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Development of a Quality, High Throughput DNA Analysis Procedure for Skeletal Samples to Assist with the Identification of Victims from the World Trade Center Attacks

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The attacks on the World Trade Center (WTC) Towers on September 11, 2001, represented the single largest terrorist-related mass fatality incident in the history of the United States. More than 2,700 individuals of varied racial and ethnic background lost their lives that day. Through the efforts of thousands of citizens, including recovery workers, medical examiners, and forensic scientists, the identification of approximately 1,500 victims had been accomplished through June 2003 (the majority of these identifications were made within the first 8-12 months). The principal role of The Bode Technology Group (Bode) in this process was to develop a quality, high throughput DNA extraction and short tandem repeat (STR) analysis procedure for skeletal elements, and to provide STR profiles to the Office of the Chief Medical Examiner (OCME) in New York City to be used for identification of the victims. A high throughput process was developed to include electronic accessioning of samples, so that the numbering system of the OCME was maintained; rapid preparation and sampling of skeletal fragments to allow for the processing of more than 250 fragments per day; use of a 96-well format for sample extraction, DNA quantification, and STR analysis; and use of the Applied Biosystems 3100 and 3700 instrumentation to develop STR profiles. Given the highly degraded nature of the skeletal remains received by Bode, an advanced DNA extraction procedure was developed to increase the quantity of DNA recovery and reduce the co-purification of polymerase chain reaction (PCR) amplification inhibitors. In addition, two new STR multiplexes were developed specifically for this project, which reduced the amplicon size of the STR loci, and therefore, enhanced the ability to obtain results from the most challenged of samples. In all, the procedures developed allowed for the analysis of more than 1,000 skeletal samples each week. Approximately 13,000 skeletal fragments were analyzed at least once, for a total of more than 18,000 analyses, and greater than 8,000 of the skeletal samples produced STR results (65%). The percentage of successful results was low in relation to previous mass fatality incidents involving airline disasters. However, when this same process was applied to the analysis of skeletal remains from the American Airlines Flight 587 disaster that occurred on November 12, 2001, the success rate was in line with expected results (ie, greater than 92% of the skeletal remains produced results). This illustrated the quality aspects of the procedure and the degree of degradation that had occurred for the remains of the WTC victims. For future mass fatality incidents, the quality, high throughput procedures developed by Bode will allow for more rapid DNA analysis of victim remains, more rapid identification of victims, and thus more rapid return of remains to family members.

Key words: *disaster planning; DNA; DNA, mitochondrial; forensic medicine; tandem repeat sequences*

DNA analysis has played a significant role in human remains identification for mass fatality incidents in the United States since the TWA Flight 800 commercial airliner crash in 1996. In the past seven years, DNA analysis has played a role in identifying victims in other commercial airline crashes, including Korean Air Flight 801 in 1997, EgyptAir Flight 990 in 1999, Alaska Airlines Flight 261 in 2000, and American Airlines Flight 587 in 2001 (see: *www.ntsb.gov*). Most of these cases involved less than 250 victims, with less than 1,500 biological samples requiring DNA analysis (1). In addition, the majority of the samples submitted for analysis were soft tissue (muscle, organ tissue, and skin), with a small percentage of bone and teeth. Furthermore, the length of time necessary to perform DNA analysis and identify the victims of these airline disasters (prior to the American Airlines Flight 587 crash) was many months, and sometimes years. Thus, when faced with the challenge of performing DNA analysis on more than 26,000 biological samples (including approximately 13,000 bone samples) recovered from more than 2,700 victims of the World Trade Center (WTC) attacks, conventional approaches were insufficient and the development of novel, high throughput methods was required.

Sampling and Extracting DNA from Bone

Historically, skeletal remains have been one of the most challenging biological samples from which to extract DNA (2-4). This is due to a number of factors, including the difficulty of preparing and sampling the skeletal material before DNA extraction,

and the complexities involved in removing polymerase chain reaction (PCR) amplification inhibitors. A number of the current procedures utilized by the forensic community for preparing and sampling bone samples for DNA extraction employ methods such as cutting and sanding with Dremel tools, and either liquid nitrogen or blenders/grinders to pulverize the bone fragments into a powder (5,6). Once the bone has been prepared and sampled, extracting the DNA currently involves the use of organic solvents or silica-based methods when performing forensic and identification casework (7-10). In some instances, modified versions of these protocols or additional steps have been required to obtain a result by short tandem repeat (STR) analysis (4,11). Nonetheless, while these sampling and extracting procedures are robust methods for obtaining DNA from bone, they are extremely time-consuming and labor-intensive, and therefore not amenable to the high-throughput requirements of the WTC DNA identification project.

In addition to the challenges of sampling and extracting DNA from bone, the co-purification of PCR amplification inhibitors has traditionally created its own complexities when attempting to PCR amplify the DNA recovered from skeletal remains. Therefore, when designing a procedure for this purpose, careful consideration must be given not only to the environmental inhibitors that often accompany bone samples found in soil or other metal ion-rich environments, such as humic acid (12-13), but also to the inhibitors that are naturally inherent in bones, such as collagen derivatives and calcium ions (14,15). Traditionally, ethylenediaminetetraacetic acid (EDTA) washes have been used to remove calcium ions, and a variety of DNA binding and/or filtration methods used to remove other inhibitors. Unfortunately, these methods have limitations that may result in the excessive loss of DNA (6) and have not been developed for a high throughput environment.

The Unique Nature of Skeletal Remains Projects

Each skeletal remains identification project has brought with it its own set of unique challenges due to the differing exposures of the bone to environmental insults. For instance, in looking at DNA identification projects from previous military conflicts, the environmental conditions in Southeast Asia were very different from those in the former Yugoslavia, and still different from those in Korea. Therefore, each of these DNA identification projects has encountered different challenges. In Southeast Asia, the remains of U.S. military personnel killed during the Vietnam War were exposed to high temperatures and moist soil conditions, making DNA analysis a considerable challenge. While occurring some 10-20 years earlier, the remains recovered in Korea following the Korean War were in excellent condition, and readily provided DNA analysis results due to the favorable environment (cooler and dryer). However, a large percentage of the remains returned from Korea and buried in the Punch Bowl cemetery in Honolulu, Hawaii, were exposed to excessive amounts of acidic preservatives that caused extreme degradation of the endogenous DNA, making DNA analysis impossible. In projects like these two from Southeast Asia and Korea, involving remains that were more than 30 years old, mitochondrial DNA (mtDNA) analysis is required, because STR analysis is rarely successful (5,16). On the other hand, the remains of civilians and soldiers killed in action in the early 1990's in the former Yugoslavia should have produced STR results in most cases. Unfortunately, the bones were exposed to soil conditions that were highly acidic, as well as deliberate attempts to degrade DNA using chemical agents. This limited the ability to perform STR analysis, and therefore, mtDNA analysis was required in a large percentage of cases (17).

In the same manner, the remains recovered from victims of the WTC disaster were exposed to a unique set of conditions that affected DNA recovery and analysis. A large percentage of the skeletal remains were highly fragmented due to the magnitude of the disaster and the sheer force of the collapse of the WTC towers (e.g., in some instances more than 200 fragments were identified as coming from the same individual). For a period of weeks or months, the majority of the remains had been subjected to intense fire and heat (>1,093 °C), as well as subsequent exposure to water as the fires were being extinguished and the recovery workers waited for the heat to dissipate. Given this exposure, some of the samples were highly charred to the point of being calcined or marbleized, while others were wet to the point of being excessively moldy. Surprisingly, a fair percentage of the remains were in pristine condition. Nonetheless, the condition of the WTC remains was the worst that these authors have seen in any mass fatality incident involving high velocity destruction. The majority of commercial airline disasters in the U.S. in the past seven years have occurred over water (e.g., TWA Flight 800, EgyptAir Flight 990, and Alaska Airlines Flight 261); however, some have occurred on land (e.g., Korean Air Flight 801 in Guam). Even though the sheer force of these airplane crashes caused extensive fragmentation of the skeletal remains, and the over-water disasters exposed the remains to potential water damage and mold, the condition of the remains in these cases was considerably better than the WTC remains.

Triage of Samples for DNA Testing

Decisions regarding the identification strategy may have a significant impact on the success of obtaining DNA results. The medical examiner responsible for the identification of the remains (and signing death certificates) must decide whether to analyze remains until all individuals have been identified, analyze only those remains that are anatomically recognizable, or analyze each remain recovered. Under normal circumstances, human remains recovered in a mass fatality incident are taken through a triage-like process in a medical examiner's facility by a qualified specialist (e.g., a forensic anthropologist or pathologist). This process allows for the assessment of the condition of the individual remains, and the suitability of the remains for DNA analysis. In doing so, a large portion of the remains are usually determined to

be unsuitable for DNA analysis (e.g., calcined and moldy samples). In the case of the WTC identification project, the medical examiner and his DNA advisors made the decision to test all remains recovered, and Mayor Rudy Giuliani reported this decision to the public. While this decision was met with skepticism and disagreement in the forensic community, it was based in part on the anticipated difficulty of finding remains from each individual lost in the WTC towers. As the project unfolded, it became quite clear that the decision to do so was correct. Nonetheless, this decision also meant that a larger percentage of the remains would not produce DNA results. Therefore, the task at hand was a daunting one: to develop a DNA extraction method that could handle the rapid processing of thousands of skeletal remains, many of which were in extremely poor condition, while maintaining the quality necessary for identifying as many of the WTC victims as possible.

Aim of the Study

This paper describes a new, high quality, high-throughput DNA analysis procedure for bone, which was used to assist in the identification of victims of the WTC disaster. The project has gone through two distinct phases thus far, in a focused effort to identify and re-associate as many fragmented skeletal remains as possible. Phase I involved rapid DNA extraction of approximately 13,000 bone samples by using the newly developed and validated procedure. Once the bone samples were extracted, they were carried through DNA quantification, PCR amplification, and STR analysis in a high-throughput manner. During Phase I, an assessment was made of the success rate of obtaining useful STR profiles when employing the newly developed extraction method. In doing so, it was determined that further modifications would be necessary to increase the quality and quantity of DNA recovered from the more challenged remains. Thus, Phase II of this project involved the re-extraction of more than 5,300 of the original 13,000 bone samples. This process utilized a modified version of the initial bone extraction procedure that was geared towards the reduction of potential inhibitors and the use of larger amounts of input bone material. Along with this modified extraction procedure, Phase II included the use of a novel pair of mini-STR multiplexes, which were designed to reduce the amplicon size of larger STR loci, thereby increasing the chances of obtaining complete STR profiles from the most degraded bone samples. While the efforts made during Phase II had a positive impact on the total number of useful STR profiles available for victim identification, and many of these profiles led to both re-association of additional remains and identification of new victims, approximately 45% of the WTC victims had not been identified at the time this manuscript was written. Therefore, it is anticipated that Phase III of this project may be initiated in the coming months. Phase III will involve the analysis of individual bone samples on a case by case basis, and may include the re-extraction of bone samples using more traditional methods, and the use of mtDNA analysis or other advanced techniques, such as single nucleotide polymorphisms (SNPs).

As of June 2003, Bode had spent 20 months completing Phases I and II of this project, processing and analyzing thousands of skeletal fragments and soft tissue extracts from samples recovered from WTC victims. From the initial challenge of developing a quality, high-throughput extraction procedure for bone to the improvements and developments made to the overall process along the way, we have gained valuable knowledge and experience from this project. It is hoped that by sharing this knowledge, Bode will help play a role in revolutionizing the way that remains from mass fatality incidents are analyzed in the future.

Material and Methods

Phase I

Due to the overwhelming number of samples to be analyzed, and the time frame in which the analysis needed to be completed, a new and more efficient process had to be developed. Each step of the process flow had to be redesigned, starting from the receipt of the samples through the return of STR profiles back to the OCME.

The majority of the samples received at Bode were contained in 50-mL conical tubes, each previously bar-coded by the OCME. Larger bone fragments were sent in sealed plastic bags, also bar-coded. The numbering system employed by the OCME had a DM (standing for Disaster Manhattan) followed by 01 for the year 2001, then five numbers to account for the possible collection of 99,999 samples (e.g., DM0123456). In some cases, a suffix was added to the number by the OCME to designate the second portion of a major remain (e.g., DM0123456-2). In other cases, Bode was instructed by the OCME to designate a tissue sample by adding a T to the number (e.g., DM0123456-T). Each sample was scanned into the custom-designed Bode Laboratory Information Management System (LIMS) database, and the electronic manifests received from the OCME were compared to ensure the receipt of the correct number and identity of specimens. Each shipment of samples received from the OCME was subdivided into groups of 84 before the extraction process. Bar-code labels for each group of samples were printed and used during the extraction process. The numbering system of the OCME was maintained throughout the testing process.

One of the most labor-intensive and time-consuming steps in the extraction process of skeletal elements is the initial cleaning and sampling of the bone. Due to the potential of having co-mingled remains, as well as the inhibitory effects of adhering dirt and debris, the outer surface of the bone must be cleaned before sampling. Prior to the WTC project, our method had involved removing the adhering tissue from the majority of the bone, and sanding the outer surface when possible. Once the surface was cleaned, a 1-2 g section of the bone was cut from the sample, and a powder-like form of the bone was generated using a blender-cup and blender. The blender-cup was then cleaned and bleached between each use. This entire process can take approximately 20 minutes per bone. Extrapolating this out for more than 18,000 extractions of approximately 13,000 bone fragments, and assuming two work stations for sampling, the time required for sampling each bone fragment would be more than 3,000 hours, 375 working days, or 1.4 working years.

In the newly developed method, the adhering tissue from a small section of the bone is removed and either a standard variable speed drill with a wide bore bit is used to remove a 2-3 mm layer of the outer surface of bone, or the bone is cleaned with a scalpel blade and then with bleach. If pre-drilling is used, the initial shavings are discarded as potential surface contaminants. A drill bit is then used to sample the inner portion of the bone. The shavings or powder are collected in a clean, bar-coded, anti-static weigh boat and taken to a transfer station. Between samples, the drill bits are cleaned with soap, bleach, and then rinsed with de-ionized water. Using this approach, the cleaning and sam-

pling process is reduced from approximately 20 minutes per bone to 3-4 minutes per bone. Thus, in two work stations and with seven staff members, 250 skeletal fragments can be cleaned and sampled in an 8 hour workday. The diagram in Figure 1 illustrates the process flow for cleaning and sampling of skeletal elements as conducted by Bode during the WTC project.

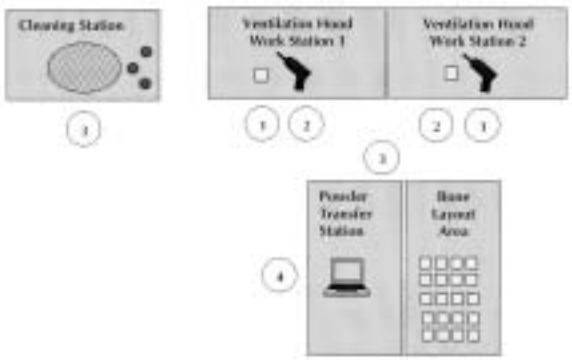

Figure 1. Diagram of the laboratory flow for preparing and sampling of the skeletal elements. 1 – driller; 2 – holder; 3 – cleaner/runner; 4 – powder transfer.

The process flow revolves around the two work stations located in ventilation hoods. The runners (station 3) ensure that all sample containers are associated with a matching weigh boat (bar-coded with the same DM01 sample number), bring samples and weigh boats to each of the work stations, make sure the DNA analysts in the hoods have the tools necessary to clean and sample the skeletal remains, and make sure supplies are stocked. The holder (station 2) assists the driller (station 1) with the cleaning and sampling process. Once a skeletal fragment is prepared and sampled, the shavings are taken to a transfer station where a DNA analyst (station 4) checks that the sample number on the container matches the number on the weigh boat. The analyst then wands the sample bar-code on the weigh boat into the LIMS, and the shavings are transferred to an appropriately labeled microtube. While this is occurring, the runner takes the used cleaning and sampling tools to a work station where a cleaner (station 3) washes the tools and prepares them for subsequent processing. Once a set of 84 samples has been transferred, they are then sent through the extraction process. This process flow was used to prepare, sample, and extract over 1,000 bones per week (Fig. 1).

To guard against the chances of mishandling a sample during the extraction process, a 96-well extraction format was developed. This extraction protocol was a combination of existing Bode methods and a modification of the QIAamp® 96 DNA Blood Kit (Qiagen Inc., Valencia, CA, USA). The extraction involves a lysis step with SDS and Proteinase K followed by the purification of the DNA using the Qiagen bind-elute technology. The amount of bone powder used for extraction was based on the capacity of the bind-elute membrane of the QIAamp 96 plate. An excess amount of sample will overwhelm the membrane and subsequently reduce the amount of DNA recovered. The diagram in Figure 2 illustrates the effects of adding too much starting material to the Qiagen system. In the validation studies conducted by Bode, this effect was reproduced.

Once the bar-code on the weigh boat is scanned into the LIMS, and approximately 25-50 mg of bone powder transferred to an appropriately labeled 1.5-mL microtube, the sample numbers for a 96-well tray are automatically populated on the extraction, quantification, amplification and STR analysis worksheets used downstream in the testing process. To the bone powder in each microtube is added 700 µL of extraction buffer (7 mL of extraction buffer solution consists of 70 µL of 1 mol/L Tris, pH 8.0, 140 µL of 5 mol/L NaCl, 700 µL of 0.5 mol/L EDTA, pH 8.0, 1.4 mL of 10% SDS, and 4.69 mL of sterile double distilled cold H_2O), and 50 µL of 20 mg/mL Proteinase K (Gibco BRL, Gaithersburg, MD, USA). Each sample is mixed briefly to suspend the bone powder and incubated overnight at 56 °C. The

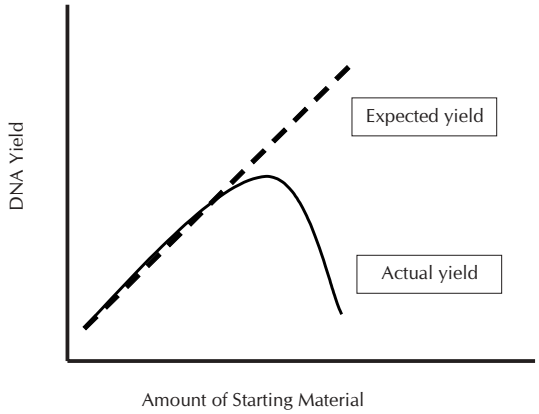

Figure 2. Diagram of effects of starting material on Qiagen system (figure reproduced from the Qiagen DNeasy Tissue Handbook, 05/2002).

next morning the samples are centrifuged for 5 minutes at 6,000 G to pellet the bone powder. Then, 600 µL of the lysate is transferred to the appropriate well of a QIAamp® 96-deep well block (S-block). To the lysate is added 600 µL of pre-warmed Qiagen Buffer AL and the solution mixed thoroughly with a pipettor. The 96-well S-block is covered with an AirPore tape strip and incubated at 70 °C for 10 minutes to solubilize any precipitate that may have formed. After incubation, 600 µL of 100% ethanol is added to each well and thoroughly mixed with the pipettor. A total of 850 µL of the lysate mixture is then transferred to a QIAamp® 96-well plate on an S-block. After all samples are transferred, the plate is covered with an AirPore tape strip and centrifuged at 5,600 G for 10 minutes. The remaining lysate is then transferred to the appropriate well of the QIAamp plate and the centrifugation repeated. The flow-through, which is collected in the S-block, is discarded and the S-block is rinsed and then placed again under the QIAamp plate. To each well is added 500 µL of Qiagen Buffer AW1. The plate is covered again with an AirPore tape sheet and centrifuged at 5,600 G for 5 minutes. This wash is followed by a second wash of 500 µL of Qiagen Buffer AW2 and centrifuged for 5 minutes at 5,600 G. The plate is then placed on a rack of 96 microtubes and incubated at 70 °C uncovered to thoroughly dry the membrane. After the plate has dried, 60 µL of Qiagen Buffer AE (preheated to 70 °C) is added to each well. The plate is covered one last time with an AirPore tape sheet and incubated at 70 °C for one minute. The covered plate is then centrifuged for 2 minutes at 5,600 G to recover the eluted DNA. The QIAamp plate is discarded and the microtubes capped.

Further development on DNA extraction from bone using the QIAamp 96-well plates was performed, resulting in modifications that increased the yield and purity of the DNA. The modifications included a 5 minute room temperature incubation of the samples after the addition of Qiagen Buffer AW2, a 1:10 dilution of the Qiagen Buffer AE, and an increase of the elution incubation from 1 minute to 5 minutes after the Qiagen Buffer AE had been added. It was also determined that additional DNA could be recovered if a second elution step was incorporated.

After extraction, the DNA for each sample is quantified. Due to the obvious potential for non-human DNA in the extracts, a human-specific quantification method was employed. The samples were quantified by using the BodeQuant method developed at Bode (*www.bodetech.com*). The BodeQuant method is ideal for this application because of its simplicity, use of a 96-well format, and ability to automate DNA concentration calculations. The basic design of the method involves three steps. First, 1-5 µL of each sample is amplified in a 96-well format with unlabeled TH01 primers (Promega Corp., Madison, WI, USA). In addition to the samples, a row of known concentration DNA standards, which range from 0.1 to 40 ng/µL, are amplified simultaneously. After amplification, Pico-green is added and the quantity of intercalated dye is measured using an automated plate reader (CytoFluor® Series 4000, Applied Biosystems, Foster City, CA,

USA). The fluorescent signal from each sample well is then compared to the standard curve created from the known DNA concentration samples, and an estimate of the human DNA concentration is automatically computed. These data can then be transferred to a Qiagen BioRobot 9604 (Qiagen, Germantown, MD, USA), or other robotic instrument to dilute each sample to the appropriate concentration.

Once diluted, each tray of bone extracts is ready for amplification and STR analysis. The samples were analyzed at the following STR loci: D3S1358, vWA, FGA, Amelogenin, D8S1179, D21S11, D18S51, D5S818, D13S317, D7S820, D16S539, TH01, TPOX, and CSF1PO, as well as the gender determining locus, Amelogenin. Amplification was performed with the Applied Biosystems ProfilerPlus and COfiler STR amplification kits (Applied Biosystems), as well as the Promega PowerPlex 16 kit (Promega Corp., Madison, WI, USA). Analysis of the amplification products was performed on the Applied Biosystems 3100 or 3700 Genetic Analyzer, and by using the Genescan and Genotyper analysis software.

The STR data generated for each instrument run underwent two independent analyses by qualified DNA analysts. Data tables were created from the analyses and compared electronically. If discrepancies were identified between the analyses, they were investigated and conflicts were resolved. The data were then run through a custom designed computer software program that looks for concurrence at overlapping loci, duplicate sample numbers, and creates an output file in a format used by the OCME for their database and matching program (MFISys; developed by GeneCodes Corporation, Ann Arbor, MI, USA; MFI – mass fatality incident). These data were also run through an MFISys database at Bode to look for discrepancies between samples with the same core sample number. For example, the program would compare two bone samples with the same core DM but different item number (ie, DM0123456-1 and DM0123456-2), as well as compare tissue and bone profiles assigned the same core DM (ie, DM0123456-3 and DM01236456-4-T).

Instrument run data, STR electropherograms, and data tables were burned onto CD ROM diskettes and sent to both the NY State Police (NYSP) in Albany, NY, and the OCME in New York City. The NYSP reviewed each set of data, inspecting the controls (extraction reagent negatives, amplification positives, amplification negatives, and allelic ladders). After the controls and samples were checked and accepted, the NYSP would inform the OCME, and the STR data would be uploaded into the OCME's master MFISys database (or other type of database). The data in MFISys was pooled with all other data (ie, sample profiles, personal effects profiles, family reference profiles, and mtDNA profiles) in order to make an identification either by direct agreement with a personal effect item or by performing kinship analysis.

Phase II

It was assumed before the start of the WTC project, and observed during the course of analysis, that the DNA obtained from a majority of the skeletal remains would be highly degraded. This became even more apparent when a comparison of results from the WTC remains was made to the AA Flight 587 remains. A typical degradation or inhibition pattern often seen in the STR results obtained from the WTC skeletal remains is shown in Figure 3. Therefore, in parallel to the initial extraction of over 12,000 skeletal elements during the first 8 months of the project, an advanced extraction method was developed to attempt to overcome potential inhibition and to increase the yield of DNA.

The advanced method involves the extraction of approximately 125-150 mg of bone powder (3-5-fold more material), and an initial soak of the bone powder in a 0.5 mol/L EDTA solution, pH 8.0 (to remove calcium ions and other potential inhibitors that will bind to EDTA). Unlike many protocols, the EDTA solution is not discarded prior to the addition of extraction buffer. It has been found that a large percentage of the DNA can be lost if the EDTA solution is discarded (6). Extraction buffer is added to the EDTA slurry, and digestion of the bone powder is initially achieved with collagenase (USB, Cleveland, OH, USA). This is followed by a typical Proteinase K digestion step. The details of the advanced extraction method are currently proprietary. However, it is anticipated that the method, and the lessons learned during development of the method will be published in the future. Nonetheless, over 2,500 bone specimens that originally produced low partial STR results, as well as over 2,800 bone specimens that produced no STR results were re-extracted using the advanced extraction protocol. This led to greater than 500 additional searchable profiles.

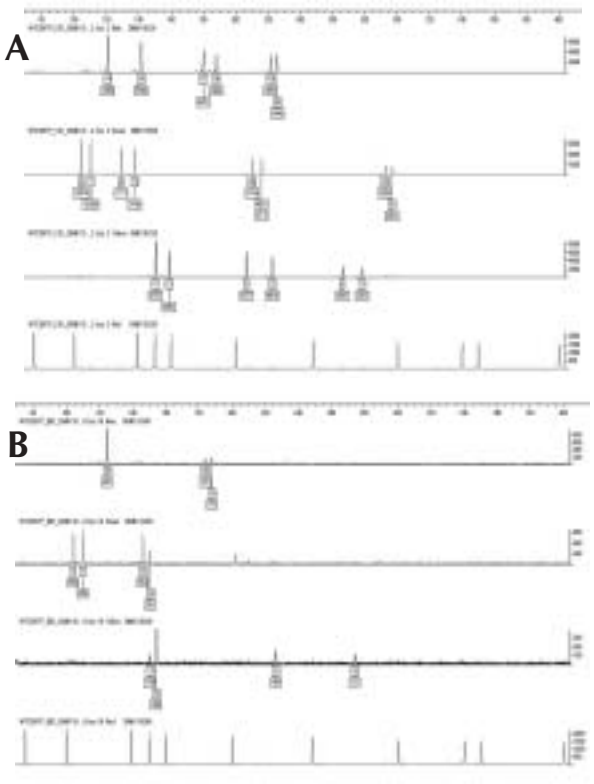

Figure 3. DNA analysis of skeletal remains from victims of the terrorist attacks on the World Trade Center on September 11, 2001. Full (**A**) and partial (**B**) short tandem repeat (STR) profile for ProfilerPlus showing DNA degradation.

Previous publications have cited the dramatic effect of shortening the fragment length of the STR product to obtain results from highly degraded samples (18-20). Since amplifiable DNA was present in the original extracts of WTC samples that produced low partial STR profiles, it was proposed that reducing the length of the larger loci would result in additional profile information. STR primers found in the literature, provided in a personal communication from John Butler of the National Institutes of Science and Technology, and novel primers designed at Bode were used to develop two operationally useful mini-STR multiplexes – BodePlex 1 and BodePlex 2. The target loci for BodePlex 1 and BodePlex 2 were the larger loci in ProfilerPlus and COfiler. BodePlex 1 consists of the D13S317, D21S11, D16S539, CSF1PO, and D7S820 loci. BodePlex 2 consists of the TPOX, FGA, and D18S51 loci, as well as the overlapping locus D7S820.

Standard PCR amplification conditions were followed for the BodePlex reactions, and 32 cycles of amplification were used (details of the amplification conditions and primer sequences are currently proprietary). As expected, a dramatic increase in sensitivity and ability to obtain results was observed. During the validation process, known DNA samples containing less than 100 pg of DNA were successfully amplified. Unfortunately, with increased sensitivity came the inherent low copy number issues previously described by Gill et al (18,21). "Allele drop-in" was occasionally observed during validation, as well as allelic drop-out. Therefore, the interpretation guidelines developed for the BodePlexes were very stringent. To account for the possibility of allele drop-in and allele dropout due to the stochastic effects caused by limited quantities of DNA, each sample was amplified and analyzed in duplicate. Any result that was not observed in both analyses was determined to be inconclusive and not re-

ported. In addition, to account for the possibility of allelic dropout, the minimum cutoff for homozygotes was raised to 400 relative fluorescence units (RFU), and the heterozygote cutoff was raised to 100 RFU. Once profiles were obtained and analyzed, customized software developed for the WTC project by Bode was used to compare and combine the duplicate results for BodePlex 1 and BodePlex 2.

Results

Phase I

The electronic accessioning process employed by Bode successfully maintained the numbering system provided by the OCME, making the tracking of samples and the reporting of STR profiles back to the OCME more efficient and convenient. Given the number of samples received, a second numbering system would have been impossible to manage. In future mass fatality incidents, the use of a single numbering system throughout the process would benefit all agencies involved.

The DNA extraction procedure for bone developed and used in Phase I was robust and effective, providing typical results seen with skeletal fragments from mass fatality incidents involving airline disasters. Table 1 provides a summary of the results for the 442 skeletal fragments of victims from the American Airlines (AA) Flight 587 disaster analyzed by Bode. The success rate of generating STR profiles from these samples was 92.1%, with 84.9% of the results considered useful profiles. Useful STR profiles were defined by the OCME as those that generated 7-13 reportable STR loci. High partial (HP) profiles were designated as 7-12 loci, and full profiles (FP) as complete 13-locus profiles. A profile of 1-6 loci was considered a low partial (LP) profile, and a sample yielding no results was designated as NR. The Amelogenin marker was not counted as a locus, so a profile with Amelogenin results alone was considered a NR sample. While the number of loci obtained in the testing process defined the category a sample would be placed into, only profiles that resulted in a likelihood ratio in favor of identification of greater than 10^{10} were considered by the OCME as positive proof of identification.

Table 1. Short tandem repeat (STR) results on skeletal remains from the AA Flight 587 crash that occurred in Queens, NY, on November 12, 2001

STR profiles	No. (%) of skeletal remains
Full (13 loci)	323 (73.1)
High partial (7-12 loci)	52 (11.8)
Low partial (1-6 loci)	32 (7.2)
No results (0 loci)	35 (7.9)
Total	442 (100.0)

Table 2 provides data for the 12,849 bones tested during Phase I of the project. As can be seen, the success rate of generating useful profiles was relatively low (44.6%), as was the total samples providing results (65.7%).

Table 2. Phase I short tandem repeat (STR) results on skeletal remains from the victims of terrorist attacks on the World Trade Center on September 11, 2001

STR profiles	No. (%) of skeletal remains
Full (13 loci)	3,500 (27.2)
High partial (7-12 loci)	2,233 (17.4)
Low partial (1-6 loci)	2,712 (21.1)
No results (0 loci)	4,404 (34.3)
Total	12,849 (100.0)

Phase II

Given the degraded nature of the WTC victim remains, advancements in the extraction protocol were necessary. The modified protocol allowed for a larger quantity of bone powder to be extracted, used EDTA to help remove CA^{2+} ions (known PCR inhibitors and components of the bone matrix), and used collagenase to help remove the major protein component of bone. As a result, this protocol allowed for greater recovery of extracted DNA, and the removal of a larger quantity of the inhibitors typically seen in bone. The vast majority of skeletal samples that had previously given LP STR results (2,528 of 2,712) were analyzed using the new protocol. In addition, a selected number of the samples yielding NR in Phase I, but through visual inspection seemed to be suitable candidates for analysis (2,807 of 4,404), were analyzed by using the new protocol (Tables 3 and 4). A total of 542 new FP or HP STR profiles were obtained from these experiments, bringing the total number of useful profiles to 6275, and the percentage of useful profiles up to 48.8%. Figure 4 illustrates the success of obtaining results from samples with LP results by using the original extraction method, and then with the advanced protocol.

Table 3. DNA analysis of skeletal remains from the victims of the terrorist attacks on the World Trade Center on September 11, 2001. Short tandem repeat (STR) results obtained by using the advanced bone extraction procedure on skeletal samples with low partial STR results in Phase I

STR profiles	No. (%) of skeletal remains
Full (13 loci)	99 (4.0)
High partial (7-12 loci)	220 (8.7)
Low partial (1-6 loci)	370 (14.6)
No results (0 loci)	1,839 (72.7)
Total	2,528 (100.0)

Table 4. DNA analysis of skeletal remains from the victims of terrorist attacks on the World Trade Center on September 11, 2001. Short tandem repeat (STR) results obtained by using the advanced bone extraction procedure on skeletal samples with no result in Phase I

STR profiles	No. (%) of skeletal remains
Full (13 loci)	55 (2.0)
High partial (7-12 loci)	168 (6.0)
Low partial (1-6 loci)	378 (13.5)
No results (0 loci)	2,206 (78.5)
Total	2,807 (100.0)

The use of the BodePlex 1 and BodePlex 2 multiplexes on the LP and NR DNA extracts from Phase II was far more successful than the results with ProfilerPlus and COfiler (Tables 5 and 6). The definition of useful profiles was modified for the number of loci in the BodePlex systems. FP's produced results at all 8 loci, HP profiles were 5-7 loci, and LP profiles were 1-4 loci.

When combining the ProfilerPlus and COfiler data generated at the time of the writing of this manuscript for the WTC skeletal remains analyzed (18

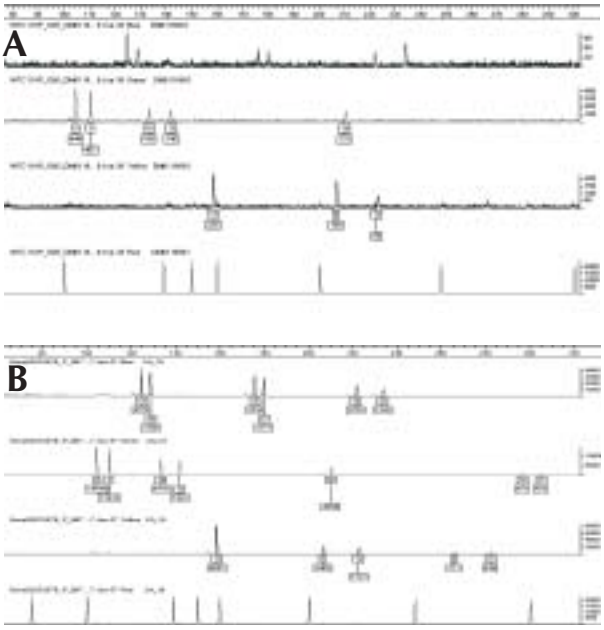

Figure 4. DNA analysis of skeletal remains from victims of the terrorist attacks on the World Trade Center on September 11, 2001. **A.** The low partial ProfilerPlus profile obtained for a sample by using the original extraction method. **B.** The full ProfilerPlus profile obtained for the same sample extracted by using the advanced method. The samples were run on the AB 3700, allowing for higher maximum values of relative fluorescence units (RFU).

Table 5. DNA analysis of skeletal remains from the victims of terrorist attacks on the World Trade Center on September 11, 2001. Short tandem repeat (STR) results obtained by using the advanced bone extraction procedure on skeletal samples with low partial STR results in Phase I, and the BodePlex 1 and BodePlex 2 multiplexes

STR profiles	No. (%) of skeletal remains
Full (8 loci)	456 (17.3)
High partial (5-7 loci)	581 (22.0)
Low partial (1-4 loci)	565 (21.5)
No results (0 loci)	1,031 (39.2)
Total	2,633 (100.0)

Table 6. DNA analysis of skeletal remains from the victims of terrorist attacks on the World Trade Center on September 11, 2001. Short tandem repeat (STR) results obtained by using the advanced bone extraction procedure on skeletal samples yielding no result in Phase I, and the BodePlex 1 and BodePlex 2 multiplexes

STR profiles	No. (%) of skeletal remains
Full (8 loci)	165 (6.7)
High partial (5-7 loci)	346 (14.1)
Low partial (1-4 loci)	547 (22.3)
No results (0 loci)	1,394 (56.9)
Total	2,452 (100.0)

,187), the WTC soft tissue extracts analyzed (5,163), the WTC family references analyzed (3,445), and the AA Flight 587 skeletal remains (442) analyzed by Bode, the total results reported to the OCME reached 27,237. Of these results, 10,871 fell into the useful category (FP and HP profiles), and another 4,102 were LP profiles.

The combined data for BodePlex results reported to the OCME as of the writing of this manuscript reached 6295. Of these results, 1,787 were considered useful for identification purposes. Unfortunately, only 239 of the 1,210 soft tissue extracts received from the OCME produced results with the BodePlex system (19.8%), compared to the results from skeletal remains (30.4%). Another 3,000 tissue extracts were being analyzed with BodePlex 1 and BodePlex 2 at the time of the writing of this manuscript. Therefore, it is expected that another 600 useful profiles will be reported to the OCME in June or July of 2003.

Discussion

A high quality DNA extraction procedure for bone was developed and incorporated into a high throughput laboratory testing process for the analysis of 12,849 skeletal fragments recovered from victims of the WTC disasters. Given that no triage of the remains was conducted to sort out those samples with little to no chance of producing STR results, the success of this project was remarkable. In addition, the reliability of the testing process was further validated through the analysis of skeletal fragments recovered from victims of the AA Flight 587 crash in Queens, NY. While the success rate of producing profile information for WTC remains was 65.7%, the rate of success for the AA Flight 587 remains was 92.1%. The success rate for AA Flight 587 was high even though very little triage was conducted to remove poor samples. This success rate was due in part to the fact that the remains were not exposed to the type of severe conditions the WTC remains were exposed to for weeks or months (e.g., temperatures of more than 1,093 °C), and also in part to the quality of the testing process. In the end, the vast majority of the data generated by Bode for the AA Flight 587 remains was returned to the OCME in approximately three weeks (the OCME staff performed the analysis of all soft tissue samples in the same time frame). As a result, the 265 individuals killed in this disaster were identified by the OCME and returned to their family members within 6-8 weeks of the incident. This is by far the fastest a case of this nature has been completed, and illustrates the robust nature of the entire identification process. The fact that this airline disaster involved the largest number of victims of any airline disaster in the U.S. in the past 10 years added to the significance of these accomplishments.

The results presented in this paper show that the BodePlex mini-STR systems are robust, sensitive, and effective. The goal of using the BodePlex systems was to obtain a higher percentage of results for the largest loci in the ProfilerPlus and COfiler multiplexes. The sensitivity of the BodePlex systems more than tripled the success rate of obtaining results when compared to the ProfilerPlus and COfiler systems. This sensitivity was gained not only through the reduction of the size of the STR loci, but also through the design of the primers and amplification parameters. At 100 pg or more of input DNA (15 copies of each allele), while some imbalance was noted, the results were reproducible. When attempting to amplify less than 100 pg

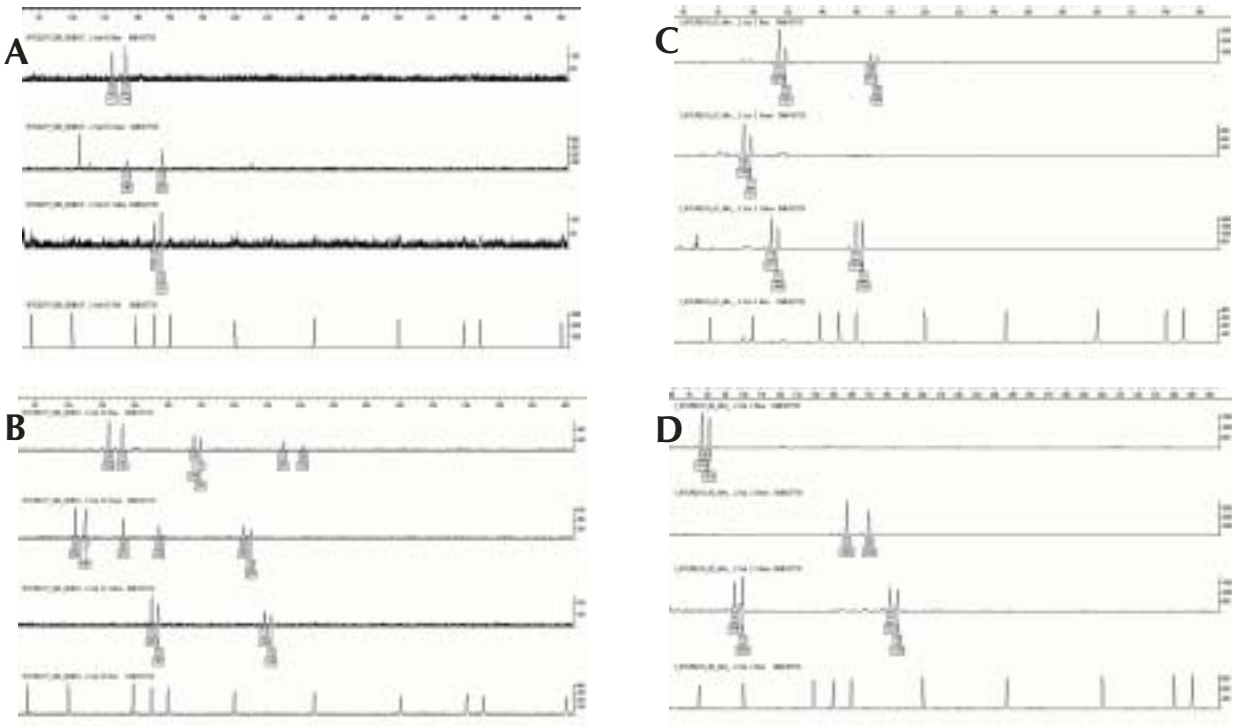

Figure 5. DNA analysis of skeletal remains from victims of terrorist attacks on the World Trade Center on September 11, 2001. **A.** The low partial ProfilerPlus profile for a sample obtained by using the original extraction method. **B.** The high partial ProfilerPlus profile obtained for the same sample extracted by using the advanced method. **C.** The BodePlex 1 full profile for the same extract as in B. **D.** The BodePlex 2 full profile for the same extract as in B.

of DNA, both allelic drop-in and dropout were observed. While this could present a problem with interpretation, the concept of low copy number (LCN) PCR has been discussed and applied on forensic casework (18,21). As long as the interpretation criteria for the analysis of LCN-like PCR systems are reliable and robust, the results of analysis can be accepted with confidence. The BodePlex systems provide a sensitive assay by reducing the amplicon size of the STR loci and enhancing the amplification parameters. This LCN-like PCR system involves conservative interpretation criteria (e.g., raised RFU cutoffs and the requirement for duplication of results), which have produced reliable results in the WTC project. Therefore, the BodePlex method may prove to be a valuable tool in forensic casework in the future for challenged samples.

Of interest is the comparison of results for samples producing LP profiles in Phase I to results for the same samples in Phase II. A fairly high percentage of the remains produced NR in Phase II (72.7%). However, it turns out that more than 55% of the LP results from Phase I involved only 1-3 STR loci. Given this fact, and that the best portion of many of the remains (e.g., ribs and other small fragments) were almost consumed during the Phase I analysis, it is understandable that these samples saw a reduction in the quality of the results during Phase II. As expected, a significant percentage of the LP samples that produced NR in Phase II using ProfilerPlus and COfiler did produce results using the BodePlex systems. Figure 5 illustrates the progression of a single sample from a Phase I LP result, to a Phase II HP result, and finally to a BodePlex result that completes the 13-locus STR profile. After two different extraction methods, and the use of mini-STR analysis, a complete 13-locus profile was reported back to the OCME.

While the number of STR loci obtained in the testing process for each WTC remain was used to define the category a sample was placed into (ie, FP, HP, LP, or NR), only profiles that resulted in a likelihood ratio in favor of identification of greater than 10^{10} were considered by the OCME as positive proof of identification. This continues to challenge the identification process, as many of the STR profiles have produced likelihood ratios of between 10^5 and 10^9. Given that approximately 1,500 of the victims have been identified thus far, and that the victims of the WTC disaster can be considered a relatively closed population, it may be that the likelihood ratio requirement will be reassessed in the future to allow for more individuals to be identified. To supplement the STR results, mtDNA data has been generated on thousands of remains and on maternal family members of the victims. Bode has played a role in this process and we expect that remains will be identified in the future using a combination of STR and mtDNA results. In addition, as the pool of missing victims decreases, and if an mtDNA profile is developed that is quite rare in the population database, it may be that mtDNA alone can support the identification of a set of remains. These suggestions are simply speculation on our part of the potential future course of action.

As the single largest forensic identification project in the history of the United States, the WTC disaster has given those involved unprecedented challenges and numerous obstacles to overcome. The staff of the OCME in NY City is to be highly commended for their dedication to this mission. The level of creativity applied to the project, the level of support given to the project, and the unwavering commitment to complete the project have been unmatched in the forensic community. Given the number of victims and the daunting number of samples tested, along with the poor condition of the remains recovered, this project has unquestionably been a success. While there may be victims of this terrible disaster that will remain unidentified, it will not be for a lack of perseverance.

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