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Developmental Validation of Reduced-Size STR Miniplex Primer Sets*

ABSTRACT: This paper describes a developmental validation study of three Miniplex sets covering 12 of the 13 CODIS loci. As these new sets will be used for the analysis of degraded and low level DNA, the validation studies were performed using 100–125 pg of DNA, the lowest input level at which peak balance, peak intensity, and allele consistency were stable. To demonstrate the applicability of the Miniplex sets to forensic casework, these validation studies were completed in accordance with the Scientific Working Group on DNA Analysis Methods (SWGDAM). A range of tests were performed including studies of concordance with standard multiplex kits, sensitivity and reproducibility, and PCR amplification conditions. Additionally, studies of mixtures, nonhuman and environmentally degraded DNA, and simulated forensic samples were performed. Our results demonstrate that Miniplex STR amplification procedures are a robust and sensitive tool for the analysis of degraded DNA.

KEYWORDS: forensic science, DNA, degraded, STR, validation, TH01, CSF1PO, TPOX, FGA, D21S11, D7S820, D5S818, D8S1179, D16S539, vWA, D18S51, D13S317

Degradation in forensic DNA samples can present a challenge to analysts. In such samples, the DNA template becomes highly fragmented due to physical, biological, and oxidative processes. In order to obtain complete amplification, the DNA target sequence must be intact at the point where the forward and reverse primers bind as well as within the region bracketed by those primers (1). When template fragmentation occurs, the possibility of finding an intact target sequence is reduced. To address this issue, new reduced-size Short Tandem Repeat (STR) primer sets, known as Miniplexes, have been developed (2). In these multiplex sets, the primers are designed to bind as close to the repeat region as possible. As primer position determines amplicon length but does not interfere with the repeat information, these new kits can be used on degraded DNA and are compatible with the current databases. Several examples of reduced amplicon size primers for various STR loci have already been published (3,4). However, the Miniplexes were the first attempt to redesign the full set of CODIS loci. Much of the design of the Miniplexes was based on previous work to redesign STR primers for analysis by time-of-flight mass spectrometry, as small amplicons are necessary for analysis by mass spectrometry due to fragmentation of larger molecules in the ion source (5).

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Four standard sets of Miniplexes covering 12 of the 13 CODIS loci were created for use with four-dye detection systems in capillary electrophoresis. The 13th locus, D3S1358 is already small in most commercial kits. Other primer sets have been designed to cover non-CODIS STR markers, and miniSTR multiplex sets for five-dye systems have also been designed (2,6). The present research focuses on a four-dye system of the common ABI310 capillary electrophoresis instrument, where the red dye (ROX) is reserved for an internal standard. Because of their reduced size, many of these fragments would overlap if amplified together, thus it is not possible to simultaneously amplify all 12 loci. Instead only one locus is assigned to each dye lane to avoid confusion. An exception to this rule exists for the Miniplex 1 and Miniplex 3 sets. The size ranges for these sets do not overlap and have been combined to form a six loci multiplex set known as "Big Mini." These kits produce a reduction in amplicon size for STR loci of up to 191 base pairs when compared to commercial kits. The size of the alleles in the Miniplex kits range from 60 to 284 base pairs.

Developmental validation studies of the Miniplex primer sets were undertaken in accordance with the Scientific Working Group on DNA Analysis Methods (SWGDAM) guidelines (7). This process involves a series of tests which can be used by a laboratory to determine the limitations of the method and to examine the different parameters that will affect the ability of the method to produce reliable results under a variety of conditions (8). A range of tests were performed, including studies testing concordance with standard multiplex kits, sensitivity and reproducibility, and PCR amplification conditions. Additionally, studies of various mixtures, tests with nonhuman and environmentally degraded DNA, and studies involving simulated forensic samples were performed.

Because these sets were designed for use with samples which, in addition to being degraded, may contain relatively low amounts of amplifiable DNA, these validation studies were performed using 100–125 pg of template DNA. This quantity of DNA is just above

the range where low copy number precautions must be taken (<100 pg) (9,10). In these studies we demonstrate through the use of sensitivity studies, peak balance measurements, and allele concordance that Miniplex sets produce consistent and reliable results at this level. The results and tables provided below demonstrate this fact and provide important details of the range of applicability of the system.

Materials and Methods

Unless otherwise stated, all amplifications were carried out with 100 pg of template DNA. Sources of DNA included purchased control standards 9947A Female DNA (Promega Corporation, Madison, WI), 9948 Male DNA (Applied Biosystems, Foster City, CA), and K562 (Promega Corporation) as well as blood stains and buccal swabs from laboratory volunteers. To maintain consistency of results, control standards were used for most experiments unless otherwise specified. Amplifications were performed in reaction volumes of 25 μL at 33 cycles using a master mix containing 1X GeneAmp® PCR Gold buffer (Applied Biosystems), 1.5 mM $MgCl_2$, 200 μM dNTPs (Life Technologies), and 2 U of AmpliTaq Gold® DNA polymerase (Applied Biosystems) unless otherwise specified (2). Three replicates of each sample for each test were performed unless otherwise specified.

DNA Extraction

Extractions of blood stain samples were performed with 300 μL stain extraction buffer (10 mM Tris–HCl pH 8.0, 100 mM NaCl, 39 mM dithiothreitol, 10 mM EDTA, 2% SDS) and 40 μg proteinase K (Fermentas, Hanover, MD) using a previously published method (11). Organic extraction was completed using 300 μL of phenol/chloroform/isoamylalcohol (25:24:1) (Invitrogen, Carlsbad, CA) and the supernatant was purified and concentrated using Microcon® YM-100 filters (Millipore Corporation, Bedford, MA). DNAzol (Molecular Research Center, Inc., Cincinnati, OH) and FTA (Whatman, Florem Park, NJ) extractions were performed according to manufacturer's protocols (12). Samples were collected in deionized water and stored at −20°C for 0–8 weeks. High level DNA samples were diluted and amplified without storage.

DNA Quantification

DNA samples were quantified using an Alu-based real-time PCR method (13) with a RotorGene RG 3000 cycler (Corbett Research, Sydney, NSW, Australia) and SYBR Green I dye (Invitrogen Molecular Probes). The DNA standards used (2, 0.7, and 0.1 ng/μL) were obtained from the Quantiblot® Human DNA Quantification Kit (Applied Biosystems). Samples were quantified prior to amplification and serial dilutions were made based on the quantification results.

Primer Set Optimization

Primer sequences and amplification conditions have been published previously and are listed in Tables 1 and 2 (2). Primer concentrations for the Miniplex 2 set and the Miniplex 4 set (Table 3) were not further optimized. For the Big Mini set, primer concentrations for TH01, CSF1PO, TPOX, FGA, D21S11, and D7S820 were further adjusted and optimized in order to improve the sensitivity and peak balance for the larger loci (FGA, D21S11, and D7S820) of the Big Mini multiplex.

TABLE 1—*Miniplex set primer sequences.*

Locus	Sequence (5′–3′)
D5S818 F	GGGTGATTTTCCTCTTTGGT
D5S818 R	AACATTTGTATCTTTATCTGTATCCTTATTTAT
D8S1179 F	TTTGTATTTCATGTGTACATTCGTATC
D8S1179 R	ACCTATCCTGTAGATTATTTTCACTGTG
D16S539 F	ATACAGACAGACAGACAGGTG
D16S539 R	GCATGTATCTATCATCCATCTCT
vWA F	AATAATCAGTATGTGACTTGGATTGA
vWA R	ATAGGATGGATGGATAGATGG
D18S21 F	TGAGTGACAAATTGAGACCTT
D18S21 R	GTCTTACAATAACAGTTGCTACTATT
D13S317 F	TCTGACCCATCTAACGCCTA
D13S317 R	GTTTCTTCAGACAGAAAGATAGATAGATGATTGA
TH01 F	CCTGTTCCTCCCTTATTTCCC
TH01 R	GGGAACACAGACTTCATGGTG
CSF1PO F	ACAGTAACTGCCTTCATAGATAG
CSF1PO R	GTGTCAGACCCTGTTCTAAGTA
TPOX F	CTTAGGGAACCCTCACTGAATG
TPOX R	GTTTCTTGTCCTTGTCAGCGTTTATTTGC
FGA F	AAATAAAATTAGGCATATTTACAAGC
FGA R	GCTGAGTGATTTGTCTGTAATTG
D21S11 F	ATTCCCCAAGTGAATTGC
D21S11 R	GGTAGATAGACTGGATAGATAGACGA
D7S820 F	GAACACTTGTCATAGTTTAGAACGAAC
D7S820 R	GTTTCTTTCATTGACAGAATTGCACCA

TABLE 2—*PCR amplification program.*

Amplification step	Temperature (°C) and time (min)
Hold 1	95 for 10
Denaturation	94 for 1
Annealing	55 for 1
Extension	72 for 1
Final extension	60 for 45

TABLE 3—*Miniplex Set optimized primer concentrations.*

Set	Locus	Concentration (μM)
Mini 2	D5S818	0.4
	D8S1179	0.4
	D16S539	0.2
Mini 4	vWA	0.4
	D18S21	0.4
	D13S317	0.56
Big Mini	TH01	0.16
	CSF1PO	0.16
	TPOX	0.2
	FGA	0.24
	D21S11	0.24
	D7S820	0.32

PCR Reaction Components and Thermal Cycling Parameters

In order to establish the valid range of conditions, several amplification parameters were tested, including *Taq* enzyme concentration, annealing temperature, magnesium concentration, reaction volume and cycle number. AmpliTaq Gold® Polymerase (Applied Biosystems) concentrations from 1 to 5 U/25 μL were tested with 100 pg of DNA template for Miniplex 2, Miniplex 4, and Big Mini. Annealing temperatures of 50, 55, 58, 60, 65°C were tested to determine the optimum temperature as well as the range of temperatures at which amplification is possible. Magnesium concentrations of 1.0, 1.5, 2.0, 2.5, and 3.0 mM were also tested with the three Miniplex sets. The GeneAmp® PCR buffer II (Applied

Biosystems) without magnesium was used and the specified amount of magnesium was then added. For the reaction volume study, reaction volumes of 5, 10, 12.5, 25, and 50 μL were tested with final DNA amounts of 100, 250, 500, and 1000 pg. For the cycle number study, 100–500 pg of DNA template were amplified at 28, 30, 33, and 36 cycles.

Sensitivity, Peak Balance, and Stutter

In order to establish the sensitivity of the method, samples with DNA amounts of 500, 250, 125, 63, and 32 pg were amplified with the Miniplex 2, Miniplex 4, and Big Mini sets. The samples were prepared by serial dilution from samples of known concentration. Three replicates were made for each template amount and the average peak height was determined. The heterozygous peak balance ratio at all five DNA amounts was also calculated. Only samples that were heterozygous for a particular locus were included in the calculations. The peak balance ratio was calculated by dividing the peak height of the smaller peak by the peak height of the larger peak. For samples with complete dropout of one allele, a zero peak balance ratio was assigned. Each STR locus in these three Miniplexes was evaluated for stutter using the amplification and analysis data from the concordance study. Stutter was determined only for alleles that differed by at least eight base pairs. Stutter percentage was calculated by dividing the peak height of the stutter peak by the peak height of its corresponding allele multiplied by 100. Stutter percentages from off-scale peaks were excluded because the height of the actual allele is underestimated and this causes the stutter percentage to be artificially high.

Standard Specimens and Reproducibility, Concordance, and Environmental and Matrix Studies

Raw (whole) blood was collected by venipuncture and immediately spotted on unbleached white cotton cloth and FTA cards and stored for 1 year. Fresh saliva from the same individuals was then obtained and applied to FTA indicator cards a few days before the aged blood samples were amplified to compare the genotype results. Three different extraction methods were used: organic extraction (phenol/chloroform) for blood stains (4), DNAzol elution for blood spotted FTA cards (14), and the standard FTA extraction protocol for saliva specimens (12) (Whatman). Concordance studies for 532 Caucasian, African-American, Hispanic, and Asian samples (208, 212, 110, and 2, respectively) have been published previously (15). Anonymous liquid blood samples with self-identified ethnicities were purchased from Interstate Blood Bank (Memphis, TN) and Millenium Biotech, Inc. (Ft. Lauderdale, FL) and extracted, quantified, and typed with the commercial kit AmpF*l*STR™ Identifiler (Applied Biosystems). For the Big Mini assay, 5 μL volumes with 2 ng of input DNA and 28 PCR cycles were used while Miniplex 2 and Miniplex 4 utilized 5-μL volumes, 2 ng of DNA template, and 26 PCR cycles (15). Sequencing of discordant alleles will be published separately for all population groups.

For the environmental and matrix studies, blood was collected by venipuncture in tubes without EDTA and immediately spotted on unbleached white cotton cloth and the matrix substrates (five to six drops per piece of cloth or substrate). Thirty-six spots were made and allowed to air dry at room temperature overnight. Three cuttings of the cloth with spots were then extracted the following day using organic extraction to serve as the control sample. The spots were then stored at −20°C, 4°C, room temperature (24°C), and 50°C for periods of 3, 7, 14, 28, 56, and 84 days. One set of spots were exposed to naturally fluctuating temperatures (inside a vehicle) for the same periods of time. Another set of spots was exposed to a UV plant "gro" light for 8 h a day at room temperature to simulate sunlight and were also sampled under the same time periods as mentioned above. For the matrix study, fresh blood stains were deposited on a variety of substrates and extracted using organic extraction. The following substrates were used: white cotton (control), white paper, blue denim, black leather shoe, rusted wrench, clean metal hammer, rubber hammer, leaf, and pine wood. The stains were allowed to dry for one week at room temperature prior to extraction. After 8 weeks of storage at room temperature, the stains were sampled again. Those on cloth, paper, and denim were sampled as cuttings and those on the rest of the surfaces were collected with swabs dampened with deionized water.

Species Specificity

To ensure that the Miniplex sets demonstrate specificity for humans, a variety of animal and bacterial species were examined. DNA samples from chimpanzee, dog, cat, pig, mouse, rat, chicken, *Escherichia coli*, *Staphylococcus aureus*, *Enterococcus faecalis*, and *Pseudomonas aeruginosa* were amplified with the Miniplex 2, Miniplex 4, and Big Mini primer set using 10, 1, and 0.25 ng of DNA template.

Mixture Studies

Mixtures of DNA samples in defined ratios were examined while keeping the template concentration constant at 100 pg. Pairs of DNA samples were mixed in the following ratio: 1:19, 1:9, 1:4, 1:3, 1:2, and 1:1. The average peak heights of the minor and major alleles were used to calculate the ratio of the minor to major component for each locus. The average peak height ratio was calculated as the intensity of the minor allele divided by the intensity of the major allele in relative fluorescence units (RFU). For each locus, only samples which were both homozygotes and both heterozygotes with alleles at least eight base pairs apart were used.

Detection and DNA Analysis

Amplified DNA was separated and detected using the ABI PRISM® 310 GeneticAnalyzer (Applied Biosystems). The GeneScan® ROX 500 (Applied Biosystems) size standard was used. Samples were prepared by adding 1 μL PCR product to 12 μL Hi-Di™ formamide (Applied Biosystems) containing 0.5 μL of GS-ROX size standard. Injection and analysis parameters were published previously (2). The detection threshold used for calling alleles was 150 RFUs which was *c.* 10 times the standard deviation of the noise (limit of quantitation).

Results and Discussion

Primer Set Optimization

At the original primer concentrations used in the Big Mini assay, three loci (FGA, CSF1PO, and D21S11) displayed poor sensitivity and reduced peak balance (<60%). In response to this problem, a series of primer concentration sets was tested in order to determine the best set. Primer concentrations were varied for the problematic loci (FGA, CSF1PO, and D21S11) in Big Mini. Initially three loci, CSF1PO, D21S11, and D7S820, were examined while keeping the other three loci, TH01, TPOX, and FGA at fixed primer concentrations of 0.16, 0.20, and 0.30 μM, respectively. Based on the results of this test, the best balance was achieved at primer concentrations

of 0.20 μM CSF1PO, 0.3 μM D21S11, and 0.35 μM D7S820. Following this initial test, primer concentrations were further optimized by examining different primer concentrations around the optimum region. The best overall peak balance was observed at the following primer concentrations: 0.16 μM TH01 and CSF1PO, 0.2 μM TPOX, 0.24 μM FGA, 0.24 μM D21S11, and 0.32 μM D7S820. At these concentrations, a low intensity peak in the K562 FGA profile could be detected (this peak, which is significantly lower in intensity than the other allele peaks, is a normal feature of the K562 cell line). These same primer concentrations also work well for amplification of samples with higher quantities of DNA template. A complete list of the primer concentrations for all assays is presented in Table 3.

PCR Reaction Components and Thermal Cycling Parameters

For the *Taq* polymerase study, 1 U of enzyme is sufficient to successfully amplify 100 pg of DNA at 33 cycles. However, better peak balance (>65%) was achieved at 2 U of enzyme. Higher concentrations of enzyme did not have an effect on the yield of PCR products (Fig. 1). A default enzyme concentration of 2 U is currently used.

At annealing temperatures of 50°C and 55°C, allele dropout was not observed and no additional artifacts were seen. Locus and allele dropout started to occur at the annealing temperature of 58°C for D5S818 of the Miniplex 2 set. At 60°C, CSF1PO, D21S11, and FGA of the Big Mini set began to drop out, and at 65°C, all loci failed to amplify (Fig. 2). For these primer sets, an annealing temperature of 55°C is recommended as this temperature provides maximal specificity without allele dropout.

Magnesium concentrations can also affect specificity and allele dropout was a problem at 1 mM magnesium concentration. Magnesium concentrations of 1.5–2.0 mM were found to provide the best peak intensity and balance. These results indicate that the availability of magnesium ions is essential for optimal polymerase activity. At higher magnesium concentrations (2.5–3.0 mM), problems with nonspecific binding ("allele drop in") became apparent. While an increase in magnesium concentration (2.0–3.0 mM) did not greatly affect PCR yield, a decrease in the amount of magnesium (<1.5 mM) caused adverse effects on the yield of the reaction.

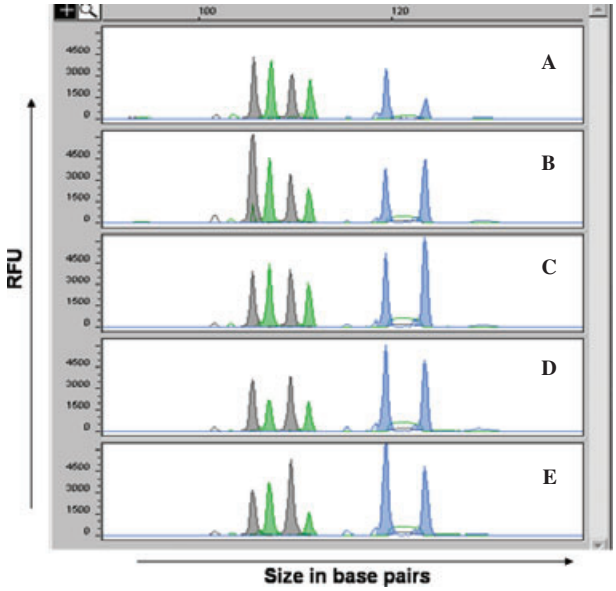

FIG. 1—*Miniplex 2 titration of AmpliTaq Gold® DNA polymerase. 100 pg/25 μL of DNA template was amplified at 33 cycles and titrated with varying enzyme concentrations. Enzyme concentration per 25 μL is as follows—Panel A: 1 U, Panel B: 2 U, Panel C: 3 U, Panel D: 4 U, and Panel E: 5 U.*

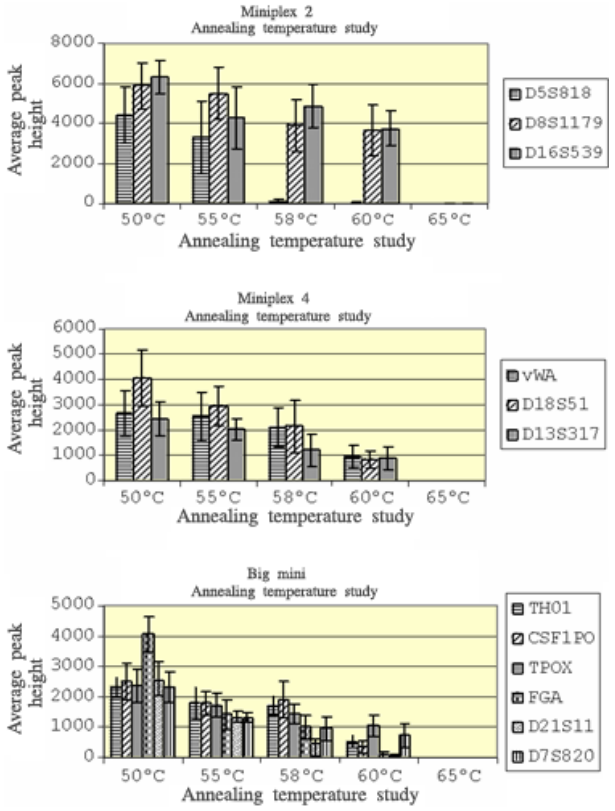

FIG. 2—*Effect of the variation in annealing temperature for Miniplex 2 (top), Miniplex 4 (center), and Big Mini (bottom). 100 pg/25 μL of DNA template amplified at 33 cycles using different annealing temperatures. The D5S818 began to dropout at 58°C followed by CSF1PO, FGA, and D21S11 at 60°C. At 65°C, no amplifications are seen for all loci. Error bars represent ±95% confidence interval for the average peak intensity.*

Miniplex 2 and Miniplex 4 gave good signal intensities (≥1000 RFU) and good peak balance ratios at 30 and 33 cycles (Fig. 3). However, at 28 cycles the signal intensity was too low (<150 RFU). For the Big Mini set, successful amplification of all the loci was only achieved at 33 cycles. At 28 cycles, only the FGA, D21S11, and D7S820 loci amplified with 100 pg of DNA template. The smaller sized loci, TH01, CSF1PO, and TPOX, required 500 pg at 28 cycles and 250 pg of template at 30 cycles. At 36 cycles, all Miniplex loci were over-amplified and problems with nonspecific amplification were encountered. Although increasing the cycle number can increase signal intensity, lower cycle numbers can achieve better peak balance. For DNA template amounts in excess of 250 pg, lower cycle numbers can be used (i.e., 30 cycles for Big Mini and 28 cycles for Miniplex 2 and Miniplex 4). However, in situations where lower amounts of DNA template are available, it becomes indispensable to use a higher cycle number. Using 33 cycles for amplifying DNA samples with 100 pg of template is recommended, as it achieves the best balance between sensitivity and profile quality.

Reaction volumes of 10–25 μL provided good amplification results. Signal intensities for the amplified alleles did not vary as the volume changed. Using a 5 μL reaction volume, allele dropout was observed for Miniplex 2 with 100 pg of DNA template (Fig. 4) and no amplification products were observed for the Big

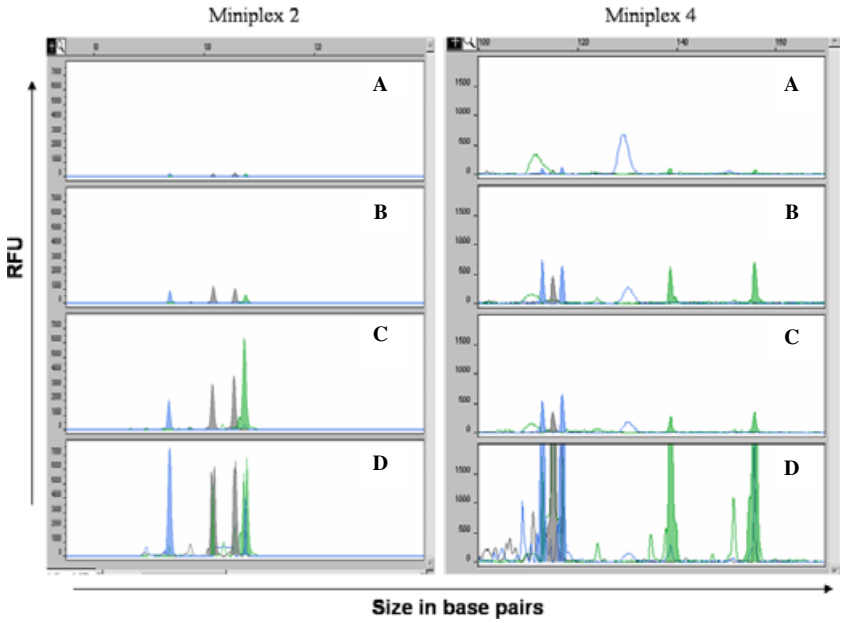

FIG. 3—*Cycle number study for Miniplex 2 and Miniplex 4. Amplifications of 100 pg/25 μL of DNA with Miniplex 2 and Miniplex 4 at different cycle numbers. Panel A: 28 cycles, Panel B: 30 cycles, Panel C: 33 cycles, and Panel D: 36 cycles. 1 μL of amplified sample was added to 12 μL formamide. Injection time used was 5 sec at 15 kV.*

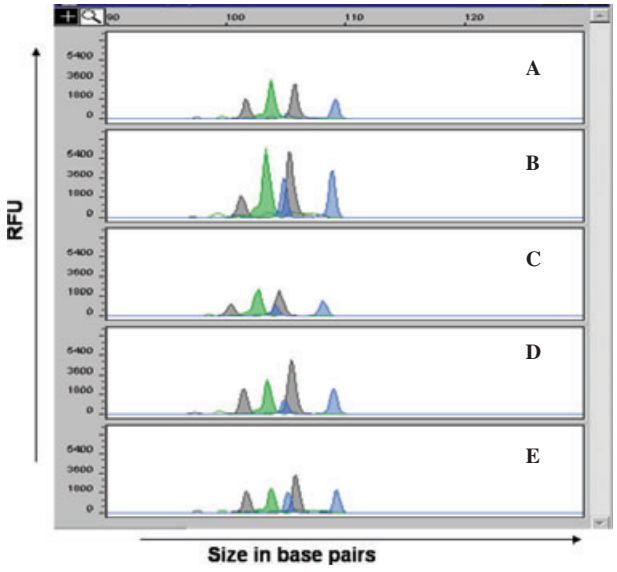

FIG. 4—*Miniplex 2 reaction volume study. 100 pg/25 μL of DNA template amplified at 33 cycles with Miniplex 2. The reaction volume for each panel is as follows—Panel A: 5 μL, Panel B: 10 μL, Panel C: 12.5 μL, Panel D: 25 μL, and Panel E: 50 μL. Allele dropout for D5S818 was observed when 5 μL of reaction volume at this concentration.*

Mini set. All other volumes and concentrations tested produced consistent profiles. For all Miniplex sets, the best results were obtained at 25 μL reaction volume for 100 pg of DNA template as larger volumes minimize pipetting errors (16).

Sensitivity, Peak Balance, and Stutter

Using the optimal conditions described above, all standard samples produced consistent genotypes, regardless of extraction method or time of blood spot storage. No allele loss was observed for any of the samples, but over amplification was observed from the full 3 mm diameter FTA saliva card sample. When smaller portions of the FTA cards were used, sample peak intensities occurred within the linear range of the instrument.

TABLE 4—*Results from sensitivity and peak balance study: template concentrations, percentage of full amplification (detection of all alleles in all loci), average RFU for all loci, and average peak balance for all loci.*

Kit	Template (pg)	Full amplification (%)	Average RFU	Average peak balance (%)
Miniplex 2 (n = 12)	500	100	3858	82
	250	100	3750	83
	125	100	2427	74
	63	92	1377	60
	32	83	808	57
Miniplex 4 (n = 20)	500	100	2595	83
	250	100	1600	77
	125	100	1267	74
	63	80	484	61
	32	70	390	57
Big Miniplex (n = 3)	500	100	5095	86
	250	100	1621	74
	125	100	1554	74
	63	100	615	60
	32	67	350	56

In the sensitivity study, interpretable amplification for all loci was achieved for template concentrations as low as 32 pg for Miniplex 2 (n = 12) and Miniplex 4 (n = 20), and 63 pg (n = 3) for Big Mini. Correct genotypes were obtained at concentrations as low as 32 pg for the majority of samples tested with Miniplex 2 and Miniplex 4. However, some instances of allele dropout occurred at this level. The overall results of this study are presented in Table 4. Based on these data, template input of 100–125 pg are optimal for Miniplex 2 (Fig. 5) and Miniplex 4 (Fig. 6). At this concentration range, the average peak heights for Miniplex 2 and Miniplex 4 are 2400 and 1200 RFU, respectively. These are well above our detection threshold of 150 RFU set for the ABI 310. Below 150 RFU peaks become unreliable as they fall below our instrument's limit of quantitation. However, it is recommended that

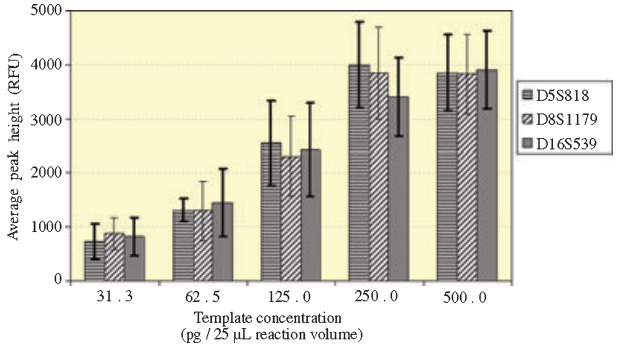

FIG. 5—*Sensitivity studies for Miniplex 2. The change in fluorescence signal intensity as a function of template concentration is shown for D5S818, D8S1179, and D16S539. Primer concentrations used were 0.4, 0.4, and 0.2 µM for D5S818, D8S1179, and D16S539, respectively. All samples were amplified at 33 cycles. No allele dropout and good signal intensities (c. 2500 RFU) were achieved at template concentrations >125 pg/25µL. Template concentrations of 100 pg/25µL are currently used with this primer set (n = 12). Error bars represent ±95% confidence interval from the average peak intensity.*

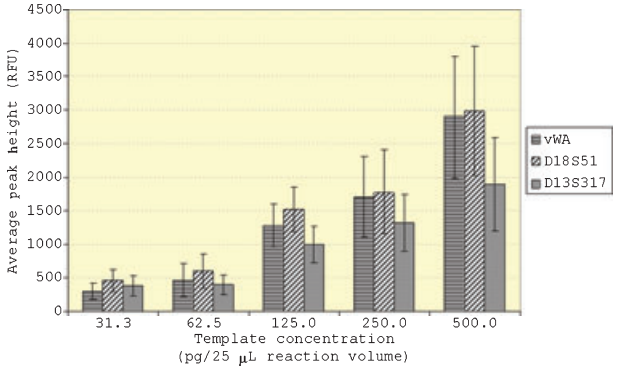

FIG. 6—*Sensitivity studies for Miniplex 4. The change in fluorescence signal intensity as a function of template concentration is shown for vWA, D18S51, and D13S317. Primer concentrations used were 0.40, 0.40, and 0.56 µM for vWA, D18S51, and D13S317, respectively. No allele dropout and good signal intensities (1200 RFU) were achieved at template concentrations >125 pg/25µL. Template concentrations of 100 pg/25µL are currently used with this primer set. Error bars represent ±95% confidence interval from the average peak intensity (n = 20).*

individual laboratories using these assays develop their own interpretational guidelines based upon peak balance and quantitation limits developed through their own internal validation studies.

Currently we utilize a template input of 100 pg for these two primer sets. For the Big Mini set, our preliminary studies showed that a template amount >250 pg (Fig. 7) was needed to avoid allele dropout and produce satisfactory signal intensity. After further experiments (discussed in a previous section), the primer concentrations of the Big Mini multiplex set were optimized to permit amplification of 100 pg of DNA template. This corresponds to *c.* 30 copies of DNA template, or 15 cells.

Good intraloci and interloci balance (≥0.6 ratio) were obtained at a concentration of 100 pg for Miniplex 2 (Fig. 8) and Miniplex 4 (Fig. 9). For the Big Mini set, poor peak balance for CSF1PO and D21S11 (0.21 and 0.41, respectively) were observed initially using the original primer ratios. This problem was resolved by changing the primer concentrations for the problematic loci of this set. After primer ratio adjustment for Big Mini, consistent amplification and good peak balance was achieved at 100 pg. It should be noted that these peak balance studies demonstrate that peak intensities well

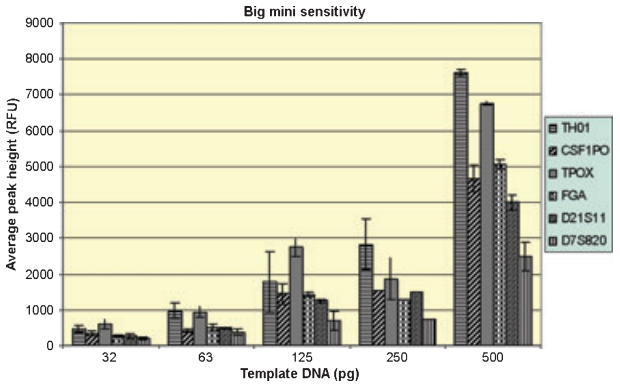

FIG. 7—*Sensitivity studies for Big Mini. The change in fluorescence signal intensity as a function of template concentration is shown for TH01, CSF1PO, TPOX, FGA, D21S11, and D7S820 Primer concentrations used were 0.16 µM for TH01 and CSF1PO, 0.2 µM for TPOX, 0.24 µM for FGA and D21S11, and 0.32 µM for D7S820. All samples were amplified at 33 cycles. Template concentrations of 125 pg/25µL or greater produced no allele dropout and achieved good signal intensity. Error bars represent ±95% confidence interval from the average peak intensity.*

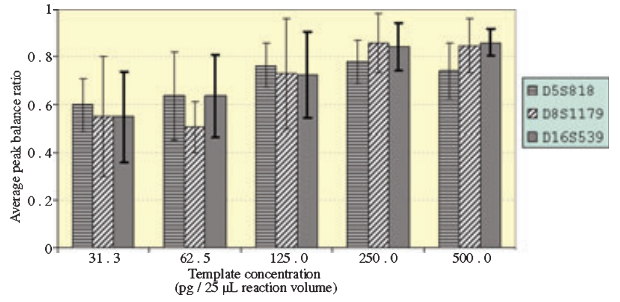

FIG. 8—*Peak balance ratio for Miniplex 2. The average peak balance ratio for D5S818, D8S1179, and D16S539 is plotted as a function of template concentration. Primer concentrations used were 0.40, 0.40, and 0.20 µM for D5S818, D8S1179, and D16S539, respectively. All samples were amplified at 33 cycles. Template concentrations >125 pg/25µL gave good peak balance ratios for this set at these conditions. At the 100 pg/25 µL of template currently used, the average peak balance ratio is still above the 0.6 criterion (n = 12). Error bars represent ±95% confidence interval from the average peak balance.*

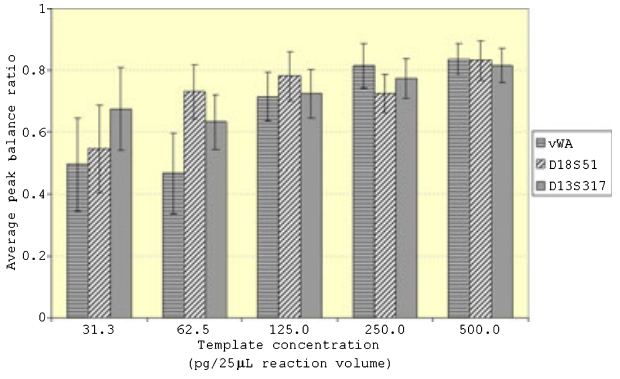

FIG. 9—*Peak balance ratio for Miniplex 4. The average peak balance ratio for vWA, D18S51, and D13S317 is plotted as a function of template concentration. Primer concentration used was 0.40, 0.40, and 0.56 µM for vWA, D18S51, and D13S317, respectively. All samples were amplified at 33 cycles. Template concentrations >125 pg/25µL gave good peak balance ratios for this set at these conditions. At the 100 pg/25 µL of template currently used, the average peak balance ratio is still above the 0.6 criterion. Error bars represent ±95% confidence interval from the average peak balance (n = 20).*

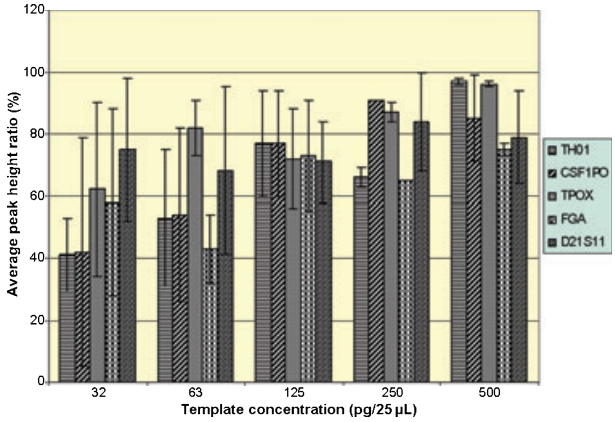

FIG. 10—*Peak balance ratio for Big Mini. The average peak balance ratio for TH01, CSF1PO, TPOX, FGA, D21S11, and D7S820 is plotted as a function of template concentration. Primer concentrations used were 0.16 µM for TH01 and CSF1PO, 0.2 µM for TPOX, 0.24 µM for FGA and D21S11, and 0.32 µM forD7S820. All samples were amplified at 33 cycles. Template concentrations >125 pg/25µL gave good peak balance ratios for this set at these conditions. Error bars represent ±95% confidence interval from the average peak balance.*

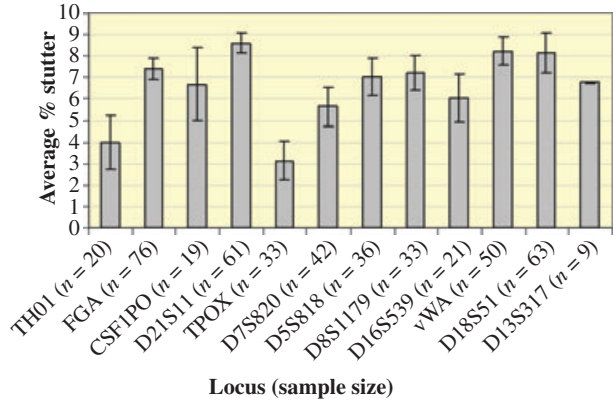

FIG. 11—*Average stutter calculated for each locus of the Miniplex 2, Miniplex 4, and Big Mini multiplex set. The sample size (n) indicates the number of samples used to calculate stutter for each locus. Error bars represent ±95% confidence interval from the average stutter value.*

above 150 RFUs may be anticipated for the Miniplex sets when amplifying low copy number DNA. This phenomena is unlike commercial STR kits where allele loss can occur when amplifying low levels of input DNA (16). Sample inputs below 100 pg should be interpreted with caution when amplifying the Miniplex sets due to the potential for peak imbalance.

Stutter is a phenomenon which occurs during PCR amplification in which a peak one repeat unit shorter than the true allele shows up at a reduced intensity. Percent stutter was calculated from the ratio of the stutter peak to that of the true allele. Stutter was <15% for all alleles observed (Fig. 11). TH01 and TPOX loci were found to have the lowest stutter percentage through the whole range of alleles. Average stutter increased as the alleles became larger, results that were observed for all loci tested and were consistent with previous reports of these phenomena for larger amplicons (17).

Standard Specimens and Reproducibility, Concordance, and Environmental and Matrix Studies

All samples, regardless of extraction method, produced consistent and reproducible genotypes with the Miniplex sets. Due to the sensitivity of the Miniplex sets, the size of the paper punches for the FTA extraction samples had to be reduced (1/4 of the 3-mm punch was used) in order to prevent over-saturation of the charge coupled device (CCD) detector. Results of the concordance studies have been published previously (15). Five hundred thirty two samples consisting of DNA extracts from Caucasian, African-American, and Hispanic individuals, previously analyzed using the Identifiler (Applied Biosystems) and Powerplex®16 (Promega) commercial STR kits were reanalyzed using the Miniplex sets. Full concordance was observed in 99.8% (6369 of 6384) of all STR allele calls compared (15). Fifteen discordant results in three loci (5-D13S317, 1-D5S818, 9-vWA) were observed. The lack of concordance for the vWA and D5 loci was likely the result of a primer binding site mutation which produced homozygous genotypes in heterozygous samples (Fig.10). The vWA mutations have been reported previously by Lazaruk et al. (18).

Samples exposed to the range of environmental conditions displayed no allele drop out, but loss of intensity (decrease in RFU values) in four of the loci of Big Mini (CSF1PO, FGA, D21S11, and D7S820) was observed for the sample exposed to the highest temperature after 14 days. Some further loss of intensity was observed up to 84 days (Fig. 12). All samples extracted from the range of matrices produced full profiles, but there was some loss of intensity for the samples on white paper. The decrease in the signal intensity of amplicons from white paper after 8 weeks of storage in room temperature could be attributed to the presence of bleach (introduced into the paper as part of the manufacturing process).

Species Specificity

Amplifications for all loci were seen for the chimpanzee sample at 1 ng of template. At 250 pg of DNA from the chimpanzee, amplification was not seen with the Big Mini loci. Higher amounts (1 and 10 ng) of mouse DNA produced peaks for the D16S539 and D13S317 loci in Miniplexes 2 and 4 (Fig. 13). The amplification products from the mouse sample at the D16S539 locus of Miniplex 2 were larger than the typical allele size range for this locus. With Miniplex 4, nonspecific amplification was observed at DNA concentrations of 10 ng for the D13S317 locus. At 1 ng, an amplification product that resembled allele 9 of the D13S317 locus was still visible. Below 250 pg no amplification was detected. For the other species samples tested, including dog, cat, pig, rat, chicken, and bacteria, no amplifications for any loci were seen.

Mixture Studies

A mixture study was performed in which the total DNA input was kept at 100 pg. For these experiments it should be noted that in mixtures individual levels of input DNA fall below our 100 pg threshold for acceptable peak balance and should be interpreted with extreme caution due to the potential for allele dropout (9,10). However, as these studies were designed to test the application of Miniplex sets at low sample inputs, and as mixtures are commonly present in forensic samples, we were interested in the ability of the technique to detect low level mixtures. The data from the experiments show that when the minor component was present at 10% of the total quantity of DNA template (10 pg minor contributor), all major and minor contributor alleles were detectable. Results for all loci also show that the relative peak height ratio for the minor component increases with the proportion of minor components in the sample; however, this relationship was not linear. Thus these

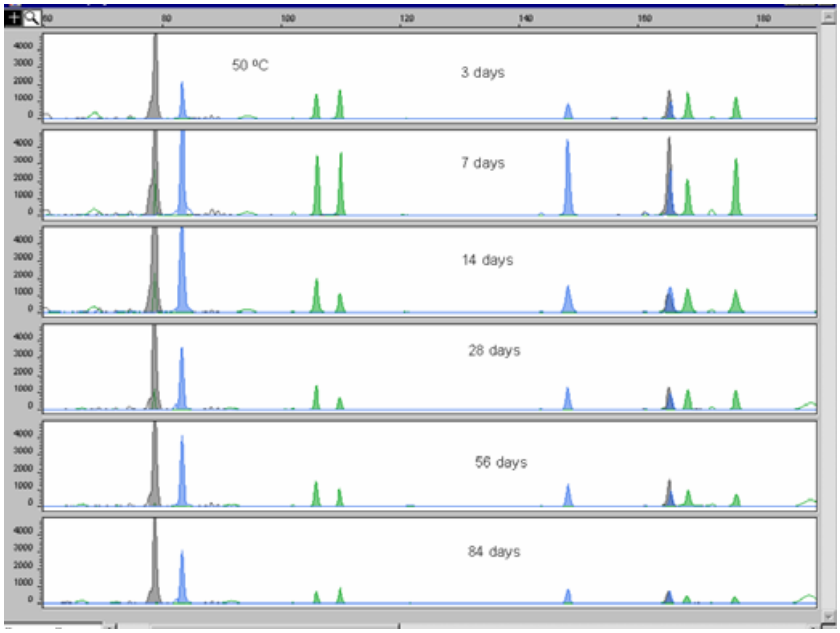

FIG. 12—*Example of results from environmental study, Big Mini amplification of 50°C samples at each time period. Loss of allele intensity for the larger loci (FGA, D21, and D7) is observed after 84 days at 50°C.*

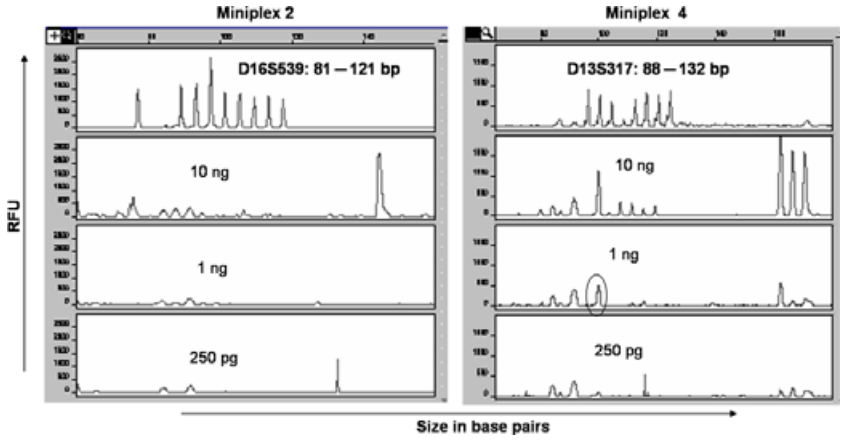

FIG. 13—*Mouse DNA sample amplified with Miniplex 2 and Miniplex 4. Amplification products from Miniplex 2 are larger than the allele size range for the D16S539 locus. Nonspecific binding was observed at D13S317 locus of Miniplex 4 at high DNA concentrations. At 1 ng/25 μL of DNA, an amplification product that resembles allele 9 of the D13S317 locus was still visible (circled peak).*

mixture studies do indicate that it is possible to detect a mixture despite the low concentration of 100 pg total input DNA template (Fig. 14). However, using data at low template concentrations to determine the exact ratio of input DNA is not advised. Instead the total quantity of input DNA should be raised to an appropriate level if a mixture is found to be present.

Simulated Forensic Samples

In two previous reports (19, 20) we have detailed the application of Miniplex sets using samples of blood degraded using DNAse I, and we have also analyzed recovered samples of bone which were highly degraded. For both types of samples, the data indicated that the Miniplex sets gave results consistent with commercial STR multiplexes, and for all samples where degradation was present, the Miniplex amplifications resulted in fewer missing alleles. In the study with recovered bone, 31 degraded bone samples were amplified with the Miniplex sets, and full profiles were produced for 94% of samples tested with Miniplex 2, 90% with Miniplex 4, and 65% with the Big Miniplex. Of these same samples only 16% produced full (12 loci) profiles with the commercial Powerplex® 16 kit (20).

Conclusions

The STR multiplex primer sets, Miniplex 2, Miniplex 4, and Big Mini contain 12 of the 13 CODIS loci and the primer binding regions of each locus are designed to produce amplicons that are as short as possible, thus providing an effective tool for the analysis of degraded DNA samples. This developmental validation study demonstrates the robustness, reliability, and reproducibility of Miniplex sets 2, 4, and Big Mini under a variety of conditions. DNA template concentrations as low as 100 pg/25μL can be successfully amplified with high sensitivity and good peak balance ratio using 33 cycles. Sensitivity studies, peak balance measurements and allele concordance data conclusively

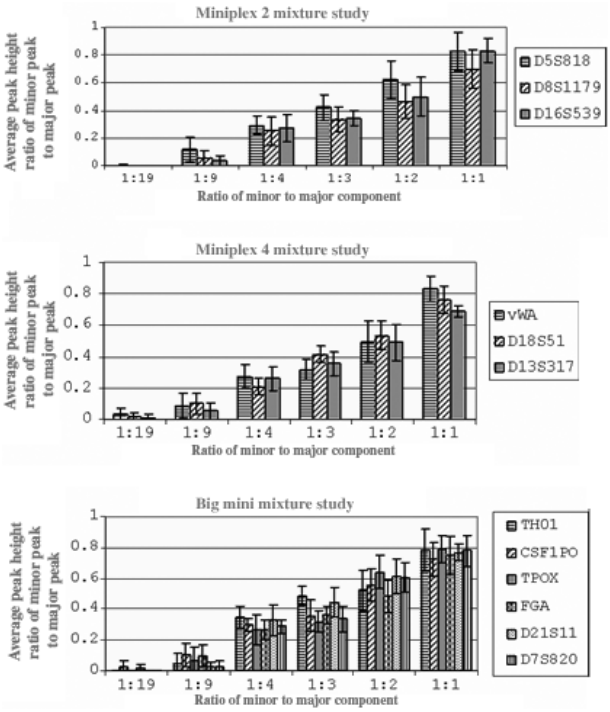

FIG. 14—*Mixture study of the Miniplex primer sets. Two DNA samples were mixed at different ratios while keeping the template concentration constant at 100 pg/25 μL. At a 1:19 ratio, the minor components of the D5S818 loci, vWA, D18S51, D13S317, CSF1PO, and FGA start to become detectable. All Miniplex loci are detectable when the minor component is present at a 1:9 ratio. Error bars represent ±95% confidence interval from the average peak intensity.*

demonstrate that Miniplex sets produce consistent and reliable results at this level.

These studies provide important data for forensic laboratories to assess the stability of the Miniplex sets under a variety of conditions that may arise in the interpretation of data from complex forensic samples. Further tests are being conducted to examine the application of these multiplexes to nonroutine forensic samples such as hair, fingernail scrapings, and low copy number DNA sources. As the Miniplex sets have been shown to work well with degraded and low copy number samples (20) it is anticipated using these primer sets for these samples would improve genotyping success rates.

In summary, these Miniplex sets are a robust and reliable tool which can provide an alternative to standard STR typing kits when allele drop out and low sensitivity of large amplicons becomes a problem due to DNA degradation.

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