Final Report

The Development of Miniplex Primer Sets for the Analysis of Degraded DNA[1]

NIJ Grant# 2002-IJ-CX-K007

Bruce McCord, (PI) Ohio University[2]

1. Portions of this report were previously published in the thesis of Dr. Denise Chung, Ohio University, 2004 and from papers listed Appendix C.

2. Current address: Department of Chemistry, Florida International University, University Park, Miami, FL 33199, mccordb@fiu.edu

3. This project was carried out at Ohio University and the National Institute of Standards and Technology. The study involved the joint effort of the following individuals: Bruce McCord, PI, John Butler (NIST), Yin Shen, Denise Chung, Kerry Opel, Jiri Drabek, Nancy Tatarek, Lee Meadows Jantz (UTK), Brittany Hartzell, and Kylie Graham. Additional work was also performed at the University of Tennessee-Knoxville.

1. Abstract

In this project we have undertaken a collaborative study with Dr. John Butler of the National Institute of Standards and Technology to develop a new set of multiplexed PCR reactions for the analysis of degraded DNA. These DNA markers, known as Miniplexes, utilize primers that have shorter amplicons for use in STR analysis of degraded DNA. In our work we have defined six new STR multiplexes, each of which consists of 3 to 4 reduced size STR loci, each labeled with a different fluorescent dye. Reductions in size of up to 300 basepairs are possible with these new amplicons.

To demonstrate compatibility with commercial STR kits, a concordance study of 532 DNA samples of Caucasian, African American, and Hispanic origin was undertaken. We achieved 99.77% concordance between allele calls with the two methods. In the 532 samples, there were 15 samples that showed discrepancies at one of 12 loci. These occurred predominantly at 2 loci, vWA and D13S317. DNA sequencing revealed that these samples had deletions between the two primer binding sites. Uncommon deletions like these can be expected in certain samples and will not affect the utility of the Miniplexes as tools for degraded DNA analysis.

Developmental validation to examine sensitivity, peak balance, PCR conditions, environmental contamination and the resolution of mixtures were next carried out. The results showed the miniplexes to perform well at template concentrations above125 pg and show excellent sensitivity for low copy number DNA. Mixtures were fully detected at levels down to 9 to 1 and stutter was under 10 % for all loci.

The Miniplexes were also applied to enzymatically digested DNA to assess their potential in degraded DNA analysis. The results demonstrated a greatly improved efficiency in the analysis of degraded DNA when compared to commercial STR genotyping kits. We have also applied these new primer sets in the analysis of human skeletal remain samples that have been exposed to a variety of environmental conditions. Sixty-four percent of the samples generated full profiles when amplified with the Miniplexes, while only sixteen percent of the samples tested generated full profiles with the Powerplex® 16 commercial kit. In addition, we obtained complete profiles for eleven of the twelve Miniplex loci which had amplicon size ranges less than 200 base pairs. These data clearly demonstrate that smaller PCR amplicons provide an attractive alternative to mitochondrial DNA for forensic analysis of degraded DNA.

Table of Contents page

2. Executive Summary

Introduction

In situations where DNA is highly degraded, poor amplification of the larger sized loci (300-500 base pairs) in standard commercial multiplex typing kits is common. 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 commonly seen, in which the peak height is inversely proportional to the amplicon length. The frequency of allele drop out and stochastic effects is also increased for large sized fragments. This can result in a partial genetic profiles leading to incomplete typing of an individual. Forensic laboratories confronted with this problem move toward mtDNA sequencing. There are 100s of copies of mtDNA in a given cell, and this technique can result in a higher likelihood of producing a result than genotyping with nuclear DNA. Unfortunately because mtDNA are haploid markers, the admixing that occurs with sexual reproduction does not take place and thus the statistics for determining identification are not nearly as definitive as is the case with STRs and nuclear DNA.

In the summer of 2001 we became interested in developing alternatives to mtDNA for the analysis of forensic and anthropological samples. We then discussed this problem with John Butler at NIST who had redesigned a number of STR loci for use with MALDI-TOF mass spectrometry(10). With his assistance, we then began developing multiplex kits containing these redesigned STR loci for use with the ABI 310. It was our hypothesis that by using smaller amplicons, we would be better able to type the degraded DNA that is common in the bone samples we were interested in analyzing. During the fall of that year, following the disaster of 9/11 in New York, we realized that these new STR kits might prove useful in identifying victims

of mass disaster, and we accelerated our work on developing the Miniplexes. This grant was awarded in the summer of the following year to permit us to more fully develop and validate the Miniplex approach.

Miniplex design

The concept behind the Miniplexes approach was to produce a set of CODIS STR primers which produced amplified PCR products as short as possible. In commercial kits this cannot be done as it is necessary to analyze several genetic loci in each dye lane. To avoid overlap in these kits, commercial STR amplicon sizes are expanded to 350 bp or more. In the Miniplex approach this problem is eliminated by placing only one STR amplicon in each dye lane. While this makes it necessary to prepare 3-4 amplifications to cover all 13 CODIS loci, this alternative may produce results for otherwise intractable samples. To produce these shorter STR amplicons primer binding sites were moved inwards towards the location of the core repeats. To avoid problems with primer binding at these new locations, every attempt was made to avoid polymorphic sites in the regions immediately adjacent to the STR repeat regions. However, there was still a danger that mutations could occur between the original primer binding sites and the Miniplex primer binding sites. This situation could result in a lack of concordance between analysis methods and was checked in extensive concordance studies.

In our experimental work, we did not design new primers for the D19S433 locus or the sex-typing marker amelogenin as these markers are already small in the widely used kits. In addition, there is minimal size reduction with the miniSTR primers for D3S1358, D21S11, D8S1179, and D5S818 relative to currently available commercial kits. However, these new primers may prove useful in checking for potential allele dropout due to primer binding site mutations as the new primer positions do not overlap the commercial kit primer locations.

Thus in the initial stages of this work, a set of 5 multiplex PCR reactions were designed. These reactions were referred to as miniplexes. Each miniplex consists of 3-4 different loci, with each locus labeled with a different colored dye. Each of the first four miniplexes can have 3 or 4 different loci depending on whether the user's DNA sequencer supports four fluorescent channels (3 dyes plus an internal standard) or five fluorescent channels (4 dyes plus an internal standard). The fifth multiplex consists of 3 non-CODIS DNA loci which would not have been available in the first four Miniplexes in a four dye sequencer.

Dye Label:	Blue	Green	Yellow
Miniplex 1	TH01	CSF1P0	TPOX
	-105	-191	-148
Miniplex 2	D5S818	D8S1179	D16S539
	-53	-37	-152
Miniplex 3	FGA	D21S11	D7S820
	-71	-33	-117
Miniplex 4	vWA	D18S51	D13S317
	-64	-151	-105
Miniplex 5	Penta D	Penta E	D2S1338
	-282	-299	-198

Table 1E: The Miniplex loci utilized in this study with the difference in size of the amplified MiniSTR compared to commercial multiplex kits. Note that Miniplex 1 and 3 are combined in a single multiplex known as Big Mini.

Table 1E lists the dye colors and locus combinations in the five Miniplex sets created with the primers described in the appendix. Other combinations are possible as all primers were crosschecked against one another in order to assure complete compatibility. As mentioned above, the principle goal in producing these Miniplexes is to keep only one STR locus in each dye lane of the Miniplexes. This permits the location of the new primers to be moved inward without regard to interference from other loci. Interestingly, it was possible to combine two of the Miniplex sets because it is not currently possible to reduce the sizes of Miniplex 3 below

140bp. This combined multiplex was designated BigMini, and is a combination of Miniplex sets 1 and 3.

Experimental work

To test the hypothesis that the size of template DNA affects DNA amplification, a series of experiments were conducted to examine the utility of the miniplexes using enzymatically degraded blood samples. We were also interested in examining the sensitivity and specificity of the amplifications using the different Miniplex loci. In these experiments, DNA extracted from whole blood samples was digested with DNase I for time periods of 2, 5, 10, 20, and 30 minutes. The degraded DNA was then separated by gel electrophoresis and regions of the gel corresponding to different states of degradation were excised, purified and amplified with the Big Miniplex, Miniplex 2, and Miniplex 4. The results were then compared to amplifications using the PowerPlex® 16 system. Consistent genotypes were obtained for all samples amplified with the miniplexes and the commercial kits. Figure 1E shows the results of the analysis of the different size ranges of degraded template DNA using both the miniplex amplifications and PowerPlex® 16 amplifications. The figure reveals amplification efficiency vs the average template size. The early dropout of specific alleles in the amplification of badly ranges of template DNA.plate DNA.es. (nplex

amplifications.

degraded DNA template is clearly seen with the larger PowerPlex® 16 loci. These larger sized loci have much lower intensity and fall below the detection threshold as average template size decreases. Similar behavior was seen withseen at other loci of

other larger amplicons. In contrast, the smaller BigMini STRs showed superior recovery as the average template size was reduced.

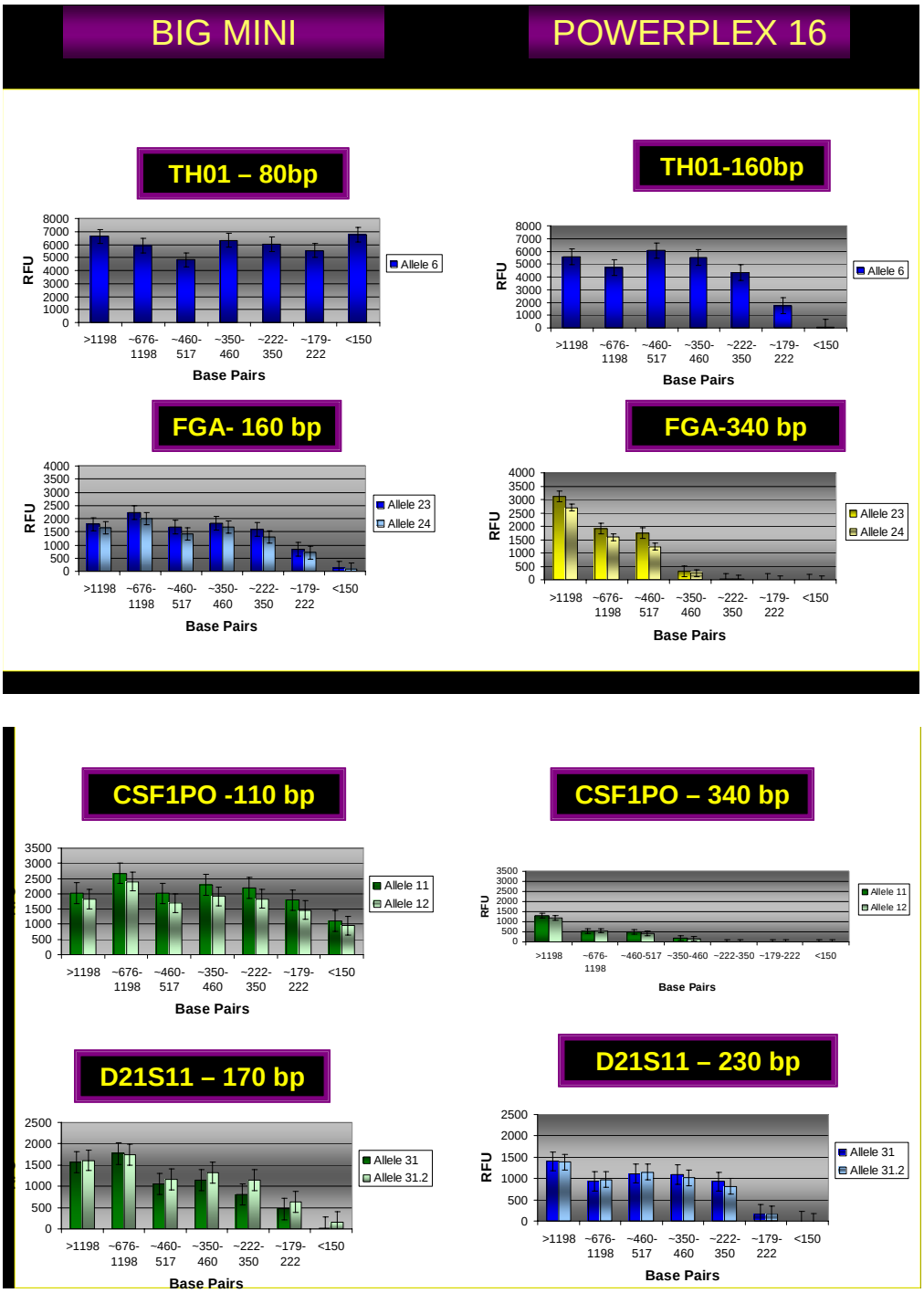


Figure 1E: A comparison of the amplification of different template DNA sizes (isolates from the gel in Figure 2) between the Miniplex kits and the PowerPlex® 16 mulitiplex, The results show the correlation between amplicon size (above each chart) and peak intensity.

Concordance Studies

The next set of experiments evaluated the capability of the Miniplex primer sets to faithfully reproduce results form the standard commercial kits. In these experiments, 532 samples were evaluated by both methods: 208 Caucasian, 212 African American, 110 Hispanic, and 2 Asian individuals. Full concordance was observed in 99.77% (6,369 out of 6,384) STR allele calls compared. The 15 differences seen are listed in Table 2E and encompass the three loci VWA (n=9), D13S317 (n=5), and D5S818 (n=1). The other 9 STR loci, CSF1PO, FGA, TH01, TPOX, D7S820, D8S1179, D16S539, D18S51, and D21S11, were fully concordant at all samples examined in this study.

	Locus	Origin	Miniplex	Identifiler	PP16	Likely Cause
1	D13S317	AA	**11**,13	10,13	10,13	deletion outside of allele 11
2	D13S317	H	**9**,14	8,14	8,14	deletion outside of allele 9
3	D13S317	AA	**10**,11	9,11	9,11	deletion outside of allele 10
4	D13S317	H	**10**,11	9,11	9,11	deletion outside of allele 10
5	D13S317	H	**10**,14	9,14	9,14	deletion outside of allele 10
6	D5S818	AA	11,**11**	11,12	11,12	primer binding site mutation
7	vWA	AA	**16**,16	12,16	12,16	primer binding site mutation
8	vWA	AA	**18**,18	13,18	13,18	primer binding site mutation
9	vWA	AA	**15**,15	14,15	14,15	primer binding site mutation
10	vWA	AA	**15**,15	14,15	14,15	primer binding site mutation
11	vWA	AA	**17**,17	14,17	14,17	primer binding site mutation
12	vWA	AA	**17**,17	14,17	14,17	primer binding site mutation
13	vWA	AA	**19**,19	14,19	14,19	primer binding site mutation
14	vWA	AA	**19**,19	14,19	14,19	primer binding site mutation
15	vWA	AA	**19**,19	14,19	14,19	primer binding site mutation

Table 2E: A list of the 15 Miniplex allele calls out of a total 6384 which were discordant with the Powerplex and Identifiler multiplex kits. Sequencing studies revealed that the allele dropouts of

at vWA were the result of an 8 bp deletion outside of the core repeats which did not affect Identifiler and Powerplex 16 loci primer binding.

A subset of the 15 loci which produced discrepant results was sequenced to determine the reasons for lack of concordance of these alleles. We suspected deletions between the two primer binding sites. DNA sequencing of the D13S317 discrepant samples revealed the presence of a TGTC deletion 24 bases downstream of the core TATC repeat. This deletion is responsible for the allele shifts with the Miniplex primer sets. One Hispanic sample revealed a TATC deletion 12 bases downstream of the core D13S317 STR repeat. The TATC deletion falls within the D13-reverse MiniSTR primer binding region causing allele dropout for this sample. The vWA allele dropouts with MiniSTR primers were caused by an eight-base pair CCATCCAT deletion 10 bases downstream of the core vWA repeat (Figure 5) which falls within the vWA reverse MiniSTR primer binding site. Interestingly, despite the fact that the reverse primer of PowerPlex kits fall within this region, primer binding site problems are not encountered because the adjacent sequence, CCATCTAT, only differs from the deleted CCATCCAT sequence by one base pair (C-T). This eight- base pair deletion actually causes commercial kits to miscount the actual number of alleles. DNA sequencing of the two D5S818 alleles confirmed the presence of polymorphic nucleotides that affect the D5S818 MiniSTR forward primer binding. Overall the sequencing studies revealed an interesting degree of sequence homology at certain primer binding sites. The results also demonstrate that differences between the MiniSTRs and commercial kits are rare and occur mainly in 2 loci, vWA and D13. The mutations we found can affect both commercial and Miniplex kits. However, for the commercial kits, the effect is latent and does not influence the significance of a match.

Developmental Validation Studies

We evaluated several factors that can potentially affect the genotyping results obtained from amplifications with the Miniplex 2, 4, and Big Mini. Because the Miniplexes were designed for the analysis of degraded and compromised samples and since low quantities of DNA template are usually recovered in these situations, most of these studies conducted with the Miniplexes were performed with 100 pg of DNA template per 25 µL of reaction volume (4 pg/µL) and 33 amplification cycles. In these validation studies, experiments were performed to assess the optimum PCR reaction conditions, to determine the sensitivity and specificity of the method, and to examine the effects of environmental contaminants. In general we found that the Miniplex kits showed superior sensitivity to the commercial kits. Miniplexes 2 and 4 were particularly sensitive, easily amplifying samples with template quantities as low as 33 pg. Peak balance was above 60% down to 100 pg of template (with the exception of 2 of the BigMini loci which required larger quantities). During these studies, we also performed a number of experiments to further optimize the results with the BigMiniplex and improve its balance and sensitivity. These experiments are continuing. Experiments with cycle number showed that at 100 pg of template, 33 cycles produced good results with minimal artifacts. Mixture studies showed good results at template ratios ratios of 9 to 1 and greater. Average stutter was below 10% for all 12 loci tested.

Simulated casework

In portion of the study, we extracted naturally degraded DNA from human skeletal remains which had been exposed to a variety of environmental conditions, and amplified the DNA with the newly developed Miniplex primers. We used real time PCR to quantitate the

amount of human DNA present and the PowerPlex® 16 system (100-480 bp amplicon size range) to confirm the extent to which these samples were degraded.

One tibia bone sample and twenty-four femur bone samples from twenty-five individuals were obtained from the Forensic Anthropology Center (FAC) at the University of Tennessee in Knoxville. Six additional femur bone samples from six individuals were obtained from the Franklin County Coroner's Office (FCCO) in Columbus, Ohio.

The above samples were extracted from bone with the assistance of EDTA to decalcify each sample, quantified via real time PCR and amplified using 100pg of template (if available) using Powerplex and Miniplex STR primers. In our results, 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), however, ten of the samples yielded only a partial genetic profile for the larger loci of the Big Mini primer set (125-281 base pairs). Only five of the samples yielded a full profile for the PowerPlex® 16 system (100-480 base pairs). With the signal intensity threshold set at 150 RFU, 13 out of the 25 samples from the Forensic Anthropology Center and 6 out of the 6 samples from the Franklin County Coroner's Office yielded complete profiles for all Miniplex loci. The amplification efficiency per locus in relation to amplicon length is shown in Figure 2E.

Amplification with the PowerPlex® 16 system confirmed that degradation had occurred with these bone samples. Most of the samples yielded complete profiles for the D3S1358, TH01, D5S818, Amelogenin, and vWA loci. 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 as seen in the figure. The loss

of intensity of the larger loci represents a “decay curve”, where the intensity of the larger size fragments is inversely proportional to fragment size in degraded DNA.

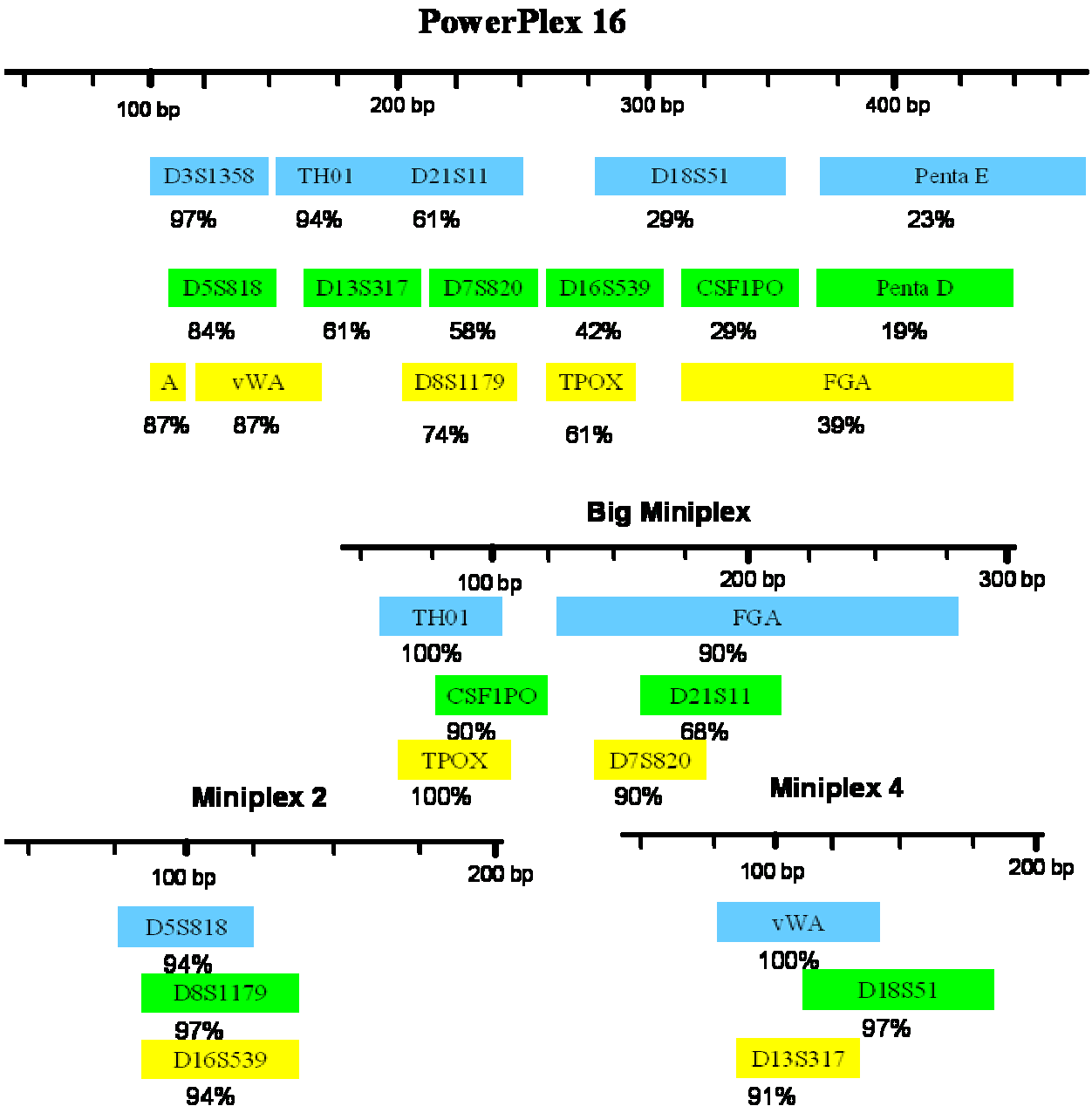


Figure 2E: Amplicon size ranges of the PowerPlex® 16 system and Miniplex primer sets showing percentages of amplification success per locus (n = 31).

The Big Mini and PowerPlex® 16 data suggest that the degradation cut-off length of template fragments predominantly occurs around 200 base pairs and is not kit-dependent.

Overall, the Miniplexes produced results for at least 11 of the 12 loci for all samples in which the quantity of DNA recovered was above 5 pg/µL of extracted DNA. The commercial kit with its larger loci averaged only ten loci for these same samples, with some samples producing as little as three amplified loci. Because only three of the six buried bone samples contained larger quantities of DNA it was difficult to assess the effect of burial (3 samples) versus surface treatment (14 samples). However it is clear from the results that the amount of DNA recovered from similar samples placed on the surface varied widely.

Conclusions

MiniSTRs are CODIS loci which utilize primers designed to keep the amplified samples as short as possible. These new primer sets provide an important new tool for the determination of DNA profiles when only partial genetic profiles are generated from standard kits due to the effects of DNA degradation. We have shown the MiniSTRs to be highly robust and generally concordant with standard STR typing kits. Sensitivity is excellent with peak balance above 60% at 100pg for most loci, and the kits have proven themselves to be superior to commercial DNA typing systems for the analysis of degraded DNA. These redesigned primer sets should have important applications in forensic science for the identification of degraded DNA.

3. Introduction and History

Short tandem repeats (STRs) are genetic loci containing tandemly repeated sequences of DNA 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 (1-4). To perform this analysis, DNA is extracted from blood, bone, or semen, and specific STRs are targeted using short strands of DNA known as primers (5). Amplification then takes place using the polymerase chain reaction and millions of copies of each STR are made, resulting in a highly sensitive and specific result.

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 (6-8). 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 commonly seen, in which the peak height is inversely proportional to the amplicon length. (3,7,9).

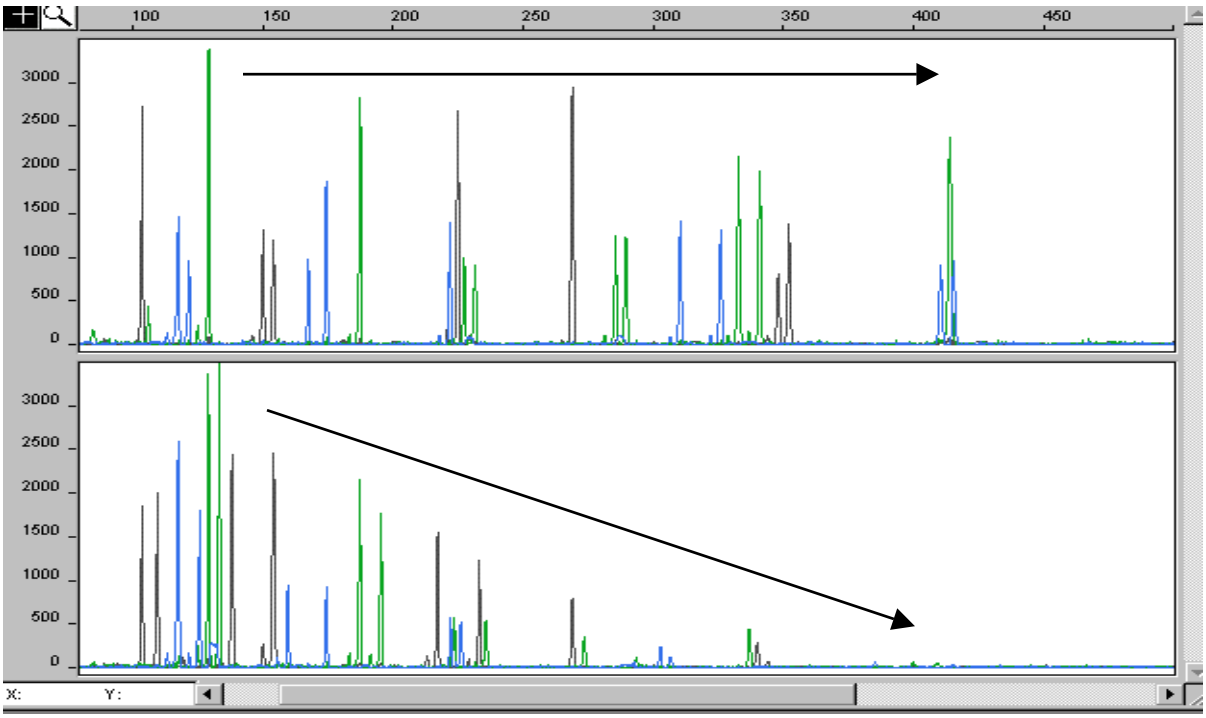


Figure 1: Characteristic ski slope effect seen in the analysis of a degraded bone sample when compared to a control. The upper panel is a control sample, and the lower panel is the amplification of a bone sample. Both samples were amplified using the PowerPlex16 multiplex kit (Promega) and following manufacturers suggested protocols.

The frequency of allele drop out and stochastic effects is also increased. This can result in a partial genetic profile leading to incomplete typing of an individual. Forensic laboratories confronted with this problem move toward mtDNA sequencing. There are 100s of copies of mtDNA in a given cell, and this technique can result in a higher likelihood of producing a result than genotyping with nuclear DNA. Unfortunately because mtDNA are haploid markers, the admixing that occurs with sexual reproduction does not take place and thus the statistics for determining identification are not nearly as definitive as is the case with STRs and nuclear DNA.

In the summer of 2001 we became interested in developing alternatives to mtDNA for the analysis of forensic and anthropological samples. We then discussed this problem with John

Butler at NIST who had redesigned a number of STR loci for use with MALDI-TOF mass spectrometry (10). With his assistance, we then began developing multiplex kits containing these redesigned STR loci for use with the ABI 310. It was our hypothesis that by using smaller amplicons, we would be better able to type the degraded DNA that is common in the bone samples we were interested in analyzing. During the fall of that year, following the disaster of 9/11 in New York, we realized that these new STR kits might prove useful in identifying victims of mass disaster, and we accelerated our work on developing the Miniplexes. This grant was awarded in the summer of the following year.

A number of prior reports have been published supporting the validity of using small amplicons for the analysis of degraded DNA. Primer pairs producing 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 (11). 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 their study, amplified fragment sizes were decreased from 205-253 bp to 125-173 bp (12). Reductions in primer pairs for TH01, D10S2325, DYS319, DYS19 (13), and CSF1PO (14) have also been reported. However none of these workers had attempted to use MiniSTRs to produce a full set of CODIS loci for general genotyping of forensic samples. This was the focus of our project.

4. Development of the Miniplex Approach

The web based Primer3 program (15) was used to design new, shorter primers for each of the CODIS STR loci. To do this the reference sequence for each STR marker found at http://www.cstl.nist.gov/biotech/strbase/seq_ref.htm was pasted into the DNA sequence window of Primer3 and a target region was defined that included the STR repeat region. Typically 80-100 bp was used as a starting point for the desired PCR product size and the melting temperature of each primer was targeted to be in the 57-63 ^{o}C range. Certain primer pairs were used that fell below the 57 ^{o}C design criteria, such as CSF1PO and D16S539 because these primers had been shown to work well empirically during previous time-of-flight mass spectrometry work (10).

For all STRs examined, attempts were made to bring primers as close as possible to the STR repeat region. However, the flanking sequences for some of these STRs contain polymorphic nucleotides that could prevent stable primer annealing. For example, the STR marker FGA has a partial repeat and mononucleotide repeat stretch “TTTC TTCC TTTC TTTTTT” immediately downstream of the core STR repeat. To avoid this region, the 3’end of the MiniSTR FGA-reverse primer was located 23 nucleotides away from the end of the repeat region. A similar rational was used to design the other primers. The primer located furthest away from its marker’s repeat region was the D7S820-reverse primer.

Every attempt was made to avoid polymorphic sites in the regions immediately adjacent to the STR repeat regions. However, there is always a danger with placing primers close to the repeat region if mutations occur between the original primer binding sites and the Miniplex primer binding sites. This situation can result in a lack of concordance between analysis methods. For example, certain D13S317 loci have a four base deletion of TGTC that is 24 bases downstream from the TATC core repeat. This 4 base deletion is located between the Miniplex

primer binding site and the Identifiler primer binding site and will cause a difference in allele call between the two methods (16). We have observed this phenomenon during our concordance study, Section 6.

It is also interesting to note that it is possible to put the 3'end of a primer into the repeat region up to two full repeats and still obtain successful amplifications. Examples in the current set of primers include the TPOX-forward primer and D8S1179 primers, which are both 4 bases into the repeat region (one full repeat).

We did not design new primers for the D19S433 locus or the sex-typing marker amelogenin as these markers are already small in the widely used kits. In addition, there is minimal size reduction with the MiniSTR primers for D3S1358, D21S11, D8S1179, and D5S818 relative to currently available commercial kits. However, these primers may be useful in checking for potential allele dropout due to primer binding site mutations as the new primer positions do not overlap the commercial kit primer locations.

Creating Combinations of MiniSTR Loci

In order to keep all of the MiniSTR loci as short as possible, mulitiplexes were designed with one STR locus in each dye lane. This is in contrast with commercial kits which place 4 or more loci in a single dye lane by adjusting the lengths of each set of amplified products to avoid overlap. Thus in the initial stages of this work, a set of 5 multiplex PCR reactions were designed. These reactions were referred to as Miniplexes. Each Miniplex consists of 3-4 different loci, with each locus labeled with a different colored dye. Each of the first four Miniplexes can have 3 or 4 different loci depending on whether the user's DNA sequencer supports four fluorescent channels (3 dyes plus an internal standard) or five fluorescent channels

(4 dyes plus an internal standard). The fifth multiplex consists of 3 non-CODIS DNA loci which would not have been available in the first four Miniplexes in a four dye sequencer. Two loci, D3S1358 and amelogenin, that are included in CODIS were not utilized in the current 4 dye Miniplex systems. These loci are already smaller in current systems, but could be implemented at a later date along with the non CODIS locus D19S443 in a 6th multiplex set.

Dye Label:	Blue	Green	Yellow
Miniplex 1	TH01	CSF1P0	TPOX
	-105	-191	-148
Miniplex 2	D5S818	D8S1179	D16S539
	-53	-37	-152
Miniplex 3	FGA	D21S11	D7S820
	- 71	-33	-117
Miniplex 4	vWA	D18S51	D13S317
	-64	-151	-105
Miniplex 5	Penta D	Penta E	D2S1338
	-282	-299	-198

Table 1: The Miniplex loci utilized in this study with the difference in size of the amplified MiniSTR compared to commercial multiplex kits. Note that Miniplex 1 and 3 are combined in a single multiplex known as Big Mini,

Table 1 lists the dye colors and locus combinations in the five Miniplex sets created with the primers described in the appendix. Other combinations are possible as all primers were crosschecked against one another in order to assure complete compatibility. As mentioned above, the principle goal in producing these Miniplexes is to keep only one STR locus in each dye lane of the Miniplexes. This permits the location of the new primers to be moved inward without regard to interference from other loci. Interestingly, it was possible to combine two of the Miniplex sets because it is not currently possible to reduce the sizes of Miniplex 3 below 140

bp. This combined multiplex was designated BigMini, and is a combination of Miniplex sets 1 and 3.

5. Experimental Studies with degraded DNA

To test the hypothesis that the size of template DNA affects DNA amplification, a series of experiments was conducted to examine the utility of the Miniplexes using enzymatically degraded blood samples. We were also interested in examining the sensitivity and specificity of the amplifications using the different Miniplex loci.

In the first set of experiments, the effects of degradation were studied on DNA extracted from whole blood samples digested with DNase I. Extracted DNA was incubated with 0.01 Units/µL of DNase I for several time periods: 2, 5, 10, 20, and 30 minutes. The degraded DNA was then separated by gel electrophoresis using 2% agarose and stained with ethidium bromide for detection. Different regions of the gel corresponding to different fragments sizes were excised, purified and amplified with the Big Miniplex, Miniplex 2, and Miniplex 4 (Figure 2).

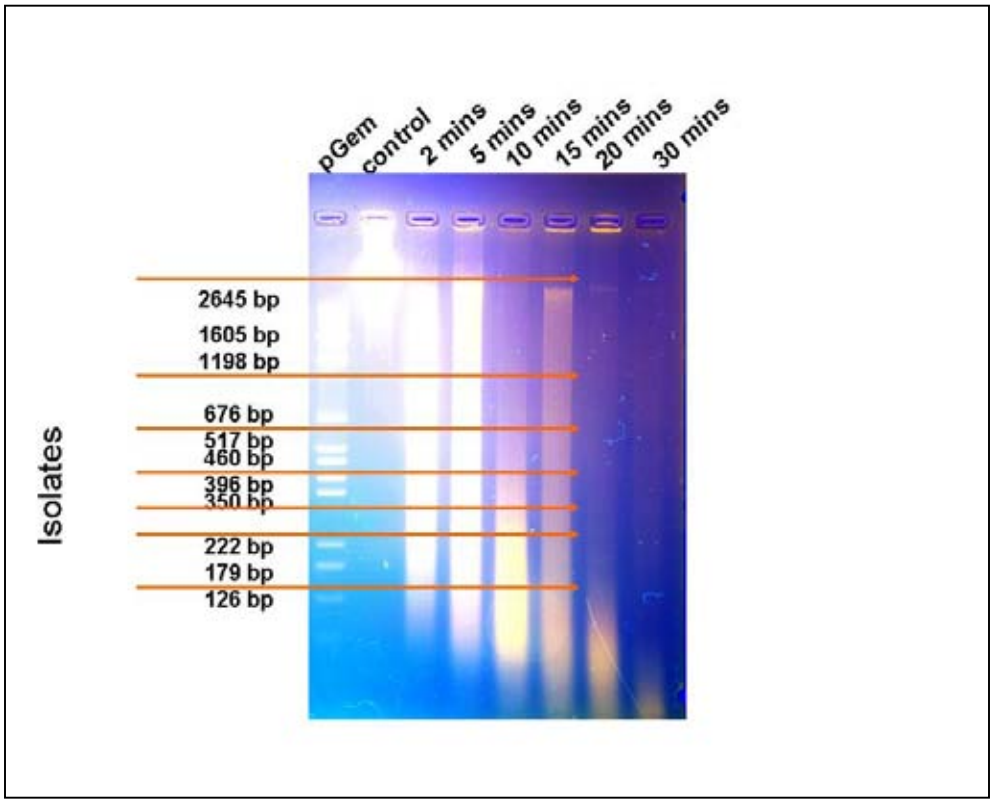


Figure 2: Agarose gel stained with ethidium bromide showing DNA extacted from blood and digested at varying times using DNase I. Isolates were then cut from the gel, quantified, and amplified using Miniplex and PowerPlex® 16 primer sets. The pGem sizing ladder was used to determine the maximum size of each isolated set of DNA template.

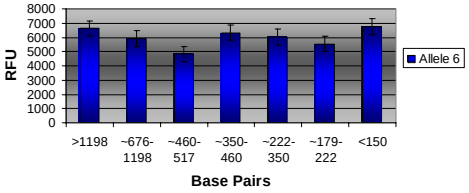
The results were then compared to amplifications using the PowerPlex® 16 system. Consistent genotypes were obtained for all samples amplified with the Miniplexes and the commercial kits. Figure 4 shows the results of the analysis of the different size ranges of degraded template DNA using both the Miniplex amplifications and PowerPlex® 16 amplifications. In the figures, the dropout of specific alleles in the amplification of badly ranges of template DNA.plate DNA.es. (nplex amplifications.

degraded DNA template is clearly seen with the larger PowerPlex® 16 loci. The larger sized loci have much lower intensity and fall below the detection threshold as average template size decreases. Similar behavior was seen withseen at other loci of

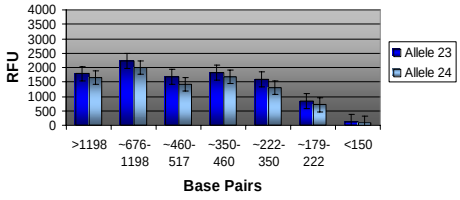
other larger amplicons. For example, results with the PowerPlex® 16 system show that the Penta D and Penta E loci began to drop out at template sizes of ~350-460 base pairs. D18S11, CSF1PO and FGA started to drop out at ~222-350 base pairs followed by D16S539 and TPOX at ~179-222 base pairs. Below 150 base pairs only the smaller sized loci (e.g TH01, D5S818, vWA) were detectable. On the other hand, the Miniplex primer sets were capable of producing complete profiles for all smaller loci even with template sizes below 150 base pairs.

BIG MINI

TH01 – 80bp

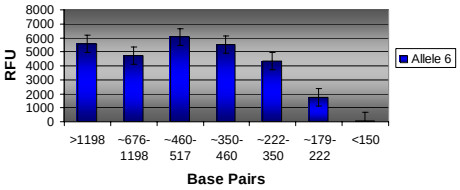


FGA- 160 bp

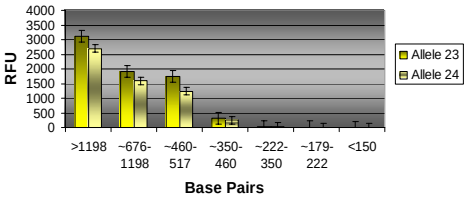


POWERPLEX 16

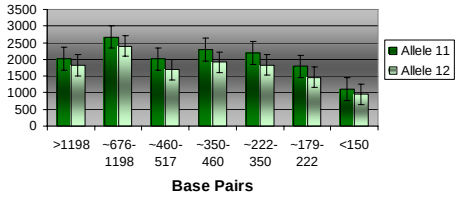
TH01-160bp



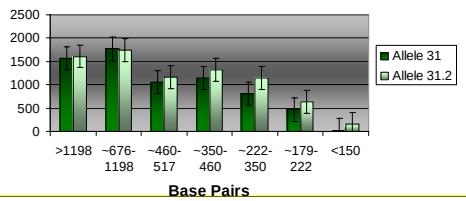
FGA-340 bp



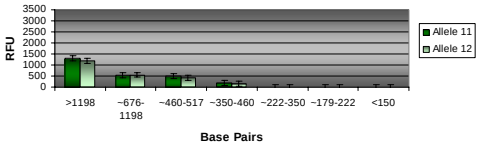
CSF1PO -110 bp



D21S11 – 170 bp



CSF1PO – 340 bp



D21S11 – 230 bp

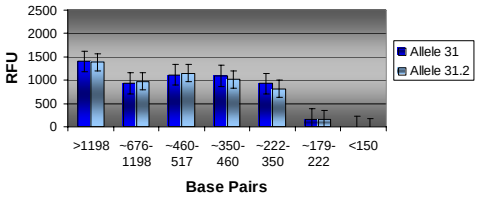


Figure 3: A comparison of the amplification of different template DNA sizes (isolates from the gel in Figure 2) between the Miniplex kits and the PowerPlex® 16 mulitiplex, The results show the correlation between amplicon size (above each chart) and peak intensity.

6. Concordance Studies and Sequencing

A total of 12 STR loci were compared between the single amplification Identifiler kit and the three separate Miniplex sets: "Big Mini" (CSF1PO, FGA, TH01, TPOX, D7S820, D21S11), Miniplex 2 (D5S818, D8S1179, D16S539), and Miniplex 4 (VWA, D13S317, D18S51). 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. PCR amplification was carried out on a GeneAmp® 9700. Note that these conditions involved high levels of template and were used solely for rapid analysis in the concordance studies. Different amplification conditions are necessary to achieve the optimum sensitivity necessary for degraded DNA samples.

532 samples were evaluated by both methods: 208 Caucasian, 212 African American, 110 Hispanic, and 2 Asian individuals. Full concordance was observed in 99.77% (6,369 out of 6,384) STR allele calls compared. The 15 differences seen are listed in Table 2 and encompass the three loci VWA (n=9), D13S317 (n=5), and D5S818 (n=1). The other 9 STR loci, CSF1PO, FGA, TH01, TPOX, D7S820, D8S1179, D16S539, D18S51, and D21S11, were fully concordant at all samples examined in this study. Discrepancies between Identifiler kit and MiniSTR assay primer sets were confirmed by re-amplification of the samples and further testing using the PowerPlex® 16 kit (Promega Corporation, Madison, WI) following manufacturer protocols.

	Locus	Origin	Miniplex	Identifiler	PP16	Likely Cause
1	D13S317	AA	**11**,13	10,13	10,13	deletion outside of allele 11
2	D13S317	H	**9**,14	8,14	8,14	deletion outside of allele 9
3	D13S317	AA	**10**,11	9,11	9,11	deletion outside of allele 10
4	D13S317	H	**10**,11	9,11	9,11	deletion outside of allele 10
5	D13S317	H	**10**,14	9,14	9,14	deletion outside of allele 10
6	D5S818	AA	11,**11**	11,12	11,12	primer binding site mutation
7	vWA	AA	**16**,16	12,16	12,16	primer binding site mutation
8	vWA	AA	**18**,18	13,18	13,18	primer binding site mutation
9	vWA	AA	**15**,15	14,15	14,15	primer binding site mutation
10	vWA	AA	**15**,15	14,15	14,15	primer binding site mutation
11	vWA	AA	**17**,17	14,17	14,17	primer binding site mutation
12	vWA	AA	**17**,17	14,17	14,17	primer binding site mutation
13	vWA	AA	**19**,19	14,19	14,19	primer binding site mutation
14	vWA	AA	**19**,19	14,19	14,19	primer binding site mutation
15	vWA	AA	**19**,19	14,19	14,19	primer binding site mutation

Table 2: A list of the 15 Miniplex allele calls out of a total 6384 which were discordant with the Powerplex and Identifiler multiplex kits. Sequencing studies revealed that the allele dropouts of

at vWA were the result of an 8 bp deletion outside of the core repeats which did not affect Identifiler and Powerplex 16 loci primer binding.

Sequencing Studies

A subset of the 15 loci which produced discrepant results was sequenced to determine the reasons for lack of concordance of these alleles. We suspected deletions between the two primer binding sites. DNA sequencing of the D13S317 discrepant samples revealed the presence of a TGTC deletion 24 bases downstream of the core TATC repeat (Figure 4). This deletion is responsible for the allele shifts with the Miniplex primer sets (9),(16). One Hispanic sample

revealed a TATC deletion 12 bases downstream of the core D13S317 STR repeat. The TATC deletion falls within the D13-reverse MiniSTR primer binding region causing allele dropout for this sample (Figure 4). The vWA allele dropouts with MiniSTR primers were caused by an eight-base pair CCATCCAT deletion 10 bases downstream of the core vWA repeat (Figure 5) which falls within the vWA reverse MiniSTR primer binding site. Interestingly, despite the fact that the reverse primer of PowerPlex® 16 kits fall within this region, primer binding site problems are not encountered because the adjacent sequence, CCATCTAT, only differs from the deleted CCATCCAT sequence by one base pair (C-T) (Figure 6).

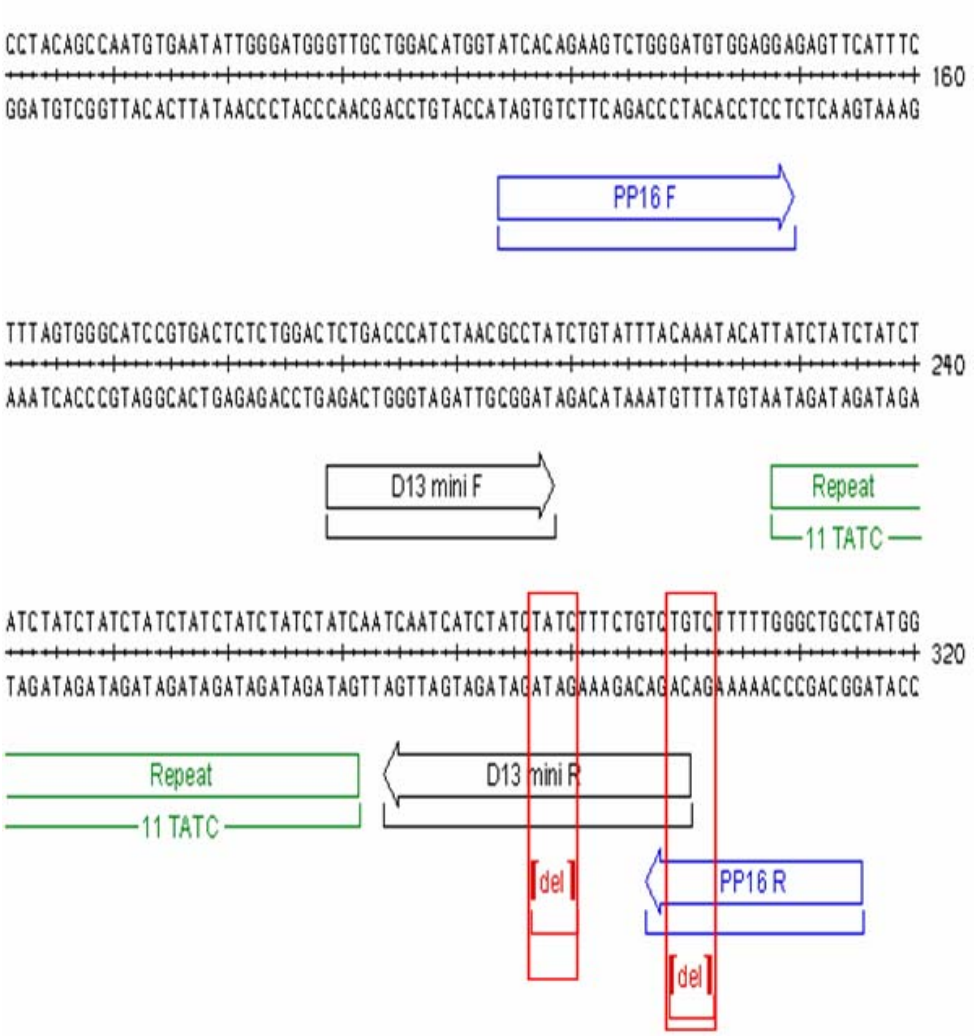


Figure 4: Four base TGTC and TATC deletion at the D13S317 locus. The TGTC deletion is found 24 bases downstream of the core TATC repeat. The TGTC deletion is responsible for the allele shifts observed with the D13S317 Miniplex primer. The TATC deletion is found 12 bases downstream of the core D13S317 STR repeat. The TATC deletion falls within the D13-reverse MiniSTR primer binding region causing allele dropout for the Hispanic sample.

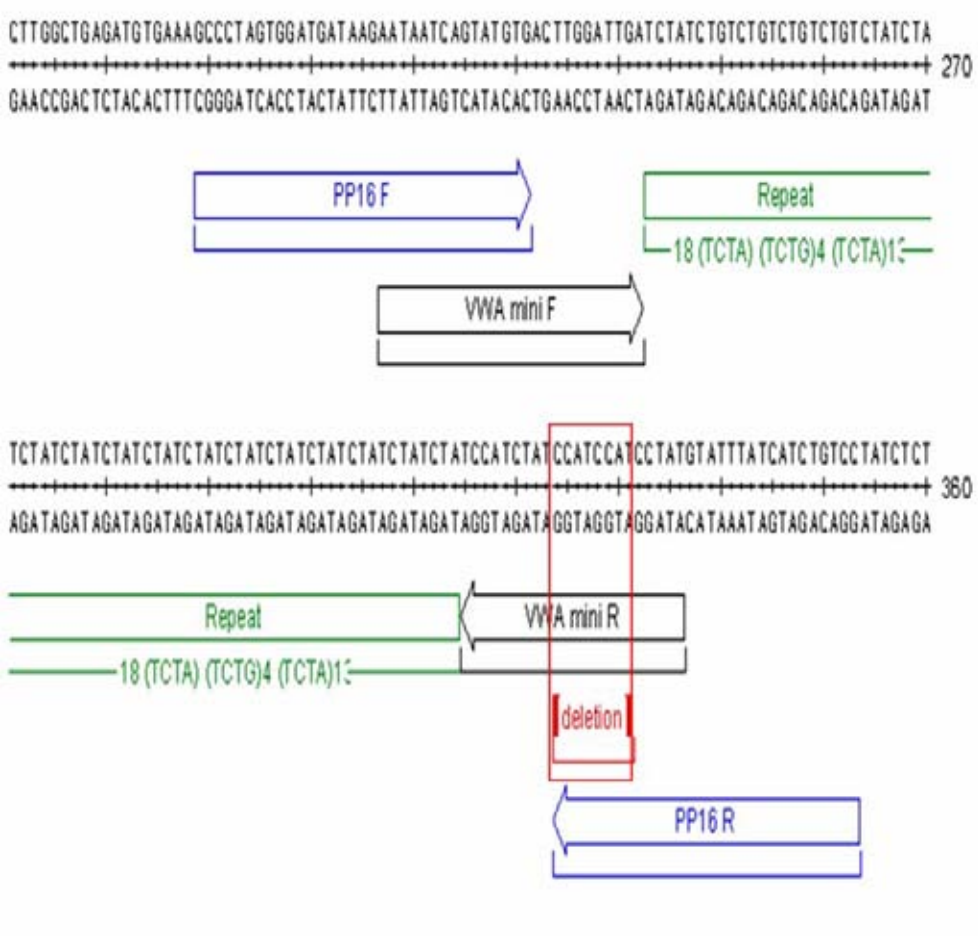


Figure 5: Eight base pair CCATCCAT deletion in the vWA locus**. The deletion is found 10 bases downstream of the core vWA repeat and falls within the vWA-reverse MiniSTR primer binding site.**

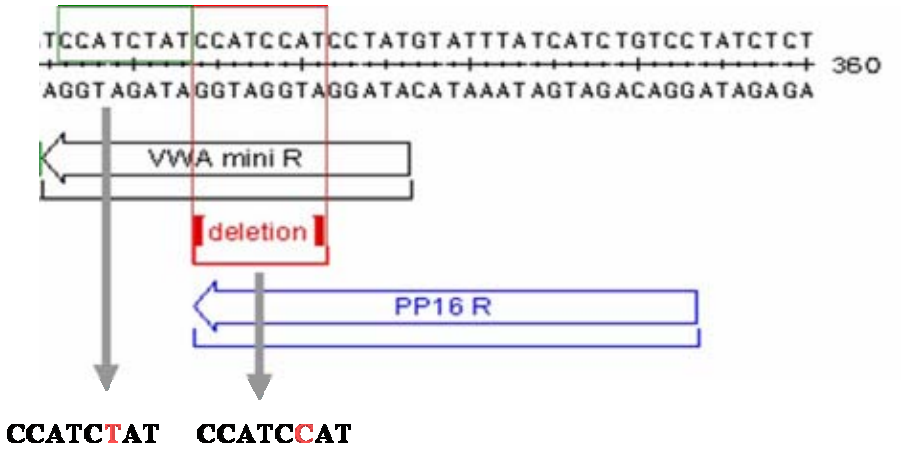


Figure 6: A closer look at the eight base pair deletion in the vWA locus. **Primer binding site problems are not encountered with the PowerPlex® 16 primers because the adjacent sequence, CCATCTAT, only differs from the deleted CCATCCAT sequence by one base pair (C-T). This deletion, however, causes the commercial kit to miscount the actual number of alleles.**

This eight- base pair deletion causes commercial kits to miscount the actual number of alleles. For example, a sample actually having 14 core repeats will be typed as a 12 because of this eight- base pair deletion. The mutation in the vWA MiniSTR reverse primer binding region could possibly be the same mutation that was previously reported by Lazaruk et al (17). DNA sequencing of the two D5S818 alleles of the African American sample confirmed the presence of two polymorphic nucleotides that affect the D5S818 MiniSTR forward primer binding. These polymorphic nucleotides may also responsible for the heterozygote peak height imbalance observed for some samples. Interestingly, sequencing results for one of the D5S818 alleles showed a four-base pair AGAG deletion immediately adjacent to the core STR repeat. This deletion impacts the D5S818 reverse Miniplex primer which led to the allele dropout of the African American sample. The forward and reverse D5S818 MiniSTR primers have now been moved farther away from the repeat to avoid these polymorphisms. The primer sequences for the new D5S818 MiniSTR primers are forward 5'-GGGTGATTTTCCTCTTTGGT-3' and reverse 5'- AACATTTGTATCTTTATCTGTATCCTTATTTAT-3'.

7. Validation Studies

The developmental validation of newly designed methods for DNA analysis is an integral process used by the scientific community prior to the adoption of the method by any other laboratory. We have begun developmental validation studies of the Miniplex primer sets in accordance with the Technical Working Group on DNA Analysis Methods (TWGDAM) guidelines to evaluate the performance of the Miniplex primer sets and to examine the different parameters that will affect the results withof the Miniplex primer sets,

forensic samples. These validation studies were also be used to examine the robustness of the Miniplex primer sets in typing compromised forensic samples.

In the subsequent sections, validation studies on Miniplex primer sets 2, 4, and Big Mini will be presented. Validation studies on Miniplex 5 have not been performed because non-specific binding problems have been encountered with the Penta E locus at low DNA template concentrations (Figure 7). This non-specific binding observed with the Penta E primers could be attributed to the potential binding of the redesigned forward and reverse Penta E primers to compatible sequences in the human genome as shown from BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) and BLAT (http://genome.ucsc.edu) results.

Several factors such as the instrumentation, reaction and analysis conditions, performance of the analyst, quality and the quantity of the DNA template can have variable effects on the outcome of results from PCR-based STR assays. In this study, we

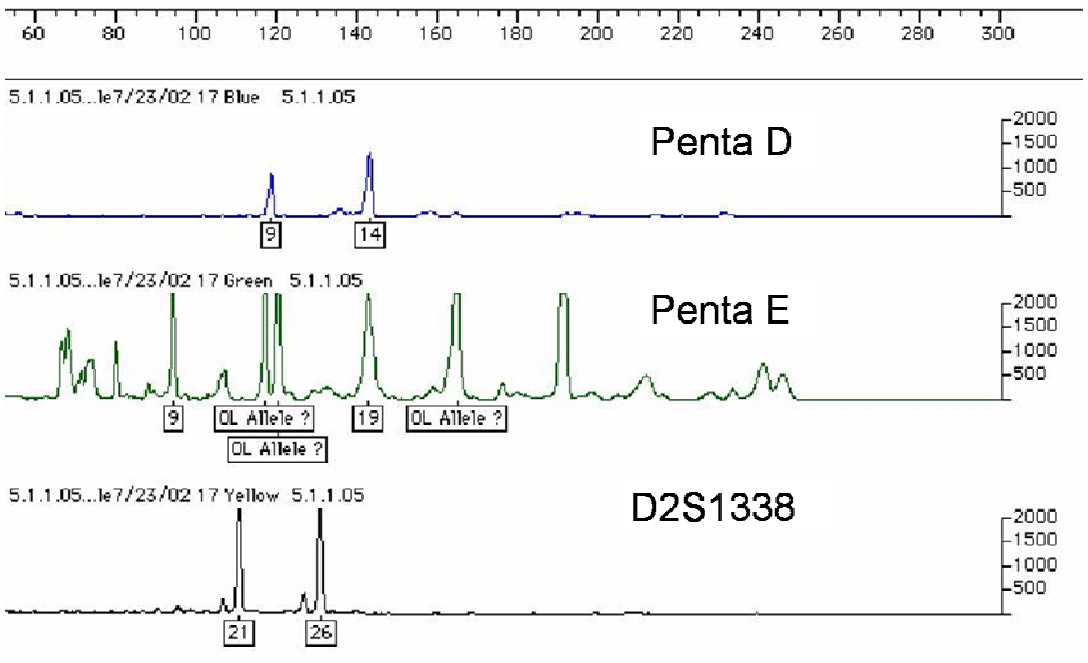


Figure 7. Non-specific binding observed at the Penta E locus of Miniplex 5. This non-specific binding could be attributed to the binding of both the re-designed forward and reverse Penta E primers to other sequences in the human genome.

evaluated several factors that can potentially affect the genotyping results obtained from amplifications with the Miniplex 2, 4, and Big Mini. Because the Miniplexes were designed for the analysis of degraded and compromised samples and since low quantities of DNA template are usually recovered in these situations, most of the studies conducted with the Miniplexes were performed with 100 pg of DNA template per 25 µL of reaction volume (4 pg/µL) and 33 amplification cycles.

Standard Specimens and Reproducibility

The standard specimen studies were conducted to ensure that fresh body fluid samples obtained and stored in a controlled manner from the same donor will produce the same genotype result. Similarly, reproducibility studies ensure that stain and liquid specimen samples would yield the same genotype. For this study, fresh (no EDTA) blood was spotted on unbleached white cotton cloth and on FTA cards. Saliva was also collected from the same individual and applied to an FTA indicator card. These three specimens were amplified with Miniplex 2, Miniplex 4, and Big Mini and consistent genotypes were obtained for all.

Genotype consistency was also tested between samples that have been stored for a year and samples that have been freshly obtained. In this study, EDTA treated blood samples were spotted on an unbleached white cotton cloth and FTA card and stored at room temperature for one year. Fresh saliva from the same individuals were then obtained and applied to FTA indicator cards a few days before the blood samples were amplified to compare the genotype results of fresh samples to those that have been stored for one year. In both cases, all three Miniplex primer sets gave consistent genotypes.

Stability studies were conducted between fresh samples and samples that have been stored for two years. In this study, EDTA treated blood was spotted on an unbleached white

cotton cloth. Some of the stains were extracted and amplified prior to storage at room temperature for two years. After two years, the remaining stains were again extracted and amplified. In both cases, all three Miniplex primer sets gave consistent genotypes.

For these studies, three different extraction methods were used. The phenol/ chloroform method was used for blood stains, DNAzol method was used for blood spotted FTA cards, and the standard FTA extraction protocol for saliva specimens. Using these different extraction methods did not affect the genotype results obtained.

Sensitivity Studies

In most situations, the DNA template recovered from the crime scene is not only degraded but the concentration of the DNA template recovered is also low. We anticipated that shortening the PCR amplicon size would improve the amplification efficiency of samples where the DNA template is present in low copy numbers. Most manufacturers of STR multiplex kits set the lower limit of sensitivity in the region of 250pg, and most multiplex kits have their optimum efficiency when 1 ng of DNA is amplified (18),(19). Because these Miniplex primer sets were designed for the analysis of highly degraded samples where only low concentrations of template are present, we explored the limits of sensitivity for Miniplex 2, 4, and Big Mini. We amplified samples with DNA concentrations ranging from 31pg to 500pg in 25 µL reaction volumes. These concentrations are below the range recommended for commercial sets. Correct genotypes were obtained at concentrations as low as 31pg/ 25 µL 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 dropout for Miniplex 4 (n=20). At 63 pg// 25 µL, 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 31pg/ 25 µL.

At 63 pg/ 25 µL, 50% of the samples (n=14) still showed allele dropout for the CSF1PO, D21S11, and D7S820 loci. Based on our results, we found template concentrations above 100pg/ 25 µL work well for Miniplex 2 (Figure 8) and Miniplex 4 (Figure 9). 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 set, template concentrations greater than 250 pg/ 25 µL (Figure 10) are needed to avoid allele dropout. As will be discussed in the next section, we conducted experiments to optimize the primer concentrations of the Big Mini multiplex set to make it work for samples with 100 pg/ 25 µL of DNA template. This corresponds to approximately 30 copies of DNA template, or 15 cells (20).

The sensitivity we have achieved with the Miniplex primer sets is better than what was reported in the validation studies of other multiplex sets (21),(22). However, it is important to note that 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 Studies

When the amount of template added to the PCR reaction is extremely low, the amplification of heterozygote alleles may be imbalanced due to stochastic effects. One

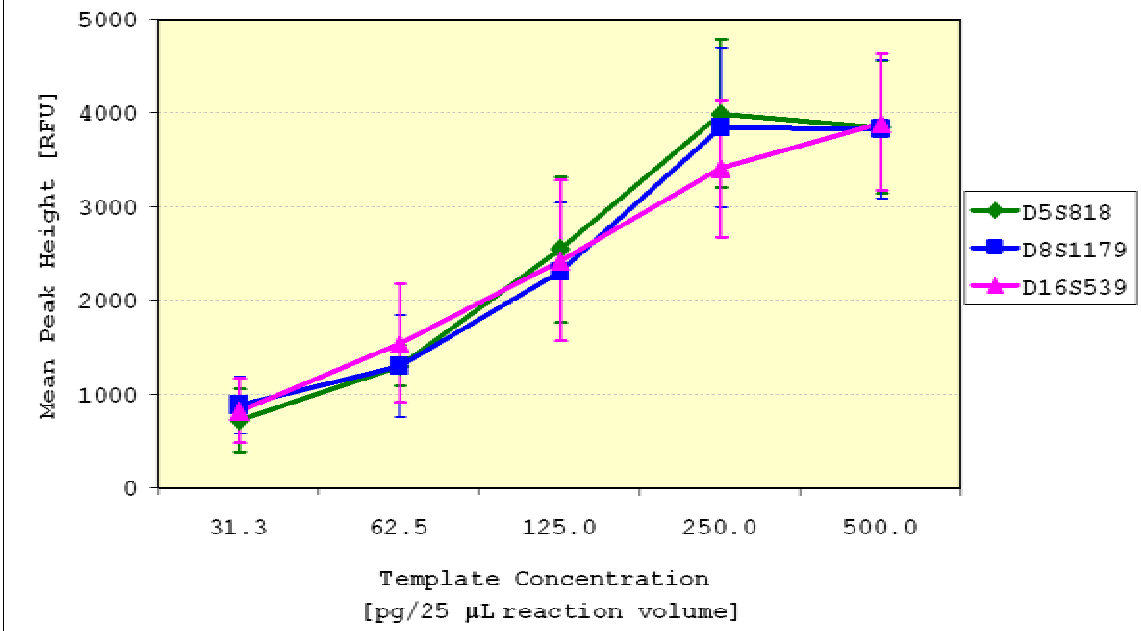


Figure 8. Sensitivity studies for Miniplex 2. The change in fluorescence signal intensity as a function of template concentration is shown for D5S818, D8S1179, and D16S539. Primer concentrations used were 0.4 µM, 0.4 µM, and 0.2 µM for D5S818, D8S1179, and D16S539, respectively. All samples were amplified at 33 cycles. Template concentrations greater than 100 pg / 25µL are optimal for this set at these conditions. (n=12)

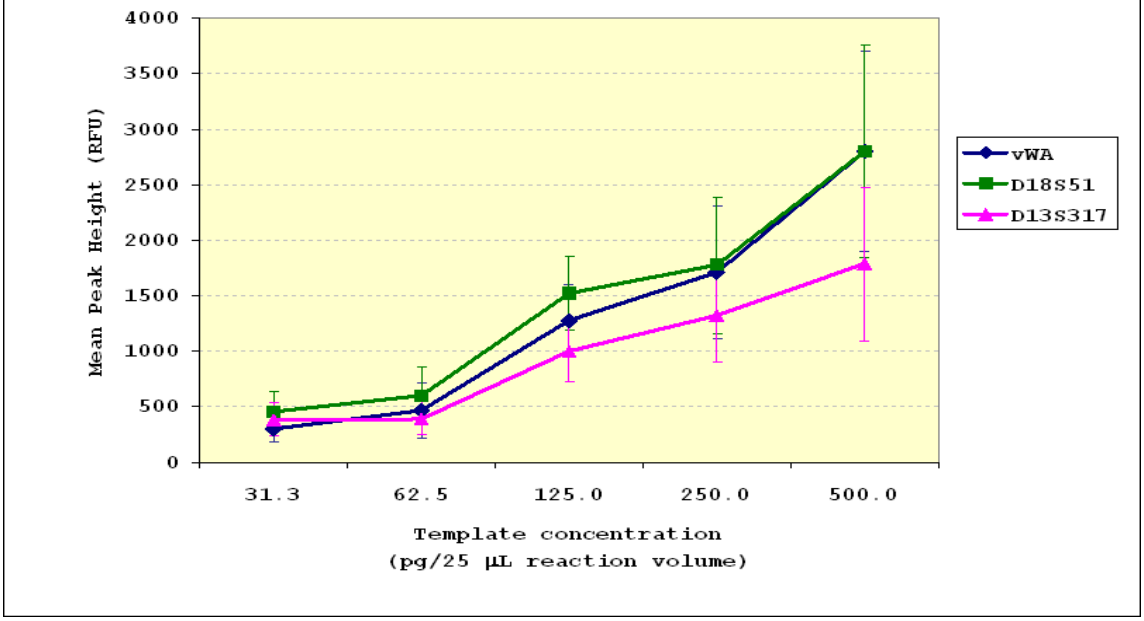


Figure 9. Sensitivity studies for Miniplex 4. The change in fluorescence signal intensity as a function of template concentration is shown for vWA, D18S51, and D13S317. Primer concentration used was 0.4 µM for vWA, D18S51, and D13S317. All samples were amplified at 33 cycles. Template concentrations greater than 100 pg / 25µL are optimal for this set at these conditions. (n=20)

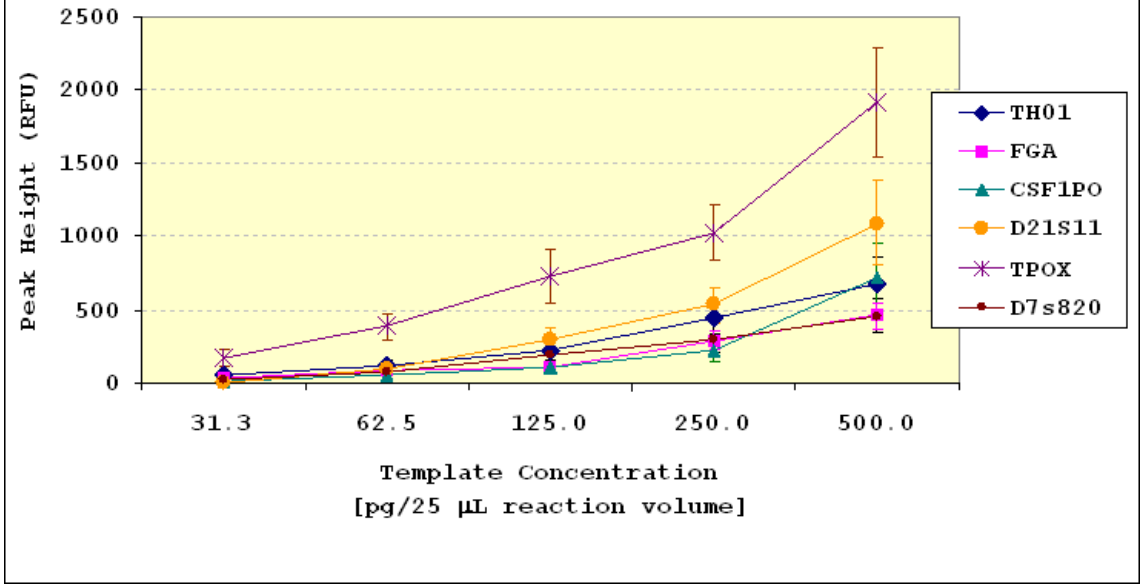


Figure 10. Sensitivity studies for Big Mini. The change in fluorescence signal intensity as a function of template concentration is shown for TH01, CSF1PO, TPOX, FGA, D21S11, and D7S820. Primer concentrations used were 0.2 µM for TH01, TPOX, FGA, 0.3 µM D21S11, D7S820, and 0.12 µM CSF1PO. All samples were amplified at 33 cycles. Template concentrations greater than 250 pg / 25µL are optimal for this set at these conditions. (n=14)

allele can be preferentially amplified over the other due to unequal sampling of heterozygote alleles during the early stages of the PCR reaction (23). Since an important requirement for accurate genotyping is to produce balanced allele peaks, we calculated the heterozygous 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 (≥ 60% ratio) were obtained at concentrations greater than 100 pg/ 25 µL for Miniplex 2 (Figure 11) and Miniplex 4 (Figure 12). For the Big Mini, good peak balance for all loci was achieved at 250 pg/ 25 µL of template (Figure 13). At DNA concentrations less than 125 pg/ 25 µL, CSF1PO and D21S11 become highly imbalanced (0.21 and 0.41, respectively). The peak balance ratio at 100 pg/ 25 µL of DNA template for the Big Mini multiplex set was improved by varying the primer concentrations as will be discussed in the next section. Although samples with less than 100 pg/ 25 µL of DNA template have been amplified efficiently with these multiplex sets, stochastic effects can be seen with some samples.

Primer Concentration

Changing the primer concentration of one locus can affect the way the other loci are amplified. Because the efficiency of synthesis and labeling of primers is different for every batch, primer concentrations must be optimized, accordingly. The difference in the DNA sequence for each locus is also responsible for the variation in the

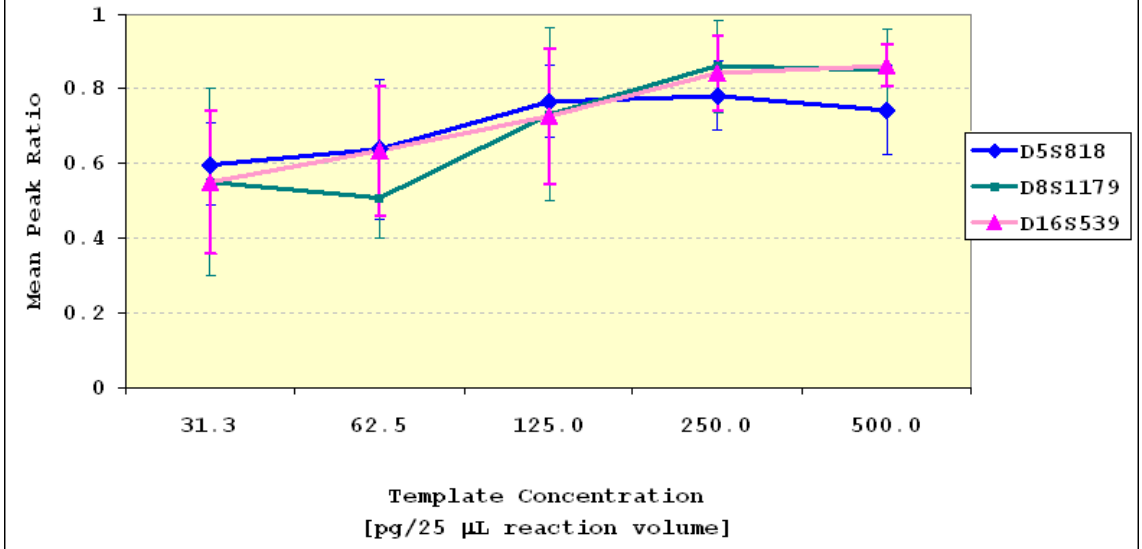


Figure 11. Peak balance ratio for Miniplex 2. The mean peak balance ratio for D5S818, D8S1179, and D16S539 is plotted as a function on template concentration. Primer concentrations used were 0.4 µM, 0.4 µM, and 0.2 µM for D5S818, D8S1179, and D16S539, respectively. All samples were amplified at 33 cycles. Template concentrations greater than 100 pg / 25µL gave the best peak balance ratio for this set at these conditions. (n=12)

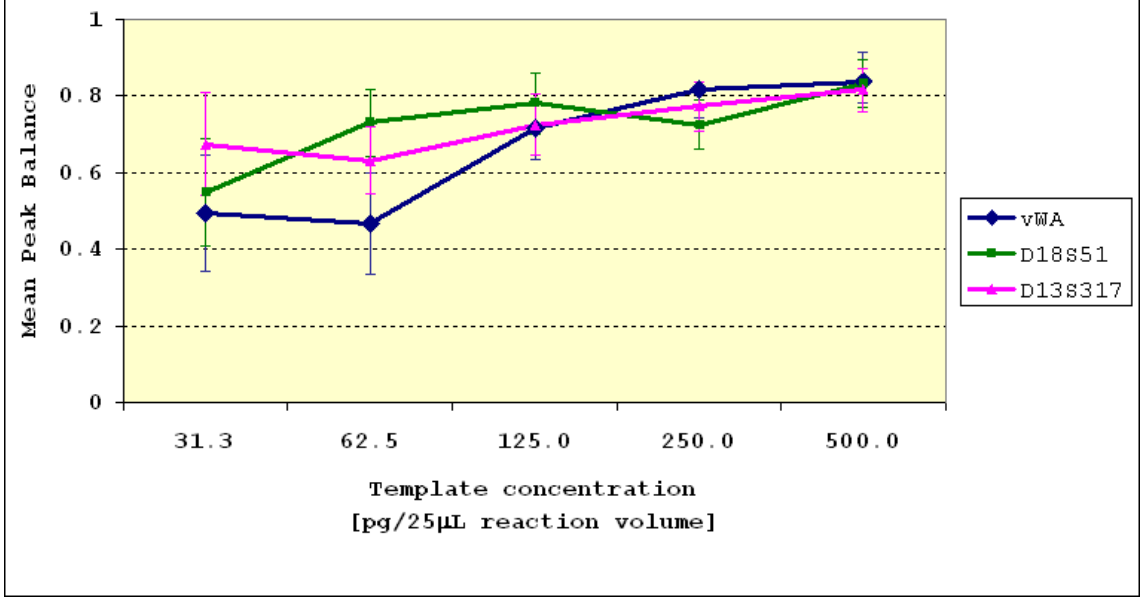


Figure 12. Peak balance ratio for Miniplex 4. The mean peak balance ratio for vWA, D18S51, and D13S317 is plotted as a function on template concentration. Primer concentration used was 0.4 µM for vWA, D18S51, and D13S317. All samples were amplified at 33 cycles. Template concentrations greater than 100 pg / 25µL gave the best peak balance ratio for this set at these conditions. (n=20)

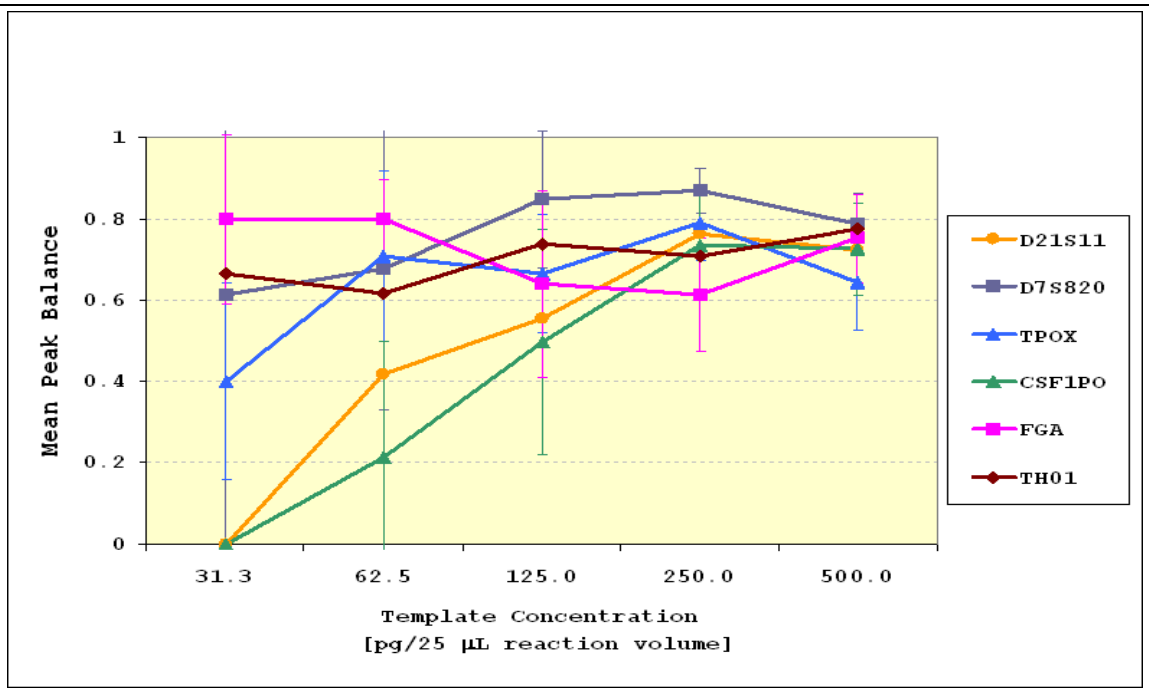


Figure 13. Peak balance ratio for Big Mini. The mean peak balance ratio for TH01, CSF1PO, TPOX, FGA, D21S11, and D7S820 is plotted as a function on template concentration. Primer concentrations used were 0.2 µM for TH01, TPOX, FGA, 0.3 µM D21S11, D7S820, and 0.12 µM CSF1PO. All samples were amplified at 33 cycles. Template concentrations greater than 250 pg / 25µL gave the best peak balance ratio for this set at these conditions. (n=14)

efficiency of primer binding. Thus, it becomes necessary to adjust the primer concentrations to obtain the best ratio that would yield good signal intensities and balanced peak height for each multiplex set. The primer concentrations were optimized to efficiently amplify DNA samples with quantities as low as 100 pg/ 25 µL of template. Optimal primer concentrations for the Miniplex 2 set are 0.4 µM, 0.4 µM, and 0.2 µM for the D5S818, D8S1179, and D16S539 loci, respectively. For the Miniplex 4 set, 0.4 µM vWA, 0.4 µM D18S51, and 0.56 µM D13S317 efficiently amplified DNA samples at 100 pg/ 25 µL. For the Big Mini set, the initial primer concentrations used were 0.2 µM TH01, TPOX, FGA, 0.3 µM D21S11, D7S820 and 0.12 µM CSF1PO. As seen from the results of the previous section, the Big Mini set has poor sensitivity and poor peak balance ratio for some loci at 100 pg/ 25 µL of DNA concentration.

We tried to improve the sensitivity and peak balance ratio of the problematic loci (FGA, CSF1PO, and D21S11) in Big Mini by varying their primer concentrations. A factorial design was used to find the optimum region of primer concentration.

We started by testing three loci, CSF1PO, D21S11, and D7S820 while keeping the other three loci, TH01, TPOX, and FGA at a fixed primer concentration of 0.16 µM, 0.2 µM and 0.3 µM respectively. A 2^3 factorial design was used. Based from the results of the factorial design, most of the samples that gave the best results had primer concentrations of 0.20 µM CSF1PO, 0.3 µM D21S11, and 0.35 µM D7S820. After the initial factorial design test, we still further optimized the primer concentrations by performing titrations of different primer concentrations around the optimum region. Optimal results for the Big Mini set was achieved at 0.16 µM TH01, 0.16 µM CSF1PO, 0.2 µM TPOX, 0.24 µM FGA, 0.24 µM D21S11, and 0.32 µM D7S820.

Table 3: Optimal primer concentrations for each Miniplex locus when using 33 cycles for amplification.

Miniplex Locus	Primer Concentration
D5S818	0.4 µM
D8S1179	0.4 µM
D16S539	0.2 µM
vWA	0.4 µM
D18S51	0.4 µM
D13S317	0.56 µM
TH01	0.16 µM
CSF1PO	0.16 µM
TPOX	0.2 µM
FGA	0.24 µM
D21S11	0.24 µM
D7S820	0.32 µM

when amplifying samples with 100 pg/ 25 µL of DNA template. These primer concentrations also work well for amplification of samples with higher quantities of DNA template. Table 3 shows the optimal primer concentration for each Miniplex locus at 33 amplification cycles.

Cycle Number Study

One way to improve the sensitivity of any PCR based method is to increase the number of amplification cycles. The improvement in sensitivity obtained by using an increased number of cycles allows for a wider range of evidence types to be analyzed. For example epithelial cells obtained after strangulation have been analyzed using 30-31 cycles of PCR(24). Fingerprints and hair shafts without the root have also been analyzed using increased cycle numbers (25). In

addition, forensic anthropologists have routinely used increased PCR cycle numbers to obtain profiles from ancient DNA. Gill et al. have used 38-43 PCR cycles to analyze STRs from 70-year- old bone samples from the Romanov family (26). Other authors have reported using 50 and 60 PCR cycles to analyze STRs from thousand years old bone samples (27,28). Samples with less than 100 pg of DNA template have been routinely analyzed with both SGM and AmpF*l*STR® SGM Plus™ using 34 cycles (29). However, in our experiments, further increases in the cycle number did not improve sensitivity but instead led to artifact production.

To test the effect of cycle number on the sensitivity of the Miniplex primer sets, we have amplified 100- 500 pg per 25 µL of DNA template at 28, 30, 33, and 36 cycles. Miniplex 2 and Miniplex 4 gave good signal intensities (≥ 1000 RFU) and good peak balance ratio with 30 and 33 cycles (Figure 14). At 28 cycles, amplification products were observed but the signal intensity was quite low. 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/ 25 µL of DNA template. The smaller sized loci, TH01, CSF1PO, and TPOX were only successfully amplified at 500 pg/ / 25 µL at 28 cycles. At 30 cycles, the samples of the smaller sized loci of the Big Mini set successfully amplified with 250 pg/ 25 µL of template. At 36 cycles, all Miniplex loci were over-amplified and problems with non-specific amplification were encountered.

The effect of cycle number on the amplification efficiency of DNA fragments were studied for fragment sizes of ~222-350 base pairs and ~350-460 base pairs. These

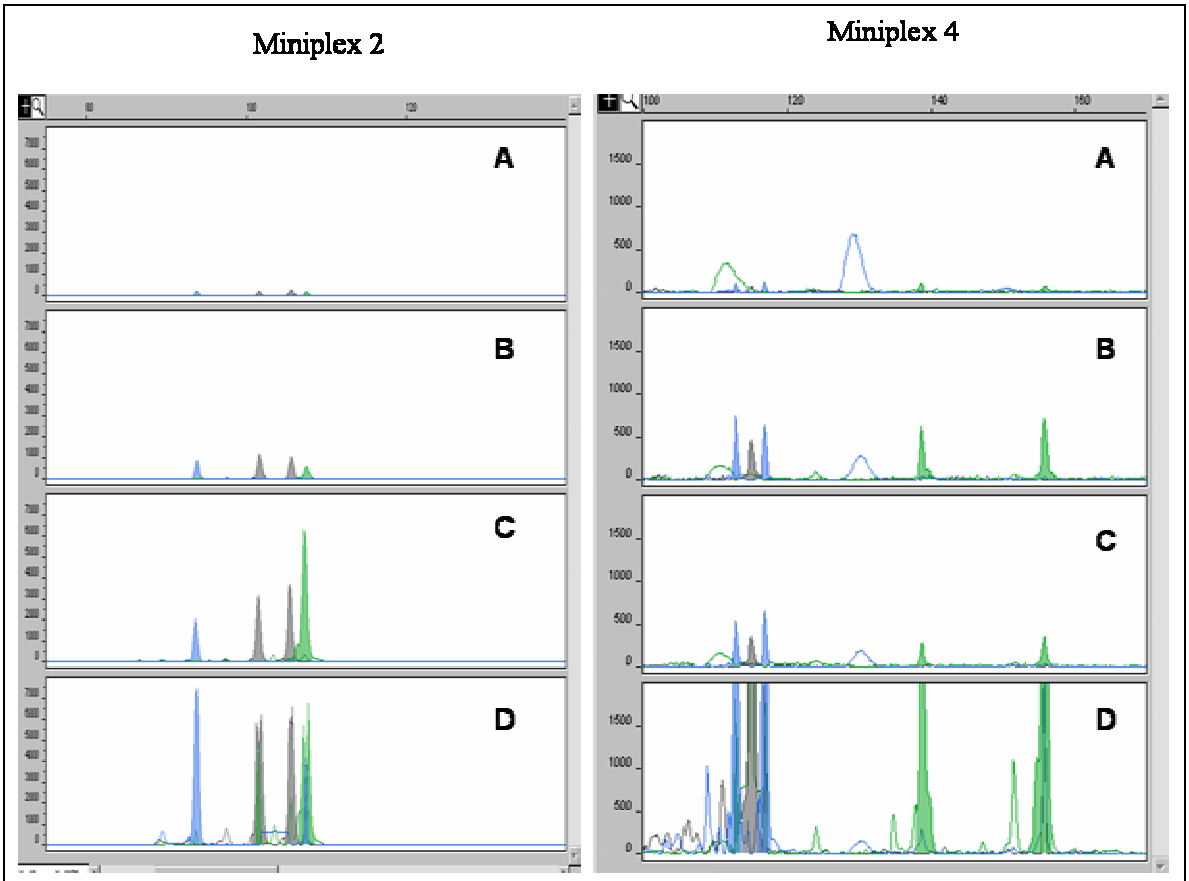


Figure 14. Cycle number study for Miniplex 2 and Miniplex 4. Amplifications of 100 pg/ 25 µL of DNA with Miniplex 2 and Miniplex 4 at different cycle numbers. Panel A: 28 cycles, Panel B: 30 cycles, Panel C: 33 cycles, and Panel D: 36 cycles. 1 µL of amplified sample was added to 12 µL formamide. Injection time used was 5 seconds at 15 kV.

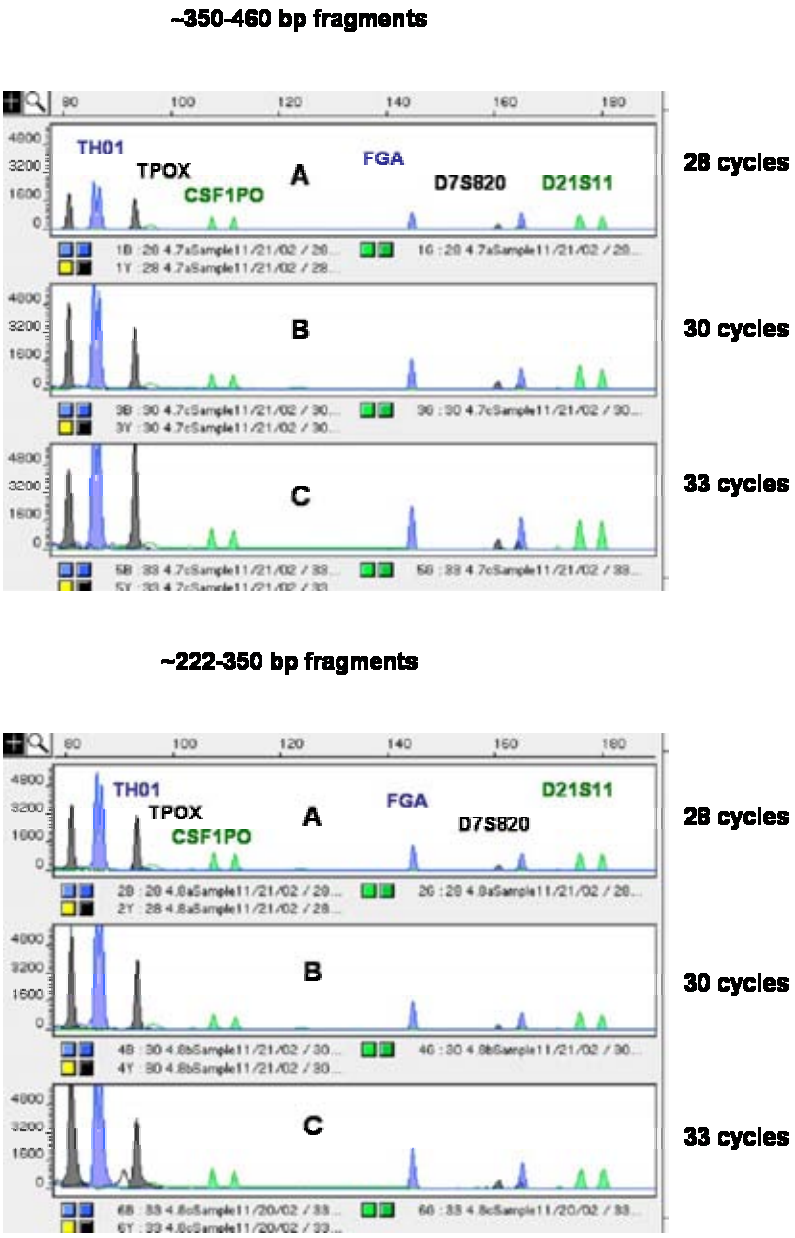


Figure 15. Effect of cycle number on the amplification efficiency of DNA fragments. 2 ng/25 µL of ~350–460 and ~222–350 base pair fragments amplified with Big Mini at 28 (Panel A), 30 (Panel B) and 33 (Panel C) cycles. Increasing the cycle number increases the average peak height with the smaller sized loci, TH01, CSF1PO, and TPOX being most affected. However, smaller cycle numbers generally give better peak balance.

were tested at 28, 30, and 33 amplification cycles with the Big Mini primer set (Figure15). We observed that an increase in cycle number increases the average peak height, with the smaller

loci (i.e. TH01, CSF1PO, TPOX) being most affected. For DNA templates in excess of 1 ng, the effect of cycle number is not that apparent. Although increasing the cycle number can increase signal intensity, lower cycle numbers can provide better peak balance. For DNA templates in excess of 250 pg/25 µL, 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 only low copy amounts of DNA template are available, it becomes indispensable to use a higher cycle number. We recommend using 33 cycles for amplifying DNA samples with 100 pg/ 25 µL of template. Using this cycle number with the Miniplex primer sets achieves the best balance between sensitivity and profile quality.

Reaction Volume Study

Decreasing the reaction volume of the PCR reaction is used as a means to save the cost of reagents. It also saves the amount of DNA template added to the PCR reaction. The use of smaller reaction volumes would be beneficial for amplifications using the Miniplex primer sets because three different sets of amplifications have to be performed for each sample to obtain profiles for 12 loci. This could pose a limitation when the amount of DNA template recovered from the crime scene is limited. Thus, reducing the reaction volume of the sample would be an effective way to reduce the amount of DNA template used for amplification.

In this study, we tested the effects of varying the reaction volume on amplification efficiency. Reaction volumes of 5, 10, 12.5, 25, and 50 µL were tested with DNA concentrations of 100, 250, 500, and 1000 pg/ 25 µL using 33 cycles. We elected to keep

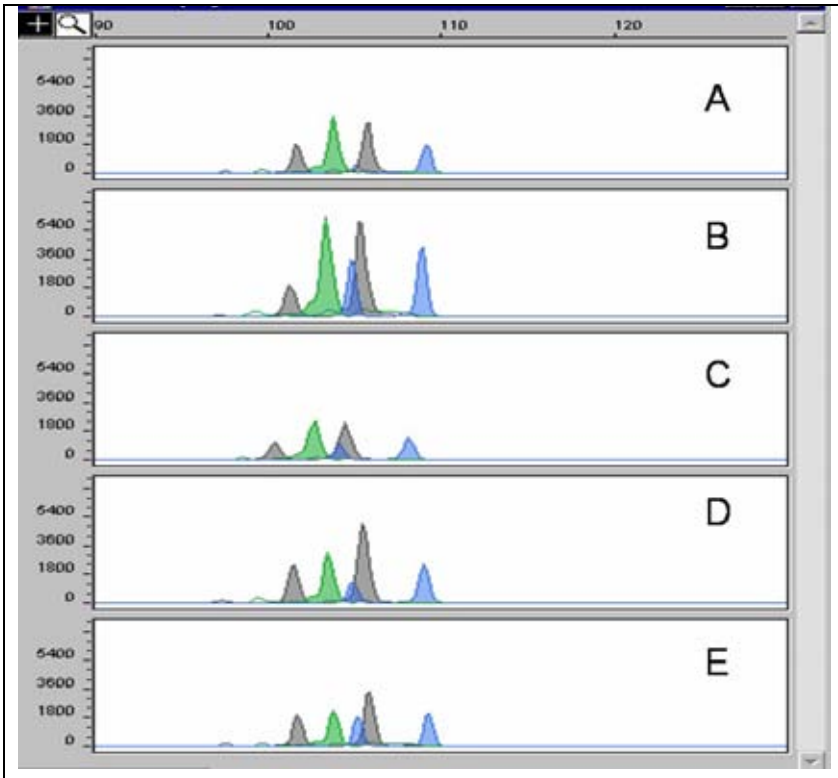


Figure 16. Miniplex 2 reaction volume study. 100 pg/ 25 µL of DNA template amplified at 33 cycles with Miniplex 2. The reaction volume for each panel is as follows: Panel A: 5 µL, Panel B: 10 µL, Panel C: 12.5 µL, Panel D: 25 µL, and Panel E: 50 µL. Allele dropout for D5S818 was observed when 5 µL of reaction volume at this concentration.

the final template concentration constant to avoid overloading the CCD camera of the instrument. Signal intensities for all reaction volumes remained the same. We observed allele dropout with 100 pg/25 µL concentration of DNA template at 5 µL of reaction volume for Miniplex 2 (Figure 16) and no amplification products were observed at this concentration and volume for the Big Mini set. All other volumes and concentrations tested produced consistent profiles. For the Big Mini set, best results were obtained at 25 µL reaction volume for 100 pg/25 µL DNA concentration. The presence of dye blobs are exacerbated at lower reaction volumes at this concentration. The EDGE Biosystems DTR spin column can be used to remove these dye blobs when lower reaction volumes are to be used at 100 pg /25 µL for the Big Mini set. When the concentration of DNA template at a 25 µL reaction volume is in excess of 250 pg, excessive amplification resulted when using 33 cycles (i.e. saturation of CCD camera).

Magnesium Titration

Magnesium ions act as a cofactor for the Taq polymerase enzyme. The concentration of this ion in the reaction can affect primer annealing, product specificity, strand dissociation temperatures of both template and products, formation of primer-dimer artifacts, and polymerase activity and fidelity (30). We have examined the effect of different magnesium concentrations on the efficiency of the PCR reaction. Ten samples plus two DNA standards (9947A and 9948) were amplified with different magnesium concentrations. Magnesium titrations of 1, 1.5, 2, 2.5, and 3 mM were tested with these three Miniplex sets at 33 cycles with 100 pg/ 25 µL of DNA template. Results for the magnesium titration of the Big Mini set is shown on Figure 17. The GeneAmp® PCR buffer II without magnesium was used and the specified amount of magnesium was then titrated.

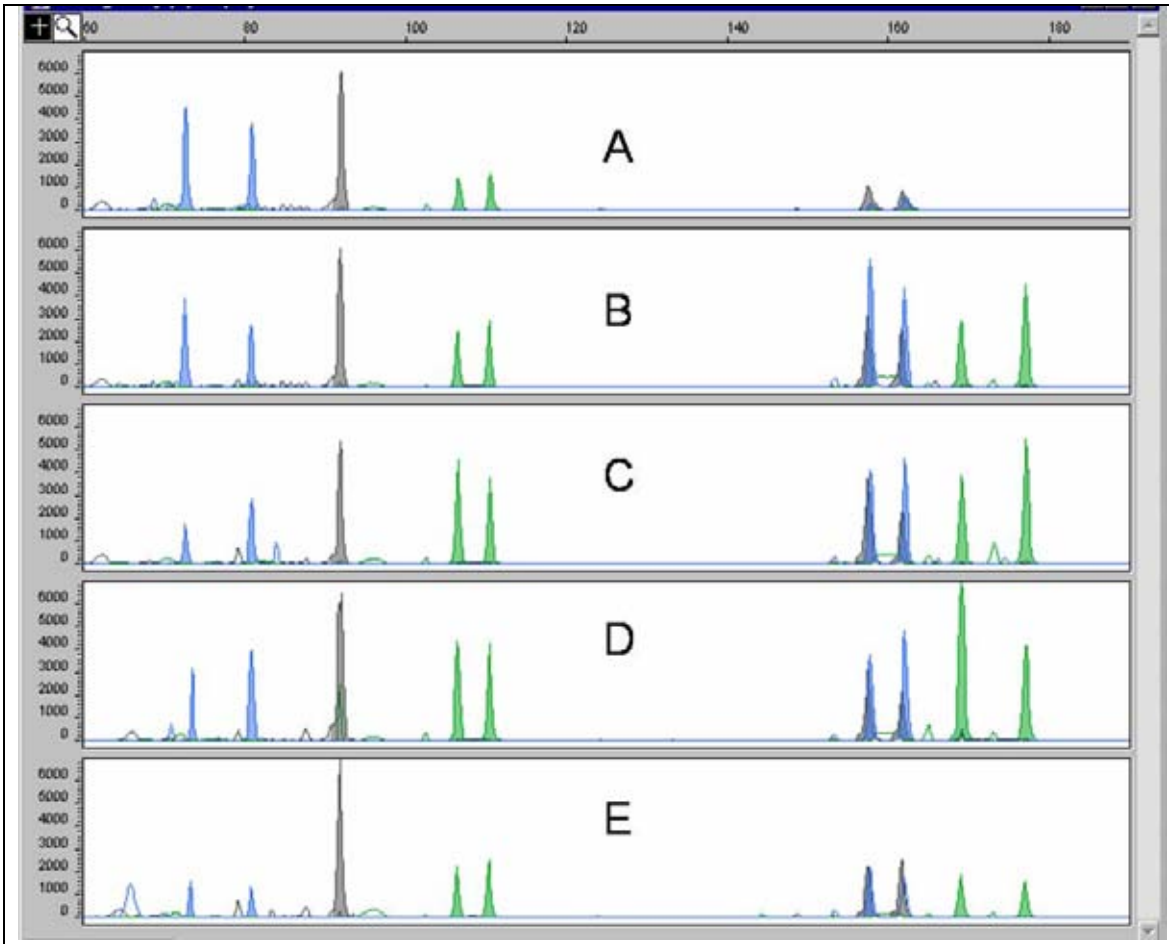


Figure 17. Magnesium titrations for the Big Mini multiplex set. 100 pg/ 25 µL of DNA template amplified at 33 cycles titrated with varying magnesium concentrations. Final magnesium concentration in each reaction is as follows: Panel A: 1 mM Mg, Panel B: 1.5 mM Mg, Panel C: 2 mM Mg, Panel D: 2.5 mM Mg, and Panel E: 3 mM Mg.

Based from our results, allele dropout was observed at 1 mM magnesium concentration. Magnesium concentrations of 1.5 – 2 mM were found to be optimal. Problems with non-specific binding start to become apparent at higher magnesium concentrations (2.5 -3 mM). These results indicate that the availability of magnesium ions is essential for optimal polymerase activity. Although increases in magnesium concentration does not greatly affect PCR yield, a decrease in the amount of magnesium can cause adverse effects on the yield of the reaction.

Variation in Annealing Temperature

The annealing temperature during the PCR cycle can affect the specificity, yield, and balance of the amplified loci. Lower annealing temperatures can increase yield while higher annealing temperatures increase specificity. We studied the effects of different annealing temperatures on the efficiency of PCR amplification. Annealing temperatures of 50, 55, 58, 60, 65 ºC were tested at 33 cycles with 100 ng/ 25 µL of DNA template (Figure 18). At an annealing temperature of 50ºC, allele dropout was not observed and no additional artifacts were seen. Locus and allele dropout started to occur at the annealing temperature of 58ºC for D5S818 of the Miniplex 2 set. At 60ºC, CSF1PO, D21S11, and FGA of the Big Mini set began to drop out. And at 65ºC, all loci failed to amplify. Increasing the annealing temperature seems to have a greater effect on the yield of the larger sized loci. We recommend using an annealing temperature of 55ºC.

AmpliTaq Gold® Polymerase Titration

The amount of DNA polymerase added to each reaction affects the yield of PCR products. Therefore, we studied the effects of varying the Taq polymerase concentration on the amplification efficiency of the reaction. Titrations of AmpliTaq Gold DNA

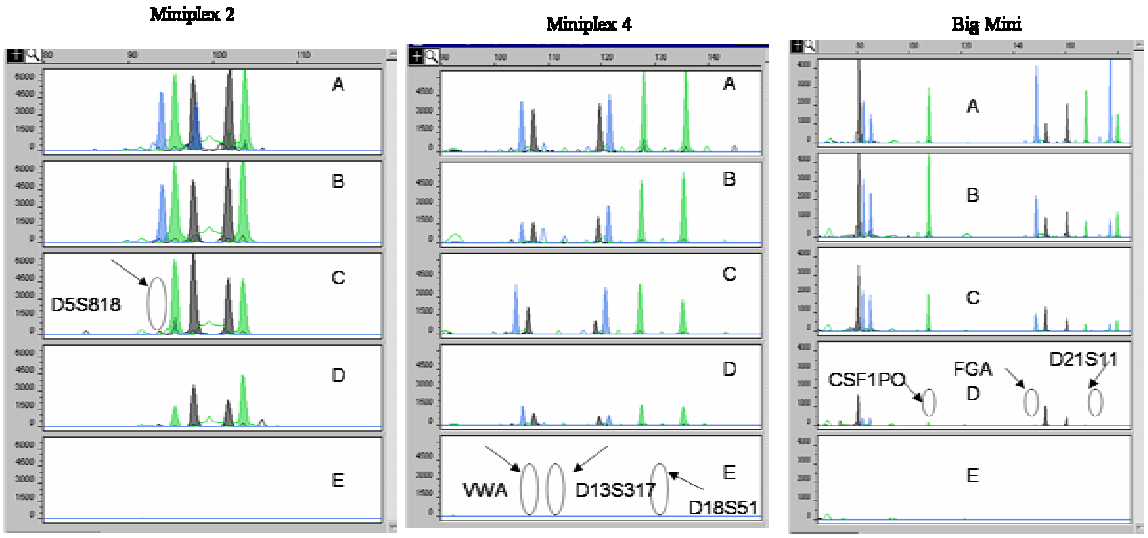


Figure 18. Effect of the variation in annealing temperature for Miniplex 2, Miniplex 4 and Big Mini. 100 pg/ 25 µL of DNA template amplified at 33 cycles using different annealing temperatures. Annealing temperature for each reaction is as follows: Panel A: 50ºC, Panel B: 55ºC, Panel C: 58ºC, Panel D: 60ºC, and Panel E: 65ºC. The D5S818 began to dropout at 58ºC followed by CSF1PO, FGA, and D21S11 at 60ºC. At 65ºC, no amplifications are seen for all loci.

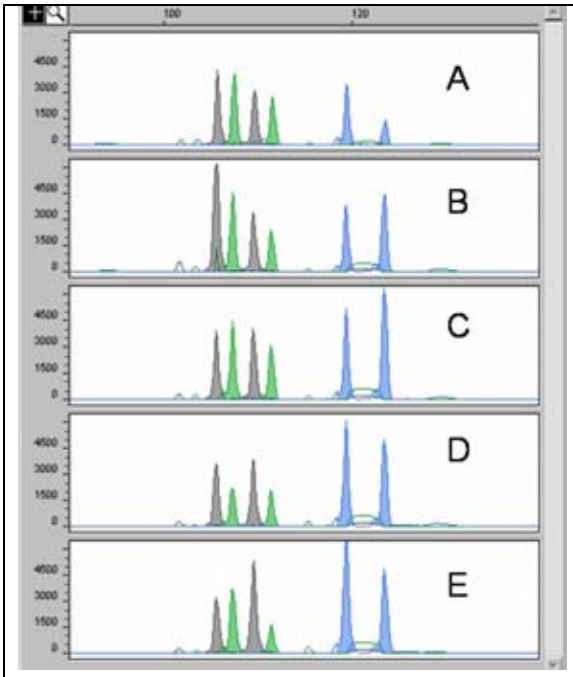


Figure 19. Miniplex 2 titration of Amplitaq Gold® DNA polymerase. 100 pg/ 25 µL of DNA template was amplified at 33 cycles and titrated with varying enzyme concentrations. Enzyme concentration per 25 µL is as follows: Panel A: 1 Unit, Panel B: 2 Units, Panel C: 3 Units, Panel D: 4 Units, and Panel 5 Units.

polymerase from 1-5 Units/ 25 µL were tested with 100 pg/ 25 µL of DNA template for Miniplex 2 (Figure 19), Miniplex 4, and Big Mini. Based from our results, 1 U of enzyme per 25 µL is enough to successfully amplify 100 pg/ 25 µL of DNA at 33 cycles. However, better peak balance was achieved at 2 U of enzyme. Higher concentrations of enzyme do not seem to have much of an effect on the yield of PCR products. We are using a default enzyme concentration of 2 U/ 25 µL.

Environmental Study

DNA samples recovered from forensic situations have usually been exposed to a variety of conditions. Environmental conditions have actually more influence on DNA preservation than does time. Depending on which condition the samples are exposed to, the template can fail to amplify due to DNA degradation or PCR inhibition.

In the previous section, we assessed the effects of DNA degradation on the amplification efficiency of the Miniplex primer sets. In the following section, the results of the Miniplex primer sets on bone samples that have been exposed to a variety of environmental insults will be discussed. However, for validation purposes, we simulated different environmental conditions in our laboratory to make the conditions more controlled.

Five to six drops of fresh blood without EDTA was spotted on an unbleached white cotton cloth. Thirty-six swatches were made and allowed to air dry at room temperature overnight. Five spots were then extracted the following day using the phenol/chloroform method to serve as the control sample. The swatches were then stored at -20ºC, 4ºC, room temperature, and 50ºC for periods of 3, 7, 14, 28, 56, and 84 days. One set of swatches were exposed to randomly fluctuating temperatures for the same periods of time. Another set of swatches were exposed to a UV plant “gro” light for 8 hours a day to simulate sunlight and were also sampled

under the same time periods as mentioned above. No allele drop out was observed for all samples tested. However, a decrease in the ratio of the amplicons of the D7S820 loci to the TPOX loci was observed for samples exposed to higher temperatures at longer times.

Matrix Studies

The substrates on which forensic samples are in contact with may affect the quality of the DNA template. The substrate can enhance DNA degradation or possibly contain PCR inhibitors that can be co-extracted with the DNA template. For instance, pigments and dyes which inhibit the PCR reaction can be co-extracted from denim and leather (8,31).

For this study, DNA from fresh blood stains deposited on a variety of substrates was extracted using the organic method. 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 eight weeks of storage at room temperature, the stains were sampled again.

In the results of this study, we did not see any significant decreases in signal intensity for all extracts except those from white paper after 8 weeks of storage at room temperature. The decrease in the signal intensity of amplicons from white paper after eight weeks of storage in room temperature could be attributed to the presence of bleach.

Mixture Studies

Many casework samples contain DNA from more than one contributor. Thus, the capability of STR multiplex sets to distinguish between major and minor components of the sample becomes important. In cases where contributors share common alleles, the genotypes may not easily be decipherable. The analysis of peak ratios becomes an important tool to

determine the amount of DNA originating from the major or minor contributor. For this study, mixtures of DNA samples in defined ratios were examined while keeping the template concentration constant at 100 pg/ 25 µL. Pairs of DNA samples were mixed in a ratio of 19:1 to 1:19 (Figure 20). The peak heights of the

alleles were used to determine the presence of the major and minor components of the sample. Most minor components for Miniplex 2, Miniplex 4, and Big Mini started to become detectable at a ratio of 1:9. Only the minor component of the D18S51 was detectable (> 150 RFU) at a ratio of 1:19. Thus, when the minor component is present at 10% of the total quantity of DNA template, it is detectable.

Stutter Peaks

The PCR amplification of STR alleles often produces stutter products. Stutter peaks are usually four base pairs smaller than the main product peak. The presence of these stutter products can complicate mixture analysis. Because these products usually have the same size as allele bands, sometimes it is impossible to identify a peak as an allele from a minor contributor or as a stutter product. We evaluated each STR locus in these three Miniplexes for stutter. Stutter was calculated 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 (Figure21). We excluded stutter percentages from off-scale peaks since the height of the actual allele is underestimated and this causes the stutter percentage to be artificially high. Based from the results, we have found that TH01 and TPOX have the lowest stutter percentage. Average stutter also increases as the alleles for a particular locus increase (Figure 22).

Figure 20. 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. The minor component only becomes detectable at a ratio of 1:9 for all loci except for D18S51 where the minor component can be detected at a ratio of 1:19.

Non-Human Studies

To ensure that the Miniplex primer sets demonstrate specificity for humans, a variety of animal and bacterial species were examined. DNA from chimpanzee, dog, cat, pig, mouse, rat, chicken, *E. coli, S. aureus, E. faecalis,* and *P. aeruginosa* were amplified with the Miniplex 2, Miniplex 4, and Big Mini primer set. Amplifications for all loci were seen with the chimpanzee sample at 1 ng/ 25 µL of template. At 250 pg/ 25 µL of DNA from the chimpanzee, amplification was not seen with the Big Mini loci. Peaks were also seen at D16S539 and D13S317 when the mouse sample was amplified with Miniplex 2 and Miniplex 4. However, the amplification products from the mouse sample were larger than the allele size range of the D16S539 locus in Miniplex 2 and the D13S317 locus in Miniplex 4 (Figure 23). No amplifications for all loci were seen with the other samples.

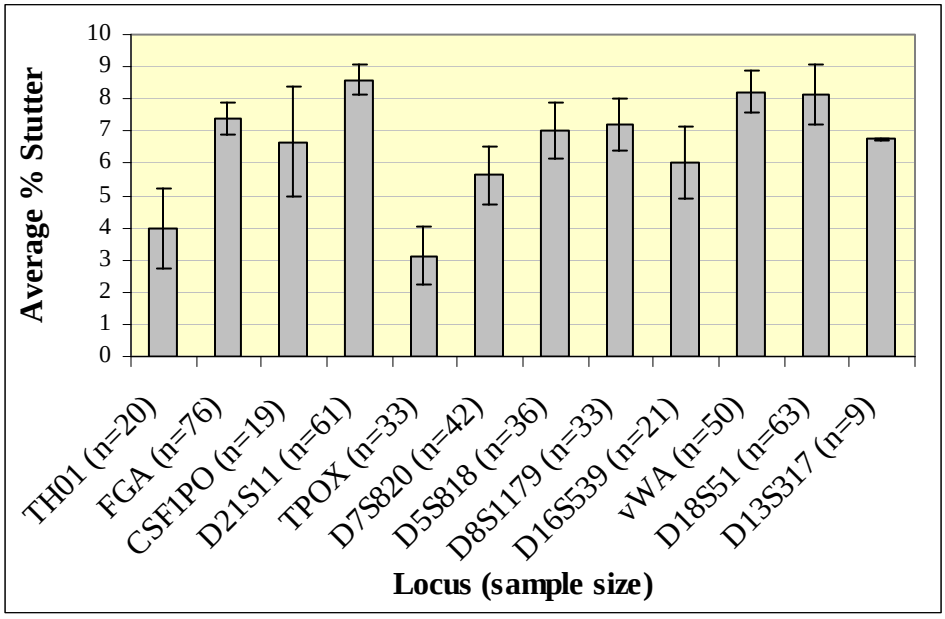


Figure 21. Average stutter calculated for each locus of the Miniplex 2, Miniplex 4, and Big Mini multiplex set. The sample size (n) indicates the number of samples used to calculate stutter for each locus. A 95% confidence interval was used to calculate the error bars.

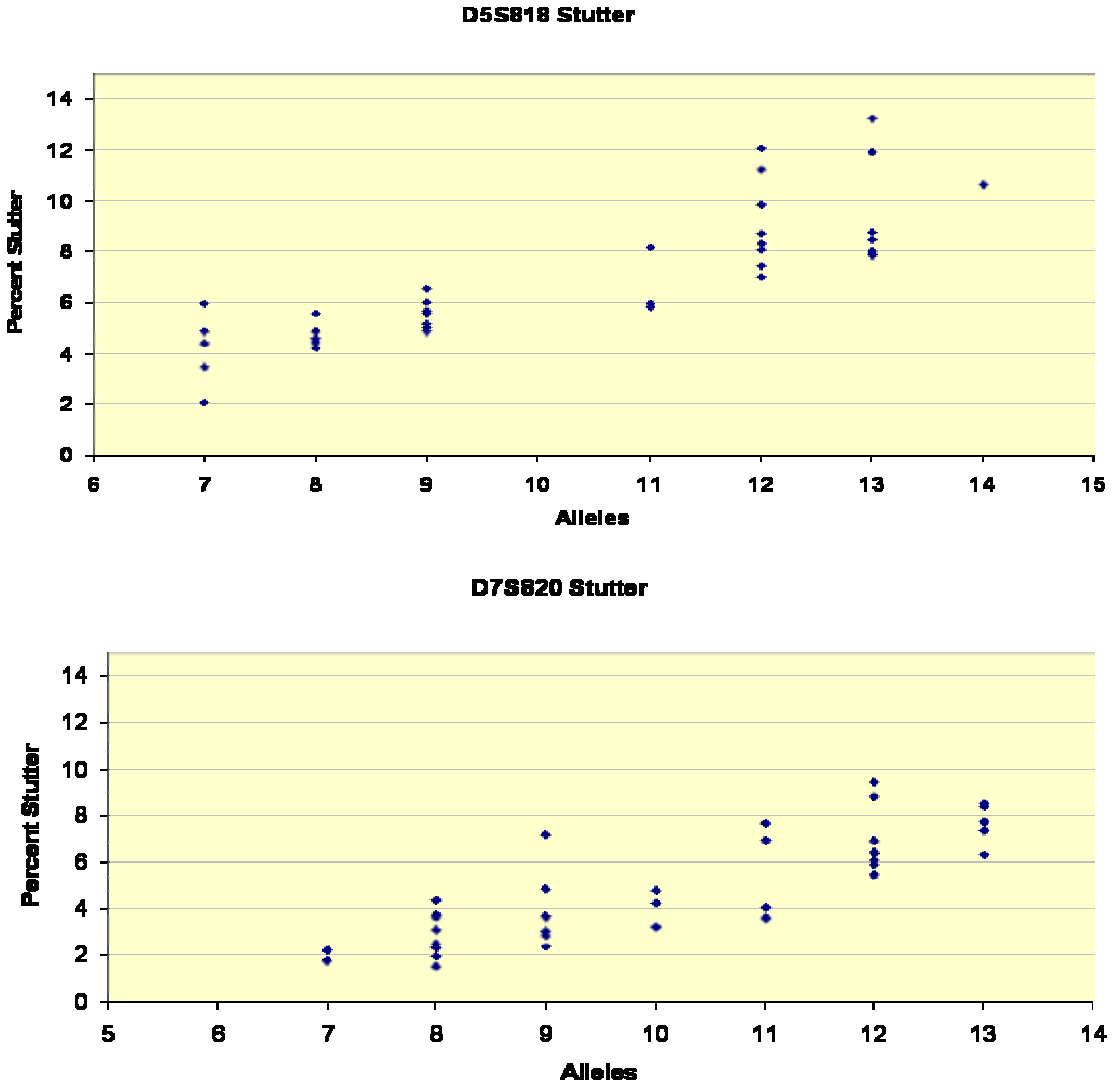


Figure 22. Stutter percentage for the alleles of the D5S818 and D7S820 loci.
Average stutter increases for the larger alleles of a particular locus.

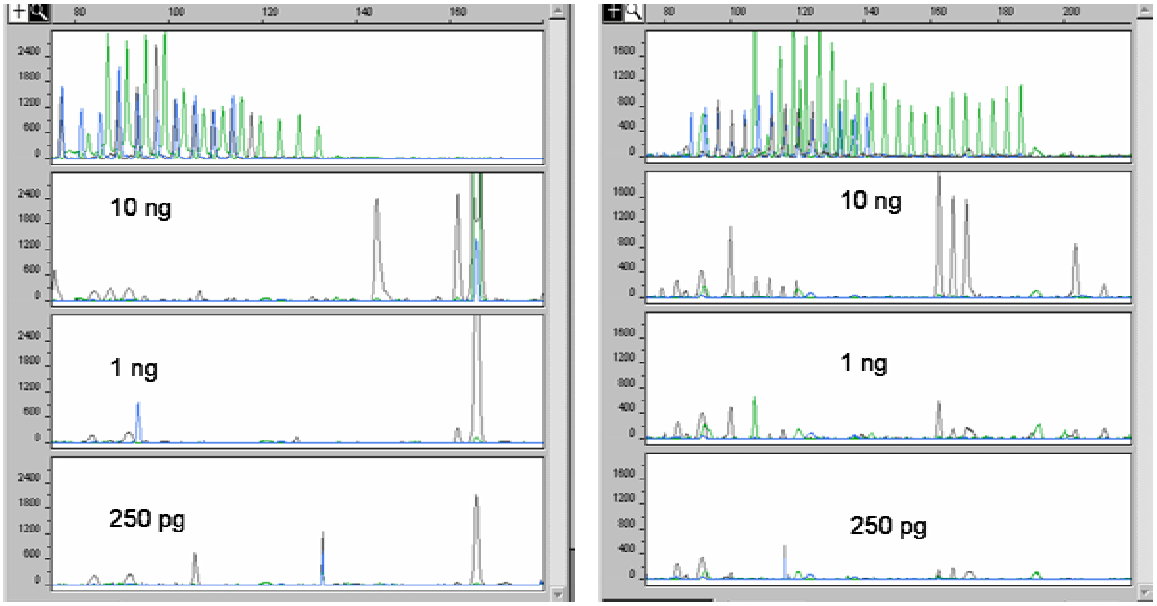


Figure 23. Mouse DNA sample amplified with Miniplex 2 and Miniplex 4. Amplification products seen from the mouse DNA sample are larger than the allele size range for the D16S539 locus of Miniplex 2 and the D13S317 locus of Miniplex 4.

Preferential and Differential Amplification

Differential amplification is defined as the difference in the amplification efficiency between loci in the multiplex set. On the other hand, preferential amplification is defined as the difference in amplification efficiency between alleles of the same locus. Differential amplification for the Miniplex primer sets have been observed when analyzing degraded DNA samples. In these situations, a decay curve is seen where the larger sized loci start to fall below the detection threshold. We also saw this effect when amplifying bone samples obtained from different environmental conditions. Profiles for the larger sized loci of the Big Mini set (FGA, D21S11, and D7S820) could not be obtained when to severe DNA degradation occurred. In the case of PCR inhibition, differential amplification was observed for some loci. The pattern which was observed was not the same as that for degraded DNA. There was no correlation seen between the amplicon size and the degree of PCR inhibition. This could be due to stronger primer binding for some loci as this phenomenon is also observed when the annealing temperature is increased. Problems of preferential amplification due to stochastic fluctuations are common when amplifying low copy number DNA. When the concentration of DNA template amplified is below 100 pg/ 25 µL, peak imbalance is observed for most of the Miniplex loci. Preferential amplification due to significant differences in base pair size was not observed since most of the Miniplex loci have small size ranges. However, in cases where the sample is degraded and has larger FGA alleles, preferential amplification can be observed.

Problems with Dye Blobs

Problems with residual dye molecules or “dye blobs” are often encountered when the PCR product size is less than 150 base pairs. These residual dye molecules can result from

improper attachment of the dye molecule during the oligonucleotide synthesis process(9). These dye blobs give rise to peaks that are wider and less intense than true alleles permitting a trained analyst to distinguish the difference between a true allele and a dye blob artifact. Figure 24 shows the results from a Big Mini amplification. The dye blobs can appear in each of the three different dye lanes and can migrate through the capillary slightly differently depending on the electrophoretic conditions used. These dye blobs can be removed by using a Gel Filtration Cartridge spin column from Edge Biosystems (Gaithersburg, MD). iosystems (Gaithersburg, MD),

The procedure can be found in Appendix I.

The presence of residual dye molecules do not always interfere with the genotyping process and most of the time the additional clean-up step using the Edge DTR spin column is not necessary. s not

necessaryt

However, when the dye interferes with the true alleles of the sample, using the spin column may be necessary to recover the sample genotype. These dye blob artifacts often affect Big Mini amplifications but are usually not a problem with Miniplex 2 and Miniplex 4 amplifications.

Sizing Precision of GeneScan-500 ROX Size Standard

The sizing precision of the internal lane standard used is important for genotyping purposes. The presence of off-ladder allele microvariants must be distinguished from true alleles.

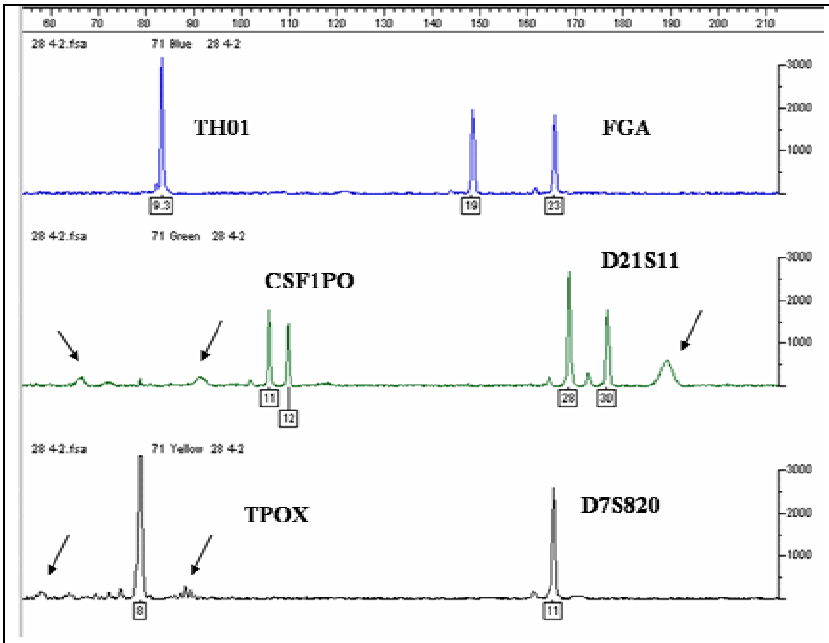


Figure 24. Dye blob artifacts encountered in Big Mini amplifications. These residual dye molecules are usually wider and less intense than the true alleles. However, sometimes they can interfere with correct genotype interpretation when it impacts the true alleles of the sample.

In this study, the sizing precision of the GS-500 ROX size standard was evaluated by comparing the sizing of alleles from 12 injections of the Big Mini ladder. Allele sizing was performed with GeneScan® Software using the Global Southern method of sizing. The local Southern sizing method was not used due to an insufficient number of peaks in the ladder to correctly perform this sizing algorithm.

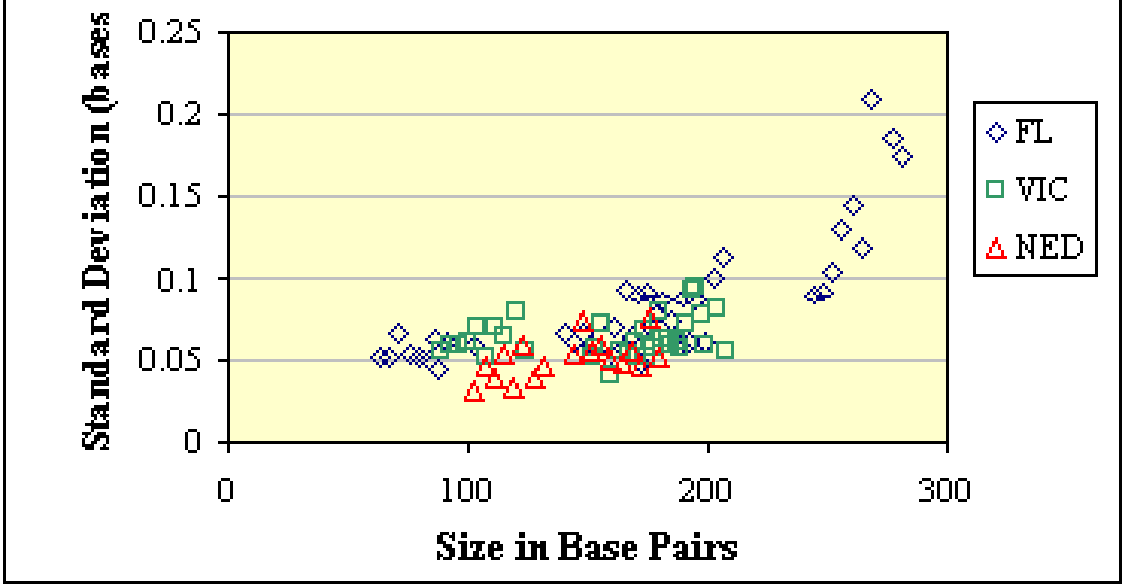


Figure 25. Sizing precision of GeneScan-500 ROX size standard. The average fragment size of each allele in the Big Mini ladder was plotted against the standard deviation obtained from 12 ladder injections.

In addition this method is less sensitive to temperature fluctuations(32). For this study, the average allele size was plotted against the standard deviation for each allele. All alleles below 200 base pairs gave standard deviations of less that 0.1bp. Increased deviation was observed for the larger alleles (> 250 base pairs) of the FGA locus (SD >0.1 bases) (Figure 25). Our results confirm the precision of the GS-500 ROX size standard in the sizing of possible off-ladder microvariants.

8. Application to Simulated Forensic Samples

Introduction

In portion of the study, we extracted naturally degraded DNA from human skeletal remains which had been exposed to a variety of environmental conditions, and amplified the DNA with the newly developed Miniplex primers. We used real time PCR to quantitate the amount of human DNA present and the PowerPlex® 16 system (100-480 bp amplicon size range) to confirm the extent to which these samples were degraded. Finally, we were able to demonstrate that an extraction method which only requires 100 mg of bone powder per sample can produce a sufficient amount of DNA for amplification with the Miniplex primer sets.

Sample collection

One tibia bone sample and twenty-four femur bone samples from twenty-five individuals were obtained from the Forensic Anthropology Center (FAC) at the University of Tennessee in Knoxville. 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. Prior to accession into the collection, the remains had been subjected to different environmental conditions, cleaned, and heated without chemicals (50°-60°C) for 6-12 hours, and analyzed by the researchers at the facility. The samples had been stored at room temperature prior to sampling.

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

Bone preparation

Bone samples were first sanded prior to brushing with 5% bleach solution and immediately rinsed with distilled water and with 95% ethanol. Bone powder was generated by Black and Decker cordless drilling into the bone using drill bits ($^{1}/_{4}$", $^{5}/_{16}$", and $^{3}/_{8}$") 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 Franklin County Coroner's Office 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 Forensic Anthropology Center in Tennessee and the Franklin County Coroner's Office are shown on Tables 4 and 5, respectively.

Sample Number	Bone	Condition	Time Out	Miniplex	Powerplex
D2003.5.1	femur	surface, clothed	11 months	100%	63%
D2003.5.2	femur	buried	35 months	83%	31%
D2003.5.3	femur	buried	36 months	92%	19%
D2003.5.5	femur	semi-buried/cleaned	unknown	100%	44%
D2003.5.6	femur	surface	3 years	100%	100%
D2003.5.7	tibia	surface	3 years	100%	100%
D2003.5.8	femur	surface	3 years	92%	44%
D2003.5.14	femur	surface/preservative	12 to 18 months	33%	13%
D2003.5.15	femur	surface	12 to 18 months	100%	69%
D2003.5.16	femur	surface	12 to 18 months	100%	94%
D2003.5.17	femur	buried	12 to 18 months	100%	75%
D2003.5.18	femur	buried	12 to 18 months	75%	13%
D2003.5.19	femur	buried in compost	12 to 18 months	100%	100%
D2003.5.20	femur	surface	12 to 18 months	92%	75%
D2003.5.21	femur	buried/preservative	12 to 18 months	92%	13%
D2003.5.22	femur	surface/clothing	12 to 18 months	92%	56%
D2003.5.23	femur	surface/clothing	12 to 18 months	100%	38%
D2003.5.24	femur	surface/clothing	12 to 18 months	100%	56%
D2003.5.25	femur	surface	12 to 18 months	92%	19%
D2003.5.26	femur	surface/sun	12 to 18 months	92%	75%
D2003.5.27	femur	surface/sun	12 to 18 months	100%	44%
D2003.5.28	femur	surface	12 to 18 months	100%	63%
D2003.5.29	femur	surface	12 to 18 months	75%	50%
D2003.5.30	femur	surface	12 to 18 months	100%	94%
D2003.5.31	femur	surface	12 to 18 months	58%	25%

Table 4: Sample information and conditions of bone samples from the Forensic Anthropology Center, University of Tennessee, Knoxville, Tennessee. Also shown are the percent of loci that gave profiles.

Sample Number	Bone	Condition	Years in Storage	Miniplex	PowerPlex
D2003.6.1	Femur	cold storage	5	100%	44%
D2003.6.2	Femur	cold storage	11	100%	100%
D2003.6.3	Femur	cold storage	6	100%	56%
D2003.6.4	Femur	cold storage	14	100%	63%
D2003.6.5	Femur	cold storage	5	100%	100%
D2003.6.6	Femur	cold storage	10	100%	81%

Table 5: Sample information and condition of bone samples from the Franklin County Coroner's Office, Columbus, Ohio. Also shown are the percent of loci that gave profiles.

DNA extraction and quantification:

DNA was extracted by modification of the QIAamp® protocol. Briefly, 100 mg of bone powder was decalcified in EDTA, digested using a stain extraction buffer and proteinase K, and purified and concentrated using the QIAamp® Blood Mini Kit (Qiagen, Inc., Valencia, CA). The samples were quantified using the Alu-based real-time PCR method (Nicklas et al., 2003) with a Corbett Research RotorGene RG3000 cycler.

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 in a 12.5 µL reaction volume. 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 due to extremely low concentrations of DNA template (1-5 pg/µL before concentration). The samples were amplified at 33 cycles in a total reaction volume of 12.5 µL. 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 system followed the procedure as specified in PowerPlex®16 technical manual (2000). Non-acetylated 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 with larger amplicons in both Miniplexes and PowerPlex 16 was used to assess DNA degradation.

Data analysis:

Amplicons were analyzed using the ABI PRISM® 310 GeneticAnalyzer. The GeneScan® ROX 500 (Applied Biosystems, Foster City, CA) size standard was used for Miniplex amplified samples and the ILS 600 size standard was used for PowerPlex®16 amplified samples. Samples were prepared by adding 1 µL PCR product to 12 µL Hi-Di™ formamide (Applied Biosystems, Foster City, CA) containing 0.50 µL of the internal lane standard. Injection and analysis parameters are listed in the appendix.

Results

The samples were quantified by real time PCR with the use of non-acetylated BSA. BSA is used as a PCR additive to reverse inhibition (33). Nine (36%) samples from the Forensic Anthropology Center and one sample from the Franklin County Coroner's Office yielded low amounts of DNA template (< 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).

Bone samples were amplified with Miniplex 2, Miniplex 4, Big Mini, and PowerPlex® 16 primers, and 0.5 µg of BSA was added to the mixes to diminish the effect of PCR inhibitors. There were 4 samples (Sample #'s 5.14, 5.18, 5.21, 5.25) that failed to amplify due to an extremely low amount of DNA template available (1-5 pg/µL). Other (modified) extraction methods were attempted with these samples (34), and the results gave similarly low yields. These samples were concentrated approximately 10 times using the Microcon YM-100 filters and amplified again. However, only partial profiles were obtained. All bone samples were able to give amplifiable DNA.

Samples from the Forensic Anthropology Center			
Primer Set	**Samples tested**	**Full Profile**	**Partial Profile**
Miniplex 2	25	23 (92%)	2 (8%)
Miniplex 4	25	22 (88%)	3 (12%)
Big Mini	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			
Primer Set	**Samples tested**	**Full Profile**	**Partial Profile**
Miniplex 2	6	6 (100%)	0
Miniplex 4	6	6 (100%)	0
Big Mini	6	6 (100%)	0
Miniplex 1		6 (100%)	0
Miniplex 3		6 (100%)	0
PowerPlex® 16	6	2 (33%)	4 (67%)

Table 6: Summary of profiling results grouped by sample source

Tests for DNA degradation

The Miniplex primers were designed to make the amplified product size as short as possible because the chances of obtaining long amplicons are lower if the DNA template is degraded. 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), ten of the samples yielded only a partial genetic profile for the larger loci of the Big Mini primer set (125-281 base pairs). Only five of the samples yielded a full profile for the PowerPlex® 16 system (100-480 base pairs). With the signal intensity threshold set at 150 RFU, 13 out of the 25 samples from the Forensic Anthropology Center and 6 out of the 6 samples from the Franklin County Coroner's Office yielded complete profiles for all Miniplex loci. The conditions of the 13 samples that yielded complete profiles with the Miniplex primer sets were surface: 3 years (n = 2); surface: 12-18 months (n = 4); burial: 12-18 months (n = 1); burial in compost: 12-18 months (n = 1); surface with clothing: 11 months (n=1); surface with clothing: 12-18 months (n=2); semi-burial: unknown (n=1); and surface under sunlight: 12-18 months (n=1). Table 6 summarizes these results. Taking a closer look at the results of the Big Mini amplifications, among the samples from the Forensic Anthropology Center that yielded partial genetic profiles, ten failed to amplify at the larger 3 loci (FGA, D21S11, and D7S820) of the multiplex set. These 3 loci have a larger range of amplicon sizes compared to all other Miniplex loci. This suggests that some form of DNA degradation has occurred with these bone samples. Although there were samples that yielded partial genetic profiles for the smaller sized loci of the Big Mini set (CSF1PO), the Miniplex 2 set, and the Miniplex 4 set, these could be attributed to the extremely low amount of DNA template available even after concentration with the Microcon filters. The amplification efficiency per locus in relation to amplicon length is shown in Figure 26.

Amplification with the PowerPlex® 16 system further confirmed that degradation has occurred with these bone samples. Most of the samples yielded complete profiles for the D3S1358, TH01, D5S818, Amelogenin, and vWA loci. 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 as seen from the figure. The loss of intensity of the larger loci represents a "decay curve", where the intensity of the larger size fragments is inversely proportional to fragment size in degraded DNA. The 3 samples (Sample #'s 5.6, 5.7, 5.19) from the FAC that yielded complete profiles with the PowerPlex® 16 system also yielded complete profiles with all the Miniplex sets, although the decay curve was observed for these samples as well. There were also 5 samples amplified with the Big Mini set

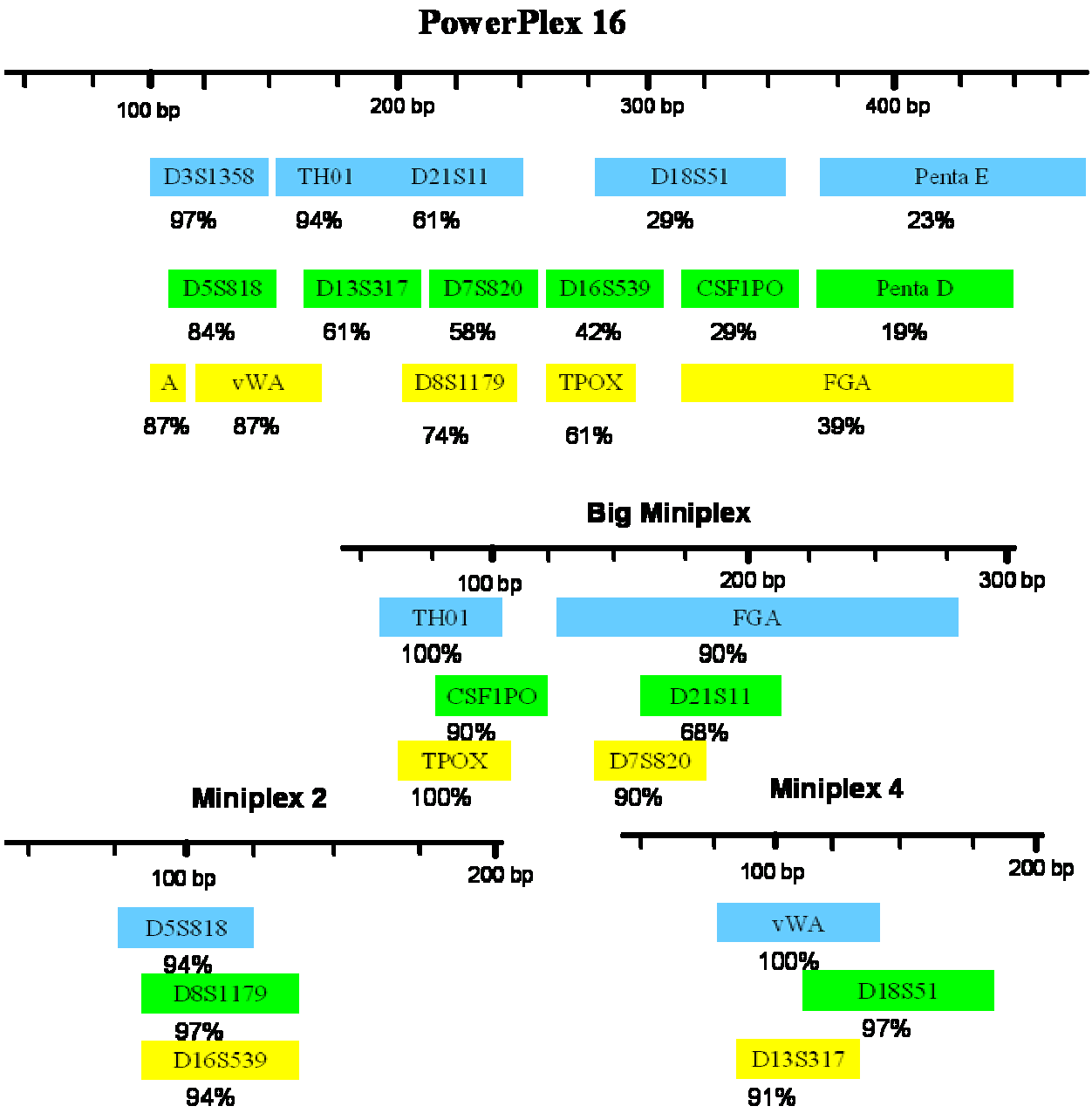


Figure 26: Amplicon size ranges of the PowerPlex 16 system and Miniplex primer sets showing percentages of amplification success per locus (n = 31).

which indicated the possibility of allele drop out in one or two loci. Since 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. The alleles that dropped out are the larger sized alleles of these loci. The Big Mini and PowerPlex® 16 data suggest that the degradation cut-off length of template fragments predominantly occurs around 200 base pairs and is not kit-dependent.

Overall, the Miniplexes produced results for at least 11 of the 12 loci for all samples in which the quantity of DNA recovered was above 5 pg/µL of extracted DNA. The commercial kit with its larger loci averaged only ten loci for these same samples, with some samples producing as little as three amplified loci. Because only three of the six buried bone samples contained larger quantities of DNA it was difficult to assess the effect of burial (3 samples) versus surface treatment (14 samples). However it is clear from the results that the amount of DNA recovered from similar samples placed on the surface varied widely. DNA degradation may be affected by a variety of factors including, humidity, temperature, soil pH, and presence of microorganisms. (28). These were not controlled in this study.

The samples kept in cold storage for 5-14 years from the Franklin County Coroner's Office were less prone to degradation and PCR inhibition. These samples were of course 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 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 test these materials using Miniplex amplifications.

9. Conclusions

MiniSTRs are CODIS loci which utilize primers designed to keep the amplified samples as short as possible. These new primer sets provide an important new tool for the determination of DNA profiles when only partial genetic profiles are generated from standard kits due to the effects of DNA degradation. We have shown the MiniSTRs to be highly robust and generally concordant with standard STR typing kits. Sensitivity is excellent with peak balance above 60% with100 pg for most loci, and the kits have proven themselves to be superior to commercial DNA typing systems for the analysis of degraded DNA. These redesigned primer sets should have important applications in forensic science for the identification of degraded DNA.

10. Appendix

a. Primer sequences

Miniplex primer sequences. Primer melting temperatures (Tm) are calculated from the Primer 3 program using default values. **The 5' tails are added to the reverse (non labled primers) to minimize problems with Taq addenylation.**

STR Locus		Miniplex Primer Sequence (5' to 3')	Tm (ºC)	5' Tail Added	Distance from Repeat (bp)
TH01	F	6FAM-CCTGTTCCTCCCTTATTTCCC	61.0		0
	R	GGGAACACAGACTCCATGGTG	62.8	GTTTCTT	1
CSF1PO	F	VIC-ACAGTAACTGCCTTCATAGATAG	52.4		14
	R	GTGTCAGACCCTGTTCTAAGTA	53.6		6
TPOX	F	NED-CTTAGGGAACCCTCACTGAATG	60.0		-4
	R	GTCCTTGTCAGCGTTTATTTGC	61.0	GTTTCTT	5
FGA	F	6FAM-AAATAAAATTAGGCATATTTACAAGC	55.9		3
	R	GCTGAGTGATTTGTCTGTAATTG	56.6		23
D21S11	F	VIC-ATTCCCCAAGTGAATTGC	55.8		2
	R	GGTAGATAGACTGGATAGATAGACGA	56.5		0
D7S820	F	NED-GAACACTTGTCATAGTTTAGAACGAAC	58.9		4
	R	TCATTGACAGAATTGCACCA	58.6	GTTTCTT	65
D5S818	F	6FAM-GGGTGATTTTCCTCTTTGGT	58.0		4
	R	AACATTTGTATCTTTATCTGTATCCTTATTTAT	58.3		-5
D8S1179	F	VIC-TTTGTATTTCATGTGTACATTCGTATC	58.5		-4
	R	ACCTATCCTGTAGATTATTTTCACTGTG	59.4		5
D16S539	F	NED-ATACAGACAGACAGACAGGTG	52.5		0
	R	GCATGTATCTATCATCCATCTCT	55.0		16
vWA	F	6FAM-AATAATCAGTATGTGACTTGGATTGA	58.1		0
	R	ATAGGATGGATGGATAGATGGA	57.3		0
D18S51	F	VIC-TGAGTGACAAATTGAGACCTT	54.8		5
	R	GTCTTACAATAACAGTTGCTACTATT	52.7		33
D13S317	F	NED-TCTGACCCATCTAACGCCTA	58.3		19
	R	CAGACAGAAAGATAGATAGATGATTGA	57.4	GTTTCTT	2
Penta D	F	6FAM-GAGCAAGACACCATCTCAAGAA	59.5		11
	R	GAAATTTTACATTTATGTTTATGATTCTCT	57.3		19
Penta E	F	VIC-GGCGACTGAGCAAGACTC	57.1		6
	R	GGTTATTAATTGAGAAAACTCCTTACA	57.6		4
D2S1338	F	NED-TGGAAACAGAAATGGCTTGG	61.0		3
	R	GATTGCAGGAGGGAAGGAAG	61.1		3
D3S1358	F	NED-CAGAGCAAGACCCTGTCTCAT	59.5		-1
	R	TCAACAGAGGCTTGCATGTAT	58.4		-1

b. Protocols

PCR amplification

Amplifications were performed in reaction volumes of 25 µL using a master mix containing 1X GeneAmp® PCR Gold buffer (Applied Biosystems), 1.5 mmol/L $MgCl_2$, 200 µmol/L deoxynucleotide triphosphates (Life Technologies, dNTPs: dATP, dCTP, dGTP, dTTP), and 2 units of AmpliTaq Gold® DNA polymerase. Optimal primer concentrations are as follows: D5S818, 0.4 µM; D8S1179 0.4 µM; D16S539, 0.2 µM; vWA, 0.4 µM; D18S51, 0.4 µM; D13S317, 0.56 µM; TH01, 0.16 µM; CSF1PO, 0.16 µM; TPOX, 0.2 µM; FGA, 0.24 µM; D21S11, 0.24 µM; D7S820, 0.32 µM

Thermal cycling was performed with the GeneAmp 9700 (Applied Biosystems) using the following conditions in 9600-emulation mode (i.e., ramp speeds of 1 ^{o}C/s):

95 ^{o}C for 10 minutes
28-33 cycles: 94 ^{o}C for 1 minute, 55 ^{o}C for 1 minute, 72 ^{o}C for 1 minute
60 ^{o}C for 45 minutes
25 ^{o}C forever

For degraded or compromised samples, we recommend using 33 cycles for amplifying DNA samples with 100 pg/ 25 µL of template. Using this cycle number with the Miniplex primer concentrations listed above achieves the best balance between sensitivity and profile quality.

Removal of residual dyes following PCR.

Removal of residual dye molecules that generated the so-called “dye blobs” in capillary electrophoresis electropherograms was accomplished with Edge Performa™ DTR Gel Filtration Cartridges from Edge BioSystems (Gaithersburg, MD) following the manufacturer’s protocol. Briefly, the Gel Filtration Cartridge was centrifuged for 2 minutes at 750 x g to remove the storage liquid in the cartridge. Then a 15 µL aliquot of the 25 µL PCR reaction was placed in the center of the slanted gel bed surface and centrifuged for 2 minutes at 750 x g. The resulting

eluate contained the purified PCR product from which 1 µL may be used for ABI 310 or 3100 sample preparation.

Analysis on ABI 310 (single capillary) Genetic Analyzer.

The ABI Prism® 310 Genetic Analyzer (Applied Biosystems) was used with filter set F in order to process the data from the 4 dyes 6FAM, VIC, NED, and ROX after an appropriate matrix had been created using materials from matrix standard sets DS-32 and DS-33 (Applied Biosystems). Each sample for analysis on the 310 was prepared by adding 1 µL PCR product to 19 µL of Hi-Di™ formamide (Applied Biosystems) containing 0.75 µL GS500 ROX size standard (Applied Biosystems). Samples were then placed immediately into the instrument for analysis without heat denaturing or snap cooling the samples prior to running them. Samples were injected for 5 s at 15,000 volts and separated at 15,000 volts for 24 minutes with a run temperature of 60 ^{o}C. Standard electrophoretic conditions were used including 310 Genetic Analyzer POP™-4, 1X Genetic Analyzer Buffer with EDTA, and a 47 cm x 50 µm capillary (Applied Biosystems).

Analysis on ABI 3100 (16-capillary) Genetic Analyzer

Prior to running any miniplex samples on the ABI 3100, a 4 dye matrix was established under the "Z filter" with the dyes 6FAM (blue), VIC (green), NED (yellow), and ROX (red) using matrix standard set DS-30 (Applied Biosystems) and substituting a VIC matrix standard for HEX. Samples were typically prepared with 9 µL Hi-Di™ formamide, 0.6 µL GS500 ROX, and with 1 µL PCR product (either filtered or unfiltered). Again, no heat denaturation, nor snap cooling was performed prior to injection on the ABI 3100. The miniSTR and commercial STR kit samples were run using the default module GeneScan36_POP4DefaultModule, which

performs an electrokinetic injection onto the 16-capillary array for 10 s at 3,000 volts. The STR alleles were then separated at 15,000 volts for approximately 30 minutes with a run temperature of 60 ^{o}C using the 3100 POP^{TM}-4 sieving polymer, 1X Genetic Analyzer Buffer with EDTA, and a 36 cm array (Applied Biosystems). Data from both the ABI 310 and ABI 3100 were analyzed using GeneScan 3.7 and Genotyper 3.7 programs (Applied Biosystems) for a Windows NT platform. In earlier work, Macintosh versions of GeneScan and Genotyper were used.

Generation of allelic ladders and Genotyper macros

Allelic ladders were created with the miniSTRs using a dilution of allelic ladders from the Identifiler™ (Applied Biosystems) or PowerPlex® 16 (Promega) kits. Briefly, a 1:1000 dilution of the kit allelic ladders was prepared and then 2 µL of these diluted ladders were amplified, either individually or as a multiplex set, using the thermal cycling parameters outlined for the PCR above, except for 15 cycles instead of the standard 28 cycles. Genotyper macros were constructed for each of the miniplex combinations to work with the new allelic ladders.

c. Papers and Reports

McCord, B.; Hartzell, B.; King, S. Separation of DNA by Capillary Electrophoresis, in "*CE applications from small ions to macromolecules,* Humana Press/ Methods and Protocols, Series *Methods in Molecular Biology*, *Molecular Medicine and Biotechnology* (Series Editor : John M. Walker), in preparation

An overview of DNA methods used in these and other studies

Opel, K. L.; Chung, D.; Drábek, J.;Tatarek, N.; Jantz, L. M.; McCord, B. The Application of Miniplex Primer Sets in the Analysis of Degraded DNA from Human Skeletal Remains, Journal of Forensic Sciences, to be submitted

A review of the application of the miniplexes towards simulated forensic samples

Hartzell, B.; McCord, B. Effect of divalent metal ions on DNA studied by capillary electrophoresis. *Electrophoresis*; in press.

An analysis of the effect of environmental contaminants on DNA typing, with particular focus on divalent metal ions.

Drábek, J.; Chung, D.; Butler, J.; McCord, B. Concordance study between Miniplex assays and a commercial STR typing kit, *J. Forensic Sciences*, **2004**, *49(4)*, 859-860.

A concordance study between Miniplex amplifications and a commercial kit for CODIS loci.

Butler, J.; Buel, E.; Crivelente, F.; McCord, B. Forensic DNA typing by capillary electrophoresis, *Electrophoresis*, **2004**, *25 (10-11)*, 1397-1412.

A review of DNA typing methods

Chung, D.; Drabek, J.; Opel, K.; Butler, J.; McCord, B., A study on the effects of degradation and template concentration on the amplification efficiency of the STR miniplex primer sets, *J. Forensic Sciences*, **2004**, *49(4)* 733-740.

A study of the efficiency of Miniplexes in amplifying degraded DNA

Butler, J.; Shen, Y.; McCord, B. The Development of reduced size STR amplicons as tools for analysis of degraded DNA. *Journal of Forensic Sciences*, **2003,** *48(5)*, 1054-1064.

A report on the development of the Miniplex approach

d. Acknowledgements

This project was supported under award 2002-IJ-CX-K007 from the National Institute of Justice. Points of view in the document are those of the authors and do not necessarily represent the official view of the U.S. Department of Justice. We would like to gratefully acknowledge the Provost Undergraduate Research Fund of Ohio University for support of Kerry Opel, the staff of the University of Tennessee Forensic Anthropology Center for allowing us to sample their collection, Dr. Dorothy Dean and Dr. Bradley Lewis from the Franklin County Coroner's Office for generously donating bone samples, Dr. Kerri Dugan from the FBI DNA I Research

Laboratory for providing the DNA extraction protocol for bone samples, and Dr. John Butler from the National Institute from Standards and Technology for his experimental design work on the Miniplexes and his technical support. This project involved the joint effort of the following individuals: Bruce McCord, PI, John Butler, co-PI, Nancy Tatarek, Lee Meadows Jantz, Jiri Drabek, Yin Shen, Denise Chung, Kerry Opel, Brittany Hartzell, and Kylie Graham.

e. Reference List

1. Lygo, J. E.; Johnson, P. E.; Holdaway, D. J.; Woodroffe, S.; Whitaker, J. P.; Clayton, T. M.; Kimpton, C. P.; Gill, P. *Int.J.Legal Med.* **1994,** *107*(2), 77-89.

2. Ruitberg, C. M.; Reeder, D. J.; Butler, J. M. *Nucleic Acids Res.* **2001,** *29*(1), 320-322.

3. Edwards, A.; Civitello, A.; Hammond, H. A.; Caskey, C. T. *Am.J.Hum.Genet.* **1991,** *49* 746-756.

4. Hammond, H. A.; Jin, L.; Zhong, Y.; Caskey, C. T.; Chakraborty, R. *Am.J.Hum.Genet.* **1994,** *55*(1), 175-189.

5. Butler, J. M.; Buel, E.; Crivellente, F.; McCord, B. R. *Electrophoresis* **2004,** *25*(10-11), 1397-1412.

6. Kriss E.J.; Furguson, T. D.; Sipes D.E.; Cotton, R. W. *Validation of the AmpFlSTR Profiler Plus anc Cofiler PCR amplification kits using the ABI Prism 310 Genetic Analyzer*; Promega Corp.: Madison, WI, 1999; pp. 58-59.

7. Krenke, B. E.; Tereba, A.; Anderson, S. J.; Buel, E.; Culhane, S.; Finis, C. J.; Tomsey, C. S.; Zachetti, J. M.; Masibay, A.; Rabbach, D. R.; Amiott, E. A.; Sprecher, C. J. *J.Forensic Sci.* **2002,** *47*(4), 773-785.

8. Wallin, J. M.; Buoncristiani, M. R.; Lazaruk, K.; Fildes, N.; Holt, C. L.; Walsh, P. S. *J.Forensic Sci.* **1998,** *43*(4), 854-870.

9. Butler, J. M.; Shen, Y.; McCord, B. R. *J.Forensic Sci.* **2003,** *48*(5), 1054-1064.

10. Butler, J. M.; Li, J.; Shaler, T. A.; Monforte, J. A.; Becker, C. H. *Int.J.Legal Med.* **1999,** *112*(1), 45-49.

11. Hellmann, A.; Rohleder, U.; Schmitter, H.; Wittig, M. *Int.J.Legal Med.* **2001,** *114*(4-5), 269-273.

12. Ricci, U.; Giovannucci Uzielli, M. L.; Klintschar, M. *Int.J.Legal Med.* **1999,** *112*(5), 342-344.

13. Wiegand, P.; Kleiber, M. *Int.J.Legal Med.* **2001,** *114*(4-5), 285-287.

14. Yoshida, K.; Sekiguchi, K.; Kasai, K.; Sato, H.; Seta, S.; Sensabaugh, G. F. *Int.J.Legal Med.* **1997,** *110*(1), 36-38.

15. Rozen, S.; Skaletsky, H. Primer 3. 1998.

16. Boutrand, L.; Egyed, B.; Furedi, S.; Mommers, N.; Mertens, G.; Vandenberghe, A. *Int.J.Legal Med.* **2001,** *114*(4-5), 295-297.

17. Lazaruk, K.; Wallin, J.; Holt, C.; Nguyen, T.; Walsh, P. S. *Forensic Sci.Int.* **2001,** *119*(1), 1-10.

18. Sparkes, R.; Kimpton, C.; Gilbard, S.; Carne, P.; Andersen, J.; Oldroyd, N.; Thomas, D.; Urquhart, A.; Gill, P. *Int.J.Legal Med.* **1996,** *109*(4), 195-204.

19. Sparkes, R.; Kimpton, C.; Watson, S.; Oldroyd, N.; Clayton, T.; Barnett, L.; Arnold, J.; Thompson, C.; Hale, R.; Chapman, J.; Urquhart, A.; Gill, P. *Int.J.Legal Med.* **1996,** *109*(4), 186-194.

20. Walsh, P. S.; Erlich, H. A.; Higuchi, R. *PCR Methods Appl.* **1992,** *1*(4), 241-250.

21. Oldroyd, N. J.; Urquhart, A. J.; Kimpton, C. P.; Millican, E. S.; Watson, S. K.; Downes, T.; Gill, P. D. *Electrophoresis* **1995,** *16*(3), 334-337.

22. Cotton, E. A.; Allsop, R. F.; Guest, J. L.; Frazier, R. R.; Koumi, P.; Callow, I. P.; Seager, A.; Sparkes, R. L. *Forensic Sci.Int.* **2000,** *112*(2-3), 151-161.

23. Findlay, I.; Taylor, A.; Quirke, P.; Frazier, R.; Urquhart, A. *Nature* **1997,** *389*(6651), 555-556.

24. Wiegand, P.; Kleiber, M. *Int.J.Legal Med.* **1997,** *110*(4), 181-183.

25. Barbaro, A.; Falcone, G.; Barbaro, A. *Progress in Forensic Genetics* **2000,** *8* 523-525.

26. Gill, P.; Ivanov, P. L.; Kimpton, C.; Piercy, R.; Benson, N.; Tully, G.; Evett, I.; Hagelberg, E.; Sullivan, K. *Nature Genetics* **1994,** *6* 130-135.

27. Schmerer, W. M.; Hummel, S.; Herrmann, B. *Electrophoresis* **1999,** *20*(8), 1712-1716.

28. Burger, J.; Hummel, S.; Hermann, B.; Henke, W. *Electrophoresis* **1999,** *20*(8), 1722-1728.

29. Gill, P.; Whitaker, J.; Flaxman, C.; Brown, N.; Buckleton, J. *Forensic Sci.Int.* **2000,** *112*(1), 17-40.

30. Henegariu, O.; Heerema, N. A.; Dlouhy, S. R.; Vance, G. H.; Vogt, P. H. *Biotechniques* **1997,** *23*(3), 504-511.

31. Larkin, A.; Harbison, S. *Int.J.Legal Med.* **1999,** *112*(6), 388-390.

32. Hartzell, B.; Graham, K.; McCord, B. *Forensic Sci.Int.* **2003,** *133*(3), 228-234.

33. Hochmeister, M. N.; Budowle, B.; Borer, U. V.; Eggmann, U.; Comey, C. T.; Dirnhofer, R. *J.Forensic Sci.* **1991,** *36*(6), 1649-1661.

34. Holland, M. M.; Cave, C. A.; Holland, C. A.; Bille, T. W. *Croat.Med.J.* **2003,** *44*(3), 264-272.