APPLICATIONS OF MOLECULAR GENETICS TO HUMAN IDENTITY

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Introduction

Each year, a large number of human remains are found that cannot be identified by traditional gross morphological methods such as visage, dental, radiological, or fingerprinting. The condition of these remains varies widely and, in many cases, a few pieces of a disarticulated skeleton are all that are ever recovered. This is, of course, because teeth and bones are the most durable portions of the human body, persisting long after soft tissues have succumbed to decay. The unique mineral composition of these structures also works to stabilize DNA, protecting it to some degree from the oxidative and hydrolytic damage that otherwise would rapidly occur in all but the coldest of climates (19). All of these properties make bone-derived DNA a natural focal point in the field of missing persons identification.

DNA has been used for forensic analysis since 1984, when Alec Jeffreys adapted David Botstein's restriction fragment length polymorphism (RFLP) technique to examine human polymorphic loci and thus identify an individual as the likely source of DNA evidence found at a crime scene (42). RFLP has an excellent power of discrimination, but it is a labor-intensive technique that requires relatively large quantities of high-quality DNA (the fragments analyzed in RFLP are on the order of thousands of base pairs long, and the analysis of multiple loci must be conducted sequentially, requiring weeks to complete even a four-locus profile). Two years after

Jeffreys' innovation, Kary Mullis developed the polymerase chain reaction (PCR), technology which was first applied to human identity using the polymorphic HLA DQα locus. The first dot blot strip test was rapid and required far less template DNA than did the RFLP process, but it only analyzed a single locus with six possible alleles. Therefore, it had a substantially reduced discriminatory power. Improvements were made to the dot strip systems, adding loci and refining the technique to provide the HLA DQA1 system and later the Amplitype® PM+DQA1 system (42). These iterations increased the power of discrimination of the approach, but it was the development of PCR primers to amplify short tandem repeat (STR) loci (and the multiplexing of primers that followed) that finally combined the ease and sensitivity of PCR amplification with a power of discrimination that approached and ultimately surpassed that of RFLP analysis. The STR typing profiles were also readily entered into databases (as were the RFLP profiles before them) that allow law enforcement to quickly compare STR profiles obtained from evidentiary samples to reference profiles obtained from convicted criminals, unsolved crime scene evidence databases, and to family members of missing persons.

In 2001, the Texas state legislature funded the profiling of DNA samples from unidentified human remains and the creation of the Texas Missing Persons DNA Database (TMPDD) at the University of North Texas Health Science Center (UNTHSC) at Fort Worth. The TMPDD began accepting samples from Texas law enforcement agencies in 2003. Under the new moniker of the University of North Texas Center for Human Identification (UNTCHI), and with federal funding, the UNT Center for Human Identification currently operates as a national facility in conjunction with the Federal

Bureau of Investigation's Combined DNA Index System (CODIS). Its mission continues, generating and associating short tandem repeat (STR) profiles from unidentified remains with reference STR profiles (50). To date, the UNT Center for Human Identification has successfully identified the remains of over 100 missing persons based upon genetic analysis of submitted samples (15).

Endogenous, PCR amplifiable nuclear and mitochondrial DNA can, under optimal conditions, persist in skeletal remains for thousands of years (23). However, the environmental milieu to which remains are exposed is often not conducive to DNA preservation. As a result, DNA from these remains may show signs of substantial degradation. The currently applied methodologies for generating nuclear genetic profiles from highly degraded samples do not always yield full STR profiles from forensic or archeological human remains. Post-mortem degradation affects both the quantity and quality of DNA which, as noted by Svante Paabo, is "invariably of a low average molecular size and damaged by oxidative processes" (38). In these cases, due to the high copy number and smaller, more durable nature of the mitochondrial genome, it is often still possible to generate a mitochondrial DNA (mtDNA) profile well beyond the point where standard nuclear STR analyses fail. Mitochondrial haplotypes can reliably exclude an individual as a possible genetic donor, but cannot include an individual to nearly the same degree as a full nuclear STR profile. In particular, mtDNA profiles are not useful to distinguish between relatives with the same maternal lineage nor are they of any use in the absence of maternal-lineage family reference samples.

New tools and techniques are constantly becoming available in the rapidly evolving fields of molecular genetics and biochemistry, many of which offer the

opportunity to obtain more genetic information from highly degraded samples. Although many techniques exist, often the bits and pieces of them that are adopted and applied are “chosen” more as the result of happenstance rather than based upon a broad, thorough effort to systematically evaluate the strengths of each portion of the available protocol(s). There are three areas of forensic DNA analysis in particular that may benefit from new technology: DNA isolation, amplification of sample DNA prior to genetic analysis via whole genome amplification, and improvement of DNA template quality using repair enzymes. The goal of this project is to identify the protocols presently available to molecular biologists in each of these three areas, compare their relative performance, and create new hybrid variant protocols based upon the strong points of each method. This should make possible an improved level of standardization for molecular-based identification of human remains, particularly those that have been recalcitrant to presently utilized DNA profiling techniques.

DNA Isolation from Bone

The first priority of any DNA isolation protocol must be to produce a DNA extract that can be successfully profiled. This means that it must yield DNA of a minimum quality and quantity, preferably at an acceptable cost. The ideal DNA isolation protocol should be streamlined and provide a maximum yield of DNA free from inhibitory compounds that can affect downstream applications. For example, when dealing with samples containing very low levels of or highly degraded DNA, it is important to minimize sample handling steps, as every manipulation provides an opportunity for

sample loss, contamination from exogenous human DNA sources or, in forensic laboratories, previously amplified STR loci/ PCR reaction products. Even small quantities of “introduced” DNA can stoichiometrically out-compete the “sample” DNA in subsequent enzymatic amplification reactions, leading to a loss (or dropout) of “sample-derived” alleles (22). Reduced sample manipulation also limits the loss of DNA through repeated extractions and transfers. Another important consideration is that bone is rife with PCR inhibitory compounds, including but certainly not limited to the Ca^{++} present in the hydroxyapatite matrix. Removal of these inhibitory compounds is required for subsequent successful generation of a genetic profile. A simplified and robust DNA isolation protocol has the added benefit of overall cost reduction, by reducing the labor involved in the initial extraction, as well as a reduced likelihood that the procedure will have to be repeated due to a failure of the STR profiling reaction. The latter benefit becomes even more critical in cases where the quantity of bone available for DNA isolation is limited.

In order to extract DNA from bone, there are five major phases: bone surface decontamination, bone pulverization, decalcification, collagen matrix digestion, and separation of nucleic acids from undesirable compounds. A variety of bone surface cleaning methods exist, both chemical and physical. Some of the most widely used chemical techniques involve treating the surface of the bone with acid, ethanol, hydrogen peroxide, sodium hypochlorite (bleach), or a commercially available product such as DNA Away™ (produced by Molecular BioProducts®, it likely consists of concentrated sodium hydroxide) (22). “Mechanical” techniques include surface sanding and irradiation of the surface of the bone with UV light. Drilling into a bone so that only

internal material is collected circumvents, to a certain degree, the issue of surface contamination. Bones that have been previously examined by a forensic anthropologist will likely already have been macerated, a process that removes any soft tissue from the bone so that signs of trauma and anthropomorphic features can be more readily observed. The specific maceration technique employed by the forensic anthropologist can affect the state of preservation of the DNA (45), but that is currently beyond the scope of this study. Once a clean section of bone is acquired, an initial reduction of the bone sample to powder vastly increases the surface area accessible to extraction reagents, enhancing the effectiveness of the subsequent steps of the isolation protocol. Bone powder can be generated by 1) grinding in a mortar and pestle, 2) drilling into the bone, 3) grinding in a coffee mill or blender, 4) pulverization in a freezer/mill®, or a range of other methods. However, each of these methods have significant weaknesses including difficulty in the decontamination of the grinding vessel (mortar and pestle, coffee mill, blender), unacceptable heating of the substrate (drilling), labor intensive processing (mortar and pestle), and relatively high initial equipment cost (freezer/mill®). The method of choice for most human identity laboratories is the freezer/mill®, which rapidly pulverizes the bone, maintains liquid nitrogen temperatures during grinding, and has only three smooth stainless steel parts plus a readily disposable polycarbonate vial in direct contact with the sample.

Decalcification is generally accomplished by incubation of the bone powder with 0.5 M ethylenediaminetetraacetic acid (EDTA), pH 8.0. EDTA chelates the Ca^{++} ions, helping to release DNA that has adsorbed to the hydroxyapatite matrix of the bone (as previously noted, high Ca^{++} levels can also inhibit PCR) (16). EDTA also inhibits

nuclease activity by chelating other metal ions (such as Mg^{++}) that are required by many of these enzymes. DNA in bone is trapped in and protected to a certain degree by the collagen matrix as well as being adsorbed to the hydroxyapatite. In fact, the amount of collagen present in a bone correlates well with the presence of DNA (16). Like Ca^{++}, collagen is an inhibitor of PCR, and so must be digested (often with proteinase K) both to release the DNA and to prevent interference with downstream applications (41).

The DNA released from the bone must then be separated from the proteins, carbohydrates, lipids, and other compounds that have co-extracted with it. Of particular significance are humic acid, fulvic acid, and melanoidins (the products of the Maillard reaction, polymers and copolymers of covalently bound sugars and proteins) (38). These brown, inhibitory products will often partition with the DNA into the aqueous phase even after multiple phenol/chloroform extractions. A myriad of purification methods exist, but ultrafiltration, organic extraction, and silica column purification are some of the most prevalent. Ultrafiltration (UF) devices such as Microcon®, Centricon®, or Amicon® Ultra 4 columns, produced by Millipore®, may be used for sample concentration, buffer exchange, and removal of contaminants smaller than the nominal molecular weight cutoff of the device's filtration membrane. These devices typically must be used in conjunction with another purification method such as organic extraction or silica column purification in order to sufficiently reduce inhibitory compounds. One of the most promising solutions for reduction of inhibitory compound is the isolation the DNA by taking advantage of a biochemical property that allows DNA to specifically bind to a silica substrate in the presence of a high-salt solution. Other mixture components are then washed away, and the DNA is eluted from the silica by a low salt solution. This

procedure also facilitates the buffer exchange necessary to eliminate EDTA used earlier in the protocol (just as EDTA inhibits nucleases by chelation of metal ions, it also effectively inhibits the polymerases in PCR). DNA adsorbs to silica particularly well in the presence of chaotropic salts such as guanidinium thiocyanate (GITC) and guanidine hydrochloride, which are also useful due to their protein denaturation properties (11). Although these types of extractions were originally performed with a slurry of diatoms or glass milk (9), there are now commercially available silica columns such as the QIAquick® and QIAamp® columns produced by Qiagen®, and the DNA Wizard Prep® columns produced by Promega®. Prototype silica columns (PMB columns), with a larger volume capacity, have been produced in collaboration with Mike Brownleader at Generon Inc., to more effectively handle typically encountered working volumes of crude DNA extract. This simplification of the protocol substantially reduces the number of handling steps and sample transfers required, as well as eliminating the use of hazardous compounds such as phenol and chloroform.

Whole Genome Amplification

Although PCR has a theoretical limit of one copy of the target DNA, the practical limit is often much higher. This becomes an even greater problem when the target DNA is in less than pristine condition, as damage (such as apurinic/apyrimidinic sites) can cause the DNA polymerase (particularly non-proofreading enzymes such as *Taq*) to dissociate from the DNA strand prematurely. Forensic samples in particular often suffer from the dual problem of low quantity and low quality. Whole genome amplification

(WGA) is a technique designed to address the issue of limited DNA template quantity by amplifying all the DNA in a sample (not just the loci that lie between specific forward and reverse PCR primers). Some WGA techniques have shown great promise, generating sufficient quantities of DNA from laser micro-dissected tumor biopsies for analysis of chromosomal copy number aberrations (21). There are several distinct approaches that each fall under the procedural umbrella of whole genome amplification: primer extension pre-amplification (PEP), tagged PCR (T-PCR), degenerate oligonucleotide primed PCR (DOP-PCR), and Φ29 isothermal amplification (also known as multiple displacement amplification or MDA). The primary concern for forensic scientists is that the product of a WGA reaction must reflect unbiased and high-fidelity amplification of the original genetic material.

Primer extension pre-amplification is a *Taq* polymerase-based WGA method that, much like standard PCR, involves a series of primer annealing and extension steps. Improved primer extension pre-amplification (I-PEP) is identical to PEP except for the use of a proofreading polymerase in addition to *Taq* polymerase (6). The primary difference between standard PCR and PEP/I-PEP is that instead of using a specific set of primers, these methods use a collection of 15-mer, completely degenerate oligonucleotides (25). This degenerate primer mix includes a total of 10^9 different 15-mer sequences that anneal at random intervals (though generally not too distantly spaced) throughout the genome (47). Although PEP/I-PEP can provide good coverage of the genome, the “random” priming creates shorter and shorter products with each subsequent cycle (due to priming within previously amplified segments) and the process does not produce the exponential amplification seen with standard PCR.

Tagged PCR, which is also *Taq*-based, combines the genome coverage of random priming with the exponential amplification of standard PCR by making use of two separate sets of amplification steps. First, T-PCR uses low-stringency annealing conditions and "hybrid" primers that have the same degenerate 3' sequences as before with a constant 5' sequence (the tag sequence). The first cycle produces large-fragment full coverage of the entire genome while "tagging" each of the amplified sequences at their 5' ends. Several subsequent cycles randomly prime on the first cycle's products to yield amplicons with the conserved primer tag sequence at both ends. The unincorporated hybrid primers are then removed and the dual-tagged amplicons are used for high-stringency amplifications using primers complementary to the tag sequence. While this method yields considerably more product than PEP, there can be a tendency toward preferential amplification of shorter tagged sequences during the high stringency round of amplification. There is also the added concern that the primer removal step can result in the selective loss of some initial amplicons (47).

Degenerate oligonucleotide-primed PCR also uses partially degenerate (hybrid) primers and amplification using low-stringency annealing conditions followed by amplification using high-stringency conditions. Here, however, there is no need for primer removal between steps. Originally a *Taq*-based application, many researchers have substituted proofreading polymerases (alone or in combination with *Taq*) in an effort to improve overall fidelity (25). This practice has also shown promise with degraded DNA samples. The proofreading polymerase can correct base pair mismatches that often occur with *Taq*, as well as bypass small replication errors, both of which promote more complete elongation products (49).

Multiple displacement amplification varies from the previously discussed methods in that it does not rely on repeated cycles of denaturation, annealing, and elongation for the enzymatic amplification of DNA. The Φ29 viral DNA polymerase is a highly processive enzyme (70,000 nucleotides incorporated following a single binding event) that simultaneously displaces the non-template strand of the DNA as it elongates the nascent strand at the rate of 25-50 nucleotides/second (8). This process is carried out at 30-31°C, uses 3' exonuclease-resistant random hexamer primers and requires that the template be denatured only before the first amplification cycle. After which, the strand displacement function of the enzyme provides ample single-stranded DNA for further priming and subsequent amplification. MDA-replicated DNA from tumor cell lines has, in one study, shown 96% concordance with original sample DNA, with only one translocation (in which the target sample had very low levels of that particular DNA fusion) escaping detection (32). Another study found 99.86% concordance between native and MDA-amplified DNA (7).

Replicative fidelity and genome coverage are primary concerns with all WGA methods. Other problems include difficulty with accurate quantitation of the DNA post-WGA. Whole genome amplified DNA is difficult to quantify with standard techniques as the telomeric and repetitive centromeric regions, targeted by Quantifiler® and Quantiblot® respectively, are not effectively amplified by WGA (4). In addition, electrophoretic and spectrophotometric DNA quantitation methods do not accurately represent the quantity of MDA amplified target DNA, due to the production of large amounts of nonspecific product (4). Though promising, the accuracy and utility of DNA profiles generated from WGA amplified templates has yet to be verified.

DNA Repair

A range of types of DNA damage begin to accumulate as soon as the living tissue loses integrity. These can be the result of activities integral to the cell such as the release of hydrolytic enzymes or as a result of environmental factors such as UV irradiation. DNA damage can negatively impact the ability to obtain STR profiles. Recently, mixtures of enzymes designed to repair DNA, such as New England Biolabs® PreCR™ product, have become commercially available. These cocktails have the ability to repair damage such as apurinic/apyrimidinic (AP) sites, thymine dimers, single-strand breaks, gaps, deaminated cytosine, 8-oxo-guanine, and to restore free 3' hydroxyl groups (36). While the reduction of such lesions would certainly improve the efficiency of subsequent PCR-based applications, the potential to inadvertently generate alterations in the sequence of the DNA template as a result of the activities of these enzymes must be carefully examined.

The PreCR™ DNA repair enzyme cocktail contains 8-oxoguanine DNA glycosylase (Fpg), endonuclease VIII, uracil-DNA glycosylase (UDG), T4 endonuclease V (T4 PDG), endonuclease IV, *Bst* DNA polymerase, and *Taq* DNA ligase. Oxidatively damaged purines (such as 8-oxoguanine) are removed from double stranded DNA by 8-oxoguanine DNA glycosylase (Fpg) to generate a single nucleotide gap flanked by a 5' phosphate and 3' hydroxyl, respectively (48). Damaged pyrimidines are released from double stranded DNA by the N-glycosylase activity of endonuclease VIII, creating an AP site. The enzyme's AP-lyase activity then cleaves 5' and 3' of the AP site, leaving a

phosphate on both sides (12). *E. coli* uracil DNA glycosylase removes uracil from DNA by cleavage of the N-glycosylic bond, creating an apyrimidinic site (29). Thymine dimers caused by UV irradiation are rectified using T4 endonuclease V, also known as T4 bacteriophage pyrimidine dimer glycosylase (T4 PDG), whose glycoslase activity cleaves at the 5' end of the dimer and AP-lyase activity cleaves the phosphodiester bond at the AP site (18). Endonuclease IV hydrolyzes AP sites, leaving an intact 5' phosphate and 3' deoxyribose on the respective adjacent nucleotides (27). The large fragment of *Bst* DNA polymerase fills gaps in one strand of double stranded DNA using its 5' $\rightarrow$ 3' polymerase activity, but lacks the domain responsible for the 5' $\rightarrow$ 3' exonuclease activity responsible for nick translation and seen in the native *Bacillus stearothermophilus* polymerase (3). *Taq* DNA ligase will seal nicks between adjacent 3' hydroxyl and 5' phosphate groups, but only if the surrounding bases are perfectly base paired to a complementary strand of DNA and no gaps are present (5). Although not all types of DNA damage are reparable by this cocktail, and indeed some types of repair (such as fragment ligation) should be avoided entirely to preserve sequence integrity of the template DNA, it is theoretically possible to achieve a significant reduction in many of the lesions that are disruptive to downstream applications.

Genetic Analysis

The UNT Center for Human Identification and CODIS databases both rely, in part, on thirteen core autosomal STR loci that make up each profile. When amplified with the Applied Biosystems Incorporated® (ABI®) Profiler Plus™ *ID*, COfiler™, and

Identifiler™, the product sizes for these STR amplicons range from 97-352 base pairs. Since DNA degradation is marked by a reduction in average strand length, the loci with larger products tend to "drop out" of STR profiles generated from severely degraded DNA. This results in the "ski slope" effect, where the electropherogram's smaller STR amplicons show significantly greater peak heights than do the larger amplicons. This is due to inequity in the relative abundance of intact DNA template for each locus, as the odds of fragmenting within a 400 base pair section of DNA are twice that for a 200 base pair section.

Objectives

This study was conducted as part of the President's DNA Initiative to advance justice through DNA technology (13). One of the goals of the President's 5-year, $1 billion initiative is to "ensure that DNA forensic technology is used to its full potential to solve missing persons cases and identify human remains" (13). In keeping with that goal, the primary objectives of this project were: 1. to develop improved methods for extraction of DNA from human skeletal remains, 2. to improve STR profiling success of low-copy DNA samples by employing whole genome amplification to amplify the total pool of DNA prior to STR analysis, and 3. to improve STR profiling success of damaged DNA templates by using DNA repair enzymes to reduce the number/severity of lesions that interfere with STR profiling. Together, all three of these objectives should aid in the development of more robust methods for analyzing DNA from degraded, aged, or otherwise compromised skeletal remains.

Materials and Methods

Sample Sectioning and Cataloguing

All bone samples were obtained from adult femoral cortical bone. Bones were sectioned with a Striker saw to produce C-shaped segments weighing from 2 grams (g) to 5 g each. The first two digits (**XX**.xx.xx) of the assigned sample number indicate the identity of the whole bone from which the sample was derived (all "03" samples are from the same bone). The second set of numbers (xx.**XX**.xx) refers to the actual section cut from the whole bone, with the 01 being closest to the proximal (hip) end of the femoral shaft (section 03.09.xx is more proximal than section 03.17.xx). Bone 03 was divided into 48 sections, with odd numbered sections coming from the anterior face of the femur and even numbered sections coming from the posterior face. Each bone section was photographed to record the gross morphology of the bone (see Figure 1).

Individual sections were pulverized in one vial during a single grind cycle. Bone sections too large to be processed in a single grinding tube were noted by assigning a letter to each batch of ground bone (for example, 03.20A.xx, 03.20B.xx). The third set of numbers (xx.xx.**XX**) defines the individual aliquot of the total bone powder obtained from a single pulverized sample (for example, all 03.09.xx samples were ground simultaneously and then separated into aliquots to generate samples 03.09.01 through 03.09.04).

FIG. 1 Representative photographic documentation of bone sections.

Bone Surface Decontamination

Surface decontamination was accomplished by either 1) UV irradiation in a CL 1000 Ultraviolet Crosslinker at 254 nanometers (nm), 120 Joules per centimeter squared (Jcm^{-2}) two times for 5 minutes each, rotating the bone 180° between exposures or 2) by soaking the bone for 15 minutes in 3% sodium hypochlorite (NaOCl, commercially available bleach is approximately 6% NaOCl), followed by repeated rinsing with sterile nuclease-free water as described by Kemp and Smith (22). UV irradiation is considered a “non-destructive” method, reducing exogenous DNA on the bone surface without damaging the bone itself. However, this method does nothing to remove/reduce PCR inhibitors that are often present on bone surfaces. These inhibitors include humic acid and fulvic acid, which are often seen as a brown color on the surface of the bone (particularly those that have been exposed to soil or other organic material) and commonly co-extract with DNA. Immersion for 15 minutes in 3% sodium hypochlorite, followed by repeated rinsing with sterile DNase/RNase free ddH_2O, can remove not only exogenous DNA but also surface-bound inhibitory compounds. The primary concern with this method is the risk of endogenous DNA destruction. Decontaminated bone sections were stored at room temperature in 50 ml conical tubes prior to pulverization.

Bone Pulverization

The bone section (and resulting powder) comes into contact with four components of the freezer/mill® during pulverization, all of which need to be free of contaminating DNA. Polycarbonate grinding vials were decontaminated by treating inside and out with a 10% bleach solution and then allowing the vial to soak for 5-10 minutes. The vial was next rinsed by liberally spraying with 70% ethanol, followed by 95% ethanol, and finally allowed to air dry in a clean dead air space. The pair of stainless steel end caps and the impactor were scrubbed thoroughly with corrosion-inhibited detergent, rinsed with distilled water, and then treated in the same manner as the grinding vials to ensure removal of contaminating DNA. Clean, dry vials and stainless steel parts were then sealed in sterile 50 ml conical tubes at room temperature until they were to be used. Individual sets of two end caps and one impactor were designated and always used together (as a contamination control measure). Bone sections were ground in a SPEX 6750 freezer/mill® at 15 impacts per second for 5 minutes following a 10 minute liquid nitrogen pre-chill. The resulting bone powder was then weighed and separated into 0.5 g (± 0.02 g) aliquots in sterile 15 ml conical tubes that were stored at -20°C until needed.

DNA Extraction

First, a commercially available silica column needed to be selected for further study. In order to directly compare the efficiency of DNA recovery using the QIAquick® and QIAamp® column kits (from 500 µl of 0.5 M EDTA, 0.5% SDS, 100 µg/ml ProK extraction buffer), known quantities of DNA were added to the buffer and then recovered following the manufacturer's directions with both types of column (39, 40). The QIAquick® and QIAamp® DNA extraction kits use different reagents in the DNA binding and washing steps, so this also served to test the compatibility between the extraction buffer and the Qiagen® chemistries (39, 40). Two DNA sources were used to evaluate both total and size-dependent DNA recovery: λ–phage DNA digested with *Hin*dIII, which consists of seven major fragments (23,130, 9416, 6557, 4361, 2322, 2027, and 564 bp), and a *Hin*fI digestion of the plasmid pBR322, which includes ten fragments (1631, 516, 506, 396, 344, 298, 221, 220, 154, and 75 bp). Either 31 or 62.5 ng of λ *Hin*dIII DNA was loaded onto each column for recovery comparisons. Recovery comparisons for pBR322 *Hin*fI DNA used 125 or 250 ng of DNA. Although each of these amounts is larger than the quantity of DNA likely to be present in 0.5 g of bone powder, these amounts were empirically set to be at the limit of detection for the smallest fragment in each digestion. Analysis of λ–phage and pBR322 DNA recovery was accomplished via electrophoresis in Tris-borate/EDTA (TBE) electrophoresis buffer using 1% and 2% agarose gels, respectively (43). The gels were stained with 1x SYBR® green and the DNA visualized with a Bio-Rad® Molecular Imager FX™.

Six DNA extraction methods were examined during the completion of this project; each starting with 0.5 g (± 0.02 g) of bone powder. The first three methods were silica-based DNA extraction protocols, modified from Yang, Eng et al. (52). These procedures began with an overnight decalcification and protein digestion. The resulting slurry was centrifuged to pellet the remaining bone powder, and the supernatant was processed through a 0.75 ml capacity QIAquick® silica column (according to the manufacturer's instructions) or through a 20 ml capacity PMB column. The last three methods coupled organic extraction with ultrafiltration. The HSC/YM100 protocol utilized two sequential overnight incubations (10). The first incubation decalcified the sample and the second digested the protein matrix. The final slurry was again centrifuged to collect undissolved bone powder and the resultant supernatant subjected to organic extraction. The aqueous phase of the organic extraction was transferred to a Microcon® YM100 ultrafiltration device for concentration and the DNA eluted in sterile, nuclease-free water. The AFDIL and modified AFDIL procedures shared the single overnight decalcification and protein digestion step with the silica-based methods, but the resulting supernatant was then subjected to organic extraction followed by further purification and volume reduction using either a Centricon® 30 or one of the Amicon® Ultra-4 ultrafiltration devices. Each allotment of powdered bone generated multiple 0.5 g aliquots, so it was possible to compare the quantity and quality of DNA obtained from each of the techniques using an identical bone powder substrate.

The QIAquick®/1 ml extraction involved single step overnight decalcification and protein digestion in 1 ml of Yang extraction buffer (YEB - 0.5 M EDTA, 0.5% SDS, 100 µg/ml ProK) at 56°C, followed by DNA recovery via QIAquick® silica gel column (as

modified from Yang, Eng et al., 1998) (52). The QIAquick® kit makes use of three buffers: PB buffer contains guanidine hydrochloride and is used to bind the DNA to the silica bed; PE buffer contains ethanol and is used to wash the undesirable mixture components from the column; and EB buffer (10 mM Tris-HCl pH 8.5) is the elutant for the DNA bound to the column (40). Approximately 0.5 g of bone powder was transferred to a 2 ml screw-cap microcentrifuge tube and 1 ml YEB was added to the bone powder. The mixture was vortexed thoroughly to suspend the bone powder. The suspension was incubated at 56°C with constant agitation overnight (up to 24 hours) in a rotating hybridization oven. The remaining bone powder was pelleted via centrifugation at 12,800 x *g* for 5 minutes. The supernatant was transferred to a 15 ml conical tube and 5 volumes of PB buffer (Qiagen®) added. The contents were then mixed well by repeated inversion (to avoid excessive foaming). In order to bind the DNA to the QIAquick® column, 0.75 ml of sample/PB mixture was added to a column that had been placed in one of the 2 ml "catch" tubes supplied with the kit. The unit was then centrifuged at 17,900 x *g* for 1 minute, after which the flow through buffer was discarded. The remainder of the sample was loaded 0.75 ml at a time, repeating the centrifugation steps until all of the sample/PB mixture had been passed through the column. The column was then washed with 0.75 ml PE buffer (Qiagen®) and centrifuged at 17,900 x *g* for 1 minute. The binding and washing processes can be accomplished more rapidly by attaching the column to a vacuum manifold using disposable connectors. The flow-through was discarded and the column centrifuged once more at 17,900 x *g* for 1 minute. This "dry" centrifugation step was necessary to remove residual alcohol. After the "dry" centrifugation, the column was then transferred to a new 1.5 ml

microcentrifuge tube and 50 µl EB buffer (10 mM Tris-HCl pH 8.5) was added directly to the unit's silica gel membrane. The column was now incubated at room temperature for 1 minute and centrifuged at 17,900 x *g* for 1 minute to collect the eluate in a microcentrifuge tube. The elution step was then repeated with a fresh microcentrifuge tube. The two elutions were stored separately at -20°C until further analyzed.

The QIAquick®/3 ml extraction method was identical to the QIAquick®/1 ml extraction, except that 3 ml of YEB was used to decalcify and digest the bone powder in a 15 ml conical tube. The remaining bone powder was pelleted by centrifugation for 5 minutes at 2545 x *g*. The increased total volume also necessitated the use of a 50 ml conical tube for the addition of the 5 volumes of PB buffer.

The PMB extraction method used single step overnight decalcification and protein digestion in 3 ml of Improved Extraction Buffer (IEB - 0.5 M EDTA, 1% sodium lauroyl sarcosinate, 100 µg/ml ProK). Sodium lauroyl sarcosinate (sarkosyl) was used instead of sodium dodecyl sulfate (SDS) because sarkosyl is soluble in 0.5 M EDTA at room temperature, while YEB must be heated before the SDS will fully solubilize. The QIAquick®/1 ml Extraction protocol was then modified to process the sample through the various PMB column types (40). Remaining bone powder was pelleted by centrifugation for 5 minutes at 2545 x *g*. Due to their larger size, the entire volume of crude DNA extract/PB buffer mixture (approximately 18 ml) could be processed with a single centrifugation step for 10 minutes 2545 x *g*. The PMB columns were constructed on the 20 ml capacity Proteus™ protein purification column platform (designed to be seated in a 50 ml conical tube during use), and contain one of two types of glass fiber filter (A or B). Type B glass fiber is more densely "woven" than type A. Five types of

experimental prototype PMB columns were used: type A constructed with type A glass fiber filter with one, three, or six layers, and type B units constructed with type B glass fiber filter with one or three layers. Volumes of PE wash buffer were initially increased to 1 ml for use with the larger columns. Poor performance of columns containing multiple layers of glass fiber led to the use of higher wash volumes and multiple washing steps. A 5 ml wash was investigated as a single step; as was a 5 ml wash repeated 2 or 3 times (for total wash volumes of 5, 10, or 15 ml, respectively). Collection of the washes was carried out by centrifugation for 5 minutes at 2545 x *g*, with a final dry column centrifugation in an empty 50 ml conical tube for 5 minutes at 2545 x *g*. DNA elution was accomplished using a 100 µl of EB treatment, which was repeated as many as four times per PMB column, and always with an extended 5 minute incubation time at 25°C prior to centrifugation for 10 minutes at 2545 x *g*.

The HSC/YM100 extraction method was the only one to include sequential overnight decalcification and protein digestion steps, followed by organic extraction and subsequent volume reduction via Microcon® YM100 micro-concentrator (100,000 nominal molecular weight limit) (34). Approximately 0.5 g of bone powder was transferred to a 1.5 ml microcentrifuge tube and vortexed to suspend the bone powder. The bone powder was decalcified overnight with constant agitation in 1 ml of 0.5 M EDTA at 25°C. The bone powder was pelleted via centrifugation, at 12,800 x *g* for 5 minutes, and the EDTA supernatant was discarded. The bone powder was washed (vortexed and collected by centrifugation) with 1 ml of sterile, nuclease-free water, which was discarded. The remaining (partially decalcified) bone powder was incubated with constant agitation in a hybridization oven at 56°C in 600 µl stain extraction buffer

(SEB: 10 mM Tris, 100 mM NaCl, 39 mM DTT, 10 mM EDTA, 2% SDS) to which was added 20 µg of ProK. Residual bone powder was pelleted by centrifugation at 12,800 x *g* for 5 minutes, and the resulting supernatant removed to a clean microcentrifuge tube. An equal volume of 25:24:1 phenol:chloroform:isoamyl alcohol (PCIA) was added to the supernatant, the tube briefly vortexed, and centrifuged for 3 minutes at 10,000 x *g* at 25°C. The organic extraction may be repeated at the technician's discretion, based upon the intensity of the extract color. The aqueous layer was transferred to a Microcon® YM100 micro-concentrator. The concentrator was centrifuged at 500 x *g* for 20 minutes, 400 µl of sterile, nuclease-free water was then added, and the centrifugation step repeated (34). The sample was then recovered from the device by placing 20-60 µl of heated sterile, nuclease-free water on the side of the filter opposite the sample and centrifuging the device upside down in a fresh 1.5 ml microcentrifuge tube for 3 minutes at 1000 x *g* (personal communication, Christina Capt (10)).

The AFDIL extraction utilizes a single step overnight decalcification and protein digestion at 56°C, followed by two rounds of organic extraction with PCIA, a single butanol extraction, and subsequent volume reduction. First, 0.5 g (± 0.02 g) of bone powder was transferred to a 15 ml conical tube, then 3 ml AFDIL extraction buffer (0.5 M EDTA, 1% sodium lauroyl sarcosinate, 1 mg/ml ProK was added to the bone powder, and the mixture was vortexed to suspend the bone powder. The suspension was incubated at 56°C with constant agitation overnight (up to 24 hours) in a hybridization oven. Organic extraction was performed using an equal volume (3 ml) of PCIA and vortexing to create a uniform emulsion. This was centrifuged at 650 x *g* in a swinging bucket rotor for 3 minutes, and the aqueous layer transferred to a clean 15 ml conical

tube. The organic extraction was repeated a second time with PCIA, followed by an extraction with butanol. The final aqueous layer was transferred to either a Centricon® 30 or one of the Amicon® Ultra-4 ultrafiltration devices. Amicon® Ultra-4 devices with three different nominal molecular weight limits (NMWL) were used: 10K (10,000 NMWL), 30K (30,000 NMWL), and 50K (50,000 NMWL) (33). The ultrafiltration devices were centrifuged at 650 x *g* until the level of retentate approximated 50 µl, monitoring the level at 30 minute intervals to prevent excessive drying of the membrane. At this time, 2 ml of TE was added, and the centrifugation process repeated until the retentate volume had again dropped to 50 µl. This TE wash step was then repeated. The final concentrated retentate was then pipetted from the ultrafiltration reservoir into a 1.5 ml microcentrifuge tube. A 50 µl aliquot of sterile, nuclease-free water was pipetted up and down in the emptied reservoir to collect any residual sample and this was pooled with the original retentate to give a final recovered volume of approximately 100 µl (30, 31). The different ultrafiltration devices varied in the number of 30 minute centrifugation intervals required to reduce the retained volume to 50 µl, as would be expected due to the variation in membrane pore size (NMWL) and membrane orientation. The Centricon® 30 has a single horizontal membrane, while the Amicon® Ultra-4 devices have dual vertical membranes designed to reduce problems associated with clogging. The Centricon® 30 required a total centrifugation time of 6.5 hours, the Ultra-4 10K 6 hours, and both the 30K and 50K required 4.5 hours.

The modified AFDIL protocol differed from the original AFDIL extraction method in four ways: pelletization of the bone powder after the overnight digestion step, reduction of the amount of ProK, an increase in the number and type of organic

extractions, and the number and composition of washes used on the sample in the ultrafiltration device. The protocol began with single step overnight decalcification and protein digestion in 3 ml of: 0.5 M EDTA, 1% sodium lauroyl sarcosinate, 100 µg/ml ProK (IEB) at 56°C and subsequent centrifugation at 650 x *g* for 10 minutes to clear the slurry of any remaining bone powder. This was followed by two rounds of PCIA extraction, one round of chloroform extraction (24:1 chloroform:isoamyl alcohol), a single butanol extraction, and subsequent volume reduction via Centricon® 30 or one of the Amicon® Ultra-4 ultrafiltration devices. The reduced extract was "washed" twice using 2 ml of TE for each device and once with 2 ml of sterile, nuclease-free water.

DNA Quantitation

The initial comparisons of DNA extraction methods were carried out using results from the Applied Biosystems® Quantifiler® real-time PCR kit for human DNA quantitation. The Quantifiler® kit uses two TaqMan® probes, one labeled with FAM™ dye that detects a 62 base pair amplicon from an intron in the human telomerase reverse transcriptase gene (hTERT, located on the "p" arm of chromosome 5) and another probe labeled with VIC® dye that detects the amplicon generated as the internal positive control (IPC) (2). All necessary reaction components required to generate the IPC amplicon are supplied in the reaction mix, and consist of a synthetic DNA template and primers complementary to its sequence. The signal generated by the FAM™ probe and the hTERT amplicon allows for quantitation of human DNA by comparison to signals generated from a dilution series of human DNA standards of known quantity.

The IPC reaction runs simultaneously in each tube, and since every reaction begins with the same amount of synthetic template, differences in the signal from the IPC reaction can be used to asses the relative levels of inhibitory compounds in each sample. The same ABI® 7300 real-time PCR system was used for all of the Quantifiler® reactions with a thermal cycling profile of 10 minutes at 95°C (once), followed by 40 cycles of 15 seconds at 95°C, 1 minute at 60°C. A dilution series of 50, 16.7, 5.56, 1.85,0.62, 0.21, 0.068, and 0.023 ng/µl was made from the ABI® supplied human DNA standard and used for each reaction plate. No template controls (NTC) and independently obtained 1.0 and 0.1 ng reference standards of 9947A female human control DNA (9947A DNA is commercially produced from lymphoblast cell cultures (26)) were also quantified with each plate. Each bone-derived DNA extract was quantified using 2 µl of template, 10.5 µl of primer mix, and 12.5 µl of PCR reaction mix (as per the manufacturer's instructions) and performed in duplicate. Standard STR typing with Profiler Plus™ *ID* followed quantitation.

Whole Genome Amplification

There are a wide variety of reasons why a sample would yield an incomplete STR profile or no profile at all. These include the failure of reaction conditions/components other than the DNA extract, the presence of high levels of inhibitory compounds, insufficient DNA resulting in STR profile failure, and DNA degraded to the point that the number of full length templates becomes limiting (especially at larger amplicons). The latter two situations may be improved through the

use of whole genome amplification (WGA), although problems with genome coverage are known to increase substantially as WGA is used in an attempt to compensate for problems associated with template quantity and quality (6). The REPLI-g® whole genome amplification kit is based on multiple displacement amplification (MDA) using the Phi 29 polymerase and exonuclease-resistant primers. The DNA is isothermally amplified with MDA. Only a single denaturation step is required, as subsequent strand displacement activity of the polymerase continuously generates single-stranded template DNA.

The REPLI-g® protocol restricts the user to a single microliter of template DNA, and is optimized for >10 ng of DNA. For many of the forensic applications where WGA would be most useful, a 10 ng/µl concentration of sample DNA is unrealistic and a total of 10 ng is more than is generally required for genetic analysis. In order to determine the efficacy of the protocol under conditions likely to be encountered using bone-derived DNA, 1 µl of DNA extract was added “as is”. The total quantity of DNA added as template ranged from 0.0043 ng to 0.0875 ng. Prior to MDA, initial denaturation of the template DNA would typically be accomplished by “melting” the DNA at 94-95°C. However, the REPLI-g® kit utilized chemical denaturation with 1 µl of the potassium hydroxide-based buffer D1 for 3 minutes at room temperature, after which the solution was neutralized with 2 µl of buffer N1. The denatured template was then combined with 15 µl REPLI-g® ultrafast reaction buffer and 1 µl REPLI-g® ultrafast DNA polymerase. All WGA reactions were incubated in a thermal cycler at 30°C for 90 minutes, after which the polymerase was heat inactivated at 65°C for 3 minutes. The reactions were then

stored on ice (or stored at -20°C) until they could be incorporated into STR typing reactions using Profiler Plus™ *ID*.

DNA Repair

Two DNA repair cocktails were investigated. The first was the commercially available PreCR™ repair mix (cocktail A), which contains *Taq* DNA ligase, endonuclease IV, *Bst* DNA polymerase, 8-oxoguanine DNA glycosylase (fpg), uracil-DNA glycosylase (UDG), T4 endonuclease V (T4 PDG), and endonuclease VIII. The second was the PreCR™ T6 repair mix (T6 cocktail), for which the precise makeup has not yet been disclosed, as it is still in the research and development phase at New England Biolabs® (NEB®). Cocktail A is marketed as a multipurpose DNA repair kit, and the T6 cocktail has been recommended for use on bone-derived DNA (14). The manufacturer recommends using 50-500 ng of template DNA in a 50 µl repair reaction. For the purposes of this project, the input was reduced to 0.066-1.34 ng of DNA in a 20 µl repair reaction. The reaction composition was the same for both cocktails: 1x NEB® ThermoPol buffer, 100 µM NEB® dNTPs, 1x NEB® NAD^+, 1x NEB® BSA, and the maximum volume of DNA possible (15.32 µl of extract, which contained 0.066-1.34 ng DNA, depending on the sample). The repair reaction was carried out for 20 minutes at 37°C with cocktail A, and for 30 minutes at room temperature with the T6 cocktail. Afterwards, the reactions were placed on ice (or stored at -20°C) in preparation for use in STR typing with Profiler Plus™ *ID*.

Genetic Analysis

All DNA preparations that contained quantifiable amounts of human DNA were subjected to STR profiling with Profiler Plus™ *ID* prior to any subsequent manipulation. Although normally combined with COfiler™ to generate a full thirteen-locus profile, Profiler Plus™ *ID* by itself is a cost-effective method for screening DNA extracts for "profilability" and success at these nine loci is predictive for success at the remaining four COfiler™ specific loci. The 9 autosomal loci represented in the Profiler Plus™ multiplex are: D3S1358 (D3), a mixture of [TCTA] and [TCTG] repeats on the p arm of chromosome 3 (28); D8S1179 (D8), a combination of [TCTA] and [TCTG] repeats on the q arm of chromosome 8 (37); D5S818 (D5), an [AGAT] tetranucleotide repeat on the q arm of chromosome 5 (20); von Willebrand factor type A (vWA), a complex [TCTA] [TCTG] [TCCA] repeat region located in the 40th intron of the vWA gene near the terminus of the q arm of chromosome 21 (24); D21S11 (D21), an often imperfect [TCTA] [TCTG] repeat on the q arm of chromosome 21 (44); D13S317 (D13), a [TATC] repeat on the q arm of chromosome 13 that can also include an [AATC] repeat (20); alpha fibrinogen (FGA), a complex $[TTTC]_3TTTTTTCT[CTTT]_nCTCC[TTCC]_2$ tetranucleotide repeat located in the 3rd intron of the FGA gene on the q arm of chromosome 4 (35); D7S820 (D7), a [GATA] repeat on the q arm of chromosome 7 (17); and D18S51 (D18), an [AGAA] repeat on the q arm of chromosome 18 (51). The amelogenin locus (AMEL) is also included for gender determination, though it is not actually an STR locus. The AMEL gene occurs on both the X and Y chromosomes, but the copy on the X chromosome has a six base pair deletion that allows for

discrimination between the amplicons (46). See Figure 2 for the range of amplicon sizes generated by Profiler Plus™ *ID*.

Profiler Plus™ STR Amplicon Sizes

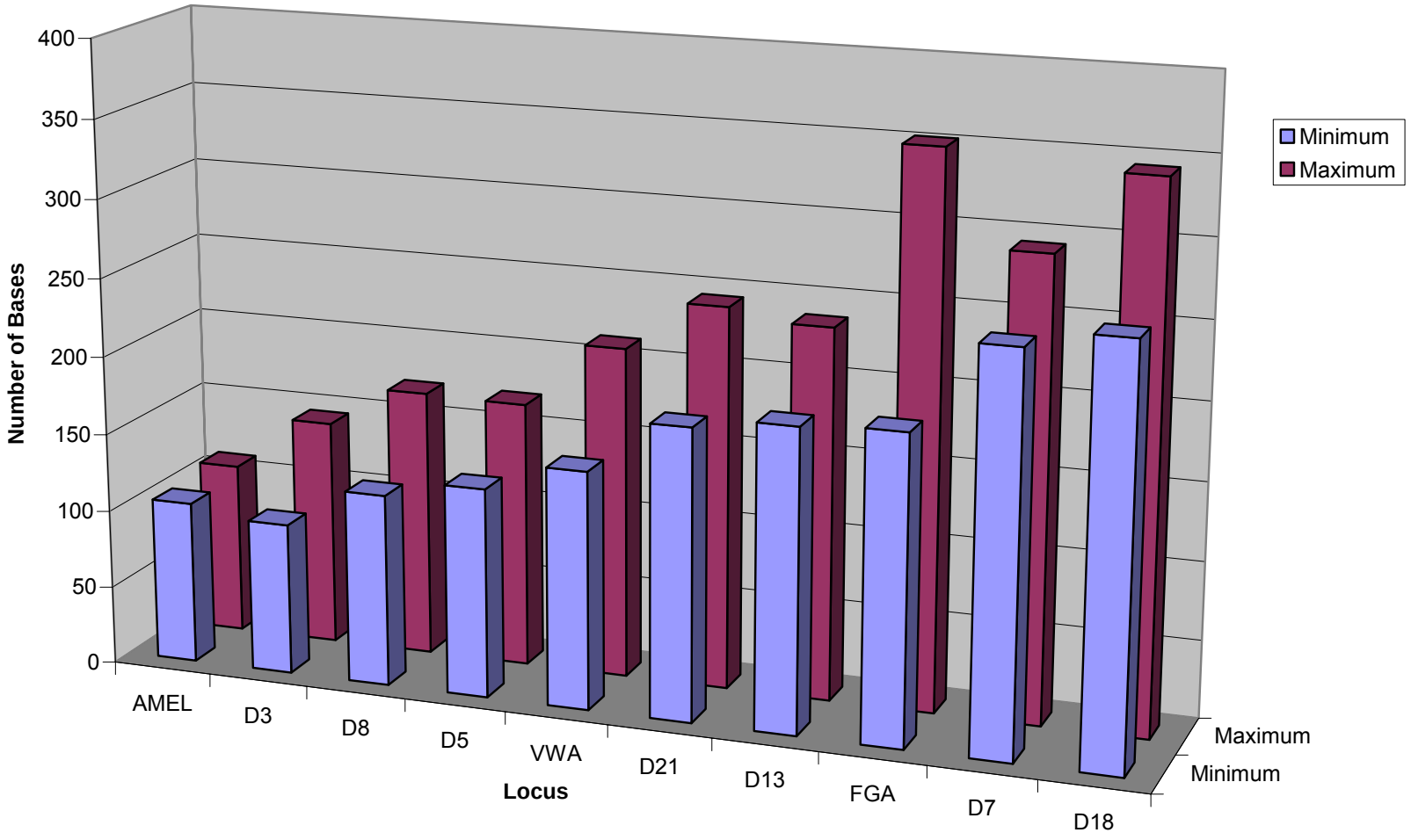


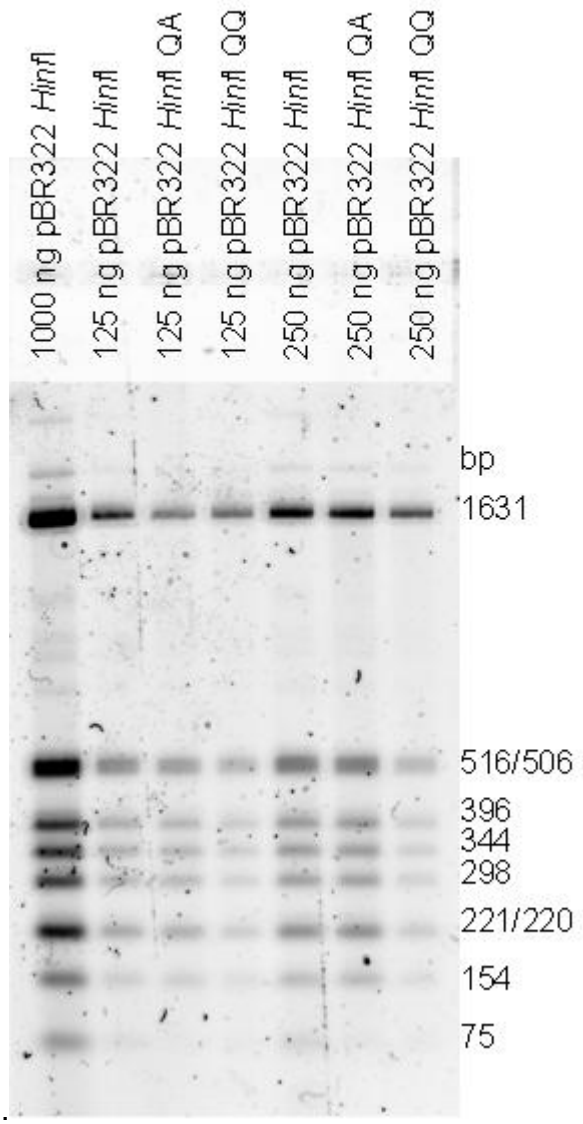
FIG. 2 The range of amplicon sizes possible at each locus when using the Profiler Plus™ *ID* multiplex.

The standard protocol for STR typing with Profiler Plus™ *ID* uses a master mix containing 21 µl AmpF*ℓ*STR® Profiler Plus PCR reaction mix, 1 µl AmpliTaq Gold®, and 11 µl AmpF*ℓ*STR® Profiler Plus Primer set for each sample to be run. This formulation includes 3 µl of overrun per sample, as only 30 µl of master mix should be added to 20 µl DNA plus water (1). In order to double the number of samples that could be typed from a single kit, all of these amounts were halved to yield a total volume of 25 µl per reaction. The manufacturer recommended thermal cycling parameters are 11 minutes at 95°C (once), followed by 28 cycles of 1 minute at 94°C, 1 minute at 59°C, 1 minute at 72°C. The 28 cycles of denaturing, annealing, and extending is followed by an unusually long final extension step of 45 minutes at 60°C, and a hold at either 25°C or 10°C (25°C if the samples were to be left in the thermal cycler for less than 18 hours, 10°C if they were to be left longer) (1). In order to compensate for the low copy number and degradation of the DNA templates, the number of cycles was increased from 28 to 32 for all Profiler Plus® *ID* reactions (10). All DNA extracts that could be successfully quantified with Quantifiler® were subjected to STR typing with Profiler Plus® *ID* (in triplicate) prior to other manipulations. The quantity of extract added varied with DNA concentration, with the goal to add 0.5-1 ng DNA total. Some DNA extracts were so dilute that even 10 µl maximum was still significantly less than 0.5 ng of DNA in the reaction. However, this is a common problem with bone-derived as well as other forensic DNA samples, and these extracts were included as representative of the low DNA copy numbers often encountered in casework.

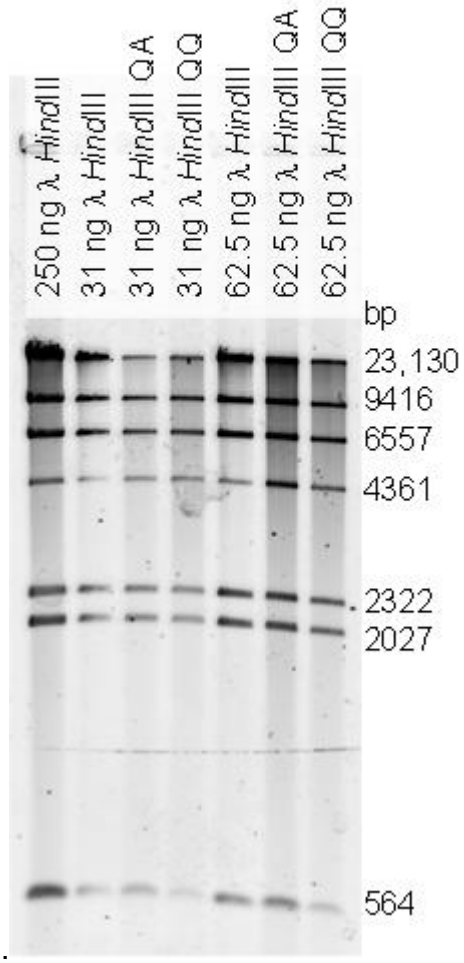
Results

DNA Extraction

Initial comparisons of the performance of the QIAquick® (QQ) and QIAamp® (QA) DNA extraction kits using pBR322 *Hinf*I and λ *Hind*III DNA showed no substantial bias for retention or loss of DNA fragments based upon size. Although, the smaller DNA fragments became more difficult to visualize as total recovered DNA decreased (see Fig. 3a and 3b for representative gel photos); this simply reflected the lower initial signal for these fragments. This effect was particularly evident for the 75 bp band as seen in Figure 3a. The reagents for both columns were compatible with the YEB extraction buffer. The DNA recovered from the QIAamp® columns was closer in band intensity to the unprocessed DNA. Even though the buffers/reagents were themselves compatible in mock extractions, the addition of ethanol (after the guanidine hydrochloride binding buffer AL was added, as per the QIAamp® kit protocol (39)) caused an interesting problem with YEB after decalcification and protein digestion of bone powder. The load of calcium carried by the buffer rapidly precipitated (likely as calcium phosphate) upon addition of the ethanol, causing the entire sample to solidify. This occurred even when the sample and ethanol were preheated to 56°C, and somewhat surprisingly this problem could not be overcome by further dilution of the sample with water. As a result, QIAquick® columns were used for all subsequent DNA extractions with a commercially available silica column.



a.



b.

FIG. 3. Comparison of recovery of known DNA quantities and size standards (*Hin*fI digested pBR322 and *Hin*dIII digested λ phage) with the QIAamp® (QA) and QIAquick® (QQ) columns. A) Electrophoresis was carried out using 2% agarose in TBE at 100 volts. Gel was stained with SYBR green and recorded using a BioRad® Molecular Imager FX™. B). Conditions as in A, but gel composed of 1% agarose. Lanes labeled only *Hin*fI or *Hin*dIII are untreated samples, lanes labeled QA show samples using QIAamp® protocol and lanes labeled QQ isolated using the QIAquick® protocol.

Quantitation

The Quantifiler® results provided two types of data: human DNA quantitation (in ng/µl), which was then multiplied by the volume of extract recovered to give total DNA recovery in nanograms (ng), and internal positive control (IPC) cycle threshold (C_T) values that are relative to the progress of a DNA amplification reaction and does not involve external template DNA. The presence of inhibitors in the reaction will often increase the IPC C_T, because the reaction (with inhibition) requires a greater number of cycles to reach the set amplification threshold. Since IPC C_T values should only be directly compared within the same Quantifiler® run, all IPC values for bone extracts are presented as the difference between the sample IPC C_T and the average of the IPC C_T values of DNA standards from the same run. If the IPC C_T shows a positive shift, then inhibition is probable.

Of the silica devices, the QIAquick® columns recovered the least DNA (0.3 ng for a 3 ml extraction and 1.8 ng for a 1 ml extraction), but also had negative shifts in IPC C_T values, indicating that there were fewer inhibitors in these samples than were present in the ABI® standards. As noted earlier, PMB column descriptions are given as the type of glass fiber (A or B), followed by the number of layers of glass fiber (1, 3, or 6). The glass fiber type B columns with three layers (B3) recovered the most DNA (from 7.8-9.8 ng), but often had positive shifts in the IPC C_T values. The other PMB columns varied considerably in DNA recovery and IPC C_T shifts depending upon the sample pretreatment method utilized, the number of washes with PE buffer, and the number of elutions (see Fig. 4-7). Figure 4 shows estimated DNA recoveries from bleach

pretreated bone using various silica devices. The QIAquick®/1 ml Ex. used 1 ml of YEB, and the QIAquick®/3 ml Ex. used 3 ml of YEB. All four types of PMB column extractions started with 3 ml of IEB and were washed with a single 1 ml aliquot of buffer PE. The DNA recovered by the QIAquick® columns was found in the first elution only, and all subsequent elutions did not yield quantifiable amounts of DNA. The quantity given as DNA recovered from the PMB columns is the sum of DNA recovered in the first and second elutions which both contained measurable amounts of DNA. While three layers of type B glass fiber captured by far the most DNA, this type of column also had the greatest IPC C_T deviation.

Figure 5 shows estimated DNA recovery from UV pretreated bone with various silica devices. The QIAquick®/1 ml Ex. used 1 ml of YEB, and the QIAquick®/3 ml Ex. used 3 ml of YEB. All four types of PMB column extractions started with 3 ml of IEB and were washed with either a 1 ml aliquot of buffer PE (1 ml W), a 5 ml aliquot of buffer PE (5 ml W), 2 separate 5 ml aliquots of buffer PE (10 ml W), or 3 separate 5 ml aliquots of buffer PE (15 ml W). Again, the DNA recovered by the QIAquick® columns was found only in the first elution and the DNA recovered from the PMB columns is the sum of DNA recovered in the first and second elutions. While three layers of type B glass fiber again captured by far the most DNA, it continued to have the greatest IPC C_T deviation, regardless of the number of washes. It is of particular interest to note that the type A glass fiber with 6 layers recovered the second largest quantity of DNA, and this sample exhibited a negative shift in the IPC C_T value when washed 3 times.

Figure 6 demonstrates that DNA was recovered in four sequential elutions (E1, E2, E3, E4) from PMB columns A1, A3, B1, and B3. All columns were washed with a

single 5 ml aliquot of PE buffer. PMB column descriptions are given as the type of glass fiber (A or B), followed by the number of layers of glass fiber (1 or 3), followed by the elution number (i.e. first elution from 1 layer of type A fiber would be A1/E1). PMB column A1 bound very little DNA to begin with, and all of it was released in the first 100 µl elution. PMB columns A3 and B1 released about 2/3 of the total DNA in the first elution, with the other third to follow in elution 2 for column A3 and elutions 2 and 3 for column B1. Column B3 released only half of the total DNA in the first elution, and the majority of the remaining DNA eluted in the second and third. Thus, in general, additional layers of glass fiber filter gave the column additional capacity but required additional elution steps to recover the DNA.

The estimated DNA recovered in four elutions (E1, E2, E3, E4) from PMB columns A3, A6, and B3 is shown in Figure 7. Column descriptions are given as the type of glass fiber (A or B), followed by the number of layers of glass fiber (3 or 6), followed by the number of times that column was washed with 5 ml of PE buffer (2 or 3), followed by the elution number (i.e. the first elution from 3 layers of type A fiber washed twice would be A3(2) E1). Regardless of the number of washes, the B3 columns captured the largest amount of DNA but also had the largest positive shifts in IPC C_T values. However, the IPC C_T values did improve in subsequent elutions. The most promising balance of high DNA recovery coupled with a negative shift in IPC C_T value was seen with column A6 when washed with 3 x 5 ml of PE buffer. It should be noted that multiple elution steps were performed on all silica devices, but only the PMB columns had quantifiable DNA in elutions two through four.

The HSC/YM100 protocol using the Microcon® YM100 ultrafiltration device recovered the least DNA (~2 ng), but as was seen with the QIAquick® columns, had negative shifts in IPC C_T values. All other ultrafiltration devices recovered between 6 and 12 ng of DNA, and had positive IPC C_T shifts of 1-3.5 cycles (see Fig. 8, 9). Figure 8 shows estimated DNA recovery from bleach pretreated bone using Amicon® ultrafiltration devices. These included the Microcon® YM100 (nominal molecular weight limit of 100,000), Centricon® 30 (nominal molecular weight limit of 30,000), and the Amicon® Ultra-4 ultrafiltration devices with three different nominal molecular weight limits (NMWL) 10K (10,000 NMWL), 30K (30,000 NMWL), and 50K (50,000 NMWL). The Amicon® Ultra-4 devices with 30K and 50K NMWL recovered the largest amount of DNA, followed by the Centricon® 30, Ultra-4 10K NMWL, and the YM100. With the exception of the YM100 (on bleach pretreated bone), the use of all ultrafiltration devices resulted in sizeable positive shifts in the IPC C_T values ranging from 0.75 to 3.5 cycles. Of these, the Ultra-4 50K had the smallest shift in IPC C_T value.

Figure 9 demonstrates estimated DNA recovery from UV pretreated bone using Amicon® ultrafiltration devices. The Amicon® Ultra-4 device with the 50K NMWL recovered the largest amount of DNA, followed by the Ultra-4 30K, Centricon® 30, Ultra-4 10K NMWL, and the YM100. With UV pretreated bone, all ultrafiltration devices yielded DNA that exhibited large positive shifts in IPC C_T values. Of the ultrafiltration devices, the YM100 and the Ultra-4 50K had the smallest positive shifts in IPC C_T value when used to extract DNA from UV pretreated bone.

Total DNA Recovery, Silica Devices, Bleach Pretreatment

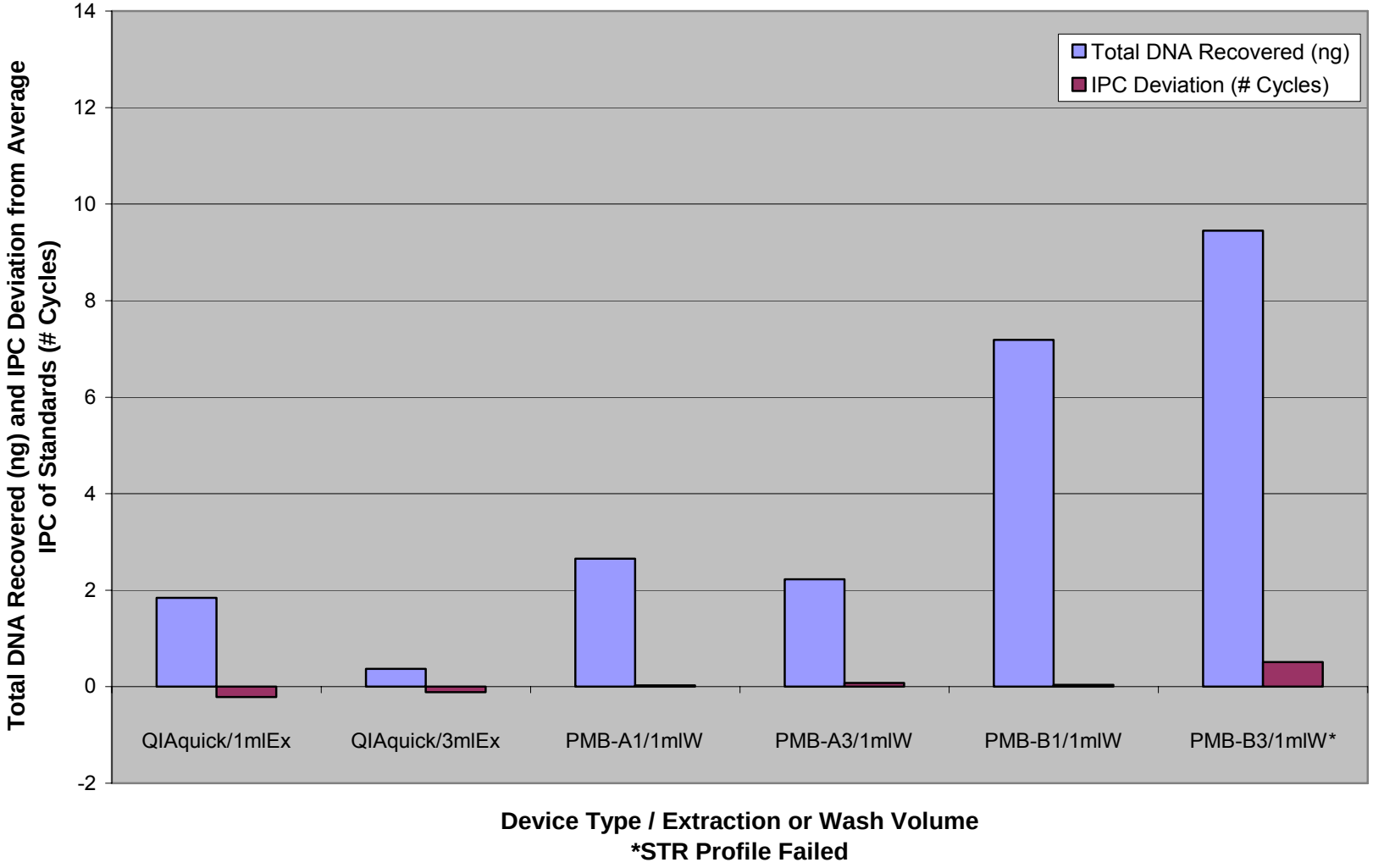


FIG. 4 DNA recovery from bleach pretreated samples using silica-based devices, estimated with Quantifiler®. The QIAquick®/1 ml Ex. used 1 ml of YEB, and the QIAquick®/3 ml Ex. used 3 ml of YEB. All four types of PMB column extractions started with 3 ml of IEB and were washed with a 1 ml aliquot of buffer PE.

QIAquick®/1 ml Ex – bone powder digested with 1 ml Yang extraction buffer, recovered with QIAquick® column, washed with 0.75 ml PE buffer

QIAquick®/3 ml Ex – bone powder digested with 3 ml Yang extraction buffer, recovered with QIAquick® column, washed with 0.75 ml PE buffer

PMB-A1 – bone powder digested with 3 ml improved extraction buffer, recovered with 20 ml PMB column containing 1 layer of type A glass fiber

PMB-A3 – bone powder digested with 3 ml improved extraction buffer, recovered with 20 ml PMB column containing 3 layers of type A glass fiber

PMB-B1 – bone powder digested with 3 ml improved extraction buffer, recovered with 20 ml PMB column containing 1 layer of type B glass fiber

PMB-B3 – bone powder digested with 3 ml improved extraction buffer, recovered with 20 ml PMB column containing 3 layers of type B glass fiber

1 ml W – washed with 1 ml PE buffer

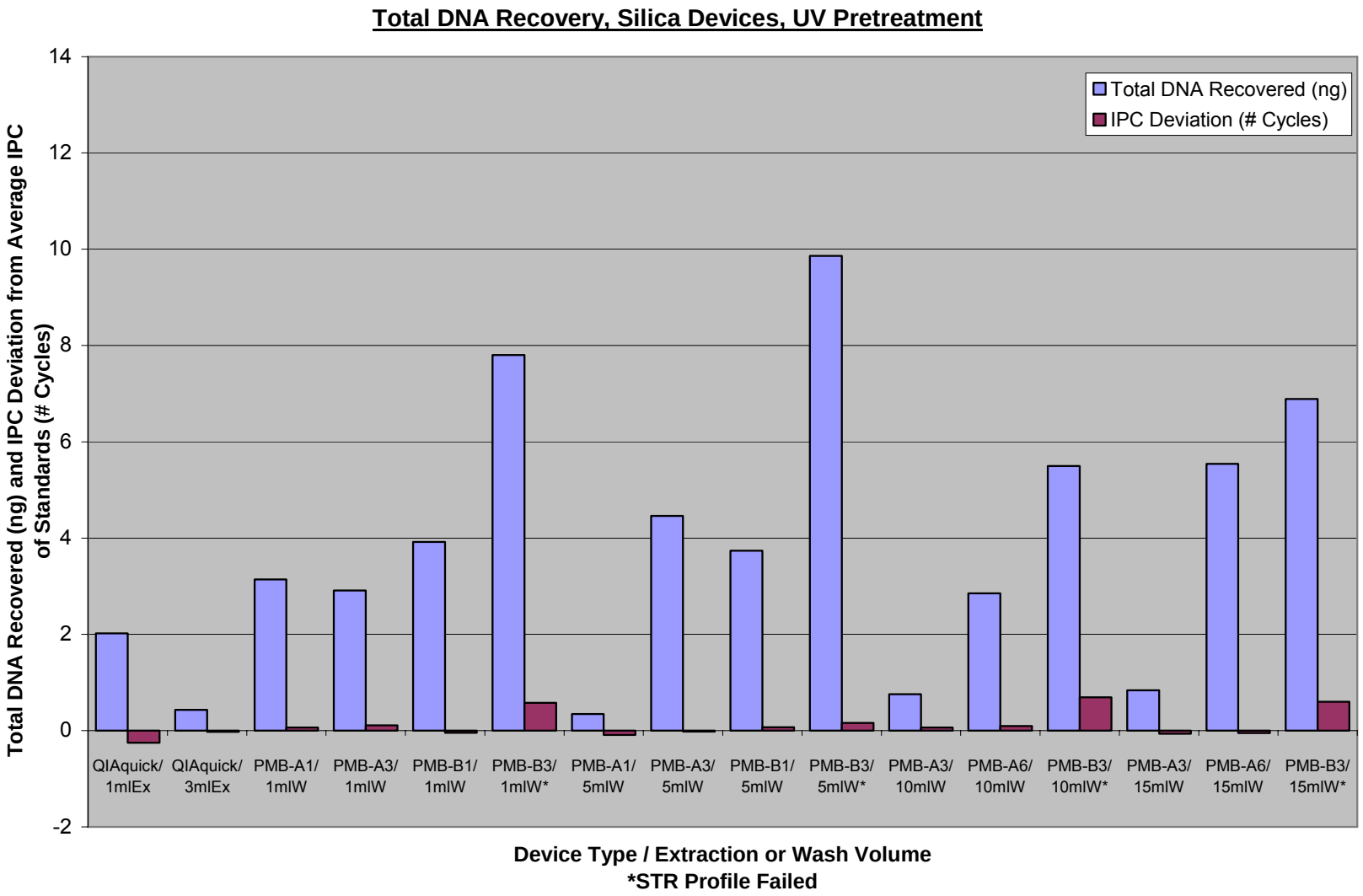


FIG. 5 DNA recovery from UV pretreated samples using silica-based devices, estimated with Quantifiler®. The QIAquick®/1 ml Ex. used 1 ml of YEB, and the QIAquick®/3 ml Ex. used 3 ml of YEB. All four types of PMB column extractions started with 3 ml of IEB and were washed with either a 1 ml aliquot of buffer PE (1 ml W), a 5 ml aliquot of buffer PE (5 ml W), 2 separate 5 ml aliquots of buffer PE (10 ml W), or 3 separate 5 ml aliquots of buffer PE (15 ml W).

QIAquick®/1 ml Ex – bone powder digested with 1 ml Yang extraction buffer, recovered with QIAquick® column, washed with 0.75 ml PE buffer

QIAquick®/3 ml Ex – bone powder digested with 3 ml Yang extraction buffer, recovered with QIAquick® column, washed with 0.75 ml PE buffer

PMB-A1 – bone powder digested with 3 ml improved extraction buffer, recovered with 20 ml PMB column containing 1 layer of type A glass fiber

PMB-A3 – bone powder digested with 3 ml improved extraction buffer, recovered with 20 ml PMB column containing 3 layers of type A glass fiber

PMB-B1 – bone powder digested with 3 ml improved extraction buffer, recovered with 20 ml PMB column containing 1 layer of type B glass fiber

PMB-B3 – bone powder digested with 3 ml improved extraction buffer, recovered with 20 ml PMB column containing 3 layers of type B glass fiber

1 ml W – washed with 1 ml PE buffer

5 ml W – washed with 5 ml PE buffer

10 ml W – washed with 2 aliquots PE buffer, 5 ml each

15 ml W – washed with 3 aliquots PE buffer, 5 ml each

DNA Recovery in 4x100 ul Elutions, PMB Columns, Single 5 ml Wash

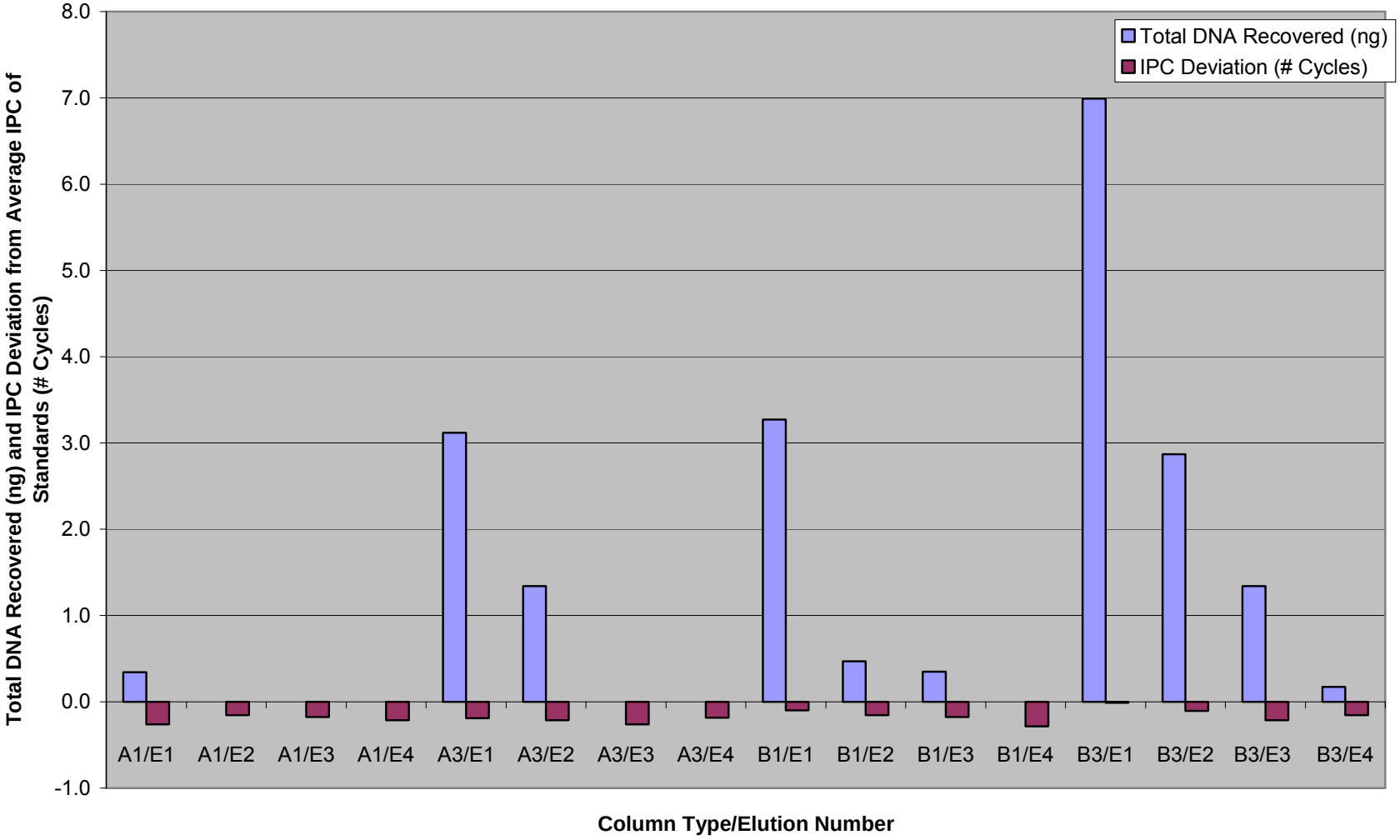


FIG. 6 DNA recovery from four elutions (E1, E2, E3, E4) from PMB columns A1, A3, B1, and B3, estimated with Quantifiler®. All columns were washed with a single 5 ml aliquot of PE buffer. PMB column descriptions are given as the type of glass fiber (A or B), followed by the number of layers of glass fiber (1 or 3), followed by the elution number.

A1 – bone powder digested with 3 ml improved extraction buffer, recovered with 20 ml PMB column containing 1 layer of type A glass fiber

A3 – bone powder digested with 3 ml improved extraction buffer, recovered with 20 ml PMB column containing 3 layers of type A glass fiber

B1 – bone powder digested with 3 ml improved extraction buffer, recovered with 20 ml PMB column containing 1 layer of type B glass fiber

B3 – bone powder digested with 3 ml improved extraction buffer, recovered with 20 ml PMB column containing 3 layers of type B glass fiber

E1 – First 100 µl elution

E2 – Second 100 µl elution

E3 – Third 100 µl elution

E4 – Fourth 100 µl elution

DNA Recovery in 4x100 ul Elutions, PMB Columns, 5 ml Wash Repeated 2 or 3 Times

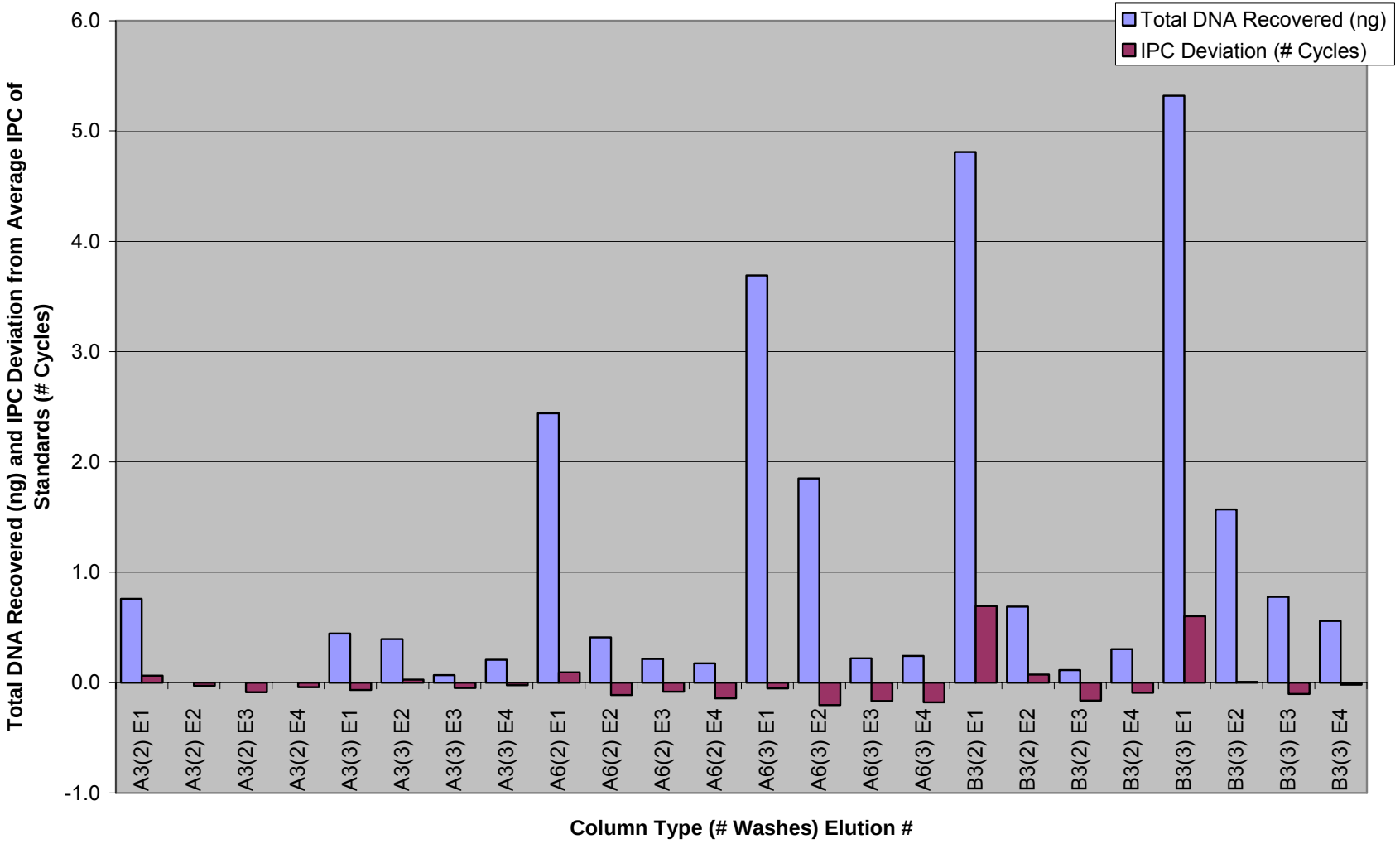


FIG. 7 DNA recovery from four elutions (E1, E2, E3, E4) using PMB columns A3, A6, and B3, estimated with Quantifiler®. Column descriptions are given as the type of glass fiber (A or B), followed by the number of layers of glass fiber (3 or 6), followed by the number of times that column was washed with 5 ml of PE buffer (2 or 3), followed by the elution number.

A3 – bone powder digested with 3 ml improved extraction buffer, recovered with 20 ml PMB column containing 3 layers of type A glass fiber

A6 – bone powder digested with 3 ml improved extraction buffer, recovered with 20 ml PMB column containing 6 layers of type A glass fiber

B3 – bone powder digested with 3 ml improved extraction buffer, recovered with 20 ml PMB column containing 3 layers of type B glass fiber

(2) – washed with 2 aliquots PE buffer, 5 ml each

(3) – washed with 3 aliquots PE buffer, 5 ml each

E1 – First 100 µl elution

E2 – Second 100 µl elution

E3 – Third 100 µl elution

E4 – Fourth 100 µl elution

Total DNA Recovery, Amicon UltraFiltration Devices, Bleach Pretreatment

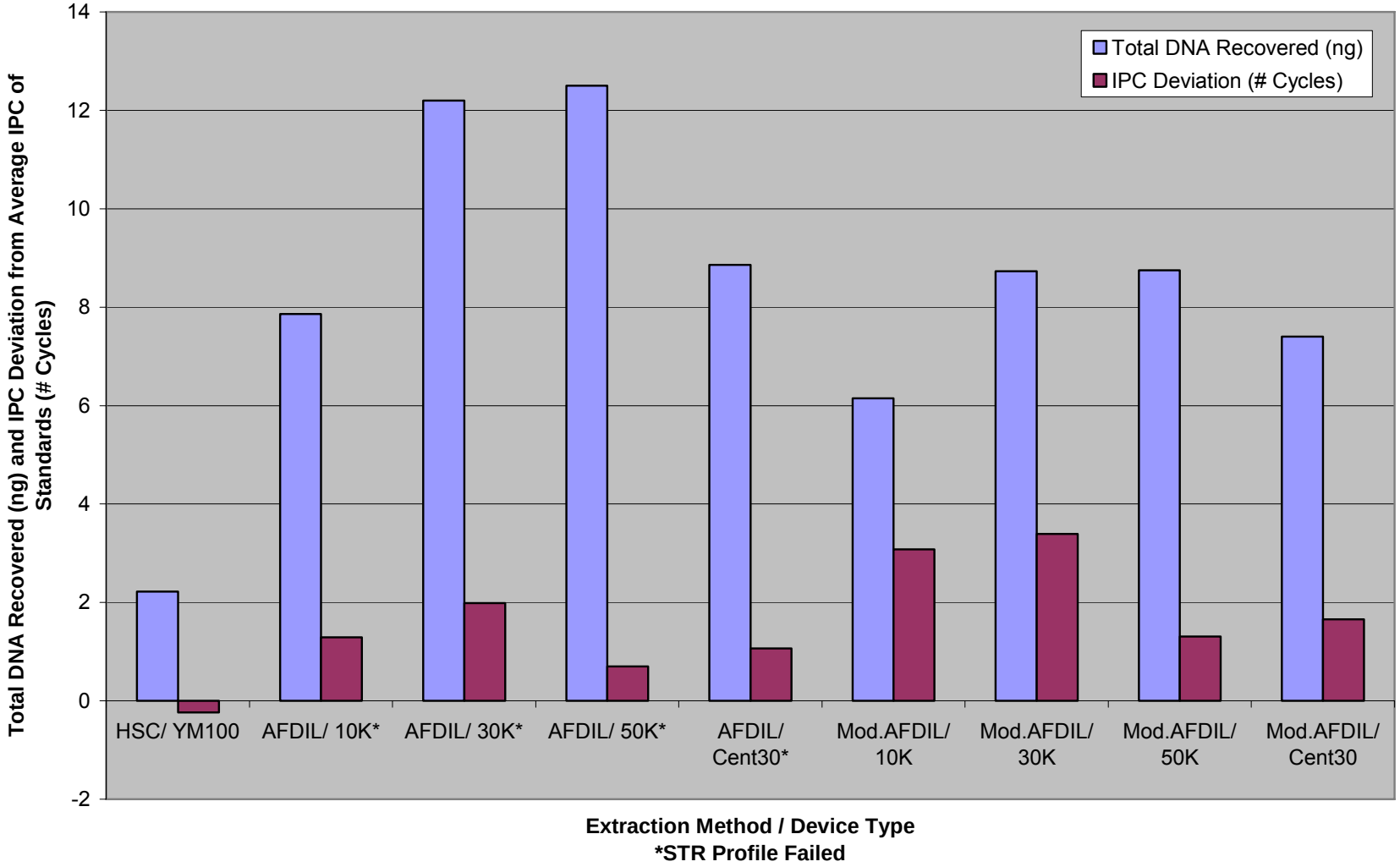


FIG. 8 DNA recovery from bleach pretreated bone using Amicon® ultrafiltration devices, estimated with Quantifiler®. These included the Microcon® YM100 (nominal molecular weight limit of 100,000), Centricon® 30 (nominal molecular weight limit of 30,000), and the Amicon® Ultra-4 ultrafiltration devices with three different nominal molecular weight limits (NMWL) 10K (10,000 NMWL), 30K (30,000 NMWL), and 50K (50,000 NMWL).

HSC/YM100 – bone powder decalcified with 1 ml 0.5 M EDTA, digested with 1 ml stain extraction buffer, subjected to organic extraction, recovered with Microcon® YM100 device, horizontal membrane, nominal molecular weight limit 100,000

AFDIL – bone powder digested with 3 ml improved extraction buffer, subjected to three rounds of organic extraction, ultrafiltrate washed once with TE

Mod.AFDIL – bone powder digested with 3 ml improved extraction buffer, subjected to four rounds of organic extraction, ultrafiltrate washed twice with TE, once with nuclease-free water

10K – recovered with Amicon® Ultra-4 device, vertical membrane, nominal molecular weight limit 10,000

30K – recovered with Amicon® Ultra-4 device, vertical membrane, nominal molecular weight limit 30,000

50K – recovered with Amicon® Ultra-4 device, vertical membrane, nominal molecular weight limit 50,000

Cent30 – recovered with Centricon® 30 device, horizontal membrane, nominal molecular weight limit 30,000

Total DNA Recovery, Amicon UltraFiltration Devices, UV Pretreatment

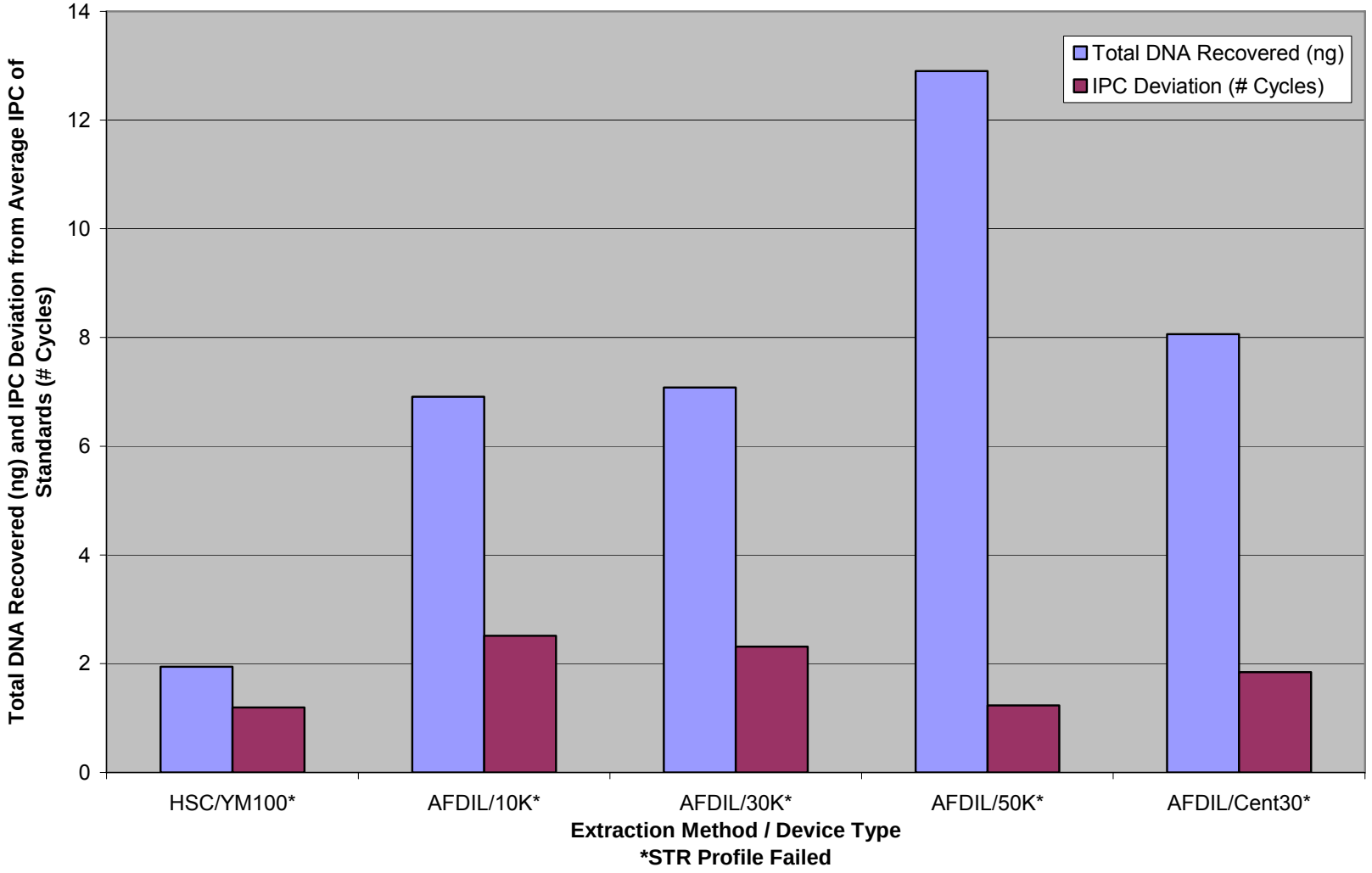


FIG. 9 DNA recovery from UV pretreated bone using Amicon® ultrafiltration devices, estimated with Quantifiler®. The columns represented here include the Microcon® YM100 (nominal molecular weight limit of 100,000), Centricon® 30, and the Amicon® Ultra-4 ultrafiltration devices: 10K (10,000 NMWL), 30K (30,000 NMWL), and 50K (50,000 NMWL).

HSC/YM100 – bone powder decalcified with 1 ml 0.5 M EDTA, digested with 1 ml stain extraction buffer, subjected to organic extraction, recovered with Microcon® YM100 device, horizontal membrane, nominal molecular weight limit 100,000

AFDIL – bone powder digested with 3 ml improved extraction buffer, subjected to three rounds of organic extraction, ultrafiltrate washed once with TE

10K – recovered with Amicon® Ultra-4 device, vertical membrane, nominal molecular weight limit 10,000

30K – recovered with Amicon® Ultra-4 device, vertical membrane, nominal molecular weight limit 30,000

50K – recovered with Amicon® Ultra-4 device, vertical membrane, nominal molecular weight limit 50,000

Cent30 – recovered with Centricon® 30 device, horizontal membrane, nominal molecular weight limit 30,000

Average STR Peak Heights

Profiler Plus™ *ID* was used to gauge the ability of each extract to provide an STR profile. Peak heights (measured in relative fluorescence units (RFU)) were averaged for all analyses of a sample, providing a method for comparison of STR profile success. The loci/alleles in each graph are ordered from the smallest amplicon (on the left of the graph) to the largest amplicon (on the right).

Average STR peak heights for bleach or UV pretreated bone extracted with either 1 ml or 3 ml of YEB extraction buffer are shown in Figure 10. All the extracts in Figure 10 were processed through QIAquick® columns. In the 1 ml extractions, both bleach and UV pretreatment had 16 out of 18 alleles successfully amplify to above threshold levels, but the bleach pretreated sample had much larger peak heights. Neither 3 ml extraction provided a complete profile. Only 11 out of 18 alleles were amplified from the bleach pretreated 3 ml extraction, and 12 alleles were amplified from the UV pretreated 3 ml extraction.

Figure 11 shows the average STR peak heights for bleach or UV pretreated bone extracted with A1, A3, or B1 PMB columns, and washed with a single 1 ml aliquot of PE buffer. The profiling reactions for the B3 column samples failed completely. The B1 columns yielded the most complete profiles (for both bleach and UV pretreated bone) when washed with 1 ml PE buffer; although none of the 1 ml washed PMB columns provided a complete profile.

Average STR peak heights for UV pretreated bone extracted with A1, A3, or B1 PMB columns, and washed with a single 5 ml aliquot of PE buffer are shown in Figure

12. The B3 columns were also used to extract DNA from bone powder using a single 5 ml wash, but these extracts were not successfully profiled. DNA recovered from the A3 columns provided the only complete profile when used in conjunction with a 5 ml wash, although the A1 and B1 columns both amplified 15 out of 18 alleles.

Figure 13 shows average STR peak heights for UV pretreated bone extracted with A3, or A6 PMB columns (washed with a 2 x 5 ml or 3 x 5 ml PE buffer). Extractions were performed with the B3 columns using both 2 x 5 ml and 3 x 5 ml wash combinations, but none of the recovered DNA samples were successfully profiled. All DNA extracts from the A3 and A6 columns washed with 10 ml or more of PE buffer provided full profiles. However, the DNA isolated using a combination of the A6 column and 15 ml wash resulted in the highest overall RFU values.

Figure 14 shows average STR peak heights for samples isolated from bleach pretreated bone using Amicon® ultrafiltration devices and the Modified AFDIL protocol. The Amicon® Ultra-4 device with the 50K NMWL yielded the only complete profile. The sample concentrated with the YM100 yielded a partial profile 15 out of the 18 possible alleles, the Centricon® 30 provided 13 of 18, the Ultra-4 30K provided 12 out of 18 alleles, and the Ultra-4 10K sample yielded only 5 of 18. When used in conjunction with the AFDIL protocol, neither the Ultra-4 devices nor the Centricon® 30 provided samples that could be successfully profiled (regardless of pretreatment method).

Average STR Peak Heights - QIAquick Columns

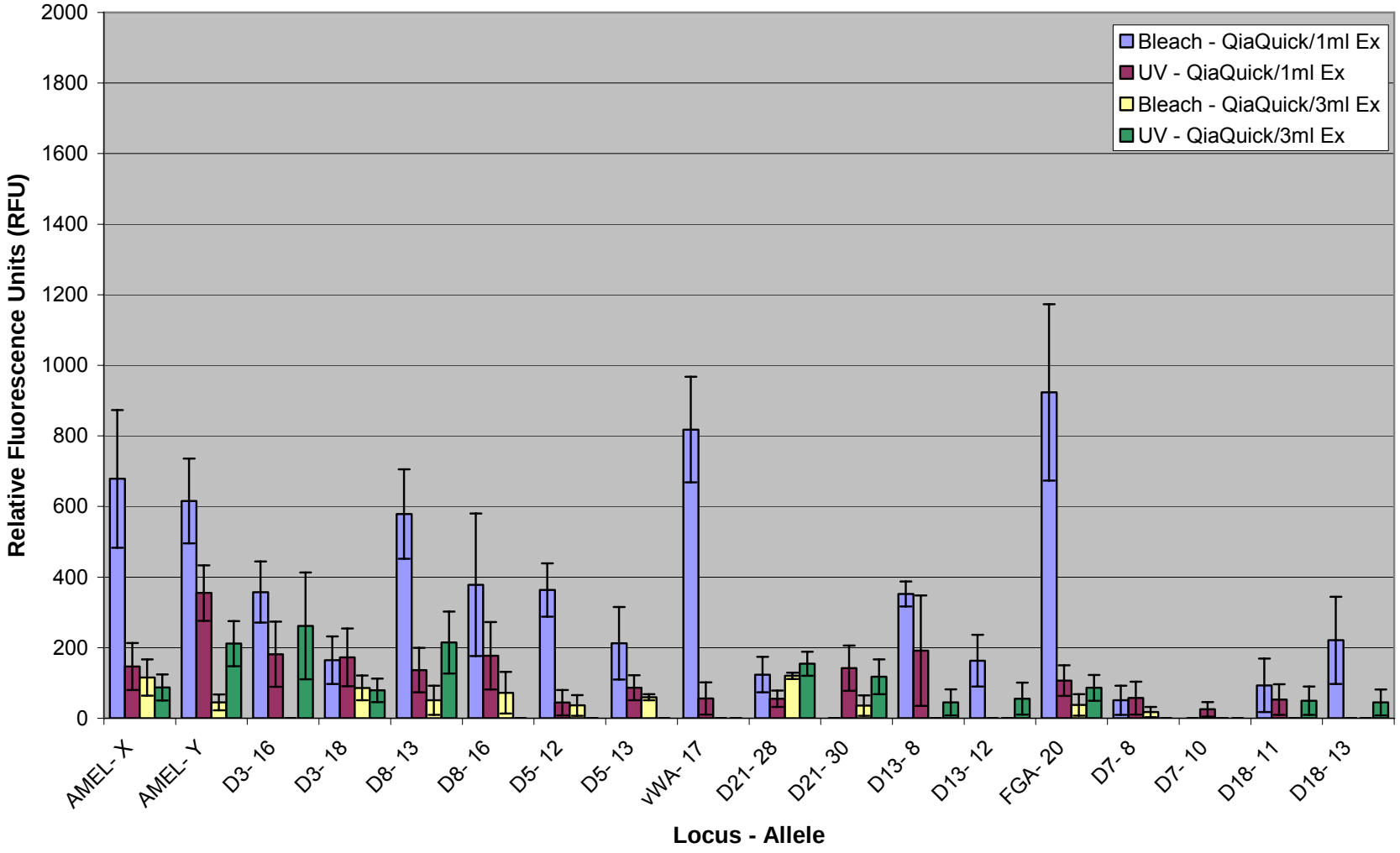


FIG. 10 Average STR peak heights for bleach or UV pretreated bone extracted with either 1 ml or 3 ml extraction buffer and processed through QIAquick® columns.

Bleach – bone section pretreated with bleach

UV – bone section pretreated with UV irradiation

QIAquick®/1 ml Ex – bone powder digested with 1 ml Yang extraction buffer, recovered with QIAquick® column, washed with 0.75 ml PE buffer

QIAquick®/3 ml Ex – bone powder digested with 3 ml Yang extraction buffer, recovered with QIAquick® column, washed with 0.75 ml PE buffer

Average STR Peak Heights, PMB Columns 1 ml Wash

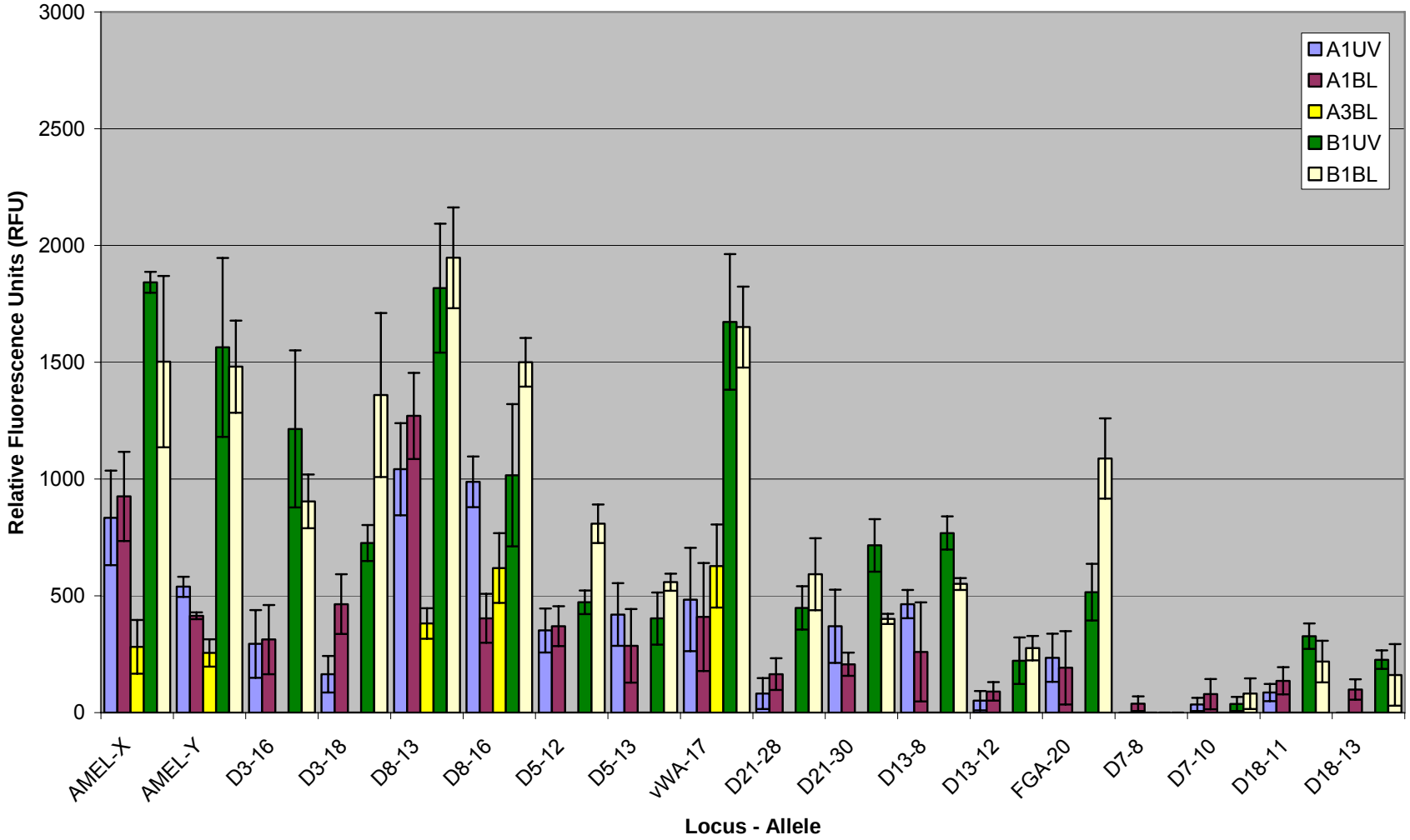


FIG. 11 Average STR peak heights for bleach or UV pretreated bone extracted with A1, A3, or B1 PMB columns (washed with a single 1 ml aliquot of PE buffer).

BL – bone section pretreated with bleach

UV – bone section pretreated with UV irradiation

A1 – bone powder digested with 3 ml improved extraction buffer, recovered with 20 ml PMB column containing 1 layer of type A glass fiber

A3 – bone powder digested with 3 ml improved extraction buffer, recovered with 20 ml PMB column containing 3 layers of type A glass fiber

B1 – bone powder digested with 3 ml improved extraction buffer, recovered with 20 ml PMB column containing 1 layer of type B glass fiber

1 ml Wash – washed with 1 ml PE buffer

Average STR Peak Heights - PMB Columns, UV Pretreat, 5 ml Wash

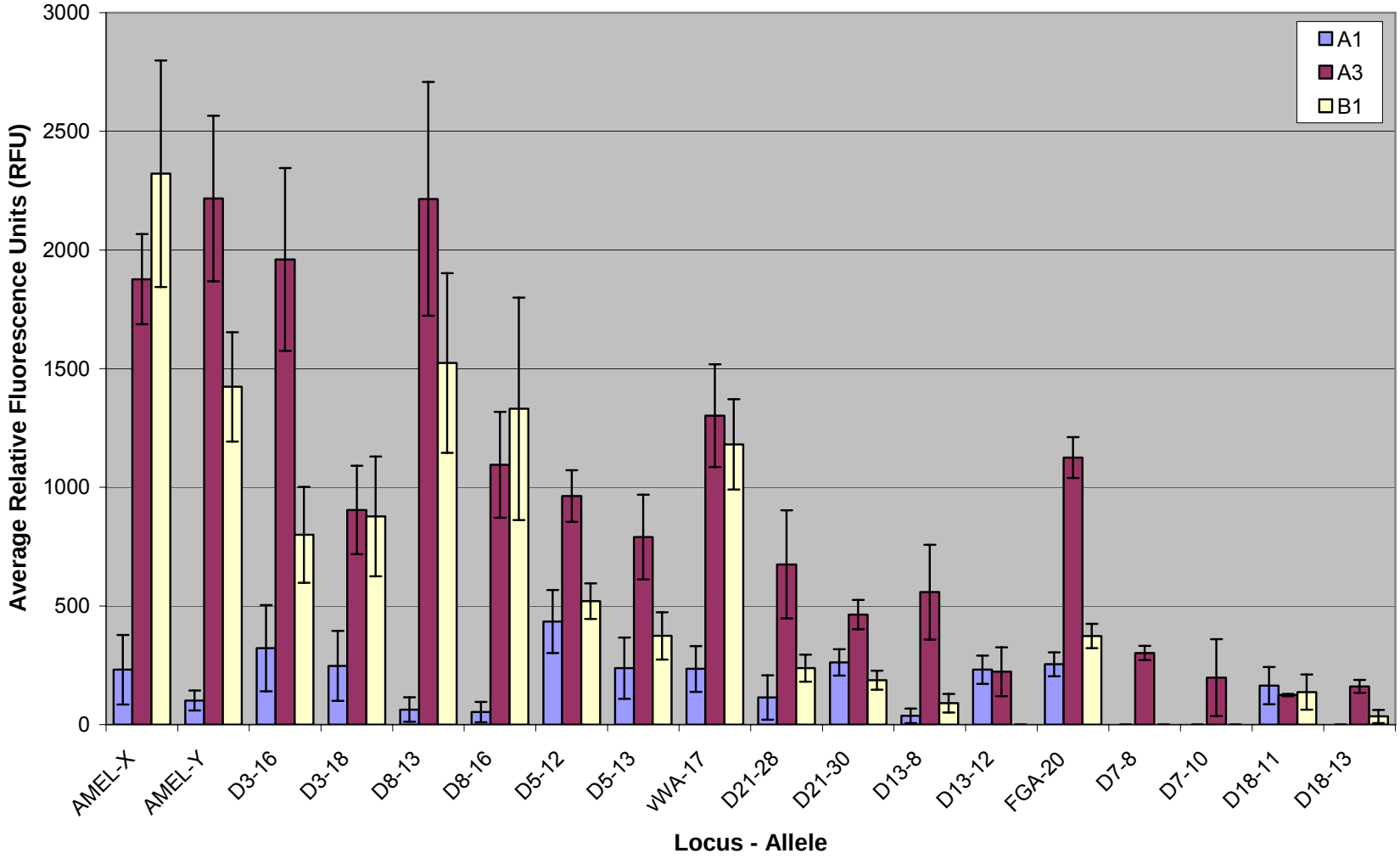


FIG. 12 Average STR peak heights for UV pretreated bone extracted with A1, A3, or B1 PMB columns (washed with a single 5 ml aliquot of PE buffer).

A1 – bone powder digested with 3 ml improved extraction buffer, recovered with 20 ml PMB column containing 1 layer of type A glass fiber

A3 – bone powder digested with 3 ml improved extraction buffer, recovered with 20 ml PMB column containing 3 layers of type A glass fiber

B1 – bone powder digested with 3 ml improved extraction buffer, recovered with 20 ml PMB column containing 1 layer of type B glass fiber

5 ml Wash – washed with 5 ml PE buffer

Average STR Peak Heights - PMB Columns, UV Pretreat, 10 or 15 ml Wash

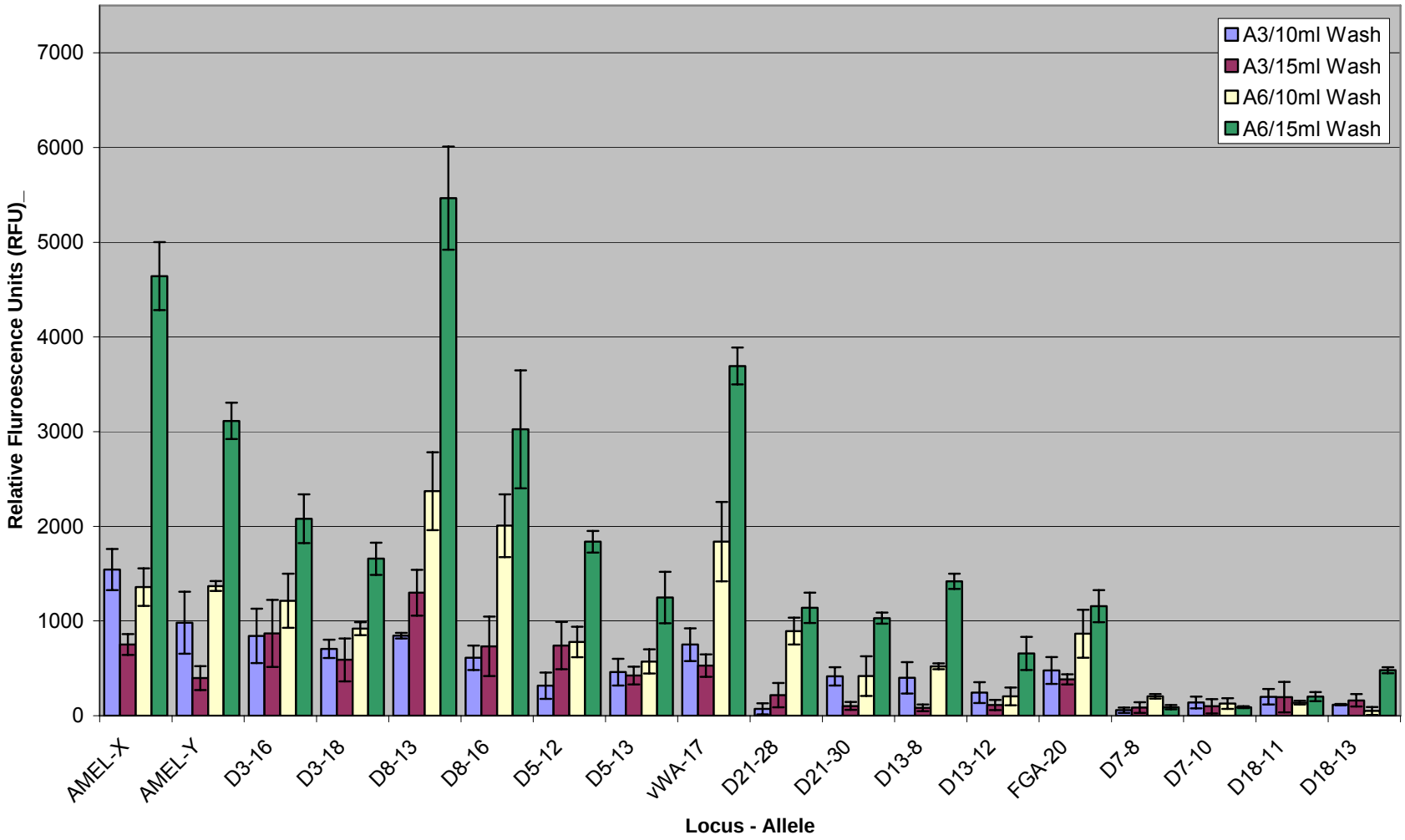


FIG. 13 Average STR peak heights for UV pretreated bone extracted with A3, or A6 PMB columns (washed with a 2 x 5 ml or 3 x 5 ml PE buffer).

A3 – bone powder digested with 3 ml improved extraction buffer, recovered with 20 ml PMB column containing 3 layers of type A glass fiber

A6 – bone powder digested with 3 ml improved extraction buffer, recovered with 20 ml PMB column containing 6 layers of type A glass fiber

10 ml Wash – washed with 2 aliquots PE buffer, 5 ml each

15 ml Wash – washed with 3 aliquots PE buffer, 5 ml each

Average STR Peak Heights - Amicon UltraFiltration Devices, Bleach Pretreatment

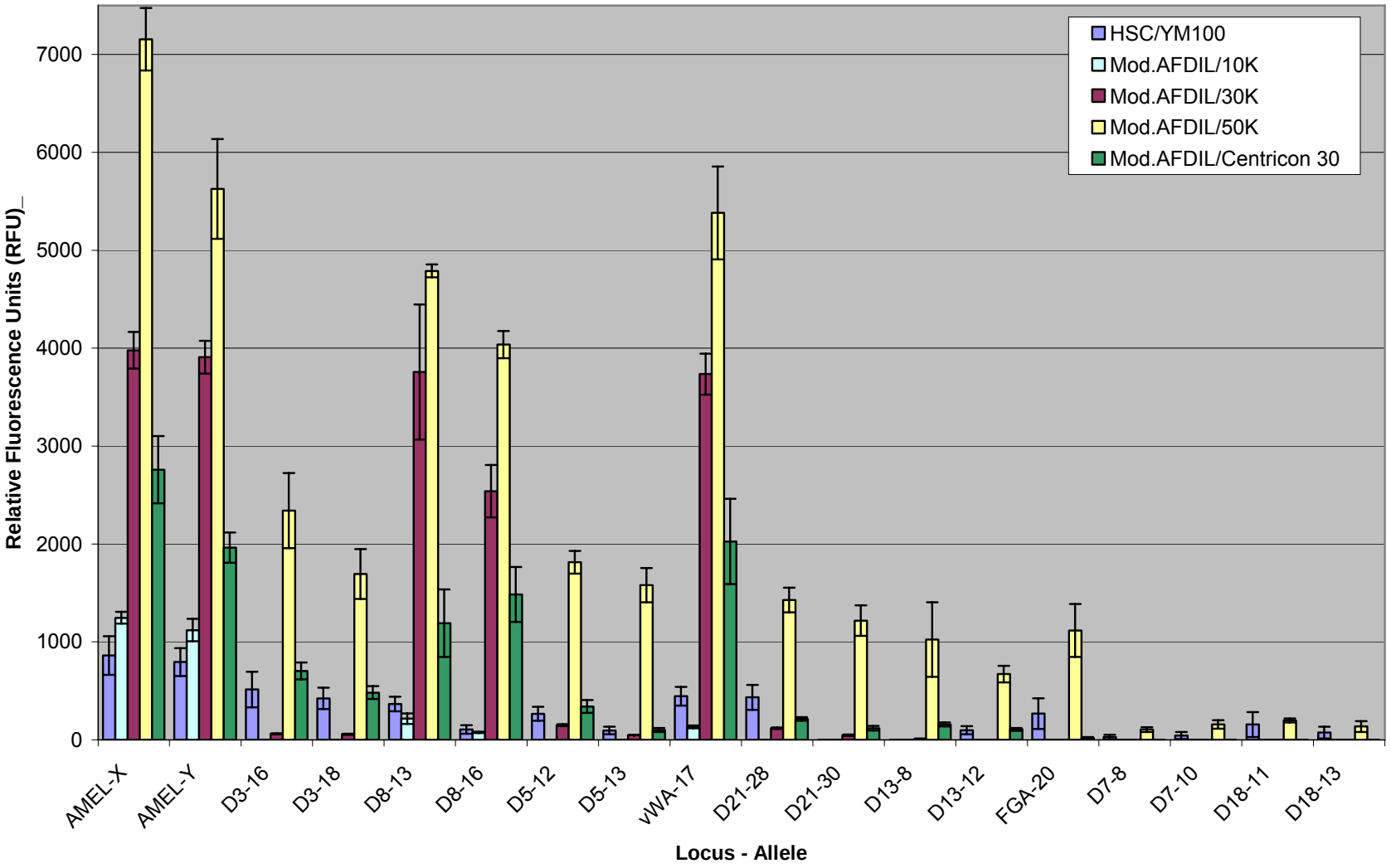


FIG. 14 Average STR peak heights for bleach pretreated bone using Amicon® ultrafiltration devices and the Modified AFDIL protocol.

HSC/YM100 – bone powder decalcified with 1 ml 0.5 M EDTA, digested with 1 ml stain extraction buffer, subjected to organic extraction, recovered with Microcon® YM100 device, horizontal membrane, nominal molecular weight limit 100,000

Mod.AFDIL – bone powder digested with 3 ml improved extraction buffer, subjected to four rounds of organic extraction, ultrafiltrate washed twice with TE, once with nuclease-free water

10K – recovered with Amicon® Ultra-4 device, vertical membrane, nominal molecular weight limit 10,000

30K – recovered with Amicon® Ultra-4 device, vertical membrane, nominal molecular weight limit 30,000

50K – recovered with Amicon® Ultra-4 device, vertical membrane, nominal molecular weight limit 50,000

Cent30 – recovered with Centricon® 30 device, horizontal membrane, nominal molecular weight limit 30,000

Whole Genome Amplification

The REPLI-g® kit was chosen as a WGA method for three reasons: recent publications have suggested that multiple displacement amplification (MDA) outperforms PCR-based methods when used with forensic samples (4), the GenomiPhi® MDA kit had already been tested on forensic-type samples as reported in multiple publications (4, 6), and the REPLI-g kit uses chemical denaturation instead of heat denaturation. (The GenomiPhi® MDA kit uses heat denaturation.)

In no case did whole genome amplification (WGA) with the REPLI-g® kit improve the STR profile of a sample. The electropherograms generated from WGA samples often contained a multitude of above threshold off-ladder peaks, particularly in the size range of 100-200 bases. The most complete profiles contained a maximum of 1-3 successfully amplified alleles. The amplified alleles were often immediately preceded by stutter peaks of up to 45% of the height of the legitimate peak.

DNA Repair

DNA repair protocols were performed with PreCR™ cocktail A and PreCR™ T6 cocktail on five different sample types. The average relative fluorescence units (RFU) were obtained by determining the mean for 3 Profiler Plus *ID* reactions; 1 each with 2, 7, or 10 µl of finished repair reaction mix as template for STR profiling. Reactions that used 10 µl of repair reaction mix contained 77% of the original amount of template used in the non-repaired profiling reactions, the 7 µl samples contained 54%, and the 2 µl

samples contained 15%. The optimum profile obtained from each sample is also noted, along with the volume of repair reaction mix that generated it. Reduced RFUs were expected with a reduced amount of template. However this was not always observed.

When repaired with cocktail A, the QIAquick®/3 ml extraction profile gained 5 alleles, lost 3 alleles, and showed an increase in overall average relative fluorescence units from 75 to 199 (Figure 15). The profile from this sample when "repaired" with the T6 cocktail failed entirely, as did the T6 profiles from all but two other sample types (Ultra-4 50K and Centricon® 30). Two sample types derived from the PMB columns with six layers of type A glass fiber were also subjected to repair. The only sample demonstrating a reduction in profile quality is shown in Figure 16, and was isolated using a PMB A6 column and washed with 2 x 5 ml of PE buffer. Three alleles were lost in comparison to its previously complete profile, and the overall average RFU dropped from 880 to 326. The STR profile in Figure 17 (from a sample prepared on a PMB A6 column and washed with 3 x 5 ml of PE buffer) showed no loss of alleles. Although the overall average RFU dropped from 1835 to 1316, the optimum profile (in which 7µl of PreCR™ A repair mix reaction was used in the Profiler Plus™ reaction) had an average RFU of 2541. The T6 profiles failed for both types of PMB column-derived samples. The Amicon® Ultra4 50K NMWL extracted sample (shown in Figure 18) had no loss of alleles after repair with cocktail A, the average RFU dropped a negligible amount from 2249 to 2172, and the optimum profile (7 µl repaired DNA mix in the STR profile reaction) showed an increase in RFU to 3224. The most complete profile from the T6 cocktail "repaired" sample (from the 50K ultrafiltration device) lost 6 alleles. Shown in Figure 19, the final sample type to undergo repair was extracted using the Centricon®

30 ultrafiltration device. The average RFU went up after repair, from 648 to 744, and one allele was gained. The optimum profile (10 µl) had an average RFU of 970.

Average STR Peak Height - UV Pretreatment, QIAquick/3 ml Extraction Before and After Repair

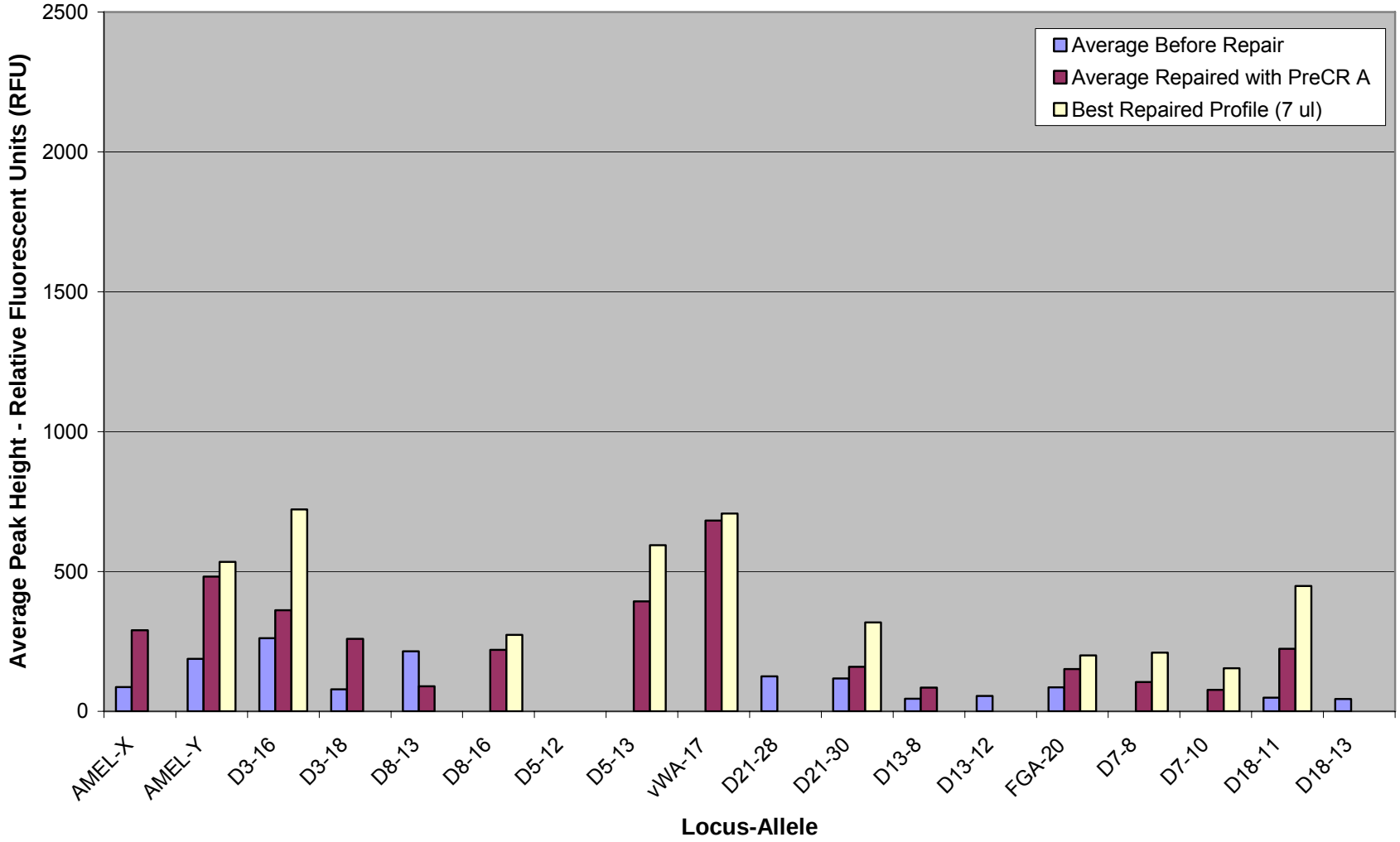


FIG. 15 PreCR™ A repair of UV pretreated, 3 ml QIAquick® extracted DNA.

QIAquick®/3 ml Extraction – bone powder digested with 3 ml Yang extraction buffer, recovered with QIAquick® column, washed with 0.75 ml PE buffer

UV – bone section pretreated with UV irradiation

Average STR Peak Heights - UV Pretreatment, PMB A6 Columns, 2 x 5 ml Wash Before and After Repair

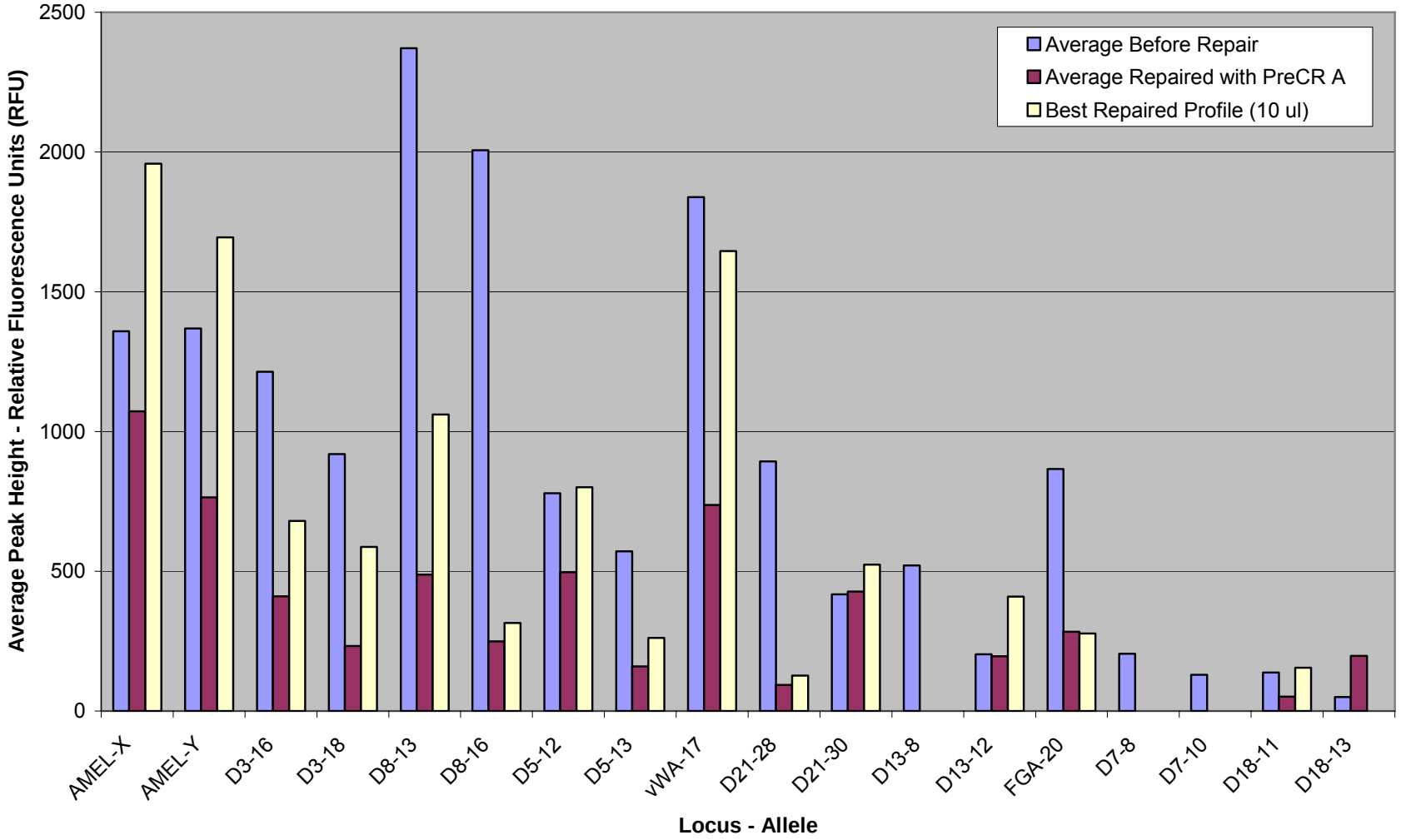


FIG. 16 PreCR™ A repair of UV pretreated, PMB A6 (2 x 5 ml wash) extracted DNA.

A6 – bone powder digested with 3 ml improved extraction buffer, recovered with 20 ml PMB column containing 6 layers of type A glass fiber

2 x 5 ml Wash – washed with 2 aliquots PE buffer, 5 ml each

UV – bone section pretreated with UV irradiation

Average STR Peak Heights - UV Pretreatment, PMB A6 Columns, 3 x 5 ml Wash Before and After Repair

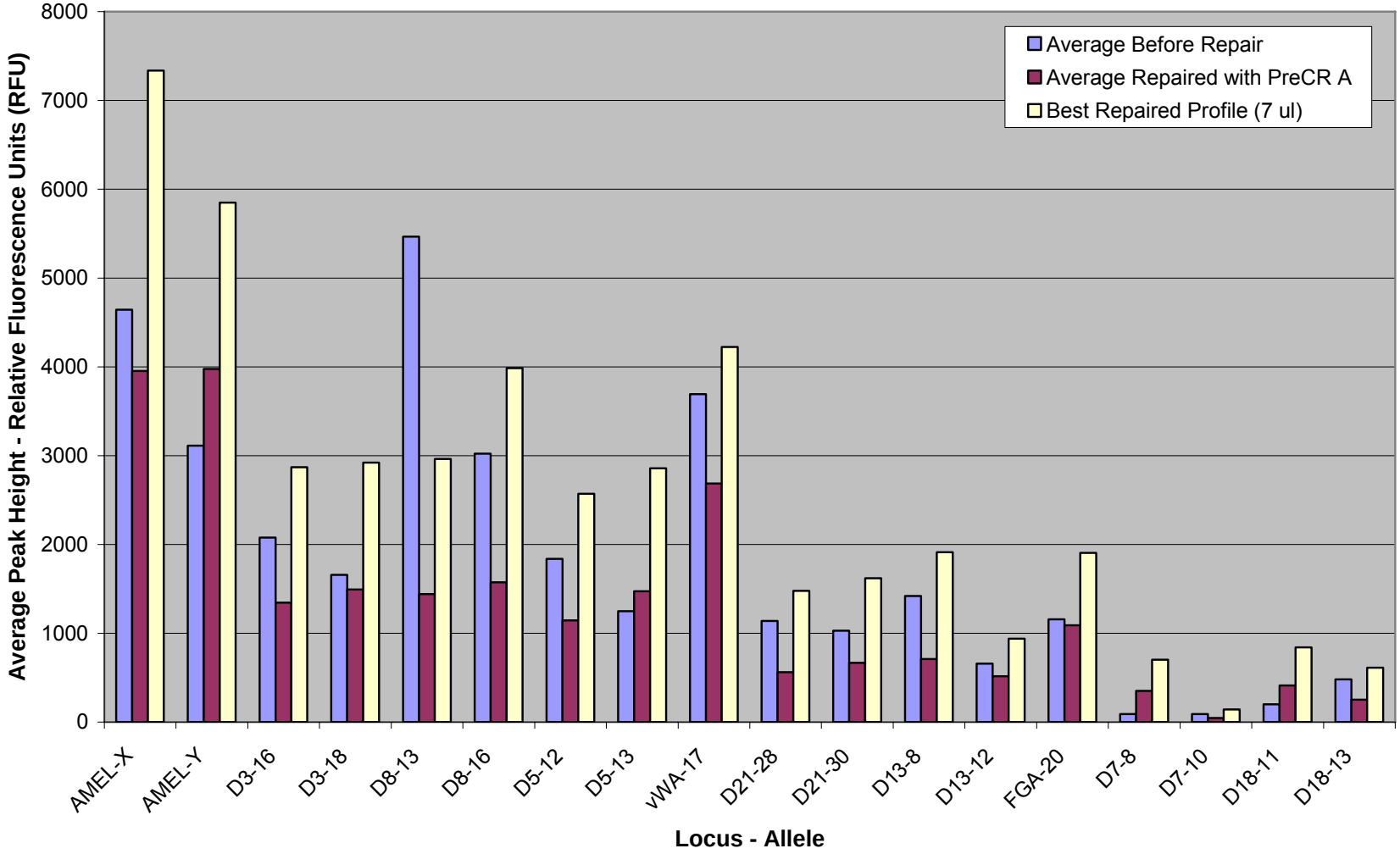


FIG. 17 PreCR™ A repair of UV pretreated, PMB A6 (3 x 5 ml wash) extracted DNA.

A6 – bone powder digested with 3 ml improved extraction buffer, recovered with 20 ml PMB column containing 6 layers of type A glass fiber

3 x 5 ml Wash – washed with 3 aliquots PE buffer, 5 ml each

UV – bone section pretreated with UV irradiation

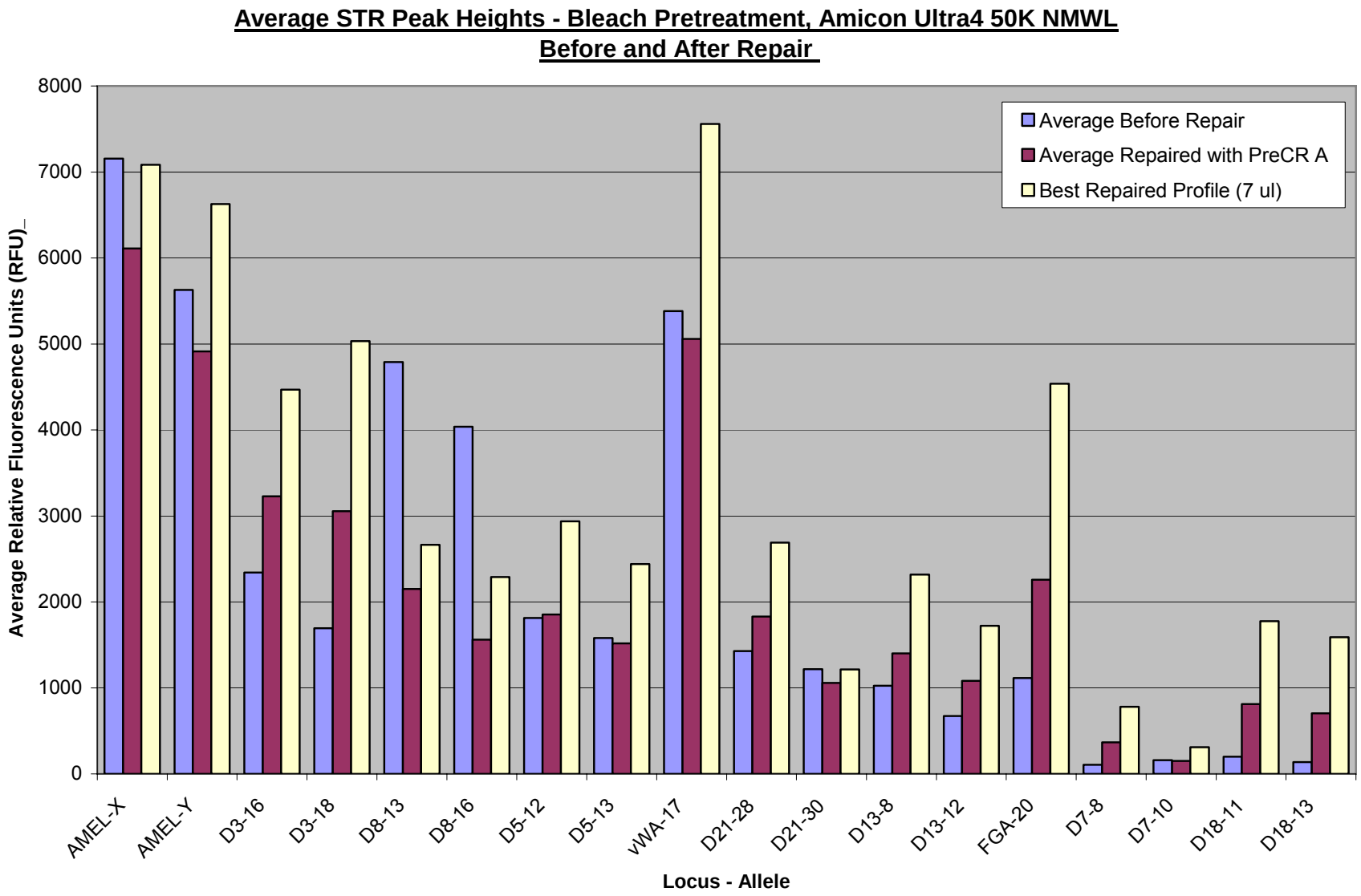


FIG. 18 PreCR™ A repair of bleach pretreated, Amicon® Ultra-4 50K NMWL extracted DNA.

Amicon® Ultra-4 50K – recovered with Amicon® Ultra-4 device, vertical membrane, nominal molecular weight limit 50,000

Bleach – bone section pretreated with bleach

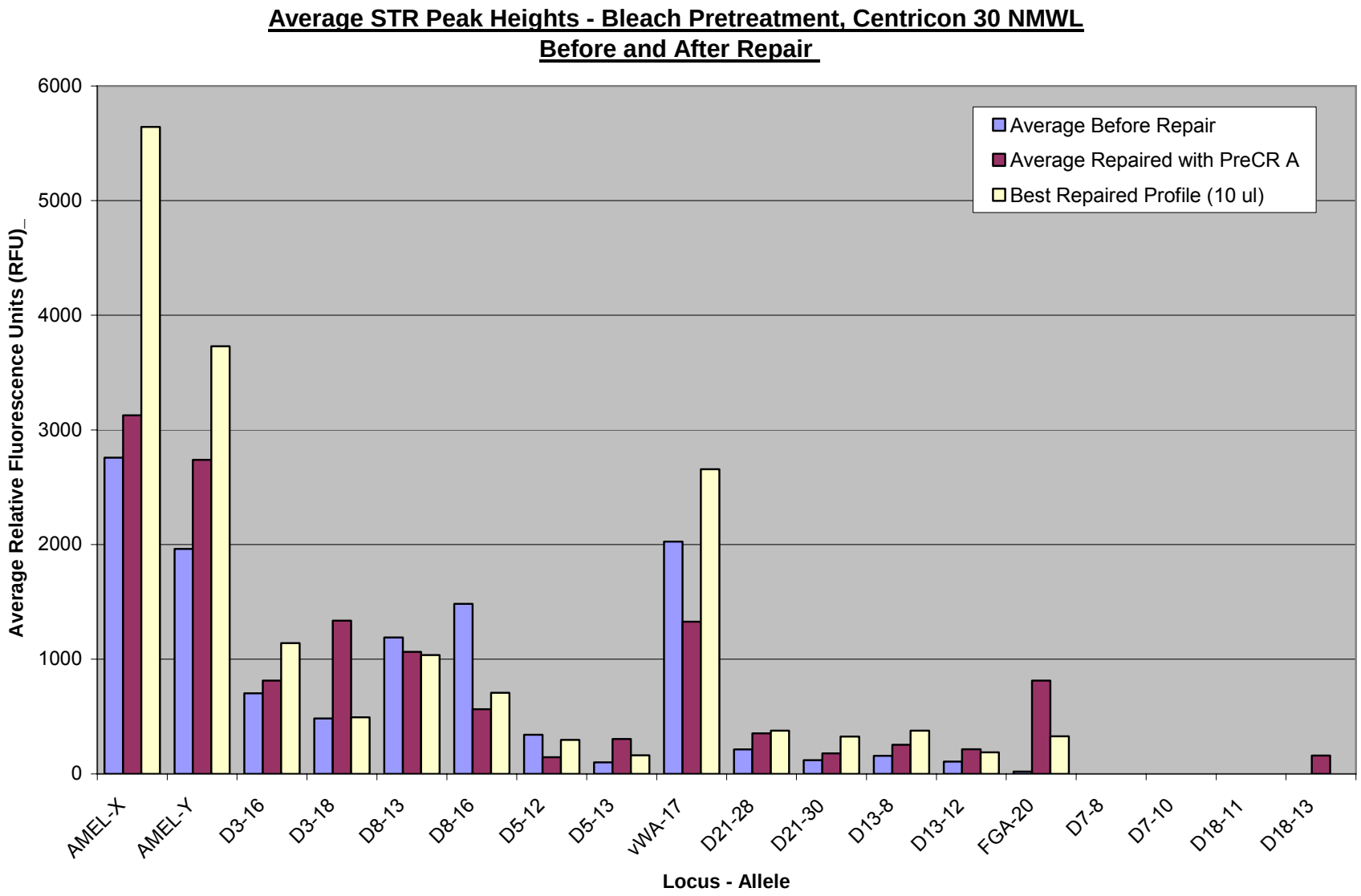


FIG. 19 PreCR™ A repair of bleach pretreated, Centricon® 30 extracted DNA.

Cent30 – recovered with Centricon® 30 device, horizontal membrane, nominal molecular weight limit 30,000

Bleach – bone section pretreated with bleach

Discussion

Virtually all extraction methods provided more DNA and better quality profiles with bleach pretreated samples than with UV pretreated samples. By using these two general approaches, it was possible to generate “high inhibitor” (UV) and “low inhibitor” (bleach) samples from the same bone, allowing for simulation of a more diverse range of sample types while retaining the benefits of comparing various methods using a single sample source. The availability of “high” and “low inhibitor” samples allows a particular method to be challenged not only with DNA recovery, but with removal of varying levels of inhibitory compounds commonly encountered in forensic samples. The varied taphonomy of forensic samples often leaves them impregnated with highly undesirable plant and microbial derived compounds. Many of the compounds, such as humic acid, are potent inhibitors of PCR-based genetic analyses. Thus, reduction of inhibition is essential for successful STR profiling. In many cases it is inhibition, and not DNA quantity, that hampers efforts to obtain a useable profile from DNA recovered from a piece of skeletal remains. Simply put, the cleaner the isolated DNA, the more efficiently downstream processes will work. Therefore, when assessing any extraction or isolation method, one must strike a balance between maximizing DNA recovery and minimizing contaminants. An additional consideration when designing the “perfect” protocol for isolating DNA from skeletalized remains is that not all skeletalized remains or even bones are the same and each may present a different set of obstacles to be overcome. Although the results in this study showed that bleach treated bones outperformed UV treated bones in virtually every case, bleach may not always prove to

be the most effective method for surface decontamination. Porous or extremely friable bone would be quite different with regard to permeability to bleach solutions and in this case it might be necessary to re-evaluate which decontamination is optimal as part of ongoing development of DNA extraction methods.

Bone, by its very nature, has a relatively low DNA content. Cell density is well below that of other common tissues and of course, most forensic bone samples are collected after a significant post-mortem interval and thus most of the original DNA has been degraded by natural processes. For this reason, it is critical to recover as much DNA that remains in a forensic bone sample as is possible. However, increased DNA recovery is unfortunately often accompanied by increased recovery of unwanted substances and thus many of the methods that recovered the largest quantities of DNA also seemed to recover the most inhibitory compounds (AFDIL / 50K UltraFiltration, PMB B3), complicating downstream applications. This co-purification of some inhibitors with DNA is not surprising since they often act in a competitive fashion, binding to many enzymatic components of the downstream processing reactions. It was therefore not surprising that the extraction methods that were most successful overall (Modified AFDIL / 50K UltraFiltration, PMB A3, PMB A6) seemed to forgo a small amount of DNA recovery in favor of increased DNA purity.

Results from the isolations from the QIAquick® columns clearly demonstrated that larger extract volumes resulted in smaller DNA recoveries and reduced quality of the subsequent STR profiles. The effect of a three-fold increase in extract volume was assessed to determine whether this might allow for greater recovery of DNA from the 0.5 g bone powder sample. It appears that, although more DNA may have been

solubilized form the bone, the overall increase in extracted material may have caused an excessive accumulation of filtrates on the membrane surface, prohibiting proper contact of the DNA with the glass fiber matrix and in the final analysis leading to lower DNA recoveries. In addition, it is likely that reduced washing efficiency also occurred for this reason, leading to poorer STR profile quality when the recovered DNA was subjected to analysis with Profiler Plus™ *ID*. Discussions with Dr. Mike Brownleader led to a collaboration with Generon Inc. to produce larger prototype silica columns (PMB columns) that would be able to more effectively handle larger volumes of crude DNA extract. As has been shown in this study, these columns allow for purification of nucleic acids directly after decalcification and protein digestion, without the need for organic extraction or sample concentration (52). This simplification of the protocol substantially reduces the number of handling steps and sample transfers required, as well as eliminating the use of hazardous compounds such as phenol and chloroform. For example, the entire volume of crude DNA extract plus binding buffer can be loaded onto the PMB column in a single step. Based upon the overall performance of these columns, we intend to continue the effort to “fine-tune” the protocol to further enhance DNA recovery as well as reduce the presence of co-purifying inhibitors.

Recovery of DNA from bone can involve a long and labor-intensive protocol. In the case of the protocols assessed in this study, most required an overnight digestion step, with the exception being HSC/YM100, which actually required two. Although this is a “set-it and forget it” step, it does increase the time required to complete the overall process substantially and in forensics time can sometimes be a critical consideration. Bench time, here defined as the analyst’s hands-on time required to complete the

extraction, is also an important factor when designing new isolation protocols particularly since it is prudent for analysts to work single cases or even single samples at a time.

Following the extended decalcification/digestion steps, each of the isolation procedures required hand-on processing time that varied from ~2.5 hours for the PMB columns to ~4+ hours for the Modified AFDIL ultrafiltration method. Here it is clear that less time spent handling an extraction translates into the ability to process more samples in a given period of time. The PMB process also eliminates the phenol and chloroform treatments. The elimination of steps involving dangerous organic compounds has several positive outcomes. Phenol is a corrosive contact hazard that anesthetizes the skin as destroys it, leaving many laboratory workers unaware there is a problem until the damage is quite severe. Phenol and chloroform are both inhalation hazards; chloroform can cause permanent, cumulative liver damage and is a known carcinogen. The inhalation hazard necessitates the use of a chemical fume hood when carrying out organic extractions and thus the elimination of this step gives substantial cost savings by eradicating several handling steps (labor), and reducing or eliminating the need for chemical fume hoods, as well as the time and expense associated with the proper disposal of hazardous organic compounds.

Forensic scientists have explored the application of whole genome amplification to improve results when dealing with low copy number samples. The goal of WGA is to increase the number of copies of DNA in a sample without substantially altering individual sequences and their relative abundance. Clearly, if this goal could be achieved, WGA would be a most useful forensic tool. For this reason, WGA was

included as one of the downstream processing applications studied using the samples generated by the isolation techniques assessed as part of this study. The MDA-based REPLI-g® kit was chosen for testing based on previous reports of some success with the GenomiPhi™ kit (Amersham Biosciences/GE healthcare), which is also MDA-based. Ballantyne *et al.* reported an 80% recovery of endonuclease digested alleles from 50 ng of template DNA, and Barber and Foran reported limited success with amplification with the of mitochondrial (but not nuclear) DNA from skeletal remains (4, 6). The primary difference between the GenomiPhi™ and REPLI-g® kits is that the former uses heat denaturation of the DNA, while the latter uses chemical denaturation to accomplish the same objective. Complete denaturation of the DNA is critical in MDA, since only those sections that become single-stranded and then bind to primers in the single denaturation/annealing step will be amplified successfully. Chemical denaturation held the possibility of ensuring that the DNA pool was completely single stranded while limiting the types of DNA damage that might be caused by extended heating. However, as observed by Barber and Foran, the improved primer extension pre-amplification (I-PEP) step and multiple displacement amplification (MDA) “perform poorly on forensically relevant samples” (6). Our findings confirm what these researchers have suggested, that the results of whole genome amplification of bone-derived DNA using the REPLI-g® MDA kit were of little utility. In addition, WGA methods generally recommend that at least 10 ng of high molecular weight template DNA be used. From a forensic perspective, a yield of 10 ng of high-quality DNA extracted from any sample represents a more than sufficient quantity of DNA for virtually any and all desired

downstream analyses. Clearly, the potential benefit, if any, is far outweighed by the potential risk of altering the endogenous sequences or their relative abundance.

Forensic DNA samples are well-known for experiencing destructive taphonomic conditions, and thus have often endured a wide range of microbial and other environmental insults. This often results in DNA molecules that have been substantially reduced in size and that contain a wide range of DNA damage. Relevant types of DNa damage range from simple single-strand breaks to missing or modified bases. The goal of incorporating a DNA repair protocol into downstream processing of samples is simply to achieve a reduction in those lesions that interfere with subsequent enzymatic amplification of the DNA during PCR-based genetic analyses. The results obtained in this study confirmed the utility of the commercially available New England Biolabs® PreCR™ A DNA repair enzyme cocktail to improve STR profile quality for almost all samples isolated using a range of protocols. This improvement could be achieved even when the repair process resulted in a moderate reduction in template quantity. Through a collaboration with scientists at New England Biolabs®, it was possible to asses an as yet commercially unavailable related DNA repair cocktail tentatively named PreCR™ T6. The T6 cocktail uniformly failed to improve the quality of all samples on which it was tested, leading to their failure to profile. This included even the undamaged 9947A control sample, whose reaction failed after treatment with the cocktail. Although this new cocktail under development may have properties that will prove useful with forensic samples in the future, it appears at this time that the T6 cocktail includes buffer components which are incompatible with downstream genetic analyses such as Profiler Plus™ *ID*. However, although it is clear that there are issues still to be resolved, it is

equally clear that the application of DNA repair cocktails to many forensic DNA isolates will substantially improve our ability to successfully generate STR or other genetic profiles.

As a final thought, for good scientific reasons all results were obtained from bone powder generated from a single femur. This provided the most consistent basis possible for comparison of various extraction methods. However, as noted above, not all forensic samples are the same even if limited to comparing those which are classified as skeletal remains. With this in mind, there will be a future need to further assess isolation techniques using a wider range of bone sources with varied taphonomic histories. This would include an assessment of our most promising extraction protocols (PMB A6, Modified AFDIL 50K ultrafiltration) using a wider range of sample types. Further testing is also needed to explore the possibility of improving whole genome amplification results by circularizing the DNA template molecules prior to multiple displacement amplification with Φ29 polymerase. In addition, the overall utility of whole genome amplification and DNA repair must be assessed using the variety of samples generated during testing of DNA extraction methods on various bone sources. This project has laid the foundation for improvement of STR profiling results via improved extraction methods and DNA repair. What remains to be done is to challenge the robustness of these methods on skeletal remains representative of the diverse preservation states likely to be encountered in forensic casework.

Summary

Forensic molecular biology has made enormous technical advances in the last two decades, allowing its practitioners to put to rest cases that would have been deemed unsolvable thirty years ago. The pace of technological advancement has not slowed, but while the methods in common practice today are powerful discriminatory tools, many are labor-intensive, technically challenging, and unable to cope with the myriad of preservation states encountered in missing persons cases. Updated, streamlined protocols can require less time from a technician, be less error-prone, and are less likely to necessitate repeated processing of the same sample – contributing to increased sample throughput and reduced case backlog.

The primary objectives of this project were: 1. to develop improved methods for extraction of DNA from human skeletal remains, 2. to improve STR profiling success of low-copy DNA samples by employing whole genome amplification to amplify the total pool of DNA prior to STR analysis, and 3. to improve STR profiling success of damaged DNA templates by using DNA repair enzymes to reduce the number/severity of lesions that interfere with STR profiling.

Regardless of extraction method, bleach pretreated samples provided more DNA and better quality profiles than UV pretreated samples. Bleach and UV pretreatment also provide an opportunity to generate “high inhibitor” (UV) and “low inhibitor” (bleach) samples from the same bone, allowing for simulation of a more diverse range of sample types while retaining the benefits of comparing various methods using a single sample source.

In the case of DNA extraction from bone, sometimes less is more. Many of the methods that recovered the largest quantities of DNA also seemed to recover more inhibitory compounds (AFDIL / 50K UltraFiltration, PMB B3). The methods that were most successful overall (Modified AFDIL / 50K UltraFiltration, PMB A3, PMB A6) seemed to forgo a small amount of DNA recovery in favor of increased DNA purity. Another example of "less is more" is that the larger the volume of extract processed through the QIAquick® columns, the smaller the amount of DNA recovered and the poorer the STR profile. This was likely caused by filtrates accumulating on the membrane surface, prohibiting proper contact of the DNA and other reagents with the glass fiber (silica).

All methods tested required at least one overnight digestion step (with the exception of HSC/YM100, which required two), but the time required to complete the extraction varied from 2.5 hours for the PMB columns (which also eliminated the use of phenol and chloroform) to 4-6 hours for the Modified AFDIL ultrafiltration methods.

Attempts to perform whole genome amplification with REPLI-g® significantly reduced profile quality. DNA repair with PreCR™ A led to STR profile improvement in all but one case, and 2-100+ fold increases in peak height for the larger amplicons in half of the samples. DNA repair with PreCR™ T6 led to a loss of loci present in the profiles. The Profiler Plus™ *ID* reaction was likely affected by inhibitors present in the PreCR™ T6 mix, as even the undamaged control DNA failed to yield a full profile when combined with the T6 cocktail.

Inhibitory compounds must be removed prior to enzymatic amplification; either during bone section pretreatment or by the DNA extraction method. Overall, bleach

outperformed UV as a pretreatment and DNA extraction using silica outperformed microconcentration and organic extraction. DNA repair with PreCR™ A outperformed both whole genome amplification and repair with PreCR™ T6. Superior DNA extraction results were achieved using the A6 PMB columns, and DNA repair with PreCR™ A led to an overall improvement in profile quality in most cases, although whole genome amplification was unsuccessful. Rapid, robust DNA isolation, successful amplification of loci from the sample-derived DNA pool, and an elimination of DNA damage and inhibitors may assist in providing sufficient genetic information from cases that might otherwise lie on the fringe of what is possible to obtain today.

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