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Testing MiniSTR Primers for Addition to a PCR-Based Forensic Specimen Identification Protocol

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16. Abstract

An assay has been developed for polymerase chain reaction (PCR)-based human identity testing using the Federal Bureau of Investigation's human Combined DNA Identity System (CODIS) primers. Recent forensic literature has identified difficulties using these primers due to amplicon size and the degraded nature of DNA from forensic samples. Primers termed mini Short-Tandem Repeat (STR) primers targeted to the same loci as the CODIS primers but which have smaller amplicons have been developed. Two of the three miniSTR primer pairs examined with our established assay were successfully tested with forensic DNA. This allowed the substitution of new primers for detection of a locus, D16S539, which was poorly defined in our assay and the addition of a primer pair for a locus, FGA, not previously included. The replacement of the D16S539 CODIS primers with the miniSTR primers will provide more accurate results for this locus. The addition of the FGA mini STR primers to the core set of tested loci will increase the overall power of discrimination of our assay.

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INTRODUCTION

DNA-based analysis of biological samples from fatal accident sites can serve as an additional and independent test for sample identification when results from routine toxicological testing conflict with preliminary sample identification. An assay has been developed (1, 2) using primers for eight Combined DNA Index System (CODIS) short tandem repeat (STR) loci (3), and two gender-determining loci (4, 5) to analyze samples from aviation accidents in support of toxicological findings. However, the D16S539 CODIS primers have been problematic due to an amplicon size with low resolution in our assay that utilizes a microfluidics platform, the Agilent 2100 Bioanalyzer, for electrophoretic separation. The size of the amplicon is at the limit of resolution for the polymer, which is best suited to the other, shorter amplicons used in the assay.

Work reported recently (6, 7) describes a series of primers designed for the CODIS STR loci that result in smaller amplicon sizes. The difficulty obtaining consistent polymerase chain reaction (PCR) results with degraded forensic DNA was the rationale for designing these additional miniSTR CODIS primers. Both weak signals and allelic dropout had been observed. Using these miniSTR primers was shown to give more efficient and consistent PCR results with degraded samples, potentially, an additional benefit due to the postmortem origin of the majority of samples used in our lab.

The purpose of the work presented here was to test the miniSTR primers for the D16S539 locus and to test miniSTR primers for two additional loci, FGA and D21S11, not currently used in our protocol. The smaller amplicons for the miniSTR version of these three loci suggested that they were in the size range that could be resolved by our system and the use of two additional loci would increase the power of discrimination of our assay. The primer sets were tested to determine optimum concentration in the PCR reactions. They were then tested at this optimal concentration against previously analyzed forensic DNA samples and human blood and tissue DNA controls. Our results showed that D16S539 and FGA miniSTR primers but not the D21S11 primers performed well in our assay and could be incorporated into the core set of loci to increase the overall power of discrimination of our assay.

MATERIALS AND METHODS

DNA samples

Initial PCR testing was performed with previously evaluated DNA samples from positive control 2221, negative control 2222, and forensic DNA samples 2215, 2216, 2217, 2218, 2219, and 2220. Additional samples used were blood positive control 12423A and forensic tissue liver sample 03010577, from which DNA was newly isolated for this study.

Blood and tissue extraction of genomic DNA

Samples were processed as described in previous reports (1, 2). Tissue and blood were stored at -20° C. Approximately 25 mg of sample 03010577 liver tissue was minced and processed using the manufacturer protocol with the Qiagen QIAamp DNA Mini Kit #51304 (Qiagen; Valencia, CA). DNA was extracted from 200 ul whole blood from control 12423A using the same kit with modifications for blood and body fluid as specified by the manufacturer.

Following DNA extraction, samples were analyzed using DNA 7500 chips on an Agilent 2100 Bioanalyzer (Agilent Technologies; Palo Alto, CA) to determine DNA quality. The NanoDrop 1000 Spectrophotometer Model ND-1000, (NanoDrop Technologies; Wilmington, DE) was used to determine DNA concentration.

PCR primers and amplification. Table 1 lists the primers used in this study. All primers were synthesized commercially (Integrated DNA Technologies; Coralville, IA).

PCR reaction setup was for 25 ul final volume. Each reaction contained 50 ng template, 200-800 nM each primer, 2.5 mM dNTPs, 0.1% TritonX-100, 1x Amplitaq Gold buffer, and 2.25 units Amplitaq gold.

A Perkin-Elmer GeneAmp 9600 (PerkinElmer; Wellesley, MA) was used for PCR according to the following protocol: one cycle at 95°C for 11 min and 96°C for 1 min;10 cycles at 94°C for 30 sec, 60°C for 30 sec (ramp time 1:08), and 70°C for 45 sec (ramp time 0:50); 24 cycles at 90°C for 30 sec, 60°C for 30 sec (ramp time 1:00), and 70°C for 45 sec (ramp time 0:50); 1cycle at 60°C for 30 min; and a 4°C hold until removal of samples.

Detection of a 1:3 dilution in distilled water of amplification products was performed on an Agilent

Table 1. Primers used in study.

Locus	Primer Sequence	Product Size Range (bp)	Reference	Size Reduction (bp)
D16S539	GGG GGT CTA AGA GCT TGT AAA AAG GTT TGT GTG TGC ATC TGT AAG CAT GTA TC	264-304	8	
D16S539 (miniSTR)	ATA CAG ACA GAC AGA CAG GTG GCA TGT ATC TAT CAT CCA TCT CT	81-121	6	152
D21S11 (miniSTR)	ATT CCC CAA GTG AAT TGC GGT AGA TAG ACT GGA TAG ATA GAC GA	153-211	6	33
FGA (miniSTR)	AAA TAA AAT GCA TAT TTA CAA GC GCT GAG TGA TTT GTC TGT AAT TG	125-281	6	71

Size reduction is comparison of the miniSTR to the corresponding CODIS primer-generated amplicon

2100 Bioanalyzer with DNA 1000 chips and Agilent Expert software version B.02.03.SI307 (Agilent Technologies; Palo Alto, CA).

RESULTS AND DISCUSSION

Initially, four primer concentrations, 200, 400, 600, and 800 nM, were tested for each of the three primer pairs using positive control 2221 template. The primers tested included mD16S539, mFGA, and mD21S11, with an "m" designating a miniSTR primer pair.

Determination of optimum concentration

mD16S539: Peak detection on the Agilent 2100 Bioanalyzer showed the expected peak sizes for all mD16S539 primer concentrations. However, the 200 nM concentration reaction result was below our lower confidence limit of 20 fluorescence units (FU). Almost equivalent results were seen at 400, 600, and 800 nM (Figure 1A), showing that peak intensity did not increase dramatically with increased concentration. There was good peak resolution, low primer dimer, and low secondary product produced at all primer concentrations tested (Figure 1A).

mFGA: The Agilent 2100 Bioanalyzer electrophoresis results displayed positive results for all FGA primer concentrations. However, the 200 nM concentration showed peaks below our lower confidence limit of 20 FU. Best results were seen at 400 and 600 nM (Figure 1B). There was good peak resolution and low primer dimer product at these concentrations. Secondary product increased with concentration, and at 800 nM, a significant primer dimer and secondary product was seen (See Figure 1B).

mD21S11: No primer concentrations showed positive results with PCR. Due to the negative result at all four primer concentrations using our established laboratory protocol, no further work was done with this primer pair.

For mD16S539 and mFGA, a concentration of 400 nM was judged to be optimal, based on maximal specific product peak heights combined with minimal secondary product formation. This concentration was used in the additional testing performed next.

Testing of previously analyzed DNA samples at optimum primer concentration

The three miniSTR primer pairs were tested against DNA from six forensic samples, 2215-2220, that had been characterized in our laboratory. Our previous results had determined that Sample 2215 and Sample 2219 came from the same source, Sample 2216 and Sample 2218 came from the same source, and Sample 2217 and Sample 2220 came from the same source. The CODIS D16S539 primer set was included for comparison purposes with the miniSTR D16S539 primers. We had previously observed poor peak resolution from the CODIS primer set on the Bioanalyzer due to sizes of the amplicons. Using both primer sets for locus D16S539 in this part of the study allowed a direct comparison.

D16S539: Primers showed results similar to those from the previous analysis. Results showed single peaks for all samples. Product sizes are listed in Table 2. Examination of the electrophoresis results only allowed discrimination of samples 2215, 2216, 2218, and 2219 from samples 2217 and 2220. These results were consistent with our previous results for this locus but inconsistent with the original analysis reported from eight loci without the inclusion of the D16S539 results.

mD16S539: PCR products migrated at sizes somewhat larger, 115-127 bp, than the reported range of 81-121 bp (6). There was no primer dimer present, but secondary product was seen for all samples. These primers provided better peak resolution than the CODIS D16S539 primers presumably due to the smaller size of the products. Samples 2215, 2216, 2218, and 2219 were heterozygous; Samples 2217 and 2220 were homozygous. Peak sizes are listed in Table 2. Overlays of the electrophoresis results showed that Samples 2215 and 2219 had the same pair

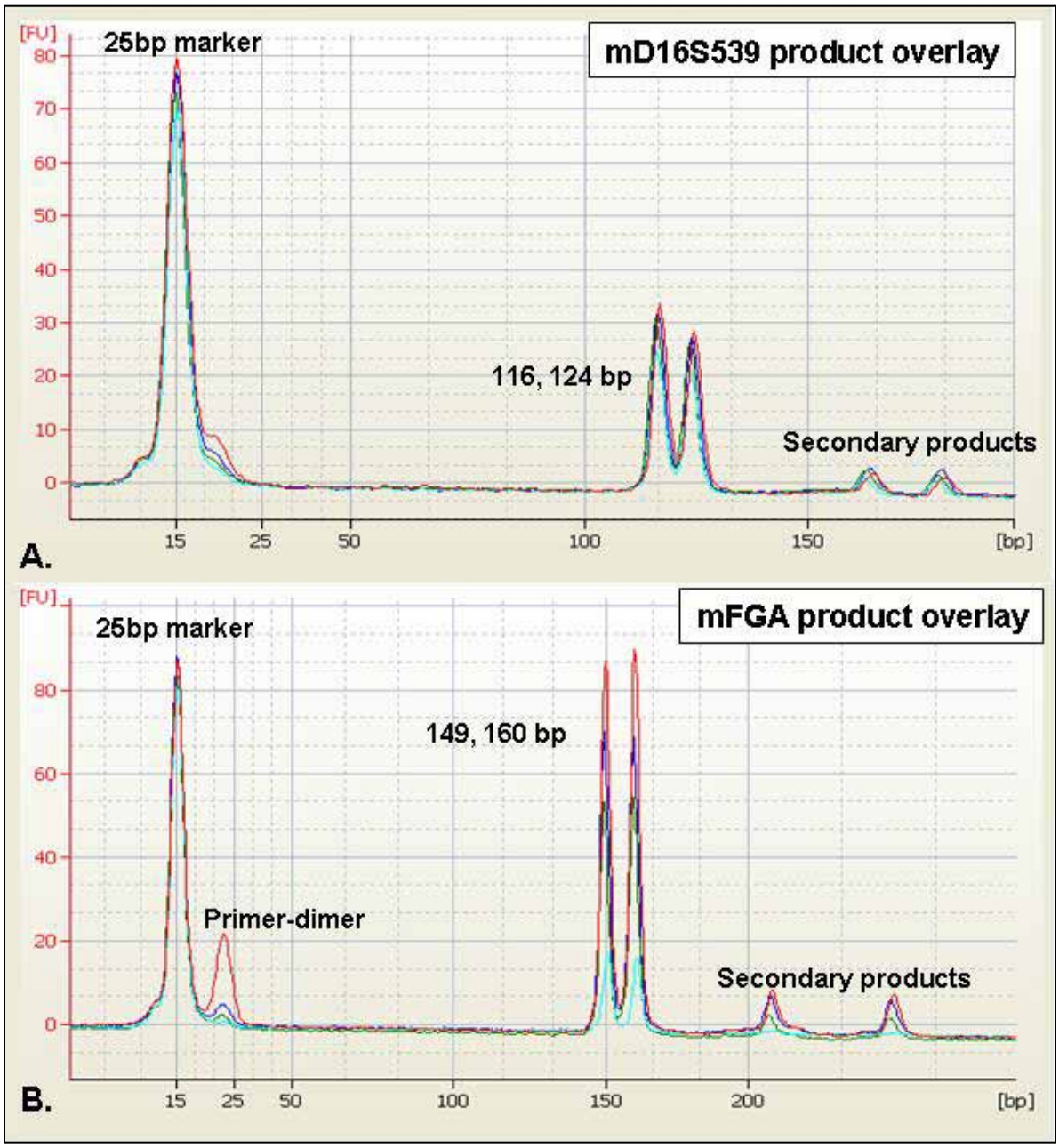

Figure 1. Overlays of PCR products from miniSTR primer titration series using positive control DNA 2221. 800 nM (Red), 600 nM (blue), 400 nM (green), 200 nM (turquoise). FU Fluorescent Units. A. mD16S539 PCR products. B. mFGA PCR products.

Table 2. PCR product sizes for the primer pairs tested with six forensic samples.

Loci	Peaks	Sample 1 2215	Sample 2 2216	Sample 3 2217	Sample 4 2218	Sample 5 2219	Sample 6 2220	Sample Comparison
mFGA	1		147		148			1=5, 2=4, 3=6
	2	154		154		155	155	
	3	160	157		158	162		
	4			168			169	
D16S539	1			290			292	1=2=4=5, 3=6
	2	295	295		296	295		
mD16S539	1	118		118		119	119	1=5, 2=4, 3=6
	2	121	122		123	123		
	3		125		126			

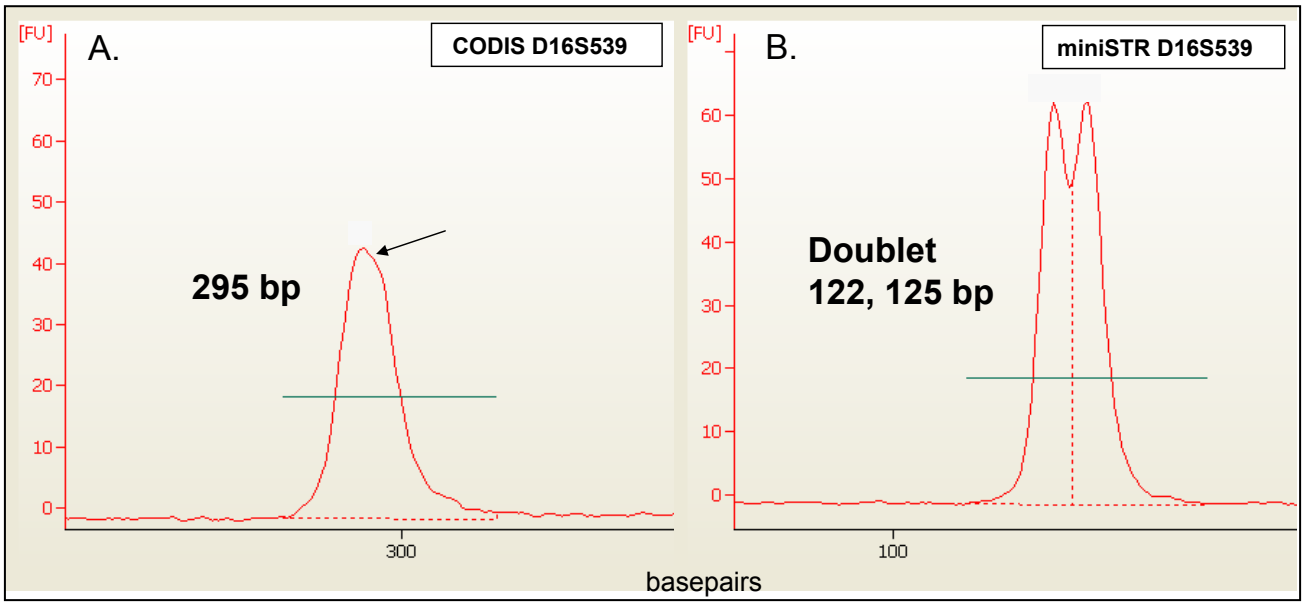

Figure 2. Comparison of CODIS D16S539 and miniSTR D16S539 for Sample 2216. Basepairs (bp). FU Fluorescent Units. A. Results from CODIS D16S539 primers showing a single broad peak with poorly defined doublet (arrow), B. miniSTR D16S539 showing well-defined doublet.

of alleles; Samples 2216 and 2218 shared the same alleles, and Samples 2217 and 2220 shared the same allele (data not shown). This is consistent with previous results for other loci used to test these samples.

Most important was the increased peak resolution for Sample 2216 with the mD16S539 primers. Electrophoresis of product from the CODIS primer pair (Figure 2A) shows a poorly resolved doublet that could be easily interpreted as a single peak from a homozygote. Due to the shorter amplicon lengths from mini-STR primer pair, the doublet is easily resolved and the true result emerges (Figure 2B).

FGA: PCR products were within the reported range of 125 - 281 bp (6) for all samples. Peak resolution and intensity were good for all samples. Very little primer dimer or secondary product was seen in all cases. All samples were heterozygous and showed product sizes as seen in Table 2. After examining the electrophoresis results, it was determined that Samples 2215 and 2219 had the same alleles, Samples 2216 and 2218 had the same alleles, and Samples 2217 and 2220 had the same alleles. This was consistent with previous results, as noted above.

Table 2 shows a summary of the results for this analysis. mD16S539 primers gave products that were better resolved on the Bioanalyzer DNA1000 chip than were the CODIS D16S539 primers and led to the correct interpretation of the sample relationships. While CODIS D16S539 primer products suggested broad homozygous peaks for Samples 2215, 2216, 2218, and 2219, mD16S539 products for the same samples were clearly heterozygous peaks on the same DNA1000 bioanalyzer chip. Consistent results with the mFGA primer set suggested that replacement of the CODIS D16S539 primer pair with the mD16S539 primers and addition

of the mFGA primer pair would dramatically increase the power of discrimination.

Analysis of primers with freshly extracted blood and tissue

DNA was extracted from forensic blood and tissue samples (Materials and Methods) to assure that template newly isolated from degraded forensic samples could be used as template with the two miniSTR primer sets, mFGA and mD16S539. DNA 7500 chip results indicated the tissue DNA was more degraded than blood DNA (See Figure 3). The quality of both samples is consistent with previously observed genomic DNA isolations performed with the method described in Materials and Methods (Data not shown). Both templates were used in PCR with mFGA, mD16S539, and CODIS D16S539 primers.

CODIS D16S539: Results for this analysis indicate good peak intensity (tissue 30 FU, blood 90 FU), with little primer dimer or secondary product (tissue 7 FU and blood 2 FU). Blood DNA showed a homozygous peak at 288 bp and tissue DNA was heterozygous, with peaks at 289 bp and 296 bp.

mD16S539: Primers showed positive results with one peak appearing beyond the expected range of 81 bp-121 bp for blood DNA. Peak intensities were good (tissue 50 FU and blood 145 FU) and no primer dimer was detected. A secondary product was present but was below our significance cutoff of 20 FU (tissue 14 FU and blood 3 FU). Blood DNA was homozygous, with a peak at 118 bp; tissue DNA was heterozygous, with peaks at 118 bp and 126 bp.

mFGA: Primers showed positive results within the expected range of 125- 281 bp for all samples. Peak intensities were acceptable (60 FU for tissue, 80 FU for blood) with little primer dimer present for all samples. However, secondary product was high in blood, slightly above our significance cutoff of 20 FU (8 FU tissue, 24 FU blood). Blood DNA showed heterozygous peaks of 150 bp and 157 bp, and tissue DNA showed heterozygous peaks of 151 bp and 161 bp.

Although results for the CODIS D16S539 primers were the same as the miniSTR primers, mD16S539 results still showed increased peak resolution. Regarding the templates used for this portion of the study, the difference in degradation of the DNA (Figure 3) appeared to have little effect on the PCR outcome except that the signal in tissue was lower than in blood.

There were three concerns with the findings in this study. First, the failure of the mD21S211 primers suggests that none of the primer concentrations used in this study were ideal under the cycling conditions used. Second, the secondary product peaks seen in the mFGA PCR products,

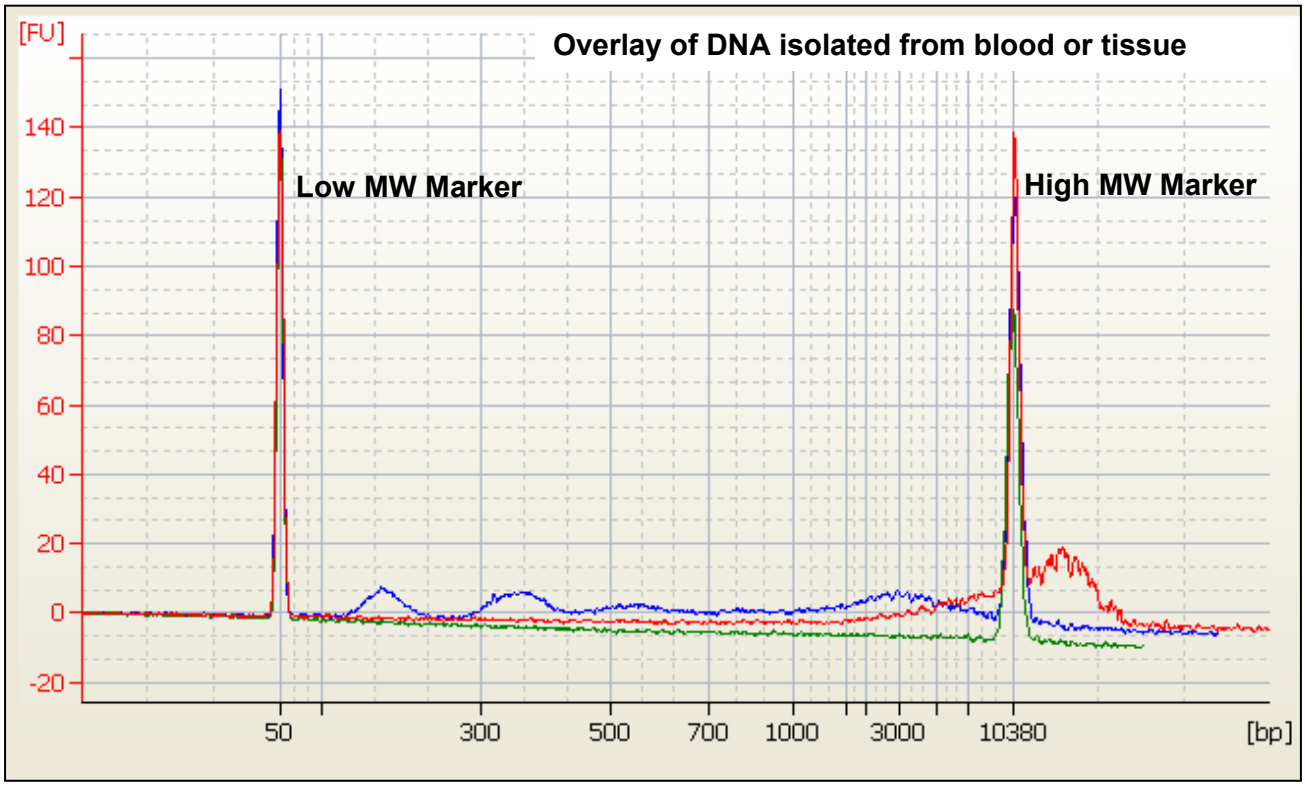

Figure 3. Electropherogram comparing DNA isolates from forensic whole blood (red) and liver (blue) with the liver sample showing overall smaller species. FU Fluorescent Units. Negative control (green).

while not unique to this locus, preclude multiplexing of reactions in a single tube. This is not a significant issue for the single locus analysis we currently use since the expected product size range is known, therefore causing little difficulty determining the primary product. However, in a multiplex reaction, secondary products may fall within the expected size ranges of other primer pairs resulting in multiple peaks, thus making correct data evaluation difficult. The results seen for both mD21S211 and mFGA suggest that additional primer concentration optimization would be useful. Finally, the higher-than-expected product sizes (seven basepairs larger than the published size) we see with the mD16S539 primers in samples 2216 and 2218 are most likely due either to an error in the sizing algorithm or a migration shift resulting from the matrix used with the bioanalyzer. We have seen this to different extents with other CODIS primers; however, because our assay is based on comparative analyses between samples and does not rely on absolute allele identification, this does not interfere with our ability to arrive at valid conclusions.

CONCLUSIONS

miniSTR D16S539 has smaller amplicons and provides dramatically better peak resolution over the CODIS D16S539 PCR products. The improved resolution increases the power of discrimination at this locus because n+1 heterozygotes can now be detected. The example provided here demonstrated that the level of resolution with the CODIS D16S539 amplicon is of such poor quality as to lead to incorrect comparison conclusions. Therefore, the miniSTR version will be substituted into our assay in the future, and miniSTR FGA will also be incorporated into the core set of loci used at CAMI, further increasing the power of discrimination of our assay. Reducing the size of amplicons by using the miniSTR primers proved to be useful in enhancing discrimination, retrieving a locus that had performed poorly in our assay and allowing us to incorporate an additional locus for which the CODIS version had a PCR product beyond the lower limit of detection for n+1 heterozygotes in our assay.

We have recently converted to the use of a robot for setup of our genotyping reactions. With the use of this instrument, the need for uniform primer concentrations in our reactions is decreased, since the increase in complexity of reaction setup can be easily programmed. This suggests to us that in the future, we could explore additional miniSTR primers, including reinvestigating the use of mD21S11.

Additionally, it should be noted that the samples used in this assay are forensic and degraded to various degrees. Smaller amplicons may be particularly beneficial when handling the degraded DNA found in forensic samples. To date, we have not noticed samples which are completely PCR-resistant using our core CODIS set of primers. However, the existence of additional miniSTR primer sets may be useful in the future if we analyze larger numbers of heavily degraded forensic samples.

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