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Denise T. Chung,[1] *B.S.; Jiří Drábek,*[1] *Ph.D.; Kerry L. Opel,*[1] *M.A.; John M. Butler,*[2] *Ph.D.; and Bruce R. McCord,*[1] *Ph.D.*[1]

A Study on the Effects of Degradation and Template Concentration on the Amplification Efficiency of the STR Miniplex Primer Sets*

ABSTRACT: In forensic DNA analysis, the samples recovered from the crime scene are often highly degraded leading to poor PCR amplification of the larger sized STR loci. To avoid this problem, we have developed STR markers with redesigned primer sequences called "Miniplexes" to produce smaller amplicons. To assess the effectiveness of these kits, we have tested these primer sets with enzymatically degraded DNA and compared the amplifications to a commercial kit. We also conducted sensitivity and peak balance studies of three Miniplex sets. Lastly, we report a case study on two human skeletal remain samples collected from different environmental conditions. In both types of degraded DNA, the Miniplex primer sets were capable of producing more complete profiles when compared to the larger sized amplicons from the commercial kit. Correct genotypes were obtained at template concentrations as low as 31 pg/25 μL. Overall, our data confirm that our redesigned primers can increase the probability of obtaining a usable profile in situations where standard kits fail.

KEYWORDS: forensic science, DNA profiling, short tandem repeat, miniplex, degraded DNA, TH01, CSF1PO, TPOX, D5S818, D8S1179, D16S539, FGA, D21S11, D7S820, vWA, D18S51, D13S317, Penta D, Penta E, and D2S1338

Short tandem repeats (STRs) are genetic loci containing tandemly repeated sequences of 2–6 base pairs in length. DNA profiling based on STRs is the most popular method of human identification due to the highly polymorphic nature of STRs and the ease of their genotyping (1–5). In comparison to minisatellites, the small amplified fragment length of STRs facilitates its utility in the analysis of degraded DNA samples (6–10).

However, in situations where DNA is highly degraded, poor amplification of the larger sized loci (300–500 base pairs) in standard multiplex typing kits is common (11–14). As the sample decomposes, the DNA template can become highly fragmented, and the yield of complete target fragments is greatly reduced. Thus, in multiplex kits with a wide range of amplicon sizes, a "decay curve" is seen, in which the peak height is inversely proportional to the amplicon length. (2,11,14). In this case, the larger amplicons often have lower sensitivity and fall below the detection threshold. This can result in a partial genetic profile. To solve this problem, redesigned primer sets were developed in which the primers were positioned as close as possible to the ends of the repeat to reduce the amplified product size. These reduced sized primer sets were called Miniplexes (15). The primer sequences were originally designed to be compatible with the use of matrix-assisted-laser-desorption-ionization time-of-flight mass spectrometry (16–18), but have been modified to function with fluorescence based sequencers for the detection of degraded DNA (15).

[1] Ohio University, Department of Chemistry and Biochemistry, Athens, OH.
[2] National Institute of Standards and Technology, Biotechnology Division, Gaithersburg, MD.

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A set of five Miniplexes with 3 STR loci per set has been designed (Table 1). The primer sets include 12 of the 13 Combined DNA Index System (CODIS) core STR loci. The D3S1358 locus was excluded because its alleles are already small in commercial sets. The Miniplexes also include 3 non-CODIS loci (Penta D, Penta E, and D2S1338), although these were not tested in this study. To avoid overlap between amplicons, one dye was used for each locus. However, Miniplex 1 and Miniplex 3 differ in size range and can be combined together to create a six-loci set called "Big Mini." The Miniplex sets allow for a reduction in product size up to 299 base pairs, with most amplicon size reductions ranging from 60–200 base pairs (15).

Several reports have been published supporting the validity of this approach for highly degraded DNA samples. Primer pairs producing small amplicons less than 110 base pairs for three STR loci, FES, TH01, TPOX, were used by Hellman et al. for typing DNA extracted from human telogen hairs (19). Ricci et al. demonstrated an increase in the success rate of typing degraded DNA samples using a new primer pair for the D12S391 STR loci. In this study, amplified fragment sizes were decreased from 205–253 bp to 125–173 bp (20). Reductions in primer pairs for TH01, D10S2325, DYS319, DYS19 (21), and CSF1PO (22) have also been reported. Because only short fragments of intact DNA are necessary, the success rate in the amplification of degraded DNA is increased.

To examine the hypothesis that the amplification efficiency of degraded DNA can be improved by reducing the amplicon size, we compared the results of amplification using Miniplex primer sets 2, 4, and Big Mini with a commercially available multiplex kit on enzymatically degraded DNA. We also examined the effect of DNA template concentration on signal intensity and peak balance ratio because we anticipated that shortening PCR amplicons would improve amplification efficiency. Lastly, we report here two case studies on DNA extracted from human skeletal remain samples that had been exposed to different environmental conditions

TABLE 1—*STR Loci in Miniplexes. Big Mini is a combination of Miniplex 1 and 3. Miniplex 5 was not tested in this study.*

		6FAM	VIC	NED
Miniplex 2		D5S818	D8S1179	D16S539
Miniplex 4		vWA	D18S51	D13S317
Miniplex 5		Penta D	Penta E	D2S1338
Big Mini	Miniplex 1	TH01	CSF1PO	TPOX
	Miniplex 3	FGA	D21S11	D7S820

and where complete profiles are not available using the standard kits.

Materials and Methods

DNA Extraction

Whole blood samples ($n = 4$) were extracted using a silica based procedure following the QIAamp® Blood Maxi Kit protocol (Qiagen, Inc., Valencia, CA). Degraded DNA was prepared by enzymatically digesting 2.5 μg of the extracted DNA with 0.01 units/μL DNase I (Fermentas, Inc., Hanover, MD) for time periods of 2, 5, 10, 20, and 30 min. The degraded DNA was separated by gel electrophoresis using 2% agarose (Sigma-Aldrich, St. Louis, MO) and stained with ethidium bromide (Fisher Scientific, Pittsburgh, PA) for detection. DNA from different regions of the gel corresponding to different fragment sizes (<126, 179–222, 222–350, 350–460, 460–517, 676–1198, >1198 bp) based on the pGEM® DNA marker from Promega were extracted using the QIAquick® Gel Extraction Kit (Qiagen, Inc., Valencia, CA). These extracted fragments were then requantitated. All blood DNA extracts were quantitated by the Quantiblot® Human DNA Quantitation Kit (Applied Biosystems, Foster City, CA), according to the manufacturer's protocol.

Bone samples ($n = 2$) were obtained from the Ohio University Department of Anthropology. Bones were brushed with 5% bleach solution and immediately rinsed with distilled water and with 95% ethanol. The bones were cut with a $\frac{1}{4}$ in. Black and Decker Wizard rotary tool using a no. 409 emery cut-off wheel (Dremel, Racine, WI). After cutting, bone samples were cleaned again with distilled water and ethanol and pulverized under liquid nitrogen using a 6750 freezer mill (Spex Certiprep, Inc., Metuchen, NJ). The milling cycle began with 15 min of pre-cooling followed by 3 cycles of 2-min grinding and 2-min resting. An impact frequency of 15 was used.

Extraction of DNA from bone powder followed a modification of the QIAamp protocol by Dr. Kerri Dugan from the FBI DNA I Research Laboratory (personal communication). A hundred milligram sample of bone powder was decalcified in 1.6 mL EDTA (0.5 M, pH 8.0) and incubated for 16 hs at room temperature with agitation. Samples were then centrifuged and washed with 1 mL distilled water three times. Pelleted bone powder was digested using 300 μL of stain extraction buffer (10 mM TRIS-Cl pH 8.0, 100 mM NaCl, 39 mM Dithiothreitol, 10 mM EDTA, 2% SDS) and proteinase K (2 μL of 20 mg/mL) and incubated for 8 hs at 56°C with agitation, 400 μL of ethanol was added and the samples were heated at 70°C for 10 min prior to purification and concentration using the QIAamp® Blood Mini Kit (Qiagen, Inc., Valencia, CA). DNA from bone samples were quantitiated using the ACES® 2.0 Human Quantitation system (Whatman Bioscience, Newton, MA), following the manufacturer's protocol. In the course of the study, we applied the Alu-based real time PCR protocol (23) to spare the amount of template available and increase accuracy.

All human samples were processed with the formal consent of Ohio University and in accordance with Ohio University's institutional review board.

PCR Amplification

An aliquot of a 1–2 ng of DNase I digested DNA from whole blood was used for the degradation study. Blood samples were serially diluted from a concentration of 500 pg to 30 pg for the sensitivity and peak balance study, and 60 pg of DNA were used for the bone samples. These were all amplified in a total reaction volume of 25 μL consisting of Miniplex primers (Big Mini: 0.2 μM TH01, FGA, TPOX, 0.3 μM D21S11, D7S820 and 0.12 μM CSF1PO primers; Miniplex 2: 0.4 μM D5S818, 0.4 μM D8S1179, and 0.2 μM D16S539; Miniplex 4: 0.4 μM vWA, D18S51, and D13S317), 200 μM of each dNTP, 1X GeneAmp® PCR buffer with 15 mM $MgCl_2$, and 1–2 U/ 25 μL AmpliTaq Gold® DNA polymerase (Applied Biosystems, Foster City, CA). The primer sequences have been published elsewhere (1) with the exception of D13S317 that was used without the GTTCTT tail in the reverse primer. Amplification reactions were performed using a 96-Well GeneAmp® PCR System 9700 thermal cycler (Applied Biosystems, Foster City, CA). Thermal cycling conditions were 95°C for 10 min, 30 cycles (DNase I digested DNA) or 33 cycles (bone and blood DNA for sensitivity and peak balance studies) at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, followed by a final incubation of 60°C for 45 min and 25°C forever. Allelic ladders were prepared by diluting commercial kit ladders (1:1000) in distilled deionized water and reamplifying with the Miniplex primers as described previously (1).

An aliquot of 1–2 ng/25 μL of enzymatically degraded DNA fragments and 60 pg/25 μL of bone extract was amplified with 10/22 and 10/23 cycles, respectively, using the PowerPlex® 16 system following the parameters specified in the technical manual (24).

For the Alu-based real time PCR quantitation, the Corbett Research RotorGene RG3000 cycler was used following the published protocol (23).

Detection and Data Analysis

The Miniplex sets uses a four dye system of 6-FAM, VIC, NED and ROX (Applied Biosystems, Foster City, CA). Amplicons were analyzed using the ABI PRISM® 310 Genetic analyzer and GeneScan® ROX 500 size standard (Applied Biosystems, Foster City, CA) with filter set F. Samples were prepared by adding 1 μL PCR product to 12 μL Hi-Di™ formamide (Applied Biosystems, Foster City, CA) containing 0.50 μL GS500 ROX internal lane standard. Samples were injected into a 43 cm × 50 μm capillary (Polymicro Technologies, Phoenix, AZ) for 5 s at 15,000 volts in POP™4 (Applied Biosystems, Foster City, CA) and separated at 15,000 V for 18 min with a run temperature of 60°C. The global Southern sizing method was employed for data analysis. Data was collected by the ABI Data collection software version 2.0 under the GeneScan run module GS POP4-F (virtual filter set F) and processed in GeneScan® software version 3.1. Allele designations were made using the Genotyper® 2.5 software program. Templates with macros are available from authors upon request.

Electrophoretic and analytical conditions for amplifications with the PowerPlex® 16 System followed the parameters and conditions specified in the PowerPlex® 16 System technical manual (23).

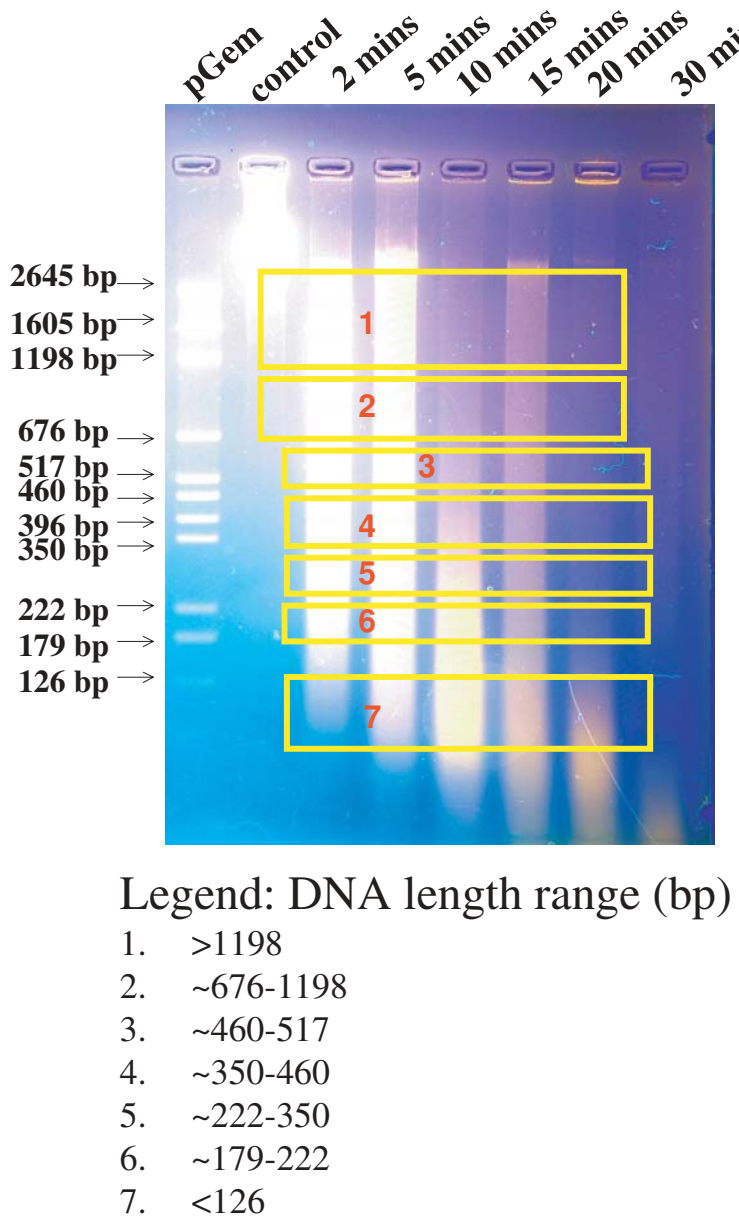

FIG. 1—*DNA degraded with DNase I over different time periods. pGEM® DNA marker (Promega corporation, Madison, WI) was used as ladder (L). Lanes 1–7 were loaded with DNA incubated with DNase for several time periods: 0 (control), 2, 5, 10, 15, 20, and 30 min, respectively.*

Results and Discussion

Degradation Study

We tested our Miniplex STR primer sets on enzymatically digested DNA to assess their potential for forensic genotyping of degraded DNA samples. The effects of degradation were studied using whole blood samples incubated with DNase I for several time periods (Fig. 1). Fragments with different lengths were excised from the gel and then amplified with the Big Mini, Miniplex 2, and Miniplex 4 primers sets and compared to amplifications with the PowerPlex® 16 system (Fig. 2a, b). We have not performed studies on the Miniplex 5 set (Penta D, Penta E, and D2S1338) because one of the loci, Penta E, showed non-specific binding at low DNA template amounts. This failure is probably caused by the potential binding of both the redesigned forward and reverse primer of Penta E to compatible sequences in other parts of the human genome as shown from BLASTN (http://www.ncbi.nlm.nih.gov/BLAST/) and BLAT (http://genome.ucsc.edu) results. For all samples tested in the degradation study, the genotypes obtained from Big-Mini, Miniplex 2 and Miniplex 4 loci were the same with the genotypes obtained from the larger amplicons in the commercial kit. However, we do not rule out the possibility of mutations that could lead to allele dropout or low sensitivity of one allele as more samples are tested, since the primer sequences are different for both kits. A full concordance study will be addressed in another paper.

When examining the results obtained with the commercial multiplex kit with amplicon sizes ranging from 100–480 base pairs, the PCR product yield for the larger sized loci began to decrease as the template DNA fragment sizes became smaller. For example, Penta D and Penta E with allele sizes in the range of ~365–480 bp began to drop out when the average template size dropped to ~350–460 bp (Fig. 1). D18S11, CSF1PO and FGA started to drop out at DNA template sizes of ~222–350 base pairs, and lastly D16S539 and TPOX dropped out at DNA template sizes ~179–222 base pairs. Below

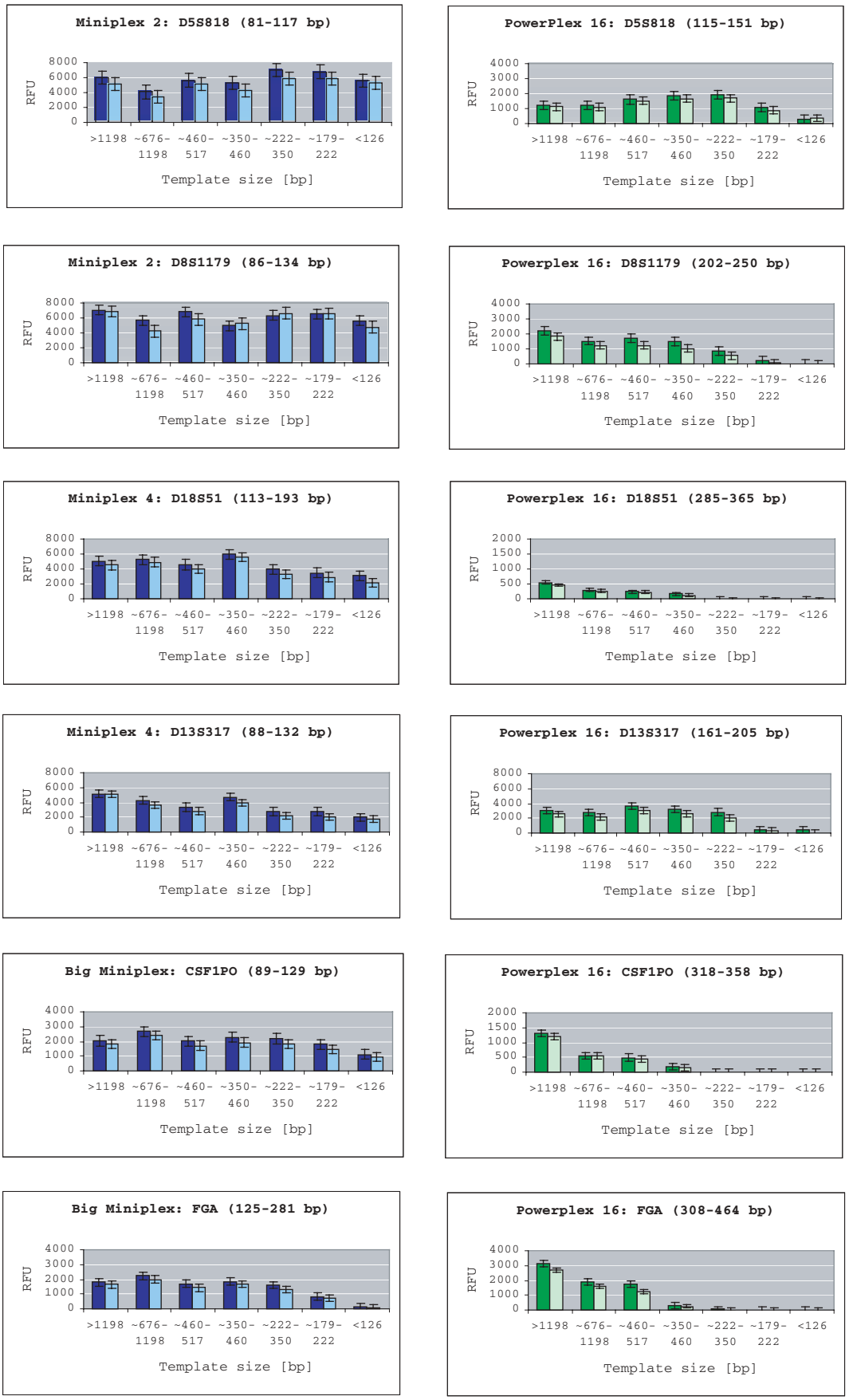

FIG. 2—*Comparison of different DNA fragment sizes excised from agarose and amplified with (a) Miniplex 2: D5S818, D8S1179, Miniplex 4: D18S51, D13S317, (b) Big Mini: CSF1PO, FGA (left) and the PowerPlex® 16 system (right). 1 ng/25 μL was used to compare Miniplex 2 and Miniplex 4 with PowerPlex®16. 2 ng/25 μL was used to compare Big Mini with PowerPlex® 16. The Miniplexes were amplified at 30 cycles, while 32 cycles were used for PowerPlex® 16.*

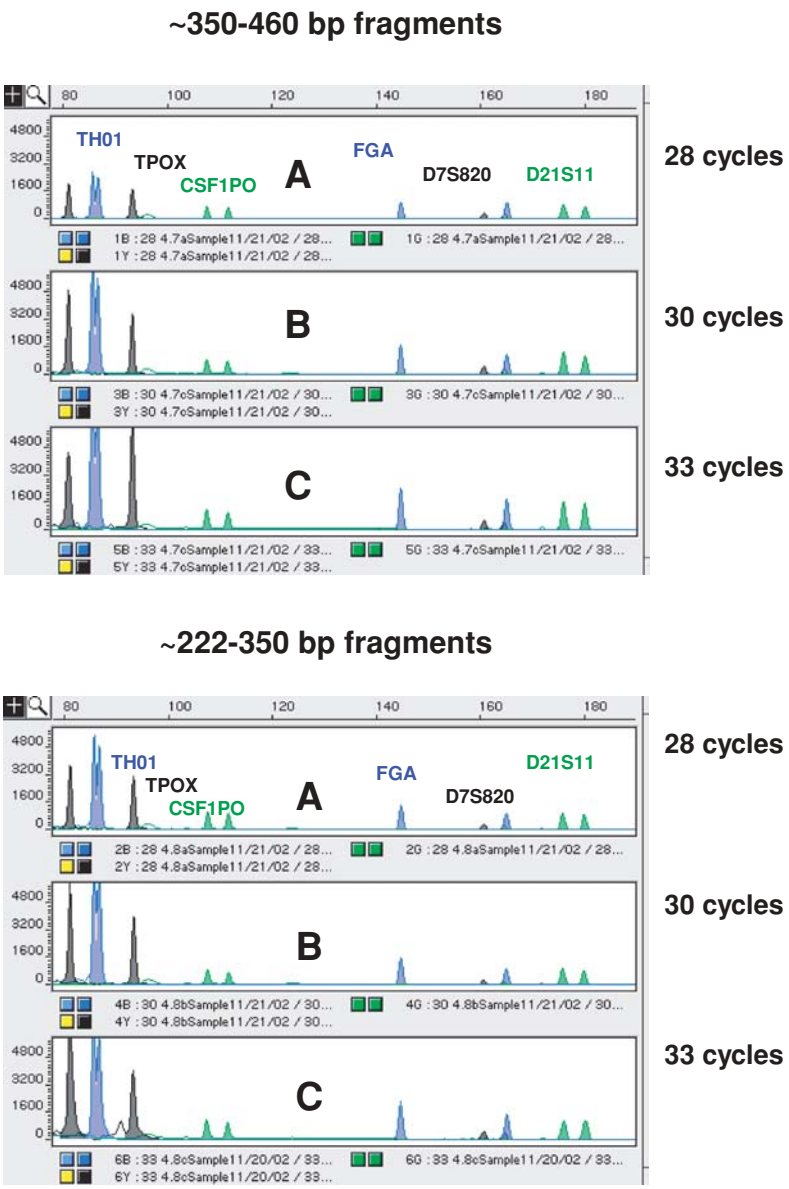

FIG. 3—*2 ng/25 μL of ~350–460 and ~222–350 base pair fragments amplified with Big Mini at 28 (Panel A), 30 (Panel B) and 33 (Panel C) cycles. Increasing the cycle number increases the average peak height with the smaller sized loci, TH01, CSF1PO, and TPOX being most affected. However, smaller cycle numbers generally give better peak balance.*

126 base pairs only the smaller sized loci (i.e., TH01, D5S818, and vWA) were detectable. The loss of the peak intensity as the amplicon sizes became larger was clearly evident for the PowerPlex® 16 system, and this behavior would be expected for any commercial kit that contains large amplicon sizes (i.e., AmpFℓSTR™ Identifiler and SGM Plus™). On the other hand, the Miniplex primer sets were capable of producing complete profiles for all tested samples even at template fragment lengths below 222 base pairs. However, it should be anticipated that allele dropout could occur for the longest alleles of the FGA locus. The physical limitation of commercial kits to amplify alleles that are larger than the available intact DNA template may be less apparent in certain forensic situations, as degraded DNA will contain a mixture of fragments of different lengths. However, these results clearly define the effect of template size on amplification efficiency. While in some circumstances it may be possible to increase template concentration to reduce allele dropout with commercial kits, this is not always an option with forensic samples because the DNA template recovery can be very low.

Sensitivity Study

The effect of cycle number and amount of DNA template on the amplification efficiency of DNA fragments were studied for fragment sizes of ~222–350 base pairs and ~350–460 base pairs, These were tested at 28, 30, and 33 amplification cycles with the Big Mini primer set (Fig. 3). We observed that an increase in cycle number increases the average peak height, with the smaller sized loci (i.e., TH01, CSF1PO, and TPOX) being most affected. Since the DNA template utilized was in excess of 1 ng, the effect of cycle number was not that apparent. Although increasing cycle number generally increases signal intensity, smaller cycle numbers generally achieve better peak balance (13). Because low concentrations of DNA template are usually obtained from highly degraded samples, we explored the sensitivity of Miniplex 2, Miniplex 4, and Big Mini with DNA concentrations ranging from 31 pg to 500 pg. These concentrations are below the range recommended for commercial sets. The standard amount of DNA recommended for commercial kits is 1 to

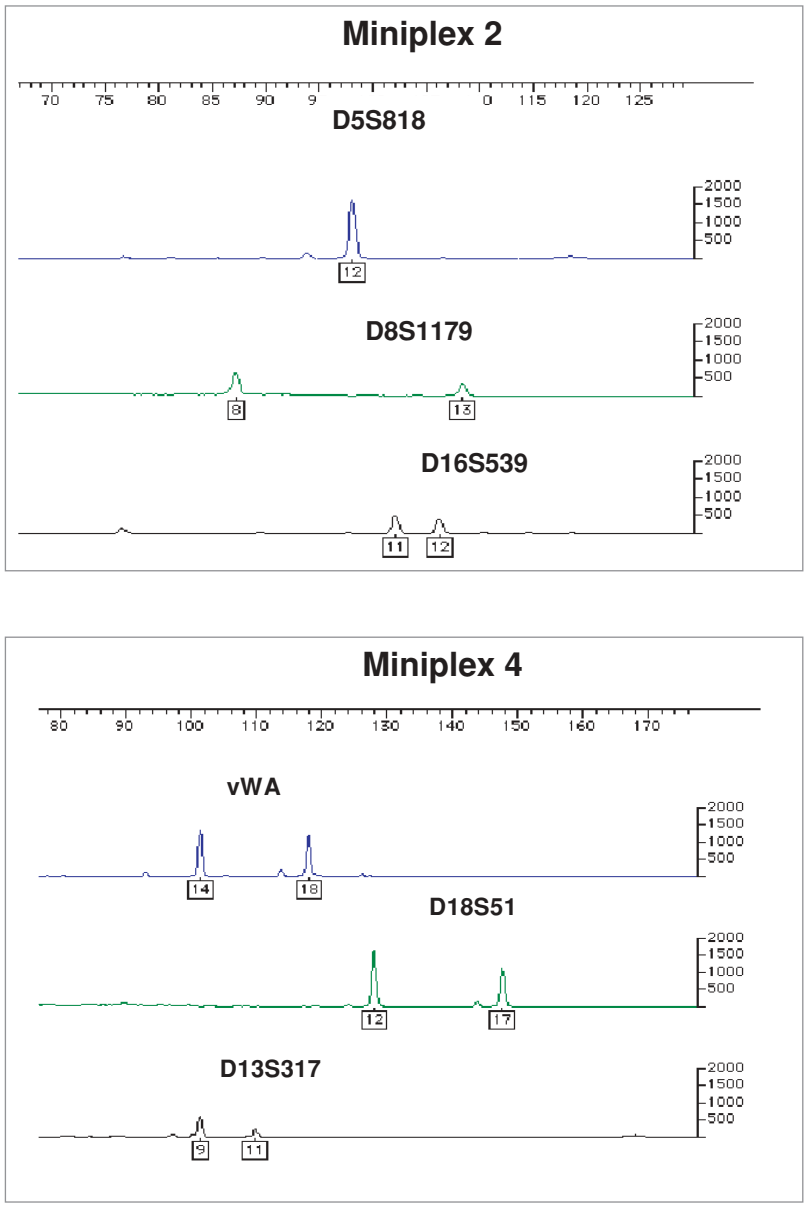

FIG. 4—*60 pg/25 μL of bone sample amplified with Miniplex 2 (a), Miniplex 4 (b), Big Mini (c), and PowerPlex® 16 (d) at 33 cycles. Typing by PowerPlex 16 resulted in a partial genetic profile at this template concentration. The Miniplex sets were able to give typable results at this concentration. Off-ladder (OL) allele is a current spike.*

2 ng (24). Correct genotypes were obtained at concentrations as low as 31 pg for most of the samples tested with Miniplex 2 and Miniplex 4. However at this concentration, there was one sample that showed allele dropout for Miniplex 2 ($n = 12$) and six samples that showed allele drop out for Miniplex 4 ($n = 20$). At 63 pg, one sample for Miniplex 2 and four samples for Miniplex 4 showed allele dropout. For the Big Mini multiplex, allele dropout was evident for most of the loci tested at 31 pg. At 63 pg, 50% of the samples tested ($n = 14$) still showed allele dropout for the CSF1PO, D21S11, and D7S820 loci. Based on our results, we found that template concentrations above 100 pg in 25 μL reaction volumes work well for Miniplex 2 and Miniplex 4. At this concentration, the average peak heights for Miniplex 2 and Miniplex 4 are 2000 RFU and 800 RFU respectively. These are well above our detection threshold of 150 RFU. As for the Big Mini, template concentrations greater than 250 pg/25 μL are needed to avoid allele dropout. Signal intensities for Miniplex 2 and Miniplex 4 were generally better compared to the Big Mini for the concentrations tested which may be due to the higher level of multiplexing in Big Mini. The sensitivity we report here is better than what was reported in validation studies for commercial kits for Mini 2 and Mini 4 but not for Big Mini (13,15). However, sensitivity is a function of cycle number, injection time, and concentration of sample injected. Thus, it is the responsibility of the analyst to develop validated procedures when sensitivity is an issue.

Peak Balance Study

Since an important requirement for accurate genotyping is to produce balanced allele peaks, we also calculated the heterozygote peak balance ratio at five DNA concentrations (500, 250, 125, 63, 31 pg per 25 μL). Only samples that were heterozygous for a particular locus were included in the calculations. For samples with complete dropout of one allele, a zero peak balance ratio was assigned. Good intraloci and interloci balance (≥ 0.6 ratio) were obtained at

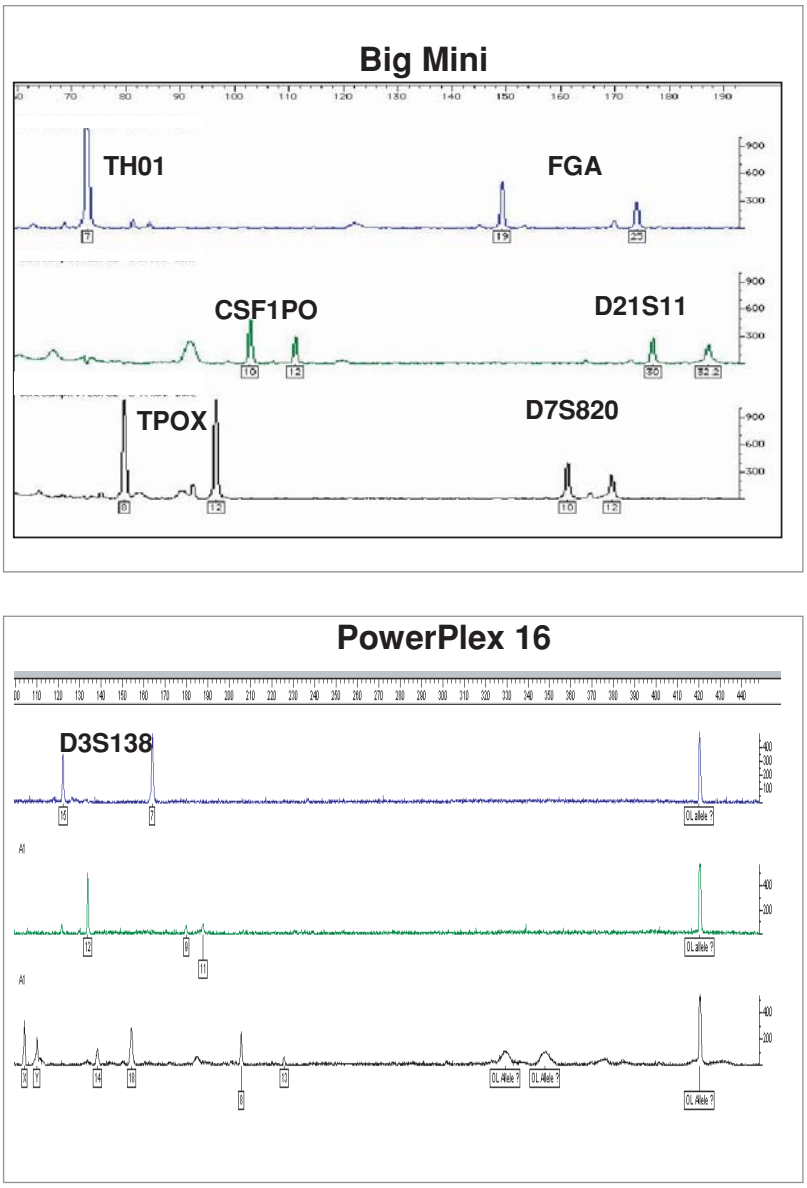

FIG. 4—*Continued*.

concentrations greater than 125 pg/25 μL for Miniplex 2 and Miniplex 4. For the Big Mini, good peak balance for all the loci was achieved at 250 pg/25 μL of template. At DNA concentrations of less than 125 pg/25 μL, CSF1PO and D21S11 become highly imbalanced (0.21 and 0.41, respectively).

To see if we can improve the sensitivity and peak balance ratio of the problematic loci (FGA, CSF1PO, and D21S11) in Big Mini, we tried varying their primer concentrations. We found that changing the primer concentration of one locus unpredictably affects the way other loci are amplified. Because the efficiency of synthesis and labeling of primers is different for every batch, primer concentrations must be optimized, accordingly. For this set of primers, increasing the primer concentration of the FGA, CSF1PO, and D21S11 loci to 0.3 μM, 0.5 μM, and 0.6 μM, respectively, improved the sensitivity and peak balance ratio at 31 pg/25 μL and 63 pg/25 μL of DNA template (data not shown). However, the dye blob that impacts allele 7 of CSF1PO and alleles 32 to 34 of D21S11 is exacerbated at these higher primer concentrations. For samples where the dye blobs can interfere with correct genotype interpretation (i.e., mixtures), using the Performa® DTR spin column from Edge Biosystems after PCR can help alleviate the problem (15).

A disadvantage of the Miniplexes compared to commercial multiplex kits is the increase in the number of amplification reactions and capillary electrophoresis injections that must be performed to obtain a profile for all 12 CODIS loci. That could pose a limit when the quantity of DNA template is minimal or sample throughput is a concern.

Case Report

Two bone samples from which complete profiles from standard kits were unavailable were re-tested with the Miniplex primer sets. The first sample was from a human body that decomposed at high temperatures in a closed environment for more than a month. The second bone sample was from a set of remains that was discovered near a stream and was believed to have been in water at some point. Complete profiles for all loci in Miniplex 2, Miniplex 4 and Big Mini were obtained at concentrations of 60 pg/25 μL (Fig. 4). Higher

concentrations were not tested due to the low amount of DNA available. Amplifications of the first bone sample with the commercial multiplex kit resulted in a partial genetic profile (Fig. 4). For the second bone sample, the results from the commercial kit were inconclusive because the peak height for the larger sized amplicons was below our detection threshold of 150 RFU. In contrast, the Miniplex sets were able to give typable results for these larger sized amplicons.

Conclusion

Our STR primer sets, Miniplex 2, Miniplex 4, and Big Mini, in which amplicon size is kept at minimum, provide an effective tool for degraded forensic samples as seen from the degradation study, sensitivity study, and the real case examples. When amplifications of enzymatically degraded DNA from the Miniplex sets were compared to the commercial kit, the improvement in the amplification efficiency for the smaller sized fragments was evident. The Miniplex primer sets were also able to successfully amplify low DNA template concentrations, 100 pg/25μL for Miniplex 2 and Miniplex 4 and 250 pg/25μL for Big Mini. Although the sensitivity is favorably comparable to commercial kits, further optimization studies will still be conducted to increase the heterozygous peak balance ratio for the Big Mini primer set. Overall, we have demonstrated that these Miniplex primer sets can provide an alternative to standard STR typing kits when allele dropout and low sensitivity of large amplicons becomes a challenge.

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Additional information and reprint requests:
Bruce R. McCord, Professor
Department of Chemistry
Ohio University
136 Clippinger Laboratories
Athens, OH 45701