International Journal of Legal Medicine
https://doi.org/10.1007/s00414-023-02980-9

ORIGINAL ARTICLE

Optimization of InnoXtract™ extraction and purification system for DNA extraction from skeletal samples

Jennifer Snedeker[1] · **Sheree Hughes**[1] · **Rachel Houston**[1]

Received: 21 October 2022 / Accepted: 14 February 2023

Abstract

The InnoXtract™ extraction and purification system is a purification method designed for DNA extraction from low-template samples, specifically rootless hair shafts. Its ability to successfully capture highly fragmented DNA suggests its suitability for use with other challenging sample types, including skeletal remains. However, the lysis and digestion parameters required modifications to successfully optimize the method for this sample type. A two-part digestion was developed utilizing a homebrew digestion buffer (0.5 M EDTA, 0.05% Tween 20, and 100 mM NaCl) and a supplemental lysis with the Hair Digestion Buffer included in the InnoXtract™ kit. Additionally, the magnetic bead volume was modified to improve DNA recovery from these challenging samples. With the altered protocol, the quality and quantity of DNA recovered from InnoXtract™ extracts were comparable to another commercial skeletal extraction method (PrepFiler™ BTA). This modified extraction method successfully purified sufficient amounts of quality DNA from a variety of skeletal samples to produce complete STR profiles. Successful STR typing from surface decomposition, burned, cremated, buried, and embalmed remains indicates the potential of this new method for challenging human identification and missing-person cases.

Keywords DNA extraction · InnoXtract™ · Skeletal remains · Short tandem repeats (STR) · Human identification (HID) · Forensic sciences

Introduction

The National Institute of Justice refers to the accumulation of unidentified remains and missing persons as “the nation’s silent mass disaster” [1]. There are multiple techniques that can be used to aid in human identification (HID), such as the examination of dental records or other anthropological techniques, but these methods are often limited and rarely provide definitive identifications [2, 3]. However, DNA typing through the analysis of traditional STR markers is considered the gold standard in HID and can provide this individualization even when the quantity and/or quality of biological material is limited. This is often the case with mass disasters, missing persons, or mass burials, when only skeletal remains are available for HID.

Compared to traditional forensic samples (blood, semen, saliva, etc.), DNA extraction from skeletal remains is a time-consuming and complicated process due to the inherent nature of the sample. Skeletal samples contain relatively low amounts of DNA that naturally reduce the extraction yield [4]. Additionally, skeletal samples contain PCR inhibitors (both endogenous and exogenous) that require an effective DNA extraction and purification method that is capable of sequestering and removing a variety of inhibitors for successful PCR amplification and subsequent analysis [4].

In addition to PCR inhibition, DNA extraction from skeletal remains is also often hindered by DNA damage and degradation; therefore, DNA extraction methods must be able to capture small fragments (< 200 bp) of DNA for a successful analysis [4]. In addition to the expected issues with processing skeletal remains, there are numerous other situations that may further impact the success of DNA typing, such as burning, cremation, embalming/chemical treatment, and burial [5–7]. Therefore, DNA extraction methods for skeletal remains must be able to overcome many challenges presented by these samples and environmental insults.

Jennifer Snedeker
jls191@shsu.edu

[1] Department of Forensic Science, College of Criminal Justice, Sam Houston State University, 1003 Bowers Blvd., TX 77341 Huntsville, USA

Because there are many factors to consider when processing skeletal samples, a wide range of DNA extraction techniques have been investigated. The protective barrier of the calcified extracellular matrix requires an additional demineralization step to successfully extract DNA from skeletal samples [4]. Total demineralization of skeletal samples uses high concentrations of a chelating agent, such as EDTA, to remove calcium ions from the sample. This method involves an overnight incubation that includes both lysis and complete demineralization by fully dissolving the bone powder, allowing maximum DNA recovery [4, 8]. While total demineralization of skeletal samples is time-consuming, incomplete demineralization methods reduce extraction times by only partially demineralizing the samples using a shorter incubation period (two hours vs. overnight) and also often uses less bone tissue.

The PrepFiler™ BTA kit is a modified version of the traditional PrepFiler™ kit that has been designed to extract DNA from bone, tissue, and adhesives (Thermo Fisher Scientific, South San Francisco, CA). Silica-coated paramagnetic bead-based chemistry is used with incomplete demineralization and a shorter incubation time [9]. Multiple studies have demonstrated sufficient removal of inhibitors and the generation of interpretable STR profiles from skeletal samples using this chemistry [10–12]. Conversely, a study by Amory et al. detected a PCR inhibitor (presumably calcium) that appeared to only affect a total demineralization method but had no impact on the partial demineralization ICMP method. This inhibition was not detected during quantification but resulted in poor downstream STR profiles when the total demineralization extracts were amplified [13]. Although complete demineralization facilitates the total release of DNA, it also releases more inhibitory agents from the extracellular matrix of the sample. Overall, partial demineralization DNA extraction methods, such as PrepFiler™ BTA, are common in many forensic laboratories due to the successful recovery of quality DNA, reduced processing time, minimal starting bone material, and compatibility with automatic platforms.

The InnoXtract™ Extraction and Purification System by InnoGenomics Technologies (New Orleans, LA) also utilizes a magnetic bead-based chemistry for successful extraction of challenging DNA samples. It is specifically marketed for use with low-level samples such as touch DNA and rootless hair shafts [14]. Gutierrez et al. analyzed rootless hair shafts utilizing InnoXtract™ and successfully extracted highly degraded nuclear DNA [15]. The ratio of surface area to volume of the magnetic beads utilized with the InnoXtract™ method targets smaller fragments and leads to greater success with these highly compromised samples, suggesting that degraded skeletal samples may also be suitable for extraction using this kit [14]. Because InnoXtract™ is typically used for hair samples, its lysis parameters require modifications and the addition of a homebrew digestion buffer for optimal DNA extraction from skeletal remains.

In this study, the original InnoXtract™ protocol was modified to include a two-part digestion similar to that of PrepFiler™ BTA methods. A homebrew digestion buffer consisting of 0.5 M EDTA, 0.05% Tween 20, and 100 mM NaCl provided the initial lysis, which was coupled with the InnoXtract™ Hair Digestion Buffer as a supplemental lysis step. The overall quality and quantity of DNA in the extracts were assessed with each modification to the extraction method. Additionally, the optimal bone powder input mass, optimal magnetic bead volume for purification, and the versatility of the kit to extract DNA from a variety of challenging skeletal samples (buried, decomposed, embalmed, burned, and cremated remains) were also assessed. Results were compared to those obtained using the PrepFiler™ BTA method. Overall, the implementation of these modifications to the currently available InnoXtract™ extraction kit can provide DNA analysts with a new alternative method to extract DNA from limited and highly degraded skeletal samples.

Materials and methods

Sample preparation and selection

Skeletal samples ($n = 33$) were collected from 23 cadavers donated to the Southeast Texas Applied Forensic Science (STAFS) Facility at Sam Houston State University. To examine the versatility of InnoXtract™, bone samples exposed to a variety of insults were studied, including cremated ($n = 2$), embalmed ($n = 7$), burned ($n = 15$), surface-decomposed ($n = 6$), and buried ($n = 3$) remains.

Maceration was performed on select samples to remove any tissue or debris present. Window cuts were taken from 18 femora, six tibiae, five humeri, two vertebrae, two teeth, and one carpal using a rotary power tool (Dremel Stylus™, Racine, WI). Next, the surface of these samples were sanded, removing any additional foreign material and exogenous DNA, and cut into 0.5 cm^2 bone chips using the Dremel and cutting disks. The chipped samples went through a series of wash steps that included 20% bleach, at least two cycles of deionized sterile water, and 100% ethanol. After drying overnight in a sterile fume hood, the samples were powdered using a freezer mill (SPEX 6770, Metuchen, NJ) and liquid nitrogen. Crushing parameters involved a 10 min pre-cool before a 1 min crush cycle, 1 min cooling cycle, and a final 1 min crush cycle.

Initial optimization of the new modified InnoXtract™ method was performed using a 1-year-old surface-decomposed femur sample (sample 32). All DNA extractions were performed in a dedicated UV-irradiated low-copy-number clean room while wearing appropriate disposable protective gear (gloves, hair net, face mask, gown, and shoe covers). Extraction blanks were performed with each method to monitor contamination.

Preliminary study: lysis and purification method comparison

Initial work involved comparing established extraction methods (PrepFiler™ BTA and Intermountain Bone/Tooth Processing and Purification utilizing EZ1® Advanced XL (QIAGEN, Hilden, Germany) instrumentation) and the coupling of these established lysis parameters with InnoXtract™ purification in triplicate, as indicated in Table 1, to determine which method provided the best quality extract when paired with the InnoXtract™ kit [9, 16–18]. DNA extracts were then quantified using the Quantifiler Trio DNA Quantification kit (Thermo Fisher Scientific) [19].

Lysis parameter optimization study

Extractions were performed in triplicate on 50 mg of bone powder by modifying the lysis parameters of the InnoXtract™ manufacturer's protocol comparing a single-digestion and two-part digestion lysis (Online Resource 1) [16].

Single-digestion Three hundred ninety µL of homebrew digestion buffer (0.5 M EDTA, pH 8.0; 0.05% Tween 20, 100 mM NaCl) was combined with 25 µL of 20 mg/mL proteinase K and 3 µL of 1 M DTT. Samples were then incubated at 56 °C for 2 h at 1100 rpm. Following incubation, 405 µL of supernatant was collected, and extraction continued following the InnoXtract™ manufacturer's protocol for binding, washing, and elution [16]. However, the sample was eluted in 30 µL of TE instead of the specified 20 µL.

Two-part digestion method Two hundred µL of a homebrew digestion buffer (0.5 M EDTA, pH 8.0; 0.05% Tween 20, 100 mM NaCl) was coupled with 25 µL of 20 mg/mL Proteinase K and 3 µL of 1 M DTT. The samples were then incubated at 56 °C for 2 h at 1100 rpm. After incubation, 200 µL of supernatant was collected, and 205 µL of InnoXtract™ Hair Digestion Buffer was added. Following this supplemental lysis, extraction continued following the InnoXtract™ manufacturer's protocol for binding, washing, and elution [16]. However, the sample was eluted in 30 µL of TE instead of the specified 20 µL to account for the loss during the final drying step.

Table 1 Pairings of lysis and purification methods tested

Lysis parameters	Purification parameters
PrepFiler™ BTA [9]	PrepFiler™ BTA [9]
PrepFiler™ BTA [9]	InnoXtract™ [16]
Intermountain bone/tooth processing and purification [17]	EZ1, large volume protocol [18]
Intermountain bone/tooth processing and purification [17]	InnoXtract™ [16]

DNA quantification and STR typing

DNA extracts were quantified via real-time quantitative PCR (qPCR) on the ABI 7500 Instrument (Thermo Fisher Scientific) using the Quantifiler Trio DNA Quantification Kit and 2 µL of sample [19]. A non-template control was included to monitor contamination. A standard curve was generated, and the data was accepted if the R^2 value was greater than 0.99.

STR amplification was performed using the Investigator 24plex QS Amplification Kit (QIAGEN) following the manufacturer's protocol on the Veriti-96 well system (Thermo Fisher Scientific), targeting 0.8 ng of large target DNA [20]. Both negative and positive controls were included. PCR products were then separated and detected using the ABI 3500 Genetic Analyzer (Thermo Fisher Scientific). Genemapper IDX v1.4 software (Thermo Fisher Scientific) was used for automatic genotype calling with an analytical threshold of 100 RFUs and a stochastic threshold of 200 RFUs.

Data analysis

To analyze the efficiency of each extraction method, the quantification results and STR profile data were analyzed using Excel software (Microsoft, Redmond, WA). Student t tests were used to assess statistical significance of large and small target DNA yield and the average peak height and average peak height ratio between lysis methods. p values $< 0.05 = \alpha$ were considered significant.

The occurrence and extent of stochastic effects, including loci and allelic dropout, were also examined. To monitor the effect of degradation on each sample, the degradation index $\left(DI = \frac{Concentration\ of\ Small\ Target}{Concentration\ of\ Large\ Target}\right)$ was assessed. The presence of inhibition was recognized using two methods. First, the change in cycle threshold (C_T) of the internal PCR control (ΔIPC) was examined when analyzing the quantification results. The IPC for standard one was not included in this calculation. Inhibition was also monitored when examining the STR profiles by analyzing the quality sensor signals and determining the ratio of QS2 to QS1 $\left(QS\ Ratio = \frac{Qulity\ Sensor\ 2}{Quality\ Sensor\ 1}\right)$. If the QS2 signal dropped 20% below the QS1 signal, then inhibition was recorded [20].

Purification parameter optimization study: magnetic bead volume

DNA extractions were performed in replicates of five on 50 mg of bone powder using increasing volumes of magnetic beads (10, 15, 20, 25, 30 μL) following the two-part digestion method. DNA quantification and data analysis followed previously described methods in "Lysis parameter optimization study."

Sample input optimization study

DNA extractions were performed in replicates of five using 25 mg or 40 mg of bone powder utilizing the two-part digestion method and 20 μL of magnetic beads. DNA quantification and data analysis followed the previously described methods in "Lysis parameter optimization study" and were compared with the 50 mg extracts collected in the magnetic bead study, above.

Comparison to established skeletal extraction method: optimized InnoXtract™ vs. PrepFiler™ BTA

Extractions were performed on 33 samples utilizing the optimized InnoXtract™ method (two-part digestion, 20 μL of magnetic beads, and 50 mg of bone powder) and the PrepFiler™ BTA method [9]. DNA quantification, STR typing, and data analysis followed the previously described methods in "Lysis parameter optimization study," except 0.8 ng of small fragment DNA was targeted, and automatic genotype calls were performed by OSIRIS v.2.16 in conjunction with ArmedXpert™ v.3.1.1 with an analytical threshold of 75 RFU and an interpretation threshold of 200 RFU. Additionally, a paired *t* test was used for comparing the small target DNA yield and allele recovery percentage for each sample between the two methods.

Results

Lysis and purification method comparison

When comparing DNA yields from the full PrepFiler™ BTA extraction protocol with DNA yields using PrepFiler™ BTA lysis coupled with InnoXtract™ purification (Fig. 1), no significant difference was observed in the large (PrepFiler™ BTA, 0.0807 ± 0.0127 ng/μL; PrepFiler™ BTA/InnoXtract™, 0.0631 ± 0.0046 ng/μL; *p* value = 0.139) or small qPCR target yields (PrepFiler™ BTA, 0.1074 ± 0.0100 ng/μL; PrepFiler™ BTA/InnoXtract™, 0.0906 ± 0.0036 ng/μL; *p* value = 0.091). On the other hand, a significantly higher yield was observed for both the large (Intermountain/EZ1, 0.0475 ± 0.0300 ng/μL; Intermountain/InnoXtract™, 0.0721 ± 0.0047 ng/μL; *p* value = 0.003) and small targets (Intermountain/EZ1, 0.0528 ± 0.0102 ng/μL; Intermountain/InnoXtract™, 0.0782 ± 0.0075 ng/μL; *p* value = 0.047) when Intermountain Forensics lysis methods were coupled with InnoXtract™ purification rather than purification using the EZ1 Advanced Instrumentation.

No significant difference between the two methods was observed when InnoXtract™ purification was coupled with either of the established lysis methods (PrepFiler™ BTA and Intermountain Forensics). However, when examining the average large fragment recovery, PrepFiler™ BTA with InnoXtract™ purification appears to recover a slightly lower yield of 0.0631 ± 0.0046 ng/μL compared to the 0.0721 ± 0.0047 ng/μL recovered when using Intermountain Forensics coupled with InnoXtract™ (*p* value = 0.123). The opposite observation was true when we examined the average small fragment recovery. PrepFiler™ BTA lysis coupled with InnoXtract™ purification appeared to provide a higher yield of 0.0906 ± 0.0036 ng/μL compared to Intermountain Forensics lysis coupled with InnoXtract™ purification, which recovered 0.0782 ± 0.0075 ng/μL (*p* value = 0.103) (Fig. 1). The average DI values (PrepFiler™ BTA, 1.3455 ± 0.0987; PrepFiler™/InnoXtract™, 1.4483 ± 0.1649; Intermountain Forensics/EZ1, 1.1284 ± 0.2895; Intermountain Forensics/InnoXtract™, 1.0959 ± 0.1806) for all methods fall below 1.5. However, both methods that involve PrepFiler™ BTA lysis resulted in

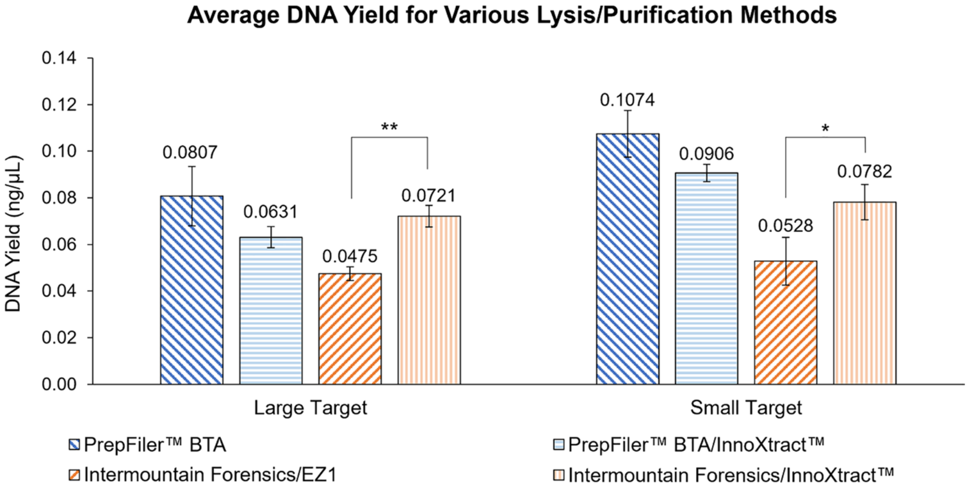

Fig. 1 Average DNA yield comparing lysis/purification methods: PrepFiler™ BTA and PrepFiler™ BTA/InnoXtract™ had similar average large and small target yields. Intermountain Forensics/EZ1 had significantly lower yields compared to Intermountain Forensics/InnoXtract™. PrepFiler™ BTA/InnoXtract™ and Intermountain Forensics/InnoXtract™ had similar DNA yields. (Data reported as $\mu \pm \sigma$; * indicates a *p* value < 0.05; ** indicates a *p* value < 0.005)

higher DI values compared to Intermountain Forensics lysis methods (n.s., p value > 0.05).

Lysis parameter development

The two-part digestion method using the InnoXtract™ Hair Digestion Buffer as a supplemental lysis resulted in a significant increase (p value = 0.003) in small target yield (0.0951 ± 0.0039 ng/μL) compared to the single-digestion method (0.0625 ± 0.0058 ng/μL) (Fig. 2). When the DI values of each extraction method were compared (single-digestion, 0.8582 ± 0.0148; two-part digestion, 1.2857 ± 0.0875), it was noted that the two-part digestion resulted in significantly higher values (p value = 0.021). Complete STR profiles were obtained for samples using both methods. When comparing key STR profile metrics, the average peak height (p value = 0.921) and peak height ratios (p value = 0.737) did not show a significant difference regardless of a single- or two-part digestion. A summary of the STR profile metrics obtained for the InnoXtract™ lysis and digestion methods can be found in Online Resource 2.

As expected with most skeletal remains, the electropherograms of both methods contained the classic "ski-slope" effect in some dye channels but appeared more prevalent in the two-part digestions [21]. Additionally, in two of the three replicates of the single-step digestion method, the DNA profiles exhibited split peaks or shoulders in the short amplicons, despite containing less than 0.8 ng of target DNA. Although no signs of inhibition appeared when comparing the ΔIPC C_T values during quantification, inhibition was detected when examining the quality sensors during STR amplification with complete dropout of the QS2 marker in a single-digestion replicate. The Investigator 24plex QS protocol states that inhibition is present when the QS2 signal drops below 20% of the QS1 signal. Therefore, following these guidelines, PCR inhibition was only detected in this replicate; however, the other replicate had a lower QS ratio of 46%. The presence of split peaks in these samples suggests inhibition regardless of whether the QS sensors fall below the inhibition threshold of 20%.

Optimal magnetic binding bead volume and sample mass

When examining the effect of magnetic binding bead volume on DNA yield, a positive correlation was present until 20 μL bead input, where the highest average small target DNA yield was recovered (Online Resource 3). By increasing the magnetic binding beads from 15 to 20 μL, a statistically significant increase in average small target DNA yield was captured (15 μL, 0.108 ng/μL; 20 μL, 0.138 ng/μL; p value = 0.008). Additionally, the 20 μL of magnetic beads provided a statistically significant increase in the small target DNA yield compared to the 10 μL of magnetic beads specified in the InnoXtract™ protocol (10 μL, 0.101 ng/μL; p value = 0.002). Further increasing the magnetic bead volume to 25 μL did not result in higher DNA yields than those recovered using the 20 μL of beads (25 μL, 0.129 ng/μL; p value = 0.349). However, when 30 μL of magnetic beads were used, a statistically significant decrease in the small target DNA yield was identified relative to the yield recovered when following the 10 μL bead input specified by the protocol (30, 0.075 ng/μL; p value = 0.011).

When modifying the sample input mass, it was determined that the addition of more bone powder provided a statistically significant increase in DNA yields. The use of 50 mg of bone powder provided the highest average large and small target DNA yields of 0.100 ng/μL and 0.138 ng/μL respectively. Online Resource 4 compares the DNA yields for each sample input.

Comparison to PrepFiler™ BTA methods

A thorough comparison between the optimized InnoXtract™ method and PrepFiler™ BTA was performed by examining 33 challenging skeletal samples. The two methods were

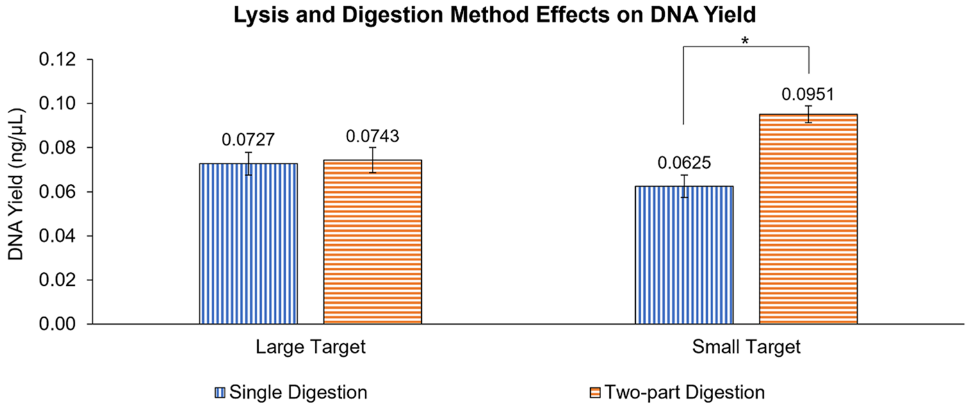

Fig. 2 Lysis and digestion method effects on DNA yield: a two-part digestion method resulted in a significantly larger DNA yield for small targets. (Data reported as μ ± σ; * indicates a p value < 0.005)

determined to produce similar small target DNA yields (p value = 0.148) (Fig. 3). The InnoXtract™ small target DNA yields ranged from 0.0085 to 56.4058 ng/µL, whereas the PrepFiler™ BTA small target DNA yields ranged from 0.0077 to 124.1780 ng/µL. To examine the versatility of InnoXtract™ with various challenging skeletal samples, sample insults included cremation, embalming, burning, surface decomposition, and burial. Both InnoXtract™ and PrepFiler™ BTA methods resulted in quantifiable DNA for all insults, with an embalmed humerus sample providing the highest small target DNA yield for both methods. However, a range of DNA yields were recovered from all insults (Fig. 3).

The overall allele call rates for each method were similar between InnoXtract™ and PrepFiler™ BTA methods (p value = 0.086) (Fig. 4). Three InnoXtract™ extracts (19, 31, 33, noted in Fig. 4) failed to amplify, indicated by the dropout of the Investigator 24plex quality sensors. PCR amplification still failed for these three samples after reamplifying utilizing different replicate extracts or a combination of the original and replicate extract to reach sufficient volume. Additionally, the QS2 marker dropped out in two samples (12 and 13). Both samples exhibited poor peak morphology, with several split peaks present, suggesting inhibition. However, no signs of PCR inhibition were detected in these five samples when examining the ΔIPC C_T values. When examining the versatility of the newly optimized InnoXtract™ method, full STR profiles were recovered from embalmed, burned, and surface-decomposed remains. PrepFiler™ BTA was also successful

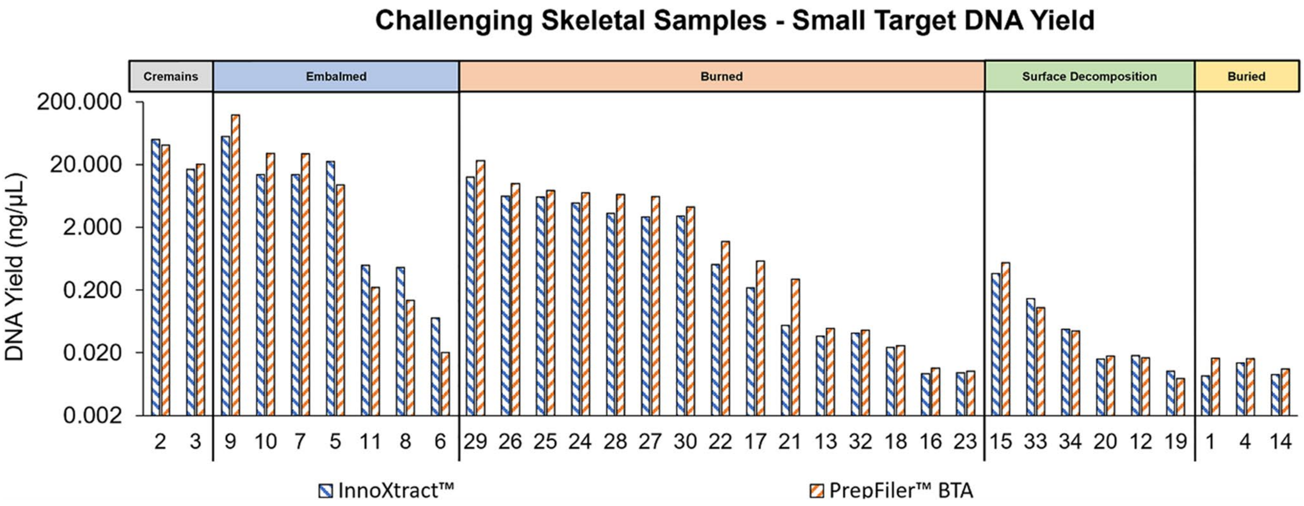

Fig. 3 Challenging skeletal samples (DNA yield): no statistically significant difference was seen when comparing the small target DNA yields for the InnoXtract™ and PrepFiler™ BTA methods

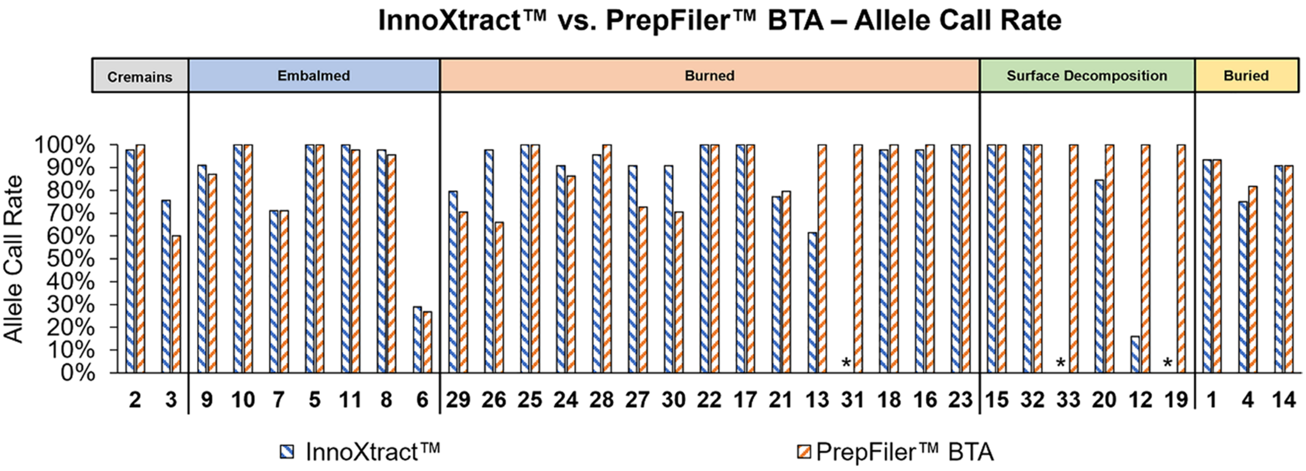

Fig. 4 Challenging skeletal samples (STR typing): no significant differences between InnoXtract™ and PrepFiler™ BTA methods. (* indicates samples that failed to amplify)

in recovering a full STR profile from cremated remains. However, both methods were unable to successfully recover a full profile from any of the buried samples. The key STR metrics for both methods can be found in Online Resource 5.

Discussion

Coupling of lysis and digestion parameters

To successfully optimize the InnoXtract™ method for skeletal samples, it was necessary to first modify the lysis and demineralization parameters. By coupling the InnoXtract™ purification with the Intermountain Forensics lysis method, a significant increase in both large and small target DNA yield was observed. This result highlights the capabilities of the InnoXtract™ purification process and the success of the magnetic bead recovery. However, this improvement was not unexpected since the traditional Intermountain Forensics method utilizes the EZ1 platform for automatic purification and the InnoXtract™ purification process is manual allowing for more analyst discretion and control.

When comparing the two methods coupled with InnoXtract™ purification, there were no significant differences in DNA recovery. However, PrepFiler™ BTA coupled with InnoXtract™ provided a slightly higher recovery of small DNA fragments. The optimal ratio of surface area to volume of magnetic beads in the InnoXtract™ purification is designed to enhance the recovery of small DNA fragments [14]. Because DNA in skeletal samples is often highly fragmented, it is important that this attribute is not diminished by the InnoXtract™ lysis parameters designed for DNA extraction from skeletal remains. Therefore, small target recovery was given precedence, suggesting that PrepFiler™ BTA lysis parameters may be more compatible with InnoXtract™ purification. The DI is another parameter that can be used to assess the quality of DNA extracted from a sample. Because these samples are collected from a homogenized mixture of bone powder, they are expected to exhibit similar levels of degradation. However, both methods that involve PrepFiler™ BTA lysis resulted in higher DI values compared to Intermountain Forensics lysis methods, indicating the ability to capture a higher number of small DNA fragments when following these lysis parameters.

Homebrew digestion buffer development

Because recovering highly fragmented DNA is crucial when processing degraded skeletal samples, it was decided that the homebrew digestion buffer should perform similarly to the PrepFiler™ BTA buffer to maximize the chance of recovering these small fragments of DNA. The homebrew digestion buffer was therefore designed to be similar to the PrepFiler™ BTA lysis buffer as coupling this lysis buffer with InnoXtract™ purification appeared to have overall higher yields of small DNA fragments (although not significantly) compared to the Intermountain Forensics method. The PrepFiler™ BTA initial lysis step involves a chelating agent, detergent, salt, reducing agent, and enzyme [22]. DTT (reducing agent at 1 M) and proteinase K (enzyme at 20 mg/mL) were maintained in the InnoXtract™ lysis parameters; however, the volume (and therefore the concentration in the working solution) of DTT was reduced to 3 μL. Most likely, the large volume of DTT specified in the InnoXtract™ protocol was necessary to break the disulfide bridges in cystine residues present in hair [23].

Similar to the PrepFiler™ BTA lysis buffer, we also maintained a chelating agent –0.5 M EDTA, a detergent –0.05% Tween 20, and a salt –100 mM NaCl in the homebrew digestion buffer. Most skeletal extractions involve the addition of EDTA as a chelating agent at 0.5 M to aid in demineralization of bone samples [4, 24–30]. Loreille et al. state that to fully dissolve 1 g of bone powder, 15 mL of 0.5 M EDTA is needed [4]. However, because InnoXtract™ involves only a partial demineralization, this ratio could not be maintained. The detergent chosen was selected according to the Dabney method [28, 29]. Several studies indicate that the Dabney method (or adaptations of this method), which involves an incubation in 0.45 M EDTA, proteinase K, and 0.05% Tween 20, successfully extracted DNA from highly challenging skeletal remains [5, 28–30].

Lysis parameter development

Because PrepFiler™ BTA lysis involves a two-part digestion and this method provided a higher small target yield compared to the single-step digestion of the Intermountain Forensics lysis method, a two-part InnoXtract™ digestion method was examined [9, 17]. The PrepFiler™ BTA lysis method involves an initial lysis using the bone, tooth, and adhesive (PrepFiler™ BTA) lysis solution specialized for these samples. A supplemental lysis is then performed using the traditional PrepFiler™ lysis buffer. This buffer is typically used for more common forensically relevant sample types (blood, saliva, semen) [9]. Following this protocol, we coupled the homebrew digestion buffer with the InnoXtract™ Hair Digestion Buffer and compared it with a single-step digestion using only the homebrew digestion buffer.

In addition to the added benefits of an additional lysis step, it is likely that the supplemental lysis using the InnoXtract™ Hair Digestion Buffer provided a significant increase in small target yields because of the improved ability to remove inhibitors commonly found within skeletal samples (Ca^{+2}, collagen, humic acid, etc.). Because InnoXtract™ was initially designed with the Hair Digestion Buffer interacting with the magnetic beads and binding solution, it is likely that maintaining this chemical environment allowed for more effective DNA capture and purification. The significant increase in the DI when comparing single- and two-part digestions also highlights the increased recovery of small DNA fragments when the homebrew digestion buffer is combined with the Hair Digestion Buffer, further suggesting that the Hair Digestion Buffer is vital for this small fragment recovery.

Detection of PCR inhibition

The ability to obtain full STR profiles for both homebrew methods indicates that InnoXtract™ can successfully extract sufficient quantity and quality of DNA for HID, despite signs of DNA degradation and PCR inhibition in some samples. The presence of split peaks in two of the single-digestion profiles is potentially due to PCR inhibition, limiting the ability of the DNA polymerase to perform complete adenylation. We hypothesized that a supplemental lysis using the InnoXtract™ Hair Digestion Buffer may be required to mitigate these PCR inhibitors, as this buffer maintains the correct chemical environment for effective DNA isolation and purification.

Despite the appearance of split peaks, no indications of PCR inhibition were detected when examining the ΔIPC C_T values during DNA quantification. However, the quality sensors within the Investigator 24plex QS kit suggested that inhibition may be present. The conflicting results in the detection of PCR inhibition using qPCR and STR amplification could be due to differences in amplicon length and volume of extract. The IPC amplicon is 130 bp compared to the 435 bp amplicon of the QS2 marker in Investigator 24plex QS [19, 20]. This length difference likely provides Investigator 24plex QS with an increased sensitivity when detecting PCR inhibition. Additionally, only 2 µL of DNA extract is added to the qPCR amplification, while up to 15 µL of DNA was added to the STR amplification allowing for an increase in the amount of inhibitory agent in the PCR [19, 20]. Other studies have also identified situations where PCR inhibition was not detected during qPCR [13, 31, 32]. Pionzio and McCord hypothesized that the length and sequence of the IPC amplicon can impact the detection of inhibition and suggested the use of a longer IPC amplicon with lower GC content to improve the detection of PCR inhibitors [31]. Holmes et al. suggest that quantification using the Investigator® Quantiplex® Pro Kit (QIAGEN) rather than the Quantifiler® Trio kit used in this current study may be more suitable for skeletal extracts, as it is more tolerant of calcium, a PCR inhibitor commonly found in skeletal samples [33]. They noted that small target yields were still accurately predicted and that PCR inhibition was flagged when higher amounts of calcium were spiked in the extracts [33].

Because a two-part digestion provided increased small target DNA yields and improved profile quality by limiting PCR inhibition, it was decided to proceed forward with this method as the optimized lysis and demineralization protocol for skeletal samples when using the InnoXtract™ kit.

Optimal magnetic bead volume

Although rootless hair shafts and skeletal remains are often both low-template samples, it was assumed that a greater volume of magnetic binding beads would be required for DNA extraction of skeletal samples because the volume of magnetic beads was optimized for the limited DNA recovery from a single rootless hair shaft (Sudhir Sinha, PhD, Personal Communication, April 12, 2021). Both 20 µL and 25 µL of magnetic beads provided significantly higher DNA yields than the 10 µL specified in the protocol. However, further increasing the volume to 30 µL caused a significant decrease in DNA yield. Potentially, this overabundance of magnetic beads resulted in sample loss due to incomplete DNA coverage on all of the beads and subsequent competition of the beads for surface area along the magnetic stand. Because no significant increase in DNA yield was observed when increasing the volume of the beads from 20 to 25 µL, it was determined that 20 µL of magnetic beads were sufficient for InnoXtract™ to successfully extract skeletal samples.

Optimal sample input mass

In addition to modifying the magnetic bead volume, the optimal sample input mass required investigation. Several studies have shown that the ratio of bone powder to lysis solution can affect the overall quantity and quality of recovered DNA. Specifically, increasing this ratio can result in greater DNA recovery, likely due to a decrease in PCR inhibition [4, 25, 34]. Additionally, increasing this ratio may allow for more interactions between the sample and reagents. Traditionally, this analysis would involve increasing the volumes of lysis solution; however, for the successful capture of small fragments with InnoXtract™ methods, it is important to maintain the volume of lysis solution and binding solution. Therefore, it was decided to reduce the bone powder sample input to examine if this reduction may allow for an increase in interactions between the homebrew digestion buffer and bone tissue, leading to improved digestion and lysis, and, therefore, to an increased recovery in DNA yield. However, it was determined that higher sample inputs of 50 mg resulted in a statistically greater DNA recovery for both large and small amplicons. Because utilizing 50 mg of sample input successfully provided sufficient quality (average DI, 1.37; ΔIPC, − 0.53) and quantity of large and small target DNA, further investigation of greater bone powder inputs was not necessary. Additionally, limiting the sample input to 50 mg conserves precious bone and provides a comparable method to PrepFiler™ BTA, which also requires only 50 mg of sample.

Comparison to an established method, PrepFiler™ BTA, using challenging skeletal samples

PrepFiler™ BTA is a widely used commercial kit for processing skeletal samples that provides a suitable comparison method for the optimized InnoXtract™ method. Similarly to the newly developed method, PrepFiler™ BTA also employs a partial demineralization and utilizes paramagnetic silica-bead-based technology during purification. Additionally, it was important to examine whether the optimized InnoXtract™ method could successfully extract DNA from challenging skeletal samples (cremated, embalmed burned, surface-decomposed, and buried remains) that are more representative of forensic samples in cases such as missing persons, skeletal recoveries, and cold cases.

The overall DNA yield and allele recovery were similar between both methods, suggesting that the newly optimized InnoXtract™ method aligns with established methods. However, the InnoXtract™ method had indications of PCR inhibition issues (amplification failure, dropout of QS2 markers, and poor peak morphology) when examining samples 12,13, 19, 31, and 33. Because the extracts of these samples were fully consumed, a dilution and reamplification were not possible to determine if PCR inhibition could be overcome. Other than the extraction method, no common feature was identified for these samples, and they all resulted in full profiles when processed using PrepFiler™ BTA methods.

Unfortunately, due to the limited availability of cadavers for certain environmental insults, it is impossible to draw definitive conclusions based on these characteristics. Nevertheless, InnoXtract™ demonstrated success with at least one sample per insult providing at least 70% allele recovery. InnoXtract™ methods also successfully extracted DNA of sufficient quality and quantity from compromised skeletal samples for HID, with 25 of the 33 samples meeting the minimum CODIS loci requirement of 8 of the original 13 loci present. Additionally, at least one sample from all insult groups met this requirement. Overall, these findings suggest that InnoXtract™ is capable of successfully extracting sufficient amounts of quality DNA from compromised skeletal remains. Furthermore, this newly optimized method is comparable in performance to the well-established PrepFiler™ BTA kit.

Conclusion

Because InnoXtract™ was designed to extract DNA from hair samples, modifications to the lysis parameters were needed to optimize InnoXtract™ for successful DNA extraction from skeletal samples. The addition of a homebrew digestion buffer composed of 0.5 M EDTA (pH 8.0), 0.05% Tween 20, and 100 mM NaCl provided the initial demineralization and lysis. A supplemental lysis using the Hair Digestion Buffer provided in the kit allowed for an increased recovery in small DNA yield. Because the mass of starting tissue and expected DNA yield of skeletal samples is presumed to be greater than that of a single rootless hair shaft (which the kit was originally designed for), the magnetic binding bead volume was increased to 20 µL, and 50 mg of bone powder was found to be optimal for DNA extractions with the InnoXtract™ kit.

Overall, this optimized InnoXtract™ method generated comparable results to another commercial partial demineralization method (PrepFiler™ BTA), and this workflow is similar to those currently implemented within crime labs, allowing for easy transitions between extraction methods. Additionally, the overall InnoXtract™ kit is available at a more affordable price than other manufactured skeletal extraction kits, and the reagents required for the homebrew digestion buffer are sourced from common forensic laboratory reagents, reducing the time and cost of purchasing new materials. Furthermore, because this method implements a partial demineralization, samples can be processed, from powder to extract, in a matter of hours rather than the days needed for complete demineralization methods. The relatively low sample input of 50 mg provides an additional benefit by limiting the consumption of precious evidence material and allowing a potential screening method for overall genotyping success. Therefore, this newly optimized InnoXtract™ method can provide DNA analysts with an affordable alternative extraction method for processing the limited challenging skeletal samples often encountered in HID cases.

Additionally, this workflow was able to extract sufficient amounts of quality DNA for successful STR typing of various challenging skeletal samples. Full STR profiles were recovered from three of the five insult groups with an overall allele recovery of 78.83%. However, many samples were still too degraded or had inadequate quantity for sufficient DNA recovery and successful STR typing. Therefore, other downstream genotyping methods for InnoXtract™ extracts could be investigated to fully utilize the high recovery of small DNA fragments. Future studies will investigate various genotyping approaches, such as next-generation sequencing assays or SNP microarrays from samples extracted using the optimized InnoXtract™ method to demonstrate its compatibility with a wide range of HID applications.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00414-023-02980-9.

Acknowledgements The authors would like to thank the Southeast Texas Applied Forensic Science Facility (a willed body donor program at Sam Houston State University), the donors, and their loved ones, without whom this research would not be possible. The authors would also like to thank InnoGenomics Technologies for providing valuable feedback and technical support.

Funding This project was partially funded by Award # 1739805 through the Center for Advanced Research in Forensic Science (CARFS), an NSF-funded IUCRC at Florida International University. The opinions, findings, conclusions, or recommendations expressed in this article are those of the authors.

Data availability The authors can confirm that all relevant data are included in the article and/or its supplementary information files.

Declarations

Ethical approval For the skeletal samples used in this study, Code 45 of the US Federal Regulations part 46102(f) exempts the requirement for Institutional Review Board (IRB) approval regarding the use of human cadaveric samples. All procedures were in accordance with the 1964 Helsinki Declaration and its later amendments.

Conflict of interest The authors declare no competing interests.

References

1. Ritter N (2007) Missing persons and unidentified remains: the nation's silent mass disaster. NIJ Journal 256:1–7
2. Krishan K, Kanchan T, Garg AK (2015) Dental evidence in forensic identification - an overview, methodology and present status. Open Dent J 9:250–256. https://doi.org/10.2174/1874210601509010250
3. Slaus M, Strinović D, Petrovecki V, Vyroubal V (2007) Contribution of forensic anthropology to identification process in Croatia: examples of victims recovered in wells. Croat Med J 48:503–512
4. Loreille OM, Diegoli TM, Irwin JA et al (2007) High efficiency DNA extraction from bone by total demineralization. Forensic Sci Int Genet 1:191–195. https://doi.org/10.1016/j.fsigen.2007.02.006
5. Emery MV, Bolhofner K, Winingear S et al (2020) Reconstructing full and partial STR profiles from severely burned human remains using comparative ancient and forensic DNA extraction techniques. Forensic Sci Int Genet 46:102272. https://doi.org/10.1016/j.fsigen.2020.102272
6. Wheeler A, Czado N, Gangitano D et al (2017) Comparison of DNA yield and STR success rates from different tissues in embalmed bodies. Int J Legal Med 131:61–66
7. Hebda LM, Foran DR (2015) Assessing the utility of soil DNA extraction kits for increasing DNA yields and eliminating PCR inhibitors from buried skeletal remains. J Forensic Sci 60:1322–1330
8. Jakubowska J, Maciejewska A, Pawłowski R (2012) Comparison of three methods of DNA extraction from human bones with different degrees of degradation. Int J Legal Med 126:173–178. https://doi.org/10.1007/s00414-011-0590-5
9. PrepFiler and PrepFiler BTA Forensic DNA Extraction Kits, Rev. B (2012) Thermo Fisher Scientific, USA
10. Stray J, Holt A, Brevnov M et al (2009) Extraction of high quality DNA from biological materials and calcified tissues. Forensic Sci Int Genet Suppl Ser 2:159–160. https://doi.org/10.1016/j.fsigss.2009.08.086
11. Hasap L, Chotigeat W, Pradutkanchana J et al (2019) Comparison of two DNA extraction methods: PrepFiler® BTA and modified PCI-silica based for DNA analysis from bone. Forensic Sci Int Genet Suppl Ser 7:669–670. https://doi.org/10.1016/j.fsigss.2019.10.132
12. Barbaro A, Cormaci P, Falcone G (2011) Validation of BTA™ lysis buffer for DNA extraction from challenged forensic samples. Forensic Sci Int Genet Suppl Ser 3:e61–e62
13. Amory S, Huel R, Bilić A et al (2012) Automatable full demineralization DNA extraction procedure from degraded skeletal remains. Forensic Sci Int Genet 6:398–406
14. InnoGenomics Technologies (2019) InnoXtract. InnoGenomics: Innovation in our genes. https://innogenomics.com/products/innoxtract/. Accessed 21 Oct 2020
15. Gutierrez R, LaRue B, Houston R (2021) Novel extraction chemistry and alternative amplification strategies for use with rootless hair shafts. J Forensic Sci. https://doi.org/10.1111/1556-4029.14763
16. InnoXtract Kit User Guide, v1.4 (2020) InnoGenomics Technologies, USA
17. Intermountain Bone/Tooth Processing and Purification (2021) QIAGEN, Germany
18. EZ1 DNA Investigator Handbook, Rev. 7 (2014) QIAGEN, Germany.
19. Quantifiler^{TM} HP and Trio DNA Quantification Kit - User Guide, Rev. H (2018) Thermo Fisher Scientific, USA
20. Investigator 24plex QS Handbook, v. HB-1860-007 (2018) QIAGEN, Germany
21. Butler JM (2009) Forensic challenges: degraded DNA, mixtures, and LCN. In: Fundamentals of forensic DNA typing, 1st edn. Academic press, Massachusetts, pp 315–339
22. Stray J, Yingjie Liu J, Brevnov M, et al (2021) Lysis buffers for extracting nucleic acids. Patent No: US 10,894,957 B2. https://patents.google.com/patent/US10894957B2/en
23. McNevin D, Wilson-Wilde L, Robertson J et al (2005) Short tandem repeat (STR) genotyping of keratinised hair Part 2. An optimised genomic DNA extraction procedure reveals donor dependence of STR profiles. Forensic Sci Int 153:247–259. https://doi.org/10.1016/j.forsciint.2005.05.005
24. Rothe J, Nagy M (2016) Comparison of two silica-based extraction methods for DNA isolation from bones. Leg Med 22:36–41. https://doi.org/10.1016/j.legalmed.2016.07.008
25. Caputo M, Irisarri M, Alechine E, Corach D (2013) A DNA extraction method of small quantities of bone for high-quality genotyping. Forensic Sci Int Genet 7:488–493. https://doi.org/10.1016/j.fsigen.2013.05.002
26. Seo SB, Zhang A, Kim HY et al (2010) Efficiency of total demineralization and ion-exchange column for DNA extraction from bone. Am J Phys Anthropol 141:158–162
27. Booncharoen P, Khacha-ananda S, Kanchai C, Ruengdit S (2021) Factors influencing DNA extraction from human skeletal remains: bone characteristic and total demineralization process. Egyptian J Forensic Sci 11:1–9
28. Xavier C, Eduardoff M, Bertoglio B et al (2021) Evaluation of DNA extraction methods developed for forensic and ancient DNA applications using bone samples of different age. Genes 12(2):146. https://doi.org/10.3390/genes12020146
29. Dabney J, Knapp M, Glocke I et al (2013) Complete mitochondrial genome sequence of a Middle Pleistocene cave bear reconstructed from ultrashort DNA fragments. Proc Natl Acad Sci 110:15758–15763
30. Rohland N, Glocke I, Aximu-Petri A, Meyer M (2018) Extraction of highly degraded DNA from ancient bones, teeth and sediments

for high-throughput sequencing. Nat Protoc 13:2447–2461. https://doi.org/10.1038/s41596-018-0050-5

31. Pionzio AM, McCord BR (2014) The effect of internal control sequence and length on the response to PCR inhibition in real-time PCR quantitation. Forensic Sci Int Genet 9:55–60. https://doi.org/10.1016/j.fsigen.2013.10.010
32. Zeng X, Elwick K, Mayes C et al (2019) Assessment of impact of DNA extraction methods on analysis of human remain samples on massively parallel sequencing success. Int J Legal Med 133:51–58
33. Holmes AS, Houston R, Elwick K et al (2018) Evaluation of four commercial quantitative real-time PCR kits with inhibited and degraded samples. Int J Legal Med 132:691–701. https://doi.org/10.1007/s00414-017-1745-9
34. Duijs FE, Sijen T (2020) A rapid and efficient method for DNA extraction from bone powder. Forensic Sci Int Reports 2:100099. https://doi.org/10.1016/j.fsir.2020.100099