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Technical Note

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The Application of Miniplex Primer Sets in the Analysis of Degraded DNA from Human Skeletal Remains*

ABSTRACT: A new set of multiplexed PCR primers has been applied to the analysis of human skeletal remains to determine their efficacy in analyzing degraded DNA. These primer sets, known as Miniplexes, produce shorter amplicons (50–280 base pairs (bp)) than standard short tandem repeat (STR) kits, but still utilize the 13 CODIS STR loci, providing results that are searchable on national DNA databases. In this study, a set of 31 different human remains were exposed to a variety of environmental conditions, extracted, and amplified with commercial and Miniplex DNA typing kits. The amplification efficiency of the Miniplex sets was then compared with the Promega PowerPlex® 16 system. Sixty-four percent of the samples generated full profiles when amplified with the Miniplexes, while only 16% of the samples generated full profiles with the Powerplex® 16 kit. Complete profiles were obtained for 11 of the 12 Miniplex loci with amplicon sizes less than 200 bp. These data suggest smaller PCR amplicons may provide a useful alternative to mitochondrial DNA for anthropological and forensic analysis of degraded DNA from human skeletal remains.

KEYWORDS: forensic science, multiplex PCR, short tandem repeat, STR, nuclear DNA, DNA typing, human skeletal remains

DNA template used for short tandem repeat (STR) profiling in forensic casework may become highly degraded because of bacterial, biochemical, or oxidative processes (1). In these circumstances, the possibility of finding an intact target sequence is greatly reduced because of extensive template fragmentation. Because of this problem, it is often not practical to use STRs to analyze such samples. Instead, mitochondrial (mt) DNA can be used. As mtDNA is present in large numbers in cells, extraction and analysis of this type of DNA has a higher probability of producing typable results (2). However, mtDNA has the disadvantages of haploid inheritance, low discriminatory power, and increased analysis time.

Genotyping with STR loci produces results quickly, and with high discriminatory power, yet there is a need to extend this technique to access degraded samples. Commercial multiplex STR kits typically have amplicon sizes ranging from 100 to 480 base pairs (bp) (3–5). Because of this wide range of amplicon sizes, allele dropout for the larger sized amplicons often results in a partial genetic profile when these kits are used to amplify highly degraded DNA samples (3,5). There are also problems because of contamination from bacteria and polymerase inhibitors from soil (6–8). Sample concentration has also been a issue, as 1–5 g of bone powder may be required to obtain sufficient DNA for analysis, and removal of this much material from the bone can be very destructive to samples that must be kept for forensic or archival purposes (6,9–11).

To help alleviate the problems associated with analyzing DNA from degraded samples, a new set of STR primers known as Miniplexes have been recently designed. The primers were created by moving the primer binding sites as close as possible to the repeat region (12,13). Previous examples of the application of reduced amplicon size for various STR loci have already been published (14–16), but these attempts have generally focused on single loci and not the full set of 13 CODIS loci recommended by the United States Department of Justice for profile data-basing (12). The Miniplexes cover 12 of the 13 CODIS loci, with three loci per set. To avoid overlapping loci, only one locus could be assigned to each dye lane for detection. However, the size ranges for two of these sets, Miniplex 1 and Miniplex 3, did not overlap, so the two sets were combined to form a six loci (in 4 dye detection) multiplex set known as "Big Mini."

In a previous study (13), it was demonstrated that the efficiency of amplification of larger sized template DNA is greatly reduced in artificially (enzymatically) degraded samples, and that the Miniplexes were able to amplify the degraded DNA to produce

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TABLE 1—*Sample information and conditions of extracted bone samples from the Forensic Anthropology Center, University of Tennessee, Knoxville. Also shown are the number of loci that gave profiles.*

Sample #	Bone	Side	Condition	Exposure Time*	Storage Time	Detected Loci Miniplex	Powerplex†
D2003.5.1	Femur	Left	Surface clothed	11 months	1 month	12 of 12	8 of 12
D2003.5.2	Femur	Left	Buried	35 months	1 month	10 of 12	3 of 12
D2003.5.3	Femur	Left	Buried	36 months	1 month	11 of 12	1 of 12
D2003.5.5	Femur	Left	Semiburied	?	15 years	12 of 12	5 of 12
D2003.5.6	Femur	Right	Surface	3 years	Unknown	12 of 12	12 of 12
D2003.5.7	Tibia	Left	Surface	3 years	Unknown	12 of 12	12 of 12
D2003.5.8	Femur	Right	Surface	3 years	Unknown	11 of 12	7 of 12
D2003.5.14	Femur	Left	Surface/preservative	12–18 months	19 years	4 of 12	2 of 12
D2003.5.15	Femur	Left	Surface	12–18 months	9 years	12 of 12	10 of 12
D2003.5.16	Femur	Left	Surface	12–18 months	10 years	12 of 12	11 of 12
D2003.5.17	Femur	Left	Buried	12–18 months	1 year	12 of 12	10 of 12
D2003.5.18	Femur	Left	Buried	12–18 months	1 year	9 of 12	1 of 12
D2003.5.19	Femur	Left	Buried in compost	12–18 months	4 years	12 of 12	12 of 12
D2003.5.20	Femur	Left	Surface	12–18 months	4 years	11 of 12	10 of 12
D2003.5.21	Femur	Left	Buried/preservative	12–18 months	19 years	11 of 12	1 of 12
D2003.5.22	Femur	left	Surface/clothing	12–18 months	1 year	11 of 12	8 of 12
D2003.5.23	Femur	Left	Surface/clothing	12–18 months	1 year	12 of 12	4 of 12
D2003.5.24	Femur	Left	Surface/clothing	12–18 months	1 year	12 of 12	7 of 12
D2003.5.25	Femur	Left	Surface	12–18 months	3 years	11 of 12	2 of 12
D2003.5.26	Femur	Left	Surface/sun	12–18 months	1 year	11 of 12	10 of 12
D2003.5.27	Femur	Left	Surface/sun	12–18 months	1 year	12 of 12	4 of 12
D2003.5.28	Femur	Left	Surface	12–18 months	3 years	12 of 12	7 of 12
D2003.5.29	Femur	Left	Surface	12–18 months	3 years	9 of 12	6 of 12
D2003.5.30	Femur	Left	Surface	12–18 months	3 years	12 of 12	11 of 12
D2003.5.31	Femur	Left	Surface	12–18 months	2 years	7 of 12	2 of 12

*The data on exposure time for certain bone samples is not available as the total amount of time the bones were exposed was not included as part of the record-keeping process until 2003.

†Only those loci represented in both kits were measured.

full profiles. In this study, naturally degraded DNA was extracted from human skeletal remains, which had been exposed to a variety of environmental conditions, and amplified with the Miniplex primers. Only 100 mg of bone powder per sample produced a sufficient amount of DNA for amplification with the Miniplex primer sets.

These results were used to demonstrate the functionality of the Miniplex primers in the analysis of simulated forensic samples. In addition, a comparison was made between the amplification results obtained between the Miniplex sets and the Promega Powerplex® 16 kit (Promega Corporation, Madison, WI).

Methods

Sample Collection

One tibia sample and 24 femur samples from 25 individuals were obtained from the Forensic Anthropology Center (FAC) at the University of Tennessee in Knoxville (Table 1). The materials sampled were part of the William Bass Donated Skeleton Collection of remains that had been processed at the facility and curated. The general outdoor environmental condition at the facility was an average temperature of 16°C and high humidity for the duration of the exposure. Burials were in clay soil at a depth of 60–120 cm. Before accession into the collection, the remains had been subjected to different environmental conditions, cleaned, and heated without chemicals (50–60°C) for 6–12 h, and analyzed by the researchers at the facility. The samples had been stored at room temperature before sampling.

Six additional femur samples from six individuals were obtained from the Franklin County Coroner's Office (FCCO) in Columbus, OH. These samples were donated to the Ohio University Department of Anthropology and stored at 4°C before cleaning and sampling.

Bone Preparation

Bone samples were sanded, then brushed with 5% bleach solution and immediately rinsed with distilled water and with 95% ethanol. Bone powder was then generated by using a cordless drill (17) (Black and Decker, Towson, MD) to bore into the bone using drill bits (1/4″, 5/16″, and 3/8″) (Black and Decker) designed for woodwork. The samples were collected on weighing paper and stored frozen in 15 mL polypropylene tubes (VWR, West Chester, PA). Some samples from the FCCO required minor soft tissue removal before sampling, and this was done using sterile forceps and scalpel blades. The identification numbers and conditions for the bone samples obtained from the FAC in Tennessee and the FCCO are shown on Tables 1 and 2, respectively.

DNA Extraction and Quantification

DNA was extracted by modification of the QIAamp® protocol as described previously (13). Briefly, 100 mg of bone powder was decalcified in EDTA, digested using a stain extraction buffer and

TABLE 2—*Sample information and condition of bone samples from the Franklin County Coroner's office, Columbus, OH. Also shown are the number of loci that gave profiles.*

Sample #	Bone	Condition	Years in Storage	Miniplex Loci	Powerplex Loci*
D2003.6.1	Femur	Cold storage	5	12 of 12	5 of 12
D2003.6.2	Femur	Cold storage	11	12 of 12	12 of 12
D2003.6.3	Femur	Cold storage	6	12 of 12	7 of 12
D2003.6.4	Femur	Cold storage	14	12 of 12	9 of 12
D2003.6.5	Femur	Cold storage	5	12 of 12	12 of 12
D2003.6.6	Femur	Cold storage	10	12 of 12	11 of 12

*Only those loci represented in both kits were measured.

proteinase K, and purified and concentrated using the QIAamp® Blood Mini Kit (Qiagen Inc., Valencia, CA). The samples were quantified using an Alu-based real-time PCR method (18) with a RotorGene™ RG3000 cycler (Corbett Research, Sydney, Australia).

PCR Amplification

DNA samples containing 100 pg of template were amplified with Miniplex 2 (D5S818, D8S1179, D16S539), Miniplex 4 (vWA, D18S51, D13S317), and Big Mini [the multiplexed Miniplex 1 (TH01, CSF1PO, TPOX) and Miniplex 3 (FGA, D21S11, D7S820)], and 250 pg of DNA sample was amplified with PowerPlex®16 (Promega Corporation) in a 12.5 μL reaction volume. The lower quantity of DNA utilized in the Miniplex amplification was necessary to avoid overamplification, as determined by our previous experiments (13). For those samples with low quantities of DNA (Sample #'s 5.14, 5.18, 5.21, and 5.25), the maximum amount (volume) of sample that could be added to the PCR mixture was used. Microcon YM-100 centrifugal filters (Millipore, Billerica, MA) were used to concentrate those samples that still failed to amplify because of extremely low concentrations of DNA template (1–5 pg/μL before concentration). All samples were amplified at 33 cycles in a total reaction volume of 12.5 μL. The Miniplex primer sequences (12) and PCR parameters (13) have been published previously. Amplifications for Miniplex 2, Miniplex 4, and Big Mini were performed with 0.5 μg of nonacetylated BSA (Sigma, St. Louis, MO) added to each PCR mixture. Amplification parameters using the PowerPlex®16 (Promega Corporation) system followed the procedure as specified in PowerPlex®16 technical manual (http://www.promega.com/tbs/TMD012/TMD012.html, 2003). Nonacetylated BSA (0.5 μg) was also added to the Powerplex® 16 PCR mix, even though the reaction mix already contains BSA, because amplification results improved when additional BSA was added to the reaction mix. The decrease in fluorescence signal for larger amplicons in both Miniplexes and PowerPlex 16 was used to assess DNA degradation (Fig. 1).

Detection and Data Analysis

Amplified DNA was separated and detected using the ABI PRISM® 310 GeneticAnalyzer (Applied Biosystems, Foster City, CA). The GeneScan® ROX 500 (Applied Biosystems) size standard was used for Miniplex amplified samples and the ILS 600 size standard (Promega Corporation) was used for PowerPlex®16 amplified samples. Samples were prepared by adding 1 μL PCR product to 12 μL Hi-Di™ formamide (Applied Biosystems) containing 0.50 μL of the internal lane standard. Injection and analysis parameters were published previously (13). The detection threshold used for calling alleles was 150 RFUs.

The percent of successful amplification was calculated for each individual locus as well as for all loci for both kits and for each of the Miniplex sets. A *t*-test was done on the average success values to compare the two kits.

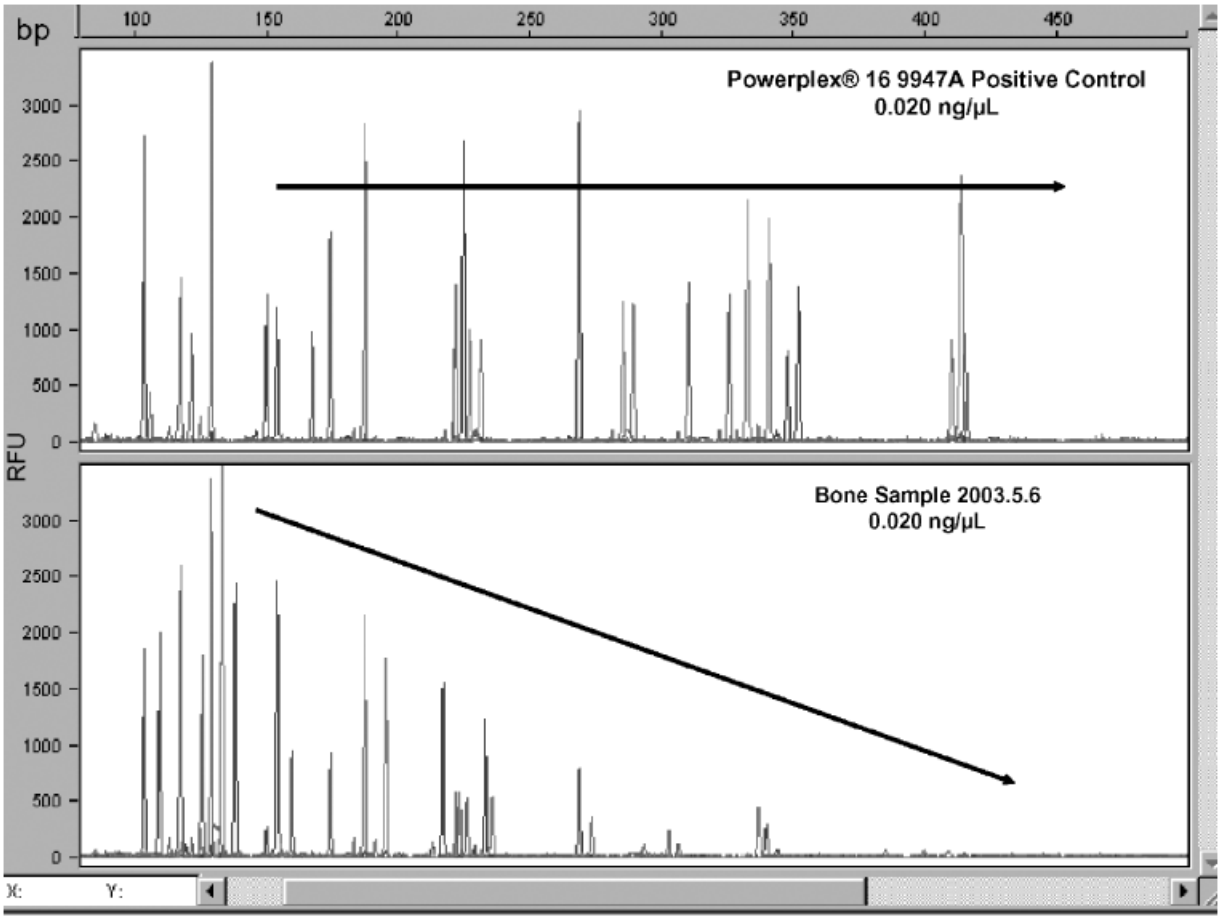

FIG. 1—*Nondegraded control sample (top) and degraded bone sample (bottom) amplified with Powerplex® 16. The characteristic "ski slope" effect is observed, where the peak intensity for the degraded sample decreases with amplicon length. Amplification and analysis conditions are provided in the text.*

TABLE 3—*Summary of profiling results grouped by sample source.*

Primer Set	Samples Tested	Full Profile	Partial Profile
Samples from the Anthropological Research Facility			
Miniplex 2	25	23 (92%)	2 (8%)
Miniplex 4	25	22 (88%)	3 (12%)
Big Miniplex*	25	14 (56%)	11 (44%)
Miniplex 1		22 (88%)	3 (12%)
Miniplex 3		15 (60%)	10 (40%)
Powerplex 16	25	3 (12%)	22 (88%)
Samples from the Franklin County Coroner's Office			
Miniplex 2	6	6 (100%)	0
Miniplex 4	6	6 (100%)	0
Big Miniplex*	6	6 (100%)	0
Miniplex 1		6 (100%)	0
Miniplex 3		6 (100%)	0
Powerplex 16	6	2 (33%)	4 (67%)

Partial profiles involve samples in which one or more loci were below the detection threshold. Amplification and analysis conditions are provided in the text.
*Big Miniplex is a co-amplified mixture of Miniplex 1 and Miniplex 3.

Results

Nine (36%) samples from the FAC and one sample from the FCCO yielded low amounts of DNA template (less than 10 pg/μL). The conditions of these samples were burials ($n = 2$), semi-burial ($n = 1$), burial with preservative ($n = 1$), surface ($n = 3$), surface with clothing ($n = 1$), surface with preservative ($n = 1$), and cold storage ($n = 1$).

Although most of the bone samples were able to produce full profiles for Miniplex 2 (81–134 bp), Miniplex 4 (88–193 bp), and the smaller loci of Big Mini (51–129 bp), 10 of the samples yielded only a partial genetic profile for the larger loci of the Big Mini primer set (125–281 bp). Only five of the samples yielded a full profile for the same 12 loci in the PowerPlex® 16 system (100–480 bp). Thirteen out of the 25 samples from the FAC and all samples from the FCCO yielded complete profiles for all Miniplex loci. Table 3 summarizes these results. There were four samples (Sample # 5.14, 5.18, 5.21, 5.25) that failed to amplify initially. This was presumably because of the extremely low amount of DNA template detected by qPCR (1–5 pg/μL). Other extraction methods were attempted with these samples (17), but the results gave similarly low yields. These samples were then concentrated using Microcon YM-100 filters and amplified again. After this treatment, these samples produced some amplifiable DNA with both the Miniplex and Powerplex® kits. However, only partial profiles were obtained. The three samples (Sample #'s 5.6, 5.7, 5.19) from FAC that yielded complete profiles with the Power-

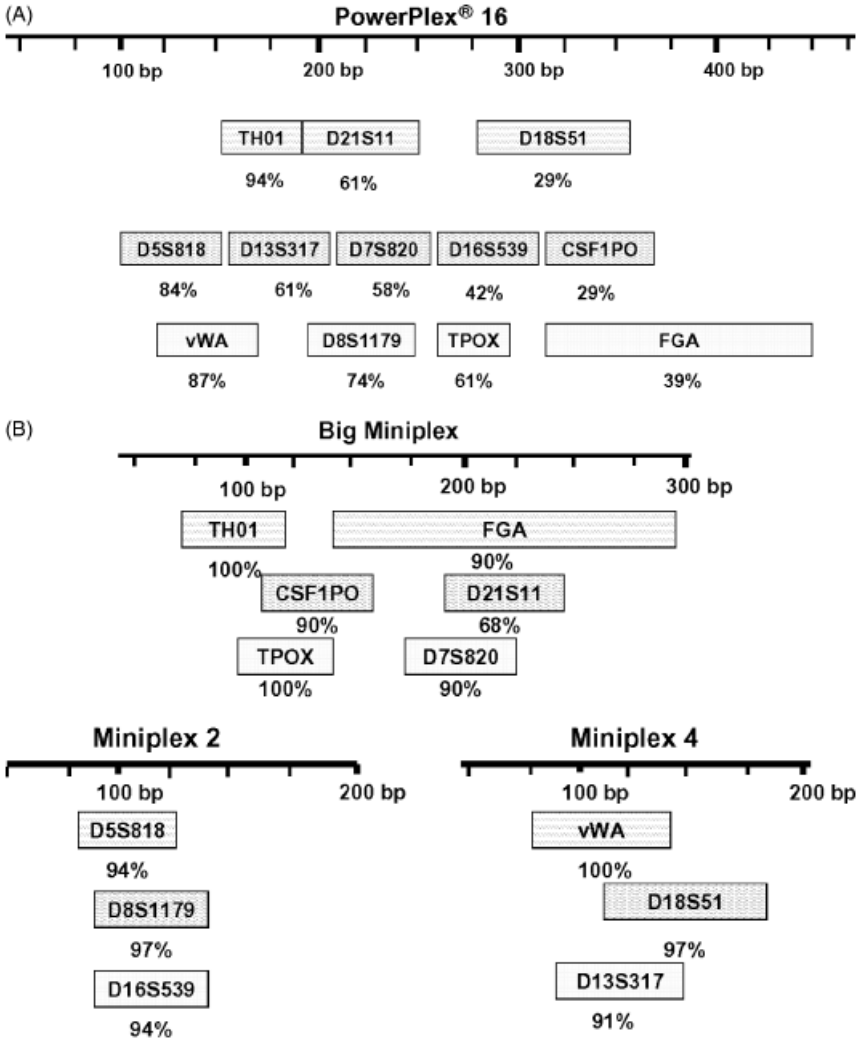

FIG. 2—*A comparison of the analysis of 31 bone samples using the Powerplex® 16 (A) and the Miniplex (B) amplification sets. The figures show the amplicon size ranges for both the Powerplex 16 system and Miniplex primer set and lists the percentage of genetic loci detected. Dye labels are: blue labeled loci, — top; green labeled loci, — middle; yellow labeled loci, — bottom. Amplification and analysis conditions are provided in the text.*

Plex® 16 system also yielded complete profiles with all the Miniplex sets, although a loss of intensity of larger alleles was observed for these samples as well. Overall, 92% of the loci produced profiles with the Miniplex kits, and 60% of the loci common to both kits amplified with the commercial kit. The standard deviations from the averages were 15% and 32%, respectively, and the calculated *t*-value for comparison of the kits was 5.41 ($p = 0.01$, the critical *t*-value for >50 is 2.58 (19)), which indicates the difference between amplification efficiency between the two kits is highly significant.

Discussion

The Miniplex primers were designed to make the amplified product size as short as possible to improve the ability to detect degraded DNA template. Because of a lack of useful primer binding sites, three of the miniplex loci, FGA, D21S11, and D7S820 have a larger size range and are combined with CSF1PO, TPOX, and HUMTHO1 to create a six-plex known as Big Mini (12). Among the samples from the FAC that yielded partial genetic profiles, 10 failed to amplify at the larger three loci of the Big Mini multiplex set. This result suggests that extensive DNA degradation has occurred with these bone samples (13).

Amplification with the Powerplex® 16 system further confirmed that degradation had occurred. Most of these samples yielded complete profiles for the TH01, D5S818, and vWA loci (Fig. 2) presumably because these loci have the smallest amplicon sizes in this multiplex kit. A sharp decrease in signal intensity of larger alleles and even complete loss of allele signal was observed with the Powerplex® 16 amplifications. The loss of intensity of the larger loci is likely the result of excessive fragmentation of the DNA template. There were five samples amplified with the Big Mini set that indicated the possibility of allele drop out in one or two loci. As we do not have reference profiles for these samples, we could not ascertain if the sample was indeed a homozygote or heterozygote for these loci. As for the samples amplified with the Powerplex® 16 system, 50% had allele drop out in one or more of the larger sized loci, and within those loci, the alleles that were lost were the larger-sized alleles. The Big Mini and Powerplex® 16 data suggest that the degradation cut-off length of template fragments predominantly occurs around 200 bp and is not kit dependent.

Overall, the Miniplexes produced results for at least 11 of the 12 loci for all samples in which the total amount of DNA recovered was above 250 pg (Fig. 3). The commercial kit with its larger loci averaged only eight loci for these same samples, with some samples producing as little as one amplified locus. Because only three of the six buried bone samples contained larger quantities of DNA, it was difficult to assess the effect of burial (six samples) vs. surface treatment (14 samples). DNA degradation may be affected by a variety of factors including humidity, temperature, soil pH, and the presence of microorganisms (20). These were not controlled in this study.

The samples kept in cold storage for 5–14 years from the FCCO were less prone to degradation and PCR inhibition. These samples were in a much better state of preservation, but their treatment prior to refrigeration was unknown. We anticipate that given a larger sample size and better control over conditions we may be able to see more of a relationship between the sample environment and the rate of DNA degradation. However, this will be the subject of future work. Instead, the goal of this study was to develop procedures for the collection and analysis of degraded DNA and to examine the efficacy of the Miniplex amplifications using simulated casework samples.

Conclusions

This paper presents the first controlled study of the utility of the reduced size STR primer sets in situations where commercial kits fail to produce full genetic profiles because of DNA degradation. The Miniplexes demonstrate a greater likelihood of producing a full profile from degraded DNA (64%) when compared with commercial kits (16%). Results from this study are consistent with previous work using enzymes to artificially degrade samples (13).

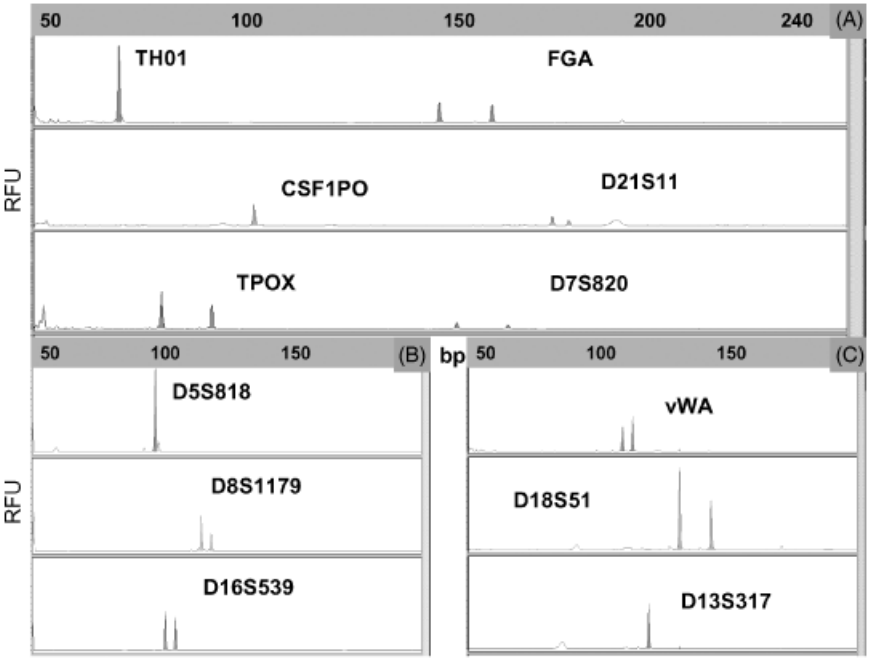

FIG. 3—*Amplification results of bone sample using the Miniplex primer sets: Big Miniplex (A), Miniplex 2 (B), and Miniplex 4 (C). Amplification and analysis conditions are provided in the text.*

It can also be concluded from these results that degradation based allele drop out is size related and is not specific to any particular locus.

In general, the Miniplex primer sets can provide a powerful new tool for the determination of DNA profiles when only partial genetic profiles are generated from standard kits because of the effects of DNA degradation. These kits should have important applications for anthropological samples and in forensic casework involving the identification of human remains, as both situations are likely to involve the analysis of degraded DNA.

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