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DNA Extraction Methods in Forensic Analysis

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Forensic biological evidence encompasses a diverse conglomerate of samples containing genetic material

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found in a variety of biological fluids and tissues such as human blood, semen, saliva, epithelial cells, hair, bone, teeth, fingernails, and putrefied tissues. In addition to human samples, nonhuman samples from plant, animal, bacteria, and fungi may also need to be processed. The samples and body fluids may be present as dried stains on an assortment of substrates, often mixed with PCR (polymerase chain reaction) inhibitors, exposed to environmental conditions and uncontrolled degradation, and are often present in limited quantities. In addition to the intrinsic characteristics of samples that include the type of body fluid (e.g. blood, saliva, semen), nature of the tissues (e.g. buccal cells, hair, bone, tooth), quantity [e.g. trace DNA (deoxyribonucleic acid), low copy number], and source (e.g. reference, evidence), they can also be grouped by their extrinsic characteristics such as the substrate (e.g. swab, clothing) and nature of the crime (e.g. sexual assault, burglary, homicide) from which they are derived.

These samples need to be processed using the most effective methods of nucleic acid extraction and purification for downstream quantification and genetic profiling by PCR. Compositionally, there are an unlimited number of combinations of sample and substrate types including the quantity and quality of the sample, substrate and conditions encountered, and contaminant and inhibitor levels. Some factors may be observed during screening, whereas many of these remain transparent to the analyst. This renders the choice of the most efficient DNA extraction methods, one of the most unpredictable steps in forensic DNA profiling.

Research has led to significant enhancements, testing, and validation of advanced extraction and amplification chemistries along with new instrumentation platforms providing systems capable of automated, sensitive, rapid, and robust extraction and genotyping and/or sequencing of short tandem repeats (STR), single-nucleotide polymorphisms (SNPs), and mitochondrial DNA. All three genetic marker classes have been accepted in the court systems worldwide and this along with legislation that has expanded both the law and funding for forensic DNA profiling has led to a high and increasing demand for forensic DNA testing.

The increased demand and new expanded capabilities have opened the door to the analysis of forensic samples that are extremely compromised in both quality and quantity. Over the past 10 years, new DNA extraction methods have been developed to reproducibly extract DNA and remove or mitigate PCR inhibitors from the majority of sample types with speed and efficiency, resulting in high-yield template free of PCR inhibition. In addition, automated processing has been tested and validated to meet the demand for high-throughput processing. This article summarizes the importance of DNA extraction, provides an update on methods for single source, compromised evidence, sexual assault evidence, automation, differential

extraction, and an overview of the most widely adopted methods and the best practices for DNA isolation from forensic biological samples.

1 INTRODUCTION

Typical genotyping workflow in forensic DNA analysis comprises activities such as examination of evidence, identification of body fluid, extraction of DNA, assessment of extracted DNA, amplification of target loci, detection of amplified products, analysis of data, and generation of a report. Of these, the DNA extraction step has the most impact on obtaining a high-quality genotype profile, which is the ultimate goal. Good quality DNA generates good quality genotype profiles, as the amplification of target loci and detection of amplified products are more or less streamlined processes. Since the 1990s, soon after demonstration of the utility of STRs in human identification, the major focus in improving genotyping workflow has been on developing robust amplification systems for challenging samples, megaplexes with greater number of STR loci to achieve higher power of discrimination, improved capillary electrophoresis systems, expert software for data analysis, developing newer genotyping markers such as single-nucleotide polymorphism (SNP), Y-chromosome STRs, and more recently, next-generation DNA sequencing protocols. Traditional DNA extraction methods such as Chelex[®] and phenol–chloroform were routinely used for a long time in forensic laboratories and still are preferred methods for some sample types. Nonetheless, the need for development of dedicated DNA extraction systems, both manual and automated, for forensic biological samples was realized in the early 2000s, leading to availability of commercial forensic DNA extraction kits from several suppliers.

Is extraction of DNA from forensic biological samples ‘an art or a science’? Well, the answer depends on the sample type. Most forensic scientists will agree that it is ‘a science’ for reference samples and ‘an art’ for most evidence samples. Forensic biological samples, unlike most clinical samples, provide multiple challenges for analysis because of nonuniformity. Evidence samples range from biological fluids, tissues, hair, bone, teeth, finger nails, and epithelial cells from trace or touch samples. Further, body fluids frequently are dried stains on many substrates that exist; often mixed with PCR inhibitor, exposed to different environmental conditions, uncontrolled degradation of DNA, and often are present in limited quantity. Forensic biological samples can be segregated on many different principles such as type of body fluid (e.g. blood, saliva, and semen), nature of tissue (e.g. buccal cells, hair, bone, and tooth), substrate

(e.g. swab and clothing), nature of the crime (e.g. sexual assault, burglary, and homicide), quantity of biological material (e.g. trace DNA and low copy number), and source (e.g. reference and evidence). The ultimate goal of DNA analysis is to obtain interpretable genotype results (profiles). Therefore, the DNA extraction chemistries are evaluated for their abilities to obtain the maximum amount of DNA from samples containing small quantities of biological material, while simultaneously resulting in high-quality DNA free of detectable PCR inhibitors.

2 OVERVIEW OF DEOXYRIBONUCLEIC ACID EXTRACTION METHODS

2.1 Introduction to Forensic Deoxyribonucleic Acid Extraction

The term ‘extraction of DNA’ is widely used for processes for isolation of DNA from biological samples. This is because phenol chloroform extraction was historically the most widely used method for purification of DNA; hence the term ‘extraction’. Cells need to be lysed before PCR for releasing DNA that can take part in the PCR. Whether an analyst uses lysate as a source of DNA for genotyping or purifies the DNA from lysate before PCR is determined by the sample type and the biology of the target loci being genotyped (STRs, SNPs, insertion–deletion, phenotype markers, etc.). At times, amplification of DNA from a lysate is referred as direct amplification as purification (extraction) of DNA is not performed. Extraction of DNA is interpreted in this article as a process for obtaining cell-free DNA; extent of the purity of DNA is determined by the method used. With this interpretation, different methods used in forensic laboratories for the extraction of DNA are classified into two groups: (i) a single step wherein the biological sample is lysed to generate PCR compatible lysate and (ii) two steps wherein samples are lysed and DNA is purified. A single method of extraction of DNA is not compatible for all sample types and, therefore, multiple methods of extraction of DNA are validated and practiced in a forensic laboratory. Needless to say that the choice of DNA extraction method will depend on the nature of the sample.

2.2 Extraction of Deoxyribonucleic Acid from Single-source Samples

Examples wherein biological samples are collected from a single donor include paternity testing, DNA database generation, reference samples in casework, and relationship testing. Single-source samples have certain advantages in that they contain ample quantity of biological material, are collected and stored in relatively controlled environments, are less likely to be degraded,

and contain minimal inhibitors of PCR. In spite of such controls, reference samples are not truly uniform exhibiting variation in type of body fluid (e.g. blood, saliva, and buccal cells) and substrates such as FTA[®] Cards (GE Healthcare Life Sciences, Marlborough, MA), Bode Buccal DNA Collection Systems (Bode Cellmark Forensics, Lorton, VA), and NUCLEIC-CARD[™] and 4N6FLOQSwabs[™] (Thermo Fisher, South San Francisco, CA). As these samples contain high quantities of DNA and relatively lower amounts of PCR inhibitors, the DNA extraction methods are restricted to lysis. Cell lysis or DNA extraction methods used for single-source-sample types include Chelex,⁽¹⁾ FTA,^(2,3) and thermal stable proteinases (ZyGEM, Hamilton, New Zealand). Direct amplification, a term promoted by commercial organizations, involves either direct amplification of biological material embedded within a substrate or lysate obtained using PCR-compatible reagents such as Prep-N-Go[™] (Thermo Fisher, South San Francisco, CA), SwabSolution[™] Kit and PunchSolution[™] Kit (Promega Corporation, Madison, WI), and Bode PunchPrep[™] (Bode Cellmark Forensics, Lorton, VA). An important factor in increasing the success of generating genotypes by direct amplification has been the development of robust STR amplification kits such as Identifiler[®] Direct, GlobalFiler[™] Express, NGM SElect[™] Express and Yfiler[™] Direct (Thermo Fisher, South San Francisco, CA), and PowerPlex[®] 18D (Promega Corporation, Madison, WI).

2.3 Extraction of Deoxyribonucleic Acid from Evidence Samples

Extraction of DNA from an evidence sample is the most critical step as a better quality and higher quantity of DNA leads to higher quality interpretable genotype. However, the amount of DNA isolated from a forensic evidence sample cannot be predicted. This is because forensic biological samples submitted for analysis are highly variable and unpredictable. Challenges in processing an evidence sample are due to variation in the source of biological samples (e.g. body fluids, tissue, hair, bone, and tooth), deposition of body fluids on a wide range of substrates, exposure to varying environmental conditions, uncontrolled degradation, contamination with inhibitors of PCR, and that these samples are often present in limited quantities.

Typically, extraction of DNA from evidence samples involves cell lysis and DNA purification. Lysis is achieved by physical, chemical, or enzymatic means. Purification of DNA is accomplished by organic extraction, precipitation, or binding and elution from activated surfaces containing silica, ionic charged, or hydrophobic moieties. Proteinase K (PK) digestion in the presence of sodium

dodecyl sulfate (SDS) followed by phenol–chloroform extraction method (commonly referred as organic extraction) is still regarded as the ‘gold standard’ and is still the preferred method for processing challenging samples.^(4–9) Interestingly, Chelex-100 resin (Bio-Rad Laboratories, Hercules, CA) that binds polyvalent metal ions thereby protecting DNA from degradation by DNase, although a crude method, was widely used in forensic laboratories for extraction of DNA from a wide variety of evidence samples such as blood stains, tissue, hair, and bone.^(1,10–14) A wide variety of DNA extraction kits are now available from manufacturers worldwide. Some examples are QIAamp Micro DNA kit, QIAamp DNA Investigator Kit, EZ1 DNA Investigator Kit, and QIASymphony DNA Investigator Kit (Qiagen, Valencia, CA); DNA IQ[™] System and DNA IQ Reference Sample Kit for Maxwell[®] 16, and DNA IQ Casework Sample Kit for Maxwell 16 (Promega Corporation, Madison, WI); PrepFiler[™] Forensic DNA Extraction Kit, PrepFiler Express[™] DNA Extraction Kit (Thermo Fisher, South San Francisco, CA); *forensic* GEM Kits (ZyGEM, Hamilton, New Zealand); MagPurix Forensic DNA Extraction Kit (Zinexts, New Taipei City, Taiwan); MPure[™] Forensic DNA Extraction Kit (MP Biomedicals, Santa Ana, CA); E.Z.N.A. Forensic DNA Kit (Omega Bio-tek, Inc., Norcross, GA); Forensic DNA Extraction Kit (GeneON GmbH, Deutschland/Germany); GF-1 Forensic DNA Extraction Kit (Vivantis Technologies, Selangor Darul Ehsan, Malaysia); *MagListo*[™] 5M Forensic Sample DNA Extraction Kit (Vivantis Technologies, Selangor Darul Ehsan, Malaysia); AGOWA[®] mag Maxi DNA Isolation Kit (AGOWA, Germany); InviMag Forensic Kit (Invitek mbH, Germany); UltraClean[™] Forensic DNA Isolation Kit (Mo Bio Laboratories Inc., Carlsbad, CA); and HiPurA[™] Forensic Sample Genomic DNA Purification Kit (HiMedia Laboratories, Mumbai, India).

2.3.1 Extraction of Deoxyribonucleic Acid from Sexual Assault Cases

Samples from sexual assault evidence may contain mixtures of male spermatozoa in semen and female vaginal epithelial cells. Typical sexual assault evidence includes a vaginal swab from the victim. Naturally, such a sample comprises many epithelial cells from the victim and few sperms from the perpetrator deposited during a sexual assault. The quantity of epithelial cells from the victim is, therefore, far greater than the number of sperms from the perpetrator, the genotype of which is of primary importance in resolving the case. Thus, it is required that DNA from epithelial cells and sperm are isolated separately for obtaining independent genotypes – each for victim and perpetrator. The extraction of DNA from

epithelial cells and sperm from sexual assault samples is achieved by differential lysis of epithelial cells and sperm in the absence and presence of dithiothreitol (DTT) generating epithelial cell lysate and sperm lysate in separate tubes.^(15,16) The DNA from these lysates is then purified using conventional DNA purification methods.

Clean separation of the two fractions (male and female) greatly assists in the interpretation and deconvolution of the donors in the mixed sample.

Several new protocols have been developed for processing sexual assault samples including Differex™ (Promega Corporation, Madison, WI) that utilizes a nonaqueous fluid to separate the sperm pellet from epithelial cell DNA during the first centrifugation step to reduce the number of sperm pellet washes, an automated differential extraction protocol on the QIAcube developed by Qiagen (Valencia, CA), and DNase I treatment to selectively remove soluble female DNA.^(17,18)

Second, strategies in processing sexual assault samples separate epithelial cells and sperm and process isolated cell fractions for extraction of DNA using standard methods. Separation of epithelial and sperm is achieved by different techniques such as flow cytometry,⁽¹⁹⁾ microfluidics,⁽²⁰⁾ and laser microdissection.^(21–25) Flow cytometry separates sperm and vaginal cells after differential immunostaining based on their differences in ploidy, major histocompatibility class I, CD45, and cytokeratin expression⁽¹⁹⁾ Additional advances in differential extraction methodology can be found in the two-step extraction method in Section 3.5.

2.3.2 Bone and Teeth Extraction

Bone and tooth are preferred samples for cases such as ancient, mass disaster, missing persons, and other compromised and severely degraded case scenarios. Skeletal elements often survive when all other tissues have vanished due to catastrophic circumstances or the end result of natural decomposition. Several publications testify to the success of obtaining interpretable genotypes from bone and teeth sample types.^(26–38) Although STRs and SNPs are used in obtaining genotypes from bone and teeth samples, mitochondrial DNA sequencing to investigate variation in hypervariable regions (HV-1 and HV-2) is preferred. The success of obtaining genotypes from such ancient and compromised samples is due to their inherent resilience and structural stability and also because the cellular material in bone and teeth is complexed with matrix components such as hydroxyapatite, collagen, osteocalcin, and minerals, resulting in better preservation in adverse conditions than for softer, noncomplexed tissues. This complexity of structure puts forth a different challenge for extraction of DNA, e.g. samples are nonuniform (not homogenous) with respect

to cellular material, small quantity of cellular material compared to the matrix, restricted access of proteases (or lysis reagents) to the cellular material, and co-extraction of matrix components that may inhibit downstream PCR.⁽³⁹⁾ Thus, optimization of the protocol for extraction of DNA from bone and teeth samples is a compromise between release of DNA, avoiding degradation of DNA during extraction procedure, and minimal co-extraction of PCR inhibitors. It is important to take extra precautions during processing bone or tooth samples as the samples contain small quantities of biological material and pulverization processes create circulating fine particles. As a rule of practice, the extraction procedure is performed either in a separate clean room or under a biological hood.^(28,37,40,41)

Different procedures used for lysis of cellular material in the bone samples are focused on overcoming the barrier of the matrix to accessibility of lysis reagents. The complex matrix structure is disturbed using chelating agents that bind the metal ions present in the matrix. Although the protocols vary in quantity of reagents added and incubation times, a central approach is the use of ethylenediaminetetraacetic acid (EDTA) as a chelating agent, SDS as a detergent, and PK as a lytic agent.^(28,36,37,40)

Loreille et al.⁽⁴²⁾ developed a total demineralization protocol for complete dissolution of the bone sample for achieving high yields of DNA, which is now adopted by forensic laboratories worldwide.⁽⁴²⁾ The total demineralization protocol offers several advantages including minimizing the loss of DNA in residual bone powder as it is completely dissolved, needs as little as 0.2 g of bone powder compared to 1–2 g for traditional protocols, minimizes the quantity of inhibitors co-extracted due to the reduced sample input requirements, and complete dissolution enables the release of DNA embedded in dense crystalline aggregates of bone matrix. DNA from the lysate obtained from traditional and total demineralization protocols is then isolated/purified by commonly used procedures such as phenol–chloroform extraction and commercial kits.

Thermo Fisher (South San Francisco, CA) developed PrepFiler BTA Forensic DNA Extraction Kit and PrepFiler Express BTA™ DNA Extraction Kit, dedicated for the extraction of DNA from bone, teeth, and certain challenging sample types.⁽⁴³⁾ Promega Corporation (Madison, WI) developed a proprietary Bone Incubation Buffer, which in combination with PK is used for lysis of bone samples. DNA is then isolated by the DNA IQ system.⁽⁴⁴⁾ Qiagen (Valencia, CA) provides protocols using the QIAamp DNA Micro Kit, EZ1 DNA Tissue Kit, and MagAttract® DNA Mini M48 Kit.⁽⁴⁵⁾

2.4 Automation of Deoxyribonucleic Acid Extraction

Acceptance of DNA results by court systems, legislation passed by government agencies, continued worldwide success of case resolution, and increased funding resulted in exponential growth in the number of biological samples processed for DNA analysis. With the increased number of samples, automation of genotyping workflows became a necessity. Automation of genotyping offers several additional advantages compared to automation in a research laboratory:

1. maintaining sample integrity
2. higher reproducibility
3. consistent performance
4. higher throughput
5. workflow integration
6. automated electronic audit trail
7. compatibility with LIMS
8. minimizes inadvertent sample switching
9. minimizes data entry errors
10. reduces hands-on time of trained forensic scientists, and
11. lowers risk of repetitive stress injuries.

DNA quantitation, PCR set-up, and preparation of plates for genetic analyzers were the first operations to be automated, as there is less variability in these steps than in extraction protocols. Automation of extraction of DNA puts forth challenges due to variability in sample types, number of protocols used in a given laboratory (or multiple laboratories), and variation in the chemistry of commercial kits. It is important to note that yield and quality of extracted DNA cannot be compromised for high throughput by automation.

One of the most significant improvements in automated analysis has been the development of beads or particle technology for solid-phase-based extraction of DNA.^(46,47) The major advantage in using magnetic beads is the speed and simplicity of magnetic separation of the particles to isolate DNA and remove debris and waste. By placing the tubes or 96-well plates next to a magnet, the particles are drawn to one side of the tube facilitating the removal of the aqueous liquid containing waste, debris, and other chemicals. The particles are modified with functional groups such as silica, polymers, or those that can be used to attract and capture DNA. In general, samples are lysed, DNA is captured on the beads, magnetic separation permits the removal of impurities, and repeated washes followed by magnetic separation permit the purification of the DNA permitting a single analyst to process approximately 100 samples in one run.

Automated DNA extraction systems developed include both low-throughput benchtop as well as high-throughput

96-sample processing robots. Some of these systems offer automation of only the isolation of DNA protocols (i.e. lysis is performed separately). Most commonly used benchtop systems are BioRobot EZ1 and BioRobot EZ1 XL (Qiagen, Valencia, CA), Automate Express (Thermo Fisher, South San Francisco, CA), and Maxwell 16 (Promega Corporation, Madison, WI). Major manufacturers of liquid handling robots such as Beckman (Brea, CA), Becton Dickinson (Franklin Lakes, NJ), Hamilton (Reno, NV), PerkinElmer (Waltham, MA), Qiagen (Valencia, CA), and Tecan (Mannedorf, Switzerland) have developed scripts and protocols for processing up to 96 samples for extraction of DNA using DNA extraction kits produced by them and/or other manufacturers. Several laboratories have implemented automated protocols for extraction of DNA for reference and casework samples.^(46,48–53)

Open liquid-handling platforms have the advantage of flexibility, permitting different workflows, chemistries and consumables, and high throughput, but the disadvantage is that they are more prone to contamination. As described previously,⁽⁵⁴⁾ several procedural guidelines can reduce contamination risk including (i) precision in the movement of liquid-handling tips, (ii) control of liquid aspiration and dispensing parameters, (iii) implementation of dedicated pathways of movement of liquid-handling tips, and (iv) avoidance of risky over-crossings of reagent reservoirs, processing plastics, and sample tubes. Some laboratories also require that at least one analyst witnesses the robotic processing to confirm that the robot follows the protocol. It should be noted that automation cannot replace human critical thinking for troubleshooting, adjusting, and modifying parameters as needed to overcome and improve the extraction process.

2.5 Considerations for an Effective Deoxyribonucleic Acid Extraction Method

In view of the foregoing discussion, considerations for an effective DNA extraction methodology are⁽⁵⁴⁾ as follows:

- achieves extraction of DNA from a variety of biological samples;
- reduces loss of available DNA from a sample resulting in the highest DNA recovery of sufficient quality for downstream analysis;
- enables isolation of DNA from samples that contain small quantities of biological material;
- isolates DNA at a high concentration so that the volume of extract used for genotyping is minimal and provides for future DNA testing;
- removes substances that interfere with PCR;
- does not introduce any contaminants;

- does not introduce inhibitors of PCR;
- maintains sample integrity: does not degrade the DNA;
- is rapid;
- is amenable for automation;
- reduces hazardous waste; and
- can be used to co-extract RNA.

The ability for the method to also extract RNA for applications in body fluid identification and tissue typing has spurred research and interest in developing extraction methods that simultaneously extract RNA.^(55,56) Considering the heterogeneity of samples encountered in a forensic DNA laboratory, a description of the commonly used DNA extraction protocols that meet several of the aforementioned considerations are discussed as follows.

3 FORENSIC DEOXYRIBONUCLEIC ACID EXTRACTION PROTOCOLS

3.1 Overview of Forensic Deoxyribonucleic Acid Extraction

DNA extraction from biological evidence samples is a pivotal step in forensic nucleic acid workflows. In general, the higher the quality and quantity of the DNA extract, the higher the likelihood of obtaining a full profile. In addition to the list of the aforementioned requirements (Section 2.5), one main challenge in forensic DNA case-work laboratories is the high degree of physical and chemical heterogeneity of forensic biological evidence samples. Heterogeneity in sample types submitted to a crime laboratory such as blood, semen, saliva, sweat, tears, vomit, hair, bones, teeth, bite marks, finger and toenails, fingerprints, urine, embalmed bodies, and feces may require that a laboratory employs different extraction methods for different types of samples depending on the case, sample type, and amount. Analysts need to consider not only the chemical and physical properties of the sample but also the need for removal of potential intrinsic (e.g. hair containing melanin or bone containing calcium) and extrinsic inhibitors introduced by substrates (e.g. indigo dye from denim),⁽⁵⁷⁾ while optimizing DNA recovery from potentially low template, degraded samples that may contain PCR inhibitors.

DNA found within cells in the nuclei and mitochondria contain a myriad of enzymes, proteins, and other organic and inorganic chemicals and compounds. These nonnucleic acid components need to be removed before analysis. Furthermore, improper storage and/or environmental exposure may lead to chemical damage of DNA. This chemical damage may result in the release of additional PCR inhibitors if these substances are not removed during the DNA extraction.

The presence of noncellular (naked) DNA in some types of evidentiary samples provides the ability to enter those samples into direct PCR methods without the need for DNA extraction. Direct PCR from low template samples has the advantage of reducing or completely eliminating multiple steps involved in several DNA extraction methods, thus reducing loss with each successive step.⁽⁵⁸⁾

Modern forensic laboratories are utilizing PCR of autosomal and/or Y-chromosome STRs, mini-STRs, mitochondrial deoxyribonucleic acid (mtDNA) sequencing, and SNPs and more recently have been validating and implementing next-generation sequencing (NGS) also known as massively parallel sequencing (MPS) of these genetic markers.^(59–73) MPS megaplexes are now available containing 231 autosomal, Y and X STRs along with identity, ancestry, and phenotype informative SNP loci and can be amplified from as little as 100 pg of DNA.⁽⁷¹⁾ These forensic DNA multiplexes require the use of efficient and scalable DNA extraction methods.

Reviews on forensic DNA extraction methods have been previously published.^(54,74–81) As these methods have been described in detail in these reviews and articles, in this article the commonly used forensic DNA extraction methods are briefly described and readers are referred to the other reviews for more information and details.

3.2 Forensic Deoxyribonucleic Acid Extraction Methods

There are several different methods of DNA extraction that can be classified into two basic categories: (i) those that simply release the DNA without purification and (ii) methods that both release and purify the DNA.

Each DNA extraction method contains one or more of the following steps:

1. Lyse the cells: Disruption of the cell membranes that is usually accomplished by both physical shearing of the tissue or stain and the treatment with detergents and heat. During this step, the release of DNA from histones and inactivation of DNAses is generally performed with proteinases and EDTA while simultaneously bringing the DNA into an aqueous phase (See more details in Section 3.2.1). For some one-tube methods such as Chelex extraction, this is the only step needed to yield crude DNA.
2. Isolation of the DNA from cellular materials and other chemicals and debris is usually accomplished by physiochemical methods. These include precipitation in isopropanol or ethanol, phenol:chloroform:isoamyl alcohol organic phase extraction, solid-phase binding such as those used in silica-based chemistries, and or electrophoresis (see details in the following methods).

3. Collection and concentration of the DNA: Collection, resuspension, and adjusting the concentration of DNA in an aqueous phase such as tris (hydroxymethyl)aminomethane (Tris)-low EDTA, to a concentration compatible with downstream PCR applications.

3.2.1 Cell Lysis

Although the main extraction step consists of the isolation of DNA before PCR, the preliminary phases, particularly that of lysis, are important. These consist of dissociating the cellular material from substrates, separating the nucleic acids from their surrounding proteins and/or tissues, and obtaining the most complete digestion in order to solubilize as much DNA as possible. Most techniques are based on incubation of the substrate in an extraction buffer (most commonly containing Tris-HCl and EDTA), PK, and a detergent (usually SDS) followed by what is usually considered the actual extraction phase or DNA isolation phase (e.g. phenol/chloroform extraction).

The role of the PK is to digest the protein, whereas the SDS is added to lyse cell membranes thus releasing the DNA. Other detergents that can be used include Nonidet and Triton X 100. Basically, many protocols in this sense are similar as they employ similar reagents and temperatures with varying incubation times (ranging from 30 min to 48 h), according to the type of substrate and protocol.

Some protocols divide the lysis phase into two steps when fresh blood is the substrate: leukocyte separation by centrifuging the sample and/or using chemical methods, including a red cell lysis buffer containing dextrose and a lysis agent, and pelleting the leukocytes. RNases have also been used in the past to eliminate any RNA present. The pH of these solutions and buffers is also important: at a pH of 5–6, the extraction of RNA is favored; at a pH of 8–9 both RNA and DNA are liberated.⁽⁸²⁾ Sodium perchlorate has also been used in these preliminary steps – it is another reagent useful both for lysing and denaturing cellular proteins and, according to some authors, can replace the organic extraction phase. This is not a method of choice, but it has been successfully used on fresh⁽⁸³⁾ and ancient material.^(84,85) Finally, guanidine-HCl is sometimes used for cell lysis as it is highly denaturing.⁽⁸⁶⁾ It has also been employed in conjunction with glass beads and sodium perchlorate. As mentioned in Section 2.5, the ability to conduct mRNA tissue profiling has increased the interest in extraction methods that yield both DNA and RNA.^(87–95)

The efficiency of each of the aforementioned steps collectively results in the final DNA recovery. For

simplicity, the DNA extraction methods have been categorized into two groups:

1. one-tube methods, without purification and
2. two-step methods, purification of DNA is an additional series of steps and generally results in higher quality DNA.

As mentioned earlier, another approach is either direct amplification of samples contained on a substrate or lysate obtained using reagents compatible with PCR STR kits such as Identifiler Direct (Applied Biosystems, part of Life Technologies, Foster City, California now Thermo Fisher, Foster City) and PowerPlex 18D (Promega Corporation, Madison, Wisconsin)^(96–99) or Y-STRs.^(96–99) Thus, the cell lysis step is included within the PCR amplification thermal cycling protocol (Thermo Fisher, Foster City, CA).

3.3 Single-tube Deoxyribonucleic Acid Extraction Protocols

During single-tube extraction procedures, chemical or physical agents achieve lysis of cells, and the cell-free extract is used for genotyping without any purification step. Single-tube indirect extraction of DNA is achieved by the selective precipitation of protein and other contaminants, or by chelation of inorganic substances. The advantages of these methods are that they are simple, quick, can be more easily scaled up and automated for high throughput, and due to the limited number of steps, have lower chances of contamination than two-step methods. One such method that has been used in many forensic DNA laboratories is Chelex resin.

3.3.1 Chelex Extraction

Chelating reagent is used to bind to inorganic substances and clear the DNA extract of inhibitors. Although simple chelating methods exist which employ the properties of substances such as EDTA to neutralize certain ions, one of the main chelating methods involves the use of Chelex. Chelex is a resin composed of styrene-divinylbenzene copolymers containing paired iminodiacetate ions that act as chelating groups in binding polyvalent metal ions. It has a high selectivity for divalent ions and differs from ordinary ion exchangers because of higher bond strength.⁽¹⁰⁰⁾ It has been postulated that boiling a sample in the presence of Chelex prevents the degradation of DNA.⁽¹⁰¹⁾ Metal ions which act as catalysts in DNA breakdown at high temperatures and at low ionic strength are chelated and inhibited from this action.

The technique is simple and rapid and does not involve multiple transfer tubes. The Chelex resin removes impurities and the alkaline pH disrupts the cell membranes

resulting in the release of DNA. It does not have a particularly selective activity on protein and can be unreliable unless relatively large amounts of DNA are present.

3.3.2 FTA Paper

A second type of single-tube method not requiring a formal purification step is the use of FTA paper (Whatmann, part of GE Healthcare, Buckinghamshire, United Kingdom) for extraction.⁽¹⁰²⁾ Room temperature collection, shipping, and storage can be performed using FTA cards (Whatmann, part of GE Healthcare, Buckinghamshire, United Kingdom).⁽¹⁰³⁾ FTA paper is made from cellulose and contains proprietary chemicals that perform several functions. The chemicals lyse the cell, deactivate nucleases, and prevent the growth of bacteria. Storage on FTA paper at room temperature has been shown to be stable for several years.⁽¹⁰⁴⁾ Following several washings, a portion of the card can be placed directly into the amplification reaction mix or the template can be eluted as is facilitated on FTA Elute cards. A two-step protocol for extraction of DNA from small (1.2 mm) punches of bloodstains on FTA and 3-mm filter paper was described (Whatmann, Piscataway, New Jersey).⁽¹⁰⁵⁾ These 1.2-mm-sized paper punches contain enough DNA while also minimizing inhibitors. They are washed in 20 mM NaOH followed by Tris buffer, pH 8.0, air dried, and then used in direct PCR. The procedure has been also modified into two steps: a 3-mm punch is first washed with 500 μ L of ultrapure water and then transferred to another tube containing 30 μ L of ultrapure water⁽¹⁰⁶⁾ where it is heated at 94 °C for 1–10 min. Conventional DNA purification methods can also be performed on the DNA from FTA paper.⁽¹⁰⁷⁾

3.3.3 Thermal Stable Proteinases

A third single-tube extraction method is facilitated by the use of thermal stable proteinases. Thermal stable proteinases are active at high temperatures and, therefore, can be used during the high temperatures used to disrupt cell membranes in the absence of detergents or reducing agents, offering a single-step DNA extraction method from several types of forensic samples. Samples are held at 75 °C at pH 7.0 with EA 1 proteinase from a thermophilic *Bacillus* strain EA 1 (ZyGEM, Hamilton, New Zealand) for 15 min followed by 15 min at 96 °C to inactivate the EA.^(108,109) Nucleases when released from the cells are inactive due to the high temperature and are then hydrolyzed by the EA 1. Successful extraction has been obtained with this method from blood, bloodstains on different substrates, saliva, swabs from beer bottles, touch trace samples, and other tissues.⁽¹⁰⁹⁾ However, samples such as cigarette butts and bloodstains on black denim did

not provide interpretable STR profiles mainly due to the presence of inhibitors. EA 1 enzyme is now available from ZyGEM, Hamilton, New Zealand, as the *forensic GEM*[™] kits family and can be used in a 96-well format or for any number of samples using PCR tubes and a thermal cycler.

Additional one-tube extraction kits with proprietary lysis solutions are also available commercially. QuickExtract[™] DNA Extraction Solution and MasterAmp[™] Buccal Swab Kit from Epicentre (Madison, Wisconsin) and Extract-N-Amp[™] Blood PCR Kit from Sigma Aldrich (St. Louis, Missouri) are examples.

3.4 Two-step Deoxyribonucleic Acid Extraction Protocols

There are several different two-step DNA extraction protocols consisting of lysis and purification. Three of these commonly used methods are described as follows: (i) organic or phenol/chloroform DNA extraction, (ii) silica-based DNA extraction, and (iii) saturated salting out methods.

These methods are based on the indirect extraction of DNA by the selective precipitation of protein and other contaminants, or by chelation of inorganic substances (Figure 1).

3.4.1 Phenol/Chloroform

Sometimes referred to as the ‘gold standard’ of DNA extraction, the organic extraction method uses phenol/chloroform⁽¹¹⁰⁾ it is still a popular method among the forensic community for challenging samples.

The first step in this method is to lyse the cells. Lysis buffers generally will contain PK, detergents such as SDS and N-lauryl sarkosine (NLS), reducing agents such as DTT, and chelating agents such as EDTA. Reviews of additional methods for hair, bone, saliva, and paraffin embedded cells were recently described.⁽⁵⁴⁾

The second step is to purify the DNA. The main mode of functioning is to remove the protein component thus purifying the nucleic acids; this is usually carried out by simply extracting aqueous solutions of the nucleic acids with phenol and/or phenol/chloroform. When dealing with complex mixtures of cell lysates and debris and this is usually the case with forensic substrates – it is sometimes necessary to employ additional measures. In these cases, more protein can be eliminated by digestion with proteolytic enzymes (such as PK) which are active against a broad spectrum of native proteins before the extraction with organic solvents. The standard way to remove proteins is to extract once with phenol, once with a 1 : 1 mixture of phenol–chloroform, and once with chloroform.

Separation using phenol–chloroform is based on the physical and chemical properties and interactions of the

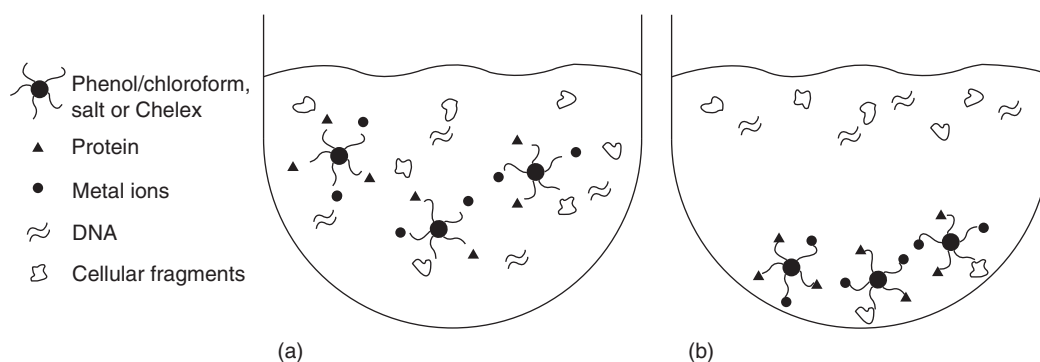


Figure 1 Mechanisms of nonaffinity methods where (a) the extracting agent (phenol/chloroform, salt or Chelex[®] resin) pull away protein, metal ions, and components of cell walls, leaving (b) DNA in solution.

reagents and cellular materials. The density of phenol is great that water causing a phase separation when mixed with the aqueous solution of lysed cells containing nucleic acids, proteins, carbohydrates, and other cellular debris. Phenol has a density of 1.07 g cm^{-3} , and water's density is 1.00 g cm^{-3} ; thus, the higher density phenol separates into the lower phase, from the lower density aqueous (water) phase forming the upper layer. In the presence of phenol, the hydrophobic cores of the proteins interact with phenol, causing precipitation of proteins and polymers (including carbohydrates) to collect at the interface between the aqueous and organic phases. Deproteinization is more efficient when two different organic solvents are used instead of one. Chloroform improves this phase separation as it is miscible with phenol and has an even higher density at 1.47 g cm^{-3} than phenol, resulting in a better, sharper separation of the organic and aqueous phases. This better, sharper interface results in a more effective recovery of the aqueous phase containing the DNA as the sharper the interface, the less chance there is of inadvertently aspirating some of the lower organic phase that would result in cross-contamination with the phenol:chloroform known to inhibit downstream PCR typing and other enzymatic assays.

The advantage of this method is that it is the most tested and proven method in forensic science with protocols (including alternatives) for a myriad of substrates. The disadvantage is that the method does not eliminate other nonprotein contaminants as efficiently and may lower yield because of the number of steps involved. In addition, when organic material is separated from the double-layered solution, some of the aqueous DNA-containing layer is lost with it, although this loss may be diminished by piercing a hole through the tube and allowing the phenol to drip out or by using a pipet where the narrow tip has been cut off.⁽¹⁶⁾ Phenol and chloroform can be harmful both for the substrate and for the operator. It is

possible that DNA may be damaged as a result of steric shearing during the multiple tube transfers necessary in this method. Furthermore, phenol is a well-known carcinogen and chloroform can cause liver damage.

In spite of these disadvantages, the resilience of the phenol:chloroform method in forensic DNA laboratories is due in part to the ability to extract DNA from extremely degraded and compromised samples along with a seemingly universal ability to extract from a variety of sample types. Phenol/chloroform organic DNA extraction has been used successfully to extract DNA from decomposed bodies, blood stains, hair shafts, maggots, saliva, dandruff, fingerprints, ancient formalin fixed tissues, teeth, bone, urine, vaginal swabs, mummies, putrefied burnt liver and brain, semen, and fire victims.^(5,111–130)

3.4.2 Silica-based Deoxyribonucleic Acid Extraction

The novelty of affinity methods is that they rely on the process of actively selecting, to varying degrees, the DNA by either a nonspecific or specific reaction, thus allowing the examiner to wash out all other substances. In this section, commonly used nonspecific affinity methods using silica or magnetic beads are described as they have gained widespread use in the forensic DNA community.

The main nonspecific affinity methods employ (i) glass (silica) beads in combination with isothiocyanate bound to guanidium or (ii) magnetic beads from Dynal coated with a substance(s) specific for DNA but which are not revealed in the manufacturer's data sheets.

The silica glass particle method (sometimes called the glass milk or silica gel method) is based on glass particles actively attaching to isothiocyanate which is itself bound to guanidium. It has been hypothesized that the positively charged amine groups on the guanidium act as the link to the negatively charged phosphate groups on DNA. Once DNA is bound to the glass particles, it can be repeatedly

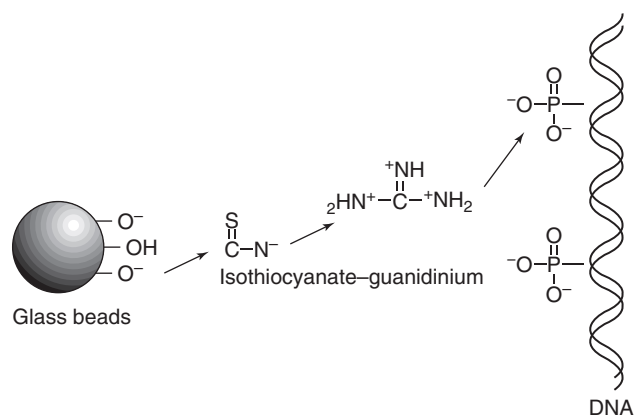


Figure 2 Chemical interaction between the silica bead and DNA.

washed and spun, as the glass particles will retain the attached DNA and allow elimination of other material and inhibitors (Figure 2). However, other mechanisms of DNA binding to silica have been suggested. These may involve chaotropic salt disruption of the water structure around negatively charged silica, allowing a cation bridge to form between it and the negatively charged phosphate backbone of DNA; this reaction is reversible in low salt concentrations.⁽¹³¹⁾

The main advantage is that, contrary to nonaffinity methods, DNA is selectively bound (even if only temporarily) to a substrate (glass) that can be repeatedly washed. Once it has been satisfactorily cleansed, the DNA can be eluted. This offers a much better chance of purifying DNA from PCR inhibitors. The main disadvantage is the fact that when a great deal of contaminant is present, it may sterically hinder the adsorption process of DNA on to the glass substrate. In addition, the silica method preferentially recovers high-molecular-weight DNA and so may fail to recover any DNA from samples in which molecules are present but in a highly fragmented form (<100 bp).

Several silica-based DNA extraction kits are now available from Qiagen and have undergone testing demonstrating successful extraction of genomic DNA from different tissue types and body fluids including bone, blood, semen, saliva, tissues, and several other sample types.^(132–136)

The silica-based extraction method is based on the affinity of DNA to silica in the presence of chaotropic agents such as NaI, NaClO₄, GuSCN, or GuHCl. The combination of GuSCN-based lysis and silica-based extraction was described in 1990.⁽¹³⁷⁾ Commonly used silica columns in forensic DNA laboratories include spin columns in QIAamp Blood kits from Qiagen, Valencia, California, silica beads within a spin column

(e.g. UltraClean. Forensic DNA Isolation Kit from MoBio Laboratories, Carlsbad, California), and silica magnetic particles (e.g. DNA IQ Systems from Promega, Madison, Wisconsin and EZ1 DNA Investigator Kit from Qiagen, Valencia, California). Low DNA recovery may be due to the presence of silica-binding inhibitors that compete with the capture of the DNA during processing.

3.4.3 Saturated Salting Out Method

Methods involving the use of salts are widely employed in blood transfusion and clinical scenarios where abundant clean samples are available. Salting out can now be used in forensic laboratories due to the exquisite sensitivity of PCR and new advances in chemistry that reduce the impact of the residue salt on amplification. Literature searches have shown them to be as valuable as phenol/chloroform with some forensic substrates and to provide DNA yields that are adequate for PCR and RFLP (restriction fragment length polymorphism). Inorganic reagents, mainly salts, will precipitate proteins, and act very similarly to phenol/chloroform. The cellular proteins are salted out by dehydration and precipitation with a saturated salt solution.

The main advantage is that compared to organic methods, the techniques are quicker and cheaper⁽⁵⁾ and much less toxic. The main disadvantage is they still do not eliminate all inhibitors and considerable amounts of protein may be retained. The literature does not provide as wide a variety of protocols for salt extraction in forensic scenarios as for the other methods. However, a previously mentioned comparative study between phenol/chloroform and saturated salt precipitation was performed for the extraction of DNA from putrefied cadavers.⁽¹³⁸⁾

3.5 Differential Extraction Methods

Several advances in the field of differential extraction have been published. A recent review on the biology, development, and proteomics of sperm and seminal fluid summarizes how these advances can improve differential extraction procedures.⁽¹³⁹⁾ As is the case with all evidentiary materials, maximizing the yield of DNA recovered while minimizing co-extraction of inhibitors is a high priority. An interlaboratory study on protocol comparisons on sexual assault samples demonstrated that male and female DNA yield was highly variable as expected, although surprisingly >90% of the male DNA present in the samples was lost.⁽¹⁴⁰⁾ Recovery of the cellular material from the substrates is the first critical step in maximizing yield. Modifications to the elution buffer can result in improvements to the recovery.^(141,142) The

quality and quantity of DNA is further impacted by the original environmental conditions as well as the storage conditions.

Several studies have focused on reducing carryover of male DNA into the female epithelial cell fraction allegedly due to the premature lysis of sperm during the epithelial cell lysis step.^(16,142–144) Modifications to the differential extraction procedures have been studied to allow improvement of separation while maximizing yield.^(139,145,146) Others have directed their efforts on separation of the cell types before differential lysis by gravitational, centrifugal, or vacuum filtration.^(139,142) In addition to the updates outlined in Section 2.3.1, advances in different microfluidic approaches,^(147–150) the use of new flocked nylon swab devices,⁽¹⁵¹⁾ and pressure cycling technologies for enhanced differential extraction have been published.⁽¹⁵²⁾

4 CONCLUSION

Advances in DNA extraction and PCR amplification chemistries for forensic analysis have occurred over the last decade. Improvements to methods for DNA capture, cleanup, and release coupled with an increase in PCR assay tolerance to inhibitors have led to a transition away for traditional organic methods to simplified, automated methods. Indeed, direct PCR applications where no DNA extraction is required are being implemented worldwide.^(96–99) Many laboratories are now utilizing magnetic beads containing DNA capture moieties that are easily sequestered for purification and resuspension removing the need for centrifugation and vacuum filtration. Magnetic separation techniques are, therefore, more easily adapted to automation and this has led to an expansion of semi- and fully automated DNA extraction platforms with benchtop and liquid handlers. It is now possible to process nearly all types of biological evidence samples containing small amounts of sample including touch evidence for genotyping and sequencing. Improved purification and new methods for differential extractions have also led to enhanced separation and analysis of sexual assault samples. Interest has developed in methods that can simultaneously extract total nucleic acids (DNA and RNA) due in great part to the relatively new RNA-based body fluid ID, tissue source of origin and age estimation assays that have been developed using mRNA profiling.^(55,56) The same extract can then be used for both RNA- and DNA-based applications⁽⁹¹⁾ including STR profiling; relatively new epigenetic methylation methods;^(153–156) and identity, phenotype, and ancestry informative MPS methods,^(157–159) while reducing the consumption of the evidentiary sample.

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ABBREVIATIONS AND ACRONYMS

DNA	Deoxyribonucleic Acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic Acid
MPS	Massively Parallel Sequencing
mtDNA	Mitochondrial Deoxyribonucleic Acid
NGS	Next-generation Sequencing
NLS	N-lauryl Sarkosine
PCR	Polymerase Chain Reaction
PK	Proteinase K
RFLP	Restriction Fragment Length Polymorphism
SDS	Sodium Dodecyl Sulfate
SNP	Single-nucleotide Polymorphism
STR	Short Tandem Repeat
Tris	Tris(hydroxymethyl)aminomethane

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