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TECHNICAL NOTE

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A Simple and Efficient Method for Extracting DNA From Old and Burned Bone

ABSTRACT: It has been a challenge to extract DNA from bones previously soaked in water, burned, or buried for a long time, due to the reduced quality and quantity of DNA in the bone samples. The dramatic degradation of the DNA and the presence of PCR inhibitors in the collagen significantly complicate the process of DNA identification in dated and charred bones. In this article, we present a novel strategy to obtain DNA from bones based on the use of cetyltrimethylammonium bromide (CTAB) lysis buffer and isoamyl alcohol-chloroform extraction with subsequent DNA purification using the DNA IQ™ System, or alternatively the QIAquick™ system. When applied to bones soaked, burned or buried for up to nine years, this method increases the purity and yield of DNA with respect to the traditional phenol-chloroform method and significantly improves multiplex STR genotyping using fluorescence-based methods. The results of this research will assist forensic scientists in the identification of DNA from victims whose bodies underwent significant trauma or burning, precluding the utilization of traditional forensic DNA identification techniques.

KEYWORDS: forensic science, cetyltrimethylammonium bromide (CTAB), old and burned bones, DNA extraction, DNA IQ™ System, QIAquick™ PCR purification, PCR inhibitors, fluorescence-based multiplex STR genotyping

Traditional forensic identification is based on fingerprint, dental or skeletal evidence. However, specimens from fires, explosions, airplane crashes, and other traumatic events, as well as old remains are difficult to identify via traditional methods. A successful alternative to these methods is to use DNA technology for identification purposes. Typically, this entails the use of short tandem repeat (STR) markers, which are characterized by a high level of polymorphism and are abundant in the human genome (1). Methods are in place to carry out multiplex genotyping of STR markers using sensitive and highly reliable fluorescent technologies, which are widely used in the field of forensics (2–4).

Compared with the DNA in soft tissue samples, DNA in bone samples is more resistant to the environment's chemical and physical erosion because of the protection afforded to it by the bone itself. However, in aged and burned bones, DNA is highly degraded and it is difficult to extract DNA of sufficient quality and quantity from the bone samples. The presence of PCR inhibitors complicates the application of STR multiplex methods. Removing the PCR inhibitors from the test samples is a critical step in order to successfully identify DNA in aged and/or burned bone specimens.

In this paper, we describe a new DNA isolation method that combines the use of cetyltrimethylammonium bromide (CTAB) buffer and isoamyl alcohol-chloroform to extract DNA from bone samples with a further DNA purification step using the DNA IQ™ System, or alternatively the QIAquick™ system. This method is based on Murray and Rogers' work in plant DNA extraction (5,6). This novel method provides an easy, rapid and efficient way to obtain high quality DNA from bone and offers important advantages over alternative methods in terms of DNA purity and yield. Following the use of the CTAB + isoamyl alcohol-chloroform DNA extraction method, bones previously soaked, burned, or buried for up to nine years were successfully identified using fluorescent-based STR multiplex methods.

Materials and Methods

Bone Samples

We examined bone samples collected 2–9 years after death. Additionally, we genotyped soaked and burned bones. All the samples were casework evidence analyzed at the Institute of Forensic Science in Beijing, China.

Treatment of Bone Prior to DNA Extraction

Bone samples were surface sterilized by washing with 0.5% sodium hypochlorite and then rinsed with running deionized distilled water (ddH_2O) for 5 min. The bone samples were then air-dried and exposed to UV irradiation for 1 h. Drilling into the material using a hand drill produced 1 to 3 g of bone powder, depending on the age and state of preservation.

Traditional Phenol-chloroform Extraction Method

Approximately 1 g of bone powder was placed in a 2.0 mL tube, and DNA lysis buffer (10 mM Tris-HCl, pH 8.0, 10 mM NaCl,

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2% SDS, 39 mM DTT) and 200 mg/mL proteinase K enzyme were added and mixed well. The solution was incubated overnight in a water bath at 37°C. DNA was extracted the following day with phenol-chloroform, according to standard procedures, and the DNA was concentrated using a Centricon™ –100 column (Millipore Corp, Billerica). Finally, the DNA solution was purified using the DNA Clean-Up System (Promega Inc., Madison).

CTAB + Isoamyl Alcohol-chloroform Extraction Method

Bone powder was placed into a mortar, and 2 mL CTAB buffer solution (2% CTAB,100 mmol/L Tris_HCl pH 8.0, 20 mmol/L EDTA, 1.4 mmol/L NaCl and 0.2% 2-mercaptoethanol) were added to the powder. The resulting paste was continually pestled until well mixed. The paste was then transferred to a 5 mL test tube, and an additional volume of CTAB buffer solution was added, the volume being dependent on the original amount of bone powder (approximately 1 mL CTAB per 1 g of bone powder). The tube was left at room temperature overnight.

The above solution was heated in a 65°C water bath for 1–1.5 h, and the sample was vortexed every 10–15 min to release the DNA from the bone tissue. The supernatant was collected after centrifugation at 5000 r/min (1000 xg) for 25 min and an equal volume of chloroform:isoamyl alcohol (24:1) was added. After the solution gently mixed, it was centrifuged at 5000 r/min for 25 min, after which the upper aqueous phase was transferred into a new tube. The aqueous contained DNA that was further purified by one of the two alternative methods described below.

Note: If the final DNA solution exceeded 750 μL, it was concentrated using a Microcon™-100 column to get a final solution not exceeding 750 μL.

Removal of PCR Inhibitors After CTAB + Isoamyl Alcohol-chloroform Extraction

PCR inhibitors were removed by further purifying the DNA sample using the DNA IQ™ system (Promega Inc., Madison) or alternatively, the QIAquick™ PCR Purification Kit (QIAGEN Inc., Chatsworth). A brief description of each method is provided below.

DNA IQ™ System Purification Method

Equal volumes of DNA solution and lysis buffer from the the DNA IQ™ Kit were added to a 1.5 mL test tube and mixed well. Seven μL of resuspended resin were then added to the sample, vortexed for 3 s and incubated at room temperature for 5 min. After incubation, the sample was vortexed again for 2 s, and placed immediately on a magnetic stand. The solution was carefully discarded without disturbing the resin. The resin was washed with 100 μL of lysis buffer, vortexed for 2 s, and the tube was placed again on the magnetic stand. The solution was carefully discarded without disturbing the resin. The same washing procedure was repeated for a total of three washes. After the last wash, the tube was allowed to air-dry on the magnetic stand with the lid open for 5 min at room temperature, 20–40 μL of deionized water were then added and the lid was closed. The mixture was vortexed for 2 s and heated at 65°C for 5 min. The tubes were removed from heat, immediately vortexed for 2 s and placed on the magnetic stand. The resulting liquid phase, containing the DNA, was then stored at a low temperature.

QIAquick™ Purification Method

Five volumes of QIAquick™ buffer PB were added to each DNA sample, loaded onto a QIAquick™ Spin Column, and microcentrifuged at 12800 xg for 1 min. The flow-through was discarded and the process was repeated until all of the extract had passed through the column. DNA was washed by adding 750 μL of QIAquick™ PE buffer and then microcentrifuged for 1 min. The flow-through was discarded and DNA was eluted from the column by the addition of 50 μL of TE bufferand microcentrifuged for 1 min. The flow-through was collected in a 1.5 mL tube, which contains the purified DNA.

STR Genotyping

STR genotyping was carried out using the AmpFℓSTR Profiler Plus PCR Amplification Kit (Applied Biosystems, Foster City). The Profiler Plus kit allows mutiplex amplification of nine tetranucleotide STRs (D3S1358, D5S818, D7S820, D8S1179, D13S317, D18S51, D21S11, FGA, and vWA) and the Amelogenin locus for gender identification. Fluorescence detection of genotypes was carried out with an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City).

Results

Comparison of DNA Extraction Methods

We compared the efficiency of DNA extraction from bone using a traditional phenol-chloroform method with that using the novel CTAB + isoamyl alcohol-chloroform method. Figure 1 shows the DNA quantitation of samples extracted from a bone analyzed three years after death using two alternative DNA extraction methods: bands A and C correspond to DNA extracted with the phenol-chloroform method and concentrated with a Centricon-100 column; with further purification using the DNA Clean-Up System band B corresponds to DNA extracted with the novel CTAB + isoamyl-alcohol-chloroform method, with further purification using a QIAquick spin column. The concentration of DNA obtained with the CTAB + isoamyl-alcohol-chloroform method was between five and ten times higher than the concentration obtained with the alternative phenol-chloroform approach. Figure 2 shows the Profiler Plus STR electropherogram of a DNA sample extracted

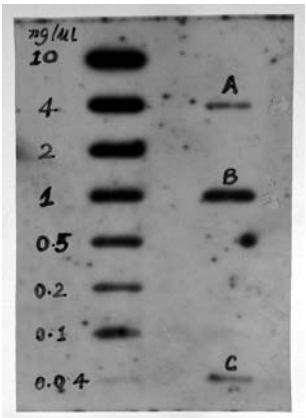

FIG. 1—*DNA quantitation of samples from bone analyzed three years after death. Bands A and C correspond to DNA extracted with the phenol-chloroform method and concentrated with Centricon-100 columns, with further purification using the DNA Clean-Up System and band B corresponds to DNA extracted with the CTAB + isoamyl alcohol-chloroform method, and further purified with a QIAquick spin column.*

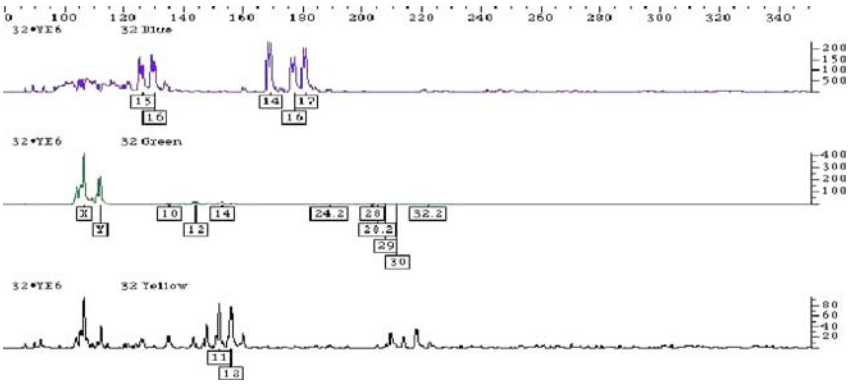

FIG. 2—*STR electropherogram of a DNA sample from bone analyzed three years after death. DNA was extracted with the phenol-chloroform method and concentrated with a Centricon-100 column, with further purification using the DNA Clean-Up System.*

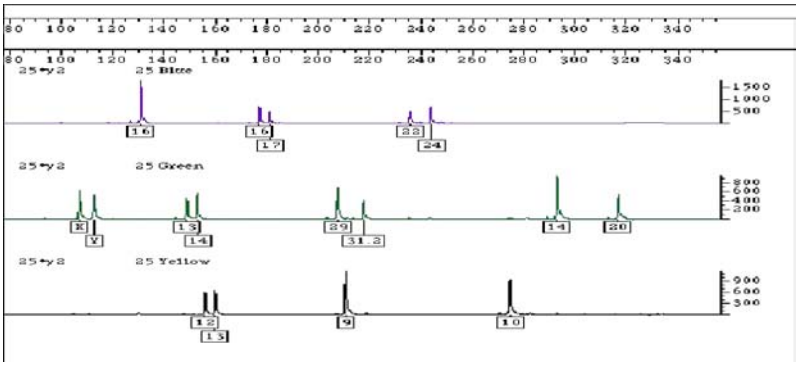

FIG. 3—*STR electropherogram of a DNA sample from the same bone as in Fig. 1. DNA was extracted with the CTAB + isoamyl alcohol-chloroform method, and further purified with a QIAquick spin column.*

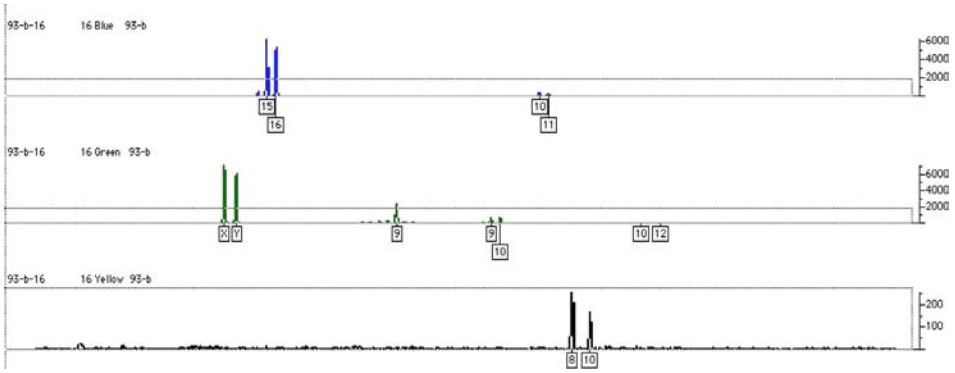

FIG. 4—*STR electropherogram of a DNA sample from a bone analyzed three years after death. DNA was extracted with the CTAB + isoamyl alcohol-chloroform method, and further purified with the DNA IQTM system.*

from a bone analyzed three years after death, using the traditional phenol/chloroform method and concentrated with a Centricon-100 column, with further purification using the DNA Clean-Up System. Figure 3 corresponds to the Profiler Plus STR electropherogram obtained when DNA from the same bone was extracted with the CTAB + isoamyl alcohol-chloroform method, and purified with the QIAquick method. STR electropherograms obtained when using template DNA extracted with the CTAB method were substantially better than electropherograms obtained using template DNA extracted with the alternative phenol-chloroform method.

Usefulness of the CTAB + Isoamyl Alcohol-chloroform Method for DNA Extraction from Bones with Different Preservation Conditions

We evaluated the usefulness of the new CTAB + Isoamyl alcohol-chloroform method for DNA extraction using casework bone samples with widely different preservation conditions. We analyzed bone samples 1, 3, 6, 8 and 9 years after death, bone samples soaked in water for approximately two years, and samples from bones burned after a traffic accident. Figures 4 and 5 show STR electropherograms of bone samples analyzed three years after death using the CTAB + Isoamyl alcohol-chloroform

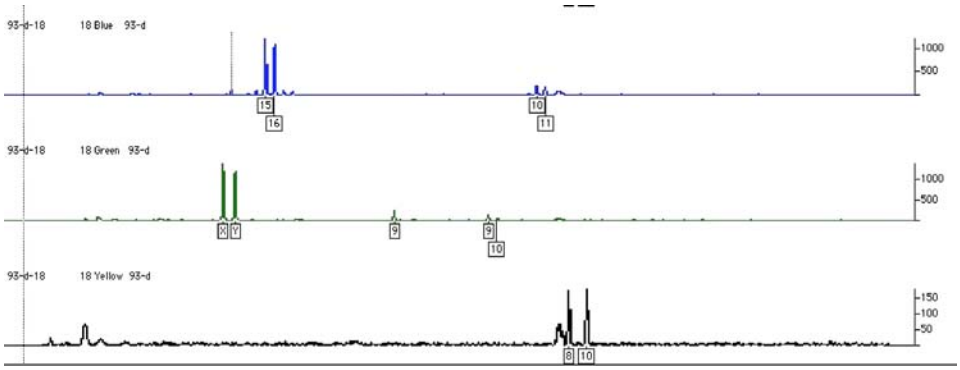

FIG. 5—*STR electropherogram of a DNA sample from a bone analyzed three years after death (same as in Fig. 4). DNA was extracted with the CTAB + isoamyl alcohol-chloroform method, and further purified with a QIAquick spin column.*

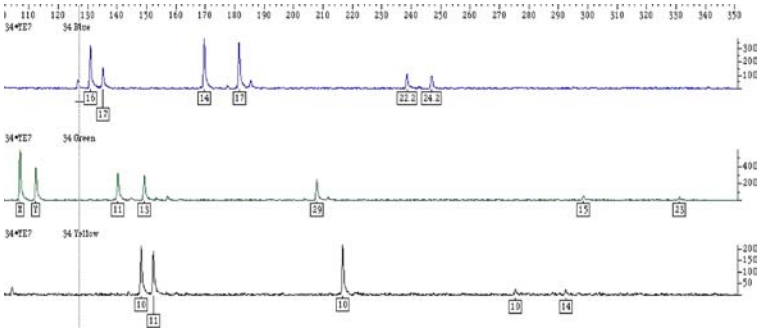

FIG. 6—*STR electropherogram of a DNA sample from a bone analyzed nine years after death. DNA was extracted with the CTAB + isoamyl alcohol-chloroform method, and further purified with a QIAquick spin column.*

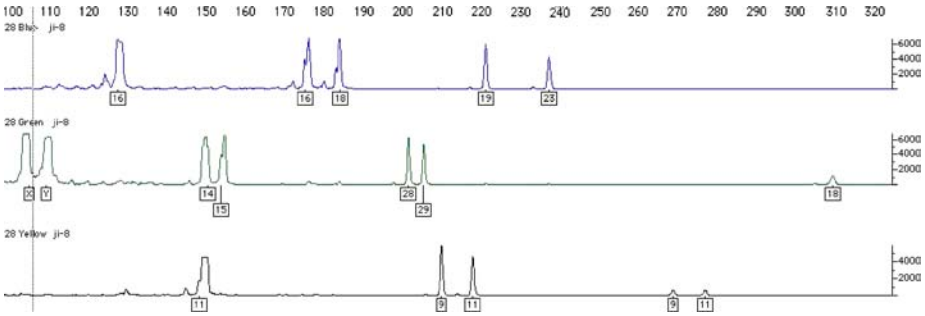

FIG. 7—*STR electropherogram of a DNA sample from a bone soaked in water for approximately two years after death. DNA was extracted with the CTAB + isoamyl alcohol-chloroform method, and further purified with a QIAquick spin column.*

extraction method, combined with two alternative DNA purification strategies: DNA IQ™ resin purification (Fig. 4) and QIAquick spin columns (Fig. 5). Figure 6 shows the STR electropherogram of a bone sample analyzed nine years after death, using the CTAB extraction method and purification with QIAquick spin columns. Figure 7 shows the STR electropherogram of a sample of bone soaked in water for two years, using the CTAB extraction method and purification with QIAquick spin columns. Finally, Figure 8 depicts the STR electropherogram of a sample of burned bone using the CTAB extraction method and purification with QIAquick. The genotype is detected with an ABI Prism 310 Genetic Analyzer.

Discussion

PCR amplification results are significantly affected by naturally-occurring impurities in aged or burned bone samples. Moreover, DNA in aged or burned bone samples is dramatically degraded, typically resulting in poor sample quantity and quality for STR examination. The major challenges when extracting DNA from old bones are to maximize DNA yield, to obtain DNA of high quality, to eliminate PCR inhibitors, and to minimize the possibility of outside contaminants.

The choice of extraction buffer is critical for the success of DNA isolation from old and damaged bones. Here we show that a mixture

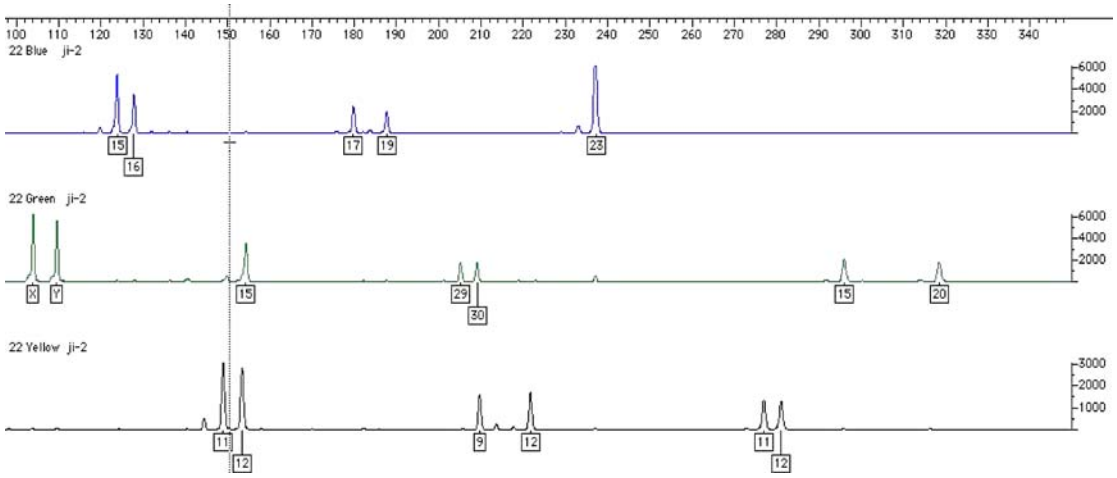

FIG. 8—*STR electropherogram of a DNA sample from a bone previously burned in a car accident. DNA was extracted with the CTAB + isoamyl alcohol-chloroform method, and further purified with a QIAquick spin column.*

of CTAB and 2-mercaptoethanol is very effective in disintegrating bone tissue, so that DNA is more completely released with this method. It has been reported that CTAB, a cationic detergent, can effectively disintegrate the cell wall and cell membrane (8). Therefore, CTAB buffer is an ideal reagent for DNA extraction from bone tissue. Using the CTAB DNA extraction method produces a higher DNA yield and substantially better STR genotyping results than the conventional phenol/chloroform method (Figs. 1–3) (7).

It is not uncommon in forensic casework that the evidence produces enough DNA to carry out multiplex STR genotyping, but PCR amplification fails due to the presence of PCR inhibitors. This is a consequence of PCR inhibitors that often co-purify with DNA. One potential strategy to solve this problem is to dilute the DNA sample, thereby diluting PCR-inhibitors presenting in the sample. However, aged and badly damaged bones contain limited amounts of DNA template, so this option is often not feasible. A better strategy to get high quality DNA is to use commercially available DNA purification kits. In the present study, we used commercially available QIAquick™ spin columns (8), which contain a silica-based membrane. The membrane is designed to bind DNA fragments that are larger than 100 bp but smaller than 10 Kb, while excluding nucleotides, proteins and salts. Based on the results presented here, the silica-based membrane appears to exclude PCR inhibitors and the substances that give rise to the brownish pigmentation observed in bone extracts. A good alternative to the QIAquick™ method is the DNA IQ System. In our experience, purification using the commercially available DNA IQ™ system (9,10) offers several advantages over the QIAquick™ system. Neither centrifugation nor rinsing is needed in this method. Therefore, it reduces the amount of DNA lost in the sample preparation. This method has been designed to isolate a maximum of 100 ng DNA. The DNA extracted from aged and burned bones is usually lower than this amount, so this method is well suited to DNA purification in these types of samples. We have systematically compared DNA extractions obtained using CTAB + isoamyl alcohol-chlorform purified with QIAquick™ spin columns, or alternatively, with the DNA IQ™ resin. Both methods can be used to obtain high-quality DNA template from aged bone (Figs. 4 and 5). However, the DNA IQ™ purification method is easier and faster to implement.

The number of osteocytes is limited in the samples. Primer-dimers frequently occur when template DNA is at a low concentration. These primer-dimer extension products act as a template for PCR amplification, and can take over as the main product of the reaction, leading to no visible yield of the target amplicon. In order to alleviate the problem of primer-dimers, we use "hot start" Taq DNA polymerase (11,12). This type of DNA polymerases remains inactive until high temperature is reached, requiring a pre-PCR heat activation step (95°C for 11 min). In addition to a dramatic reduction in primer-dimer artifacts, hot-start procedures provide other benefits, including room-temperature reagent assembly, increased yield and better specificity. Another modification introduced in our PCR reactions is that we did not add water to the sample. The absence of water increases the concentration of DNA, which is helpful in amplification and improves the success rate of DNA identification.

In summary, the DNA extraction method that we report in this paper is a stable, reliable, robust, and efficient strategy to use in the identification of aged, soaked and burned bones (Figs. 4 to 8). This method will help forensic scientists to accurately identify the victims of airplane, automobile, fire, and explosive accidents, as well as of criminal acts, such as murders and kidnappings.

Acknowledgments

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