated RNA molecules. These differences were non-significant within the groups of samples aged for 0 to 90 days and one to eight years, respectively, but they were significant between those groups.

Also, we present a robust methodological framework for the quantitative assessment of the potential of RNA candidates for forensic time estimation that can be employed in future research.

KEY WORDS

Sample age, Time estimation, Forensic RNA analysis, RNA degradation, Quantitative PCR

INTRODUCTION

DNA analysis can yield information usable only to identify individuals or to associate suspects, victims and crime scene to one another but does not support temporal contextualization of trace material. In contrast, ribonucleic acid (RNA) based forensic analysis is much more versatile and offers a variety of different approaches to assess, for instance, the cause of death [1,2], the composition of mixed stains [3,4], the age of a wound [5-7], and the presence of pregnancy [8].

While STR genotyping is employed comprehensively for DNA based identification because of its robustness, sensitivity and high power of discrimination, RNA has long been considered too labile and too susceptible to degradation to be of use in the investigation of most forensic type samples. However, several studies over the past decade have relativized this view and demonstrated that RNA in ex vivo samples can remain stable and accessible to analysis for years [9-11].

Research on the forensic applications of RNA analysis has surged considerably during the last two decades and provided recent advancements in forensic casework [11,12]. Numerous studies now document the practicability of messenger RNA (mRNA) and microRNA (miRNA) analyses for body fluid and organ tissue identification based on the detection of differentially expressed mRNA and miRNA [14-19] or the estimation of the post mortem interval [20,21].

Knowing the composition of biological material from a crime scene, e.g., whether a stain contains blood or semen, is complementary to DNA based identification and can be essential in reconstructing the course of events in criminal investigations. The point in time when a crime has happened is another critical aspect in criminal investigation and will very frequently coincide with the point in time when crime related trace material has been deposited at the crime scene. Thus, if the former is unknown, it may be inferred from the latter. Precise estimation, however, of the age of biological trace material still is a difficult and unresolved issue in forensic science and is complicated by the fact that samples encountered in forensic routine casework frequently comprise aged and/or degraded trace material in low amounts and of reduced quality. In the last decades, different research approaches were taken to develop methods for determining the age of bloodstains. Enzymatic [22,23], spectroscopic [24-26], entomological [27], and chromatographic methods like liquid chromatography (HPLC) [28-30] as well as electron paramagnetic resonance spectroscopy (EPR) [31] have all been tried. Notably these methods failed to accurately determine the age of a bloodstain and in some instances also require elaborate preparation and complex instrumentation.

Until today, numerous molecular genetic studies have been performed in an effort to determine the age of a bloodstain or other biological trace material [32-37]. RNA is hypothesized to degrade in a non-random manner and more rapidly than DNA in dried stains [38] and several forensic studies report on differential degradation of abundant RNA species in various kinds of samples. Thus, systematic analysis of RNA degradation may be a valuable approach to determine the age of biological trace material.

In this study, we introduce a robust methodological framework to test RNA candidates for their potential to determine the age of biological samples via the measurement of their time-wise degradation and apply it to evaluate the suitability of *ACTB, ALOX5AP, COX16, HBB_g1* and *SCGB1C1* for RNA based age determination.

MATERIAL AND METHODS

Blood collection for standardized samples and ethics statement

Samples of EDTA-free blood drawn by venipuncture were obtained from six healthy Caucasian individuals (three females and three males). Blood samples from all individuals were collected within a 1 h interval and were transferred to sterile Pur-Zellin® cellulose swabs (Hartmann, Heidenheim, Germany) within 30 seconds. RNA was extracted at fifteen different time points: immediately after being sampled (day 0) and on days 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 75, and 90.

Furthermore, bloodstains (1, 1.5, 3, 4, 6 and 8 years) from four different donors (two females, two males) had also been sampled and aged on sterile Pur-Zellin® cellulose swabs (Hartmann). All samples were stored at room temperature in a dry, clean and dark environment.

All volunteers provided informed consent and the study protocol was reviewed and approved by the ethics committee of the Hospital of the University of Bonn, Germany.

Sample collection from firearms (casework samples)

12 samples from six cases of suicide involving the use of firearms were included in the study (Table 1). All samples were collected using sterile, DNA-free forensic swabs (Sarstedt AG & Co., Nümbrecht, Germany) moistened with DNA-free water. After sampling from different locations of the weapon, swabs were dried and stored for from 11 month to four years in a dark place at room temperature. To counter potential bias the investigations were performed by experimentators blinded to sample age, weapon type, entry wound, sampling area etc.

RNA extraction, quantification and quality assessment

Prior to all RNA processing, all surfaces, instruments and devices employed in the processes were thoroughly cleansed using RNase-Zap® (Ambion, Austin, TX, USA) and Roti®-Nukleinsäurefrei

(Carl Roth, Karlsruhe, Germany) to remove all traces of ambient RNases and nucleic acid contaminations. Only RNase-free reagents and plastic consumables were used.

Nucleic acids were extracted using the NucleoSpin® miRNA Kit (Macherey-Nagel, Düren, Germany) according to the provided total RNA protocol which includes a DNase treatment. Minor changes in the protocol were applied which had been shown to be well suited for co-extracting RNA and DNA from forensic type samples, which also applies for mRNA extraction [39]. Briefly, cuttings of 1.0 cm^2 were incubated for 10 min with 350 µl lysis buffer at room temperature and NucleoSpin® Forensic Filters (Macherey-Nagel) were then used to separate the liquid lysate containing the nucleic acids from discardable solid material by centrifugation at 21,000 x g for 3 min. The lysate was then processed according to the protocol and total RNA was finally eluted in 40 µl of nuclease-free water pre-heated to 95 °C. All RNA extracts were stored at −80 °C until further processing.

Quantity and quality of total RNA were measured using QuantiFluor® RNA Dye with the Quantus™ Fluorometer (both Promega, Mannheim, Germany) and the RNA 6000 Pico Kit with an Agilent 2100 Bioanalyzer (both Agilent, Böblingen, Germany), respectively. For both methods, 1 µl of RNA extract was used and all measurements were performed according to the manufacturers' prescriptions. RNA quality is represented by the 'RNA integrity number' (RIN) [40]. Based on quantification results, all individual samples were diluted to a final concentration of 4 ng/µl with RNAse-free water. If RNA yield was under 4 ng/µl, the maximum input volume for reverse transcription reactions was used instead.

Selection of mRNA and reference genes for qPCR data normalization

Based on a survey of literature and publicly accessible databases (e.g. 'Expression Atlas' [41]), five candidate genes were chosen as targets of interest to assess the time-wise degradation of RNA molecules (Table 2).

TATAA Universal RNA Spike I (TATAA Biocenter AB, Göteborg, Sweden) was chosen as a reference to serve as an endogenous control for the normalization of mRNA expression data.

Note: the recommended approach of qPCR data normalization which involves the use of empirically determined co-extracted reference genes is not suitable in this experimental setup,

because the investigated variable, i.e. aging of the samples, will arguably also affect the integrity of the reference genes which is an exclusion criterion.

Reverse transcription (RT) and qPCR

Complementary DNA (cDNA) was synthesized from mRNA using the High Capacity Reverse Transcription Kit with random hexamer primers (Life Technologies) according to manufacturer's protocol and in a total volume of 20 µl. Each mRNA RT reaction consisted of 20 ng of total RNA, 1 x RT Buffer, 100 mM dNTPs, 1 x Random Primers and 50 U MultiScribe™ Reverse Transcriptase. 2 µl of TATAA Universal RNA Spike I template ($2*10^7$ copies) were added to each RT reaction.

All RT reactions were performed on a T3 Thermocycler (Biometra, Göttingen, Germany) with the following cycling conditions: 25 °C for 10 min, 37 °C for 120 min, and 85 °C for 5 min.

To detect potential cross-reactivity with genomic DNA or DNA contamination of our reagents, RNA extraction negative controls, RT(-) controls (i.e. RT reactions without reverse transcriptase) and H_2O controls (i.e. RT reactions with water instead of RNA template) were set up, respectively. RNA was kept on ice during processing and RT reaction products were stored at -20 °C until further proceedings.

QPCR was performed using target-specific TaqMan® Assays for the genes of interest (Table 2) and the TaqMan® Universal PCR Master Mix, No AmpErase® UNG (all Life Technologies), according to manufacturer's prescription. 4 µl of the corresponding RT reaction product were used in each qPCR with 1 x TaqMan® Universal PCR Master Mix and 1 x specific TaqMan® Assay adding up to a total reaction volume of 20 µl. All reaction components were kept on ice during processing.

All sample/assay combinations were run in technical triplicates. All qPCR reactions were conducted in MicroAmp® Optical 96-Well Reaction Plates on an ABI Prism 7500 Sequence Detection System (both Life Technologies) with the following qPCR cycling conditions: 95 °C for 10 min, followed by 40 cycles with 95 °C for 15 sec and 60 °C for 1 min.

To normalize inter-run variation between qPCR reaction plates the internal positive control (IPC) from the PowerQuant™ System (Promega) was used as an inter-plate calibrator. Raw fluorescence data was collected during the 60 °C step by the SDS software v2.0.6 (Life Technologies) and then exported for further analysis.

Data analysis, normalization, statistics, and MIQE compliance

To calculate quantification cycle (C_q)-values and amplification efficiencies from raw data, i.e. SDS spread sheet exported R_n-values, the LinRegPCR analysis program v2015.1 [42] was used. For the calculation of C_q-values a common threshold value was set to −0.7 log (fluorescence). Sample replicates exhibiting C_q-values deviating more than one unit from the mean of the triplicates were excluded from further processing. Normalization of computed C_q-values of target specific mRNA was performed using the GenEx software v6 (multiD Analyses). Pre-processing of qPCR encompassed the following steps in the given order: efficiency correction, averaging oftechnical qPCR replicates and normalization with TATAA Universal RNA Spike I resulting in ΔC_q-values ($=C_q\text{-value}_{(\text{target gene})}$ / $C_q\text{-value}_{(\text{TATAA Universal RNA Spike I})}$).

Data analysis, interpretation, and statistical calculations were performed using MS EXCEL and SPSS software v.22 (SPSS Inc., Chicago, IL, USA). The Kolmogorov-Smirnov test was applied to test for normal distribution; the means/medians of normally and non-normally distributed sample groups were compared applying the Student's t-test and the Mann-Whitney-U-test, respectively.

To facilitate reliable and unequivocal interpretation of the qPCR results reported herein, all information that is rated 'essential' according to the MIQE guidelines [43] is reported, where applicable.

RESULTS

Quantity and quality of RNA

Total RNA extracted from up to 90 days old and up to eight year old dried bloodstains exhibited variation in terms of quantity and considerable variation in terms of quality (RIN) among all samples over time. Quantification results and RIN values for the standardized samples and for the casework samples are presented comprehensively in Supplementary Table 1 and Table 1, respectively. Overall, RNA concentrations of standardized samples varied between two orders of magnitude (0.16 ng/µl (4 years; sample G) - 11 ng/µl (day 60; sample C)). RNA yields of samples obtained from inner surfaces of gun barrels fired in real cases ranged from 0.13 ng/µl (case #6; -2) to 3.0 ng/µl (case #3; -2) and in some cases was below the limit of detection.

No decrease of RNA yield was observed with increasing age of the bloodstain.

For the assessment of total RNA quality, RIN values were determined. RIN values did not exhibit a continuous but instead a 'three-phased' course. Up to day 40 RIN values varied

between 2.6 and 2.4 on average. Between days 45 – 90 the mean of RIN values was 1.8 and between 1 – 8 years RIN values averaged around 1.4 (Supplementary Table 1, Figure 1). When detectable, RIN values in casework samples ranged from 1.0 to 1.6 (Table 1)

The participants' sex did not influence RIN values or quantification results in the standardized samples.

Degradation analysis of mRNA markers

Downstream qPCR analysis was employed to assess the extent of time-wise degradation in selected mRNA markers (Table 2).

Based on preliminary results the marker *COX16* was excluded from further analysis because no evaluable ΔC_q-values could be generated. For the remaining four marker genes (*ACTB*, *ALOX5AP*, *HBB_g1* and *SCGB1C1*) different expression values ($=C_q\text{-value}_{(\text{target gene})}$ / $C_q\text{-value}_{(\text{TATAA Universal RNA Spike I})}$) were obtained. These were averaged for all six and four participants per time point and were obtained for the 12 casework samples. RNA degradation for standardized samples at any given time point was defined as an increase of ΔC_q-values as compared to ΔC_q at day 0 representing pristine and non-degraded RNA.

a) Standardized samples

The degradation of RNA did not increase significantly neither over the 90 days period nor over the 8 years period for any of the four markers (Supplementary Table 2, Figure 2). Therefore, mRNA degradation of *ACTB*, *ALOX5AP*, *HBB_g1* and *SCGB1C1* is not suitable to base upon a model for age estimation between 1 and 90 days and 1 and 8 years old bloodstains, respectively.

We observed, however, a significant difference in RNA degradation between samples of up to 90 days and of up to 8 years old (*p-value* <0.05) (Table 3).

These results suggest a kind of tipping point in the degradation of the RNA molecules between 3 months and 1 year of age. Furthermore, participants' sex did not influence RNA degradation. Thus, degradation of one or a combination of these mRNA markers as determined via qPCR and normalized by TATAA Universal RNA Spike I may be usable for a rough estimate of the age of a bloodstain (younger than 90 days, older than 1 year).

b) Casework samples

To demonstrate the applicability of the analysis of time-wise RNA degradation even in highly compromised and environmentally challenged casework material, samples obtained from inner surfaces of gun barrels after shots at biological targets were included in the study. The results demonstrate that measuring mRNA degradation in real casework samples corresponds to standardized samples and were suitable to correctly classify the samples' age as younger than 90 days or older than 1 year.

Additionally, to exclude any possibility of interference between assays in multiplex reactions, we investigated mRNA and TATAA Universal RNA Spike I assays in separate reaction wells and compared the resulting values to the values from multiplexed reactions. The C_q-values of all tested samples were comparable and exhibited no significant differences (data not shown). Moreover, all negative controls (extraction negative, RT(-) and H_2O) showed no or unspecific results ($C_q > 35$) for all tested samples.

DISCUSSION

In the investigation of criminal acts it is important to make maximum use of the information contained in biological trace material to be found at crime scenes. Assessment of the state of mRNA degradation in a sample can help determine the age of a biological stain and thus to understand and reconstruct the conditions and circumstances of the events that led to their respective generation. In the present study, we examined if degradation of selected mRNAs can be correlated to the time elapsed since deposition of forensic biological stains, involving the examination of the extent of degradation of said mRNAs in fresh and dried bloodstains stored for up to 90 days and between one and eight years.

First, the assessment of RNA integrity is an important step in obtaining meaningful expression data. Using intact RNA is a key element for successful downstream analyses e.g. microarray or RT-PCR analyses if small differences in expression are to be detected [44]. RNA samples with RIN values of 1.0 are considered as completely degraded, whereas RIN values of 10 indicate fully intact, pristine RNA [40]. One aspect of the present study was the assessment of the influence of time elapsed since stain deposition on the RIN. We found that RIN values significantly decreased between the time intervals of 0-90 days and 1-8 years of stain age. This suggests that considerable degradation occurs in total blood-RNA between 3 months and 1 year while it remains to be elucidated whether this is a continuous or a punctuated process.

An early study by Bauer et al. [32] demonstrated a correlation between sample age and the ratio of electropherogram peak areas of two housekeeping gene products, β-actin and cyclophilin, in up to 15 year old bloodstains. In 2005, Anderson et al. [33] focused on the examination of bloodstains aged under controlled conditions for up to 150 days. By employing species-specific probes to increase the assays' specificity in the PCR amplification step, a ratio of relative quantities was determined. Their results showed a linear relationship between sample age and the signal ratio of the two RNA species β-actin mRNA and 18S rRNA suggesting that 18S rRNA degraded slower than β-actin mRNA with an increase of the 18S:β-actin signal ratio corresponding to the time elapsed since deposition. Furthermore, this method allows for the co-extraction of DNA and RNA and can potentially be applied to various other tissue types [33]. In a follow-up study also carried out by Anderson et al. [34] they determined the relative stability of different sized fragments of the same RNA molecule to eliminate inter-molecule variation in degradation. Hampson et al. [45] in their study investigated the relative signal ratio of β-actin mRNA and 18S rRNA in hair samples (follicular tag) over a 90 day period. They, too, reported a linear relationship for up to 60 days between the relative expression ratio of the two RNA species and the age of a sample.

In contrast, the expression levels of the mRNA markers, normalized by TATAA Universal RNA Spike I, investigated herein, did not exhibit such a linear relationship with elapsed time eligible for regression analysis but instead (with the exception of *COX16)* exhibited a 'two-phased' characteristic with considerable decrease of the ΔC_q-values between 0-90 days and 1-8 years of age. These findings suggest that time-wise degradation of RNA is no uniform and strictly continuous process but may be dependent on external factors, the kind of sample as well as the particular physicochemical properties of individual RNA molecules comprising not only their base sequence and emerging secondary structure but also packaging into or association with accompanying protein components. This assumption is corroborated by recent evidence that several specific genes are activated and transcribed even after organismal death which may generate the appearance of no or even reversed degradation [46,47]. Thus, taking into account specific differences in mRNA degradation patterns is important because every biological stain is unique in its composition, size, origin and minutiae of formation and will therefore exhibit individual rather than uniform molecular properties and our results support the hypothesis that there are gene specific differences in the rates of ex vivo RNA degradation.

In the present study, we investigated blood stains from six persons (thee male and three females) at 15 different time points, and from four persons (two male and two females) at six

different time points under identical environmental conditions. Furthermore, to demonstrate the applicability of this method to measure RNA degradation in realistic casework material samples from casework was applied to real samples collected from firearms used to commit suicide. Therefore, this method can be used for a rough estimation of sample age in real cases, especially for cold cases.

It is known that the average RNA integrity differs between tissue types and donors and that it is affected by several different parameters. Our observation that the participants' sex had no influence on degradation are confirmed by the findings of both Anderson et al. [33] and Hampson et al. [45] who reported no significant differences between sexes. Furthermore, no significant inter-donor specific differences were confirmed by a larger population study [30]. Also in a study applying reflectance spectroscopy by Bremmer et al. [48] no significant inter-donor variation was found among 40 bloodstains from eight donors. In contrast, Qi et al. [36] reported that their investigated samples exhibited significant difference between sexes. It is plausible to assume that observed inter-donor variation to a large extent depends on the measurement technique. Forensic scientists deal with a broad variety of biological sample types and for the purpose of applying RNA based sample age estimation in casework it would be ideal to streamline RNA analysis by using a multiplex of easy-to-use tissue-specific assays. However, to use such methods as described for instance by Bauer [32] and Anderson [33] who did provide initial evidence for a correlation between RNA degradation and sample age in forensic routine casework, further refinement and thorough validation is required.

Another approach to estimate time since sample deposition or, in some studies, the PMI is to assess RNA degradation as a continuous decrease in RNA integrity as represented by the RIN. Sampaio-Silva et al. [49] and Inoue et al. [50] analyzed different murine tissues sampled from 1 to 11 hours and up to three days after death, respectively. While most studies evaluate RNA degradation as an indicator of PMI in samples aged for short time periods, initial research has been conducted on RNA stability in tissues over an extended PMI. A study by Young et al. [51] assessed the stability of RNA over 140 days. They compared the degradation rates of a large segment sensitive for degradation and a smaller, non-overlapping and more stable segment of β-actin RNA from the tooth pulp of buried pigs. Using this method the PMI could be estimated for up to 84 days supporting the possible use of RNA degradation as an estimator of PMI over an extended time period. Due to the random nature of RNA degradation it is commonly assumed that larger RNA molecules degrade faster than smaller ones and the findings by Anderson et al. [34] confirm this assumption. Their results indicate that differential degradation exploiting differences in molecule size can be used to estimate the

age of a bloodstain: the larger the RNA molecule, the shorter the time until the signal derived from it disappeared. To account for this finding, we chose RNA targets up to a maximum length of 177 bp. Notably, however, the largest mRNA (*COX16*) in our panel produced no evaluable signal whereas for the smallest mRNA (*SCGB1C1*) considerable differential degradation was observed. This suggests that given sufficiently long time periods degradation will affect even comparably small sized RNA molecules.

Previous studies focusing on the association of RNA degradation with PMI have not been as conclusive as studies involving the age of a biological stain instead. Some of these studies reported a correlation of RNA degradation with PMI [50,52-54] while others found no such correlation [55-57]. For instance, in their study, Sampaio-Silva et al. [49] analyzed eleven transcripts by qPCR analysis and detected a correlation between RNA degradation and the PMI in tissues of femoral quadriceps and liver, but not in skin, spleen, pancreas, stomach, and lung tissue. Up to date there is no systematic evidence to predict which tissues will show such a correlation and which will not as the stability of RNA appears to vary tissue-wise [50,56].

One of the most sensitive and reproducible methods for quantitative gene expression analysis is quantitative real-time PCR. However, to obtain reliable and unbiased data from qPCR, an accurate normalization strategy to eliminate non-biological variance is required [58-60]. The use of one or more endogenous reference genes selected based on their empirically demonstrated suitability is considered gold standard for qPCR normalization [61-63]. However, the variable in question in this study has been the extent of RNA degradation mediated by the age of the samples i.e. a process that will arguably also affect the integrity and hence the C_q-values of the reference genes which is an exclusion criterion for this method of normalization. An alternative approach to data normalization is the use of a known external standard which is spiked into the sample extract and is then co-transcribed and co-amplified with the target gene in the same reaction tube, which using a specific assay and applying set cycling conditions, will generate an index C_q unaffected by a sample's age but deviations of which will indicate non-biological variation that can then be taken account for by normalization. Therefore, we used the TATAA Universal RNA Spike I assay for normalization which amplifies a 300 bp region towards the 3'-end of a synthetic RNA template barring interference with the genes of interest. The ratio of standard to target then represents the extent of degradation: the lower the ratio the higher the starting amount of intact mRNA present in the sample. Based on C_q-values, *COX16* was excluded from further analysis because no evaluable results could be obtained from TATAA Universal RNA Spike I.

In summary, more research clearly is needed to reproduce results obtained so far and to identify more and better suited RNA markers exhibiting degradation profiles closely correlating with sample age. While previous studies provided initial evidence for a direct correlation between RNA degradation and sample age, much of this work was focused on a limited selection of sample types (i.e. blood). Also, systematic research on the comparability of RNA degradation patterns across multiple sample types as well as on the influence of different environmental conditions is still lacking.

CONCLUSION

In conclusion, the mRNA markers investigated herein do not exhibit a steady and time-wise RNA degradation process suitable for a robust correlation with the time elapsed since deposition and thus permitting a reliable calculation of the age of the trace based on regression analysis. However, the degradation of *ACTB*, *ALOXAP*, *HBB_g1* and *SCGB1C1* can still be used as a basis for the binary assessment of the age of a bloodstain as "older than one year" or "younger than 90 days". While this does not replace an exact determination of the age of a bloodstain, it can be used as a rough estimate of the time elapsed since deposition. Furthermore, this method was successfully used on real casework samples to correctly infer their age in one of these two categories.

In addition, the methodological framework presented herein which is based on a highly standardized and reproducible qPCR procedure with robust and independent TATAA Universal RNA Spike I-normalization can be adapted for further research to assess time-wise degradation in any other potential RNA candidate.

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COMPLIANCE WITH ETHICAL STANDARDS

The study protocol was reviewed and approved by the ethics committee of the Hospital of the University of Bonn, Germany.

CONFLICT OF INTEREST

All authors declare that they have no conflict of interest.

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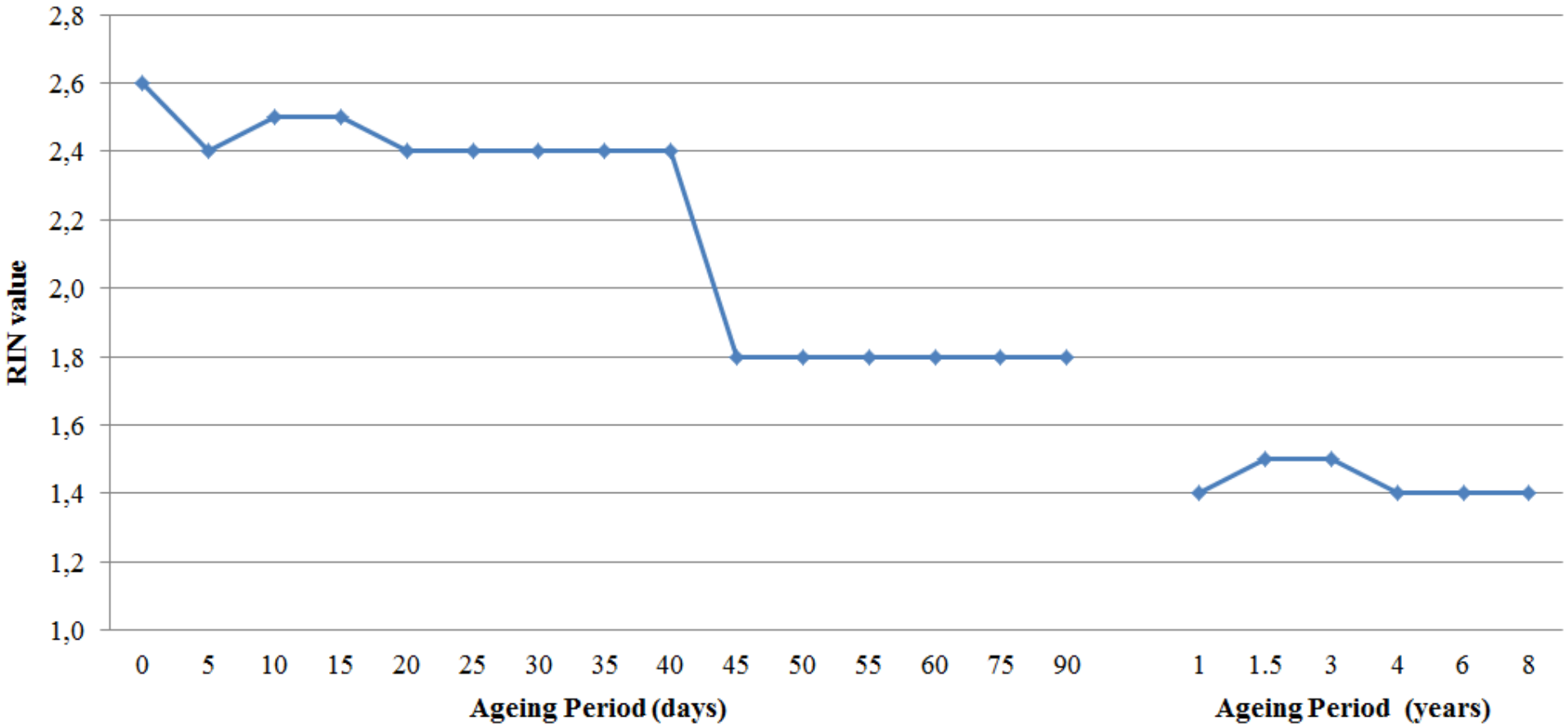

Figure 1 Temporal development of mean RIN values over time

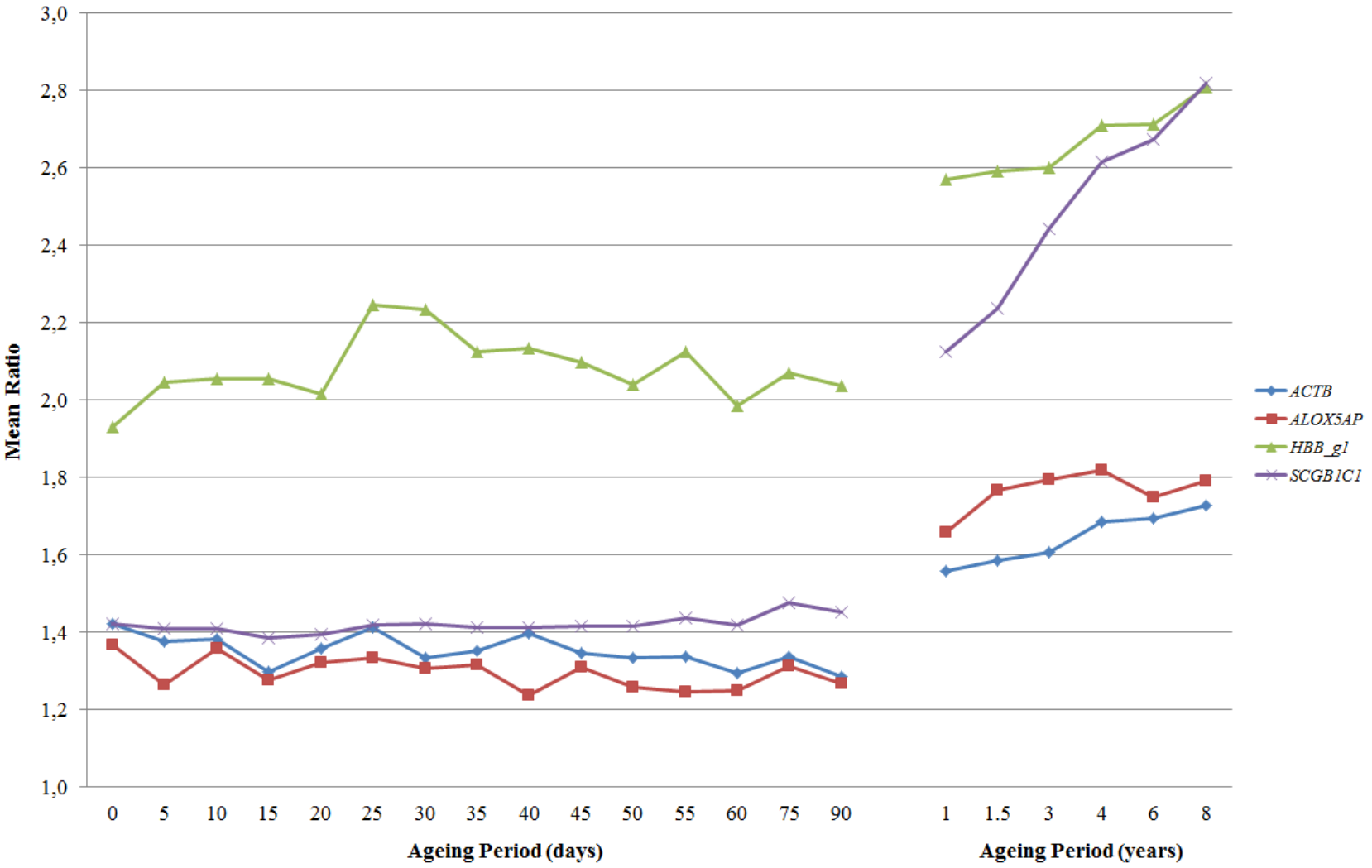

Figure 2 Development of the ΔC_q-value ratio (ordinate) of all six respondents averaged over the respective days and years (abscissa) for the genes *ACTB, ALOX5AP, COX16, HBB_g1* and *SCGB1C1*

Table 1

Overview of casework samples and RNA results

									RNA results					
									Mean ratios of ΔC_q-values per bloodstain mRNA marker					
Case	No.	weapon	caliber	entry wound (localization)	sex of victim	storage	estimated age	sampling location	[a]RNA concentration	RIN	***ACTB***	***ALOX5AP***	***HBB_g1***	***SCGB1C1***
#1	**-1**	R	.38 special	temple	m	11 mon	**older than 1 year**	a	1,15	1.5	1,49	1,70	2,51	2,15
	-2							p	0,70	1.0	1,53	1,68	2,52	2,17
#2	**-1**	R	.320	temple	m	2 y	**older than 1 year**	i	--	--	1,54	1,75	2,57	2,31
	-2							ec	--	1.2	1,54	1,73	2,57	2,32
#3	**-1**	R	.367 magnum	mouth	m	3 y 1 mon	**older than 1 year**	a	1,83	1,2	1,70	1,77	2,63	2,43
	-2							p	3,00	1,6	1,79	1,77	2,61	2,41
#4	**-1**	R	.38 magnum	temple	m	3 y 7 mon	**older than 1 year**	i	--	--	1,65	1,78	2,60	2,42
	-2							ec	--	1.2	1,69	1,79	2,59	2,41
#5	**-1**	RI	-	mouth	m	3 y 8 mon	**older than 1 year**	a	1,20	1.6	1,73	1,77	2,64	2,49
	-2							p	0.35	1.4	1,73	1,79	2,63	2,48
#6	**-1**	P	7.65 mm	temple	m	3 y 8 mon	**older than 1 year**	a	0,56	1.2	1,64	1,74	2,59	2,52
	-2							p	0,13	--	1,62	1,75	2,57	2,47

R: revolver; *RI:* rifle; *P:* pistol; -: not available; *m*: male; *y*: years; *mon:* month; *bold*: correct age estimated; *a:* anterior barrel half; *p:* posterior barrel half; *i:* inner barrel half; *ec:* all empty chambers;

[a]values in [ng/µl]*; RIN: RNA integrity number; -- :not detectable; ACTB:* actin beta; *ALOX5AP:* arachidonate 5-lipoxygenase activating protein; *HBB:* ß-hemoglobin; *SCGB1C1:* secretoglobin family 1C member 1

Table 2

Specifications of the RNA Assays

Official Gene Symbol	NCBI-Alias	NCBI sequence accession	TaqMan® Assay ID	amplicon length (base pairs)
ACTB	*BRWS1, PS1TP5BP1*	NM_001101.3	Hs03023880_g1	139
ALOX5AP	*FLAP*	NM_001204406.1	Hs00970920_m1	90
COX16	*C14orf112, HSPC203*	NM_001204090.1	Hs04234010_m1	177
HBB_g1	*CD113t-C, beta-globin*	NM_000518.4	Hs00747223_g1	106
SCGB1C1	*RYD5*	NM_145651.2	Hs00377337_m1	81

NCBI: National Center for Biotechnology Information; *ACTB:* actin beta; *ALOX5AP*: arachidonate 5-lipoxygenase activating protein; *COX16:* cytochrome c oxidase assembly homolog; *HBB:* ß-hemoglobin; *SCGB1C1:* secretoglobin family 1C member 1

Table 3

Mean ratios of ΔC_q-values per bloodstain mRNA marker for 15 and 6 time points

		ACTB	ALOX5AP	HBB_g1	SCGB1C1
1-90 days	*AM*	1.35	1.29	2.08	1.42
	SD	0.04	0.04	0.08	0.02
1-8 years	*AM*	1.64	1.76	2.66	2.48
	SD	0.07	0.06	0.09	0.27
p-value		<0.05*	<0.05*	<0.05*	<0.05†

AM: arithmetic mean; *SD:* standard deviation; *: Student's t-test; †: Mann-Whitney-U-test

Supplementary Table 1
Overview of RNA results of aged blood samples

Day	Sample	[a]RNA concentration	RIN	AM
0	A	7.65	2.4	2.6
	B	6.30	2.7	
	C	6.45	2.4	
	D	5.95	2.6	
	E	5.20	2.8	
	F	9.50	2.7	
5	A	6.15	2.6	2.4
	B	7.00	2.6	
	C	6.10	2.5	
	D	3.40	2.5	
	E	1.55	2.0	
	F	6.70	2.4	
10	A	9.10	2.4	2.5
	B	6.30	2.4	
	C	8.60	2.6	
	D	7.65	2.4	
	E	7.90	2.5	
	F	8.00	2.4	
15	A	8.30	2.3	2.5
	B	5.35	2.3	
	C	6.40	2.6	
	D	4.35	2.5	
	E	4.20	2.5	
	F	6.50	2.5	
20	A	7.75	2.4	2.4
	B	6.00	2.3	
	C	9.75	2.3	
	D	8.20	2.4	
	E	5.50	2.4	
	F	6.65	2.4	
25	A	5.75	2.4	2.4
	B	5.35	2.4	
	C	5.20	2.3	
	D	5.40	2.4	
	E	5.95	2.4	
	F	5.55	2.4	
30	A	7.40	2.4	2.4
	B	5.35	2.4	
	C	7.00	2.6	
	D	7.40	2.4	
	E	5.20	2.4	
	F	5.55	2.4	
35	A	3.75	2.4	2.4

	B	5.70	2.4	
	C	9.10	2.6	
	D	6.60	2.4	
	E	6.10	2.4	
	F	7.35	2.4	
40	A	10.00	2.3	2.4
	B	5.05	2.4	
	C	3.60	2.3	
	D	3.75	2.5	
	E	6.20	2.3	
	F	4.00	2.4	
45	A	6.95	2.0	1.8
	B	9.05	1.4	
	C	8.75	2.0	
	D	7.40	1.4	
	E	8.55	2.1	
	F	8.75	2.0	
50	A	6.60	1.0	1.8
	B	5.75	2.0	
	C	5.65	2.0	
	D	7.55	2.1	
	E	5.10	1.8	
	F	5.90	2.1	
55	A	6.75	2.0	1.8
	B	6.60	2.0	
	C	5.05	1.8	
	D	6.65	2.1	
	E	5.75	1.6	
	F	6.60	1.4	
60	A	6.45	2.2	1.8
	B	6.15	1.6	
	C	11.00	2.0	
	D	6.55	1.6	
	E	4.65	1.8	
	F	6.00	1.8	
75	A	7.70	2.0	1.8
	B	3.85	1.8	
	C	4.85	2.0	
	D	5.55	1.6	
	E	5.95	1.6	
	F	6.35	2.0	
90	A	9.60	1.6	1.8
	B	7.45	2.0	
	C	7.65	2.0	
	D	5.75	2.0	
	E	5.10	1.4	
	F	6.95	2.0	

Years	Sample	[a]RNA concentration	RIN	AM
1	G	5.75	1.4	1.4
	H	4.60	1.2	
	I	4.10	1.4	
	J	3.25	1.6	
1.5	G	5.55	1.2	1.5
	H	4.20	1.6	
	I	3.90	1.4	
	J	3.30	1.4	
3	G	5.90	1.4	1.5
	H	4.55	1.4	
	I	2.25	1.6	
	J	2.60	1.4	
4	G	0.70	1.2	1.4
	H	1.20	1.2	
	I	3.50	1.6	
	J	3.10	1.4	
6	G	5.75	1.4	1.4
	H	4.85	1.4	
	I	2.90	1.2	
	J	2.20	1.4	
8	G	3.50	1.4	1.4
	H	2.10	1.4	
	I	1.75	1.2	
	J	1.60	1.2	

A-C, G-H: female; *D-F, I-J:* male; [a]values in ng/µl; *RIN:* RNA integrity number; *AM:* arithmetic mean

Supplementary Table 2

Mean ratios of ΔC_q-values per bloodstain mRNA markers between 0 – 90 days (N=6) and 1 – 8 years (N=4)

		ACTB	ALOX5AP	HBB_g1	SCGB1C1
Day	0	1.42	1.37	1.93	1.42
	5	1.38	1.26	2.04	1.41
	10	1.38	1.36	2.06	1.41
	15	1.30	1.28	2.05	1.39
	20	1.36	1.32	2.01	1.39
	25	1.41	1.33	2.24	1.42
	30	1.33	1.31	2.23	1.42
	35	1.35	1.32	2.12	1.41
	40	1.40	1.24	2.13	1.41
	45	1.34	1.31	2.10	1.42
	50	1.33	1.26	2.04	1.42
	55	1.34	1.25	2.12	1.44
	60	1.29	1.25	1.98	1.42
	75	1.33	1.31	2.07	1.47
	90	1.29	1.27	2.04	1.45
Year	1	1.56	1.66	2.57	2.12
	1.5	1.59	1.77	2.59	2.24
	3	1.60	1.79	2.60	2.44
	4	1.68	1.82	2.71	2.61
	6	1.69	1.75	2.71	2.67
	8	1.73	1.79	2.81	2.82

ACTB: actin beta; *ALOX5AP*: arachidonate 5-lipoxygenase activating protein; *HBB:* ß-hemoglobin; *SCGB1C1*: secretoglobin family 1C member 1

6.3 Zusammenfassung

Bei der Untersuchung von Tatorten ist es wichtig, alle Informationen aus dem asservierten biologischen Spurenmaterial zu nutzen. Die Beurteilung des Zustands des mRNA-Abbaus in einer Probe kann helfen das Alter einer biologischen Spur zu bestimmen, um die Ereignisse die zu ihrer jeweiligen Generation geführt haben, zu verstehen und zu rekonstruieren. Im vorliegenden Manuskript wurde untersucht, ob die mRNA-Degradation mit der Zeit korreliert werden kann, die seit der Deponierung forensisch biologischer Spuren vergangen ist.

Hierzu wurde von freiwilligen, aufgeklärten Spendern entnommenes Blut auf sterilen Watteträgern gesammelt, bei Raumtemperatur sowie Dunkelheit getrocknet und gelagert. Zur Bestimmung der Degradation von RNA-Molekülen wurden dafür zwei Standard-Zeitreihen mit Proben mit 15 und 6 verschiedenen Messpunkten (Tag 0 (frisch), 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 75 und 90 Tage sowie 1, 1,5, 3, 4, 6 und 8 Jahre) angelegt. Des Weiteren wurden für die Überprüfung der Eignung dieser Methode Proben von sechs Suizidfällen mit Schusswaffengebrauch in die Studie aufgenommen. Parallel wurde auf Grundlage von Literatur- und Datenbankrecherche [383] fünf als Marker-Gene in Frage kommende RNA-Kandidaten für Blut zusammengestellt.

Die Gesamt-RNA, die aus bis zu 90 Tage alten und bis zu acht Jahre alten getrockneten Blutflecken extrahiert wurde, wies im zeitlichen Verlauf zwischen allen Proben deutliche Unterschiede in der Ausbeute und der Qualität (gemessen als RIN) auf. Insgesamt streuten die RNA-Konzentrationen der Proben der Standardreihe über zwei Größenordnungen, die RNA-Ausbeute der Proben, die von inneren Oberflächen der Suizid-Schusswaffen entnommen wurden, lag in einigen Fällen unter dem Detektionsgrenzwert. Mit zunehmendem Alter des Blutflecks wurde dennoch keine korrelierbare Abnahme der RNA-Ausbeute beobachtet. Zur Beurteilung der Gesamt-RNA-Qualität wurden RIN-Werte bestimmt. Die RIN-Werte zeigten keinen kontinuierlichen, sondern einen „dreistufigen“ Verlauf. Bis zu Tag 40 lagen die RIN-Werte im Durchschnitt zwischen 2,6 und 2,4. Zwischen den Tagen 45 bis 90 betrug der Mittelwert der RIN-Werte 1,8 und zwischen 1 und 8 Jahren betrugen die RIN-Werte im Durchschnitt 1,4. Wenn nachweisbar, lagen die RIN-Werte der Fallproben im Bereich von 1,0 bis 1,6. Das Geschlecht der Probanden beeinflusste weder die Qualitätswerte noch die Quantifizierungsergebnisse der Standardreihen.

Für die Datennormalisierung wurde ein zum Referenzgen-Standard alternativer Ansatz gewählt. Das „TATAA Universal RNA Spike I" wurde verwendet und mit dem Marker-Gen in demselben Reaktionsansatz ko-transkribiert und ko-amplifiziert. Der standardmäßige Ansatz zur qPCR-Datennormalisierung, der die Verwendung empirisch bestimmter, ko-extrahierter Referenzgene vorsieht [322], ist in dieser Versuchskonstellation nicht eignet. Die untersuchte experimentelle Variable, hier das Alter der Proben, betrifft voraussichtlich auch die Qualität aller internen RNA-Referenz-Marker, was ein Ausschlusskriterium darstellt. Daher wurde das extern zugegebene TATAA Universal RNA Spike I zur Normalisierung verwendet, eine synthetische RNA-Matrize, die am 3'-Ende eine 300 Bp Region enthält, die in der qPCR durch spezifische Primer amplifiziert wird, wodurch eine Interferenz mit der Amplifikation der entsprechenden Zielgene verhindert wird. Die erhaltenen C_q-Werte wurden pro Probe effizienzkorrigiert, die RT- und qPCR-Replikate gemittelt und anschließend gegen das arithmetische Mittel der C_q-Werte der TATAA Universal RNA Spike I-Replikate normalisiert. Auf diese Weise wird pro Probe-Assay-Kombination und Person und Zeitpunkt einen ΔC_q-Wert ermittelt. Das Verhältnis von Referenz-Marker zum Zielgen stellt das Ausmaß des Abbaus dar: Je niedriger das Verhältnis, desto höher ist die Ausgangsmenge an intakter mRNA, die in der Probe vorhanden ist.

Basierend auf den ermittelten C_q-Werten wurde der Marker *COX16* von der Auswertung ausgeschlossen. Dieser interferierte, wahrscheinlich aufgrund seiner Länge bzw. Sequenz, mit dem Assay zur Amplifikation der artifiziellen Sequenz der TATAA Universal RNA Spike I. Des Weiteren wies die qPCR eine deutliche reduzierte Effizienz auf, wonach keine auswertbaren ΔC_q-Werte erhalten werden konnten. Für die verbliebenen vier Marker-Gene ergaben sich unterschiedliche Expressionswerte. Die Degradation der mRNA zeigte jedoch für keinen der vier Marker einen statistisch signifikanten Anstieg weder während der 90-Tage Periode noch während der 8-Jahres Periode. Daher erscheint die Messung des mRNA-Abbaus von *ACTB*, *ALOX5AP*, *HBB_g1* und *SCGB1C1* als ungeeignet, um darauf ein Modell für die Altersschätzung für Blutproben zwischen 1 und 90 Tagen bzw. 1 und 8 Jahre zu gründen. Allerdings konnte ein signifikanter Unterschied in der RNA-Degradation zwischen den Proben im Altersintervall von bis zu 90 Tagen und den Proben im Altersintervall von 1 bis 8 Jahren festgestellt werden. Diese Ergebnisse deuten auf eine Art Wendepunkt beim Abbau der RNA-Moleküle zwischen drei Monaten und einem Jahr hin. Daher kann die Degradation von einem oder einer Kombination dieser mRNA-Marker, die durch qPCR bestimmt und

durch TATAA Universal RNA Spike I normalisiert wurden, für eine grobe Schätzung des Alters einer Blutspur (jünger als 90 Tage, älter als 1 Jahr) verwendbar sein.

Diese Ergebnisse legen nahe, dass der zeitliche Abbau von RNA kein gleichmäßiger und streng kontinuierlicher Prozess ist, sondern abhängig von externen Faktoren, der Art der Probe sowie den individuellen physikochemischen Eigenschaften einzelner RNA-Moleküle, die nicht nur ihre Basensequenz und daraus abgeleitete Sekundärstruktur, sondern auch ihre Verpackung in oder in Verbindung mit begleitenden Proteinkomponenten umfassen. Diese Annahme wird durch neuere Belege bestätigt, denen zufolge einige Gene sogar noch nach dem Tod des Organismus aktiviert und transkribiert werden, was den Anschein eines ausbleibenden oder sogar „umgekehrten" Abbaus erwecken kann [384, 385]. Somit ist die Berücksichtigung spezifischer Unterschiede in mRNA-Degradationsmustern bedeutsam, da jede biologische Spur in ihrer Entstehung, Ursprung, Zusammensetzung und Größe einzigartig ist und daher eher individuelle als einheitliche molekulare Eigenschaften aufweist. Diese Ergebnisse stützen die Hypothese, dass genspezifische Unterschiede in den Raten des ex vivo-RNA-Abbaus existieren.

Um die Anwendbarkeit dieser Methode zur Messung des zeitlichen RNA-Abbaus selbst in hochgradig kompromittiertem und durch Umwelteinflüsse beeinträchtigtem Fallmaterial zu demonstrieren, wurden Proben, die von inneren Oberflächen von Schusswaffen nach Schüssen auf biologische Ziele gesammelt wurden, in die Studie einbezogen. Die Ergebnisse zeigen, dass die Messung der mRNA-Degradation in realen Fallarbeitsproben die den standardisierten Proben entsprechen, geeignet war, das Alter der Proben korrekt als jünger als 90 Tage bzw. älter als 1 Jahr zu klassifizieren. Daher kann diese Methode für eine grobe Schätzung des Probenalters in realen Fällen, insbesondere für „Cold Cases", verwendet werden.

Zusammenfassend zeigen die hier untersuchten mRNA-Marker keinen stetigen und mit ihrem Alter korrelierbaren Degradationsprozess, der eine zuverlässige Berechnung des Alters der Spur basierend auf einer Regressionsanalyse ermöglichen würde. Der Abbau von *ACTB*, *ALOXAP*, *HBB_g1* und *SCGB1C1* kann jedoch als Grundlage für die binäre Beurteilung des Alters eines Blutflecks als „jünger als 90 Tage" oder „älter als ein Jahr" verwendet werden. Dies ersetzt nicht eine exakte Altersbestimmung einer Blutspur, kann aber als grobe Abschätzung der seit der Ablagerung verstrichenen Zeit dienen. Dies wurde anhand von realen Fallbeispielen erfolgreich demonstriert, indem ihr Alter korrekt einer dieser beiden Kategorien zugeordnet werden konnte. Darüber hinaus kann der hier vorgestellte

methodische Rahmen, der auf einem hochstandardisierten und reproduzierbaren qPCR-Verfahren mit robuster und unabhängiger universeller TATAA Universal RNA Spike I-Normalisierung basiert, für weitere Untersuchungen angepasst werden, um den zeitlichen Abbau bei weiteren RNA-Kandidaten zu bewerten.

7 Fazit und Ausblick

Die in dieser Dissertationsschrift zusammengefassten Studien belegen, dass die systematische Analyse von Rückschleuderspuren aus dem Waffeninneren sowie von Händen tatbeteiligter Personen eine sinnvolle Erweiterung des Repertoires der Nukleinsäure-basierten Untersuchungen in der forensischen Molekulargenetik darstellt. Dadurch wird die Erkenntnis begründet, dass Proben, welche von ballistischen Modellschüssen oder im Rahmen der Ermittlungen zu realen Schusswaffendelikten gesammelt wurden, eine umfassende Analyse des biologischen Materials aus dem Waffeninneren und/oder der (Schuss-)Hand ermöglichen. So kann etwa vorhandenes biologisches Material dem Spurenverursacher bzw. der Trefferlokalisation zugeordnet werden. Dennoch kann bis dato mittels molekulargenetischer Analysen anhand eines vorliegenden Spurenbilds nicht unterschieden werden, ob es sich bei einem Schuss um einen absoluten oder relativen Nahschuss oder einen Fernschuss gehandelt hat. Hierzu sollten in weiterführenden Studien verschiedene Aspekte und Variablen des Schussvorgangs, vor allem unter dem Einfluss der Schussdistanz, untersucht werden, um die Möglichkeiten für eine evidenzgestützte und objektive Aufklärung von Schusswaffendelikten zu verbessern.

Ein für die Routinearbeit eingesetztes Spurensicherungsverfahren sollte sich ohne große Probleme in den Arbeitsalltag der Erkennungsdienste integrieren lassen. Das biologische Spurenmaterial kann u.a. mittels topographischer Verfahren wie Spurensicherungsfolien oder der PVAL-Methode gesichert werden. Ein entscheidender Aspekt, der die arbeitsintensivere PVAL-Methode gegenüber den Spurensicherungsfolien bevorzugenswert erscheinen lässt, ist die deutlich höhere Ausbeute an Nukleinsäuren und das exakt abbildbare Spurenmuster auf der Schusshand. Diese Methode birgt ein großes Potential für den routinemäßigen Einsatz bei Spurensicherungsarbeiten und könnte für die Zukunft eine gegenüber den Spurensicherungsfolien konkurrenzfähige Methode darstellen [386]. Aus diesem Grund sollte in weiterführenden Forschungsarbeiten angestrebt werden, das methodische Repertoire der

Spurensicherung stetig zu erweitern und zu optimieren, um sich an neue etablierte Untersuchungsmethoden wie die forensische RNA-Analytik anzupassen.

Aufgrund des in der forensischen Routinearbeit häufig vorkommenden minimalen und/oder degradierten Spurenmaterials ist es sinnvoll, neben bereits erfolgreich etablierten Spurensicherungsverfahren die methodischen Arbeitsabläufe im Laborprozess zu optimieren und weiterzuentwickeln. Hierzu zählt zunächst die Auswahl der für das vorliegende Material bestgeeigneten Nukleinsäure-Isolationsmethode. Diese sollte mitunter den Anforderungen des jeweils angestrebten Analyseprozesses genügen und somit eine mit Möglichkeit hohe Ausbeute und Qualität der Nukleinsäuren erbringen. Ein Vorteil der miRNA-basierten Spurenartidentifikation aus Rückschleuderspuren gegenüber den klassischen Verfahren besteht in der Möglichkeit der simultanen Extraktion von RNA und DNA aus ein und derselben Probe. Um die Methode der Körperflüssigkeits- und Organidentifikation im forensischen Labor-Routinebetrieb zu integrieren wäre es, durch Multiplexierung der RT-Reaktion und/oder der nachfolgenden qPCR-Reaktion, von Vorteil den Verbrauch an Probenmaterial sowie Zeit- und Kostenaufwand zu reduzieren. Sauer et al. [133] zeigten bereits, wie sich mehrere Referenzgene in derselben RT-Reaktion multiplexieren lassen. Eine Einschränkung, die bis dato gegen ein Zusammenführen mehrerer Marker in einer qPCR-Reaktion spricht, ist die begrenzte Anzahl simultan detektierbarer Fluoreszenzfarben der industriell verfügbaren qPCR-Geräten.

Darüber hinaus sollte bedacht werden, dass bei degradiertem und minimalem Spurenmaterial häufig die DNA-Genotypisierung scheitern und die Analyse der mtDNA als Alternative genutzt werden kann. Hierbei zeigt sich, dass die Methode grundsätzlich als Erweiterung der Analysemöglichkeit von biologischem Material aus dem Inneren von Schusswaffen anzusehen ist. Neben der geringen erforderlichen DNA-Menge, die für eine effiziente Sequenzierung einer Probe ausschlaggebend ist, besteht ihr Nutzen darin, dass altes und degradiertes Spurenmaterial aus dem Schusswaffeninneren erfolgreich untersucht werden kann. Generell ist die Analyse mitochondrialer DNA selten als Mittel der Wahl bei kriminalistischen Untersuchungen anzusehen, da sie im Vergleich zu der STR-Genotypisierung eine geringere Unterscheidungskraft und letztlich einen geringeren Beweiswert besitzt. Dennoch verspricht diese Methode erfolgreiche Ergebnisse, vor allem bei der Analyse von „Cold Cases“ [387], vorrangig solchen, in denen lediglich geringe DNA-Mengen aus Knochen oder Haaren vorliegen, um Personen durch Nachweis beispielsweise von Abstammungsverhältnissen zu identifizieren.

Um letztlich den Bogen von den Erkenntnissen bezüglich des Spurenverursachers, über die Spurenart und bis zum Alters einer Spur, zu spannen sollten weitere Untersuchungen durchgeführt werden, um andere und geeignetere RNA-Degradationsmarker zu identifizieren, deren Ausmaß ihrer Degradation eng mit dem Alter einer Spur korrelieren. Ferner fehlt die systematische Untersuchung zur Vergleichbarkeit von RNA-Degradationsmustern über mehrere Spurenarten hinweg sowie zum Einfluss verschiedener Umweltbedingungen. Obwohl bereits unterschiedliche forensische Anwendungen vorgeschlagen wurden, bleiben dennoch viele Fragen bezüglich des Verhaltens von RNA in begrenzten Mengen und degradiertem Material bestehen. Dennoch sollte es künftig möglich sein, mehr Erkenntnisse bzw. Informationen aus einer an einem Tatort gefundenen Spur zu ziehen.

Nicht zuletzt durch und in Anbetracht der zunehmenden Verbreitung und Etablierung moderner und hochsensibler Methoden wie der massiven parallelen Sequenzierung (MPS) [388], mitunter auch als Sequenzierungsverfahren der „nächsten Generation“ (engl. „next generation sequencing“, NGS) bezeichnet, können sich für die forensische DNA- sowie RNA-Analytik in naher Zukunft enorme Fortschritte ergeben. Diese Technik birgt für die forensische Routinearbeit ein großes Potential zur Erweiterung der molekulargenetischen Einsatzmöglichkeiten, der Effizienzsteigerung und nicht zuletzt des Erkenntniszuwachses. Mittels des MPS-Verfahrens ist es im Gegensatz zu den klassischen STR-PCR-basierten Analysen möglich, in einem Reaktionsansatz parallel mehr forensisch relevante Informationen aus einer tatrelevanten Spur zu generieren, als lediglich ein für Individualisierungszwecke nutzbares DNA-Profil. Zusätzlich zu den gängigen autosomalen und gonosomalen STR-Systemen [389] lassen sich simultan, in einem einzigen Reaktionsansatz, eine Vielzahl von Single-Nukleotid-Polymorphismen (SNPs) sowie Insertions- und Deletionspolymorphismen (InDels) detektieren. Zudem besteht die Möglichkeit, von jeder untersuchten Probe deren gesamtes mitochondriales Genom sowie spezifische Bereiche zu analysieren [390–394], was vor allem bei altem und stark degradiertem Spurenmaterial von Vorteil ist [395]. Eine Reihe erster Studien [396–399] und eine Empfehlung zur Verwendung einer einheitlichen Nomenklatur [400] von NGS zu forensisch relevanten STR-Systemen liegen vor sowie industriell hergestellte Kits die bereits erhältlich sind [401]. Des Weiteren wurden erstmals in Pilotstudien [402, 403] Möglichkeiten zur Kombination von DNA- und körperflüssigkeitsspezifischen RNA-Markern vorgestellt, die eine Befürwortung und somit die Eingliederung RNA-basierter Verfahren in die forensisch-spurenkundliche Routinearbeit bestärken.

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