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CONSTRUCTION OF A COST EFFECTIVE NESTED-PCR

REACTION FOR USE WITH THE APPLIED BIOSYSTEMS

AMPFLSTR IDENTIFILER KIT

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CONSTRUCTION OF A COST EFFECTIVE NESTED-PCR REACTION FOR USE WITH THE APPLIED BIOSYSTEMS AMPFLSTR IDENTIFILER KIT

INTERNSHIP PRACTICUM REPORT

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CHAPTER 1

INTRODUCTION AND BACKGROUND

1.1 STR analysis:

Human STR analysis has greatly increased the ability to perform identity testing for many different situations. These situations include, but are not limited to, the identification of individuals involved in violent crimes, establishing paternity, and identification of unknown human remains. The most common type of DNA information currently used for identity testing is the short tandem repeat, or STR. STR testing utilizes the number of repeating units in the DNA to assign an allele. Alleles from several different loci are used to establish a genetic profile. Currently, the United States used a standard of 13 different DNA loci to establish identity. These 13 loci can be typed by using a number of different multiplex kits such as the Applied Biosystems Profiler Plus, Cofiler, and Identifiler Kits [1,2]. The 13 loci were selected based on a number of parameters. Each locus was required to be polymorphic, and a tetranucleotide repeat. The loci also could not display any linkage between each other and extensive population studies had to be conducted to both verify the absence of linkage and to establish allelic frequencies [1].

1.2 PCR and Nested PCR:

The polymerase chain reaction (PCR) is perhaps one of the most important breakthroughs in forensic science. Discovered in 1985, this method has allowed scientists to obtain a DNA profile on very minute or degraded samples. PCR utilizes a thermostable polymerase enzyme coupled with specific temperature cycles to make multiple copies of specific regions of the DNA. DNA profiles can be obtained from less than 1ng of DNA. To simplify the DNA profiling process, PCR allows for multiple loci to be amplified simultaneously in a multiplex reaction. This is accomplished by adding two or more primer sets to the reaction mixture. To achieve a multiplex reaction, the primers must be compatible with one another, having similar annealing temperatures and limited complementary regions to each other, as well as DNA regions that one does not wish to amplify. One drawback to multiplex PCR is the time involved in optimization. Because multiple primer annealing events must occur simultaneously without interfering with each other, the reaction components, the primer concentrations and thermal cycling conditions must be optimized, or adjusted in order to have a reproducible and balanced reaction [1]. This process is not, however required for all laboratories that conduct DNA testing; various optimized and validated kits available on the market such as Applied Biosystems Profiler Plus, Cofiler, and Identifiler Kits [2].

Nested PCR is another technique that can be used in various avenues of research and DNA testing. It is essentially two PCR reactions run one after another. The first reaction amplifies specific region(s) of DNA to make many copies. The second PCR reaction involves the amplification of a target within the product of the first reaction. This double amplification makes many times more copies of the product and also has the added

benefit of a more specific final product. Currently Nested PCR is rarely used in human identity testing, but is often used in the identification of viruses, such as human papillomavirus (HPV), HIV and other microorganisms [3, 4, 5

].

1.3 Applied Biosystems AmpflSTR Identifiler Kit:

The AmpflSTR Identifiler Kit is a commercially available DNA typing kit that includes the primers, reaction mix (buffer, dNTPs, $MgCl_2$) and AmpliTaq Gold Polymerase needed to obtain a DNA profile. The kit tests for 16 different allelic loci including D8S1179, D21S11, D7D820, CSF1PO, D3S1358, THO1, D13S317, D16S539, D2S1338, D19S433, vWA, TPOX, D18S51, D5S818, FGA and Amelogenin. Identifiler is used mainly in paternity testing, reference sample testing, and forensic testing. The kit is expensive; at $3000 a kit, under manufacturer recommendations it has the ability to test 200 samples. The cost per samples is approximately $15 [2]. There is a need in many different laboratories, especially paternity laboratories to reduce the cost of testing.

1.4 Purpose:

The goal of this research was the construction of a more cost effective method of utilizing the Applied Biosystems Identifiler Kit. Across the country there is a large backlog of samples that need to be processed in order to obtain a genetic profile. If these samples could be tested using a more cost effective method, more funding could be directed to other endeavors. Paternity testing, as well as some research endeavors could be conducted at a fraction of the cost, leaving resources for other projects or additional staff. Although it would be inadvisable to use this technique on forensic samples, the implications on paternity and research samples would be positive. This research

attempted to design a nested PCR reaction and subsequently dilute the Applied Biosystems Primers in order to reduce the cost. The first step was to design new primers for the first round of PCR, followed by testing of those primers. The new primers then required optimization so that they all worked effectively together. After optimization was accomplished, the Identifiler primers were diluted until loci began dropping out of the genetic profile.

CHAPTER 2

MATERIALS AND METHODS

2.1 Primer Design:

Genetic sequences for the 16 loci found in the ABI AmpflSTR Identifiler Kit, D8S1179, D21S11, D7D820, CSF1PO, D3S1358, THO1, D13S317, D16S539, D2S1338, D19S433, vWA, TPOX, D18S51, D5S818, FGA and Amelogenin, were obtained using STRbase [6] and GenBank [7]. Amplification primer sequences were also obtained using STRbase and were located on the DNA sequence. New primers that would amplify a larger region of DNA than the Identifiler primers were designed using the Primer3 online interface [8]. The new primers were given a limit of an annealing temperature (Tm) of greater than 57°C and less than 62°C, a limit of product size to between 500 and 600 bases, and a limit of length between 20 and 30 bases. Once designed, a Blast [7] search was conducted on each of the primers to insure that there were no complementary or homologous sequences in the genome that would cause unspecific amplification products and that the primers were human specific. Following the evaluation, the primers were ordered from Operon Biotechnologies Inc. [9]. Primer sequences and product sizes are listed in Table 1. The custom primers were rehydrated and diluted to a concentration of 100μM, from which concentrations of 50μM, 5μM and 0.1μM were produced [10].

Table 1: Primer sequences and product sizes of the oligonucleotides designed to amplify the Applied Biosystems AmpflSTR Identifiler loci.

Primer	Sequence	Product Size
D8-U	taggggagatagcagctgga	568
D8-L	gtgcattgttgttgggaatg	
D21-U	caagttctctggcctttgga	545
D21-L	tgtctggcacccagtaaaaa	
D7-U	ttacacgatggaaggcatca	523
D7-L	ttgggtcaagtggttctcct	
CSF-U	gaggagagtgatggggtcag	553
CSF-L	ctctaggggcagtgatgctc	
D19-U	ggtggatggtgaacaaaagg	549
D19-L	tcccagcactttggaagact	
VWA-U	tcatttacttcaagcccctca	538
VWA-L	cacaggttagatagattagacagacag	
TPOX-U	cttccccaagctaactgtgc	532
TPOX-L	gtgtgctcacaccaagcact	
D18-U	cgacaaaaggctatgggcta	573
D18-L	cacgtgcctgtagtctcagc	
D3-U	cccataccctgagccataga	581
D3-L	agcactttggaaggctgaga	
THO1-U	cggaatgtggaaacaaatcc	557
THO1-L	tccctgagaaggtacctgga	
D13-U	tttgggtaggaaaaagagtgga	539
D13-L	aggctgactcattggaaagc	
D16-U	caagcgaaagtgatgccata	505
D16-L	accgggaggatgactgtgt	
D2-U	ttggggagctgtttcagaac	521
D2-L	gtccttcgattgctttgagc	
D5-U	ccatctggatagtggacctca	568
D5-L	acgcctttcctctgaagtga	
FGA-U	agctggcaagtgagtgatcc	597
FGA-L	actttgcgcttcaggacttc	
AMEL-U	tcctttaatgtgaacaattgcat	581
AMEL-L	agcagaggcaagcaagagac	

2.2 Touchdown PCR and Primer Quality Test:

Each primer pair was added into singleplex reaction with the following reagents: 5µl of 0.1 µM primer pair mix; 2.5µl master mix (includes 10X buffer, 10 µM dNTP, Taq polymerase); 2.5µl template DNA (ABI control DNA 9947A). The reaction mixes were then amplified using PCR with the following parameters: 95°C for 1 min; 10 cycles of 93°C for 45 seconds, 60°C for 1 minute, and 70°C for 1 minute; 30 cycles of 94°C for 45 seconds, 55°C for 1 minute, and 70°C for 1 minute. Following PCR, the amplified products were electrophoresed on a 1% agarose gel in order to verify that all primers would produce a PCR product.

2.3 Gradient PCR:

Gradient PCR is a method that uses a thermal cycler capable of running PCR programs with different temperatures in each column of sample wells. The ability to use different temperatures makes gradient PCR a good tool to find the optimal PCR annealing temperature for multiple primers. Because of this capability, gradient PCR was used to establish a common annealing temperature that would amplify all of the newly designed primers. The temperature could then be used to design a multiplex PCR reaction. In the gradient PCR reaction, a 384 well plate is used and each column of the plate can be set at a different temperature during the amplification cycle(s). Twelve different annealing temperatures were tested using the gradient PCR method. The temperatures used were 54.8°C, 55.1°C, 55.9°C, 57.1°C, 58.6°C, 60.3°C, 62.1°C, 63.8°C, 65.4°C, 66.7°C, 67.6°C, and 68.0°C.

PCR was completed on an Eppendorf ep384 Gradient Mastercycler using the master mix described in section 2.2 and with the following PCR reaction conditions:

95°C for 1 minute; 30 cycles of 94°C for 45 seconds, gradient temperature for 1 minute, and 70°C for 1 minute; 72°C for 5 minutes. Subsequent to the completion of PCR, each of the products was processed using 1% agarose gel electrophoresis.

2.4 Creation of a PCR Primer Multiplex for Nested PCR:

The initial PCR multiplex was created using a concentration of 0.1μM of each primer pair as recommended by Butler [10]. Because an annealing temperature of between 62°C and 64°C amplified the DNA in all primer pairs, a temperature of 63°C was chosen for the annealing temperature in this initial reaction. Applied Biosystems Control DNA was amplified in this reaction under the following reaction conditions: 95°C for 1 minute; 30 cycles of 94°C for 45 seconds, 63°C for 1 minute, and 70°C for 1 minute; 72°C for 5 minutes. The reaction was a 10 μl reaction volume consisting of 2.5μl master mix, 5μl primer and 2.5μl DNA. The master mix was made using the following formula:

Number of samples X 5.5μl water

Number of samples X 4.5μl 10X buffer

Number of samples X 0.9μl 10mM dNTP

Number of samples X 0.3 μl Hotmaster Taq Polymerase

After completion of PCR, an ExoSAP-IT (US Biochemical) reaction was conducted by adding 4μl of ExoSAP-IT to the amplified product. ExoSAP-IT cleans up the reaction by dephosphorlating the dNTPs and digesting single stranded DNA (i.e. unbound primers). The samples were placed on the Applied Biosystems 9700 thermal cycler and the following reaction conditions were used: 37°C for 15 minutes and 80°C for 15 minutes.

Following ExoSAP-IT treatment, 10µl of each sample was used as the template DNA for a PCR reaction using the Applied Biosystems AmpflSTR Identifiler kit. The thermal cycling conditions and reagents used were those specified in the manufacturer recommendations. The following PCR parameters were used: 95°C for 11 minutes; 28 cycles of 94°C for 1 min, 59°C for 1 minute, and 72°C for 1 minute; 60°C for 60 minutes. The resulting profile was electrophoresed using an Applied Biosystems (ABI) Prism 3700 Genetic Analyzer and analyzed with the GeneScan/Genotyper software package.

2.5 Optimizing the PCR Reaction:

In order to get somewhat balanced peaks, the concentrations of primers used in the first round of PCR were adjusted. Primer concentrations were increased for loci with peaks that displayed a low RFU (i.e. less than 500) and the concentrations were decreased for loci displaying a high RFU (i.e. above 6000). In addition to the adjustment of primer concentrations, the annealing temperature and the dilution of the first round PCR product was also adjusted. Table 2 depicts the primer concentrations for each nested PCR experiment. The reaction conditions for each of the Nested-PCR experiments are listed below. Each of the experiments used the formula listed previously in section 2.4 for the master mix in the first round of PCR and each of the experiments used manufacturer guidelines for the Identifiler portion (second round). Applied Biosystems Control DNA 9947A was used as the DNA template for all reactions. The amplification products for all experiments were electrophoresed on the ABI 3700 Genetic Analyzer and analyzed with GeneScan/Genotyper Software.

Experiment 1 used primer mix 1 and the procedure as described in section 2.4. Primer concentrations can be found in Table 2. In Experiment 2, DNA was amplified on

the first round of PCR with a multiplex primer mix (primer mix 1) as described in Table 2. A singleplex reaction of the THO1 locus was also amplified with a 0.1μM primer concentration. The THO1 singleplex was amplified alone to verify that the primer pair worked. The multiplex, as well as the THO1 singleplex, were amplified for the first round of PCR under the following parameters: 95°C for 1 minute; 30 cycles of 94°C for 45 seconds, 63°C for 1 minute, and 70°C for 1 minute; 72°C for 5 minutes. To the first PCR product, 4μl of ExoSAP-IT was added and the reaction was completed using the following conditions: 37°C for 60 minutes and 99°C for 15 minutes. After completion of the ExoSAP-IT reaction, the PCR products were diluted to 1:5, 1:10 and 1:20 with diH_20. The samples were then processed using the Identifiler kit under manufacturer guidelines.

In Experiment 3, the primer concentrations were adjusted for the first round of PCR (primer mix 2) as shown in Table 2. The annealing temperature was increased in an attempt to reduce unspecific product. The PCR reaction parameters are as follows: 95°C for 1 minute; 30 cycles of 94°C for 45 seconds, 64°C for 1 minute, and 70°C for 1 minute; 72°C for 5 minutes. The ExoSAP-IT reaction conditions were not changed. The PCR products were then diluted sequentially to 1:20, 1:30, 1:40, 1:50, 1:60, 1:70 and 1:80 with diH_20. The diluted products were processed using the Identifiler Kit under manufacturer recommended conditions.

Experiment 4 used adjusted primer concentrations (primer mix 3, Table 2) for the first round of PCR. PCR reaction conditions were identical to those in experiment 3 and ExoSAP-IT reaction conditions remained unchanged. In order to decrease the RFU values of the final product, the PCR products were then diluted sequentially to 1:100,

1:200 and 1:300 with diH_20. The diluted products were processed with the Identifiler kit as before.

Two different primer mixes were used in Experiment 5. The first (primer mix 4) greatly increased the concentration of the primers that failed to generate amplified product, while the remainder of the primers were kept at the concentrations used in Experiment 4. The second primer mix (primer mix 5) used small adjustments to the concentrations used in Experiment 4. The primer concentrations in primer mix 4 and primer mix 5 are listed in Table 2. In addition, three singleplex reactions of primers that failed to amplify product (D7S820, D21S11 and THO1) when included in a multiplex were run. The samples were amplified and following this an ExoSAP-it reaction was conducted. All PCR products were then diluted to 1:100, 1:250, 1:500, 1:750 and 1:1000 with diH_20. The diluted products were processed with the Identifiler Kit as previously described.

The primer concentrations in Experiment 6 were adjusted based on the results of Experiment 5. Primer concentrations for primer mix 6 are listed in Table 2. PCR was completed using a reduced annealing temperature in an attempt to recover alleles that dropped out at 64°C. Reaction conditions are as follows: 95°C for 1 minute; 30 cycles of 94°C for 45 seconds, 63.5°C for 1 minute, and 70°C for 1 minute; 72°C for 5 minutes. The samples were then put through an ExoSAP-IT reaction. The second round of PCR used the Identifiler kit as previously described.

Table 2: Primer concentrations used in the PCR optimization experiments. Primer sequences are described in Table 1

Locus	Primer Mix 1 (μM)	Primer Mix 2 (μM)	Primer Mix 3 (μM)	Primer Mix 4 (μM)	Primer Mix 5 (μM)	Primer Mix 6 (μM)
D8S1179	0.1	0.05	0.05	0.05	0.05	0.05
D21S11	0.1	0.1	0.12	1.0	0.2	0.2
D7S820	0.1	0.1	0.08	0.1	0.2	0.2
CSF1P0	0.1	0.05	0.05	0.05	0.08	0.08
D3S1358	0.1	0.1	0.12	0.1	0.1	0.15
THO1	0.1	0.1	0.12	1.0	0.15	1.0
D13S317	0.1	0.05	0.12	0.12	0.12	0.11
D16S539	0.1	0.05	0.08	0.08	0.08	0.07
D2S1338	0.1	0.05	0.08	0.08	0.08	0.08
D19S433	0.1	0.1	0.1	0.1	0.08	0.07
vWA	0.1	0.1	0.1	0.1	0.1	0.09
TPOX	0.1	0.05	0.05	0.05	0.05	0.07
D18S51	0.1	0.1	0.1	0.1	0.1	0.12
Amelogenin	0.1	0.1	0.12	0.15	0.15	0.13
D5S818	0.1	0.1	0.12	0.12	0.12	0.11
FGA	0.1	0.1	0.12	0.1	0.1	0.1

2.6 Dilution of Applied Biosystems AmpflSTR Identifiler Primers:

Dilution of the Identifiler primers consisted of three different experiments. All PCR reactions were carried out using the same reaction parameters. Round one of PCR is as follows: 95°C for 1 minute; 30 cycles of 94°C for 45 seconds, 63.5°C for 1 minute and 70°C for 1 minute; 72°C for 5 minutes. The first round of PCR used a 10μl reaction volume as described in section 2.4. The ExoSAP-IT reaction used the following parameters: 14μl reaction volume; 37°C for 60 minutes and 99°C for 15 minutes. Round two PCR (Identifiler) used manufacturer recommended PCR parameters: 95°C for 11 minutes; 28 cycles of 94°C for 1 minute, 59°C for 1 minute, 72°C for 1 minute; 60°C for

60 minutes. Round two PCR also used a 25µl reaction volume as detailed later in this section.

In the first Identifiler experiment, the first round of PCR used primer mix 6 as describe in Table 2. Ten DNA samples underwent the first round PCR (reaction conditions as described above). After amplification, five samples were diluted to 1:2000 with diH_20 and five were left undiluted. The AmpflSTR Identifiler primers were diluted with diH_20 to 1:2, 1:4, 1:8, 1:16 and 1:32. Each of these Identifiler primer dilutions was used to amplify one each of the undiluted first PCR product and the 1:2000 dilution of the first PCR product. The reaction volume for the second round of PCR was kept consistent with that described in Applied Biosystems manufacturer recommendations, with 15µl of master mix and 10µl of template DNA. The formula for the master mix is as follows:

Master Mix:

Number of samples X 10.5µl reaction mix

Number of samples X 5.5µl primers (diluted)

Number of samples X 0.5µl AmpliTaq Gold Polymerase

The second experiment with the dilution of Identifiler kit primers had the purpose of further diluting the primers to find a point at which the reaction failed. This experiment involved the production of two new primer mixes (primer mix 7 and primer mix 8) for the first round of PCR, in addition to primer mix 6. Primer concentrations for theses primer mixes can be found in Table 3. A total of 9 samples were tested. Three samples using each of the primer mixes 6, 7 and 8 were amplified on the first PCR reaction as previously described. The products of the first round of PCR were not diluted due to poor results in the previous experiment. In the second round of PCR, the three

samples for each primer mix were run with the AmpflSTR Identifiler primers diluted to 1:32, 1:64 and 1:128 with diH_20.

The third and final Identifiler dilution experiment utilized a new primer mix (primer mix 9) in addition to primer mix 6. The individual primer concentrations for these are detailed in Table 3. In this experiment, 16 samples were processed. The first round of PCR was conducted as previously described with 8 samples using primer mix 6 and 8 samples using primer mix 9. The second round of PCR consisted of 8 samples using the Applied Biosystems master mix and 8 samples using the Identifiler primers, but the same reaction mix as used in round one of PCR (as described in section 2.4). The master mix for the Eppendorf HotMaster Taq was kept at 25µl (10µl reaction mix, 5µl primer and 10µl template DNA). The reactions are listed below:

1. Primer mix 6; 1:30 primer dilution; ABI master mix
2. Primer mix 6; 1:50 primer dilution; ABI master mix
3. Primer mix 6; 1:70 primer dilution; ABI master mix
4. Primer mix 6; 1:90 primer dilution; ABI master mix
5. Primer mix 9; 1:30 primer dilution; ABI master mix
6. Primer mix 9; 1:50 primer dilution; ABI master mix
7. Primer mix 9; 1:70 primer dilution; ABI master mix
8. Primer mix 9; 1:90 primer dilution; ABI master mix
9. Primer mix 6; 1:30 primer dilution; Hotmaster Taq (Eppendorf) master mix
10. Primer mix 6; 1:50 primer dilution; Hotmaster Taq (Eppendorf) master mix
11. Primer mix 6; 1:70 primer dilution; Hotmaster Taq (Eppendorf) master mix
12. Primer mix 6; 1:90 primer dilution; Hotmaster Taq (Eppendorf) master mix

13. Primer mix 9; 1:30 primer dilution; Hotmaster Taq (Eppendorf) master mix

14. Primer mix 9; 1:50 primer dilution; Hotmaster Taq (Eppendorf) master mix

15. Primer mix 9; 1:70 primer dilution; Hotmaster Taq (Eppendorf) master mix

16. Primer mix 9; 1:90 primer dilution; Hotmaster Taq (Eppendorf) master mix

Table 3: Primer concentrations of the first round PCR primer mixes used in the Identifiler dilution experiments (section 2.6)

Locus	Primer mix 6 (μM)	Primer mix 7 (μM)	Primer mix 8 (μM)	Primer mix 9 (μM)
D8S1179	0.05	0.05	0.05	0.05
D21S11	0.2	0.2	0.2	0.2
D7S820	0.2	0.2	0.2	0.2
CSF1P0	0.08	0.08	0.08	0.08
D3S1358	0.15	0.15	0.15	0.15
THO1	1.0	1.0	1.0	1.0
D13S317	0.11	0.11	0.11	0.11
D16S539	0.07	0.07	0.07	0.07
D2S1338	0.08	0.08	0.08	0.07
D19S433	0.07	0.07	0.07	0.07
vWA	0.09	0.09	0.09	0.09
TPOX	0.07	0.07	0.07	0.06
D18S51	0.12	0.5	1.0	0.5
Amelogenin	0.13	0.5	1.0	0.2
D5S818	0.11	0.11	0.11	0.11
FGA	0.1	0.1	0.1	0.11

2.7 Reproducibility Study:

An abbreviated reproducibility study was conducted to verify the results from previous experiments and to insure that the results were indeed reproducible. All parts of the reproducibility study used primer mix #6 as described in Table 2 and Table 3 and reaction parameters as follows: 95°C for 1 minute; 30 cycles of 94°C for 45 seconds, 63.5°C for 1 minute and 70°C for 1 minute; 72°C for 5 minutes. The first round of PCR

was followed by an ExoSAP-IT cleanup reaction before the second round of PCR which used a 1:50 dilution of the AmpflSTR Identifiler primers.

The first reproducibility study utilized 18 samples; 9 using the Applied Biosystems reaction mix and AmpliTaq Gold and 9 using the Eppendorf Hotmaster Taq Polymerase and master mix (both described in section 2.5). The second reproducibility study used 24 samples from three different sources (8 samples each). Four of each sample was amplified for the second round of PCR using the Applied Biosystems reaction mix and four of each was amplified using the Eppendorf Hotmaster reaction mix. Results from both experiments were obtained using the Applied Biosystems 3700 Genetic Analyzer and were analyzed using the GeneScan/Genotyper software package.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Testing the Primers:

The first test of the primers involved touchdown PCR as described in section 2.2. Most of the loci yielded positive results for the 40 cycle reaction; FGA, D21S11 and D5S818 had no product on the gel indicating that the temperatures used were not conducive for amplification. The loci D16S539, D18S51, D2S1338, D13S317, Amelogenin, THO1, TPOX, vWA, D3S1358, and D8S1179 produced strong electrophoretic bands, an indication that the temperatures used in the touchdown PCR amplify the product. The loci D19S433, CSF1P0 and D7S821 produced amplified product, but there were some unspecific bands. All successful amplifications products produced electrophoretic bands in the desired range of 500 to 600 base pairs. The results of the electrophoresis can be seen in Figure 1.

3.2 Gradient PCR:

PCR was conducted in a gradient thermal cycler to evaluate 12 different annealing temperatures. All primers amplified the DNA at a temperature between 62°C and 64°C, indication that the optimal multiplex annealing temperature would be in this temperature range. Results from this experiment are summarized in Table 4 and an example of the agarose gel results are shown in Figure 2. All amplified products were found to be in the

desired size range of between 500 and 600 base pairs. Some unspecific product bands were observed, but they were outside of the 62°C to 64°C optimal range.

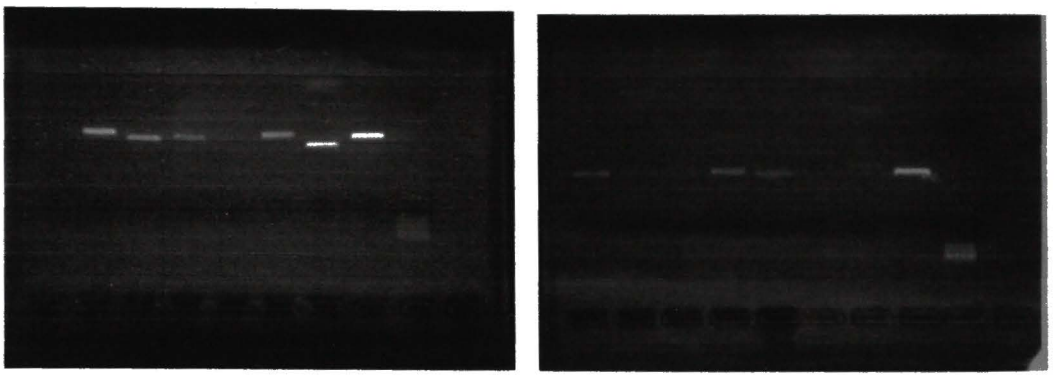

Figure 1: Agarose gel electrophoresis of amplified product from touchdown PCR. Loci are as follows: Gel #1—Lane 1: FGA, Lane 2: D16S539, Lane 3: D18S51, Lane 4: D19S433, Lane 5: D21S11, Lane 6: D2S1338, Lane 7: CSF1PO, Lane 8: D13S317, Lane 9: Ladder, Lane 10: blank. Gel #2—Lane 1: Amelogenin, Lane 2: THO1, Lane 3: TPOX, Lane 4: vWA, Lane 5: D3S1358, Lane 6: D5S818, Lane 7: D7S821, Lane 8: D8S1179, Lane 9: Ladder, Lane 10: blank

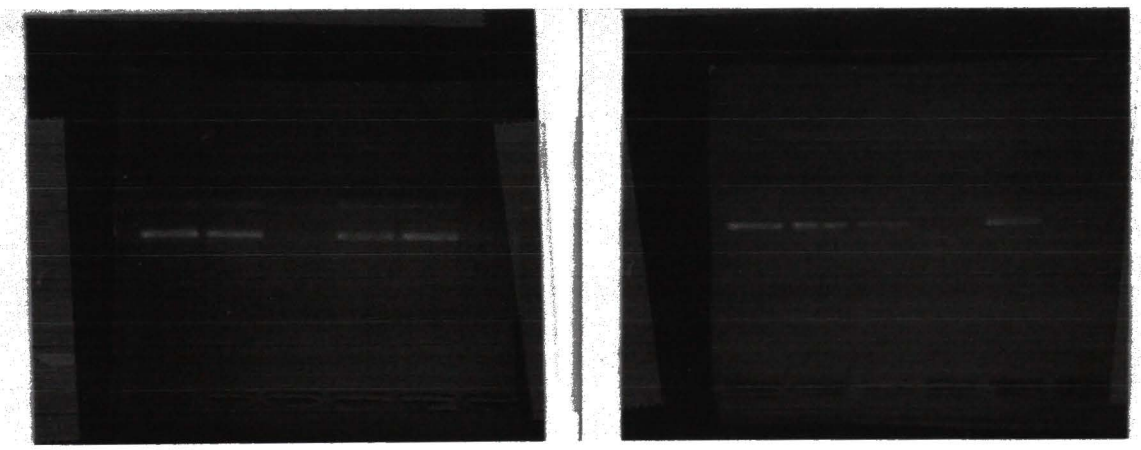

Figure 2: Example of the agarose gel results of gradient PCR. The locus shown is vWA and the temperatures in the lanes are as follows: 54.8°C, 55.1°C, 55.9°C, 57.1°C, 58.6°C, 60.3°C, 62.1°C, 63.8°C, 65.4°C, 66.7°C, 67.6°C, 68.0°C.

Table 4: Agarose gel electrophoresis results from the gradient PCR reaction. + indicates a weak band, ++ indicates a moderate band, +++ indicates a strong band, and --- indicated that no band/result was observed.

	TEMPERATURE (°C)											
LOCI	54.8	55.1	55.9	57.1	58.6	60.3	62.1	63.8	65.4	66.2	67.6	68.0
D13	+++	+++	+++	+++	+++	++	+++	+++	+++	+++	+++	+++
CSF	++	---	---	++	++	+++	+++	+	+	++	++	++
D2	---	+++	+++	+++	+++	+++	+++	+++	++	+++	+++	++
D21	---	+	+	+	+	+	++	++	++	++	+	+
D19	+++	+++	+++	---	+++	+++	+++	+++	+++	+++	+++	---
D18	+	+++	++	++	+	++	+	+++	+++	+++	---	++
D16	++	+++	+++	++	+	+	++	+++	+++	+++	+++	+
FGA	+++	+++	+++	+++	+++	++	+++	+++	+++	+++	+++	+
D8	+++	+++	+	+++	+++	+++	+++	+++	+++	+++	+++	+++
D7	+++	+++	+++	+++	+++	+++	++	++	++	+++	+++	+++
D5	+	+	++	++	++	++	++	+	+	++	+	++
D3	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
VWA	+++	+++	---	+++	+++	---	+++	++	++	---	+++	---
TPOX	---	---	---	---	---	---	+	+	+	+	+	+
THO1	---	---	---	---	---	---	+	+	+	---	---	---
AMEL	---	+	+	---	+	+	+	+	---	---	---	---

3.3 Primer Optimization:

When designing the multiplex, all primers were added in the standard starting concentration of 0.1μM as described by Butler [10]. The first round of PCR was the only portion of the experiment that underwent adjustments to decrease the number of variables affecting the results. The second round PCR reactions utilized the Identifiler kit under Applied Biosystems manufacturer recommendations. The results of the first experiment were promising; all loci with the exception of THO1, D21S11, and D18S51 were successfully amplified. There were however, a number of unspecified products, high stutter peaks (some of the stutter peaks were called by GeneScan/Genotyper), and very high RFU values of greater than 10,000 (Figure 3). It was determined that the first amplification product required dilution to decrease stutter and the RFU values.

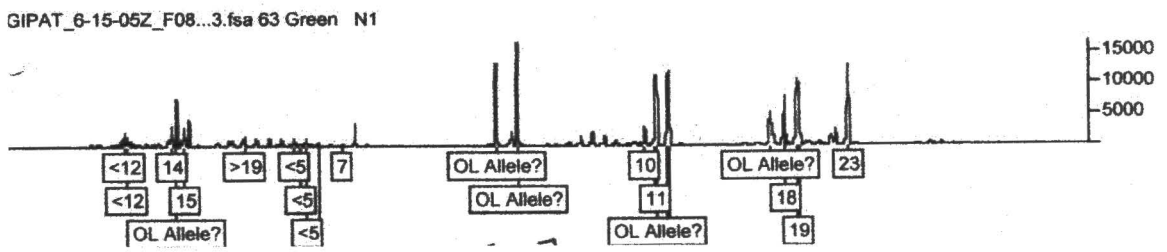

Figure 3: Results for the Green Dye in primer optimization experiment 1. Loci are D3S1358, THO1, D13S317, D16S539 and D2S1338.

In the second experiment, the first round PCR products were diluted to 1:20 with diH_20 or less. In addition to the multiplex reaction, THO1 was run as a singleplex reaction to determine if correct allele calls were being made. As in the previous multiplex experiment THO1, D21S11, and D18S51 did not yield the desired results and the stutter as well as the RFU values were again very high. The THO1 locus amplified

correctly in a singleplex reaction, indicating that the primer concentration was either too low, or that there were primer interactions (Figure 4). It was determined that the primer concentrations would require adjustment and that the initial product would have to be diluted further.

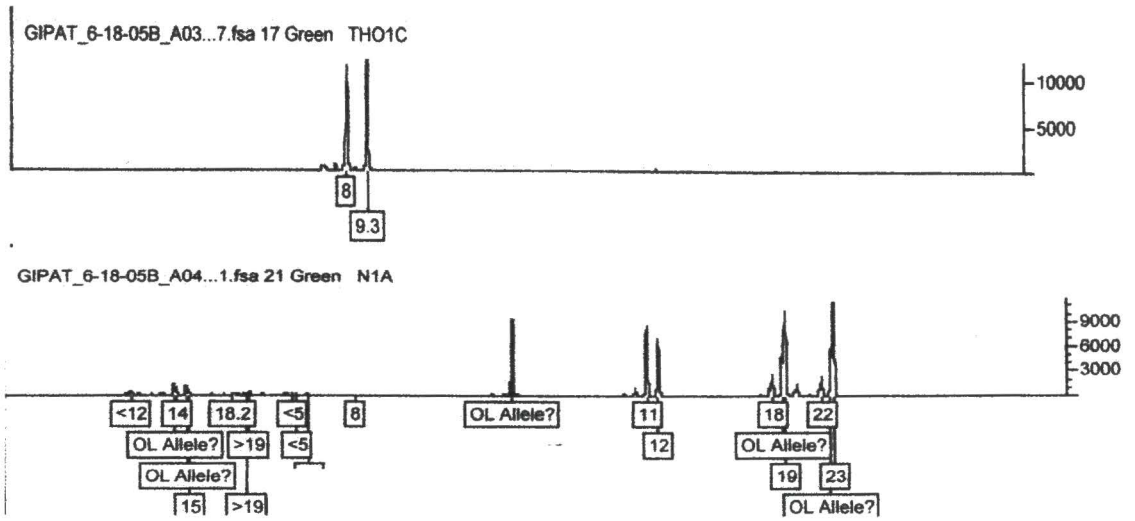

Figure 4: Results for the green dye 1:20 dilution in primer optimization experiment 2. The first electropherograms is the result for singleplex THO1. Loci in second electropherogram are D3S1358, THO1, D13S317, D16S539, and D2S1338.

In experiment 3, the primer concentrations were adjusted by +/- 0.05μM. The loci with weak signal were increased to a concentration of 0.15μM, the loci with a very high RFU value were decreased to a concentration of 0.05μM and the moderate signal loci were unchanged at 0.1μM. The product from the first round of PCR was diluted to 1:80 or less. To increase the specificity of the reaction, the annealing temperature was increased to 64°C for the first round of PCR. Some of the same loci remained problematic. D21S11 completely dropped out, perhaps due to the temperature change, and the signal and specificity of THO1 remained inadequate. D13S317 and D16S539

showed a larger amount of unspecific product than previously, but improvement was seen in D18S51. The stutter percentage remained very high, above 20% on most loci as did the RFU values of most loci (Figure 5). As before, primer concentration adjustments and further dilution was determined to be required.

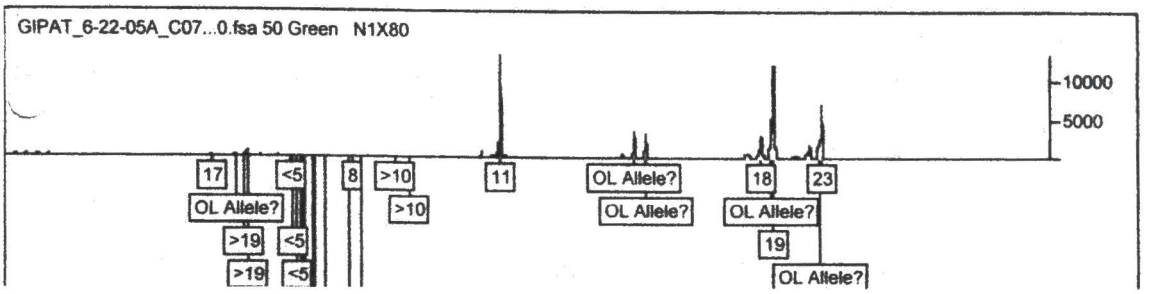

Figure 5: Result for the green dye 1:80 dilution in primer optimization experiment 3. Loci are D3S1358, THO1, D13S317, D16S539, and D2S1338.

Smaller adjustments were made in experiment four with primer concentration changes of +/- 0.03μM or less. The first PCR products were also diluted to a 1:300 or less. The locus D21S11 still displayed dropout and dropout also occurred in D7S820. THO1 locus remained problematic with unspecific product. The D3S1358 locus displayed unspecific product, probably a product of bleedthrough from another locus. Bleedthrough was assumed because the locus D19S433 had a very high RFU (above 15,000 RFU) and was in the same size range. All other loci displayed great improvement. Stutter was less prevalent, being below 20% in most cases, but still significant and RFU values remained very high (above 8000) for most loci (Figure 6). It was determined that even further dilution was needed as was more adjustments to primer concentrations.

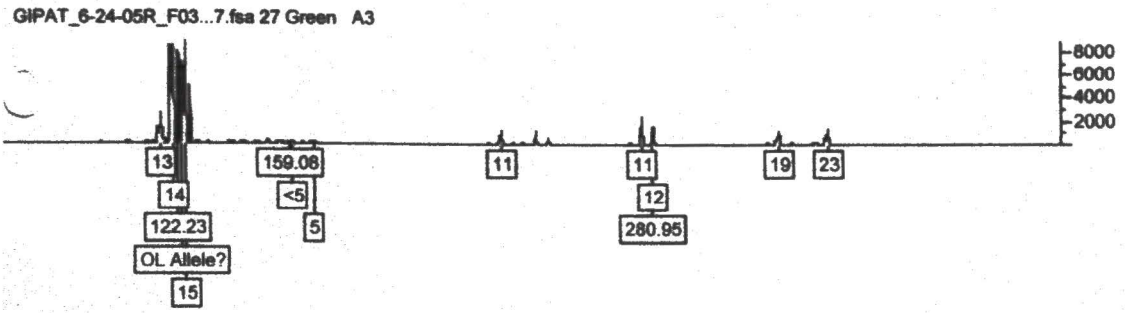

Figure 6: Results for the green dye 1:300 dilution in primer optimization experiment 3. Loci are D3S1358, THO1, D13S317, D16S539 and D2S1338.

Experiment five involved two different experiments. For the first, because successful amplification had not yet occurred on D21S11 and THO1, their primer concentrations were increased 10 fold from the original 0.1μM to 1.0μM. Other adjustments to the overall multiplex were also made, increasing the primer concentrations of loci with a weak signal and decreasing the concentration of the loci with very high RFU values. The second experiment adjusted the primer concentrations by small amounts, 0.1μM adjustment or less. The annealing temperature was decreased to 63.5°C to recover lost alleles. The dilution used of 1:1000 was the most successful, although the RFU values remained high (above 8,000 on some loci) as did the stutter values (above 15% in most cases). Because of the higher primer concentration for D21S11, the locus was amplified successfully. The THO1 locus alleles were also correctly called (Figure 7).

Experiment 6 was by far the most successful. Primer concentrations were adjusted again in small increments and the first round amplification product dilution of 1:2000 was the most successful. Both THO1 and D21S11 were recovered and correctly called, but in some instances, the signal for the loci D18S51 and Amelogenin became very weak.

For an example of the results, see Figure 8. Due to time constraints and despite the problems with D18S51 and with Amelogenin, the research was taken to the next step: dilution of the ABI AmpflSTR Identifiler primers.

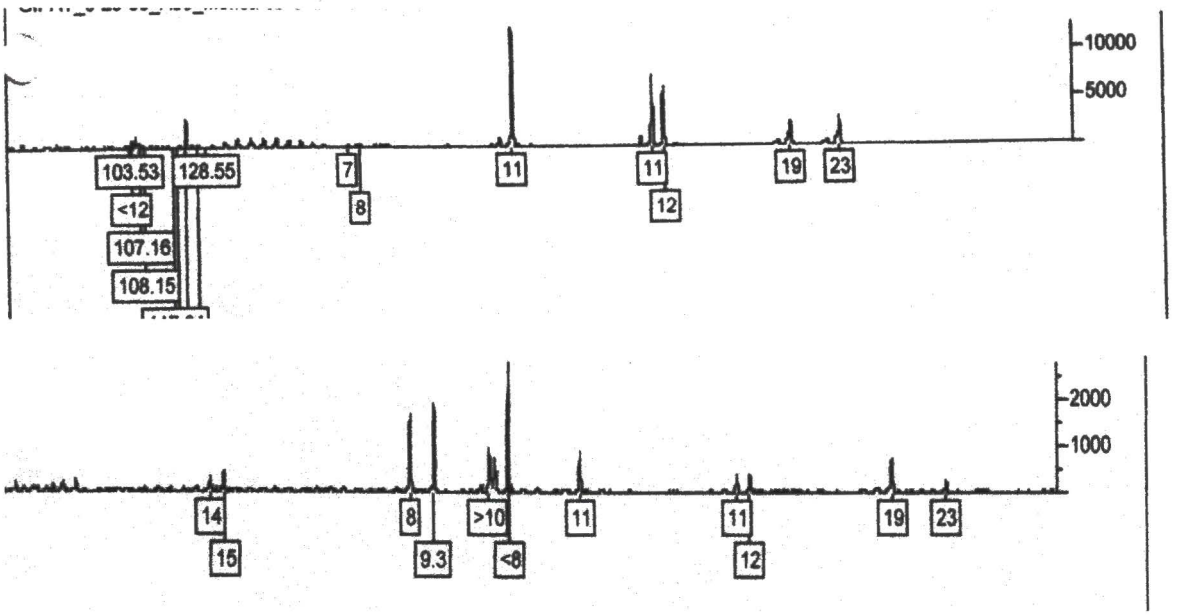

Figure 7: Results for the green dye in primer optimization experiment 5. The first electropherogram is the result for the modest adjustment of primer concentrations. The second electropherograms is the result of the large increase of the non-working primer concentrations

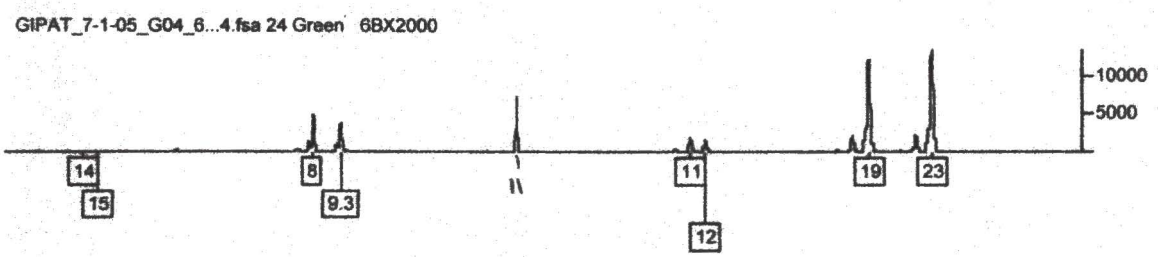

Figure 8: Result for the green dye 1:2000 dilution in primer optimization experiment 6. Loci are D3S1358, THO1, D13S317, D16S539, and D2S1338

3.4 Dilution of Applied Biosystems AmpflSTR Identifiler Primers

The overall goal of this research was to reduce the amount of Identifiler primers used to obtain a DNA profile. In all experiments, primer mix #6 (Table 3) was the most successful and will therefore be the only one reported. The first experiment involved running the samples with diluted Identifiler primers and undiluted first round PCR product as well as samples with diluted Identifiler primer and diluted (1:2000) first round PCR product. The samples with diluted first round PCR product failed in most cases (all except for the 1:2 and 1:4 Identifiler dilutions) with a very weak RFU value (200 RFU or less) and allelic dropout. The samples with undiluted first round PCR product were successful in all cases up to the maximum dilution in this experiment, 1:32 dilution. The samples using the more dilute Identifiler primers actually displayed less stutter and had overall cleaner results (Figure 9), probably because less product was being amplified in the second round of PCR. All loci amplified in this experiment, although Amelogenin and especially D18S51 gave weak signals.

The second dilution experiment utilized Identifiler primers diluted to 1:32, 1:64 and 1:128. The 1:32 dilution was successful for all loci and the 1:64 dilution was successful for all loci with exception to D18S51. In the 1:128 dilution, all loci dropped out except D7S820, CSF1P0, D13S317, D16S539, and D2S1338. The loci D19S433, vWA and TPOX were also present in the 1:128 dilution, but had RFU values of 150 or below and were therefore not called. At a later time, and after further optimization of the first round of PCR, it could be possible to use a dilution of 1:64 or greater. The results for the 1:64 dilution is shown in Figure 10.

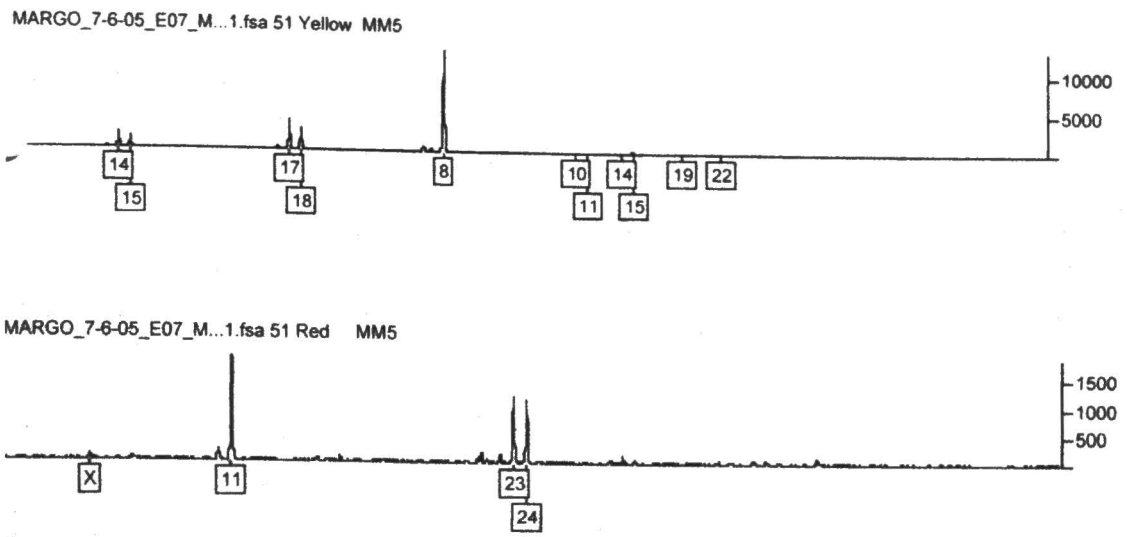

Figure 9: Result of Yellow and red dye for Identifiler dilution experiment 1, undiluted first round PCR products and 1:32 dilution of Identifiler primers. Loci displayed are: first electropherogram—D19S433, vWA, TPOX, and D18S51; second electropherogram—Amelogenin, D5S818, and FGA.

In the third dilution experiment, it was determined that testing was needed to determine if the reaction would be successful with an alternative reaction mix. The reason for this is that the Applied Biosystems reaction mixture (which is available in the Identifiler kit) is not available separately and the reaction mixture would run out prior to the exhaustion of the primer set. Using the Applied Biosystems reaction mixture, all reactions were successful for all loci (with the exception to a weak RFU value for D18S11) in dilutions of 1:50 or less. The 1:70 and 1:90 dilutions were unsuccessful with most of the alleles displaying dropout or very low RFU values (200 RFU or less). The Eppendorf HotMaster Taq reaction actually gave better success than the Applied Biosystems mastermix with reduced stutter and generally cleaner electropherograms (Figure 11).

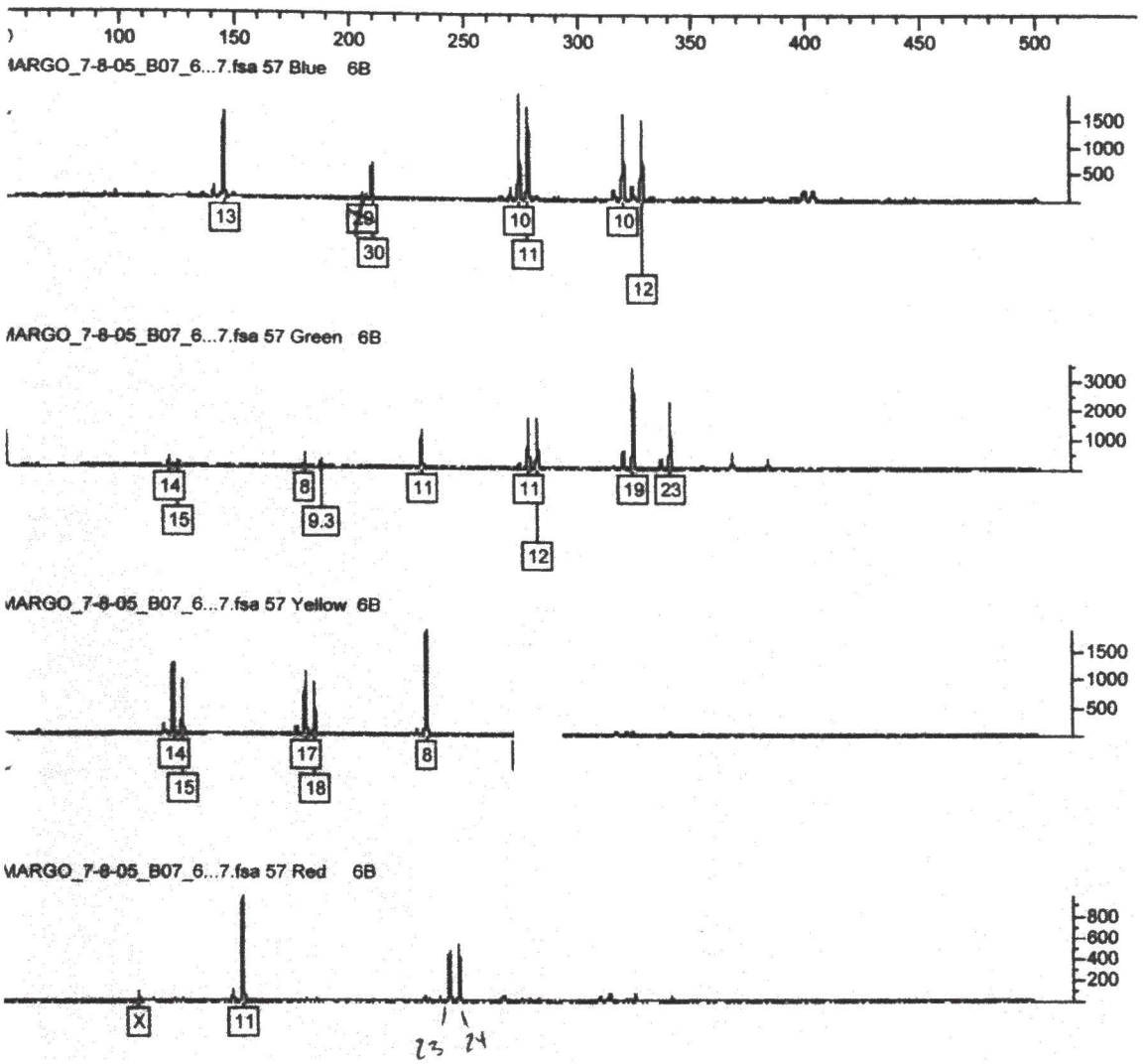

Figure 10: Full genetic profile of 1:64 dilution of Identifiler Primers

In the third dilution experiment, it was determined that testing was needed to determine if the reaction would be successful with an alternative reaction mix. The reason for this is that the Applied Biosystems reaction mixture (which is available in the Identifiler kit) is not available separately and the reaction mixture would run out prior to the exhaustion of the primer set. Using the Applied Biosystems reaction mixture, all reactions were successful for all loci (with the exception to a weak RFU value for D18S11) in dilutions of 1:50 or less. The 1:70 and 1:90 dilutions were unsuccessful with

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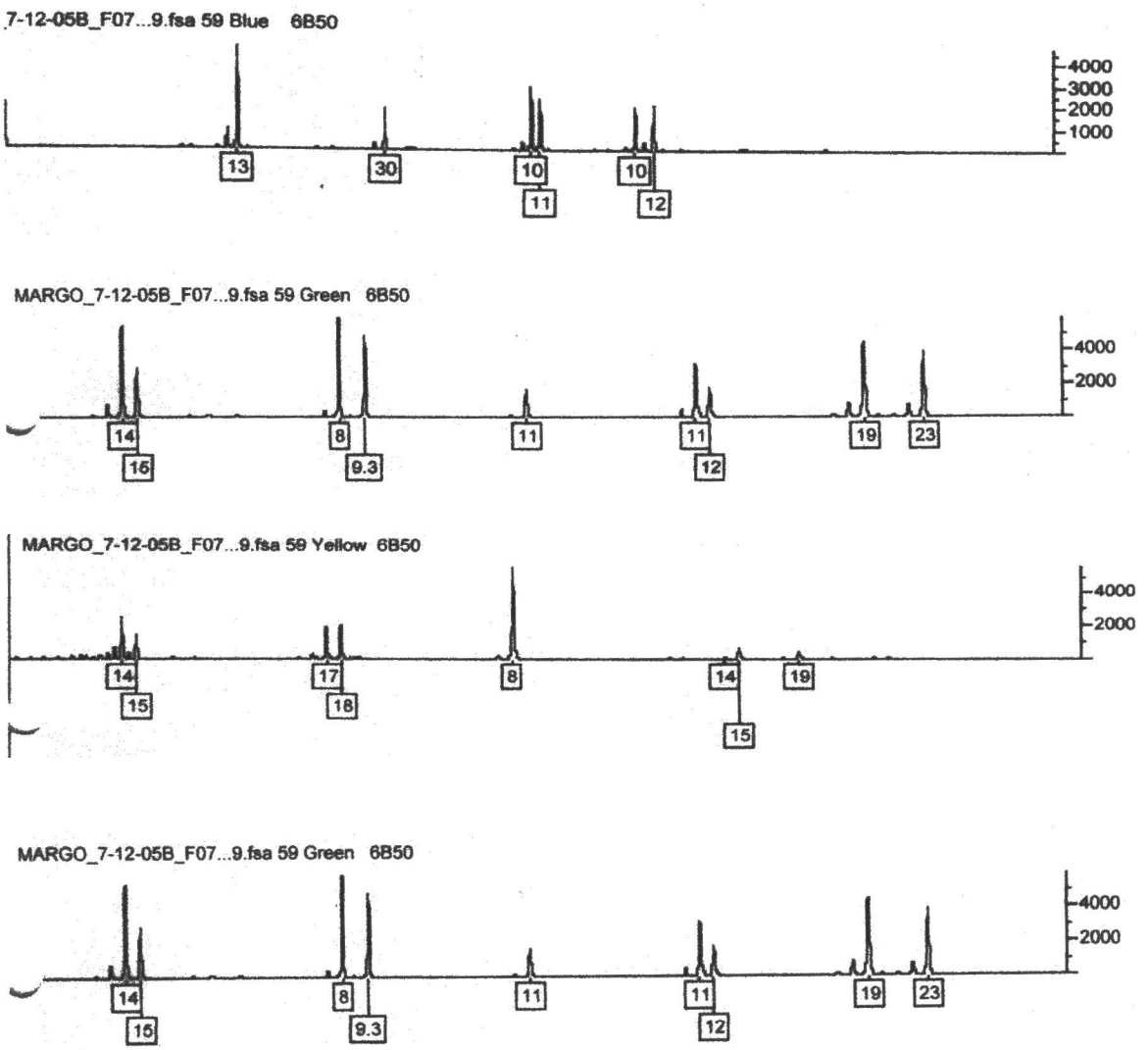

Figure 11: Full genetic profile results for the 1:50 dilution of Identifiler primers and Eppendorf Master Mix.

3.5 Reproducibility

The first experiment yielded somewhat reproducible results, with all of the loci being called correctly in all cases. There was however large discrepancies in the RFU values between the same loci. For example, the D18S11 locus had RFU values ranging from 500 to 6500 using the Eppindorf Hotmaster Taq master mix and from 350 to 5500 using the Applied Biosystems master mix. These discrepancies are thought to be due to differing sensitivities in the capillaries (96 total) of the ABI Prism 3700 Genetic Analyzer and not to the reaction itself. Problems with the instrument had previously been noted in the laboratory and were thought to be caused by inadequate usage. For example, due to inconsistent results, the left side portion of the 96 well plate was not used in the analyzer.

The second experiment yielded very similar results as the previous one. The RFU values were very different in the different capillaries of the analyzer, but all alleles were successfully amplified. One new problem did surface, however with the Amelogenin locus. Before this point, no male templates had been used, only the female control DNA. Allelic dropout was seen with the Y chromosome in all samples utilizing the male DNA template. The Y chromosome dropout could have been caused by primer binding site mutations, or it could have been necessary to have separate X and Y primer pairs for Amelogenin.

3.6 Cost Analysis:

Because a major goal of this study was to reduce the amount of Applied Biosystems AmpflSTR Identifiler Kit used to obtain a genetic profile and thus reduce the cost, a cost analysis was conducted. Costs were calculated for the reagents used in the reactions for the undiluted Applied Biosystems Identifiler and compared to the cost of the

diluted primers using the Eppindorf mastermix. The costs for the extra time involved were not taken into account. A single Identifiler kit has the approximate cost of $3000 and has the ability to test 200 samples, making the cost of testing one sample $15. Using the alternate nested PCR method, the cost of each sample is approximately cost per sample is $3.29. This is a savings of 11.71 per sample. The cost could be reduced even further by using the Applied Biosystems master mix until it is exhausted and then using the Eppindorf reaction mixture. The cost of each reaction component per sample is listed below:

1. Eppendorf HotMaster Taq Polymerase (includes 10X reaction buffer): $1.31 [11]
2. Operon custom primers: $0.0013 [9]
3. dNTP: $0.43 [11]
4. ExoSAP-IT: $1.25 [12]
5. ABI Identifiler Primers: $0.30 [2]

As an example, if a laboratory runs one plate of samples per day (96 samples per plate) five days a week for one year, they would type approximately 23,040. Using the Applied Biosystems Identifiler Kit under manufacturer recommendations, this would have the cost of about $345,600. Using a 1:50 dilution of the Identifiler primers and the Eppindorf reaction mix, the same number of samples would cost approximately $75, 801 for a savings of $269,799 per year.

3.7 Avenues for Improvement:

There are many ways in this research could be continued and or improved upon. First, the primers used in the first round of PCR need to be further optimized in order to

balance the peak heights and increase the signal of some loci such as D18S11. The Amelogenin also needs to be reexamined to correctly type the male DNA; perhaps another primer set specific for the Y chromosome is needed due to primer binding site mutations. The reproducibility needs to be retested on a properly functioning instrument to verify that the reproducibility problem was caused by the differing capillaries of the ABI Prism 3700 and not from flaws in the PCR reaction. Also, before laboratory use, a population study must be conducted to insure that no primer binding mutations are present and that the primer does not bind in a polymorphic region. The population study that could identify true mutation rates would also need to be conducted because the primer binding site mutation would cause allelic dropout. Other validation studies such as sensitivity studies to determine the optimal template DNA concentration would also be required before use.

Another possibility for improvement upon this research would be to even further reduce the cost of the reaction. The reaction volumes could be reduced in a similar manner as described by Leclair, B. *et al.* [13]. If the reaction volumes could be reduced and used with the diluted Identifiler primers, the cost could be reduced even further, possibly to as low as $1.00 per sample.

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