CO-EXTRACTION METHODS OF DNA AND TOTAL RNA FOR BODY FLUID IDENTIFICATION: A REVIEW

By

Anh Tran

A thesis submitted in fulfilment of the requirements for the degree of

Master of Forensic Science (Professional Practice)

in

The College of Science, Health, Engineering and Education:
Medical, Molecular and Forensic Sciences

Murdoch University

Supervisor:

Mr Brendan Chapman (Murdoch University)

Declaration

I declare that this manuscript does not contain any material submitted previously for the award of any other degree or diploma at any university or other tertiary institution. Furthermore, to the best of my knowledge, it does not contain any material previously published or written by another individual, except where due references has been made in the text. Finally, I declare that all reported experimentations performed in this research were carried out by myself, except that any contribution by others, with whom I have worked is explicitly acknowledged.

Signed: Anh Tran Dated: 26 June 2020

Acknowledgement

Firstly, I would like to express my sincere gratitude to my supervisor, Mr. Brendan Chapman. His mentorship and guidance have been invaluable and very much appreciated.

I am particularly grateful for the chance to study in Australia that was given to me by the Australia Awards Scholarships program.

Finally, I wish to thank my parents and friends for their encouragement and support throughout my studies. Without them, this journey would not have been possible.

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Part One

Literature Review

Co-extraction methods of DNA and total RNA for body fluid identification: a review

Abstract

Body fluid identification is a crucial step in forensic investigation, and investigators have used various techniques. With the discovery of cell-specific mRNA and miRNA markers as well as their stability in certain conditions, co-analysis of DNA and RNA from the same sample is possible and can bring multiple benefits. The workflow can be streamlined and the frequent lack of forensic sample will no longer be an obstacle. The need for a robust co-extraction method of DNA and total RNA is clear and there are several kits on the market to serve this need. This work aimed to establish an overview of nucleic acid extraction and dual extraction processes for body fluid identification as well as the use of commercial co-extraction kits.

Abbreviations

ALS	Alternate light source
AP	Acid phosphatase
CTAB	Cetyltrimethylammonium bromide
DE	Diatomaceous earth
DNA	Deoxyribonucleic acid
EDNAP	European DNA profiling group
EDX	Energy Dispersive X-Ray Spectroscopy
ELISA	enzyme-linked immunosorbent assay
KM	Kastle-Meyer
PSA	Prostate-specific antigen
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulphate
SEM	Scanning Electron Microscopy
TMB	Tetramethylbenzidine
UV	Ultraviolet light

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1. Introduction

Body fluid identification plays an important role in forensic science, being a key step in reconstructing crime scene and linking individuals to the crime scene. Crucial supporting evidence can be obtained from the process of tracing back a stain at the crime scene to its origin (1). Historically, chemical or catalytic tests are good for fast screening of body fluids but lack in specificity, whereas immunological or microscopic method can be used for confirmation (2). Body fluids of importance in forensic include blood, semen, saliva, and vaginal secretions. The assays to identify these fluids can be widely different, depend on the type of body fluid. Additionally, many of those were not designed to preserve the biological samples, which are already limited and often in poor quality (2).

Recently, messenger RNA (mRNA) and micro RNA (miRNA) have been reported as alternative body fluid identification markers (3). Numerous studies have demonstrated that RNA is stable in certain conditions, can be recovered from body fluid stain as well as post-mortem tissue, and can be used to identify aged stains (4). miRNA was also shown to have tissue-specific expression patterns and be even less prone to degradation by environmental factors than mRNA (5). RNA analysis is also useful in determining the age of injuries or the post-mortem interval (6). The RNA-based methods offer a tremendous benefit in that the same procedure format can be applied regardless of body fluid type (4).

Short tandem repeat analysis, a powerful technique in forensic science, can distinguish samples from different individuals and help identify the donor with a high statistical power of discrimination (7). However, DNA profiling cannot identify the type of body fluid, and

extraction techniques generally can only allow the isolation of one type of nucleic acid (8). Therefore, to apply mRNA-based and miRNA-based techniques in routine forensic analysis, it is desirable to co-extract DNA and RNA from the same body fluid stain. The process must be able to provide DNA of sufficient quality and quantity for STR profiling without compromising RNA yield and integrity.

1.1. Aims

This literature review presented an overview of the body fluid identification methods as well as various techniques of nucleic acid extraction throughout the development of forensic science. The aims of this work were to clarify the need for the development of new techniques for DNA and RNA co-extraction and to identify if there was a favoured method for dual extraction in the literature, with a focus on the available commercial kits.

1.2. Methodology

The review was conducted using online resources, using literature that detailed techniques and method for body fluid identification, extraction and co-extraction of nucleic acids. To achieve a comprehensive view, different types of resources were used, including, journal articles, textbooks, patents, factual databases, reports from various research organisations and empirical studies. These documents are selected from databases such as Scopus, PubMed, SciFinder, and Wiley Online as well as the resource from the Murdoch University Library and search results from Google Scholar. Keywords used for searching include DNA

RNA co-extract*, co-isolat*, dual extract*, simultaneous extract*, co-analysis, body fluid identification, commercial kits, co-extraction kits, and their combination.

2. Body fluid identification

2.1. Introduction

Body fluid found at the crime scene is one of the most crucial pieces of evidence. Except for identical twins, the DNA profile of every person is unique (5). In many cases, tracing back the origin of a fluid can be enough to determine the outcome of a case, such as exonerating a wrongfully convicted individual or proving a suspect was involved (5). Furthermore, identifying the origin and type of the biological fluid can help to reconstruct a sequence of events as well as evaluating the statements of witnesses and suspects. For instance, the existence of semen at the crime scene can indicate the involvement of sexual assault. Another example is the entire discipline of blood pattern analysis, which based on the distribution of bloodstains to interpret the events that led to their creation (9). Other types of body fluids that can be found at the crime scene include saliva, sweat, vaginal fluid, and urine.

However, body fluid identification is neither an easy nor straightforward task. Many stains are invisible to the naked eye (sweat/saliva) or similar in appearance to other substances (rust stain and bloodstain have the same colour). An experienced forensic investigator might be able to determine a fluid based on the available information; however, to be usable in court as evidence, the fluid needs to be identified with absolute certainty. Besides, fluids mixture is always possible. A stain could consist of various types of fluid from different donors (10).

When a stain is discovered, a wide range of techniques can be applied to visualise or identify it. Fast-working presumptive tests are normally used first to get an indication of what fluid might present, and then exact confirmatory tests can be applied to identify the sample (10). For each body fluid, various analysis techniques can be utilized. Techniques such as luminol for detection of bloodstain (11) or microscopic detection of spermatozoa have remained almost the same since their inception. Methods for detecting the heme group in blood, amylase in saliva or acid phosphatase in semen have changed along with the advancement of technology and new discoveries. For example, benzidine used to be a catalytic test for blood (12) but has lost its use nowadays because it was classified as a carcinogen (13). On the other hand, new methods have been developed thanks to the recent advances in genetics. Most of these methods are based on the detection of specific messenger RNA (mRNA) (14)(15)(16) or micro RNA (17)(18). However, mRNA is normally not stable due to the presence of ribonucleases and an additional sample is required if RNA and DNA are extracted separately (5). More recent advances in whole-genome epigenetic analysis have rendered DNA methylation profiling a promising method of body fluid identification in forensic science (19).

2.2. Alternate light source

One of the simplest and quickest methods for crime scene investigators to detect suspected body fluids stain is an alternate light source (ALS) such as UV (ultraviolet light) (20) since almost all body fluids are fluorescent. Instead of checking a large area or chemically testing a large piece of furniture, a forensic investigator can use ALS to select the precise location of

stains for confirmatory tests later. There are numerous commercial ALS devices available, including Wood's Lamp (WL), Bluemaxx™ BM 500, which are specific for semen stain detection (21)(22). The more versatile and widely used Polylight® can detect a wider range of fluid such as seminal fluid, saliva, bloodstain and even stains under a paint layer (23). A similarly versatile and higher-powered device called Lumatec® Superlight can even be used in daylight (24). However, a study about the effect of ALS on fingerprint has reported that exposure of more than 30 seconds to shortwave UV (255nm) can damage the DNA in the sample to the point where none can be obtained after the polymerase chain reaction (PCR) (25). Therefore, this method must be used with caution.

2.3. Chemical and immunological methods

2.3.1. Blood

The heme group, which is a component of the haemoglobin protein in blood, has an ability to catalyse the breakdown of hydrogen peroxide similar to peroxidase (12). The resulted oxidizing product can then react with several substrates to create a visible change in colour (26). The most commonly used substrates include benzidine and its derivatives, phenolphthalein, and leucomalachite green. Benzidine can indicate the presence of blood by changing into a blue-colored substance. However, benzidine is a carcinogen and has been largely replaced by one of its derivatives, namely tetramethylbenzidine (TMB) (12). TMB can turn into a bluish-green shade when reacting with blood under an acidic condition and is the main components of the commercially available Hemastix® test. Another non-carcinogenic and popular technique is the phenolphthalein test, otherwise known as the Kastle-Meyer

(KM) test. A positive result of this test yields a pink colour as the result of phenolphthalein being oxidized into phenolphthalein. A major advantage of the KM test is that it is non-destructive to the DNA, and so other tests can be applied to the sample (27)(28). Another test that has been gaining popularity in forensics recently is leucomalachite green. It produces a green colour and has a similar sensitivity with the KM test, which is 1 part in 10,000 (29). False positive results can happen with the presence of vegetable and fruit peroxidase or other chemical oxidants (28).

The luminol test, which has a higher sensitivity, works a little differently from the above-mentioned tests. It uses iron in haemoglobin to accelerate the oxidation of luminol, resulting in a luminescence substance of a blue-green colour (30). It can detect trace amount of blood and proves most useful when used to visualise the blood that had been cleaned up or minute amount of blood that is undetectable by visual examination (11). The Luminol test is not hazardous but can only be used in dark environments (31).

There are also tests that are relied on specific antibody-antigen interaction, most notably the SERATEC®HemDirect (SERATEC® GmbH, Germany) and the OneStep ABAcard®Hematrace® from Abacus Diagnostics® (West Hills, CA, USA). These test are not confirmatory for human blood as the antibodies can cross react with antigen in the blood of mustelidae and primate (28)(32)(33). The newer Rapid Stain Identification™-Blood test (Independent Forensics, Hillside, IL, USA), which identify glycophorin A in red blood cell membranes, has better selectivity and no recorded cross reaction (34). Another immunology method that is suitable

for aged and degraded material is Hexagon OBTI (10). However, this method shows a positive match with primate blood and has been observed to give false negative results (10)(28).

2.3.2. Semen

The presumptive test for seminal acid phosphatase (AP) is the most frequently used test. AP is found in seminal fluid with an activity of 500 to 1,000 times higher than other body fluid (12). The hydrolysis of phosphatases use AP as its catalyst and the resulting product can change the colour of various reagent (35). Brentamine Fast Blue reagent is the most commonly used substrate and will yield a dark purple colour in the presence of AP. Naturally, the test is only presumptive because AP is also detectable in other body fluids or plant materials (12). Another well-known method is the test for choline based on the formation of needle-like choline crystals. This technique is known as the Florence test and has lost its popularity due to the high possibility of false negative result (36).

Direct microscopic identification remains the most reliable confirmatory test. Stains can be applied to increase the contrast of the sample, as the DNA that concentrates in the head of the sperm cell can be easily stained. The most well-known method is the Christmas tree stain, which makes the head red and the tails green. The sample can also be treated with a solution of proteinase K that is able to break down epithelial cells to reduce background staining and render the sperm cells more visible (10). In cases where spermatozoa cannot be microscopically detected (for example, semen from a male individual who has zero sperm count or vasectomy), test for prostate-specific antigen (also known as P30, kallikrein 3, or simply PSA) can be used. This method has been used for some time and remained a reliable

one (37). Current and frequent immunological test include ABAcard® p30, SERATEC® PSA semiquant test, and Biosign® PSA test, with the advantage of being more economic to utilize than the original ELISA method (38)(39). These tests are still not exclusive because false positive results have been reported with other body fluids such as breast milk, urine, vagina secretions, and even rectal swabs that had no prior contact with semen (37)(40)(41). Other immunological techniques using various constituents of semen or techniques involving isozyme components have also been developed, but none of them can serve as a better alternative of the PSA tests in term of sensitivity or effectiveness (42)(43)(44)(45).

2.3.3. Saliva

ALS can be used to look for saliva at a crime scene, similar to blood and semen. However, the blue-white colour that saliva stain emit under ultraviolet light is not enough for it to be distinguished from other stains (12). The activity of α-amylase is often used as the basis for presumptive test of saliva as amylase in its salivary (AMY1) and pancreatic (AMY2) form is present in numerous other body fluids such as perspiration, breast milk, semen, and vaginal secretions (46)(47). α-amylase is abundant in saliva and its function is to cut down polysaccharides into simpler sugar molecules (46). The starch-iodine test work on the principle that iodine and starch react creating a dark blue colour, and when α-amylase starts breaking down the starch, the mixture will gradually lose its colour. An alternative test that is quick, cheap, and sensitive is the Phadebas® test (Magel Life Sciences, Lund, Sweden)(48). However, this test can still give false positive with face lotion, hand cream, or urine (49)(50).

An even more sensitive and specific test than the Phadebas® is the RSID™-saliva test. This is an immunochromatographic strip test that based on anti-alpha amylase antibodies (51). Despite the high selectivity, false positives have been reported with several body fluid samples from rat (52).

2.3.4. Vaginal secretions and menstrual blood

Despite being not as common as blood or semen at crime scenes, vaginal secretions and menstrual blood can still prove useful in cases of sexual assault. These vaginal fluids are of mixed composition, and the constituents of them, even from one individual, are constantly changing and not well defined (12). There are not many tests available to identify these fluids due to this difficulty for detecting specific components. Lugol's method of staining glycogenated epithelial cells from the vaginal wall is deemed unreliable because glycogenation varies largely during the menstrual cycle or completely absents in pre-pubescent or postmenopausal females (53). The ratio of citric acid and lactic acid of a sample can be determined in order to detect the presence of vaginal secretions since vaginal secretions have a higher lactate concentration and citric acid is present in larger quantities in semen (54).

2.3.5. Urine

Largely constituted of water as well as urea, protein, hormones, salts and numerous metabolites, urine found at crimes scene might be helpful in cases of sexual assault, abuse, mischief, or harassment. However, owing to the fact that the fluid is generally pale, diluted

and can spread out, it can be problematic to localize urine (10). Presumptive tests for urine are usually based on organic compounds that are present in high concentration in urine such as urea or creatinine. A popular method by Rhodes and Thornton (55) that has recently been validated is the para-dimethylaminocinnamaldehyde (DMAC) test, which detects ammonia from the breaking down of urea by urease (56). The Jaffe colour test uses a solution of picric acid in toluene/benzene that becomes a red substance in the presence of creatinine (12). An immunological approach for urine detection is the test for Tamm–Horsfall glycoprotein protein (TMP), which has recently been integrated into the RSID™-Urine test (Independent Forensics, Hillside, IL, USA) (57). Other techniques such as calculating the ratio of uric acid to urea nitrogen (58) and high-performance liquid chromatographic (HPLC) analysis of five 17-ketosteroid conjugates (59) are available but less frequently used, being expensive and time-consuming (56).

2.3.6. Sweat

Comparing to other body fluid mentioned above, sweat is the least commonly found fluid at crime scenes. There has been little research and there is no rapid chemical presumptive test for sweat to date. The only reported presumptive technique is the SEM-EDX (Energy Dispersive X-Ray Spectroscopy coupled with Scanning Electron Microscopy) that detect the concentration of chlorine, sodium, potassium, and other elements present in sweat (60). However, due to the complexity of the method, it is not a practical way to screen for sweat. ELISA-based assays for two proteins secreted by sweat gland, dermcidin and G-81, have been developed to be the confirmatory tests for sweat (61)(62).

3. Modern techniques

3.1. mRNA profiling

RNA is known for being short-lived due to the presence of ribonucleases, especially in post-mortem tissues and in vitro processes, thus has hindered its usefulness in forensic science for a long time. However, recent studies have recognized that RNA can be quite stable in certain conditions such as in dried stains of biological fluids and mRNA can be isolated in sufficient amount (in both quality and quantity) while retaining surprisingly high stability. A study by Zubakov et al. (63) identified fourteen stable tissue-specific mRNA markers from nine genes for blood and five genes for saliva. These markers can provide results for identification for stains up to 180 days old, and subsequent study indicated that these markers can still be used to identify saliva stains and blood stains up to 6 and 23 years old, respectively (64)(65). Another study looked at the effect of the environment to mRNA transcripted from eight tissue-specific genes and found that while mRNA can be destroyed quickly in harsh condition like high heat and humidity, it can survive in samples stored at room temperature up to 547 days (66). Numerous other studies also show a stability of RNA in different types of sample, including various body fluids and epithelial cells (67)(68)(69). A study by Meyer et al. proposed that different type of mRNA may have different degradation time due to several intrinsic factors (70) such as the cellular localization and protein complex properties (71)(72), however, the evidence is lacking and more investigation is required. Establishing the source for stable RNA is a crucial step for the application of RNA in forensic. One prominent benefit of using mRNA for identification of body fluids is that the dual extraction of DNA and RNA can

be incorporated into one workflow, thus allow different downstream processes such as DNA and mRNA profiling of a same limited amount of sample (5). To exploit this advantage, several co-extraction methods have been developed (73)(74)(75). An ideal method should be able to yield nucleic acids insufficient quality and quality, require a minimum amount of resource and time, and can be automated. Different methods, as well as commercial kits, have been evaluated against each other in several studies (4)(76)(3)(77). These methods and comparisons will be discussed further in subsequent sections of this review.

3.2. miRNA-based techniques

MicroRNAs (abbreviated miRNA) are non-coding RNA that contain 18 to 22 nucleotides with the function of regulating gene expression at the post-transcription level (78). Recently, interest in miRNA have been increasing and numerous studies have been looking at miRNA as an alternative of mRNA for body fluid identification (18)(79)(1).Currently, the miRNA database has approximately 38,500 entries of which 2,812 are listed as human's (80).

4. Nucleic acids extraction

4.1. Solution-based Method

Extraction of biomolecules is a crucial step in many disciplines of science, including forensics. This is the first step before downstream forensic procedures like DNA and RNA profiling can be performed. Nucleic acids can be extracted from almost all biological material, usually human tissue and body fluid in forensic contexts for analytical purposes (8). In most cases, the following steps are involved in the purification of nucleic acids: breaking the cell or tissue,

deactivating nucleases, and separating the debris and collecting target molecule (7). While the ubiquitously present ribonuclease can complicate the extraction of RNA, there are several methods to isolate RNA. The most commonly used methods utilize phenol and SDS or 4 M guanidinium thiocyanate (8)(81).

4.1.1. Chelex® Extraction

Chelex® is a type of chelating material used in forensics for DNA extraction from various sources such as FTA® cards or buccal swabs. The Chelex resin can bind to inhibitors of the polymerase chain reaction (PCR) and protect DNA from the activity of DNases. The sample prepared with Chelex can be stored for 3-4 months at 4°C (82).

4.1.2. Guanidinium thiocyanate-phenol-chloroform extraction

This is a widely used method involving the use of a solution of guanidinium thiocyanate in phenol and chloroform. Extraction of RNA using guanidinium thiocyanate was first described by Ullrich et al. (83) and then enhanced to be a 'single-step' method by Chomczynski and Sacchi (81). The acidic solution consists of guanidinium thiocyanate, sodium acetate, phenol and chloroform separate RNA from DNA. After centrifugation, the acidic condition allows total RNA to remain in the upper aqueous phase while DNA and protein remain in the interphase or lower organic phase of the mixture. RNA can then be precipitated with isopropanol and recovered for other applications (84).

4.1.3. Other methods

Alkaline lysis, or alkaline extraction, is mainly used to extract plasmid DNA from bacteria (84). It works by selectively denaturing chromosomal DNA but leaving plasmid DNA intact. Alkaline extraction can also be used to isolate plant genetic material (85). However, with the propensity for damaging DNA and its specific use, it has no use in forensic contexts. Cetyltrimethylammonium bromide (CTAB) is a non-ionic detergent that can serve as a resuspension buffer for purification of nucleic acids from plants thanks to the ability to precipitate nucleic acids from low ionic strength solution (84). The CTAB extraction method has limited use due to the low frequency of cases that require analysis of plant material (86).

4.2. Solid-phase Extraction

Solid-phase extraction is used in almost all commercial extraction kits nowadays and considered to be the most efficient technique (87). It is based on liquid and stationary phases, which solve the main issue of liquid-liquid extraction in which the phases are not separated completely (8). Generally, solid-phase extraction of nucleic acids involves the four following steps: cell lysis, adsorption of target molecules, washing, and elution (88).

4.2.1. Silica Matrices

In 1979, Vogelstein and Gillespie discovered that silicate could selectively bind DNA under alkaline and high-salt conditions (89). The silica materials in silica matrices can be either silica particles, glass particles, glass microfibers, diatomaceous earth, or a mixture of them (90). The high affinity between the positively charged silica particles and negatively charged

phosphate backbone is the basis of this fast and effective extraction technique. Since silica surface is covered by positive ions, DNA is tightly bound, and other cell components can be washed out. The DNA molecules can then be eluted from the silica matrices by Tris-EDTA or distilled water (91). This method does not require a high level of skill and can yield DNA at high purity. However, small fragments of DNA can bind excessively tightly to the silica matrix and might not be recovered (84).

4.2.2. Glass Particles

Glass particles in powder or beads form can be used for nucleic acids isolation. The mechanism of adsorption of nucleic acid onto glass particles is suggested to be similar to adsorption chromatography (92). Additionally, a mixture of silica gel and glass mixture can also be used for nucleic acid purification (90).

4.2.3. Diatomaceous Earth

Diatomaceous earth (DE), also known as diatomite or kieselguhr, has a varied composition of 80% to 95% silica, 2% to 4% alumina, and 0.5% to 2% hematite (93). In the presence of a chaotropic agent, nucleic acids bind to the silica in DE, similar to the silica matrices methods (94). The resulting complex will then be wash with an alcohol-based buffer, and DNA will be eluted in distilled water or a low salt buffer (95). This technique is less time-consuming but rather costly so it is not commonly used (96).

4.2.4. Magnetic Beads-Based Nucleic Acid Purification

In this method, nucleic acids is separated from the mixture via complementary hybridization (97). Numerous magnetic carriers that have high affinity to the target nucleic acid are commercially available. Magnetic particles can be made either from inorganic magnetic materials, synthetic polymers, or biopolymers (8). Magnetic materials in bead form are more commonly used thanks to their higher binding capacity (98). After the binding process, a magnet is applied on the side of the container to collect and hold the target particles, and then the remaining liquid can be pour away (99). This method does not require centrifugation and therefore can yield longer fragments of genomic DNA (96). Recently, functionalised magnetic particle and an appropriate buffer system are combined to create a rapid and efficient purification of nucleic acids directly after their extraction from unprocessed cell extracts. Magnetic beads-based purification can process a larger sample size and is considered to be the best choice for automation and high-throughput processes (99).

4.2.5. Anion Exchange Material

Similar to silica matrices, anion exchange resins are commonly used to extract DNA and RNA (96). This method is based on the interaction between the phosphate group (negative charge) on the DNA backbone and the diethylaminoethyl cellulose groups (positive charge) on the resin's surface. The material can operate on a wide range of pH and salt concentration, which allow for the optimal separation of DNA from RNA (100). DNA extracted using anion exchange is purer than the silica method, and the resin can be reused (96). However, since this method

require elution of DNA with a high-salt concentration buffer (8), desalting is needed for downstream processes.

4.2.6. Cellulose Matrix

Paper made from cellulose matrix with absorbent property is a unique method of storing and purifying nucleic acids. Under peculiar chemical conditions, cellulose produces a high polar attraction that can bind nucleic acids. This is the working basis of the FTA® card (Whatman Inc., USA) (101). When a sample is applied, the detergent in the FTA® paper lyses the cell membranes and the chelating agent prevent the activity of nucleases as well as the growth of contaminating organisms (102). FTA® cards are easy to use and can reliably preserve different types of sample (103)(104). However, it can be problematic to extract a high yield of nucleic acids from FTA® cards, especially in dilute samples (105).

5. Co-extraction of DNA/RNA

5.1. Methods for co-extraction

The need for co-extraction of DNA and RNA first arise in biomedical research. Due to the lack of sample, researchers started looking for methods for simultaneously isolating DNA and RNA without the heavy degradation of either. In 1893, Krieg et al. designed a new method for the dual extraction of DNA and RNA, with a key step of adding an extraction buffer (106). By combining purification steps in separate protocol for DNA and RNA isolation, Meese and Blin successfully collected both nucleic acids, but not without minor loss of quantity (107).

TRIzol® (Sigma-Aldrich Company, USA), or TRI reagent, is a single-phase solution of phenol and guanidine isothiocyanate and developed as a co-extraction reagent for DNA, RNA, and proteins from samples of cell and tissue (108). This reagent works by solubilizing biological material coupled with phase separation at the addition of chloroform. It has been reported to be suitable for isolating small RNAs (109). Riol et al. (110) modified it by adding a step of DNA purification and was able to collect DNA and RNA from blood and semen stain suitable for quantitative studies. Other authors have also described other modified protocol to reduce the amount of biological material needed (111)(112). However, at the time, these methods were not specifically optimized for usage in forensic contexts, where samples usually lack in both quality and quantity.

One notable study that addressed this issue was conducted by Alvarez et al. (74). Their proposed method employed standard method of DNA and RNA isolation frequently used in forensic laboratories, with the addition of SUPERase-In RNase Inhibitor (Ambion, Thermofisher), a robust RNase inhibitor to improve the quality of the RNA collected. The method was specifically tested on samples that are frequently found in forensic cases, namely vaginal secretion, blood, saliva, and semen stains. Although the extracted nucleic acids were sufficient for forensic use, there was still a notable loss of DNA compared to DNA-only isolation methods (74).

The gradual transition to automation in forensic laboratories raised the need for a new method that can integrate DNA/RNA co-extraction into an automated process. Bowden et al. describe a successful combination of the Promega DNA IQ™ system with the Zymo Research

Mini RNA Isolation Kit™ II (Ngaio Diagnostics, New Zealand). RNA was extracted in the lysis buffer discard after the sample had been run through the Promega DNA IQ™. The major advantage of this step comparing to organic extraction methods was that isolating RNA did not significantly affect the amount or integrity of DNA (75).

Recognising the benefits of automated systems in term of low contamination risk and a possibility of saving time, Kulstein et al. reported another attempt at automating DNA and RNA co-extraction, this time with a focus on miRNA (113). One automated, one semi-manual, and one manual procedure were assessed by applying on aged blood sample and sample from real cases. The fully automated procedure using the Maxwell® 16 platform (Promega, Mannheim) was shown to provide the highest yield in most cases.

While there have been numerous studies of DNA/RNA co-extraction process in other fields (114)(115)(116)(117)(118), those that can be adopted for use in forensic cases are few and far between. A protocol that co-extracts DNA and RNA from animal cells (114) was modified by Louriro et al. (119) and compared to a commercial extraction kit, ExtractME RNA & DNA (BLIRT S.A., Poland). The modification was made to the lysis step to be suitable for the semen sample that the authors used. Being originally developed for use in animal cells, this adapted manual method was inferior regarding DNA/RNA concentration and quality, but advantageous in term of cost and complexity (119).

The solution-based TRIzol method has been successfully combined with the DNA IQ™ with an increase in quantity and quality of the DNA (120). More recently, in 2019, Farivar and Ghazimoradi proposed a new approach of extracting DNA and RNA from a particularly low

amount of blood sample using TRI reagent (121). The most important step when using TRIzol was the immediate pipetting after the addition of the reagent, as clot formation can hinder the reagent from performing its function. Although previously being considered not suitable for working with blood samples, this new method is reported to yield sufficient amount of DNA and RNA for a variety of tests from as low as 2 µl of sample, and even better result with just 10 µl. The minuscule amount of cell actually facilitates this method, as it solves the problem of clotting (121). Therefore, this method could be used to overcome the obstacle of insufficient quantity of sample.

5.2. Comparative study reviews

There are a few commercially available kits for dual extraction of DNA and RNA that provided an all-in-one solution for the extraction of forensically significant biomolecules (8). For example, AllPrep DNA/RNA/miRNA Universal Kit (QIAGEN, Germany) is a column-based extraction kit that optimized to purify genomic DNA and total RNA (including miRNA) from all types of cells and tissue (122). Watanabe and Akutsu (77) compared this kit to a co-extraction kit (AllPrep™ DNA/RNA Mini Kit) and three RNA extraction kits, RNeasy Mini Kit, miRNeasy Mini Kit, and QIAamp DNA Mini Kit. The AllPrep DNA/RNA/miRNA Universal Kit, which does not have a high-temperature incubation step, was demonstrated to be better than other kits in term of DNA yield. The author suggested that this kit was a better choice in both mRNA/DNA and miRNA/DNA extraction (77). In a previous collaborative study of the European DNA profiling group (EDNAP), the AllPrep™ DNA/RNA Mini Kit was used by the

majority of participant (123). The kit gave a satisfactory result; however, since the study focused on the analysis of mRNA marker, the efficiency of this kit was not evaluated (123).

In a relatively new study, Schweighardt et al. (4) compared four commercial kits for the dual extraction of DNA and RNA, namely Zymo Research ZR-Duet™ DNA/RNA MiniPrep Kit (Zymo Research, USA), Fisher SurePrep™ RNA/DNA/Protein Purification kit (Thermo Fisher Scientific, USA), Qiagen AllPrep™ DNA/RNA Mini Kit (Qiagen, Germany), and Norgen Biotek RNA/DNA/Protein Purification Plus Kit (Norgen Biotek Corp., Canada). The kits will be referred to as Zymo, Fisher, Qiagen, and Norgen kit. All of the kits utilise silica spin column-based isolation of nucleic acids. However, there is a minor difference in the sequence of isolation, in which the Zymo kit allows for DNA and RNA to be extracted in any order or even simultaneously, while the isolation of RNA in the Fisher and Qiagen kits precedes that of DNA, and in the Norgen kits the order is reversed. The authors validated various aspects of the kits against each other, using dried blood and saliva samples on cotton-tip swabs that imitate those from real casework. The Zymo and Qiagen kits gave the best result with DNA yield, and the Zymo kit outperformed all other kits in term of RNA yield (12 times higher than the second-best, Qiagen kit). The result also indicated a limited correlation between RNA yield and RNA profiling success; however, the sample size was too small to suggest a clear trend and even large-scale studies did not conclude on this correlation (124)(125). Furthermore, Zymo was also reported to be the most cost-effective co-extraction kit of the four, although still more costly than organic method (4).

6. Research Gaps

Methods for nucleic acids extraction, especially DNA isolation, have been developed and evaluated extensively in the literature. On the contrary, the number of studies that evaluate DNA/RNA co-extraction are quite small. Additionally, most of the studies about co-extraction did not use mock forensic sample, and therefore are hard to adapt to the forensic context. A majority of studies in a forensic setting regarding the dual extraction evaluated 'in-house' methods against commercial kits while studies that compare the commercial kits against each other are infrequent. The comparison is often inconsistent because these studies used different generation of the extracting kits, lacked sample size, and used different types of sample. With the increased interest in miRNA marker in forensic science, more studies to compare and evaluate the commercial kits in term of isolating miRNA are also needed.

7. Conclusion

Determining the existence and subsequently identifying a body fluid in a crime scene is an essential step in an investigation (10). Despite the practicality, the current popular visual, chemical, and immunological method for presumptive and confirmatory tests might not be the optimal methods for body fluid identification (2). The regular shortage of forensic sample and the stark difference between these methods also hinder their usefulness.

RNA-based methods allow for the simplification of identifying body fluids, in that the need of stocking different reagent and conducting validation for various assay maybe render obsoletes, as they offer a common workflow for different body fluids even in the mixture

state (4). Ongoing research by a number of authors has been reporting new cell-specific mRNA and miRNA markers. RNA-based identification of body fluid is highly advantageous in that it can be coupled with DNA analysis to streamline the workflow (5).

The usefulness of DNA/RNA co-analysis can only be realised with the availability of an efficient co-extraction method, since forensic samples are often limited, and the separate isolation of DNA may preclude that of RNA and vice versa. As previously detailed, there is a wide range of solution-based and solid-phase extraction method. However, optimized and commercialized kits are usually considered to be better in term of nucleic acids yield and quality (3).

In the forensic context, there is a need for a method of co-extraction that can obtain total RNA without compromising the quality of DNA for profiling and can be use in routine forensic work. This method also needs to be economical in term of processing time, free of hazardous chemicals and able to be integrated into automation process (4). Despite the need, studies in the field of forensic science are still lacking. Future research should examine various co-extraction methods using mock forensic sample or sample from real casework if suitable. An larger range of latest commercial co-extraction kits, using appropriate sample size, should be compared to establish the best method for frequent use.

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Part Two

Manuscript

Co-extraction methods of DNA and total RNA for body fluid identification: a review

Abstract

Body fluid identification is a critical step in forensic investigation, and investigators have used various techniques. With the discovery of cell-specific mRNA and miRNA markers as well as their stability in certain conditions, co-analysis of DNA and RNA from the same sample is possible and highly advantageous. Body fluid identification can be coupled with DNA profiling and the obstacle of the frequent lack of forensic sample will be removed. The need for a robust co-extraction method of DNA and total RNA is clear and there are several kits on the market to serve this need. This review has identified the two kits in the literature that have the highest performance in term of yield and quality of both nucleic acids and suggested further research to identify the best kit(s) for routine forensic use.

Abbreviations

ALS	Alternate light source
CTAB	Cetyltrimethylammonium bromide
DNA	Deoxyribonucleic acid
EDNAP	European DNA profiling group
ELISA	enzyme-linked immunosorbent assay
KM	Kastle-Meyer
PSA	Prostate-specific antigen
RNA	Ribonucleic acid
UV	Ultraviolet light

Contents

1. Introduction

Important information that supports crime scene reconstruction or link individual to a crime scene can be obtained from the type and origin of body fluid (1). Historically, chemical or catalytic tests are a fast method to identify forensically significant body fluids like blood, semen, saliva, and vaginal secretions. These techniques are often destructive and the other analysis cannot be applied after the sample was consumed (2). On the contrary , body fluid identification techniques that involve messenger RNA (mRNA) and micro RNA (miRNA) can be combined with short tandem repeat analysis, which enables the capacity to identify both the body fluid and its donor in a streamlined workflow from a limited amount of sample (1).

This review summarises body fluid identification methods as well as various techniques of nucleic acids extraction and co-extraction with a focus on commercial kits. To reach a comprehensive view, various types of resources were used, including, journal articles, textbooks, patents, factual databases, reports from various research organisations and empirical studies. The focal point will be studies that compare and evaluate commercial kits for the simultaneous extraction of DNA and RNA.

2. Body fluid identification in forensic

2.1. Blood

Bloodstains that are not immediately visible, especially those that are on a dark background, can be detected by using an alternate light source (3). Polylight® is one of the more versatile and popular portable ALS used in forensic science for the detection of blood as well as other body fluid (4). ALS is a simple and fast way to look for blood stains but prolonged use may have a detrimental effect on DNA and subsequent analysis (5).

Most of the presumptive tests for blood are based on the catalytic activity of a blood component. The non-hazardous Luminol test, which gives a luminescent blue-green colour at the presence of blood, can be very effective in detecting blood in an area that has been cleaned (6). Other popular techniques include the Kaster-Meyer test, which has a lower sensitivity but non-destructive to DNA, and the commercialized Hemastix® test, which is extremely rapid and non-hazardous. Since these are presumptive methods, vegetable and fruit peroxidase can give false-positive result (7). A more modern method called Hexagon OBTI was developed for aged and degraded bloodstain with a lesser selectivity (7).

Confirmatory test for human blood that is currently used in forensic lab involved the use of anti-human haemoglobin. The same antibody-antigen interaction principle is also used to identify blood from other species (8). Crystal test and spectroscopic methods are also reliable in confirming the presence of blood (7).

2.2. Semen

Semen can be detected with an ALS at the wavelength of 450 nm (9). The most frequently used presumptive test for semen is based on seminal acid phosphatase (AP). In the presence of AP, Brentamine Fast Blue reagent change to a dark purple colour. Methods based on other substances in semen such as choline or spermine are now outdated (2).

The presence of semen can be confirmed directly by microscopic identification of sperm cells, either unaided or stained. If sperm cells are not available, a test for prostate-specific antigen (PSA) can be used. Although a very low level of PSA does exist in other body fluids, test kits are often designed to be not too sensitive to avoid false positive. Popular test kits for PSA include Biosign® PSA and OneStep® ABAcard (2).

2.3. Saliva

Similar to blood and semen, saliva can emit light under ALS. However, the colour is not distinctive enough for it to be distinguished from other stains (8). Chemically presumptive tests for saliva are mostly based on the activity of α-amylase, and the most well-known is the starch-iodine method. Other techniques include the Phadebas® test and the recently developed RSID™-saliva test, which is an immunochromatographic strip test using anti-alpha amylase antibodies (10)(11).

2.4. Other body fluids

Other body fluids that are of interest to forensic investigators are vaginal secretions, urine, and sweat. Although they can be useful in some cases, there are not many tests available due to

several reasons. Vaginal fluids generally have mixed composition and there is too much variety in its constituents. Frequently used methods for vaginal secretions include Lugol's glycogenated epithelial cells staining technique and determining the citric acid/lactic acid ratio (12)(13). Urine is hard to detect because it is generally pale, diluted and can easily spread out. Chemical approached are usually based on organic compounds in urine such as urea or creatinine (14). A newer immunological technique, the RSID™-Urine test, used Tamm–Horsfall glycoprotein protein for urine detection (15). Sweat is the least common body fluid found in crime scenes and also the least researched. Currently, there are no effective screening method for saliva, however, confirmatory test using ELISA assays are available (16)(17).

2.5. RNA-based methods

Due to the ubiquitous presence of ribonuclease, RNA is known for being unstable. However, under certain condition, RNA can be isolated from stains of body fluid such as blood or saliva (18). This stability of RNA was also found in various samples, including body fluids and epithelial cells (19)(20). This step of identifying the source of stable RNA was a key step for RNA application in forensic. There are several techniques that have been developed for the identification of blood, semen, and saliva using RNA (21). In 2005, Juusola and Ballantyne (22) developed a method involving multiplex reverse transcription-polymerase chain reaction (RT-PCR) to identify body fluid. This process is based on the detection of specific mRNA markers and is able to identify blood, semen, and vaginal fluid with high sensitivity (22). The genes for identifying semen, saliva, and vaginal fluid were also detected using the co-extraction technique proposed by Alvarez et al. (22)(23) More recently, numerous miRNAs have been shown to have tissue-specific expression pattern (24). They are a family of small RNA molecules that are 18 to 22 nucleotides in length and

show higher stability than mRNA. Hanson et al. (24) introduced miRNA profiling to forensic science in 2009. A notable advantage of RNA-based method is that RNA analysis for stain identification and DNA analysis for stain donor identification can be performed together from a limited amount of forensic sample (25).

3. Nucleic acids extraction

3.1. Solution-based methods

Chelex® is a chelating material often used for DNA and RNA extraction from different sources. The resin works by trapping other contaminants, leaving DNA and RNA in the water solution above the resin. The use of Chelex® involves a heating step that results in single-stranded DNA molecules, which are less stable for storage (26).

Another common method for extracting RNA (and sometimes DNA and protein) in molecular biology involves the use of a solution of guanidinium thiocyanate in phenol and chloroform. Chomczynski and Sacchi developed this method into a single-step co-extraction of DNA, RNA, and protein (26). Although this method can yield total RNA with high purity, the major drawbacks are the hazardous reagents that involve, namely phenol and chloroform (27).

Other methods such as alkaline extraction or cetyltrimethylammonium bromide extraction are suitable for extracting DNA from bacteria or plant material (28). Due to their limited use in the infrequent nature of their target samples, these methods are not commonly applied in forensic laboratories.

3.2. Solid-phase Extraction

Solid-phase extraction is utilized in the majority of commercial extraction kits and considered to be more efficient than liquid-liquid extraction (29). Solid-phase extraction of nucleic acids is

based on liquid and stationary phases, and involve the four steps of cell lysis, adsorption of target molecules, washing, and elution (30).

In 1979, Vogelstein and Gillespie (31) reported that under alkaline and high-salt conditions, silicate could selectively bind DNA. The surface of silica is covered by positive ions and can tightly bind to the negatively charged phosphate backbone of DNA. This method can yield highly purified DNA with a minor drawback of tightly bound small fragments of DNA that are hard to recover (27). Silica particles, glass particles, glass microfibers, diatomaceous earth, or their mixtures can be used in a silica matrix (32). Glass particle in different forms such as silicate glass, flint glass, and borosilicate glass can bind DNA with the use of chaotropic salt. The mechanism of this adsorption process is suggested to be like adsorption chromatography (33). Similarly, nucleic acids bind to the silica in diatomaceous earth in the presence of a chaotropic agent. The technique that involves diatomaceous earth is less time-consuming but rather costly (34).

Anion exchange resins have a similar working mechanism to silica matrices and can be used to isolate both DNA and RNA (34). Same as silica matrices, the diethylaminoethyl cellulose groups on the surface of the resin are positively charged and can bind to the backbone of DNA. Anion exchange method is more advantageous in that it can operate on a wide range of pH, allow the resin to be reused, and yield purer DNA (34).

The mechanism of magnetic beads-based nucleic acid purification is a bit different in that is does not require centrifugation and thus can yield longer fragments of genomic DNA. This method uses complementary hybridization of separate nucleic acid from the mixture (35). Magnetic particles are usually made into bead form to achieve higher binding capacity. After the binding

process, a magnet is applied directly to the side of the container to hold the beads and the remaining liquid can be removed. This purification technique can process a larger sample size and is considered to be the best choice for automation and high-throughput processes (36)

4. Co-extraction methods and commercial kits

In 1983, Krieg et al. suggested the first method for the simultaneous extraction of DNA and RNA from solid tumours (37). The first attempt to extract both DNA and RNA from limited amounts of cells was reported by Meese and Blin (38). Since the technique was a combination of separate protocol for DNA and RNA isolation, loss of yield was not prevented. Riol et al. (38) and Chevillard (39) modified the single-step extraction method from Chomczynski (40) to extract from blood and saliva and extract miRNA, respectively. However, these methods were still not specifically optimized for forensic use. In 2004, Alvarez et al. described a method to improve the quality of RNA in a dual extraction (25). By adding a robust RNase inhibitor, they were able to isolate high-quality RNA with a trade-off of a lower yield of DNA (25). Most recently, in 2019, Farivar and Ghazimoradi proposed a new approach of extracting DNA and RNA from a very low amount of blood sample (< 50 µl) using TRI reagent, a commercialized kit of the Chomczynski method (41). This new method is reported to yield sufficient amount of DNA and RNA for a variety of tests from as low as 2 µl of sample, however, it has only been tested with blood sample (41).

The attempts to integrate co-extraction into an automated system have gained good results. Bowden et al. combined DNA extraction by Promega DNA IQ™ system with RNA extraction by Zymo Research Mini RNA Isolation Kit™ II (Ngaio Diagnostics, New Zealand) and collected DNA of high integrity. However, the extraction of RNA had to be done manually and the quality of total RNA was not reported (42). Kulstein et al. reported another successful automated technique using the Maxwell® 16 platform (Promega, Mannheim). However, the automated process only extracted miRNA, and extraction of RNA still had to be done manually if needed (42).

There are several kits for the co-extraction of DNA and RNA available on the market (43). Although commonly used in molecular biology for the co-extraction from various type of biological material including body fluid, the studies that evaluated these kits against each other are limited. Schweighardt et al. (44) compared the four kits of Zymo Research ZR-Duet™ DNA/RNA MiniPrep Kit (Zymo Research, USA), Fisher SurePrep™ RNA/DNA/Protein Purification kit (Thermo Fisher Scientific, USA), Qiagen AllPrep™ DNA/RNA Mini Kit (Qiagen, Germany), and Norgen Biotek RNA/DNA/Protein Purification Plus Kit (Norgen Biotek Corp., Canada). All of the kits were optimized for soft tissues and cell pellets and utilise silica spin column-based isolation with a minor difference in the sequence of nucleic acids isolation. The authors suggested that the Zymo kit was the best overall for DNA and RNA yield as well as profiling success. The Qiagen kit came close in performance and even outperformed the Zymo kit in several mRNA marker analysis when compared in a per-donor basis (44). It should be noticed that this research was done with sample stored in a controlled environment, thus did not reflect the reality of casework. Furthermore, it did not evaluate the yield and profiling success of miRNA.

A study by Watanabe and Akutsu (45) suggested that the AllPrep DNA/RNA/miRNA Universal Kit (Qiagen, Germany) outperformed the Qiagen AllPrep™ DNA/RNA Mini Kit (Qiagen, Germany) in term of DNA yield and quality of mRNA and miRNA from a co-extraction. However, the study focus was on body fluid identification and the authors only assessed the quality and quantity of DNA in term of DNA methylation analysis of regions with blood- or saliva-specific methylation.

5. Discussion/Conclusion

Identifying a body fluid in a crime scene is an essential step in an investigation that enable reconstruction of criminal events (2). STR analysis combined with body fluid identification by RNA profiling is the new approach in forensic science, as it has numerous advantages, compared to the traditional approach (46). To realise the full potential of DNA/RNA co-analysis approach, an efficient co-extraction method is needed. The ideal kits for routine use in forensic work need to be able to yield a sufficient amount of DNA and RNA for subsequent analysis without compromising the quality of either nucleic acids. Despite the wide range of manufacturers of commercialized kits, the number of studies that compares and evaluates these kits is still lacking.

The review on the literature shows that the kits named ZR-Duet™ DNA/RNA MiniPrep Kit from Zymo Research and AllPrep DNA/RNA/miRNA Universal Kit from Qiagen are the viable options for co-extraction of DNA and RNA. However, these studies either compared a small number of kits or did not evaluate all the RNA targets for body fluid identification. To increase the sample size, the scope of the study can be expanded similar to the interlaboratory format used by the European DNA Profiling Group (EDNAP) (47)(48)(49). Forensic samples from real cases should be used to evaluate the performance of the kits. With the increased interest in miRNA marker in forensic science, the kits should be evaluated in term of success in isolating the miRNA markers for body fluid identification. Additionally, since magnetic beads-based extraction of nucleic acids is more suitable for automation high-throughput purification, this type of kits should also be tested.

6. References

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