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Extraction of Challenging Forensic Samples

Using the MicroGEM DNA Extraction Kit

A Thesis Presented in Partial Fulfillment of the Requirements for the Degree of

Master of Science in Forensic Science

John Jay College of Criminal Justice

The City University of New York

Falyn Vega

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Extraction of Challenging Forensic Samples

Using the MicroGEM DNA Extraction Kit

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This Thesis Has Been Presented to and Accepted by the Office of Graduate Studies, John Jay College of Criminal Justice in Partial Fulfillment of the Requirements for the Degree of Master of Science in Forensic Science.

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Abstract

In forensic science, DNA extraction can be a tedious and resource-intensive process. Extraction with Proteinase K is an industry standard but has its drawbacks, such as requiring multiple ionic detergents and washing steps. MicroGEM has developed a new enzyme called *forensic*GEM that is temperature-dependent and compatible with mesophilic enzymes, offering complete DNA extraction in about 20 minutes in a single tube, limiting contamination, loss of sample, and working time. *Forensic*GEM can extract DNA from highly degraded samples, potentially leading to more complete STR profiles. Highly degraded tissue and bone samples were collected and extracted with the *forensic*GEM kit, altering different parameters to assess the efficiency and potential uses of *forensic*GEM. The STR profiles of samples extracted with *forensic*GEM were compared to those extracted with a standard organic extraction. Half of the degraded samples extracted with *forensic*GEM had detectable DNA, with the highest success rate observed for bone samples. Success with bone profiling was notable given that there was much less sample input for *forensic*GEM (10 mg) compared to the organic extraction (2 g). The *forensic*GEM kit yielded a 22-locus and a 15-locus profile on two highly degraded samples from the 9/11 World Trade Center attacks. The bone preparation method of scraping yielded higher DNA quantities and better-quality profiles compared to samples treated with the standard method of milling. Future work will focus on further investigation of the bone scraping method and continued optimization of experimental parameters in the MicroGEM extraction protocol.

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1. Introduction and Literature Review

For forensic scientists, it can be extremely important to conserve the samples being worked with as they may be low in quantity, degraded, and subjected to environmental damage. It is vital to be able to extract DNA from even the most challenging samples. The general process of DNA extraction involves first lysing the cells and solubilizing the DNA, after which unwanted macromolecules, lipids, RNA, or proteins are eliminated through chemical or enzymatic means (Gupta, 2019). DNA extraction has historically been a tedious process: entailing the use of multiple steps that take a longer time, the use of inhibitory chemicals, and the excessive use of plastics and consumables. Organic and solid phase extraction methods take many hours to perform and often result in loss of sample due to multiple transfer steps and abrasive washes with sodium dodecyl sulfate (SDS), mercaptoethanol, or dithiothreitol (DTT). Recent advancements have demonstrated that extraction efficiency may be improved with shorter protocols, new chemistries, and fewer transfers.

As previously mentioned, there are several methods for DNA extraction, including organic extraction, solid phase extraction (SPE), and Chelex extraction. These methods differ in their underlying principles, steps involved, and advantages and disadvantages. A common technique is organic extraction, which separates DNA from other cellular components using a series of organic solvents. Cell lysis, protein and other contaminant removals, DNA precipitation, washing, and resuspension of the DNA are the fundamental steps in the organic extraction method (Gupta, 2019). The organic extraction technique is typically successful and economical, and it can produce DNA of high

quality. However, it is time-consuming, labor-intensive, and uses abrasive chemicals. Organic extractions can be utilized for both tissue and bone samples.

Solid phase extraction (SPE) is another technique for obtaining DNA. In this procedure, DNA is separated from other biological components using a solid-phase matrix. In SPE, DNA is bound to a solid-phase matrix, impurities are removed, and then the purified DNA is eluted. Magnetic bead-based techniques, which utilize magnetic beads coated with DNA-binding substances for the isolation of DNA, are variations of SPE (Li, 2021). SPE techniques can be automated, need less time than organic extraction, and can be very effective. Yet, this method might not produce DNA of high quality, may result in the loss of analyte due to many washes (i.e. DNA), may be expensive, and may call for special equipment. Similar to an organic extraction, this extraction type can be used for both bone and tissue samples.

Another method is the Chelex extraction technique. In this method, a chelating resin called Chelex is used to bind to metal (Mg^{2+}) ions in the cell. Cell lysis, inactivation of DNA-degrading DNases, and contaminant removal by washing are the fundamental processes of the Chelex extraction procedure. Small-scale DNA extractions can be done using the relatively inexpensive and straightforward Chelex extraction technique (Gautam, 2022). However, it may not produce DNA of high quality, may need further purification procedures, and may not be appropriate for complex samples -- such as bone, a sample with a hardy matrix.

To address the extraction issues faced by scientists, MicroGEM developed a DNA extraction kit that utilizes a proteinase that is not as abrasive as Proteinase K and is famed

to have a faster reaction time. The proteinase utilized was found in a hydrothermal vent at Mount Erebus (Saul et al., 1999). The enzyme is stabilized by divalent cations and lyses cells and degrades proteins while leaving nucleic acids intact. *Forensic*GEM is a temperature-driven extraction, with the enzyme's optimal activity achieved at 52˚C. As a result, the enzyme is compatible with mesophilic enzymes and is thermostable which lowers the chance of the enzyme being denatured and thus inactive (Saul et al., 1999). Typical DNA extraction with Proteinase K takes place between temperatures of 20-65˚C. It is typically not possible to mix enzymes with Proteinase K because it will destroy any other proteins or enzymes present. However, with the use of *forensic*GEM, DNA extraction takes place at around 75˚C without the use of abrasive ionic detergents. At this temperature, proteins are denatured, and nucleases are inactivated. The temperature-driven workflow utilizes this characteristic to improve DNA extraction. *Forensic*GEM is inactive below temperatures of 45˚C and above 95˚C. At 95˚C, the enzyme is inactivated and eventually self-digests. The advantage of this step is that DNA is released only at temperatures where nucleases are inactive and hydrolyzed by the proteinase (Moss et al., 2003). In addition, one of the other reagents used, Histosolv, is eliminated during the 75˚C incubation step by the *forensic*GEM. The different buffers made by MicroGEM depend on the sample from which DNA is extracted. The Blue and Red buffers are generally recommended for samples that are less susceptible to degradation and known to be abundant in DNA quantity. In this experiment, we utilize the ORANGE+ buffer which is specially formulated to overcome biological inhibitors found in bones.

With *forensic*GEM, the extraction process is shortened, requiring only 20 minutes to reach completion. This extraction technology is relatively new and the manufacturer

has not yet optimized the parameters for work with severely degraded tissues and bones. Lounsbury et al., (2012) altered the incubation times as well as the amount of enzyme used in their MicroGEM reactions to optimize results. According to *forensic*GEM's manufacturer, increasing the incubation time can increase DNA yield, but in turn, will also increase the level of inhibition. Areas of the protocol subject to modification include incubation times, the amount of enzyme added, bone preparation technique, and extract purification. These modifications are described in section 2, titled "Materials and Methods."

Inhibition is another necessary consideration when performing forensic DNA profiling. PCR inhibitors in forensic procedures are biological inhibitors most often found in bone samples. The known inhibitors within bones and connective tissues are collagen and calcium ions. Collagen binds to the DNA template which alters the way Taq polymerase can bind to the template itself. Burkhart et al., (2022) described a study in which collagenous material was found to be an inhibitor of Taq polymerase. Also found within bones, calcium ions inhibit by being a competitive inhibitor of Mg^{2+} which is required for Taq polymerase to be activated (Opel et al., 2010). An additional consequence of calcium is that its presence may lead to inaccurate quantitative measurements when quantitative PCR is used. Calcium reduces the efficiency of the amplification in qPCR thus reducing the amount of product (Opel et al., 2010). Another possible inhibitor to consider is humic acid, which is predominantly found in soil and thus could be present in any specimens that are found partially or completely buried. Humic acid inhibits PCR by binding to a specific sequence on the DNA itself, limiting the template available to be amplified (Opel et al., 2010). Additionally, the traditional

DNA extraction procedures with Proteinase K and phenol/chloroform contain known PCR inhibitors. Many inhibitors may be removed using a post-extraction purification step. Compared to other extraction methods, *forensic*GEM may have a superior ability to overcome inhibitors that remain even after purification.

Another factor that was altered was the bone preparation technique. Bone is historically one of the most challenging substrates from which to develop a DNA profile - the DNA enclosed within the bone may be well protected, but it is also very difficult to access. Perhaps this explains why many recent studies have focused on improving DNA extraction from bone. Typical preparation of bone for DNA extraction involves turning it into a fine powder. To do so, a cryogenic freezer mill is utilized. This instrument uses liquid nitrogen to maintain extremely cold temperatures, thus making bone more brittle and therefore susceptible to mechanical breakdown. The bone is placed in a capped cylinder with a metal impactor. Once inside the freezer mill, the cylinder containing the bone is shaken rapidly. The mechanical disruption combined with the cool temperature helps to pulverize the bone into a fine powder (NYC-OCME, 2017). Today, bone milling is widely used in forensic science and other fields where DNA extraction from bones is required.

Recent research has indicated that there may still be room for improvement in bone preparation techniques. In a study conducted by Sahoo et al., (2021), bone samples that were scraped, as opposed to milled, that underwent DNA extraction had a much higher success rate of obtaining a complete DNA profile. The motivation behind this new preparation technique is that DNA extracted from bones is held within osteocytes which tend to be concentrated toward the surface of the bone (Dallas et al., 2013). Osteocytes

are the cells within bones that contain DNA. As a result, scraping a bone yields a higher percentage of osteocytes compared to a milled bone where the ratio of osteocytes would be lower since the entire depth of the bone was ground. This new technique could be very promising for degraded bone samples where milling processes were previously unsuccessful in obtaining a full DNA profile. To date, there has been no published research on scraped bones extracted with the *forensic*GEM kit.

Lastly, to assess the extraction of MicroGEM's kit, one sample set was processed without the purification step to determine exactly how the DNA results compared to the prior *forensic*GEM runs that consequently were purified. The purification of DNA is an integral step to ensure proper performance in downstream applications. To ensure a greater yield and greater quality of the DNA, purification is a process in which any contaminants -- such as unwanted nucleic acids, proteins, inhibitors, and more -- are removed from the target DNA. Different methods of DNA purification can be used: these methods can be physical, chemical, or enzymatic. The general isolation procedure includes five main steps: disrupting the cellular structure and thus creating a lysate, separating the DNA from insoluble materials and unwanted cell debris, binding the target DNA to a matrix for purification, washing the contaminants away from the matrix, and lastly -- eluting the DNA (Promega, 2023). For the final experiment, the purification step on the EZ1 was not performed.

For this study, the EZ1 Advanced XL by QIAGEN was utilized for DNA purification. The EZ1 is an automated instrument that can perform DNA purification on up to 14 samples at a given time in a span of approximately twenty minutes. The EZ1 Advanced XL has an extensive application range: the instrument can purify DNA from

blood, tissues, human identity, and forensic samples. The EZ1 purifies DNA, RNA, and viral nucleic acids through the use of EZ1 Kits. Prefilled, foil-sealed reagent cartridges are included in the EZ1 Kits, and these stay sealed until the instrument door is closed and the protocol run is initiated to reduce contamination risk. Nucleic acids that have been purified are of high quality and are prepared for usage in downstream applications immediately. When the run is finished, an ultraviolet lamp is utilized to decontaminate the inner surfaces of the EZ1 instrument. The purification of nucleic acids can be completed efficiently with automated sample preparation. Reliable sample processing and the lack of manual sample handling ensure both accuracy and safety. (QIAGEN, 2023).

Tissues and bones may often be crucial pieces of evidence that lead to the identification of decomposed human remains. This can sometimes be strictly for identification purposes, as in missing persons cases, or it can also be for criminal purposes. In instances of an unidentified homicide victim, the identification of the decedent may be the catalyst that helps start the investigative process. For this reason, there is a strong need for an efficient extraction method that is specifically optimized for handling degraded samples. The need for rapid and efficient extraction is even more apparent with a large-scale mass disaster such as the September 11th attack on the World Trade Center (WTC). In the entire history of the United States, 9/11 is known to be the largest forensic investigation yet. The September 11th attacks "claimed 2,753 lives; [and] more than 220 other people were killed in Washington, DC, and outside of Shanksville, Pennsylvania" (Hanna et al., 2021). When these tragedies occur, there is a need to promptly and accurately identify remains. This effort often represents a nearly insurmountable challenge, given the extremely poor condition of recovered remains. To

appreciate the difficulty of obtaining DNA profiles from WTC remains, one must understand the unique challenges presented by such an environment. On September 11th, American Airlines Flight 11 struck the North Tower, and United Airlines Flight 175 struck the South Tower (National Transportation Safety Board, 2002). Both planes were westbound flights and were fully loaded with fuel. Collectively the two planes had 24,000 gallons of jet fuel. Both towers collapsed in less than two hours due to fire-induced structural failure (Miller, 2002). Most of those who perished were at or above the aircraft's point of impact (National Institute of Standards and Technology, 2021). The fires at Ground Zero burned for 100 days in a disaster scene that spanned 16 acres (*Guardian News and Media*, 2001). Along with the Towers, numerous other buildings were destroyed or damaged. The Towers that once stood 1368 feet high were reduced to a 70-foot-high pile of rubble (FEMA, 2002).

All debris collected at Ground Zero was transported by barge to the Fresh Kills Landfill on Staten Island. There, a massive sifting operation commenced. Ultimately over twenty thousand bone and tissue fragments were recovered. From the beginning, there were many obstacles to successful DNA profiling. Probably the most significant was that the remains were very small and had undergone severe degradation. To provide some context, one must carefully consider the unique environment at Ground Zero. Thermal images collected by the U.S. Geological Survey recorded a surface temperature of 1340 F at the WTC rubble pile (Clark, 2016). The source of this heat was a much hotter fire deep below the surface. Because of this extremely hot environment, most of the WTC remains were recovered as bones, some so severely degraded that they had essentially been cremated. Although DNA profiling has been successfully achieved on samples dating

back 100,000s of years, the extremely unforgiving conditions at Ground Zero would have undoubtedly caused irrevocable damage to the WTC remains. Excessive heat, moisture, and pressure - all elements present at Ground Zero - have made it difficult to make positive identifications.

The New York City Office of Chief Medical Examiner (OCME) has made an ongoing commitment to families of victims of the WTC attacks that every effort will be made to identify all those who perished. This is not an unreasonable goal when one considers the extraordinary scientific developments that have occurred in the years since the WTC disaster. While some question the benefit of ongoing testing, it is “a sacred obligation” to the agency and it is a matter that has been quietly investigated by seasoned analysts for many years. In a 2021 interview for *The New York Times*, former New York City Chief Medical Examiner Dr. Barbara Sampson stated, “We committed back then to do whatever it takes, as long as it takes and that’s what we’ll continue to do” (Kilgannon, 2021). Mark Desire, Assistant Director of Forensic Biology at the OCME, in an interview for *NorthJersey* stated in 2018 that “our commitment to making these identifications is as great today in 2018 as it was in 2001” (Shkolnikova, 2018). Twenty-one years after the disaster, the OCME has continued to promise to families of victims that the most updated and advanced technologies and techniques will be applied to identify the victims. Approximately 40% of WTC victims are still unidentified, although more identifications are projected for the future (Adams et al., 2022). The culmination of this study was to apply the MicroGEM technology to WTC remains intending to aid the victim identification effort.

2. Materials and Methods

2.1 Sample Preparation

2.1.a Tissue Samples

Tissue samples, IRB approved, were collected from five decedents placed in the open at the University of Tennessee Forensic Anthropology Center (UTFAC) and were granted by the NIJ Funding Award 2014-DN-BX-K014 for NYC-OCME research. Tissue samples were collected after sixty days of exposure. Samples were stored frozen and shipped to the NYC Office of Chief Medical Examiner on dry ice where they were then stored at -80°C. When the samples were ready for examination, they were thawed and then cut to approximately 1-2 mm^3 for use in this study. Table 1 lists the array of samples utilized.

Table 1. Description of the degraded muscle samples in terms of donor, weight, date collected and days since, and sample location.

Individual	Sample Number	Approximate Weight (g)	Date Collected	Days Since	Sample Location	Age	Sex
F	T1-F11	0.076 0.057	07/09/15	60	Right lateral-posterior leg at knee - skin	53	F
G	T2-G12	0.045 0.034	07/11/15	60	Left posterior midsection, medial side - skin	79	M
I	T3-I42	0.062 0.043	10/12/15	60	Right posterior shoulder	42	M
J	T4-J21	0.032 0.039	11/21/15	60	Left lower buttock, lateral	90	M
L	T5-L13	0.072 0.028	03/08/16	60	Right posterior upper tricep	64	F

2.1.b Bone Samples

Bone samples were taken from specimens collected at the New York City Office of Chief Medical Examiner that were later designated as waste to be used for research purposes. Bone samples initially underwent a multi-step cleaning process involving

scraping of soft tissue, brushing, rinsing, and sonication. The bone was rinsed with water and placed on a weigh boat in a 56˚C incubator for 3 hours or until completely dry. Large bone fragments were then cut under a biological hood into approximately 5x5x5mm size pieces with a Dremel tool (10.8 V) to obtain a weight of about 2-3g.

A 5% tergazyme solution was then prepared in a 50 mL conical tube. The powder was first suspended with inversion and then transferred to an Erlenmeyer flask with a stir bar. This was then placed on a heat plate and stirred to ensure the powder was dissolved forming a clear solution. Tergazyme is an enzyme-active powdered detergent used to clean off exogenous cells or tissue from the exterior of the bones. Cut bones were placed into 50mL tubes and covered with the 5% tergazyme solution. The tubes were then placed into the tube rack within a sonicator (Ultrasonic Cleaner, Symphony VWR, Radnor, PA) water bath. Once a weighted ring was placed over the rack to submerge samples, the sonicator ran for 30 to 45 minutes, ensuring the water level was 1-2 inches from the top. Following sonication, the bones were again placed in a 56˚C incubator overnight to ensure complete drying. Cut bone samples were then milled in the SPEX Certiprep 6870 Freezer Mill (SPEX Sample Prep LLC, Metuchen, New Jersey) until sufficiently pulverized. After pulverization, the bone samples were placed into 50 mL conical tubes. They were then measured out to approximate weights of 0.09 g - 0.11 g.

The seventh experiment was done to assess how different bone preparation techniques could affect DNA yield. For this experiment, the bone samples were prepared by scraping. In this protocol, the same sample bones were obtained after being cleaned, sonicated, and cut, before the milling step. A scalpel was used to scrape the exterior surface of the bone until enough bone scrapings were collected (see figure 2). For each of

the 5 bone samples scraped, an aliquot between 0.09-0.11 grams was obtained for use with the *forensic*GEM reagents. Table 2 describes the average approximate sample weight for each of the different experimental replicates utilized.

Table 2. B1, B2, B3, B4, and B5 bone replicate samples and their respective approximate weight for each experimental replicates 1-8.

Bone Sample	Average approximate sample weight for each experimental replicates 1-8 (g) ± standard deviation
B1	0.01088 ± 0.0003
B2	0.01075 ± 0.0004
B3	0.01013 ± 0.0002
B4	0.01048 ± 0.0003
B5	0.01038 ± 0.0004

2.2 Organic Extraction Protocol

To accurately assess the efficiency of the *forensic*GEM extraction, all of the 10 samples were also extracted following standard organic extraction protocol. The New York City Office of Chief Medical Examiner's (NYC-OCME) protocol was followed for organic extraction. Following the guidelines, 2 grams of bone dust were used. Per sample, 2370 μL of organic extraction buffer, 300 μL of 20% SDS, 120 μL of 1.0 M DTT, and 210 μL of Proteinase K (20 mg/mL) were combined to create the mastermix. 3000 μL of mastermix was added to each bone sample, vortexed, and sealed with

parafilm. The bone samples were then incubated in a 56˚C heat block (Isotemp Incubator, ThermoFisher Scientific, Waltham, MA) overnight.

The samples were then vortexed and centrifuged for 5-10 minutes at 1000 RPM. Eppendorf Phase Lock Gel (PLG) tubes were obtained, one per sample, and centrifuged at max speed for 30 seconds. Microcon filters (Millipore Sigma, Burlington, MA) were obtained for each sample and 100 µL of TE was added to the filter side of each concentrator. 400 µL of Phenol Chloroform Isoamyl Alcohol (PCIA) was added to each sample. The supernatant of the sample was pipetted to the PLG tubes obtained, and the tubes were shaken vigorously by hand. The tubes were then centrifuged for 2 minutes at maximum speed. The aqueous phase was then transferred to the Microcon concentrator and spun for 12-24 minutes at 2500 RPM, ensuring the fluid had passed through the filter. The concentrators were placed into a new collection tube and 400 µL of TE was added to the filter side of each Microcon concentrator. Tubes were then spun for 12 minutes at 2600 RPM. 10-20 µL of TE was then added to the filter side of each Microcon concentrator and the sample reservoir was inverted and then placed into a new collection tube and then spun at 3500 RPM for 3 minutes. Samples were transferred to new 1.5 mL microcentrifuge tubes and stored at 2 to 8˚C.

2.3 ForensicGEM DNA Extraction from Bones and Tissues

Eleven samples were run for each experiment: 5 tissue samples and 5 bone samples in addition to a singular extraction negative on behalf of each experiment. The *forensic*GEM DNA extraction kit provided by MicroGEM (Charlottesville, VA) includes the following reagents: 10x ORANGE+ Buffer utilized for tissues, BLUE+ Buffer for general use, *forensic*GEM, and Histosolv which is utilized for high protein samples or if

there is difficulty extracting DNA in a hardy matrix -- such as bone. The reagents employed for this study include DNA-free water (Invitrogen Ultrapure Distilled Water, ThermoFisher Scientific, Waltham, MA), 10x ORANGE+ Buffer, *forensic*GEM, and Histosolv. BLUE+ Buffer was not used for this study. Upon arrival, the reagents were stored at 4˚C. A master mix combining the previously mentioned reagents was created within a 1.5 mL microcentrifuge tube. The amounts of reagents for the created master mixes are indicated as follows in table 3.

Table 3. Reagents utilized and their respective amount of mastermix for experiments. The amount of *forensic*GEM enzyme in the "MM for Exp 2" column was doubled as it was a goal to test the impact of increasing the amount of *forensic*GEM.

Reagent	Amount Per Sample	MM per Exp (N+1, N=11)	MM for Exp 2
DNA-free water	79 µL	948 µL	936 µL
10x ORANGE+ Buffer	10 µL	120 µL	120 µL
*forensic*GEM	1 µL	12 µL	**24** µL
Histosolv	10 µL	120 µL	120 µL

After the master mix was prepared, 100 µL of the master mix was added to each sample tube, ensuring that only one sample was open at a given time. After this, samples placed on an amp tray were vortexed (ThermoFisher Scientific, Waltham, MA) for

approximately 10 seconds at high speed. Following vortexing, the samples were placed on the Mastercycler X50s (Eppendorf, Hamburg, Germany) and run at the manufacturer's standard thermal protocol, incubating at 52˚C for 5 minutes, followed by 75˚C for 10 minutes, and finished at 95˚C for 3 minutes. Figure 1 depicts the standard protocol workflow.

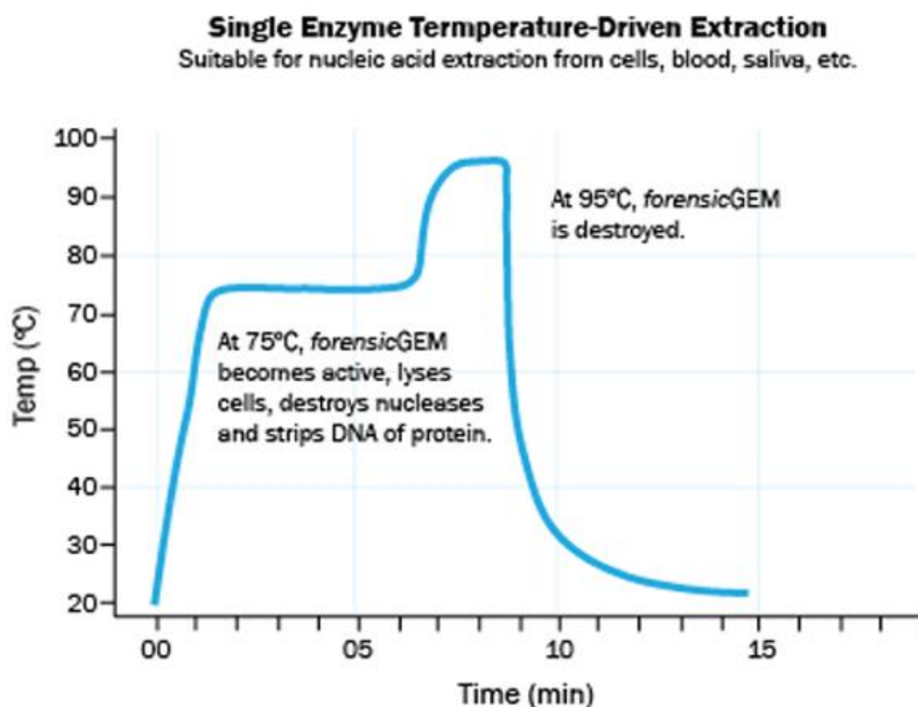

Figure 1. *Forensic*GEM MasterCycler X50 Standard Protocol (MicroGEM, 2023)

To assess the efficiency of the manufacturer's protocol, different Mastercycler X50 parameters and other recommended procedures were altered to optimize DNA yield. The first experiment was conducted following the manufacturer's protocol. The second experiment was conducted with twice the recommended amount of the novel proteinase, *forensic*GEM, in each reaction tube. The third experiment was conducted with the initial 52˚C incubation time doubled from 5 to 10 minutes. The fourth experiment had the 52˚C

incubation tripled from 5 minutes to 15 minutes. The fifth experiment was conducted with the 75˚C incubation step increased from 10 minutes to 15 minutes. The sixth experiment was conducted with the 75˚C incubation step increased from 10 minutes to 20 minutes. The seventh experiment was run with the manufacturer's protocol, but the bone samples used were prepared with scraping rather than milling of bones as done in the previous experiments (described above). The eighth experiment was run without a purification step on the EZ1 Advanced xL (Qiagen, Hilden, Germany). The ninth experiment run was a standard organic extraction, a control run, to assess how the STR profiles obtained from the *forensic*GEM experiments compared to normal STR profiles obtained with organic extraction. A summary table of the parameters changed for each experiment is below as demonstrated in table 4.

Figure 2. Demonstration of the bone scraping method as used in experiment 7. Bones were scraped with the blunt end of a sterile scalpel and shavings were produced.

Table 4. Summary of experiments performed with the *forensic*GEM extraction kit

Experiment	Altered Parameter	Initial 52˚C Incubation Length	Second 75˚C Incubation Length	Amount of Enzyme	Bone Prep Method
1	N/A	5 minutes	10 minutes	1 µL (per sample)	Milling
2	Enzyme amount	5 minutes	10 minutes	2 µL (per sample)	Milling
3	First incubation length	10 minutes	10 minutes	1 µL (per sample)	Milling
4	First incubation length	15 minutes	10 minutes	1 µL (per sample)	Milling
5	Second incubation length	5 minutes	15 minutes	1 µL(per sample)	Milling
6	Second incubation length	5 minutes	20 minutes	1 µL (per sample)	Milling
7	Bone preparation method	5 minutes	10 minutes	1 µL (per sample)	Scraping
8	Extractions not purified on EZ1	5 minutes	10 minutes	1 µL (per sample)	Milling

2.4 DNA Purification of Tissue and Bone Samples with EZ1 Large Volume Protocol

A bottle of MTL buffer was placed in the incubator (Lab Line Imperial III Incubator, ThermoFisher Scientific, Waltham, MA) for approximately 10 minutes at a temperature of 65˚C. Both the samples and extraction negatives were incubated in a

separate incubator (Eppendorf Thermomixer, Hamburg, Germany) at 56˚C for an estimated 10 minutes. While the MTL buffer and samples were being warmed in their respective incubators, QIAGEN's EZ1 Advanced XL (Qiagen, Hilden, Germany) instrument was prepared for DNA purification. 2 mL screw cap tubes and QIAGEN 1.5 mL screw cap elution tubes were obtained and appropriately labeled. EZ1 reagent cartridges were obtained and thus inverted until the magnetic particles were mixed. After mixing, the EZ1 instrument was loaded with the appropriate tips and reagent strips.

After the extraction negatives and samples were incubated for 10 minutes, the 2 mL tubes were centrifuged (Eppendorf Centrifuge 5424, Hamburg, Germany) briefly to remove any residual condensation. Then, the warmed MTL buffer was removed from its respective incubator and added to each sample tube in 400 µL aliquots. After adding the buffer, 1 µL of carrier RNA was added to each tube in addition. Following this, the tubes were centrifuged once again.

Immediately after all the necessary contents were added to the extraction negative and sample tubes, the tubes were subsequently placed into the EZ1 instrument for DNA purification. The previously labeled QIAGEN 1.5 mL screw cap elution tubes were placed in the EZ1 instrument as well. After it was verified by a witness that all proper samples, racks, and reagents were appropriately loaded within the instrument, the EZ1 protocol for sample purification was run. The EZ1 was run with the large volume protocol, TE buffer elution, and an elution volume of 40 µL. Following the 20-minute purification, the QIAGEN 1.5 mL elution tubes were removed and subsequently placed into a 20˚C refrigerator for storage. The rest of the instrument contents were removed for appropriate cleaning.

2.5 Quantifiler DNA Trio Quantitation

Three quantitations were performed: one based on the organic extraction (control) another based on *forensic*GEM extractions 1-4, and another based on *forensic*GEM extractions 5-8. The steps for the Quantifiler Trio kit (ThermoFisher Scientific, Waltham, MA) were followed according to the protocol provided by the NYC-OCME, 2023.

The following reagents were obtained before quantitating the samples: Quantifiler THP PCR Reaction Mix, Quantifiler HP Primer Mix, Quantifiler DNA Dilution Buffer, and Quantifiler THP DNA Standard (100 ng/µL). The amount of the Primer Mix and PCR Reaction Mix for the master mix was calculated to be 501.6 µL and 627 µL respectively. The reagents were gently vortexed and briefly centrifuged before preparing the master mix. The master mix was then also vortexed and centrifuged.

2.5.a Standard Curve Creation

To accurately quantify the amount of DNA in each sample, a standard curve was generated using known DNA concentrations for an accurate comparison. These standards were created by serial dilution from the 100 ng/µL Quantifiler THP DNA Standard, which was vortexed and centrifuged briefly before dilution. The standards made were 100 ng/µL, 50 ng/µL, 5 ng/µL, 0.5 ng/µL, 0.05 ng/µL, 0.005 ng/µL and NTC (no template control). For tubes labeled 50 ng/µL and NTC, 10 µL of Quantifiler DNA Dilution Buffer was added. For tubes labeled 5 ng/µL, 0.5 ng/µL, 0.05 ng/µL, and 0.005 ng/µL, 90 µL of Quantifiler DNA Dilution Buffer was added. 16 µL of the Quantifiler THP DNA Standard (100 ng/µL) was aliquoted into the first tube. To commence the serial dilution, 10 µL was pipetted from the 100 ng/µL tube to the 50 ng/µL tube. Then,

10 µL was pipetted from the 50 ng/µL tube to the 5 ng/µL tube, and then so on (see figure 3). Each new dilution was vortexed and centrifuged briefly for no more than 3 seconds at a speed no greater than 3000rpm.

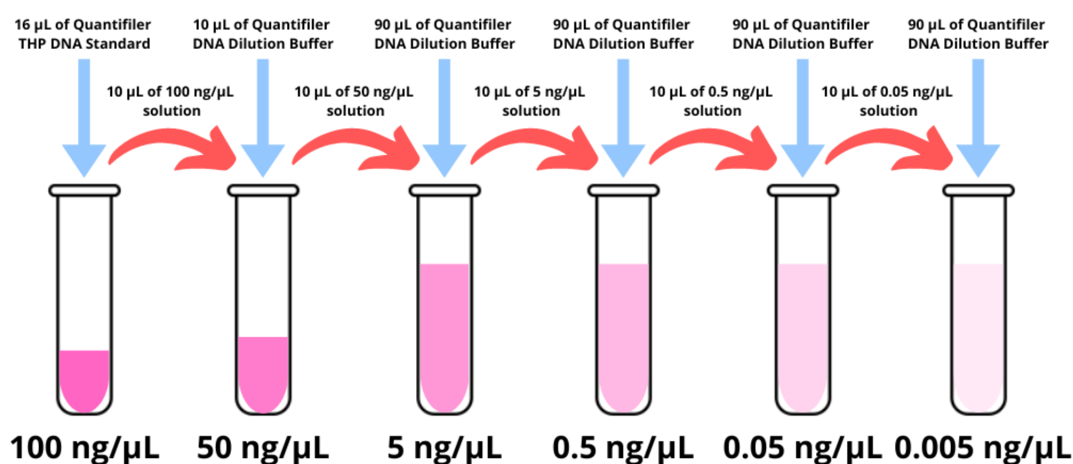

Figure 3. Quantifiler THP DNA Standard serial dilution scheme.

The purpose of the standard curve is to serve as a basis for comparison of the level of fluorescence in an unknown -- which is representative of the amount of DNA in a sample. Each of the standards has a known concentration and thus will emit a known level of fluorescence. When an unknown sample crosses the cycle threshold, its fluorescence is recorded, serving to tell how much DNA is within that sample in comparison to the known standards. Once the standards were created, the extraction samples were vortexed and centrifuged briefly for no more than 3 seconds at a speed no greater than 3000rpm.

In an Applied Biosystems MicroAmp Optical 96 Well Reaction Plate (ThermoFisher Scientific, Waltham, MA), 18 µL of the mastermix was allotted into each appropriate well. Then, 2 µL of each extraction sample, the standards, and the NTC were aliquoted into their respective wells within the plate. The reaction plate was then sealed with an Optical Adhesive Film. The plate was then run on an Applied BioSystems 7500 Real-Time PCR System (ThermoFisher Scientific, Waltham, MA) with the Quantifiler Trio run selected within the HID Real-Time PCR Analysis Software. The run started at a holding stage of 95˚C for 2 minutes. Following this, a denaturation step was performed at 95˚C for 9 seconds and an annealing step at 60˚C for 30 seconds, being repeated for 40 cycles.

2.6 DNA Amplification with PowerPlex Fusion Sample Preparation

Following the amplification protocol designated by the NYC-OCME (2021), extraction samples were retrieved from the 4˚C fridge. Dilutions were made if necessary to obtain an input amount of around 525 pg. Reagent tubes were vortexed and centrifuged briefly to ensure proper mixing. PowerPlex Fusion 5X Primer Pai Mix and PowerPlex Fusion 5X Master Mix (Promega, Madison, WI) were added together in a 1.5 mL tube, with 2.5 µL of each added per reaction. Before aliquoting, each of the samples and the master mix were vortexed and centrifuged briefly. 5 µL of the master mix was added to each of the necessary amplification tubes. Following this, an amplification negative was made, containing only 7.5 µL of amplification-grade water, along with an amplification positive containing 375 pg of control DNA. According to the amplification sheet, samples were aliquoted, for a total volume of 7.5 µL of DNA and water. Amplification-

grade water was used to dilute some samples as needed prior to aliquoting into the PCR reaction tube.

Samples were spun down at 1000 RPM (Eppendorf Centrifuge 5810, Hamburg, Germany) for one minute and then placed on the thermocycler with the following conditions: 1-minute soak at 96˚C, then denaturation at 94˚C for 10 seconds, annealing at 59˚C for 60 seconds, and extension at 72˚C for 30 seconds. 29 cycles of this were performed and then followed by a 10-minute incubation at 60˚C and then a storage soak indefinitely at 4˚C.

2.7 Capillary Electrophoresis

To prepare the samples for injection, they were spun down on a centrifuge at 1000 RPM for one minute. Mastermix was prepared with PowerPlex Fusion WEN ILS 500, PowerPlex Fusion Allelic Ladder, and HiDi Formamide. A 96-well HID plate was prepared with 10 µL of master mix in each sample according to the manual at NYC-OCME. 1 µL of the allelic ladder, positive/negative control, and the samples were added to their respective wells. The plate was then spun again at 1000 RPM for one minute, and then denatured at 95˚C for 3 minutes on a thermocycler and then placed on another thermocycler immediately for 3 minutes at 4˚C. The plate was spun again at 1000 RPM for one minute. The plate was then placed in the autosampler tray on the 3500xL Genetic Analyzer (ThermoFisher Scientific, Waltham, MA). Amplified DNA was separated with the following parameters: the oven temperature was 60˚C, the pre-run voltage was 15.0 kV, the pre-run time was 180 seconds, the injection voltage was 1.2 kV, the injection time was 24 seconds, the run voltage was 15 kV, and the run time was 1500 seconds.

3500 Series data collection software was used according to protocols laid out by NYC-OCME.

2.8 Analysis and Interpretation

For a validated analysis, a range of 37.5pg to 525pg is the optimal DNA input amount suited for amplification. The laboratory's analytical thresholds, or ATs, were determined for each dye color: blue (Fluorescein - 85 rfu), green (JOE - 120 rfu), yellow (TMR-ET - 130 rfu), and red (CXR-ET - 160 rfu). "The AT is the minimum rfu value at which peaks can be reliably distinguished from background noise" (NYC-OCME, 2022). In addition to this, the stochastic thresholds, or STs, were determined for each dye color as follows: blue (Fluorescein - 900 rfu), green (JOE - 1000 rfu), yellow (TMR-ET - 900 rfu), and red (CXR-ET - 900 rfu). It is reasonable to surmise that allelic dropout of a heterozygous genotype has not taken place at a certain autosomal site within a single-source profile if the stochastic threshold is greater than the designated value.

As designated by OCME 3500 instrument validation, the saturation point was determined to be 30,000 rfu. The saturation point is a threshold at which many overblown peaks are observed, indicating too much DNA in a sample. Lastly, using PowerPlex Fusion data on 3500s, the drop-in rate was determined to be 0.87% and the distribution parameters were determined to be $\alpha = 22.31$ and $\beta = 2.65$. The detection of non-reproducible, unexplained, low-level peaks in a DNA profile is known as drop-in. For the STRmix analysis, a drop-in cap of 300 rfu is applied.

GeneMarker version 3.0.0 (Softgenetics, State College, PA) was used to evaluate the size standard in each sample and ensure the passing status of the allelic ladders and controls. Alleles were called by the software standards to generate an electropherogram.

Each sample profile was assessed individually and classified by its number of loci, the number of fully deconvoluted loci, as well as the number of alleles. The number of loci is identified as any locus with at least one allele called. A fully deconvoluted locus is one where both alleles are called and identified. The number of alleles refers to the total number of alleles called on the electropherogram (not including the sex-determining loci). The more alleles that were called, the more complete a DNA profile for a given sample was. For the NYC-OCME, a full profile consists of 22 loci, each consisting of typically two alleles. These loci are chosen because they are highly variable and reliable genetic markers. These markers are known as short tandem repeats (STRs) and consist of repeating units of DNA that vary in number between individuals, thus allowing analysts to distinguish one person's DNA from another's (Fan, 2007).

2.9 World Trade Center Remains Testing

Five sample remains from the World Trade Center attacks were obtained, all of which had previously been submitted for DNA extraction but had failed to yield any DNA profile. They were initially cleaned with a scalpel to remove the outer layer of dirt and contaminants. The samples were then cleaned with water and a toothbrush and placed in an incubator at 56˚C to dry overnight. Because the results from experiment 7, where the bones were scraped rather than milled, yielded higher amounts of DNA, this was decided to be the most optimized method of bone preparation. The remains were then scraped to obtain about 20 mg of sample for further testing. The scrapings were separated into two tubes and measured (table 5). Each sample had a duplicate and was extracted with standard *forensic*GEM protocol. The only difference was that the master mix was prepared for 12 samples, versus the typical 11. One of each sample then underwent the

purification protocol described above on the EZ1 while the other replicates were not purified.

Table 5. WTC remains used and their respective weights

Sample	Purified (Y/N)	Weight (g)
WTC 1	Y	0.010
WTC 1	N	0.009
WTC 2	Y	0.011
WTC 2	N	0.010
WTC 3	Y	0.010
WTC 3	N	0.011
WTC 4	Y	0.011
WTC 4	N	0.009
WTC 5	Y	0.017
WTC 5	N	0.014

**Sample 5 was very charred and porous so additional scrapings were used in the extraction*

3. Results

3.1 forensicGEM Results

3.1.a Standard Curve Reports

Three quantitations were performed and all met the laboratory's requirements for passing. To pass the standard slope for each of the three curves generated (small autosomal, large autosomal, and the Y target) must be between -3.0 to -3.6, the y-intercept must be between 24.5 and 29.5 (inclusive), and the R^2 value must be greater than or equal to 0.98. All of these parameters were met for each of the quantitations that

were run. In addition, all of the negative controls, the NTC, and each extraction negative need to be less than or equal to 0.2 pg/uL. No DNA was detected in any negative controls. The small autosomal target was used for DNA quantitation of the unknowns.

3.1.b DNA Yield

DNA yield was classified into two categories: DNA yield below 0.2 pg/µL and DNA yield above 0.2 pg/µL (see figure 4). Any sample with a DNA yield above 0.2 pg/µL was considered admissible for amplification. When protocols three and four were followed, 3 samples had a DNA yield that was more than 0.2 pg/µL. When protocols one, two, five, and six were followed, 4 samples had a DNA yield that was more than 0.2 pg/µL. When protocol seven was followed, 5 samples had a DNA yield that was more than 0.2 pg/µL. And finally, when protocol eight was followed, 6 samples had a DNA yield that was more than 0.2 pg/µL. Overall, protocols seven (scraping of the bone) and eight (no purification) yielded the highest amount of DNA.

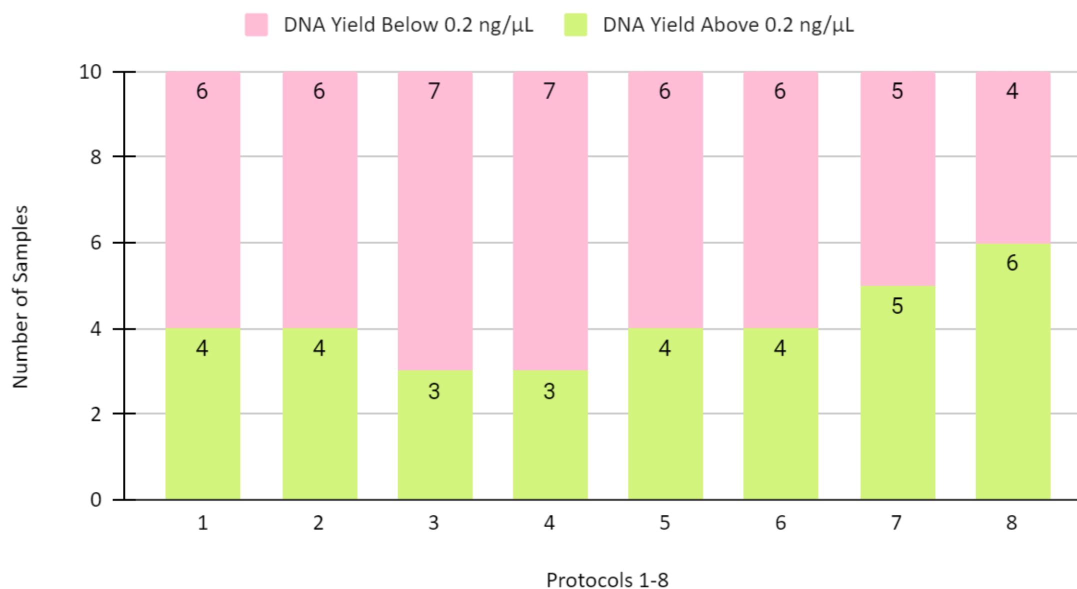

Figure 4. DNA yield from optimized MicroGEM protocols one through eight for all 10 samples. The pink portion of the bar discerns the samples with DNA yield below 0.2 pg/µL, while the green portion of the bar discerns the samples with DNA yield above 0.2 pg/µL.

3.1.c Degradation Index and Inhibition

The only sample that showed high degradation in most of the experiments (1, 2, 7, 8, and in the organic extraction 9) was B2. This bone sample yielded a degradation index of 21.45, 16.07, 5.46 (low degradation seen in experiment 6), 38.35, 28.71, and 14.54 respectively. All tissue samples were collected 60 days after placement, but the degradation values determined for one successful tissue -- tissue L, were consistently low.

The IPC (internal positive control) is used to determine if there is inhibition present within the sample (NYC-OCME, 2021). Like the degradation index, inhibition

can also help predict DNA profiling success. No inhibition is indicated by a value from 26-29, high inhibition is less than 24, greater than 31, or blank. The values for low inhibition are 24-26 and 29-31. All tissue and bone samples showed either no or low inhibition. The main objective of experiment 8 (no purification) was to compare the inhibition values with those reported for extracts that had been purified. *Forensic*GEM promises to overcome biological inhibitors for PCR and this was supported by the data. There was no distinct difference between the IPC values for experiments 1-7 where each sample was purified after extraction, compared to experiment 8 with no purification (table 6).

Table 6. IPC values for experiments 1 through 8

Experiment	1	2	3	4	5	6	7	8 (not purified)
IPC average	27.63	27.60	27.64	27.57	27.64	27.67	27.51	27.91

3.1.d STR Analysis

Bone sample B3 did not yield any alleles, but B1 performed substantially resulting in 36 alleles. Out of the five bones tested, it was apparent that the bone scraping technique tended to result in more alleles being called (table 7). While tissue samples F, G, I, and J yielded no alleles for the *forensic*GEM extractions, sample L performed notably better in all the *forensic*GEM extractions than with organic extraction. Sample L's *forensic*GEM performance on average is compared to the rest of the tissues is demonstrated in table 8. The highest allele count of 44 was observed in experiment 2 where the amount of *forensic*GEM was doubled, and in experiment 7 where the parameters for tissue samples remained the same as in experiment 1. It is worth noting that at the time of the experiment, *forensic*GEM was optimized for tissue samples and

performed better than organic extraction across all trials, even though results can only be analyzed from one tissue sample.

Table 7. Bone samples and their respective called alleles for the *forensic*GEM extraction resulting in the highest amount of called alleles

Bone Sample	Number of alleles called for *forensic*GEM extraction
B1	36 (experiment 7, bone scraping)
B2	10 (experiment 7, bone scraping)
B3	0 (for all *forensic*GEM experiments)
B4	4 (experiment 7, bone scraping)
B5	9 (experiment 5, 75˚C incubation step increased from 10 minutes to 15 minutes)

Table 8. Tissue samples F, G, I, and J, L and their respective average number of called alleles for between all *forensic*GEM extractions

Tissue Sample	Average number of alleles called between all *forensic*GEM extractions (standard deviation ± 2.425)
F	0
G	0
I	0
J	0
L	41

3.2 Organic Extraction Results

This study aimed to determine the potential of *forensic*GEM as a replacement for the time-consuming organic extraction protocol. To achieve this, we subjected all samples tested with *forensic*GEM to standard organic extraction as well. The results showed that organic extraction technically yielded more complete DNA profiles for all

bone samples as shown in table 9. However, it did require 200 times more input material compared to *forensic*GEM. Due to the vast difference in input material amounts, it was challenging to make an accurate comparison between organic extraction and *forensic*GEM for the bone samples.

Table 9. Bone and tissue samples and their respective called alleles for the organic extraction

Sample	Number of alleles called for organic extraction
Bone B1	38
Bone B2	27
Bone B3	21
Bone B4	41
Bone B5	41
Tissue F	0
Tissue G	1
Tissue I	1
Tissue J	6
Tissue L	25

Interpreting the results of the tissue samples proved difficult, as only a single sample (tissue L) provided information. To conclusively determine whether *forensic*GEM DNA extraction is equal to or superior to standard organic extraction, further experiments must be conducted. For degradation analysis, bone samples B1, B3, and B5 had degradative indices of 4.46, 6.67, and 4.0, respectively, while B2 yielded 14.54 and B4 showed a degradation index of 2.65. Importantly, all samples reported no inhibition (IPC), indicating that the PCR amplification was successful.

3.3 World Trade Center Remains Results

After extraction with *forensic*GEM, the WTC samples were tested with the same quantitation procedure stated prior. All 5 samples that were not purified did not yield any detectable DNA. Out of the five samples that were purified, three yielded non-zero

amounts of DNA and were subsequently moved forward for amplification. For these three samples, the quantitation values were 0.55 pg/µL, 0.16 pg/µL, and 1.38 pg/µL respectively. The quantitation results illustrate the importance of performing post-extraction purification on severely degraded samples. The three samples that yielded small amounts of DNA had a degradation index of 1.94, 2.56, and 2.07 while the IPC values for all had an average of 27.69. The results also demonstrate the advantage of *forensic*GEM, having been the only extraction method successful with these samples. The WTC samples utilized were extremely brittle and porous where some samples began to fragment when scraped. After electrophoresis, one of the three World Trade Center samples had no peaks called. However, two World Trade Center profiles had some peaks visible, yet not called. Since the alleles that were called were low in intensity and other peaks observed were not called, the analytical threshold was lowered to approximately 40 RFUs (specific values dependent upon locus). This change is only permitted for analyzing WTC samples and would not be permitted for typical casework purposes. This value was chosen so that peaks already present but lower than the established analytical threshold on the electropherogram would be called. After lowering the standard analytical threshold, one of the remains yielded a 19-locus profile and another yielded a 15-locus profile.

The initial results of the World Trade Center remains were promising so it was decided to re-extract them by tripling the input amount of bone scrapings. Scrapings of the 5 samples were weighed out again and then extracted with the standard *forensic*GEM protocol. With results from the first trial in mind, all extracts were purified. After re-extraction and purification, the extracts were quantified. Upon re-quantification, only one

out of the five samples resulted in a non-zero amount of DNA -- going from its previous value of 1.38 pg/µL to 3.00 pg/uL. Re-amplification of all samples only produced partial profiles for the two WTC remains that had earlier yielded partial profiles. After conducting a preliminary evaluation of the data and again lowering the analytical threshold to approximately 40 RFUs, the profiles of these two remains either stayed consistent or were improved. Ultimately the sample originally having a 19-locus profile had become a 22-locus profile and the 15-locus profile had remained the same. It must be noted that the now-22-locus profile represents a situation where data was taken from both rounds of WTC remains testing to build a "composite" profile. These two profiles were searched through the WTC database maintained by the NYC OCME. The outcome of the search has allowed each of the remains from which these profiles were developed to be re-associated with previously identified victims of the WTC disaster.

4. Discussion

The original sample size was 10, consisting of 5 bones and 5 tissues but the *forensic*GEM extraction was only successful on 5 out of the 10 samples. "Success" was defined by a non-zero quantitation value and subsequently the generation of a partial STR profile. Bone B3 and tissues F, G, I, and J never yielded DNA when extracted with *forensic*GEM. However, B3, tissues G, I, and J were successful in obtaining DNA from the standard organic extraction. Due to the absence of detectable DNA in several samples, plus the small sample size, no statistical testing could be performed to assess the efficiency of *forensic*GEM. The data obtained does, however, provide the opportunity to make a commentary on the success rate. MicroGEM's extraction kit yielded an 80% success rate for bones and a 20% success rate for tissues for the presence of a partial STR

profile and a non-zero quantitation value. While re-extraction of the two World Trade Center remains revealed one profile to have the same number of loci, there was a significant increase in the 19-locus profile expanding into a 22-locus composite profile. Although this expansion may seem small, there are 22 loci total in a standard DNA profile, making this profile complete. In addition, the locus which determines if an individual is biologically male or female, amelogenin, was complete. This provides further support for the success of the extraction. For those who have the ability, budget, and time, re-extraction may prove to be beneficial in some cases. Since the re-extraction involved three times as much bone scraping and ultimately served to increase the quantitation value of one sample and expand a profile, this perhaps will affect the future of recommended bone input amounts.

4.1 Design Parameters

Experiment two (doubled amount of enzyme) returned what were perhaps the most surprising results. All other previous studies showed a correlation between increasing the amount of *forensic*GEM and the increase of DNA yield, but this was not observed in experiment two. Compared to the manufacturer's standard protocol in experiment one, the DNA yield was lower with the doubled enzyme amount for the bones. Theoretically, doubling the enzyme amount in chemical reactions reduces the activation energy, making the reaction progress faster. While faster reactions can seem desirable, the amount of DNA extracted during the process depends on several factors, including the type of sample, the method of DNA extraction used, and the quality of the DNA extracted. It is important to note that DNA extraction is a complex process that involves several steps and each step can affect the amount and quality of DNA extracted,

therefore, the speed of the reaction is not always the most critical factor. A faster reaction certainly does not necessarily translate to more extracted DNA. Since only one tissue sample yielded DNA, it was difficult to assess the significance of the results obtained. However, the DNA yield did slightly increase in the one successful tissue sample with the increased enzyme amount, by about 11 pg/uL.

In experiments three and four, where the initial incubation step was increased to 10 and 15 minutes respectively, the amount of extracted DNA was lower compared to experiment one, for both bones and our tissue sample. The *forensicGEM* enzyme does not achieve its optimal activity until the temperature reaches at least 75˚C. It is still possible that below 75˚C, the enzyme can still have some activity, although it is likely that its performance is less than optimal. It is common that when enzymes work below their optimal temperature, they work inefficiently. In this case, it is possible that extending the time that the reaction was held at a sub-optimal temperature caused the enzyme to be used up before it ever had the chance to reach the temperature of its optimal activity.

Experiment five seemed to have the most optimized experimental design. In this run, the 75˚C incubation step was increased from 10 minutes to 15 minutes. Overall, there was an increase in extracted DNA in this run compared to experiment one in both sample types. At this temperature, proteins and nucleases are denatured. The additional time may aid in this process, thus resulting in more DNA. This is a reasonable result considering that 75˚C is within the *forensicGEM* enzyme's range of optimal activity.

In experiment six, the 75˚C incubation step increased to 20 minutes. The bone samples showed a decrease in DNA yield compared to the first experiment -- however, the one successful tissue sample showed about a 1.5x increase in DNA yield.

In experiment seven, a bone scraping technique was employed as opposed to the typical bone preparation protocol. In this case, many bone samples had an increase in DNA yield. This was significant because this new bone preparation technique is beneficial due to its ease and decreased preparation time compared to standard cleaning and milling. The increase in DNA yield is sensical due to the concentration of osteocytes being higher toward the surface of the bone. When analyzing the STR data of the experiments, this increase in DNA yield was very evident in B1, with an average peak height (APH) about 10x greater when the bone was scraped. The average peak height for all samples in this experiment was 658 RFU compared to 235 RFU for experiment 1. In addition, the average amount of loci obtained from scraping was 8.5 compared to 6.25 loci with milling.

Experiment eight was run without the purification step. In this case, this was very beneficial for a singular tissue sample. Compared to experiment 1 there was 323x more DNA extracted when there was no purification step. Although intriguing, it is difficult to draw statistically meaningful conclusions from the results of just one sample. For the bone samples, a sample where there had previously been no successful extractions (besides the organic control extraction), yielded DNA. The other bone samples showed some increase and some decrease in DNA yield. After the non-purified samples underwent STR analysis it was evident that the purification step is crucial to obtaining a better DNA profile.

5. Concluding Remarks

5.1 Research Limitations

It was difficult to accurately compare the DNA extracted from the bones in the organic procedure with the *forensic*GEM extractions. The organic extraction protocol at the Office of Chief Medical Examiner requires 2.0 g of bone dust for input. *Forensic*GEM extractions take place in a small amplification PCR tube -- so only about 10 mg were used. As a result, there was 200x more sample input into the organic extraction -- which is why the quantitation values are much higher than the values from the *forensic*GEM extractions. While most of the bone samples were successful in DNA extraction, the comparison of DNA yield was difficult due to this discrepancy.

MicroGEM's extraction kit aims to work very well on highly degraded tissue samples, but this was not always supported by the experimental data. Only one of the five tissue samples utilized yielded DNA. While the data obtained from this one specimen was insightful it is difficult to draw conclusions with only one specimen. In experiment nine -- the organic extraction -- three of the other tissue samples yielded DNA but all less than 1 pg/µL. One tissue sample never yielded DNA regardless of the extraction method.

5.2 Final Conclusions

With this study, we have demonstrated that *forensic*GEM's success was not consistent, but its use may prove to be an applicable technique when there is little sample to test and in laboratories where there is a high demand for DNA testing with rapid turnaround time. Ultimately, the organic method of extraction DNA extraction performed better, resulting in more consistent and complete profiles -- but it is noteworthy to mention that a large amount of sample input was necessary.

Even though the organic extraction did produce more complete profiles, it must not be forgotten that the MicroGEM kit did produce a 22-locus profile and a 15-locus profile and new reassociations from two sample remains from the World Trade Center attacks. These samples previously had undergone DNA extraction at the NYC-OCME but did not produce DNA profiles whatsoever. Considering that these samples were of extensive degradation, this is a monumental find not only for the method of testing World Trade Center remains going forward but also for the remains from modern casework that were found in less-than-ideal conditions.

Not only this, but in comparison to the organic extraction, the *forensic*GEM extraction does not contain the harmful detergents or washes that the organic extraction entails -- such as phenol-chloroform or mercaptoethanol -- nor does the method require any transfer steps, the loss of sample, or the excessive waste of plastics and consumables. MicroGEM's single-tube, 20-minute incubation time is highly beneficial, as the ability to decrease the processing time of DNA extraction is the aim of the novel enzyme and would aid immensely in forensic casework.

Lastly, an ancillary finding of this study is that the bone preparation method of scraping yielded higher DNA quantities and better-quality profiles compared to samples treated with the standard method of milling. Bone scraping is a far simpler method than bone milling. Therefore, if this technique of bone preparation was introduced into the standard operation of procedure, it is possible that in the future we could simplify and make more efficient the DNA extraction process, all the while not risking the quality of the profiles behind the samples in question.

5.3 Future Research

By altering certain experimental parameters, it is possible to gain a better understanding of the modifications by assessing DNA yield and profiling success. In this study, the amount of enzyme was varied, incubation times were varied, the method of bone preparation was varied (milling or scraping), and DNA purification versus no DNA purification was assessed. There are many other potential modifications to the manufacturer's protocol to increase DNA yield and profile quality for future research purposes. Some of these modifications include the alteration of the Histosolv amount by +/- 5 µL or by increasing the amount of *forensic*GEM used in 1 µL increments. In addition, increasing or decreasing the length of the 52˚C/75˚C incubation time may be an option. Lastly, another suggested modification is instead of utilizing a singular buffer, one could combine different *forensic*GEM buffers such as the ORANGE+ and the BLUE+ to obtain a final buffer concentration of 1X within the entire master mix solution. In this study, the ORANGE+ buffer was used because the samples employed contain DNA that is difficult to extract. The BLUE+ buffer is known to be the least aggressive buffer of the two. Another reagent developed by MicroGEM is called an “enhancer” which is typically utilized for plants and is known to bind inhibitors proficiently; if inhibition were a greater problem this could be introduced. In a study conducted by Lounsbury et al., 2012, the researchers decided to alter the amount of cellular material which, too, increased DNA yield. In addition, in the study conducted by Sahoo et al., (2021) a sanding step was included before scraping. It would be beneficial to compare the results of bones that were sanded before scraping versus those that were not sanded. One major parameter to be changed for future studies should be the sample size. To perform

proper statistical testing to assess how *forensic*GEM compares to other extraction methods a sample size of about 100 would be preferable. A larger sample size would allow more meaningful conclusions to be drawn.

6. References

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