DNA isolation from human remains

34

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Contents

Introduction 756
Sample Pretreatment 758
  Decontamination 758
  Pulverization 761
DNA Isolation 762
  Manual DNA Isolation 762
  DNA Isolation with Commercial Kits 764
Conclusions 767
References 768

Abstract

The DNA analysis of human remains can be challenging despite the strong matrix in bones and teeth that helps preserve DNA. To isolate DNA, the correct procedures need to be applied. Factors such as temperature, pH, and humidity affect DNA degradation, while polymerase chain reaction inhibitors can affect or even prevent DNA amplification. If a sufficient quantity and quality of genetic material is obtained during DNA extraction, the key stage in DNA typing, a genetic profile can be obtained, thereby helping to identify the human remains. The aim of this chapter is to show various pretreatment strategies for bones and teeth (surface washing, chemical washing, enzymatic predigestion, milling and sanding, and

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ultraviolet radiation), as well as pulverization methods (mortaring, freezer milling, or tissue lysis), manual DNA isolation protocols (total demineralization, organic use of Chelex resin, and manual purification), and available commercial kits for DNA extraction from human remains.

Keywords

Ancient DNA · Automated DNA extraction · Human remains · Manual DNA extraction · Sample pretreatment

Introduction

When discussing human remains, we usually refer to corpses or skeletons, and samples are most frequently found in bones and teeth. Both types of samples protect DNA from degradation and biological processes due to their physical and chemical robustness. However, accessing the DNA is not as easy as in other tissues due to these protective characteristics.

Bone tissue consists primarily of proteins (mainly collagen and osteocalcin) and minerals. Approximately 70% of the mineral component of bone comprises hydroxyapatite, which includes calcium phosphate, calcium carbonate, calcium fluoride, calcium hydroxide, and citrate. The DNA in bones is located in the osteoblasts, osteocytes, and osteoclasts. Osteons are the functional unit of bones and include osteocytes (located in spaces within the dense bone matrix called lacunae) and haversian canals, which contain blood vessels and nerves and are formed by concentric layers called lamellae. This structure favors the deposit and storage of mineral salts, which gives bone tissue its strength. Osteoblasts produce the organic components of the bone matrix and are situated at the surface of the bone matrix. Osteoclasts are responsible for bone remodeling and resorption during bone growth and are located on the surface of the bone matrix (Mescher 2018b) (Fig. 1).

Teeth consist of dentin, a calcified material harder than bone that forms a large part of the structural axis of the tooth and surrounds the internal pulp cavity. Dentin consists of 70% hydroxyapatite, type I collagen, and proteoglycans. Dentin in the dental crown is covered by enamel, an extremely mineralized, hard, acellular, avascular tissue. Enamel is the hardest component of the human body and consists of 96% calcium hydroxyapatite, very few proteins, and no collagen. The dentin at the tooth root is covered by cementum, another type of calcified connective tissue that resembles bone. The soft tissue in the dental pulp is highly vascular and innervated and consists of odontoblasts, fibroblasts, endothelial cells, peripheral nerves, undifferentiated mesenchymal cells, and other nucleated blood components, making it rich in DNA (Muruganandhan and Sivakumar 2011; Mescher 2018a). DNA is recovered from the tooth pulp, where it is abundant and unlikely to be contaminated by nonhuman DNA (Girish et al. 2010) (Fig. 2).

Bone and tooth tissues are compact and hard structures that preserve the DNA in their matrix. Isolating DNA from bones and teeth therefore requires several pretreatment steps before the DNA can be recovered from these cells.

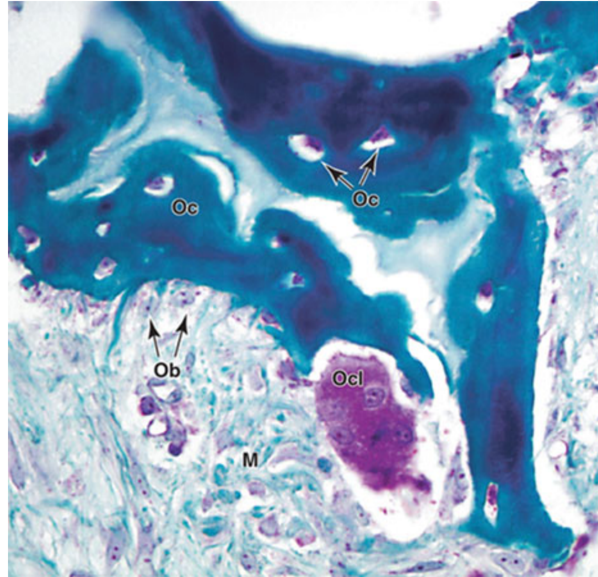

Fig. 1 Bone matrix. (M) Mesenchymal regions, (Ob) osteoblasts, (Oc) osteocytes, and (Ocl) osteoclasts (Mescher 2018b)

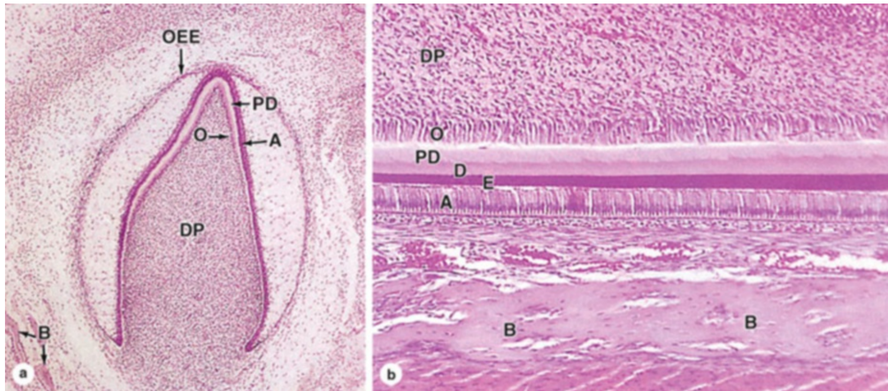

Fig. 2 Tooth formation. (A) Ameloblasts, (B) bone, (D) dentin, (DP) dental papila, (E) enamel, (PD) predentin, (O) odontoblasts, and (OEE) outer enamel epithelium (Mescher 2018a)

There are three main problems to solve at this stage: the introduction of modern DNA, the presence of too few DNA molecules to serve as templates for polymerase chain reaction (PCR) testing, and the co-purification of inhibitory substances that result in false negatives. The isolation of ancient DNA therefore has three requirements: 1) the samples have to be pretreated to reduce contamination, 2) extraction techniques that do not damage the DNA need to be employed, and 3) these techniques need to have a high purification power to reduce or eliminate the presence of inhibitors.

Sample Pretreatment

Decontamination

Bone and tooth samples are typically obtained from excavations and are often improperly and carelessly handled. There is, however, a growing awareness among archaeologists and anthropologists regarding the importance of wearing protective clothes when working with ancient DNA samples. Conducting a pretreatment stage prior to DNA isolation is extremely important for eliminating any possible contamination by exogenous DNA and the remains of putrilage and impurities.

There are several strategies for reducing or eliminating the possible superficial contamination of bones and teeth such as surface washing, acid washing, highly concentrated ethanol washing, bleach washing, hydrogen peroxide washing, milling and grinding, ultraviolet irradiation, and sampling of the inner part of the compact bone and combined techniques (Kemp and Smith 2005).

Surface Washing

Vigorous surface washing can remove the external layer of exogenous material from the bone (Holland et al. 1993) and involves using sterile water prior to cutting (Merriwether et al. 1994) or repeated rinsing of the cut pieces in distilled water, with a final air dry step (Alonso et al. 2001). However, humidity has been widely reported as a factor in damaging DNA because it facilitates mineral dissolution and increases hydrolytic damage. Moreover, the interdependence between the organic and mineral components of bone supports the hypothesis of bone susceptibility to chemical and biological effects due to the increase in porosity (Emmons et al. 2020).

Sodium Hypochlorite Washing

For forensic and ancient DNA samples, one of the most common methods for eliminating exogenous DNA from bone and teeth is washing the surface or even the powdered bone or tooth with bleach. Although washing significantly reduces exogenous human DNA, it also results in a loss of endogenous DNA (Dabney and Meyer 2019). Sodium hypochlorite, an active component of bleach, rapidly attacks nucleic acids in a nonspecific manner, degrading purine and pyrimidine bases through oxidation reactions such as chlorination (Hayatsu et al. 1971).

Given the destructive nature of bleach, the possibility of replacing it with other compounds such as phosphate buffer has been explored. The use of phosphate buffer is based on the competition between free phosphate ions and DNA phosphate groups attached to hydroxyapatite. Phosphate buffer has been shown to be less aggressive toward endogenous DNA than bleach washing and, although it can eliminate some microbial DNA, it is less effective with exogenous DNA (Dabney and Meyer 2019).

Ethanol Washing

An alternative to bleach washing is the cleaning of bone and teeth surfaces with sterile cotton previously moistened with a 95% (Fisher et al. 1993) or 70% ethanol solution (Stone and Stoneking 1998) in ultrapure water. Although ethanol exerts no degradative

activity on DNA, its use for precipitating DNA is well known, and its usefulness for cleaning surfaces in forensic laboratories has long been accepted (Kampmann et al. 2017).

Acid Washing

Similar to bleach and ethanol washing, the use of weak acids to remove exogenous DNA from samples is also common, as 30% acetic acid diluted in ultrapure water (Montiel et al. 2001). This technique is based on the power of denaturation of a low pH solution hydrolyzing the glycosidic and phosphodiester bonds of DNA. However, the effectiveness of this solution is lower if the DNA is rich in guanine and cytosine (Shapiro et al. 1978).

Hydrogen Peroxide Washing

Immersing human remains in a 3% (Ginther et al. 1992) or 3–30% hydrogen peroxide solution for 10–30 min (Merriwether et al. 1994) has also been shown to be an effective method for removing the exogenous component of samples. The decomposition of hydrogen peroxide into water and free oxygen radicals causes oxidative damage to DNA by radical-ionic mechanisms (Mouret et al. 1991).

Enzymatic Predigestion

One of the main problems with chemical decontamination methods is that they also attack the sample's endogenous DNA. A less aggressive method is the predigestion of the samples with ethylenediaminetetraacetic acid (EDTA) lysis buffer and proteinase K, which significantly reduces the contaminating DNA without affecting the sample's endogenous genetic material (Schroeder et al. 2019).

Milling and Sanding

The pretreatment technique of milling and sanding the surface of human remains consists of applying mechanical abrasion to their outermost part to eliminate the adhering exogeneous material. There are various approaches with these techniques such as air abrasion with 100 μm aluminium oxide particles (Richards and Sykes 1995), the use of sandpaper discs (Kalmár et al. 2000), scraping with a sterile scalpel (Lalueza-Fox et al. 2001), and the increasingly widespread use of precision rotary tools such as those manufactured by Dremel® (Gaudio et al. 2019).

The main problem with milling and sanding pretreatment is the formation of bone dust, which can contaminate the working surface, tools, other samples, and even the operator. The Laboratory of Genetic Identification of the University of Granada (Spain) developed a milling, sanding, and cutting methacrylate enclosure with a removable lid and two lateral holes to insert the operator's hands and the milling/sanding tool. The bottom of the enclosure is covered with filter paper, and the entire milling and sanding operation is conducted in a fume hood (Álvarez et al. 2001). The enclosure not only prevents the clogging of the filters of the hoods or cabinets in which the operation is performed but also facilitates the cleaning of the equipment. The external and internal surfaces of bones can be milled and grinded, and the bone can then be cut into fragments, being 0.5–1 cm^2 fragments recommended (Fig. 3).

Fig. 3 Methacrylate enclosure

Inner Part of the Compact Bone Sampling

To minimize the risk of contamination with modern DNA, a 2×2 cm section of compact bone from long bones, such as the posterior femoral diaphysis, can be cut, followed by scraping of the inner and outer surfaces with a scalpel (Palmirotta et al. 1997), leaving only the innermost part of the compact bone, which might be free of or less exposed to exogenous DNA.

Ultraviolet Irradiation

Short-wave ultraviolet (UV) light (254 nm) induces the covalent bonding of thymine bases, preventing denaturalization of DNA double strands during PCR, making it inaccessible to polymerase during amplification. Therefore, exposing the surface of the samples to UV light for a few minutes is useful for eliminating exogenous DNA (Latham and Miller 2019).

Variations in the UV exposure time, ranging from 10 min to up to 2 days (Carlyle et al. 2000; Kalmár et al. 2000; Matheson and Loy 2001), have been described and are sufficient to affect exogenous DNA but not endogenous DNA. The distance between the irradiation source and the irradiated surface is also important: the closer the source is to surface, the greater the irradiation power (Champlot et al. 2010; Hall et al. 2014). However, 10 min for both sides of the sample is the most frequently employed exposure time.

Combination of Techniques

Many laboratories combine two or more of the previous decontamination protocols to reduce potential exogenous contamination. The Laboratory of Genetic Identification of the University of Granada first mills, sands, and cuts the bone samples into pieces measuring approximately 0.5 cm^2 and then irradiates them with UV for 10 min on both sides before pulverizing the samples.

Pulverization

After decontaminating the sample, each laboratory employs its own extraction method, processing the bone and tooth samples in small pieces or grinding them into powder through various processes.

Manual Mortar

Manual mortars and pestles have typically been employed to grind bones and teeth. Numerous laboratories still use Teflon pestles in ceramic mortars to generate the fine bone powder needed for extractions (Cafiero et al. 2019).

Freezer Mill

Freezer/Mill® cryogenic grinders are widely used in laboratories to grind samples such as teeth, bones, and other animal and human tissues. Samples are placed in a sealed cryogenic grinding vial in the grinder and then immersed in liquid nitrogen. The samples are cooled to cryogenic temperatures and then pulverized by magnetically shuttling a steel impactor back and forth against two stationary end plugs (Fig. 4).

TissueLyser

To grind and disintegrate the tissues, a TissueLyser II system is recommended. The TissueLyser II grinds bone and teeth samples by shaking them with metallic balls without requiring liquid nitrogen. However, liquid nitrogen can be used with this technique to prevent the samples from heating, thereby facilitating the grinding (Fig. 5).

Fig. 4 Grinding vials and Freezer/Mill ®

Fig. 5 Grinding vials and TissueLyser II sytem

DNA Isolation

DNA isolation is the most important stage in the DNA analysis process, because it will determine the outcome of the entire process. If there is insufficient starting DNA, amplification will fail, yielding no results. Therefore, the key is to obtain as much DNA as possible. Molecules of genetic material have to be isolated from other cell components before the genetic material can be analyzed, because the cell proteins that package and protect the DNA can inhibit the analysis (Butler 2005). The other major problem is the presence of inhibitors that need be eliminated or minimized (Barrio-Caballero 2012), because they either inactivate DNA polymerase or compete with other components of the DNA synthesis reaction. The presence of extrinsic substances from bone such as humic and fulvic acids from the soil and intrinsic substances such as calcium have to been eliminated (Eilbert and Foran 2009).

Manual DNA Isolation

Total Demineralization

The total demineralization method was first developed in 1991 with well-preserved animal and human bones from archaeological sites. The method employs 0.5 M EDTA, proteinase K, and N-lauroylsarcosine at 37 °C for 18–24 h, followed by an extraction with phenol-chloroform. Bone decalcification is made possible by incubating cut samples with EDTA for 72 h, despite this approach reducing the total DNA yield (Hagelberg and Clegg 1991). The basis for this method is the chelating activity of EDTA, which binds to iron and calcium ions. The problem of reduced DNA yield is solved by making EDTA part of the lysis buffer, so that DNA can be purified with phenol-chloroform-isoamyl alcohol in a 25:24:1 proportion after an overnight lysis. The resultant is then concentrated with centrifugal filter units (Edson et al. 2004). A hybrid protocol of these two methods can be used by incubating

0.6–1.2 g of bone powder with 15 mL 0.5 M EDTA, 1% lauroyl-sarcosinate, and 20 mg/μL of proteinase K in a rotatory shaker overnight. The bone powder is thereby completely dissolved. Organic extraction with phenol-chloroform, filtration with centrifugal filter units, and two washes with ultrapure water are then performed (Loreille et al. 2007).

Organic Extraction

The well-known phenol-chloroform-isoamyl alcohol method, also known as organic extraction, was developed in 1991. Using this approach, 5 g of bone are powdered and decalcified with 0.5 M EDTA for 3–5 days, washed three times with ultrapure water, lysed with proteinase K and extraction buffer for 2 h, extracted with phenol-chloroform-isoamyl three times, and concentrated and purified in a Centricon centrifugal filter (Hochmeister et al. 1991). The mixture is typically stabilized with 10 mM of Tris-EDTA, the proteins are unfolded with phenol, empowered augmented by chloroform. The chloroform also denatures lipids, while the isoamyl alcohol stabilizes the interphase and increases DNA purity. DNA will be trapped in the upper aqueous phase.

Modified protocols introduce sodium dodecyl sulphate (SDS) and dithiothreitol (DTT) into the lysis buffer (Ferreira et al. 2013). SDS is an anionic detergent that linearizes the proteins present in chromatin, while DTT is a reducing agent that reduces the disulphide bonds present in proteins.

Although the phenol-chloroform method yields a large amount of DNA, the main issue is that it is a dangerous reagent, both for the analyst and the environment. The solution should therefore be used in a fume hood and its residues properly treated and disposed of. The method is also time-consuming and requires significant hands-on time.

Chelex® Resin

In 1998, a simple, chelating, single-tube, resin-based procedure, using minimal steps was suggested. The Chelex®-100 (Bio-Rad Laboratories, Hercules, CA) is a chelating resin composed of styrene divinylbenzene copolymers and iminodiacetate ions that bind to polyvalent metal ions. In the basic procedure, samples are boiled in a 5% Chelex®-100 suspension (Willard et al. 1998). A prior 30 min incubation at 56 °C is recommended for bone samples. An adapted protocol for ancient bone samples starts with a 3 h incubation at 56 °C of 100 mg of bone powder in Chelex®-100, followed by a 20 min boiling period (Coulson-Thomas et al. 2015). To yield more DNA, proteinase K can be added prior to incubation (Tsuchimochi et al. 2002). Despite being fast and environmentally friendly, Chelex® cannot remove PCR inhibitors.

Manual Purification of DNA Extracts

To maximize the chances of success, DNA extraction protocols need to obtain the largest amount of target DNA possible while reducing or even eliminating the presence of PCR inhibitors. To this end, there are two classical methods for analyzing ancient DNA (Yang et al. 1998): centrifugal filter units and silica particle

columns. The first method uses Centri-con™ filters (Hagelberg and Clegg 1991) that consist of an anisotropic membrane that retains macrosolutes, such as DNA, while letting low-molecular-weight compounds pass through, which can also occur with PCR inhibitors. Other protocols include further washing with approximately 1 mL of distilled water or 2 mL of TE buffer with 0.01 M Tris and 0.001 M EDTA at a pH of 7.5 (Hochmeister et al. 1991). The second method uses silica particles (Höss and Pääbo 1993) with a high binding capacity for DNA molecules and are therefore retained while the inhibitors are washed out; however, the silica particles are themselves potential PCR inhibitors. The silica pellets are therefore washed twice with a 10 M guanidine thiocyanate and 0.1 M Tris-HCl buffer at a pH of 6.4, washed twice with 70% ethanol, and washed once with acetone. Another approach is to precipitate out any material that is nonnucleic by adding saturated sodium acetate (a process known as salting-out [Cattaneo et al. 1995]), adding 1 mL of the solution to the tube, shaking it manually for 30 s and centrifuging it for 10 min at 4000 g.

Several DNA purification commercial kits are available, such us DNA IQ™ System purification (Promega, MA, USA), or QIAquick™ PCR purification kit (QIAGEN, Hilde, Germany) (Ye et al. 2004).

DNA Isolation with Commercial Kits

There are currently numerous commercial isolation kits for bone DNA analysis (see Table 1), although most require mechanical pretreatment. Most of these kits can be automated with the appropriate equipment, which offers several advantages such as maintained sample integrity, increased reproducibility, constant performance, greater throughput, workflow integration, electronic audits, compatibility with laboratory information management systems (LIMS), sample switching and data entry error minimization, reduced hands-on time, and lower repetitive stress injuries (Lee and Shewale 2017). In this section we will describe some of these advantages.

Table 1 Commercial kits for bone DNA analysis

Kit	Format	Lysis time	Protocol time	Automatization
PrepFiler™ BTA (ThermoFisher)	100 reactions	~2 h	~2–3 h	AutoMate™ Express
Bone DNA Extraction (Promega)	100 reactions	~2,5 h + ~2,5 h	~2 h	Maxwell®
QIAamp DNA Investigator (Qiagen)	50 reactions	Overnight	~30-60 min	QIAcube Connect
EZ1 Investigator (Qiagen)	48 reactions	~24–48 h	~20 min	EZ1
Cells and Tissue DNA Isolation (Norgen Biotek)	50 reactions	~2 h	~60 min	–
CrimePrep (Ademtech)	96/48 reactions	~2 h	~60 min	Automag

PrepFiler ™ BTA Forensic DNA Extraction Kit

PrepFiler™ BTA Forensic DNA Extraction Kit (Thermo Fisher Scientific Inc., MA, USA) was developed for isolating DNA from bone, teeth, and other forensic samples with adhesives (cigarette butts, envelope flaps, tape lifts, and chewing gum). The extraction kit uses a format that provides for 100 reactions, and the protocol can be performed in approximately 2–3 h, which reduces the processing time by requiring a shorter lysis time than standard methods. The use of phenol-chloroform with this kit is not necessary. To release DNA from calcified tissues, the kit employs a sequence of washes, with various buffers and filter columns. DNA isolation is performed with a magnetic bead. The protocol is divided into four parts: lysis (PrepFiler® BTA lysis buffer and DTT), DNA binding (PrepFiler® magnetic beads), purification (PrepFiler® wash buffer), and DNA reconstitution (PrepFiler® BTA lysis buffer). The PrepFiler® lysis buffer is composed of a thiocyanic acid compound with guanidine (1:1), while the PrepFiler® BTA lysis buffer is based on sodium hydroxide. There are several variations to the method according to the samples' complexity, ranging from increasing the quantity of powdered sample (and thus the volume of lysis reagents) to extending the lysis time to overnight. The elution volume can also be customized to concentrate the DNA extract.

There is an automated option for these kits: the AutoMate Express™ (Thermo Fisher Scientific Inc., MA, USA), which is based on the above protocol. First, lysis is performed in a thermoshaker and then the lysate is automatically purified. The AutoMate Express™ uses prefilled buffer cartridges that reduce the handling of samples, thereby reducing potential contamination by the operator (Applied Biosystems 2012).

Bone DNA Extraction Kit

The Bone DNA Extraction Kit (Promega Corporation, WI, USA) is actually the joining of two protocols: a preprocessing protocol and a subsequent purification protocol. The kit was developed as a combination of classical purification protocols, created by various genetic identification laboratories, and uses a demineralization buffer (0.5 M EDTA, pH 8.0, 1% lauroylsarcosine) and an organic extraction protocol (proteinase K and 1-thioglycerol) to effectively and efficiently extract DNA from the calcium matrix. The first step can be performed using manual or automated Promega methods (using the Maxwell® extraction instrument), using DNA IQ™ for DNA purification. The kit's format provides for 100 reactions, and the estimated time to completion is more than 7 h due to the demineralization and subsequent digestion requiring an incubation time of 2.5 h each. Performing an extraction within a single working day is therefore problematic (Promega 2019).

QIAamp DNA Investigator Kit

As with the Promega protocols, the Qiagen protocol (Qiagen N.V. Hilden, Germany) has two differentiated phases: a pretreatment protocol for bones and teeth (which describes the decalcification and lysis using EDTA and proteinase K) and a subsequent DNA purification using MinElute spin columns (QIAamp DNA Investigator Kit) to obtain purified genomic DNA. The success of this purification phase depends

on the combination of the selective binding properties of a silica-based membrane. The DNA purification can be automated using QIAcube Connect, an instrument widely used in genetic identification laboratories to fully automate the purification of nucleic acids and proteins.

EZ1 DNA Investigator Kit

Qiagen developed the EZ1 DNA Investigator kit, which uses a similar DNA extraction protocol for powdered bone and tooth to that of the PrepFiler™ BTA Forensic DNA Extraction Kit. The Qiagen protocol is based on a lysis phase performed manually and an automated purification phase in the EZ1 Advanced automated sample preparation system. The lysis requires a decalcification step with 0.5 M EDTA (not included in the kit) for 24–48 h and digestion with proteinase K for 3 h. The lysate is then divided into various aliquots, and buffer MTL is added to load the sample into the device. The protocol supports three different quantities of powered bone or tooth, which can vary the volume of reagents and the protocol on the device. The automated system uses prefilled buffer cartridges, which reduces the handling of samples and thus potential contamination by the operator (QIAGEN 2013).

Cells and Tissue DNA Isolation Kit

The Cells and Tissue DNA Isolation Kit (Norgen Biotek Corp., Ontario, Canada) employs a protocol that purifies DNA from various tissue types using a magnetic bead system. The manufacturer recommends a decalcification step prior to isolating genomic DNA to improve the efficiency of the DNA recovery. To perform decalcification, the bone or teeth are crushed, incubated with EDTA at 4 °C for 24 h, and centrifuged several times. The supernatant is then removed, and 20 mg of the sample is used as the substrate to perform the kit's protocol. The kit's format provides for 50 reactions, and the estimated hands-on time is 1 h and more than 24 h for the incubations (Norgen Biotek Corp 2015).

Crime Prep Adem-Kits for Casework

Ademtech (Pessac, France) commercial kits are based on calibrated particles with high magnetic content and controlled surfaces, specially designed for molecular biology. The Crime Prep protocol starts with 100 mg of bone powder and a 2h lysis, followed by binding, washing, drying, and eluting. Alternative protocols employ an overnight lysis. Crime Prep comes in a 96-sample format and an estimated hands-on time of 1 h plus 2 h of lysis. The process can also be automated with an Automag device for 48 samples with a reduced time cost (Ademtech 2019).

Incorporation of Manual Pretreatment Protocols to Commercial Kits

Over the years, various DNA extraction protocols have been developed to extract DNA from skeletal remains by incorporating manual pretreatment to the commercial kits. This section discusses the protocols capable of extracting DNA without requiring that the samples be pulverized. In 2007, a new DNA extraction procedure that did not require pulverization of samples was described (Kitayama et al. 2010). In their article, the authors presented a new experimental kit that combined a

conventional phenol-chloroform DNA extraction procedure with the QIAamp DNA Mini Kit for DNA isolation kit. In this protocol, mechanical grinding was replaced with gentle stirring during overnight incubation. The results were inconclusive due to the low number of samples and differences in the quality of the extracted DNA with respect to grinding protocols. However, there is certain value in exploring protocols that do not require the pulverization of samples.

In 2020, De Donno et al. described a DNA isolation from a saponified sternum from a limbless human body recovered at sea. The authors extracted DNA using a Macherey-Nagel kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany), modifying the usual procedure described for NucleoSpin® DNA Trace Kit 2. The authors made numerous modifications to the original protocol, halving the quantity of bone material, volumes of reactant, proteinase K, and lysis buffer (B3 buffer, included in the NucleoSpin DNA Trace bones buffer set). The authors also changed the number and type of columns used for binding DNA to the silica membrane (Piglionica et al. 2012; De Donno et al. 2020).

Cartozzo et al. described a similar DNA isolation method for waterlogged bones. The extraction was based on an organic isolation method followed by the use of a Thermo Fisher kit. The extraction began with digestion with proteinase K, purification with phenol-chloroform-isoamyl alcohol and subsequent drying of the aqueous phase using a Speed Vac Concentrator. The dried pellet was reconstituted with deionized water. This eluate was the substrate for starting the DNA extraction using the ChargeSwitch® gDNA Plant Kit (Thermo Fisher Scientific Inc., MA, USA). The protocol was performed according to the manufacturer's procedures (Pagan et al. 2012; Cartozzo et al. 2018). After the experiments, the authors concluded that the magnetic bead technology of the ChargeSwitch® gDNA Plant Kit might be the most efficient method for recovering DNA from waterlogged bones, a surprising statement after using a kit recommended for fungi and plants.

In any case, each bone presents its own set of challenges, requiring manual procedures and commercial kits to be adapted to ensure the success of the DNA extraction. Embalmed bones, for example, not only involve issues with extracting DNA from bones but also bring to the table the exposure to various compounds such as glutaraldehyde and formalin, which can induce molecular cross-linking. In these cases, modifying the existing grinding techniques and combining them with decalcification buffers, phenol-chloroform treatment, and commercial kits will produce efficient methods for extracting sufficient high-quality DNA (Gielda and Rigg 2017).

Conclusions

In conclusion, this review shows how to overcome the drawbacks of isolating DNA from mineralized tissues, in order to identify them through forensic genetics. Bone and tooth tissue consist primarily of proteins and minerals, which are a major inconvenience in the laboratory; however, these tissues protect against degradation of the large DNA molecules. Several techniques aimed at preventing the introduction

of exogenous DNA into the study samples have been reported. These techniques reduce or prevent the loss of the scarce DNA molecules and also reduce or eliminate the co-purification of inhibitory substances.

The complications caused by the time elapsed between death and the laboratory procedures are increased by the characteristics of where and how the body was found. The success of DNA extraction and isolation is affected by variables such as relative humidity, temperature, UV light exposure, and microbiome (amount and type of microorganisms). These factors significantly affect the degradation of the cadavers and their skeletal remains.

These variables that increase degradation and alter the mineral concentration make each bone an enigma. There is therefore no single solution for extracting DNA from bones. Although there are many valid solutions, several of which have been covered in this chapter, there is no ideal protocol for extracting DNA from bones and teeth, as this will depend on the circumstances surrounding each sample.

The most advisable strategy is to use more than one extraction method. There are protocols that eliminate all contaminants and inhibitors from the sample. Due to purification, however, there is an excess of DNA loss, and the final concentration obtained is therefore low. Other methods that obtain more DNA can contain mineral remains or DNA from other organisms. As described in this chapter, the most effective approach is to use different protocols depending on the origin of each bone or, as numerous authors have done, combine stages from different procedures. Nevertheless, this approach should only be taken with a deep understanding of each step and the reactions in each stage. This is the only way to successfully obtain extracted DNA without an excessive number of attempts.

To ensure success, certain tests should be performed before extracting DNA from bones belonging to the same mass grave, same burial type, or similar types of catastrophes. If the skeleton is highly valuable and its identification is imperative, it is highly recommended that the sample be fragmented into 1- to 2-g pieces, so that more than one test can be performed. Problems occurring during the first DNA extraction can thereby be solved in subsequent extractions.

Lastly, the use of commercial kits for the last stages of DNA extraction or for the purification of the isolated DNA is recommended. This approach is very common in laboratories and has been described by numerous authors in the literature. These kits become highly recommended due to their capacity for preventing PCR inhibition and obtaining genetic profiles, which is the ultimate goal in identifying victims.

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