FINAL TECHNICAL REPORT

Development and Evaluation of a Whole Genome Amplification Method for Accurate Multiplex STR Genotyping of Compromised Forensic Casework Samples

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Abstract

Low copy number (LCN) DNA evidence (≤100pg) can become difficult to analyze with traditional STR analysis due to allele drop-out and increased stochastic effects. To overcome these limitations, whole genome amplification (WGA) has been investigated. Degenerate oligonucleotide-primed PCR (DOP-PCR), one form of WGA, uses a partially degenerate primer and a low annealing temperature (30ºC) to generate non-specific DNA fragments from throughout the genome. Data from preliminary studies using the DOP-PCR pre-amplification technique showed small increases in STR allele amplification; however, stochastic issues affecting data interpretation were prevalent. Thus, a modified DOP-PCR technique has been developed, dcDOP-PCR. Experiments included varying the number of non-specific cycles in the initial phase of the DOP-PCR reaction, as well as altering the degeneracy of the DOP-PCR primer and adding proofreading polymerases to the reaction mixture. All samples were DOP-PCR amplified using 3, 4, 7, 9, 12, and 15 cycles during the initial non-specific amplification round as well as using both a 10N and 16N degenerate primer. Additionally, several proofreading enzyme combinations, including *Taq:Pfu*, *Taq:Deep Vent*, *Taq:Tgo*, Platinum Pfx, and ABI GeneAmp High Fidelity (TaqGold and an unknown proprietary enzyme(s)) were evaluated. Data generated under these experimental conditions were compared to data collected using the standard, previously described DOP-PCR approach which includes a 6N degenerate primer, 5 non-specific cycles, and *Taq* polymerase only. Serially-diluted, QIAamp-extracted DNA samples ranging from 0.25ng down to 7.8pg were evaluated for all initial studies. All DOP-PCR products were amplified with the AmpF*l*STR® Profiler Plus® STR kit followed by separation and detection by CE (ABI 3100*Avant*). The 10N degenerate primer, 12 non-specific cycles, and the addition of *DeepVent* proofreading enzyme in the DOP-PCR reaction all significantly increased the number of alleles successfully amplified and detected. Further, these modifications, when combined, lowered the rate of sporadic additional allele occurrence (drop-in), when compared to the previously published DOP-PCR results. Additionally, intra-locus heterozygote peak ratios were consistently ≥0.6 for most low copy number DNA samples examined. These results show that the modifications incorporated into the DOP-PCR technique allow for a more complete, balanced STR amplification from low-level DNA samples. However, in order to fully evaluate the utility of this newly described technique (dcDOP-PCR), the method was used to pre-amplify DNA from mock and non-probative casework samples, including aged and environmentally-exposed bloodstains, bones, teeth, hair shafts, dermal ridge fingerprints, and fired cartridge cases. The dcDOP-PCR method significantly improved STR allele success when compared to traditional STR analysis (without pre-amplification), producing strong partial or full profiles in many cases where little to no STR data was previously obtained. Further, dcDOP-PCR data quality was generally equivalent or superior to traditional STR analysis. This method will provide the forensic DNA community with a relatively easy, inexpensive alternative for analyzing compromised and/or low copy number DNA evidence.

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Executive Summary

Although the genetic analysis of deoxyribonucleic acid (DNA) has proven to be an invaluable tool in forensic science, it has also proven to become problematic when DNA samples are of either low quality or low copy number (LCN), defined as those samples which have ≤ 100 picograms of DNA. These include samples which have a low quantity of DNA because they are not very cellular such as some trace evidence, fingerprints, telogen phase hairs, and bone. However, LCN samples may also include low quality samples which in their original state are very cellular but have been degraded due to some external factor such as aging or exposure to severe environmental conditions such as heat, humidity, and precipitation. Since many evidentiary samples are compromised, there is a need for a technique which can be used to analyze samples of low quantity or low quality with less template DNA than is required for commercially available STR multiplex amplification kits (0.5-2.5 ng). Several techniques have been developed in an effort to overcome the limitations of low copy number sample analysis including mitochondrial DNA analysis (mtDNA), low copy number-polymerase chain reaction (LCN-PCR), and whole genome amplification (WGA).

One such WGA technique, degenerate oligonucleotide primed-PCR (DOP-PCR), has been explored for use with STR testing from low yield samples. This technique can be used to pre-amplify large sections of the genome prior to downstream STR analysis. First, a partially degenerate primer is utilized that binds at a low annealing temperature (30ºC) non-specifically throughout the genome. Then, during specific cycling, the temperature is increased (62ºC) to preferentially amplify fragments from previous cycling rounds. However, there has been limited success reported using the traditional DOP-PCR method with forensic samples due to

insufficient coverage of the genome and random stochastic effects which often overshadow the increase in allele success reported. As a result, DOP-PCR would require modification to make it more amenable for use with forensic samples.

The goal of this research project was to provide the forensic DNA community with a Whole Genome Amplification (WGA) tool that can readily increase the success rate of the analysis of severely degraded, aged, or otherwise compromised biological evidence samples. Two WGA methods, Degenerate Oligonucleotide-Primed PCR (DOP-PCR) and Multiple Displacement Amplification (MDA), along with LCN-PCR were evaluated. The DOP-PCR method was fully optimized for its performance in multiplexed STR analyses such as those that are typically utilized for human identification in forensic casework and parentage analysis or relationship studies. In order to fully understand the forensic potential for this method it was first critical that a "home brew" method be established that performs similar to, or better than the commercially available Roche DOP-PCR kit. Next, for optimization, a variety of novel parameters were examined, including (1) DOP-PCR thermalcycling conditions, (2) DOP-PCR reaction components, (3) post-PCR purification, and (4) CE analysis settings for the analysis of compromised DNA samples. The best method was fully validated and documented. Finally, the performance of this method was tested using non-probative and mock case samples. Upon completion of all analyses, the final DOP-PCR method will be compared to existing methods (LCN-PCR and MDA) for use with STR analysis of low copy number and/or compromised biological evidence samples.

In the first study, Multiple Displacement Amplification (MDA), a WGA method, and Low Copy Number PCR (LCN-PCR), were examined for their ability to create complete, balanced STR profiles with minimum stochastic effects. MDA was performed using the

GenomiPhi DNA Amplification Kit. LCN-PCR was performed using the Amp*Fl*STR® Profiler Plus™ STR amplification kit, increasing the number of cycles from 28 to 34. For MDA amplified samples, average STR locus success was 88.3% for 0.25 ng DNA inputs, decreasing to 24% for 0.016 ng inputs. For LCN-PCR, average STR success was 95% for 0.25 ng DNA inputs, decreasing to 45% for 0.016 ng inputs. However, a large number of extraneous alleles were seen with both MDA and LCN-PCR for higher DNA input amounts >0.062 ng, along with severe allele drop-out at lower inputs (<0.031 ng). Overall, while MDA did not produce the same random allele peak patterns as LCN-PCR, it did seem to produce more random alleles than LCN-PCR altogether. Thus, to be of maximum benefit to the forensic DNA community, both methods would require further optimization.

Prior to optimization of the DOP-PCR procedure, it was necessary to create a "home-brew" protocol that would perform comparable to the commercially available Roche DOP-PCR Master kit. The "home-brew" master mix was based on the Roche reaction mix and consisted of BioRad *iTaq* 10X reaction buffer, *iTaq* polymerase, $MgCl_2$, dNTPs, and 40uM 6N degenerate primers for each sample. DOP-PCR thermalcycling parameters recommended by Roche were used. There was no significant difference in STR allele success or data quality between the Roche DOP Master kit and the "home brew" DOP reaction. The use of the "home brew" DOP-PCR reaction for all subsequent DOP-PCR reactions in the Dawson Cruz lab allowed this research to be completed at a lower cost. Further, the ability to control the components of the DOP-PCR reaction created the ability for optimization of all DOP-PCR reaction parameters included this research project, including evaluation of additional degenerate primers, thermal cycling parameters, and amplification enzyme combinations.

The primary objective of this project was to optimize the DOP-PCR method specifically for the improvement of downstream multi-locus, multiplex STR analysis from low copy number DNA samples (≤0.100ng – equivalent to ~15 diploid cells or less). Previous studies have most heavily focused on clinical or biobanked tissue samples for *single*-locus diagnostics from low copy, low yield samples, or from multiple loci of higher yield samples. Experiments involved altering the degeneracy of the DOP primer, the non-specific cycle number, and adding proofreading polymerases. Low input DNA quantities were examined for the primer and the cycle number studies using standard DOP-PCR parameters. The optimized DOP-PCR technique was then implemented for the polymerase study. All DOP-PCR products were amplified using a multiplex microsatellite amplification kit to evaluate products from multiple chromosomes followed by separation and detection by capillary electrophoresis. The 10N primer, 12 non-specific cycles, and the addition of *DeepVent* proofreading enzyme all significantly increased the number of STR alleles successfully amplified. All modifications also lowered the rate of allele drop-in, or sporadic additional allele occurrence, when compared to previously published DOP-PCR results. Further, an average of >0.50 intra-locus heterozygote peak ratios were observed for most DNA input quantities examined. These results show that modifications of the traditional DOP-PCR reaction to include the use of a more degenerate primer (10N), 12 non-specific cycles, and a proofreading enzyme allows for a more complete, balanced chromosome amplification from limited and/or compromised clinical and biological samples.

Instances where there is a defined peak for an allele, but the relative fluorescent units (RFUs) are not above threshold and thus would not be called as an allele, have been previously described in forensic DNA analysis research evaluating the DOP-PCR method. However, it is well known that an increase in electrokinetic injection time will increase the amount of DNA

loaded on the CE, consequently increasing the peak heights of the electropherogram obtained (RFUs). Further, allele shifting was observed in preliminary DOP-PCR studies, indicating that high concentrations of salts or other impurities may be competing with the STR products for injection into the capillary. Thus, a subset of DOP-PCR samples used in this study were post-STR purified and analyzed with increased injection times (20s vs. the traditional 10s). When comparing the 10 second electrokinetic injected DOP-PCR samples that were purified (and eluted into formamide) to those that were not purified, there was no noticeable difference in the final STR profile results. However, it is important to note that the combination of a longer injection time (20 seconds) with a post-STR amplification purification consistently produced the best STR allele success at all low copy DNA input values tested.

The final aim of this study was to evaluate the newly described method designed for this purpose ("dcDOP-PCR") using mock and non-probative casework samples like those frequently encountered in the forensic laboratory. Samples were amplified using the dcDOP-PCR method which features a 10N degenerate primer (22-mer, 5'-CTCGAG**NNNNNNNNNN**ATGTGG-3', N=A, T, G, C), and *Platinum® Taq* High Fidelity polymerase, a *Taq*/proofreading enzyme combination of *Taq:DeepVent*. All samples were also purified using the Qiagen MinElute® Post-PCR Purification Kit and analyzed with increased CE injection times. Compared to traditional STR analysis, the dcDOP-PCR method had the greatest STR allele success producing strong partial or full profiles in many cases where little to no STR data was obtained from traditional STR analysis. Further, dcDOP-PCR data quality was generally equivalent or superior to traditional STR analysis. Unfortunately, samples extracted organically, particularly those that were environmentally challenged, displayed significant CE artifacts. Thus, it is recommended that this method be used selectively with non-organically extracted DNA samples.

The genome-wide coverage from DOP-PCR improved when a more degenerate primer (10N) was used along with additional nonspecific cycles (6) and a *Taq:*proofreading polymerase combination (*Taq:DeepVent*). Further improvements were also noted when a post-STR purification step was added prior to CE analysis along with an increased CE injection time. Low level DNA samples amplified using the optimized DOP-PCR protocol, **dcDOP-PCR**, resulted in a ~45% increase in the number of detected STR alleles when compared to traditional DOP-PCR ($p = 0.0003$) and a ~34% increase when compared to traditional STR testing without WGA ($p<0.0001$, Figure 16). On average, these increases would be expected to improve the power of discrimination by ~1 in 1.82 million (3), which could provide enough additional information to confirm a human identification. Overall, dcDOP-PCR has proven to be a relatively easy and inexpensive method for improving allele success and data quality of compromised biological evidence that might have otherwise not produced a profile using traditional STR methods. A finalized protocol will now be made available to other agencies so that it can be evaluated externally (Appendix 1).

Main Body

I. Introduction

Although the genetic analysis of deoxyribonucleic acid (DNA) has proven to be an invaluable tool in forensic science, it has also proven to become problematic when DNA samples are of either low quantity or low quality. Low copy number (LCN) samples are defined as those samples which have ≤ 100 picograms of DNA (approximately 15 diploid cells) (1). These include samples which have a low quantity of DNA because they are not very cellular such as some trace evidence, fingerprints, telogen phase hairs, and bone. However, LCN samples may also include low quality samples which in their original state are very cellular but have been degraded due to some external factor such as aging or exposure to severe environmental conditions such as heat, humidity, and precipitation. Since many evidentiary samples are compromised, there is a need for a technique which can be used to analyze samples of low quantity or low quality with less template DNA than is required for commercially available STR multiplex amplification kits (0.5-2.5 ng) (2-4).

Low Copy Number DNA Techniques

Several techniques have been developed in an effort to overcome the limitations of low copy number sample analysis including mitochondrial DNA analysis (mtDNA), low copy number-polymerase chain reaction (LCN-PCR), and whole genome amplification (WGA) (1,5-8). The hypervariable regions within the D loop of mitochondrial DNA can be amplified and sequenced. After sequencing, differences observed between the DNA sequence and the Anderson reference sequence can be used for identification. There can be more than 1000 copies

of maternally inherited mitochondrial DNA, whereas only 2 copies of nuclear DNA exist per cell, one copy inherited from each parent (5). This makes mitochondrial DNA analysis a desirable alternative when faced with compromised biological samples; however, this technique also presents several issues. For example, it is very expensive, time-consuming, tedious, and is much less discriminatory than nuclear DNA. Despite these factors, mitochondrial DNA is currently the most widely used technique for extremely degraded biological evidence samples.

Another technique used to analyze low copy number DNA, LCN-PCR, increases the number of STR amplification cycles from the normal ~28 cycles to 30-34 cycles (8). While this may produce more copies of target DNA, as the cycle number increases, the efficiency of *Taq* polymerase decreases. This often results in a lower total product yield and an increase in stochastic effects (9). Stochastic effects frequently observed in this technique include allele and/or locus drop out, an enhanced amount of stutter, and heterozygous peak imbalance (8, 9). Additionally, because of the technique's increased sensitivity there is an increase in sporadic contamination and drop-in alleles (8). This makes the implementation of LCN-PCR in forensic laboratories difficult because the presence of unexplainable alleles in a profile produces many challenges. All of these issues combined make the interpretation of LCN-PCR profiles difficult.

Other techniques have also recently been evaluated that could potentially be used for low copy number DNA samples including Y-STRs, SNPs, and miniSTRs. Y-STRs produce male haplotype profiles. Although these types of kits are commercially available to the forensic community, Y-STRs have a far lower power of discrimination than STR profiles obtained from autosomal DNA because the Y-STR haplotype is not individualizing (5). Additionally, the gains in sensitivity from Y-STRs are substantial only when there is an excess of female DNA present.

There are millions of bi-allelic single nucleotide polymorphisms (SNPs) per individual (5). Although there are many advantages to this technique, a forensic database for SNPs does not yet exist, making it difficult to integrate this approach into the forensic community without expending a large amount of time and money. Because they are bi-allelic, individually they are not as powerful as an STR locus and, therefore, the development of a system comprised of multiple SNPs (~50-100) would be necessary if this were to be implemented in laboratories. Most importantly, SNPs have limited, if any, utility with mixed DNA profiles which is a frequent issue in forensic laboratories.

The most recent development in LCN analysis to be commercialized for the forensic community is "miniSTR" multiplex STR kits. In these kits, some of the target STR primers used in traditional kits have been redesigned to be closer to the STR repeat region resulting in a reduced amplicon size (10). Therefore, this increases the chance that an entire locus is amplified in low copy number and/or degraded DNA samples. Additionally, the reaction mixture includes proprietary components that may reduce or eliminate the effects of PCR inhibitors (10). Unfortunately, due to the reduced size range for the targets, fewer loci can be amplified simultaneously with the kits currently available.

Whole Genome Amplification Methods

Whole genome amplification (WGA) is a technique which was originally utilized in clinical applications and has recently been considered for use in the forensic community (1,9,11-15). WGA theoretically preamplifies the entire genome using random or degenerate primers (1,6,7). Using this approach, high quality and high yield samples can be obtained from low quantity/low quality samples, increasing the success of downstream applications such as forensic

STR analysis (11-15). However, several stochastic effects have been associated with this method such as allele and/or locus drop in or drop out, stutter, pull-up, heterozygous peak imbalance, and a decrease in the signal-to-noise ratio (1,11,12,16). There are numerous methods which have been developed that utilize whole genome amplification such as multiple displacement amplification (MDA), primer extension preamplification (PEP) including its improved (iPEP) and modified (miPEP) versions, and degenerate oligonucleotide primed polymerase chain reaction (DOP-PCR) (6,7,17,18). To date, none of these methods as described in the literature have proven to be effective for low quantity and/or compromised biological evidence samples.

MDA is an isothermal strand displacement reaction which utilizes the highly processive Phi 29 polymerase and exonuclease resistant random hexamer primers. Studies report that Phi 29 can replicate the genome exponentially resulting in products greater than 10 kb in length (11,12,19). Although this method is capable of producing a large amount of DNA, it also requires a large amount of initial template DNA which may not be available in forensic casework. In addition, the PCR reaction takes a large amount of time, drop-in alleles can become particularly problematic, and this method is expensive (9).

PEP utilizes a totally degenerate 15-mer primer. Using this method, partial STR profiles from approximately 5 pg of DNA and full profiles from >5 pg of DNA have been reported (1). Unfortunately, because the primer is completely degenerate, the primer binds too frequently throughout the genome resulting in short products and low product yield. Further, the PCR reaction requires 50 amplification cycles which makes this procedure very time-consuming (1).

Degenerate Oligonucleotide Primed Polymerase Chain Reaction

The traditional DOP-PCR reaction uses a primer 22 bases in length with a six-nucleotide degenerate region (6N) which is used to preamplify large sections of the genome (6). Originally, the 5' end of the standard DOP-PCR primer was designed to provide a Xho I restriction endonuclease recognition site which could be used for cloning while the 3' end was a GC-rich stretch used for efficient primer annealing and subsequent polymerization (20,21). The goal of this reaction is to generate a greater amount of initial starting material to improve further downstream analyses such as multiplex STR profiling. DOP-PCR begins with five cycles at a low initial annealing temperature of 30^{o}C which facilitates random primer annealing, producing fragments which theoretically span the entire genome (Table 1). Because of the possible combinations of nucleotides that could be inserted into the six-nucleotide degenerate region of the primer, *Taq* would be expected to prime at approximately 10^6 sites throughout the genome (22). Pre-amplification is then followed by 34 cycles at an increased annealing temperature of 62^{o}C which facilitates the preferential amplification of the fragments produced during the initial five cycles of nonspecific amplification (Table 1).

One of the most important benefits of DOP-PCR as opposed to other low quality/low quantity DNA analysis methods is that the techniques used for DOP-PCR are very similar to the standard STR PCR amplification. In fact, this method would require very little personnel training and no new equipment if a forensic lab chose to implement it. Automation using standard forensic DNA analysis robotics could also be accomplished for set up of this method.

However, there has been limited success reported using the traditional DOP-PCR method with forensic samples due to insufficient coverage of the genome and random stochastic effects which often overshadow the increase in allele success reported (9, 14). As a result, DOP-PCR would require modification to make it more amenable for use with forensic samples.

Table 1: Standard thermalcycling parameters for DOP-PCR amplification (20,21)

Standard DOP-PCR Parameters			
Step 1:	**Step 2: 5 cycles (non-specific amplification)**	**Step 3: 35 cycles (specific amplification)**	**Step 4:**
5 minutes, 95°C	1 minute, 94°C	1 minute, 94°C	7 minutes, 72°C
	1.5 minutes, 30°C	1 minute, 62°C	Hold at 7°C
	3 minutes, 30→72°C	2 minutes, 72°C	
	3 minutes, 72°C	(+14 seconds at each cycle)	

Project Goals

The goal of this research project was to provide the forensic DNA community with a Whole Genome Amplification (WGA) tool that can readily increase the success rate of the analysis of severely degraded, aged, or otherwise compromised biological evidence samples. Two WGA methods, Degenerate Oligonucleotide-Primed PCR (DOP-PCR) and Multiple Displacement Amplification (MDA), along with LCN-PCR were evaluated. The DOP-PCR method was fully optimized for its performance in multiplexed STR analyses such as those that are typically utilized for human identification in forensic casework and parentage analysis or relationship studies. For optimization, a variety of novel parameters were examined, including (1) DOP-PCR thermalcycling conditions, (2) DOP-PCR reaction components, (3) post-PCR purification, and (4) CE analysis settings for the analysis of compromised DNA samples. The best method was fully validated and documented. Finally, the performance of this method was tested using non-probative and mock case samples. Upon completion of all analyses, the final DOP-PCR method will be compared to existing methods (LCN-PCR and MDA) for use with

STR analysis of low copy number and/or compromised biological evidence samples. The rationale for this research and the experimental approach are described below.

DOP-PCR Methodology

The standard amplification reaction for DOP-PCR and the one used most often in the published literature is available as a commercial kit from Roche Molecular Biochemicals (20). This kit provides the primer and a single reaction mixture that includes all other components for simplification of reaction set-up. However, several studies have found that by altering buffering conditions and/or polymerases used for WGA methods, the performance of these methods improves and is, in fact, superior to DOP-PCR using the Roche kit and manufacturer's protocol (23 – 25). Thus, in order to fully understand the forensic potential for this method it was first critical that a "home brew" method be established that performs similar to, or better than the commercially available Roche DOP-PCR kit. This will allow us the opportunity to individually manipulate the reaction components and will keep the cost of the procedure very low since many of the PCR reagents needed are commonly available. Ideally, when/if a successful DOP-PCR method is developed specifically for the purpose of forensic STR amplification, this new method can then be commercialized and marketed to the forensic community.

DOP-PCR Amplification Reaction

The traditional DOP-PCR includes a 6N (nucleotide) degenerate primer that contains specific sequences on the 3' and 5' ends (20). This can result in incomplete genome coverage and could lead to the STR locus drop-out seen in some downstream multiplex amplifications (9, 14). Therefore, the specific 5' nucleotide sequences adjacent to the six degenerate nucleotides in the traditional primer (6N) were partially and completely replaced with additional degenerate nucleotides (10N and 16N, respectively) in order to increase the number of potential binding

sites. Increasing the degeneracy of the primer for DOP-PCR may aid in producing a more thorough amplification of the human genome, thereby reducing allele drop-out.

In addition, the traditional DOP-PCR technique employs a *Taq* (*Thermus aquaticus*) polymerase for extension (20). *Taq* is a highly processive enzyme that has only 5'-3' activity. AmpliTaq Gold (ABI, Foster City, CA), the *Taq* polymerase used in preliminary DOP-PCR studies, makes an error every 38,000 bases and has no way to correct nucleotide mismatches (26). As a result, when this enzyme is used in the DOP-PCR, the resulting products are only ~3-5 kb in length (27, 28). In contrast, polymerases that possess proofreading ability have a 3'-5' exonuclease activity that allows the enzyme to proceed in the opposite direction of DNA synthesis. This allows the proofreading enzymes to remove misincorporated bases and replace them with the appropriate complimentary base. This correction results in longer PCR products, which can increase genome coverage if used in a WGA reaction (7). Unfortunately, the exonuclease activity of proofreading enzymes reduces their overall processivity rate; thus, combining proofreading enzymes with *Taq* polymerase should increase genome coverage (improve allele amplification) without compromising the speed of the reaction. Therefore, four combination enzyme conditions were evaluated in the DOP-PCR for comparison with *Taq* alone, including *Taq:Pfu* (*Pyrococcus furiosus)*, *Taq:DeepVent* (*Pyrococcus species GB-D*), *Taq:Tgo* (*Thermococcus gorgonarius*), and GeneAmp High Fidelity (ABI, Foster City, CA) (a proprietary mixture). Additionally, *Pfx* (*Thermococcus kodakaraensis*), a proofreading enzyme whose processivity and elongation rates are higher than *Taq* (28, 29), was used alone in the DOP-PCR for comparison to *Taq*.

DOP-PCR Thermalcyclying Parameters

The traditional DOP-PCR includes 5 initial cycles using low annealing temperatures for non-specific amplification, as described above (Table 1) (20). Increasing the number of non-specific cycles for the DOP-PCR should subsequently increase opportunities for the primer to anneal. Thus, a range of non-specific cycle numbers was evaluated (3, 4, 5, 7, 9, 12, and 15 cycles) to determine if downstream multiplex STR analysis could be improved.

CE Analysis & Post-PCR purification

Instances where there is a defined peak for an allele, but the relative fluorescent units (RFUs) are not above threshold and thus would not be called as an allele, have been previously described in forensic DNA analysis research evaluating the DOP-PCR method (9). As described earlier, an easy and effective correction for below threshold peaks can be to increase the electrokinetic injection time. Having RFU values below threshold may be a result of a low amount of DNA being injected onto the CE. It has been published that an increase in electrokinetic injection time will increase the amount of DNA loaded on the CE, consequently increasing the peak heights of the electropherogram obtained (RFUs) (30).

Since DNA injection also depends on ionic concentrations, it is imperative that extra ions are not present in the STR PCR product or buffer. There are several components, including some ions, present in the STR products that may hinder DNA injection by competing for injection into the capillary, thus resulting in potentially less DNA loaded onto the CE. These competing components include excess primers and magnesium and chlorine ions , all of which contain ionic charges. The primers and ions have a higher charge to mass ratio, which increases their mobility (μ_{ep}) over that of the DNA fragments. For this reason, smaller fragments of DNA may also be injected preferentially over larger ones if excess ions are present. When this occurs, the sample RFU intensity may be diminished, since less DNA will pass by the CCD camera and less

fluorescence is detected. In this case, several steps could be taken in order to increase the DNA signal intensity observed. The standard injection parameters using the default STR typing protocol for ABI CE instruments are 15,000 Volts (V) for 5 seconds (5) (this may differ according to individual laboratory and instrument validations). However, more sample can be injected by increasing either the electrokinetic voltage or injection time. In addition, purification of the sample prior to CE analysis to remove competing substances and/or purification/dilution of the sample directly into formamide could improve fluorescent detection of potentially limited STR products (5).

Spin columns are commonly used to remove impurities from samples by eliminating all substances in the sample smaller than a specified size (measured in bp). This method can effectively remove the primers, as well as excess ions, deoxynucleotide triphosphates (dNTPs) and enzyme (31, 32).

Specifically, the allelic shifting that was noted in our preliminary data could be attributed to a high concentration of salt being present during electrophoresis. It has been previously reported that a purification of the post STR amplification product shows more accurate STR profiles with less baseline noise for other applications (30). However, using spin columns as a post-amplification purification technique can be time consuming and expensive for a large number of samples, which explains why it is not instituted in forensic casework for every sample tested. Instead, it has been shown that a dilution of the sample directly into deionized formamide can effectively reduce the ionic strength of the sample as compared water alone. The use of formamide in conjunction with a 95ºC denaturation step followed by a snap freeze on ice prepares DNA for the CE by separating the DNA into single strands and preserves the

fluorescent activity of the dyes, so that the fluorescently labeled DNA fragments can be more easily excited and detected (30, 33).

Non-probative/Mock Casework Study

Ultimately, what was created from the above-mentioned studies is a combination of the optimal DOP-PCR conditions specifically for the purpose of multiplex STR typing from DNA samples of low quantity and/or low quality. This altered, optimized method will be referred to as dcDOP-PCR. In all experimental studies described above, serially diluted reference DNA samples were used for optimizing each step of this technique. However, for the final validation study, mock and non-probative casework samples similar to those frequently encountered in the forensic laboratory were analyzed. Samples tested included true low copy number DNA sources as well as challenged/degraded forensic DNA evidence sources. This step is a common requirement for validation as stated in the DNA Advisory Board Quality Assurance Standard 8.1.3 and in the Scientific Working Group on DNA Analysis Methods Validation Guideline 2.6 (5, 34).

II. Methods

After all basic technical procedures are detailed, a thorough description of each individual study (including experimental design, methods, and statistical analyses) is provided.

Optimization Procedures

Sample Collection and Preparation

Cotton tip swabs were used to collect reference buccal epithelial cell samples from 21 random individuals. Students, technicians, and other lab personnel working on this project were not included as sample donors for any experiments described herein.

DNA Extraction

DNA was extracted from all buccal swab samples using the Dawson Cruz Laboratory standard protocol for the Qiagen QIAmp® DNA blood mini kit (35).

DNA Quantitation and Dilution

Following extraction, the samples were quantified using the Applied Biosystems Quantifiler® Human Quantitation Kit (using the Applied Biosystems Quantifiler® Human DNA Quantification Kit and the ABI 7500 Real-Time PCR System according to the manufacturer's instructions (Applied Biosystems, Foster City, CA). The sample extracts were each serially diluted to 0.00156ng/ul using distilled, deionized water based on the quantitation valued obtained.

Multiple Displacement Amplification (MDA)

Samples amplified using MDA included a positive control consisting of 1 ng of K562 DNA (Promega, Madison, WI, USA), a negative control consisting of 5uL of TE, 0.25ng, 0.125ng, 0.062ng, 0.031ng, 0.016ng, and 0.0075ng of input DNA. MDA was performed according to the GenomiPhi™ DNA Amplification Kit protocols and published studies (GE Healthcare, Piscataway, NJ, USA) (17, 36, 37). A total of 6 replicates were completed for this experiment (n=6). Denaturation, incubation, and post-amplification heat inactivation steps were performed on the GeneAmp PCR System 9600 thermalcycler (PerkinElmer, Wellesley, MA, USA) in order to maintain consistent temperatures during each step.

Low Copy Number (LCN) STR amplification

LCN PCR STR amplification was performed using the AmpFlSTR® Profiler Plus® PCR Amplification Kit (Applied Biosystems, Inc) as described above (31) with one modification to cycling number as described in the literature (38, 39). Samples to be amplified using LCN PCR were a positive control consisting of 0.5ng of 9947A DNA (Applied Biosystems, Inc), a negative control consisting of 5uL of TE, and the full range of DNA inputs (0.25 – 0.0075ng) as described above. A total of four replicates were completed for this experiment (n=4). Thermalcycling parameters were as described above for the AmpFlSTR® Profiler Plus® PCR Amplification Kit (Applied Biosystems, Inc), except for an increase in cycle number from 28 cycles to 34 cycles (38).

Roche DOP-PCR Master Kit Amplification

Roche DOP-PCR Master kit (Mannheim, Germany) amplification was performed in a GeneAmp PCR System 9600 (n = 4 buccal swab samples). For each sample analyzed, 5µL of each of the DNA dilutions were added to the DOP-PCR reactions so that the following DNA quantities were input into the reaction: 0.25ng, 0.125ng, 0.062ng, 0.031ng, 0.016ng, and 0.0078ng. Five microliters of 0.1ng/µL 9947A DNA was used as the positive control. Five microliters of ddH_2O was used as the negative control. The reaction mix for each sample included 5µL of the provided DOP primer, 50µL of the provided DOP master mix and 40µL ddH_2O. The Roche DOP-PCR Master kit thermal cycling parameters were as follows: 95°C for 5 minutes; 5 cycles of 94°C for 1 minute, 30°C for 1.5 minutes, ramp to 72°C for 3 minutes and hold at 72°C for 3minutes; 35 cycles of 94°C for 1 minute, 62°C for 1 minute, 72°C for 2 minutes (an additional 14 seconds were added with each cycle); 72°C for 7 minutes; and 7°C hold indefinitely.

Degenerate Oligonucleotide Primed PCR ('home-brew')

The buccal swab DNA extracts were DOP-PCR amplified using 5µL (0.0078ng - 0.25ng or ~2 to 36 cells) of each diluted sample. Five microliters of 9947 positive control (0.1ng/µl) and

5µl of TE buffer, which served as the negative control, were also DOP-PCR amplified. The DOP-PCR reaction mix was based on the Roche DOP-PCR Master Kit (Mannheim, Germany) (20). The master mix consisted of 10uL of BioRad *iTaq* 10X reaction buffer (Hercules, CA), 2.5U BioRad *iTaq* polymerase (Hercules, CA), 3uL of 50mM $MgCl_2$, 5uL of 4mM each of dNTPs, and 5uL of 40uM 6N degenerate primers (5'- CCGACTCGANNNNNNATGTGG-3') for each sample. TE^{-4} buffer (pH = 7.6) was added to each sample amplified to bring the total reaction volume to 100µL. The Roche recommended DOP-PCR parameters previously described were used as shown in Table 1 (20). The samples were amplified in the Perkin Elmer 9600 GeneAmp PCR system (Waltham, Massachusetts). The initial non specific amplification parameters were 95°C for 5 minutes, followed by 5 cycles of 94°C for 1 minute, 30°C for 1.5 minutes, 3 minute ramp to 72°C and 72°C for 3 minutes. This was followed by a specific amplification step of 35 cycles of 94°C for 1 minute, 62°C for 1 minute and 72 °C for 2 minutes (add 14 seconds to each cycle) (20). To ensure that *Taq* completed extension and adenylation, an additional step of 72°C for 7 minutes was included followed by a hold at 7°C.

Sample Concentration Post DOP-PCR

Following DOP-PCR amplification all samples were concentrated using YM 10 Microcon centrifugal filters (Millipore, Billerica, MA) (40). Each Microcon filter was wet with 25µl of distilled, deionized water and loaded with the entire DOP-PCR product sample. The unit was centrifuged at 14,000 x g for 20 minutes and 12,000 x g for 25 minutes. Samples were then washed with 100µl of distilled, deionized water. Following the wash, 25µl of distilled, deionized water was added to each unit for reconstitution with a 5 minute room temperature incubation. Finally each filter was inverted into a new tube and centrifuged at 1000 x g for 3 minutes. The DNA was retained in the new tubes and stored at -20°C.

Evaluation of Sample Quality

The maximum fragment size obtained after dcDOP-PCR was determined for some samples using gel electrophoresis. A 1% agarose gel was prepared and 5µl of each concentrated sample was run at 80V for 5hrs in 1X TAE buffer. A 1Kb extension ladder was used to determine the maximum fragment size of the product. Bromophenol blue loading dye was added to all samples, including the ladder, before loading on the gel. After electrophoresis, the gel was stained with ethidium bromide (EtBr) for 10 minutes and then viewed under the BioDoc-It™ UV imaging system. From the agarose gel results, the mean and standard deviation was calculated for the maximum fragment size obtained for a given enzyme condition at each input value. The mean and standard deviation was also calculated for the overall maximum fragment size for each enzyme condition, regardless of the input value. For instances where no value is visualized, no value is recorded; a zero value is not recorded in this instance since there may be product that falls below the lower limit of detection for this technique.

Evaluation of Sample Quantity

Following DOP-PCR amplification and concentration, 5ul of each sample was quantified for total human DNA as described by *Walsh et al*. (41), using Applied Biosystem's Quantiblot® Human DNA Quantitation kit (ABI, Foster City, CA) in order to determine the total DNA yield (ng) following the DOP-PCR.

Profiler Plus™ Multiplex STR Amplification

Based on the Quantiblot® quantitation (ABI, Foster City, CA) values obtained, all samples were either concentrated or diluted to 0.4ng/µl. The DOP-PCR samples were concentrated using YM-10 Microcon centrifugal filter devices (Millipore, Billerica, MA) (40) as described above or diluted by adding the appropriate amount of distilled, deionized water. In cases where no quantity or <2ng was obtained, the entire sample was concentrated to 5µl. The ABI AmpFλSTR® Profiler Plus™ PCR STR Amplification kit (ABI, Foster City, CA) was used to amplify the DOP-PCR DNA samples (3, 31). This is a multiplex STR amplification kit which allows for amplification of 9 separate STR loci that are located on 9 different chromosomes. Five microliters of 9947A positive control (0.1ng/µl) and 5ul of TE buffer, which served as a negative control, were also STR amplified. A PCR master mix was prepared and 10µl was added to each sample including the positive and negative controls. The master mix consisted of 5.70µl of AmpFλSTR® PCR Reaction Mix, 2.0µl of AmpFλSTR® Profiler Plus™ Primer Set, 2.10µl PCR-TE, and 0.2µl of AmpliTaq Gold DNA polymerase (5U/µl) per sample.

The samples were amplified using the Perkin Elmer 9600 GeneAmp PCR system (Waltham, Massachusetts). The amplification parameters were 95°C for 11 minutes, and 28 cycles of 94°C for 1 minute, 59°C for 1 minute, and 72°C for 1 minute, followed by a hold at 60°C for 90 minutes.

DNA separation, detection and analysis

The STR amplified DNA samples were size-separated and detected on an ABI 3100*Avant* Genetic Analyzer (ABI, Foster City, CA) using 1.2µL of the STR product, along with 12µL Hi-Di Formamide and 0.5µL GS 500-ROX internal lane standard (ABI, Foster City, CA). One µL of Profiler Plus ™ allelic ladder (ABI, Foster City, CA) was also included in at least two wells of every plate analyzed. All samples were denatured at 95°C for five minutes and then immediately cooled on ice for five to ten minutes. All electrophoresis was performed using ABI 3100 POP-4™ (performance optimized polymer) (ABI, Foster City, CA) and a 36cm capillary using the standard (default) injection parameters. The samples were injected for a standard 20 seconds and data was collected using ABI Prism® 3100*Avant* Genetic Analyzer Data Collection Software version 2.0 (ABI, Foster City, CA).

STR data was sized and typed using the ABI GeneMapper ™ *ID* Software v. 3.2 (ABI, Foster City, CA) with an analysis threshold of 75 relative fluorescence units (rfu). Percent allele success or percent of chromosomal locations accurately represented (determined by comparison to known profiles) and intra-locus heterozygote peak ratios were recorded for each sample. Intra-locus heterozygote peak balance was calculated by dividing the lower allele peak height (rfu) of a heterozygote pair by the higher peak height (rfu). Any stochastic effects seen, such as incomplete 3'-adenylation by *Taq* polymerase, the presence of additional alleles, high stutter (an artifact of PCR STR amplification caused by slippage of the polymerase), and any other unknown artifacts were also noted (5).

Non-Probative/Mock Casework Procedures

Sample Collection and Preparation

Fresh whole blood samples were collected from three donors and stored at 4°C for approximately 24 hours. For each donor, a bloodstain was prepared on a white cotton t-shirt. After drying, each bloodstain was cut into quarters. Each quarter was then placed into its respective condition for the study: room temperature, 56°C, 80°C, and in an outdoor,

unprotected environment (central Virginia). Samples from each were collected at the following time points and then stored at -20°C in a pre-PCR freezer until analysis: 0 months, 1 week, 1 month, 3 months, and 4 months. For each time point, three samples were collected from each condition, resulting in a total of 12 bloodstain samples per time point. Samples collected from the outdoor, unprotected environment condition were dried in a hood overnight before storing at -20°C. During the course of this study (4 months), the average temperature was 45°F (SD= 10.0) and the total precipitation (including snow and rain) was 12.78".

Other samples collected for analysis included cigarette butts (n=9), bone (n=2), teeth (n=2), dermal ridge fingerprints (n=3), fired cartridge cases (n=3), hair roots (n=3), and hair shafts (n=3). Three cigarette butts were collected from each of three individuals and stored in a 4°C pre-PCR refrigerator until use with the exception of two of the cigarette butts. These two random exceptions were from the same individual and had been stored in a sealed envelope in a car for approximately five months before use. After collection, all of the cigarette butts were left outside for one week in Northern Virginia. During this week, the average temperature was 35°F (SD= 6.8) and the total precipitation was 0.57". The cigarette butts were collected and dried in a hood overnight. After drying, the cigarette butts were stored at 4°C until extraction.

Bone samples (n=2) were received in powder form from the Virginia Department of Forensic Science. The Virginia Department of Forensic Science *Large Volume DNA IQ Extraction Method for Bone Samples* protocol was followed to obtain the powder (42, 43). A cleaning solution of 1.2 mL TNE, 75 µL 20% Sarkosyl, and 225 µL of Type I water was prepared and preheated in a heat block at 56°C. After preheating, 15 µL of Proteinase K (20 mg/mL) was added to the solution. The cleaning solution was applied to a pad of Kimwipes and the Kimwipes were then applied to the surface of the bone to be drilled. The bone was placed in a Ziploc bag and incubated for 30 minutes. Following incubation, the area which was cleaned was wiped with 95% ethanol and allowed to dry. The bone powder was obtained by drilling the bone with an electric drill. The drill bits (3/32") were cleaned with 10% bleach followed by 95% ethanol before use. A weigh boat was used to collect the powder. The powder was stored at 4°C until extraction.

The teeth samples used in this study were a child's molars (n=2). The Virginia Department of Forensic Science *Organic Extraction Method for Teeth* protocol was followed (43). The outer surface of the tooth was first cleaned using a Kimwipe and 10% bleach followed by 70% ethanol. A dremel tool was used to remove the upper crown portion of the tooth. The tool and the bits were cleaned with 10% bleach and 70% ethanol before use. After the crown was removed, the tooth was then placed into a sterile Ziploc plastic bag. This plastic bag was then placed into several other sterile Ziploc plastic bags. A hammer (covered with sterile bags) was used to pulverize the tooth taking care to not puncture the plastic bags. The pulverized tooth was transferred to a sterile 1.5 mL microcentrifuge tube and stored at 4°C until extraction.

To obtain the dermal ridge fingerprint samples, each individual was given a 50 mL conical tube (n=3). The individual grasped the conical tube with a full fist and held it for 10 seconds. Each conical was then swabbed using the double-swab method (44). The swabs were stored at -20°C until extraction.

Fired olive steel cartridge cases (7.62 x 39 mm) (Wolf®, Placentia, CA) were obtained from the Virginia Department of Forensic Science. The cartridge cases were cleaned with water and isopropanol before firing. Three cartridge cases were loaded by each of two individuals (JW and DG) and five were loaded by the third individual (BC). After each firing, the cases were

retrieved with a swab and placed into a sterile plastic bag. Each case was swabbed using the double-swab method and stored at 4°C until extraction (44).

Three Caucasian females were randomly chosen as hair donors. Each donor individually pulled several of their own hairs. The hairs were stored at 4°C until use. Prior to extraction, the hairs were microscopically examined using a Carlsan CS700 stereomicroscope (Carlinville, IL) and a 40X objective. Morphological characteristics of all hairs were recorded. None of the hairs were colored or chemically treated. Each hair was in the telogen growth phase, exhibited no medullation, and had predominately eumelanin pigmentation.

Students, technicians, and other lab personnel working on this project were not included as sample donors for any experiments described herein. A schematic showing the proposed dcDOP-PCR workflow for casework samples is shown in Figure 1.

DNA Extraction

DNA was extracted from the aged bloodstains, cigarette butts, bones, teeth, and fingerprints using standard organic extraction. The samples were incubated overnight in a 56°C heat block using 400 µL of stain extraction buffer and 15 µL of Proteinase K (20 mg/mL). Following incubation, the solid material was placed into a spin basket (Alltech® Forensic Spin Filter, Deerfield, IL). The basket was inserted back into the original tube and centrifuged at 10,000 rpm for 5 minutes. Five hundred microliters of phenol:chloroform:isoamyl alcohol (25:24:1) was added to the extract. After vortexing and centrifugation at 13,000 rpm for 5 minutes, the aqueous layer was removed and a subsequent organic extraction was performed on that layer by adding 500 µL of chloroform:isoamyl alcohol (24:1) and centrifuging at 13,000 rpm for 5 minutes. The aqueous layer was transferred to a Microcon® YM-100 concentrator (Millipore, Bedford, MA). The samples were concentrated to ~30 µL with TE^{-4} buffer using the manufacturer's protocol for Microcon Purification.

DNA was extracted from hair roots and hair shafts using the Qiagen QIAamp® DNA Micro Extraction kit (Qiagen Inc., Valencia, CA) following the manufacturer's protocol. A 1-cm piece starting from the hair bulb was cut for extraction from the hair root. Subsequently, three additional 1-cm pieces were cut for extraction from the hair shaft and were combined into one tube. The samples were then incubated at 56°C for 1 hour in a heating block using 300 µL of Buffer ATL, 20 µL of Proteinase K, and 20 µL of 1 M DTT. During incubation, each tube was vortexed for 10 seconds every 10 minutes. Following incubation, 300 µL of Buffer AL with 1 µg of dissolved carrier RNA was added to each tube. The samples were incubated again at 70°C in a heating block for 10 minutes. Each tube was vortexed for 10 seconds every 3 minutes during incubation. The samples were then centrifuged for 1 minute at 13,000 rpm and the supernatant was transferred to a QIAamp MinElute® Column (Qiagen, Inc.). The MinElute® Column was centrifuged at 8,000 rpm for 1 minute and then placed into a clean collection tube. Five hundred microliters of Buffer AW1 was added to each column, centrifuged at 8,000 rpm for 1 min, and then each column was placed into a clean collection tube. The samples were washed again by repeating the previous step with 500 µL of Buffer AW2. The columns were centrifuged at 13,000 rpm for 3 minutes and then placed into a clean 1.5 mL microcentrifuge tube. For elution, 50 µL of Buffer AE was added to the center of the membrane; the tubes were incubated at room temperature for 5 minutes, and centrifuged at 13,000 rpm for 1 minute.

Fired cartridge cases from three individuals (JW, DG, and BC) were used as donors for this portion of the study. JW and DG cartridge cases were manually extracted at the Virginia Department of Forensic Science using the Virginia Department of Forensic Science protocol for

Promega DNA IQ manual extraction (43). BC cartridge cases were extracted similarly using the Virginia Department of Forensic Science protocol for *DNA IQ extraction using the Biomek™ 2000 Automation Workstation* (45).

DNA Quantitation and Dilution

Following extraction, the samples were quantified using the Applied Biosystems Quantifiler® Human Quantitation Kit (using the Applied Biosystems Quantifiler® Human DNA Quantification Kit and the ABI 7500 Real-Time PCR System according to the manufacturer's instructions (Applied Biosystems, Foster City, CA).

Profiler Plus™ Multiplex STR Amplification

All samples were amplified using the Dawson Cruz laboratory protocol for ABI AmpF*l*STR® Profiler Plus™ PCR Amplification prior to whole genome amplification for an initial evaluation of sample quality. A concentration of 1 ng of DNA in 5 µL was targeted for STR amplification. Those samples that had concentrations less than the target were concentrated to 10 µL prior to STR amplification using Microcon® YM-100 centrifugal filter devices per the manufacturer's protocol; from these samples 5 µL (~0-100 pg) was used for STR amplification. Positive and negative controls (0.5 ng 9947A DNA and 5 µL of TE^{-4}, respectively) were also amplified. For each sample, 10 µL of PCR master mix was added to the DNA sample for amplification. The master mix consisted of the following components for each sample: 5.7 µL of AmpF*l*STR® PCR Reaction Mix, 2.0 µL of AmpF*l*STR® Profiler Plus™ primers, 0.20 µL of 5U/ µL of *AmpliTaq® Gold* DNA polymerase (Applied Biosystems), and 2.1 µL of TE^{-4} buffer. The amplification was performed in a Perkin Elmer 9600 GeneAmp PCR system (Foster City, CA) using the following parameters: 95°C for 11 minutes; 28 cycles of 94°C for 1 minutes, 59°C for 1 minute; 72°C for 1 minute; 60°C for 90 minutes; hold at 4°C.

Capillary Electrophoresis

Following STR multiplex amplification, products were size-separated via CE using the ABI 3100*Avant* Genetic Analyzer. The samples were prepared for CE analysis by adding 1.2 µL of amplified sample or 1 µL of Profiler Plus™ allelic ladder to 0.5 µL of GeneScan™ 500 ROX™ size standard and 12.0 µL of Hi-Di™ Formamide (Applied Biosystems) in a MicroAmp™ optical 96-well reaction plate (Applied Biosystems). The plate was denatured at 95°C for 5 minutes, snap-cooled on ice for 5 minutes, and then loaded into the ABI 3100*Avant* Genetic Analyzer. Electrophoresis was performed using default STR conditions, ABI 3100 POP-4™ polymer, a 36 cm capillary, and a 10 second injection. The STR fragment data for high quantity samples were sized and typed using the ABI GeneMapper® ID version 3.2 software with an analytical threshold of 50 relative fluorescent units (RFU). For low quantity samples, an analytical threshold of 75 RFU was used.

dcDOP-PCR Amplification

Samples were dcDOP-PCR amplified using 0.5-5 µL of the sample extract for a targeted input of ~50-100 picograms depending on the concentration and the severity of degradation. Positive and negative controls (0.5 ng 9947A DNA and 5 µL of TE^{-4}, respectively) were also dcDOP-PCR amplified. For each sample amplified, the dcDOP-PCR reaction mix consisted of 10 µL of *Platinum® Taq* 10X reaction buffer (Invitrogen Corporation, Carlsbad, CA), 0.5 µL of *Platinum® Taq* High Fidelity DNA polymerase (5 U/ µL) (Invitrogen Corporation), 4 µL of

Platinum® Taq $MgSO_4$ (2 mM) (Invitrogen Corporation), 5 µL of 4 mM each of dNTPs, and 5 µL of 10N degenerate primers (40 µM, 22-mer, 5'-OH CTCGAGNNNNNNNNNNATGTGG OH-3', N=A, T, G, C) (7,9,11,12). TE^{-4} buffer (pH= 7.6) was added to the reaction to bring the total volume to 100 µL. The amplification was performed in a Perkin Elmer 9600 GeneAmp PCR System (Foster City, CA) using the DOP-PCR thermalcycling parameters previously described (Table 1) with the exception of 12 non-specific cycles as opposed to the recommended 5 cycles (9,13,20) (Table 2). Following dcDOP-PCR amplification, the samples were concentrated to ~5 µL using Microcon® YM-10 centrifugal filter devices (Millipore) according to the manufacturer's protocol.

Following dcDOP-PCR amplification and concentration, samples were then amplified for STRs using the entire volume of the concentrated amplification product (~5 µL) and the AmpF*l*STR® Profiler Plus™ PCR Amplification procedure described above.

Post-PCR Purification and CE Analysis

Following STR amplification, the dcDOP products were post-PCR purified using the Qiagen MinElute® Post-PCR Purification Kit according to the manufacturer's recommendations with modifications described by Smith and Ballantyne (16). Samples were washed 3 times and, at the final step, eluted into 10 µL of 100% Hi-Di™ formamide (Applied Biosystems). Finally, the samples were prepared for capillary electrophoresis as previously described with modifications. In lieu of the default injection time (10 seconds), dcDOP-PCR products were injected for 20 seconds and positive controls were injected for 5 seconds. The default injection time was used for negative controls and allelic ladders. A mixture of Hi-Di™ formamide (Applied Biosystems) and GeneScan™ 500 ROX™ size standard (Applied Biosystems) was prepared for each sample. For high quantity/high quality samples, 0.25 µL of the size standard was used per sample; for low quantity/low quality samples, only 0.125 µL was used per sample. Formamide was added to bring the total formamide:ROX mixture to 12.5 µL per sample. The STR fragment data were sized and typed using the ABI GeneMapper® ID version 3.2 software.

Experimental Design

MDA vs. LCN PCR

DNA was diluted using distilled, de-ionized water (ddH_2O) to the following concentrations (each per 5µL volume for LCN PCR reactions or per 1µL volume for MDA reactions): 0.25ng, 0.125ng, 0.062ng, 0.031ng, 0.016ng, and 0.0075ng. All MDA and LCN PCR sample STR data was checked for concordance to expected profiles. Number of alleles per sample and number of loci accurately typed were evaluated for each sample. Successful loci counted included those with at least a partial profile. A locus was counted as "successful" as long as the expected profile was seen – even if additional alleles or stochastic effects were noted. Means and standard deviations were calculated for all samples. For agarose gel fragment results and all quantitation results, zero values were not included in these calculations due to the fact that "zero" values could simply indicate that the sample tested is below the lower limits of detection for the method being used. Where applicable, a correlation coefficient (ρ) and/or P value was calculated to determine significance. A ρ -value of 1.0 is interpreted as a perfect correlation, >0.80 a strong correlation, and <0.50 a weak correlation. All P values were determined using a one-way ANOVA using a 0.05 α value. P values less than 0.05 are used to indicate significance.

Home-Brew DOP-PCR

DNA was diluted using distilled, de-ionized water (ddH_2O) to the following concentrations (each per 5µL volume): 0.25ng, 0.125ng, 0.062ng, 0.031ng, 0.016ng, and 0.0075ng. The average maximum fragment size and standard deviation was calculated for each sample input volume for both the "home brew" DOP-PCR reaction and the Roche DOP Master kit reaction. P-values for significance were calculated using the one way ANOVA test using Microsoft Excel and an α value of 0.05, where a value below 0.05 indicates significance. After STR multiplex amplification, all "home brew" and Roche DOP Master kit DOP-PCR samples were analyzed for concordance with expected profiles using the ABI GeneMapper *ID* Sofware version 3.2 and analyzed as described above; however, the minimum relative fluorescence unit (RFU) threshold for detection was set at 50 RFUs. Additional stochastic effects were not considered in this evaluation. When possible, the mean values, standard deviations, and *P*-values for significance were calculated. *P*-values were calculated using the one way ANOVA test using Microsoft Excel and an α value of 0.05, where a value below 0.05 indicates significance.

Primer Degeneracy

Two DNA input quantities (0.125ng and 0.062ng), from each of four samples, were examined as described above. However for the DOP-PCR amplification three different degenerate primers (6N, 10N, and 16N) were evaluated. The standard (6N) degenerate DOP-PCR primer has the following sequence: 5' – CCGACTCGAGNNNNNNATGTGG – 3'. The sequence of the modified 10N primer used was 5'-CTCGAGNNNNNNNNNNATGTGG -3'; the sequence of the 16N degenerate primer was 5-'NNNNNNNNNNNNNNNNATGTGG -3'.

The mean and standard deviations for allele success (number of chromosomal locations correctly represented) and intra-locus heterozygote peak balance were calculated for all primer degeneracy conditions tested. A two-way ANOVA (α = 0.05) was calculated to determine if there was a significant difference in allele success with relation to the primer used for DOP-PCR amplification. Finally, the correlation coefficient (r) was calculated to determine if a correlation existed between intra-locus heterozygote peak balance and the degeneracy of the primer.

Non-Specific Cycle Number

DNA input quantities of 0.25ng, 0.125ng, 0.0625ng, 0.031ng, 0.016ng, and 0.0078ng, from each of five samples, were examined as described above. However, for the DOP-PCR non-specific amplification rounds (Table 1, Step 2), several different cycle numbers were evaluated. The cycle numbers evaluated included 3, 4, 5, 7, 9, 12, or 15 cycles.

The mean and standard deviations for allele success (number of chromosomal locations correctly represented) and intra-locus heterozygote peak balance were calculated for all cycle number conditions tested. A one-way ANOVA (α = 0.05) was calculated to determine if there was a significant difference in allele success with relation to the cycle number used for DOP-PCR amplification. If a significant difference was observed, then linear contrast analyses (α=0.05) were calculated to determine if there was a difference in means between cycle number conditions tested and the control 5 cycles. Additionally, the correlation coefficient (r) was calculated to determine if a correlation between intra-locus heterozygote peak balance and the non-specific cycle number used.

Proofreading Enzymes

DNA input quantities of 0.25ng, 0.125ng, 0.0625ng, 0.031ng, 0.016ng, and 0.0078ng, from each of six samples, were examined as described above. However, for the proofreading enzyme study specifically, the 10N degenerate primer with 12 non-specific cycles and 2.5U of either AmpliTaq Gold(ABI, Foster City, CA), Platinum *Pfx* (Invitrogen, Carlsbad, CA), GeneAmp High Fidelity (*Taq*:unknown proprietary enzyme(s)) (ABI, Foster City, CA), *Taq:Pfu* (Stratagene PicoMaxx High Fidelity PCR system (La Jolla, CA)), *Taq:DeepVent* (Invitrogen Platinum Taq High Fidelity DNA polymerase (Carlsbad, CA)), or *Taq:Tgo* (Roche FastStart High Fidelity PCR system (Indianapolis, IN) were utilized. Additionally, in cases where Mg^{2+} was not provided in the 10X PCR buffer, additional $MgCl_2$ or $MgSO_4$ was added to the master mix to obtain the final concentration recommended by the manufacturer of the enzyme (39-43). Further, following STR multiplex amplification, the samples were all purified and eluted in 100% HiDi formamide using the Qiagen MinElute kit (Valencia, CA)(44,45). The DNA samples were eluted in 100% HiDi formamide to a final volume of 10µl. Finally, following multiplex STR amplification, the samples were size-separated and detected on an ABI 3100*Avant* Genetic Analyzer (Foster City, CA) using 1.2µL of the STR product, along with 12.25µL Hi-Di Formamide and 0.25µL GS 500-ROX internal lane standard (ABI, Foster City, CA).

The mean and standard deviations for allele success (number of chromosomal locations correctly represented), intra-locus heterozygote peak balance, inter-locus peak balance, and peak height across DNA input quantities were calculated for all proofreading enzyme conditions tested. To determine if there was a significant difference in allele success among the enzyme conditions tested, a two-way ANOVA (α=0.05) was calculated. If a significant difference was observed, then linear contrast analyses (α=0.05) were calculated to determine if there was a difference in means between enzyme conditions tested and AmpliTaq Gold (ABI, Foster City, CA). A two-way ANOVA (α = 0.05) was also performed to determine if there was a significant difference in inter-locus peak balance and in peak height across DNA input quantities for all enzyme conditions tested. If a significant difference was observed, then linear contrast analyses (α=0.05) were calculated to determine if there was a difference in means between each enzyme conditions tested and AmpliTaq Gold (ABI, Foster City, CA). Additionally, a one-way ANOVA (α=0.05) was also calculated to determine if there was a significant difference in peak height at each DNA input quantity tested for each enzyme tested.

CE Analysis & Post-PCR purification

DNA was diluted using distilled, de-ionized water (ddH_2O) to the following concentrations (each per 5µL volume): 0.25ng, 0.125ng, 0.062ng, 0.031ng, 0.016ng, and 0.0075ng. After STR multiplex amplification, all DOP-PCR samples were analyzed for concordance with expected profiles using the ABI GeneMapper ID Software version 3.2 and analyzed as described above; however, the minimum relative fluorescence unit (RFU) threshold for detection was set at 50 RFUs. Additional stochastic effects were not considered in this evaluation. When possible, the mean values, standard deviations, and P-values for significance were calculated. P-values were calculated using a one way ANOVA using Microsoft Excel and an α value of 0.05, where a value below 0.05 indicates significance.

Non-probative/Mock Casework Study

All non-probative/mock casework amples were separated into two categories following the initial data quality evaluation: high quality/high quantity samples and low quantity and/or

low quality samples (Table 3). For each category, several parameters were used to measure success of STR results. First, STR allele success was determined by dividing the number of correct/expected alleles present by the total number of expected alleles and multiplying by 100. Alleles were designated as correct were those whose type was concordant with the known type at that locus. Next, heterozygote peak balance was calculated by dividing the height of the minor peak (in RFU) by the height of the major peak (in RFU) and multiplying by 100. To determine if there was a significant difference between the traditional STR and dcDOP-PCR results in heterozygote peak balance and allele success, a one-way ANOVA test ($\alpha = 0.05$) was calculated. A two-way ANOVA test ($\alpha = 0.05$) was calculated to determine if there was a significant difference in peak height across all loci for the two methods. A linear contrast analysis was performed to determine if there was a difference in peak height between dcDOP-PCR and traditional STR analysis.

Figure 1: dcDOP-PCR Workflow

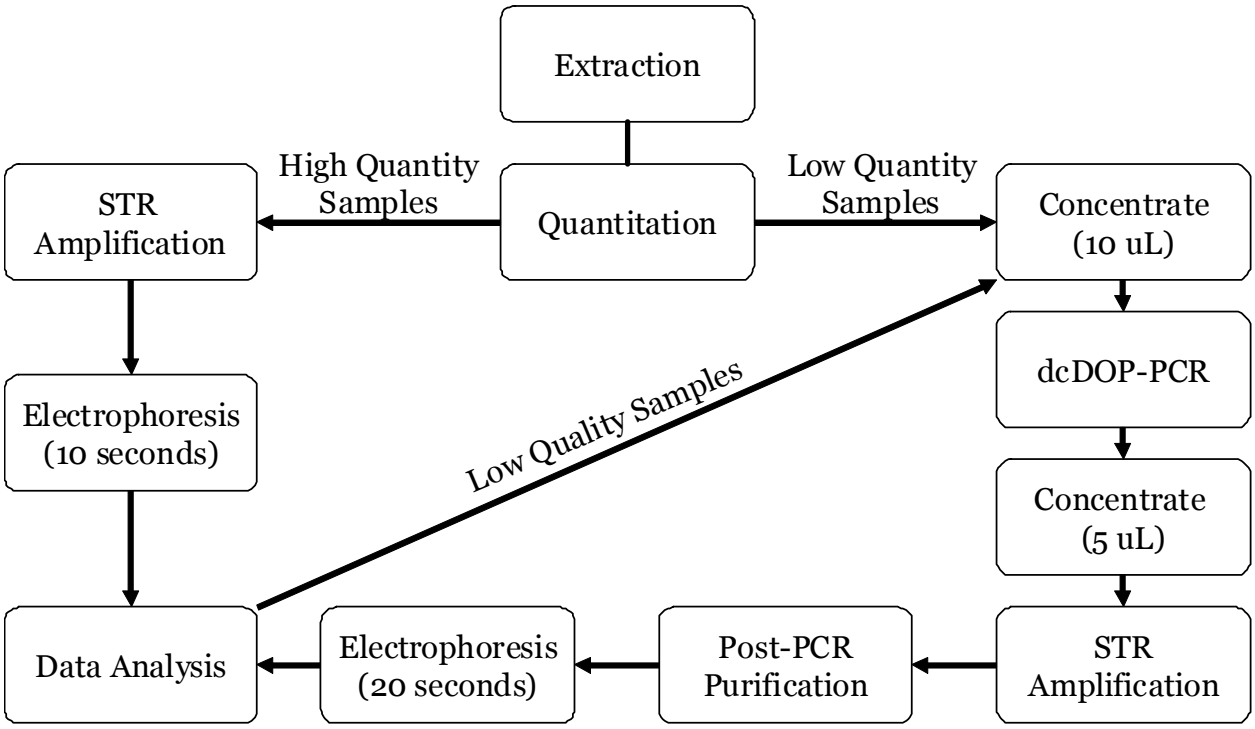


Table 2: Traditional DOP-PCR parameters compared to dcDOP-PCR parameters

	Traditional DOP-PCR	**dcDOP-PCR**
Extension Time	3 minutes	3 minutes
Primer Degeneracy	6N primer	10N primer
Enzyme	*Taq* polymerase	*Platinum Taq* High Fidelity Polymerase
Non-specific cycles	5 cycles	12 cycles
Post-PCR Purification	--	Qiagen MinElute
CE injection time	10 seconds	20 seconds

Table 3: Sample groups for analysis

High Quantity/High Quality Samples	**Low Quantity and/or Low Quality Samples**
Room temperature bloodstains (1 week-4 months)	80 degree bloodstains (1 month-4 months)
56 degree bloodstains (1 week-4 months)	Environment bloodstains (1 month-4 months)
80 degree bloodstains (1 week)	Fingerprints
Environment bloodstains (1 week)	Bones and Teeth
Hair Roots	Hair Shafts
Cigarette Butts	Cartridge Cases

III. Results

Multiple Displacement Amplification vs. Low Copy Number-PCR

When MDA products were visualized on 1% agarose gels, fragments >40kb were seen regardless of sample input amount. However, DNA fragments >40kb were also consistently seen in negative control sample lanes (data not shown). Total yields observed after MDA products were highly variable, with no specific trend noted. When examining the STR success rate of samples after MDA (including partial profiles at loci), the trend seemed to show a decrease of locus success with decreasing input amounts, as expected. At 0.25ng, the success

rate was 88.3%; this gradually decreased to a low of 24% for 0.016ng inputs ($\rho = 0.8729$) (Figure 2). Data quality was observed by calculating average heterozygote peak balance. Complete heterozygous loci produced after MDA were highly imbalanced, with overall averages being below 50% for all inputs except 0.125ng, which averaged a slightly higher heterozygote balance rate of 56.8%. In addition to the expected alleles, a number of additional alleles were produced in the MDA products upon STR amplification. For these samples, the number of alleles per locus often exceeded the expected 1.8 alleles per locus for the stock sample used. An increased number of alleles that typed as "Off-ladder" were the primary contributor to this increased average of alleles per locus (Table 4). However, none of these were determined to be due to instrumental anomalies (such as spikes or spectral failure "pull-up") nor to typical stochastic effects such as –A or high stutter. Additionally, although all negative control samples failed to produce any quantifiable DNA, all produced at least one allele in the STR analysis.

Figure 2: STR results for samples pre-amplified with MDA. Average percentage of successful loci, where success was determined by counting loci with at least a partial profile ($n = 6$). There was a trend of decreasing STR success that correlated to decreasing sample input for loci that contained both expected alleles above threshold.

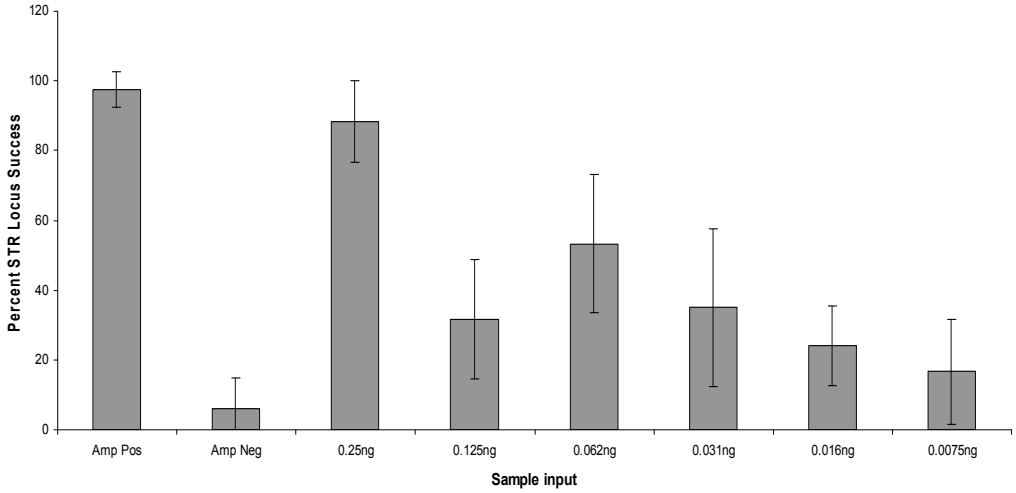


Table 4: Average number of allele peaks detected per locus for MDA and LCN amplified samples. The expected average number of alleles per locus for all samples tested is 1.8 alleles per locus.

Input Amount (ng)	Allele Peaks Detected per Locus (expected = 1.8)		
	MDA	LCN PCR (5 sec)	LCN PCR (2 sec)
Amp Pos	2.68	11.28	5.80
Amp Neg	0.30	0.28	0
0.25	4.02	9.68	4.08
0.125	1.75	4.95	2.93
0.062	3.05	2.10	1.73
0.031	1.05	1.18	0.98
0.016	1.04	0.70	0.50
0.0075	0.98	0.40	0.33
Overall Average	2.0	3.17	1.76

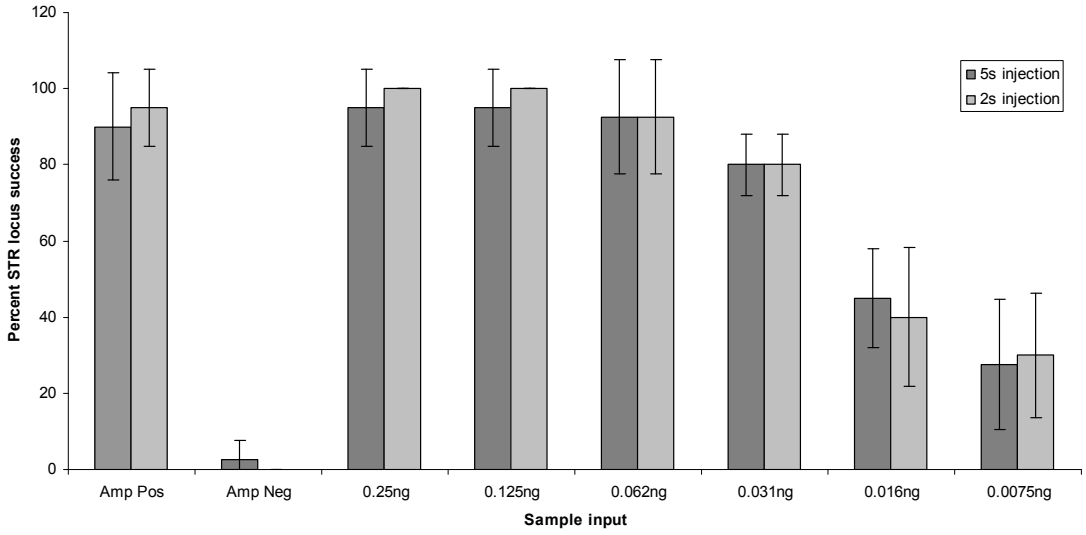
STR locus success for LCN-PCR samples with inputs at or above 0.031ng produced at least 80% success (including partial profiles at loci using the default five second injection time). However, below this input, success decreased to 45% for 0.016ng inputs and 27.5% for 0.0075ng inputs ($\rho = 0.6757$ for all). Results were comparable when the injection time was decreased to two seconds (Figure 3a). Ideally, STR profiles from pristine sample sources are expected to display heterozygous peak balances of >50%; however, average balance >50% was only obtained from the 0.25ng and 0.125ng input samples. All other inputs had an average peak balance below 50% using the five second injection time (Figure 3b). Analysis of LCN PCR products showed that the average number of alleles per locus for samples with inputs at or above 0.062ng was greater than the expected 1.8 alleles per locus (Table 4). A large number of these additional alleles typed as off-ladder alleles. In most cases these off-ladder alleles could be attributed to stochastic effects such as high –A occurrence or high stutter produced from the –A peaks; however, there were also peaks that were unable to be attributed to stochastic effects,

instrument anomalies, nor alleles contributable to persons who had worked with these samples. For example, at the D21S11 locus with a five second injection, the allele 23.2 appeared in fifteen out of 32 samples and was not attributable to any stochastic effect or contamination from the sample handlers.

A comparison of the STR results from both methods is shown in Table 5.

Figure 3: STR results for samples amplified through LCN PCR**.** (A) Average percent locus success (including partial profiles at loci) ($n = 4$). There was a moderate correlation between sample input and locus success, with a sharply increasing amount of allele drop-out occurring for samples with less than 0.062 ng of input DNA ($\rho = 0.6757$ for five second injections; $\rho = 0.7177$ for two second injections). (B) Average percent peak balance for heterozygous loci. While the average peak balance is below the desired 50% level for all samples with a five second injection, decreasing the sample injection length increased the average peak balance to above 50% for all samples.

A)



B)

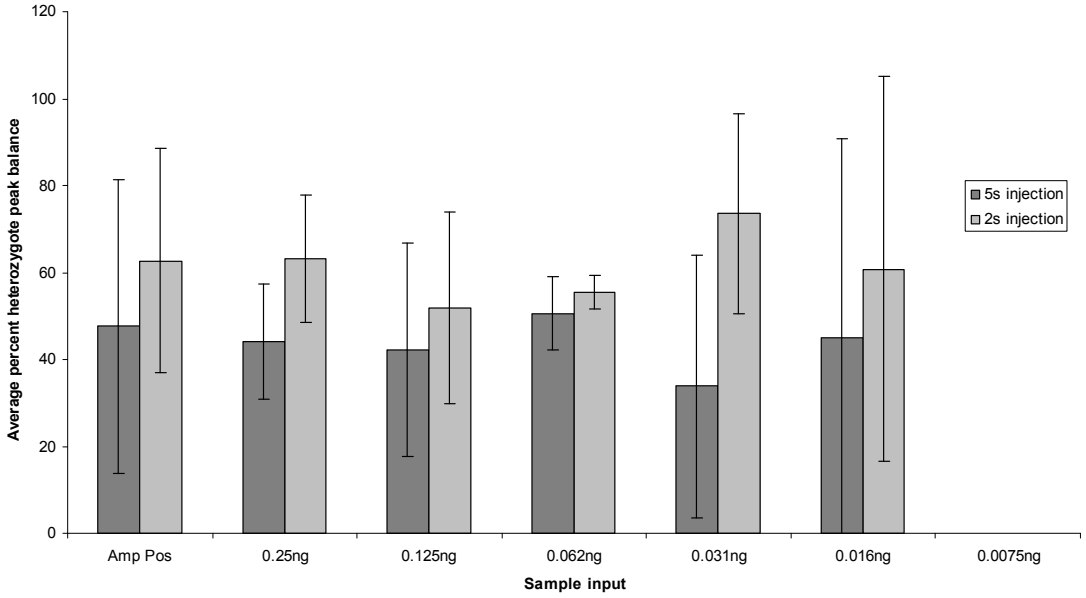


Table 5: Comparison of means between MDA and LCN-PCR for five and two second injections. While MDA did not produce the same random allele recurrence patterns, it did seem to produce more random alleles than LCN PCR altogether. (* p= 0.0006, ** p = 0.0004, † p = 0.0055, †† p = 0.0010)

	MDA	LCN-PCR (five second injection)	LCN-PCR (two second injection)
Percent STR Locus Success	47.69	75.00*	76.79**
Average Heterozygote Peak Balance	40.76	45.60	55.78†
Average Number of Alleles per Locus	2.01	3.17††	1.75

DOP-PCR Methodology

No significant difference in average maximum fragment size was observed between the "home brew" DOP-PCR and commercially available Roche DOP Master kit methods (p= 0.7191) (Figure 4). The quantification results showed a comparable increase in total DNA yield

after DOP-PCR for both the "home brew" DOP-PCR reaction and the Roche DOP Master kit reaction (Figure 5). Though some variation was noted, there was no significant difference between the "home brew" DOP-PCR and commercially available Roche DOP Master kit methods when examining total yield ($p = 0.1222$). After completion of genotyping, the average percent STR allele success was calculated for both the "home brew" DOP-PCR reaction and the Roche DOP Master kit. The lower DNA input amounts produced lower percent STR allele success. The percent allele success for the 0.062ng inputs were 8.8% and 0.5%; 0.031ng inputs were 0% and 1.9%; 0.016ng inputs were 0% and 0.25%; and 0.0078ng inputs were 3.1% and 0.75% for the "home brew" DOP and Roche DOP Master reactions, respectively (Figure 6). The positive and negative controls worked as expected. There is not a significant difference in percent STR allele success between the Roche DOP Master kit and the "home brew" DOP reaction ($p = 0.3252$). The average percent balance of heterozygote peak heights was calculated on all heterozygote peaks produced by both methods. When heterozygous peaks were present, they appeared to be well balanced (>50%) for both the "home brew" DOP-PCR reaction and the Roche DOP Master kit reaction (data not shown) ($p = 0.6539$). It was noted that allelic drop-in did occur on occasion for both methods however, the frequency of occurrence was similar for both the "home brew" DOP-PCR and the Roche DOP Master kit (Figure 7).

Figure 4: Gel electrophoresis for "home brew" dilution series. "Home brew" dilution series size separated on an agarose yield gel, stained with ethidium bromide, visualized by ultra violet light, and captured digitally. Post DOP-PCR products loaded by lane: 1kb extension ladder (Invitrogen), Positive, Negative, 0.25ng sample input, 0.125ng sample input, 0.062ng sample input, 0.031ng sample input, 0.016ng sample input, and 0.0078ng sample input, and 1kb extension ladder (Invitrogen).

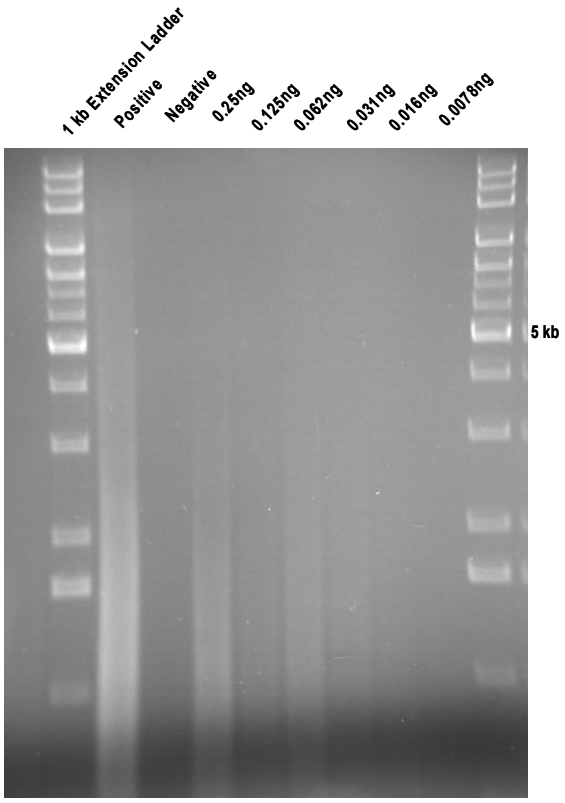


Figure 5: Total yield data for all "home brew" and Roche Master DOP kit DOP-PCR amplified samples. The data shows the average total yield values after the "home brew" amplification are comparable to the commercially available Roche DOP Master kit for most DNA sample input values. ($P = 0.1222$).

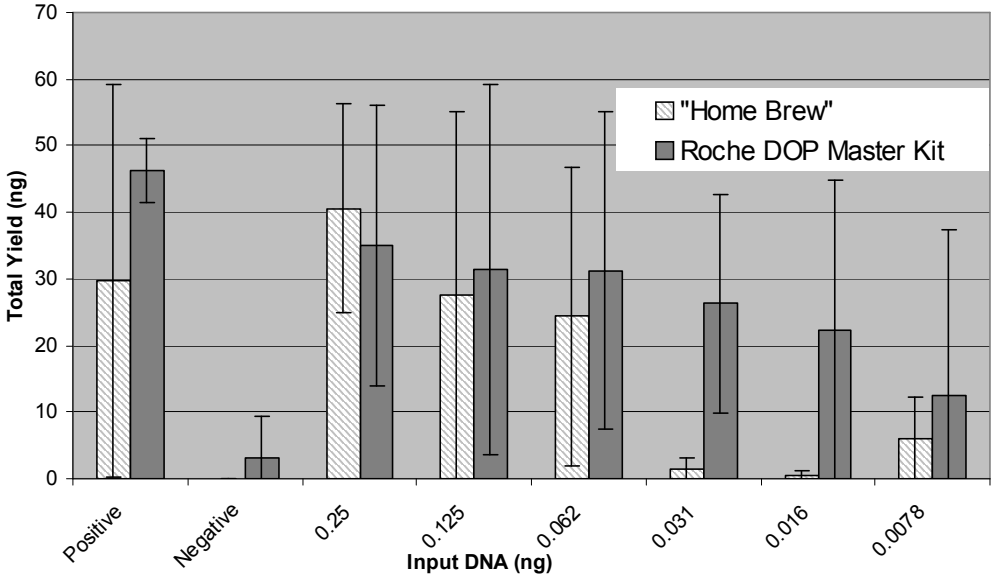


Figure 6: Percent STR allele success for all "home brew" and Roche Master DOP kit DOP-PCR amplified samples. The data shows the average percent correct allele calls for the "home brew" fragment size comparable to the commercially available Roche DOP Master kit for most DNA input values. ($P = 0.3252$)

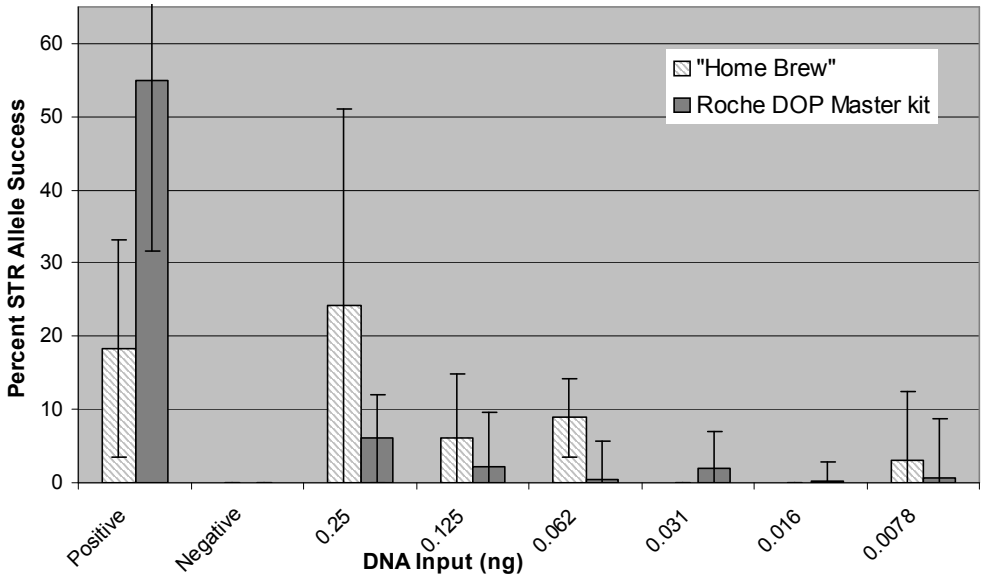
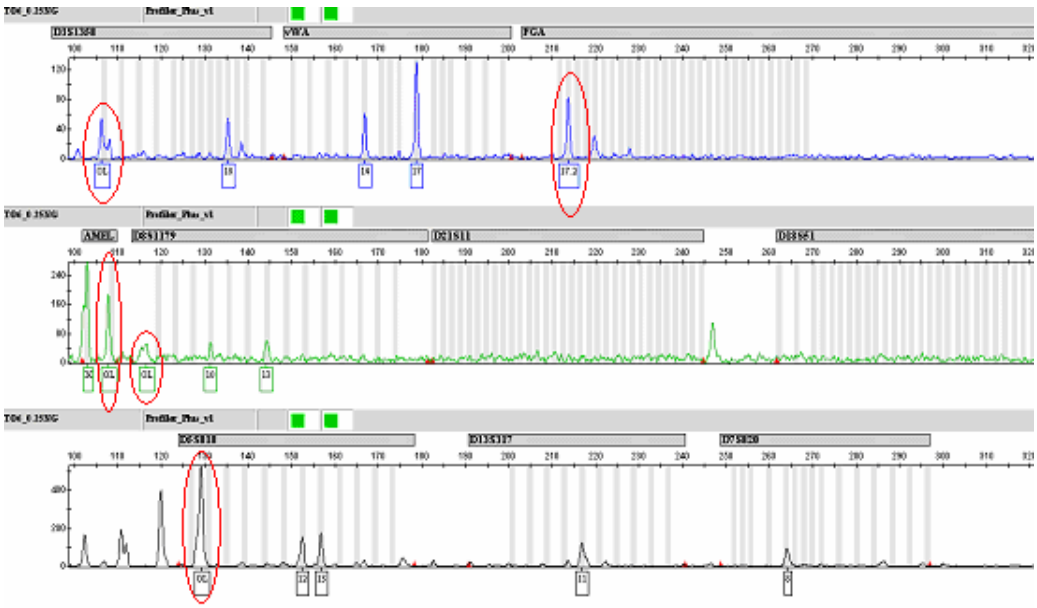
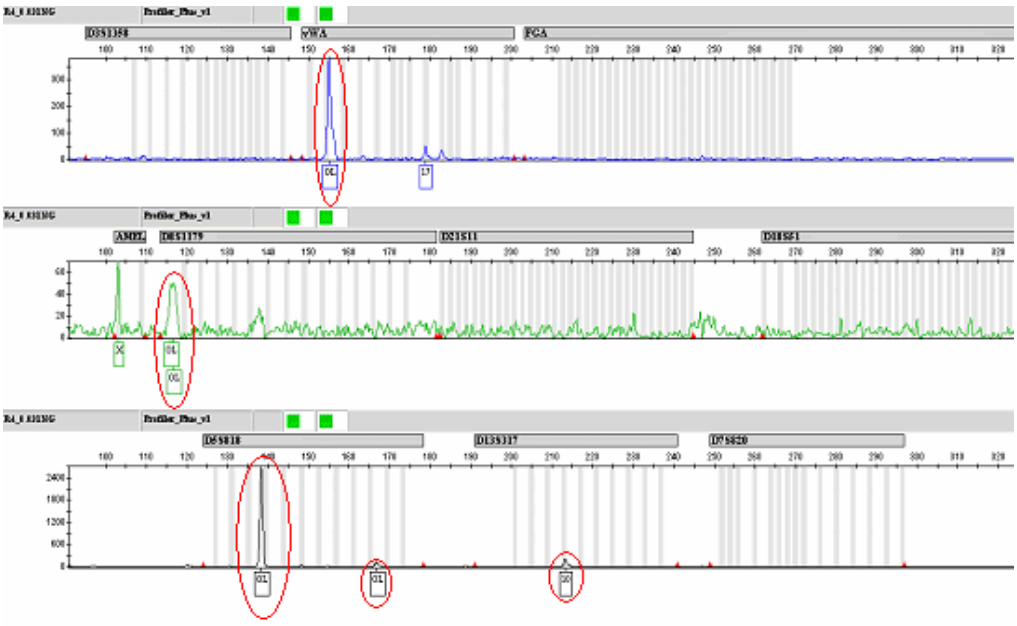


Figure 7: Electropherograms after DOP-PCR reactions displaying stochastic effects. A.) Electropherogram of a "home brew" DOP-PCR amplified sample showing allelic drop-in (circled). B) Electropherogram of a Roche DOP Master kit amplified sample showing allelic drop-in (circled).

A)



B)



DOP-PCR Amplification Reaction

Primer Degeneracy

When the 10N and 16N degenerate primers were used with the traditional DOP-PCR thermalcycling parameters, an increase in allele detection was observed when compared to the control 6N primer, regardless of DNA input ($p = 0.9320$) (Table 6). Furthermore, the 10N degenerate primer produced more expected alleles (37.5%) than the 16N degenerate primer (26.4%), and was the only primer that improved STR allele detection over traditional STR amplification (30.0%). As expected, more alleles were detected from samples with greater quantities of template DNA (0.125ng) than those with lower template DNA inputs.

Unexpected, sporadic (drop-in) alleles were seen less frequently in sample data using each experimental primer (10N, 16N) compared to the control 6N primer. Alternatively, high stutter was seen more frequently in the 10N primer and 16N primer samples than in the control

6N primer samples. However, when compared to each other, the 10N and 16N primer samples had comparable low levels of stochasticity, including the production of products with incomplete 3'-adenylation (minus A). Additionally, there was a strong positive correlation between intra-locus heterozygote peak balance and primer degeneracy, with balance improving as the primer degeneracy increased ($r = 0.953$, Figure 8). It should be noted that variation seen in the 6N primer data is likely due to the relatively small data set, as fewer heterozygote loci were successfully amplified when using this primer. Overall, given the number of alleles amplified and quality of the data produced, the 10N primer was the best performing primer of those tested in the DOP-PCR.

Proofreading Enzymes

The addition of proofreading polymerases generally improved the performance of the DOP-PCR technique ($p=0.005$). However, the Platinum *Pfx* (Invitrogen, Carlsbad, CA) enzyme had a negative effect on the DOP-PCR technique, significantly reducing the number of STR alleles detected ($p=0.0066$); whereas, FastStart High Fidelity enzyme (Roche, Indianapolis, IN) failed to significantly alter the STR results altogether ($p=0.0648$). Platinum Taq High Fidelity (Invitrogen, Carlsbad, CA) provided the best STR results when used for the DOP-PCR, producing the highest number of STR alleles amplified and detected and showing the most improvement over the control DOP-PCR with AmpliTaq Gold (ABI, Foster City, CA) ($p<0.0001$) and over traditional STR (Table 7). Though not as effective as Platinum Taq High Fidelity (Invitrogen, Carlsbad, CA), both the GeneAmp High Fidelity (ABI, Foster City, CA) and PicoMaxx High Fidelity (Stratagene, La Jolla, CA) enzymes showed a significant improvement in STR alleles detected when used for the DOP-PCR ($p=0.0013$, 0.0248 respectively) (Table 7). However, neither of these (GeneAmp High Fidelity nor PicoMaxx High

Fidelity) showed a significant improvement in subsequent STR allele success when looking exclusively at data where template DNA was in the low copy number range (≤0.100ng) (p=0.0969 and 0.3090, respectively, Table 7).

For all polymerases tested, the DOP-PCR products from alleles within a single locus were adequately balanced, producing intra-locus heterozygote peak ratios of ≥0.60 (Figure 9). Furthermore, there was no difference in peak height as STR product size varied for all enzyme conditions tested (p=0.6741, Figure 10). Among the DOP-PCR polymerase conditions that improved STR allele success (Platinum Taq High Fidelity, GeneAmp High Fidelity, and PicoMaxx High Fidelity), only GeneAmp High Fidelity significantly altered peak heights as template DNA input varied, reducing peak height as template DNA input decreased (p=0.0254) (data not shown). Additionally, when compared to the control AmpliTaq Gold (ABI, Foster City, CA), there was no difference in peak height for any polymerase tested at any DNA template quantity analyzed, except for Platinum Taq High Fidelity (Invitrogen, Carlsbad, CA) at 0.0625ng template DNA (p=0.0362). Finally, very few stochastic effects were observed regardless of DNA template input or enzyme condition. Of the 9 loci examined for each sample (n=324 loci examined per enzyme condition), no more than 5 sporadic allele drop-ins were observed in a single data set (enzyme condition). Overall, the Platinum Taq High Fidelity (Invitrogen, Carlsbad, CA) polymerase consistently produced the best STR results of all enzyme conditions tested for use in the DOP-PCR (Figure 11).

Table 6: Average percent STR allele success based on primer degeneracy.

	Traditional STR	**Primer Degeneracy**		
DNA Input	(n=10)	6N (n=10)	10N (n=8)	16N (n=8)
0.125ng	33.8	7.5	41.2	31.9
0.0625ng	26.3	3.7	33.8	20.8
Overall Average	30.0	5.6	37.5	26.4

Figure 8: Intra-locus heterozygote peak ratios for each primer tested. A correlation between heterozygote peak balance and primer degeneracy is shown (r = 0.953), with both experimental primers (10N and 16N) generating profiles with average intra-locus heterozygote peak balance of > 0.50. The box plot shows a five number summary of data. The box represents 50% of the data. The line in the center of the box represents the median. The top of the box, or the upper quartile, indicates the 75th percentile. The bottom of the box, or the lower quartile, indicates the 25th percentile. The minimum and maximum data value is indicated by the whiskers. n=30 for 6N primer and n=8 for 10N and 16N primer.

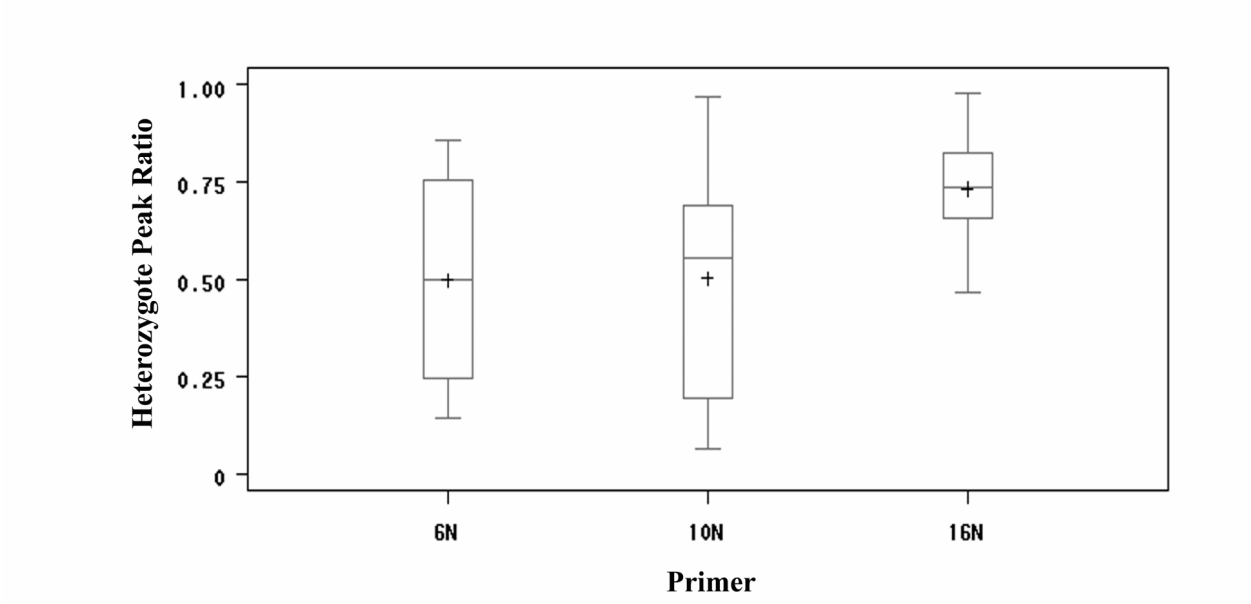


Table 7: Performance of DOP-PCR with proofreading enzyme *vs.* AmpliTaq Gold

		Avg. % STR Allele Success	% Improvement *vs.* AmpliTaq Gold (Template DNA ≤0.100ng)
Controls	Traditional STR	28.8	N/A
	AmpliTaq Gold	26.4	N/A
Proofreading Enzymes	Platinum Taq High Fidelity	56.8*	+21.1
	GeneAmp High Fidelity	45.1*	+8.3
	PicoMaxx High Fidelity	39.4*	+5.1
	FastStart High Fidelity	15.7	-0.2
	Platinum *Pfx*	10.7*	-13.9

p≤0.05; n=36 for each enzyme condition

Figure 9: Intra-locus heterozygote peak ratios for each DOP-PCR polymerase tested. Enzymes tested include: FastStart High Fidelity (FS), GeneAmp High Fidelity (GA), PicoMaxx High Fidelity (PM), Platinum *Pfx* (PP), Platinum Taq High Fidelity (PT), and AmpliTaq Gold (TG). All conditions tested produced average intra-locus heterozygote peak ratios of ≥0.60. The box plot shows a five number summary of data. The box represents 50% of the data. The line in the center of the box represents the median. The top of the box, or the upper quartile, indicates the 75th percentile. The bottom of the box, or the lower quartile, indicates the 25th percentile. The minimum and maximum data value is indicated by the whiskers. n=36 for each enzyme tested.

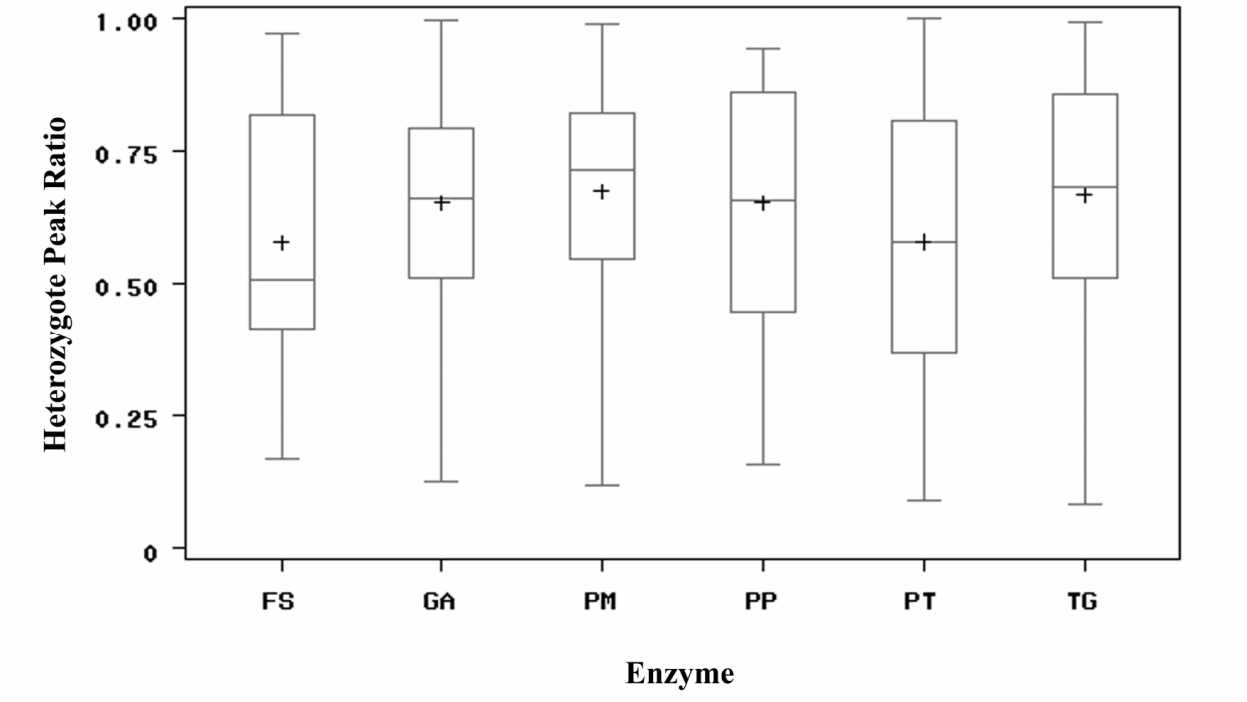


Figure 10: Inter-locus peak height for each enzyme condition. Data shown is from low copy number DNA samples only (< 0.100ng).Loci are arranged from largest expected product size (base pair) to smallest. No difference in peak height was observed regardless of product size or enzyme condition (p=0.6741). No error bars at a locus for a specific enzyme condition indicates that only one sample produced results. No data present at a locus for a specific enzyme condition indicates that no results were obtained. n=24 for each enzyme condition.

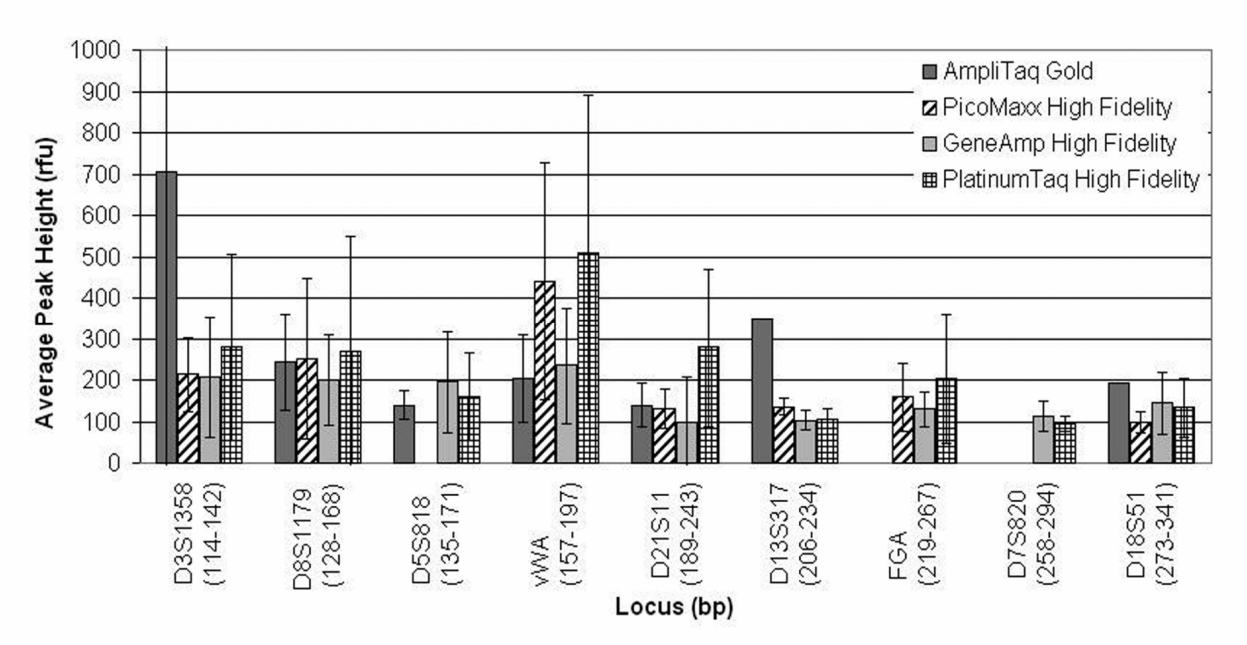
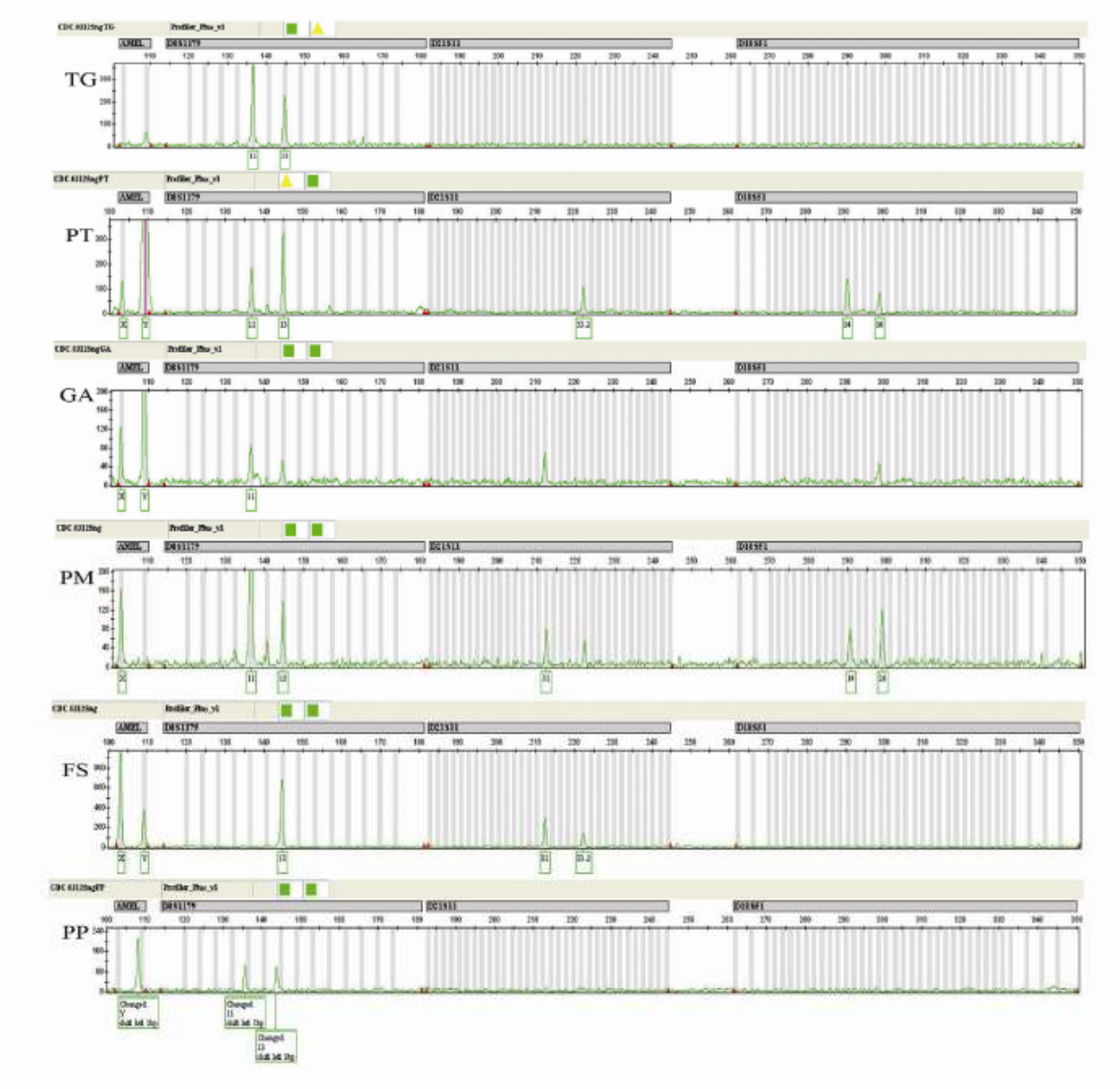


Figure 11: STR electropherograms for all enzyme conditions tested. Data shown represents three STR loci and Amelogenin from a single sample at a single template DNA input of 0.03125ng. Enzymes include: AmpliTaq Gold (TG), Platinum Taq High Fidelity (PT), GeneAmp High Fidelity (GA), PicoMaxx High Fidelity (PM), FastStart High Fidelity (FS), and Platinum *Pfx* (PP). True alleles are marked with a number indicating the STR allele call.



DOP-PCR Thermalcyclying Parameters

Changing the number of non-specific cycles significantly improved the DOP-PCR technique at all template DNA quantities tested ($p<0.0001$). Specifically, changing the cycle number to 12 resulted in the greatest number of STR alleles amplified, on average, when compared to the previously published control 5 cycles ($p=0.0002$) (Table 8). Other non-specific cycle numbers tested for the DOP-PCR did not result in a significant improvement when compared to the previously published 5 cycles. As expected, the higher template DNA quantities amplified (≥0.125ng) were consistently better at generating typeable alleles (regardless of cycle number). Further, when five or fewer non-specific cycles were used, a large degree of STR allele drop-out was consistently observed, particularly for samples in the low copy number DNA range (<0.100ng).

Data quality issues, such as sporadic allele drop-in, incomplete 3'-adenylation (minus A), and high stutter product for all non-specific cycle numbers tested were comparable to the control 5 cycles. As expected, most anomalies noted were in samples with higher template DNA inputs (>0.100ng). Although 12 non-specific cycles produced the highest overall STR allele success, there were fewer overall data quality issues or stochastic effects seen in the STR products from samples amplified with nine non-specific cycles in the DOP-PCR thermalcycling (data not shown). For the specific measure of intra-locus heterozygote peak balance, there were no observable trends as cycle number increased ($r = -0.73$, Figure 12). However, samples tested with 12 or 15 non-specific cycles had a slightly lower intra-locus heterozygote peak balance compared to the other conditions tested (Figure 12). Overall, samples amplified with 9 non-specific cycles in the DOP-PCR produced the best STR data quality; while samples amplified with 12 non-specific cycles produced the greatest number of STR alleles. It should be noted that

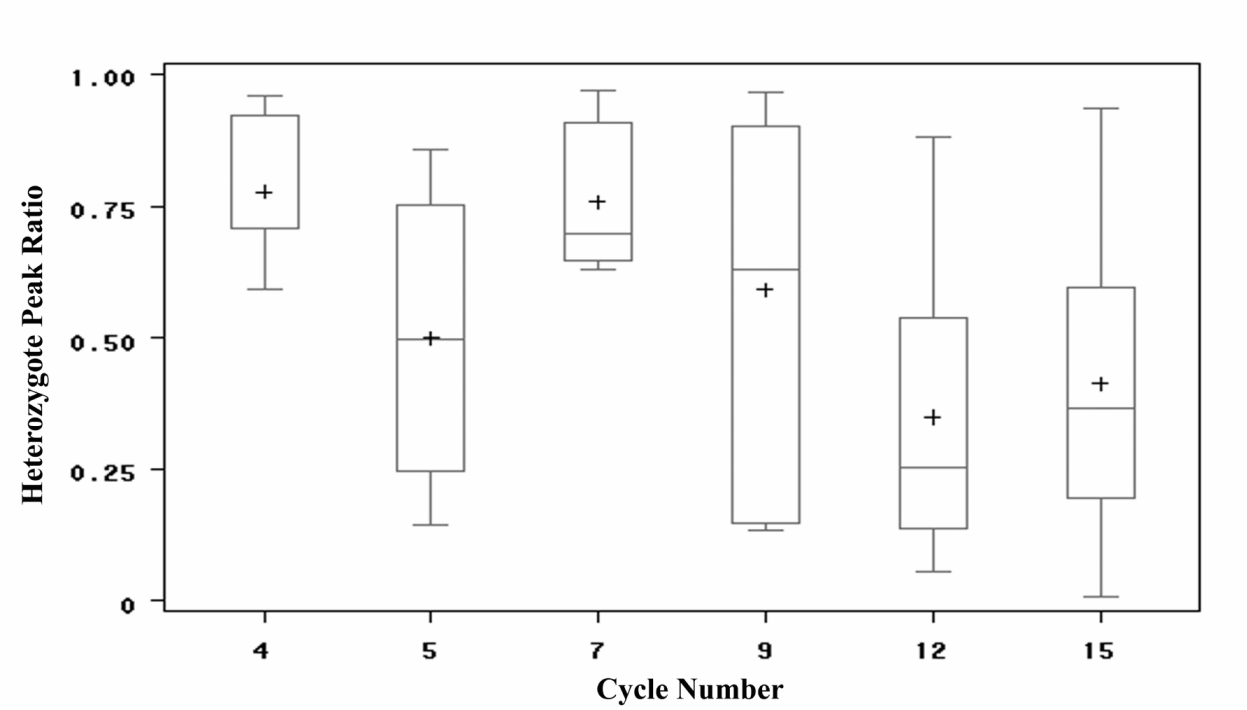
while altering cycle number improved the performance of the DOP-PCR, neither of these individual changes (thermalcycling conditions, 9 or 12 non-specific cycles) improved STR allele detection over traditional STR analysis methods (8.3% and 14.1% vs. 28.8%).

Table 8: Average percent STR allele success for each cycle number tested.

Cycle Number	Average STR Allele Success (%) ($p<0.0001$)
3	1.7
4	4.9
5	5.0
7	5.7
9	8.3
12	14.1*
15	7.0

* $p \leq 0.0002$ *vs*. control 5 cycles; n=20 for each cycle number

Figure 12: Intra-locus heterozygote peak ratios for each non-specific cycle number tested. There was no trend in heterozygote peak balance with increasing cycle number (r = -0.73). Most conditions tested produced intra-locus heterozygote peak ratios of ≥0.50. The box plot shows a five number summary of data. The box represents 50% of the data. The line in the center of the box represents the median. The top of the box, or the upper quartile, indicates the 75th percentile. The bottom of the box, or the lower quartile, indicates the 25th percentile. The minimum and maximum data value is indicated by the whiskers. n=30 for each cycle number tested.



CE Analysis & Post-PCR purification

Surprisingly, the samples which were not post-STR purified and were electrokinetically injected for both 10 and 20 seconds showed no significant difference in percent STR allele success between injection times tested for all DNA input values (data not shown) (p= 0.6723). The comparison of the samples using the standard 10 second electrokinetic injection without purification versus the samples using the standard 10 second electrokinetic injection with purification showed no significant difference in percent STR allele success for all DNA input values tested (Figure 13) (p = 0.7394). The samples which were purified and eluted directly into

formamide and electrokinetically injected for both 10 and 20 seconds also showed no significant difference in percent STR allele success between injection times for all DNA input values (Figure 14) ($p = 0.4039$). However, it should be noted that, though not significant, the 20 second injection with purification showed improved STR results at every DNA input value tested and produced more complete STR profiles ($p = 0.6524$).

The average percent balance of heterozygote peak heights was calculated for all samples on all heterozygote peaks produced by all four conditions tested. When heterozygous peaks were present, they appeared to be well balanced for both all four conditions and were well within the range acceptable within the forensic DNA community (Figure 15). It was noted that allelic drop-in did occur on occasion for both methods however, the frequency of occurrence was similar for all four conditions tested.

Figure 13: Average percent STR allele success comparing samples that have not undergone purification and samples that have undergone purification and elution directly into formamide. The standard 10 second electrokinetic injection was applied to all samples. ($p= 0.7394$).

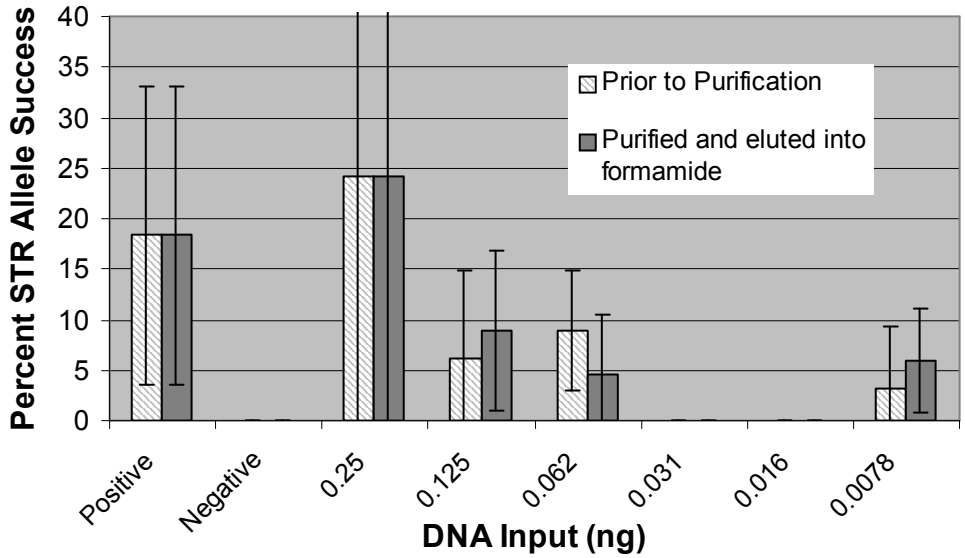


Figure 14: Average percent STR allele success comparing 10 and 20 second electrokinetic injection times for samples that have undergone post-STR amplification purification and elution directly into formamide. (p= 0.4039).

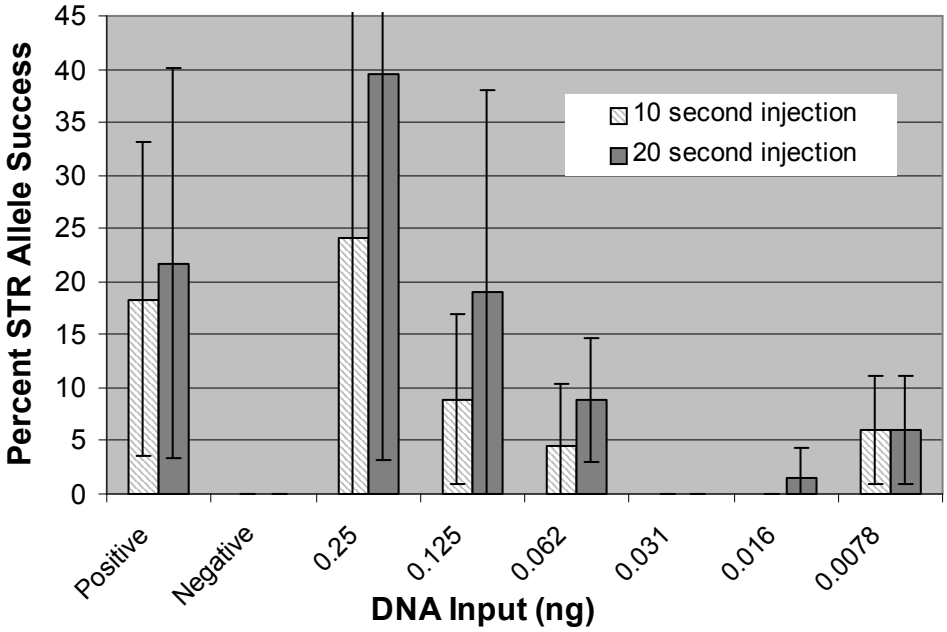
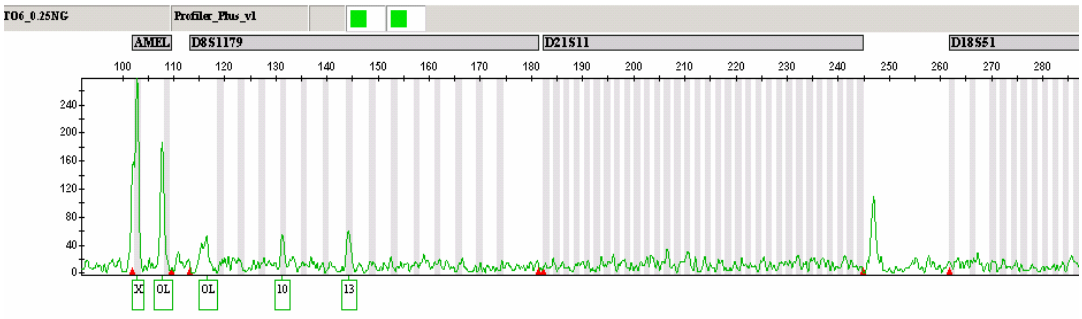
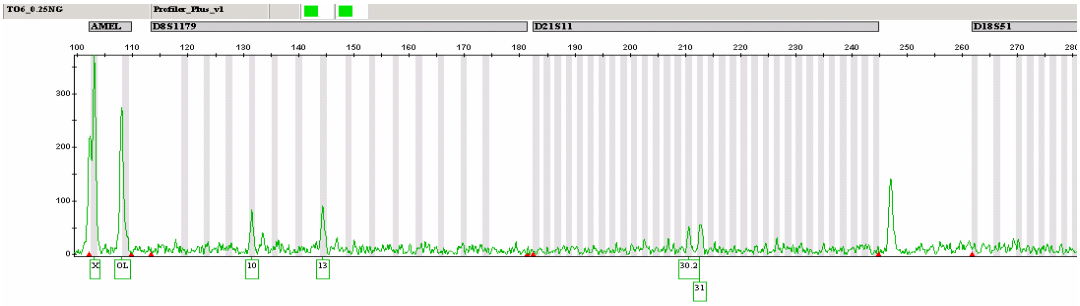


Figure 15: Electropherograms displaying an example of heterozygous peak balance for all four conditions tested. A) 10 second injection prior to purification. B) 10 second injection after purification and elution directly into formamide. C) 20 second injection prior to purification. D) 20 second injection after purification and elution directly into formamide.

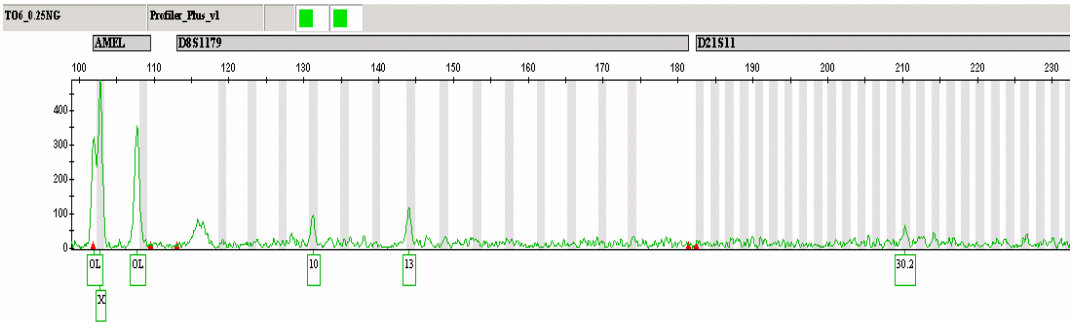
A)



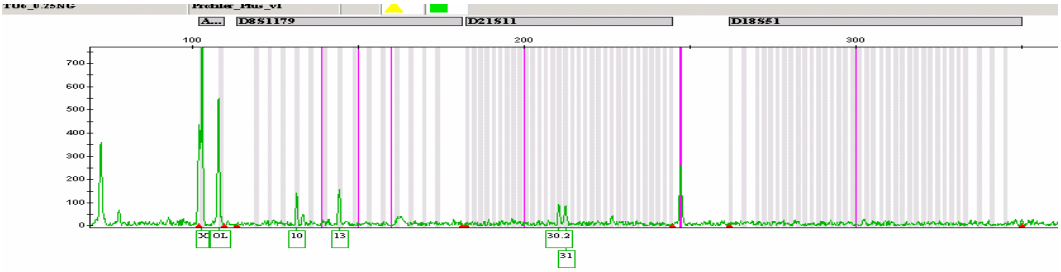
B)



C)



D)



Non-probative/Mock Casework Study

The ABI Quantifiler® Human DNA Quantitation kit and the ABI 7500 Real-Time PCR System was used to determine the concentration of DNA present in each sample. Quantitation values ranged from "undetermined" (0ng/µL) to 0.765ng/µL. The overall average quantitation values for 80 degree bloodstains and environment bloodstains were 0.286ng/µL (SD=0.25) and 0.000685ng/µL (SD=0.001555), respectively. A trend was not observed in total DNA yield over time in the aged bloodstains (data not shown). The average total DNA yields for samples tested in this study are listed in Table 9. Of these samples, the 80°C aged bloodstains had the greatest total yield (28.6ng) while the other samples had very low yields (≤0.41ng).

dcDOP-PCR performed significantly better than traditional STR analysis in overall allele success (p=0.0282) (Table 10). An overall average allele success of 21.2% (of known, expected alleles) was obtained with the dcDOP-PCR method with an average gain of 1.6 alleles. In contrast, these samples had only 9.9% allele success with traditional STR analysis.

The average heterozygous peak balance of the dcDOP-PCR samples was 69.2% (Figure 18). With traditional STR analysis, the average heterozygote peak balance was 69.9%. Thus, no significant difference in heterozygote peak balance was observed between these methods (p= 0.8910). Furthermore, a two-way ANOVA test determined that there was not a significant difference in peak height across all loci for either method tested (dcDOP-PCR or traditional STR analysis) (p=0.7325) (Figure 19). Additionally, there was no significant difference in overall peak height between the two methods, dcDOP-PCR and traditional STR analysis (p=0.1031) (Figure 19). It should be noted that these samples show a large variation in peak heights as has been shown previously (15,16).

Overall, dcDOP-PCR had greater allele success than traditional STR analysis (Table 10). Additionally, dcDOP-PCR data quality was generally equivalent to traditional STR analysis (Figures 18-19). Also, in several samples where traditional STR analysis yielded no results, strong partial profiles (with few stochastic effects) were obtained with dcDOP-PCR (Figure 20).

Table 9: Average total nuclear DNA yield from challenged and non-probative casework samples

Sample	Average Total DNA Yield (ng) ± Standard Deviation
80°C Bloodstains (n= 12)	28.6 ± 25.3
Bone/teeth (n=4)	0.41 ± 0.47
Fingerprints (n=3)	0.08 ± 0.13
Environment Bloodstains (n= 13)	0.07 ± 0.16
Hair shafts (n=3)	0.07 ± 0.11
Cartridge cases (n=3)	0.06 ± 0.10

Table 10: Average percent allele success for challenged and non-probative casework samples

Sample Type	Average Percent Allele Success	
	Traditional STR	dcDOP-PCR
Bloodstains: 80°C 1 month (n= 3)	11.1	35.2
Bloodstains: 80°C 3 months (n= 3)	24.1	35.2
Bloodstains: 80°C 4 months (n= 3)	14.8	22.2
Bloodstains: 80°C 6 months (n= 3)	1.9	13.0
Bloodstains: Env. 1 month (n= 3)	3.7	16.7
Bloodstains: Env. 3 months (n= 3)	0	7.4
Bloodstains: Env. 4 months (n= 3)	0	3.7
Bloodstains: Env. 6 months (n= 3)	0	25.9
Cartridge cases (n= 3)	0	9.3
Hair shafts (n= 3)	0	3.7
Prints (n= 3)	18.5	44.4
Bones/teeth (n= 4)	38.9	27.8
OVERALL*	**9.9**	**21.2**

*p=0.0282 Env.= environment.

Figure 18: Heterozygous peak balance for challenged and non-probative casework samples. n= 38 for each method. p=0.8910

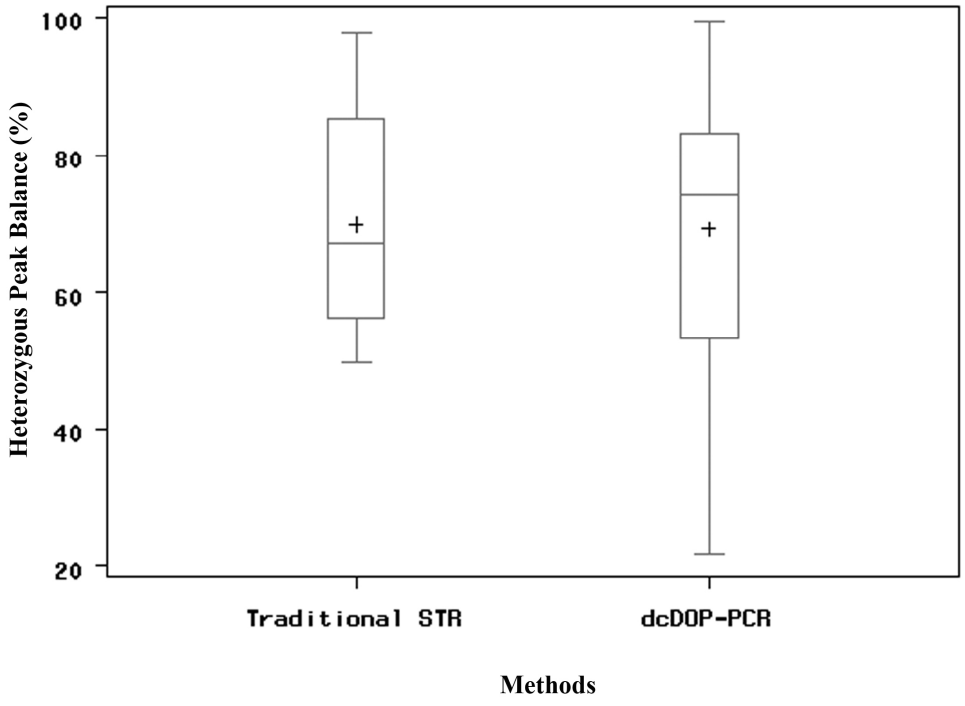


Figure 19: Average peak height across all loci for challenged and non-probative casework samples. n= 38 for each method. Loci are shown in order of expected product size range, with larger loci to the right.

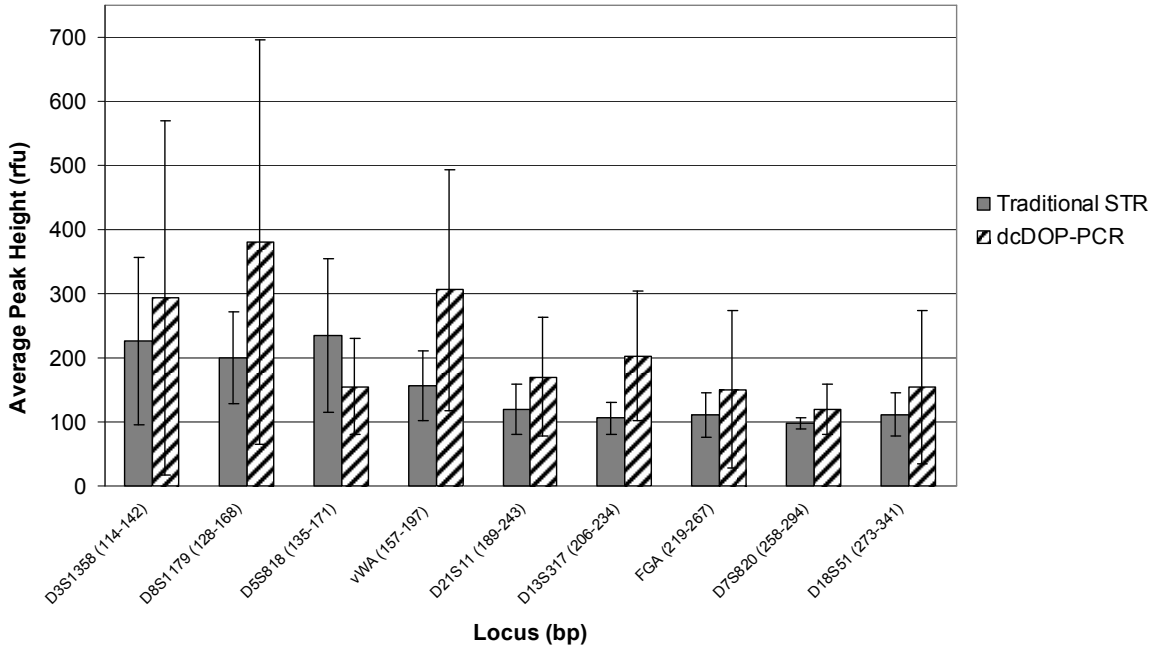
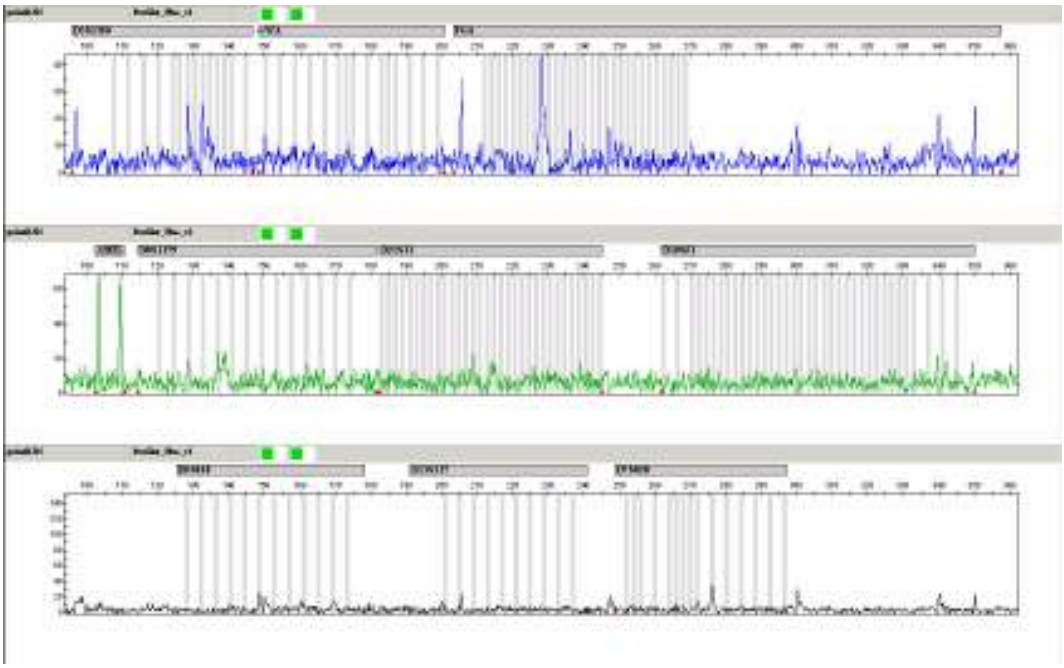
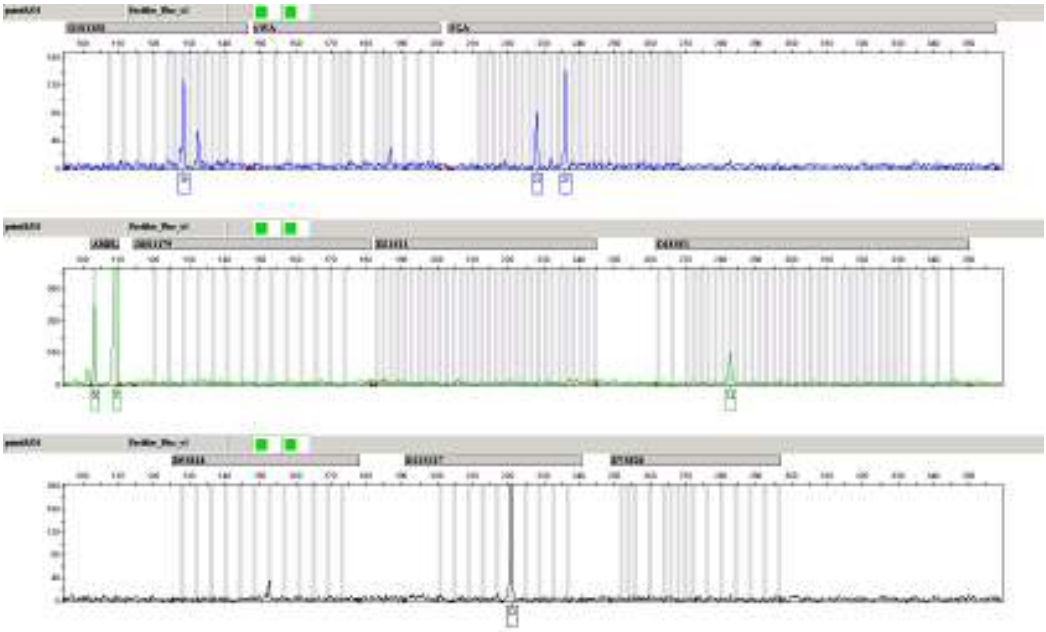


Figure 20: Electropherograms of a single dermal ridge fingerprint sample. A.) With traditional STR analysis, 0% of the expected alleles were generated. B.) With dcDOP-PCR, 33.3% of the expected alleles were generated, with few stochastic effects.

A.



B.



IV. Discussion & Conclusions

Multiple Displacement Amplification vs. Low Copy Number-PCR

Although both MDA and LCN PCR do have potential value for low copy number DNA evidence samples, both would need significant optimization to be useable in a forensic DNA laboratory setting. While LCN PCR has the highest success rate for achieving the expected alleles in a given profile (Table 5), the large numbers of extraneous alleles created and observed as stochastic effects must first be overcome. Stutter peaks for LCN PCR are often so high that they are called as alleles, which could lead to an examiner making a determination that the profile is a mixture of two or more DNA profiles rather than a single profile. In addition, the frequent reoccurrence of non-profile alleles seen with LCN PCR could prevent an accurate deduction of the proper profile for a sample. This could be especially problematic if this occurs

in multiple amplifications from the same DNA source. Both the NYC-OCME and United Kingdom's Forensic Science Service (FSS) use a consensus method of evaluating LCN PCR produced profile, where a sample is divided into multiple portions, amplified, and the resulting profiles combined to form a composite profile.

Based on this data, it would be difficult to recommend that one procedure be chosen over the other for further pursuit in obtaining complete, balanced STR profiles with minimal stochastic effects from very low copy number DNA samples (<0.100ng). However, as both would require extensive optimization, it would perhaps be best to pursue the LCN PCR for future studies. The financial burden for labs that desire to implement the LCN PCR procedure would be less than with MDA, which would require specialized reagents and/or kit purchase, additional hands-on time for laboratory personnel, and an additional reaction that involves long amplification times. Further, our data suggests that LCN PCR results in fewer extraneous alleles (stochastic effects) and an increased overall rate of STR success. Future analyses will include a direct comparison of this data with data from the newly optimized dcDOP-PCR reaction described herein.

DNA Methodology

Overall, the "home brew" DOP-PCR performed at the same level as the commercially available Roche DOP Master kit, with no significant differences seen for any of the evaluation parameters analyzed. In forensic laboratories, it is both time consuming and costly to complete employee training for forensic DNA analysis. However, DOP-PCR utilizes the same technologies and methodologies as traditional forensic DNA analysis, and requires little additional theory to be understood. Additionally, no new instrumentation or expensive reagents

are required. Based on these considerations, DOP-PCR is a cost effective method for low quantity/low quality forensic DNA analysis. Additionally, in a research laboratory, funding is typically very limited. The use of the "home brew" DOP-PCR reaction for all subsequent DOP-PCR reactions in the Dawson Cruz lab allowed this research to be completed at a lower cost, thus allowing for more analysis to be completed with the available funds. Further, it would be impossible to evaluate and optimize DOP-PCR parameters if the exact components of the kit where unknown or could not be modified. The ability to control the components of the DOP-PCR reaction created the ability for optimization of all DOP-PCR reaction parameters included this research project, including evaluation of additional degenerate primers, thermal cycling parameters, and amplification enzyme combinations.

DOP-PCR Amplification & Thermalcycling Optimization

The largest goal of this study was to determine if modifying the primer degeneracy, the non-specific cycle number, or the polymerase used for the DOP-PCR method would be advantageous for downstream multi-locus, multi-chromosome genetic analysis from low quantity DNA samples. There are many occasions, particularly for forensic and clinical genetic applications, whereby these types of analyses must be performed from an extremely limited DNA sample. Therefore, it is essential that pre-amplification techniques, such as DOP-PCR, provide true whole genome coverage and an even amplification of all loci that will be targeted in downstream testing, even when only a few cells or low copy number DNA is available ($\leq$0.100ng). In an attempt to assess whether the DOP-PCR amplification provides an unbiased genome-wide amplification, the DOP-PCR amplifications in this study were followed with a second amplification using a commercially-available multiplex STR kit (ABI AmpFλSTR®

Profiler Plus™ PCR Amplification Kit). This kit is designed to amplify a homologous region of the Amelogenin gene (found on the X and Y chromosomes) as well as nine tetrameric repeat loci on nine separate chromosomes, generating DNA fragments ranging in size from 107 to 341bp (16, 46). These repetitive DNA sequences are known to be more difficult to accurately copy compared to traditional DNA sequences. This characteristic, along with the multi-chromosome, multiplex nature of the amplification, makes the ABI AmpFλSTR® Profiler Plus™ PCR Amplification Kit (ABI, Foster City, CA) an appropriate test for determining the overall performance and genome coverage of WGA methods for forensic and clinical diagnostic applications.

The DOP-PCR method of whole genome amplification seems to work more efficiently for multiplex STR analysis from low copy number DNA samples. The traditional 6N DOP-PCR primer contains a specific and rare restriction-endonuclease recognition site as well as additional nucleotides for added specificity (26) that are not necessary for all genotyping applications. When removed and replaced with additional degenerate nucleotides, resulting fragments provide more complete genome coverage. While the 16N DOP-PCR primer provided slightly improved intra-locus peak ratios, the samples amplified using the 10N DOP-PCR primer produced a larger number of detectable alleles, indicating that a moderate amount of primer degeneracy works best to increase the number of potential binding sites during the first rounds of DOP-PCR. These data indicated that too much primer specificity limits the ability to attain true whole genome amplification, yet too little primer specificity may also lead to problems such as shortened amplification product (47).

The data generated in this study further demonstrate that increasing the number of cycles for nonspecific primer annealing in the DOP-PCR provides a more complete, genome-wide

coverage. With each additional cycle, more DNA product is generated, increasing the possibility that that target loci will be amplified. In this study, 12 nonspecific cycles generated the most complete STR profiles, especially in amplifications where less template DNA was added. While most nonspecific cycle numbers tested produced relatively similar levels of stochastic effects and low levels of sporadic allele drop-in, intra-locus allele amplification was more balanced when nine nonspecific cycles were used for the DOP-PCR. However, for human identity applications, it seems most beneficial to sacrifice a small degree of heterozygote product balance in exchange for the acquisition of additional genotype data.

The inclusion of a proofreading enzyme in the DOP-PCR reaction mixture further improved the overall performance of this WGA method. Platinum Taq High Fidelity (Invitrogen, Carlsbad, CA), a *Taq*:*DeepVent* enzyme combination, was determined to be the best polymerase for DOP-PCR amplification. Platinum Taq High Fidelity (Invitrogen, Carlsbad, CA) had the highest STR allele success rate and the best STR data quality of all enzyme conditions tested (Figure 5), showing the most significant improvement over *Taq* polymerase (when used alone). Several factors could have let to the superior performance of Platinum Taq High Fidelity (Invitrogen, Carlsbad, CA). When the *DeepVent* proofreading enzyme is combined with *Taq*, it reportedly produces fragments that range from ~12-20kb (27, 48). This is larger than what is reported for any of the other polymerase conditions used in this study. Additionally, *DeepVent* is an extreme thermophyle; it is stable in temperatures as high as 104°C, allowing it to withstand long denaturation steps, such as those used in the DOP-PCR (49). Finally, the addition of high concentrations of ammonium sulfate in the PCR buffer could have further enhanced the efficiency of Platinum Taq High Fidelity (Invitrogen, Carlsbad, CA) by preventing depurination of DNA, resulting in more stabilized template (50).

CE Analysis & Post-PCR purification

When comparing the 10 second electrokinetic injected DOP-PCR samples that were purified (and eluted into formamide) to those that were not purified, there was no noticeable difference in the final STR profile results. However, while the statistical calculation did not show significance, it is important to note that the combination of a longer injection time (20 seconds) with a post-STR amplification purification and elution directly into formamide consistently produced the highest percent STR allele success at all low copy DNA input values tested.

It is important to discuss the significance of the lack of additional stochastic effects in the data obtained after a 20 second electrokinetic injection as compared to the standard 10 second injection. Typically, when the injection time is increased, the RFU values of the peaks in a profile will increase proportionally, which may cause there to be so much fluorescence being excited by the laser, and captured by the CCD camera, that the filter cannot function optimally. The lack of additional pull-up in the 20 second injected samples over that seen in the 10 second injected samples is likely due to the lower fluorescence seen for these samples, in general. The RFU values obtained after the standard 10 second injection are not high enough to cause spectral failure when increased due to an injection time of 20 seconds.

Although the combination of a 20 second injection with a post-STR amplification and elution directly into formamide did produce a higher percent of STR allele success, there may be several disadvantages to implementing an additional purification after STR amplification. First, because the DOP-PCR reaction volume is so high (100µL), the DOP-PCR reaction products must be concentrated *prior* to STR analysis already, which initially increases the cost per sample by $2.28. A second Microcon purification/concentration will raise the cost per sample an

additional $2.28. For a high throughput lab that could potentially process thousands of low copy DNA samples annually, this can become quite expensive. Additionally, Microcon purification requires a combination of pippetting and centrifugation, which cannot be automated using standard liquid handling robotics available at this time. For these reasons, the final studies and all future studies will include the use of the Qiagen MinElute® Post-PCR Purification Kit according to the manufacturer's recommendations with modifications described by Smith and Ballantyne (16). Finally, an additional purification step introduces additional room for contamination and error, as there are more tube transfers and manual pippetting than without the additional purification step. In casework, there is no room for either error or contamination; therefore laboratories generally avoid procedures that introduce too many manual steps.

Considering the advantages and disadvantages, of a post-purification step, each individual laboratory will need to evaluate the benefits of incorporating this modification to low copy number DNA analysis via dcDOP-PCR to determine if the increase in success is worth the extra costs and risks. This method, along with increased CE injection times and in conjunction with all aforementioned DOP-PCR modifications, may prove to be of great value.

dcDOP-PCR vs. Traditional STR Testing

The genome-wide coverage from DOP-PCR improved when a more degenerate primer (10N) was used along with additional nonspecific cycles (6) and a *Taq:*proofreading polymerase combination (*Taq:DeepVent*). Further improvements were also noted when a post-STR purification step was added prior to CE analysis along with an increased CE injection time. Low level DNA samples amplified using the optimized DOP-PCR protocol, dcDOP-PCR, resulted in a ~45% increase in the number of detected STR alleles when compared to traditional DOP-PCR ($p = 0.0003$) and a ~34% increase when compared to traditional STR testing without WGA

($p<0.0001$, Figure 16). On average, these increases would be expected to improve the power of discrimination by ~1 in 1.82 million (3), which could provide enough additional information to confirm a human identification. Further, when dcDOP-PCR was used, nearly complete STR profiles were produced from as little as 0.03125ng of template DNA (Figure 17). Interestingly, when dcDOP-PCR was used, some aspects of the resulting data quality also improved. For example, there was no significant difference in inter-locus peak height as fragment size increased. This indicates that the expected drop-out of larger alleles (i.e., ski slope effect) that is often observed from multiplex amplification of low level DNA samples was not observed with this technique. Additionally, allele drop-out that has been observed with the traditional DOP-PCR technique as well as other WGA techniques (including MDA and PEP) (12, 14) was not observed when the dcDOP-PCR protocol was used. Further, very few extraneous drop-in alleles were observed with the improved DOP-PCR method, which is in stark contrast to what has been reported with other low copy number methods (21,50). Despite these results, proper precautions should continue to be taken to avoid the potential for amplification of extraneous DNA when WGA is used.

Figure 16: STR allele success (%) comparing the traditional STR test (no WGA) *vs.* STR with the traditional DOP-PCR technique and the dcDOP-PCR optimized technique. The DOP-PCR technique utilized a 6N degenerate primer with 5 non-specific amplification cycles and a *Taq* polymerase only. The dcDOP-PCR technique utilized a 10N degenerate primer, 12 non-specific cycles, and Platinum Taq High Fidelity enzyme. These conditions were combined to produce a ~34 increase in STR allele detection when compared to the traditional STR test and ~45% increase in STR allele detection when compared to STR amplification with traditional DOP-PCR technique. n=25 for traditional STR, n=25 for DOP-PCR, n=31 for dcDOP-PCR.

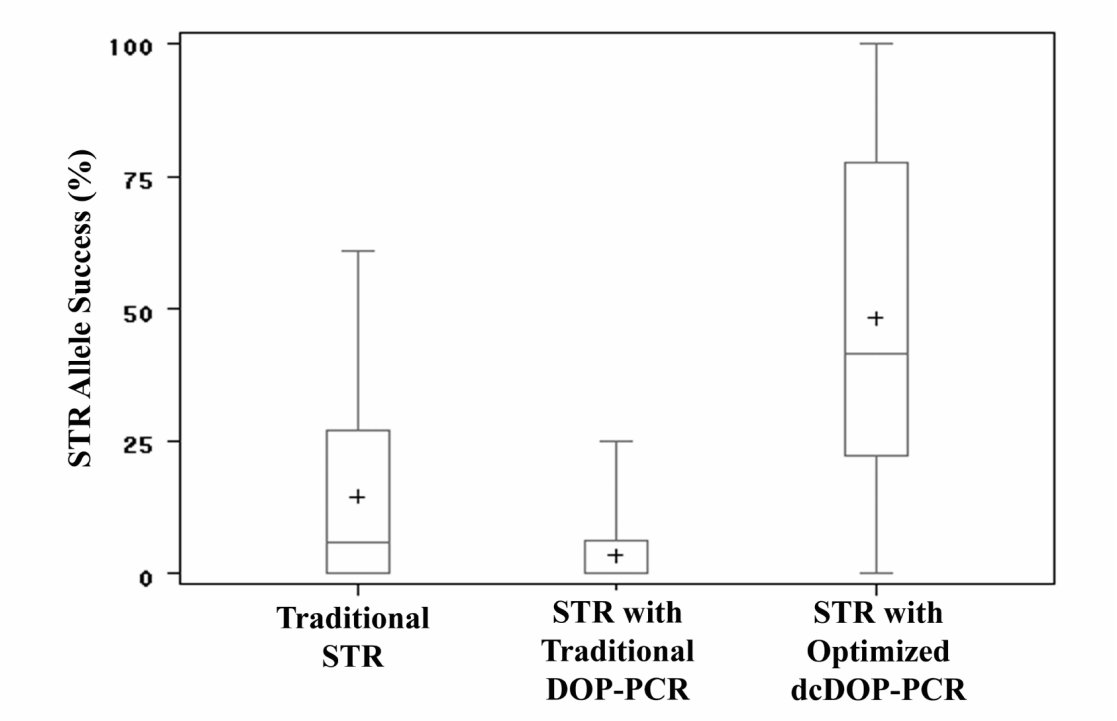
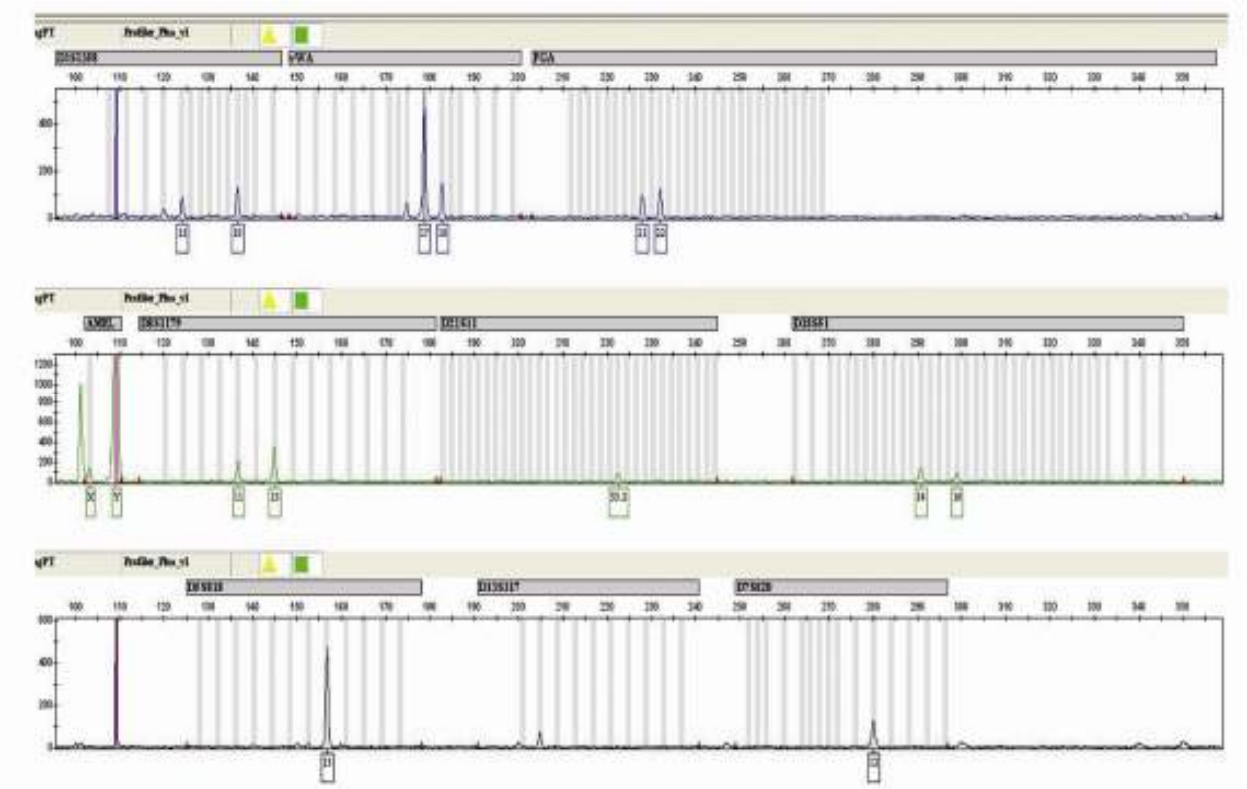


Figure 17: STR electropherogram from dcDOP-PCR amplified sample. Data shown represents all nine STR loci from a single sample (0.03125ng of template DNA amplified). True alleles are marked with a number indicating the STR allele call. For this sample, 13 of 18 of expected STR alleles were detected (72%).



Non-probative/Mock Casework Study

The final portion of this project was the testing of the fully optimized dcDOP-PCR method using mock and non-probative casework samples similar to those frequently encountered in the forensic laboratory. dcDOP-PCR improved allele success with degraded and low copy number samples as seen in previous studies (Table 10) (9,13-15). Further, from severely compromised samples, dcDOP-PCR was able to produce strong partial profiles from samples that had little to no results with traditional STR analysis (Figure 20).

It is interesting to note that an increase in CE electrokinetic injection time from the default 10 seconds to 20 seconds can significantly increase allele success and peak height (Table

11). However, it has also been shown that severe genotyping issues and artifacts increase in samples which have been exposed to uncontrolled environmental conditions samples and in samples which have been organically extracted, concentrated and dcDOP-PCR amplified. Therefore, by increasing the injection time, this can also significantly increase the amount of artifacts present in those types of samples and may also decrease heterozygous peak balance (51, Table 11).

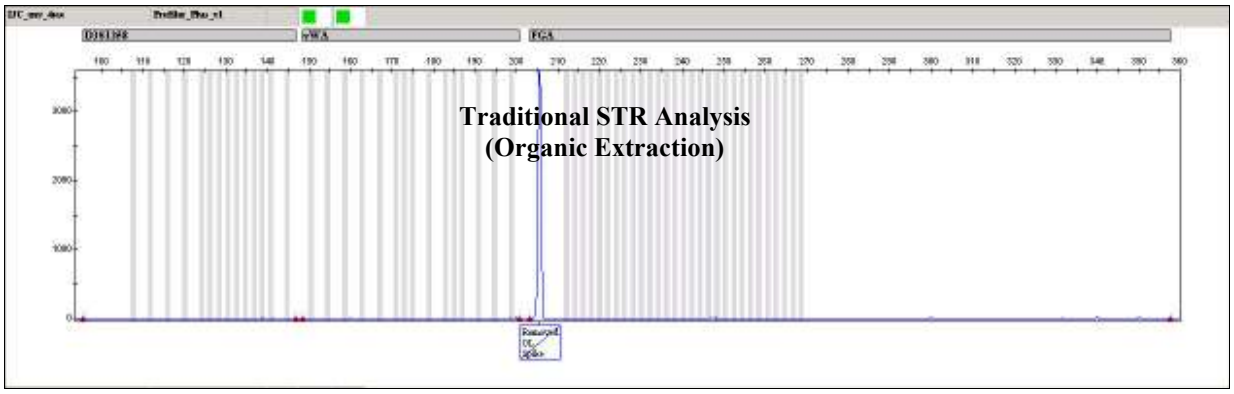
Genotyping issues and artifacts are not as frequently observed in samples that have been extracted using silica-based extraction technologies. In comparison to the organic extraction method, Qiagen DNA extraction kits have proven to be more efficient at eliminating some inhibitors and contaminants, producing fewer stochastic effects upon CE analysis (Figure 21) (52-55). Therefore, Qiagen DNA extraction kits or other silica-based methods are recommended for use with the dcDOP-PCR method.

Table 11: Comparison of traditional STR analysis to dcDOP-PCR with a 10 second CE electrokinetic injection and a 20 second CE electrokinetic injection

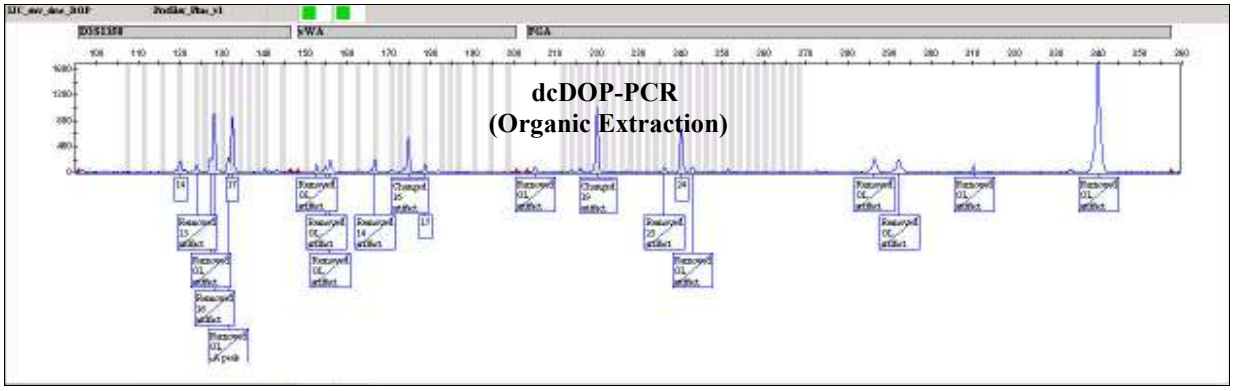
	Traditional STR Analysis	**dcDOP-PCR**	
		10 second injection	**20 second injection**
Allele Success	9.9%	21.2% (p= 0.0282)	39.2% (p<.0001)
Heterozygous Peak Balance	69.9%	69.2% (p= 0.8910)	57.8% (p=0.04)
Average Peak Height (RFU)	151.3	214.8 (p=0.1031)	827.6 (p= 0.0013)

Figure 21: A.) Electropherogram of a sample extracted organically and amplified using traditional STR analysis. B.) Electropherogram depicting the same sample that was extracted organically but was amplified using dcDOP-PCR. C.) Electropherogram of the same sample extracted using the Qiagen QIAamp® DNA Mini Kit and amplified using dcDOP-PCR.

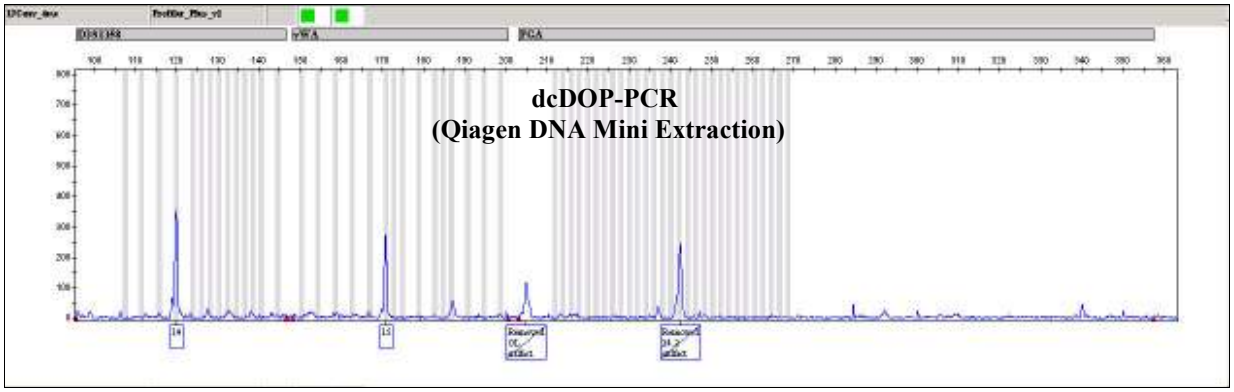
A.



B.



C.



Further Research

In the future, the aged bloodstains should continue to be collected so that further studies can be conducted with samples that were aged at longer time points than what were tested in this

study. Additionally, this method's performance should be compared to other methods designed for the analysis of compromised biological evidence samples, including MDA, LCN-PCR, and the recently available AmpF*l*STR® MiniFiler™ PCR Amplification kit (Applied Biosystems) which utilizes smaller STR amplicon sizes for the analysis of low copy number, degraded, and inhibited DNA (10).

Summary

DOP-PCR, a WGA pre-amplification method, has been thoroughly evaluated for use with forensic casework samples. However, preliminary data using the traditional DOP-PCR method with forensic samples indicated only limited success due to insufficient coverage of the genome and random stochastic effects which often overshadowed the increase in allele success reported (9, 14). As a result, DOP-PCR required modification to make it more amenable for use with forensic samples, and specifically for downstream multiplex STR typing. Successful modifications included increasing the degeneracy of the primer, increasing the number of non-specific cycle numbers used, including the use of a proofreading polymerase in the reaction mixture, post-STR purification, and an increase in electrokinetic injection time. While the majority of these individual modifications only slightly improved STR allele detection, combining the best-performing experimental conditions into a single procedure (dcDOP-PCR) significantly improved STR allele detection when compared to traditional STR analysis methods. A finalized protocol will now be made available to other agencies so that it can be evaluated externally (Appendix 1).

It is expected that when little to no traditional STR results are obtained from low copy or challenged forensic samples, the dcDOP-PCR method may assist in gaining enough valuable

information to significantly increase the power of discrimination for forensic investigations. It will be most useful for those samples which do not require organic extraction and samples that are not severely environmentally challenged. Overall, dcDOP-PCR has proven to be a relatively easy and inexpensive method for improving allele success and data quality of compromised biological evidence that might have otherwise not produced a profile using traditional STR methods.

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VI. Dissemination of Research Findings

Publications

Brown, K.L., Davis C.P., Pavlova V.R., Illescas M.J., **Dawson Cruz, T**. dcDOP-PCR for the Analysis of Mock and Non-Probative Casework Samples. (2008) *Journal of Forensic Science* (In preparation; To be submitted October 2008).

Pavlova VR, Bonnette MD, Rodier DN, Thompson LP, Boone EL, Brown KL, Trevino MB, Champagne JR, and Dawson Cruz T. (2008) Improvement of Degenerate Oligonucleotide Primed-PCR from Limited Quantities of DNA for Multi-Locus, Genome-wide Analysis. *Diagnostic Molecular Pathology* (Accepted September 2008, publication pending).

Rodier D.N., Bonnette M.B., and **Dawson Cruz T**. (2006) Comparative Evaluation of Multiple Displacement Amplification and Low Copy Number PCR for Forensic DNA Analysis of Low Yield DNA Samples. *FSI: Genetics* (Revisions in progress, To be resubmitted October 2008).

Presentations

National Institute of Justice, Annual Meeting, Washington, D.C., July, 2008

- Poster Presentation: "Degenerate Oligonucleotide Primed-Polymerase Chain Reaction with Taq/Proofreading enzyme combinations for Forensic DNA analysis" V. R. Pavlova and T. Dawson Cruz

Mid-Atlantic Association of Forensic Scientists, 2008 Annual Meeting, Huntington, W.V., April, 2008

- Platform Presentation: "Degenerate Oligonucleotide Primed-PCR with Taq/Proofreading Enzyme Combinations for Forensic DNA Analysis" V.R. Pavlova (*speaker*), K.L. Brown, L.P. Thompson, T. Dawson Cruz
- Platform Presentation: "An Evaluation of dcDOP-PCR for the Analysis of Mock and Non-Probative Casework Samples" K.L. Brown (*speaker*), C.P. Davis, M.J. Illescas, V.R. Pavlova, T. Dawson Cruz

American Academy of Forensic Sciences, 60th Annual Meeting, Washington, D.C., February, 2008

- Platform Presentation: "Primer Modifications for the Improvement of Degenerate Oligonucleotide Primed-PCR-Based WGA from Limited Quantities of DNA" M.D. Bonnette (*speaker*), D.N. Rodier, K.L. Brown, M.B. Trevino, J.R. Champagne, T. Dawson Cruz
- Poster presentation: "Optimization of DOP-PCR for Forensic DNA Analysis using Taq/Proofreading Enzyme Combinations" V.R. Pavlova, K.L. Brown, L.P. Thompson, T. Dawson Cruz

Mid-Atlantic Association of Forensic Scientists, 2007 Annual Meeting, Washington, D.C., May, 2007

- Platform Presentation: "Primer and Cycle Number Modifications for the Improvement of STR Analysis after Degenerate Oligonucleotide Primed-PCR" M.D. Bonnette (*speaker*), K.L. Brown, M.B. Trevino, J.R. Champagne, T. Dawson Cruz
- Poster presentation: "Low Copy Number Methodologies: A Comparison Study of Low Copy Number PCR and Multiple Displacement Amplification" D.N. Rodier, M.D. Bonnette, T. Dawson Cruz

American Academy of Forensic Sciences, 59th Annual Meeting, San Antonio, TX, February, 2007

- Poster presentation: "Low Copy Number Methodologies: A Comparison Study of Low Copy Number PCR and Multiple Displacement Amplification" D.N. Rodier, M.D. Bonnette, T. Dawson Cruz

National Institutes of Justice, DNA Grantees' Meeting, Washington, D.C., June, 2006

- Platform Presentation: "Degenerate Oligonucleotide Primed-PCR: Thermalcycling Optimization for forensic DNA analysis." D. N. Rodier, K.M. Meyer, M.D. Bonnette, T. Dawson Cruz (*speaker*)

Mid-Atlantic Association of Forensic Scientists, 2006 Annual Meeting, Richmond, V.A., May, 2006

- Platform Presentation: "Degenerate Oligonucleotide Primed-PCR: Thermalcycling revisions for forensic DNA analysis." D. N. Rodier (*speaker*), K.M. Meyer, M.D. Bonnette, **T. Dawson Cruz**

American Academy of Forensic Sciences, 58th Annual Meeting, Seattle, W.A., February, 2006

- Poster presentation: "*Taq*/Proofreading enzyme combinations: A method to enhance Degenerate Oligonucleotide Primed- PCR results in Forensic DNA analysis" L.P. Thompson, D.N. Rodier, K.E. Lewis, K.M. Meyer, **T. Dawson Cruz**

American Academy of Forensic Sciences, 57th Annual Meeting, New Orleans, L.A., February, 2005

- Platform Presentation: "Degenerate Oligonucleotide-Primed PCR: 'Proofreading' a Method for Forensic DNA Analysis" D. N. Rodier, K.M. Meyer, K.E. Lewis, and **T. Dawson Cruz** (*speaker)*

Other Invited Presentations

"Improving Forensic DNA Analysis: Making the most of what you have", University of Mississippi, Forensic Chemistry Program, Department of Chemistry, Oxford, M.S., April 2008

"Improving Forensic DNA Analysis: Adaptation of Whole Genome Amplification Methods", Virginia Commonwealth University, Molecular Biology & Genetics Program Seminar Series, Richmond, V.A., November, 2006

"Improving Forensic DNA Analysis: Adaptation of Whole Genome Amplification Methods", University of Richmond, Department of Biology & the Willie Reams Lecture Fund, Richmond, V.A., September, 2006

"Updates on Forensic DNA Research & Development", Virginia State University, Student Biology Club, Petersburg, V.A., April 2006

VII. Appendix 1

DAWSON CRUZ LAB:

dcDEGENERATE OLIGONUCLEOTIDE PRIMED (dcDOP)-PCR AMPLIFICATION PROTOCOL

Date: 05/2008

INTRODUCTION

dcDOP-PCR is a modified version of a previously published Whole Genome Amplification (WGA) technique, known as degenerate oligonucleotide primer-PCR (DOP-PCR) (1-3)(Figure 1). With this procedure, large sections of the genome are pre-amplified, producing additional template DNA that can be used for downstream genetic analysis, including multiplex STR analysis and/or mitochondrial DNA analysis. This technique is designed for use with **low copy number samples (<100pg) or severely degraded samples only**. In this optimized procedure, samples are dcDOP-PCR amplified and concentrated to a final volume of 5 μL, followed by a traditional STR or mitochondrial amplification. Following STR/mito amplification, the products are post-PCR purified and electrophoresed by traditional CE methods (3100*Avant* Genetic Analyzer).

Optimization of this technique has included changing the degeneracy of the primer used during the dcDOP-PCR amplification to 10N (4) and evaluation of the extension time (3 minutes) (5). The non-specific amplification cycle number of the dcDOP-PCR amplification was also changed to 12 cycles (2) and *DeepVent* (*Pyrococcus* species GB-D) enzyme, a proofreading enzyme, was added to the PCR reaction (6) to increase fidelity and product length. Lastly, experimental results observed suggest that all products obtained after dcDOP-PCR be post-PCR purified *after* the downstream amplification (STR and/or mito) and eluted directly into formamide (CE diluent). Lastly, it is also recommend that CE injection time be increased to 20 seconds for all dcDOP-PCR samples (7). All individually optimized steps have been combined to produce this protocol.

Because dcDOP-PCR is a non-specific amplification technique that targets very low quantities of DNA, it is a very sensitive technique that is more prone to suffering contamination from low-level ambient DNA. The following guidelines should be carefully adhered to:

- Take all possible steps to avoid contamination:
    - Remember to setup amps *in the laminar flow (biological) hood*
    - Clean hood with 10% bleach & 70% ethanol before and after use
    - Change tips between each sample
    - Change gloves as often as needed
    - Clean all instruments (pipets, etc) prior to and after use

  - If using genomic DNA, amplification set-up should be on the pre-PCR side of the lab and using pre-PCR equipment
  - Never open more than one sample DNA tube at once
  - Always keep your workspace neat and clean.
- Most PCR reagents are temperature sensitive, especially enzymes (polymerases). All reagents and samples (after addition of reagents) should be kept on ice until placed into the thermalcycler.
- In order to prevent amplified DNA from contaminating reagents, equipment, and samples, the procedures outlined below *must* be followed.

SAMPLE EVALUATION

DNA that has been organically extracted is not ideal for use with this method. Organic extraction can lead to artifacts that can interfere with STR interpretation (8). After dcDOP-PCR amplification, the presence of artifacts is magnified by the concentration steps included in this protocol, particularly with organically extracted samples. Thus, it is recommended that this method be utilized when DNA has been obtained via a column-based, silica extraction method (Qiagen, DNA IQ, etc.).

Low Yield DNA Samples:

Samples with low yields should be concentrated to a smaller volume prior to dcDOP-PCR amplification (protocol below).

Dawson Cruz Lab Recommendation:

- Samples with total DNA yields ≤ 0.6 ng should be concentrated to 10 µL using Microcon YM-100 concentrators.)
- Refer to Dawson Cruz lab protocol files for this procedure – "MICROCON PROTOCOL"

High Yield, Poor Quality DNA Samples:

If high yield samples have poor data quality upon initial multiplex STR amplification, these samples can also be dcDOP-PCR amplified to improve results.

Dawson Cruz Lab Recommendation:

- Dilute these samples so that ~60 pg can be targeted in the dcDOP-PCR amplification (protocol below).

dcDOP-PCR:

REAGENTS

- Invitrogen **Platinum Taq DNA Polymerase High Fidelity**, includes:
  - Platinum Taq High Fidelity DNA polymerase (5U/µL)
  - 10X High Fidelity PCR Buffer

  - $MgSO_4$ (2mM)
  - Store all at *-20°C*
- DNA control: AmpFℓSTR Control DNA 9947A (0.1ng/µL)
- **10N** degenerate primer (Invitrogen)
  - 5'-[OH-CTGGAGNNNNNNNNNNATGTGG-OH]-3'
  - ***Note:*** Primer comes as a concentrated powder. Add 1000 µL of Tris-EDTA (TE) to make stock solution.
  - ***Order Information:***
    - Primer name: DOP primer
    - Sequence (5'-3'): (DNA) – OHC TGG AGN NNN NNN NNN ATG TGG OH
    - Primer length: 26
    - Scale of Synthesis: 200N
    - Purity: desalt
    - ***NOTE***: Primers need to be further diluted to 40µM for use with the dcDOP-PCR technique
- dNTPs (4mM each)
- Tris-EDTA (TE) buffer
- ***Note:*** the dcDOP-PCR system is <u>only designed for low copy number targets</u>
  - 7.5pg-100pg have been successfully amplified with this method
  - Input targets >100pg (0.100ng) should ***not*** be used

PROCEDURE

Amplification Set-Up:

1. On ice, arrange a set of sterilized PCR strip tubes in a PCR tray. Label sides of tubes and the tops of corresponding strip caps. Assure that there is at least one tube & cap for each sample and control to be amplified. ALWAYS amplify a positive and negative control! Label a 0.5 mL or 1.5 mL tube with "MM" for Master Mix.

2. Obtain a PCR setup plate map and label wells in the appropriate order and position (corresponding to your strip tube sample labels).

3. Add 5 µL of positive control 9947a DNA (0.1ng/µl) to the appropriate tube. For the negative control, add 5 µL of TE to the appropriate tube (negative control).

4. For each sample being amplified, add 1-50 µL DNA to the corresponding tube.
   a. ***Note:*** Procedure works best with DNA input amounts ≤ 0.1ng
   b. For low yield samples, use half of the concentrated volume (i.e. 5 µL if sample is initially concentrated to 10 µL).
   c. For high yield/poor quality samples, ~60 pg should be used.

5. Master Mix: Using the dcDOP-PCR worksheet, calculate total volumes needed for each of the following Master Mix components (amount to add ***per sample*** is given):

a. 10.0 µL 10X High Fidelity PCR Buffer
b. 4.0 µL $MgSO_4$ (2mM)
c. 5.0 µL dNTPs (4mM each)
d. 5.0 µL 10N Degenerate Primer (40µM)
e. 0.5 µL Invitrogen Platinum Taq High Fidelity DNA polymerase (5U/µl)

6. Prepare Master Mix in 0.5 mL or 1.5 mL tube labeled "MM". Prepare enough for the number of samples to be amplified + ~5% for error (or at least 2 extra samples). Mix thoroughly by flicking and/or briefly vortexing. Spin tube down quickly to assure all contents are in the bottom of the tube (<10 sec).

7. Using new tips with each sample, add 24.5 µL of Master Mix to each sample tube.

8. Using new tips with each sample, add 25.5 – 74.5 µL of TE to each sample tube ***to bring the total amplification volume to*** **100 µL**. Assure that all components added to the strip tubes are in the bottom of each tube. If not, tap strip tubes on the bench and/or spin down strips briefly in post-PCR mini-centrifuge.

Thermalcycling:

1. Place samples in the thermalcycler by placing the entire PCR tray in the thermalcycler base. Location of tubes in the thermalcycler should match the location documented on the PCR setup plate map. Strip tubes should NOT be removed from the tray!

2. Turn on thermalcycler and run the "DOP 12 cycles" program:

95°C for 5 minutes

12 cycles:
94°C for 1 minute
30°C for 1.5 minute
Ramp to 72°C for 3 minutes
72°C for 3 minute

35 cycles:
94°C for 1 minute
62°C for 1 minute
72°C for 2 minute
Add 14 seconds with each cycle

72°C for 7 minute

4°C Hold (forever)

Microcon Concentration & Purification:
Refer to the Dawson Cruz Lab protocol files for this procedure – "MICROCON DNA/RNA CONCENTRATION AND PURIFICATION PROTOCOL".

When completing this step, please make note of the following:

- Use Microcon filter size YM10 for this procedure
- Use 25 µL of ddH_2O to prewet the membrane
- Use ddH_2O to wash and concentrate samples to a **5 µL total final volume**
- Centrifuge Microcon assembly as specified for YM10 filters

Multiplex STR amplification:
The concentration dcDOP-PCR DNA samples may be used for *any* downstream analysis process desired. However, for forensic work, this will most often be either Multiplex STR amplification OR mitochondrial DNA sequencing.

Refer to the Dawson Cruz Lab protocol files for these procedures – "MULTIPLEX PCR AMPLIFICATION OF ABI AmpFℓSTR LOCI" OR ""MITOCHONDRIAL AMPLIFICATION, PRODUCT GEL, & CLEAN-UP-MELTON".

Upon completing these next steps, please make note of the following:

- Use entire 5 µL of sample after Microcon for the downstream procedure
- For Mito amplifications, the post-PCR purification described below should be utilized *instead* of the Microcon procedure described in the mitochondrial DNA analysis protocol.

Post-PCR purification:
Refer to the Dawson Cruz Lab protocol files for this procedure – "POST-PCR PURIFICATION USING QIAGEN MINELUTE PCR PURIFICATION KIT"

Upon completing these next steps, please make note of the following:

- Do not purify STR amp positive and negative (ONLY purify dcDOP-PCR samples)
- For mitochondrial DNA analysis, the "MITOCHONDRIAL CYCLE SEQUENCING & PURIFICATION" protocol should be followed next, prior to CE analysis.

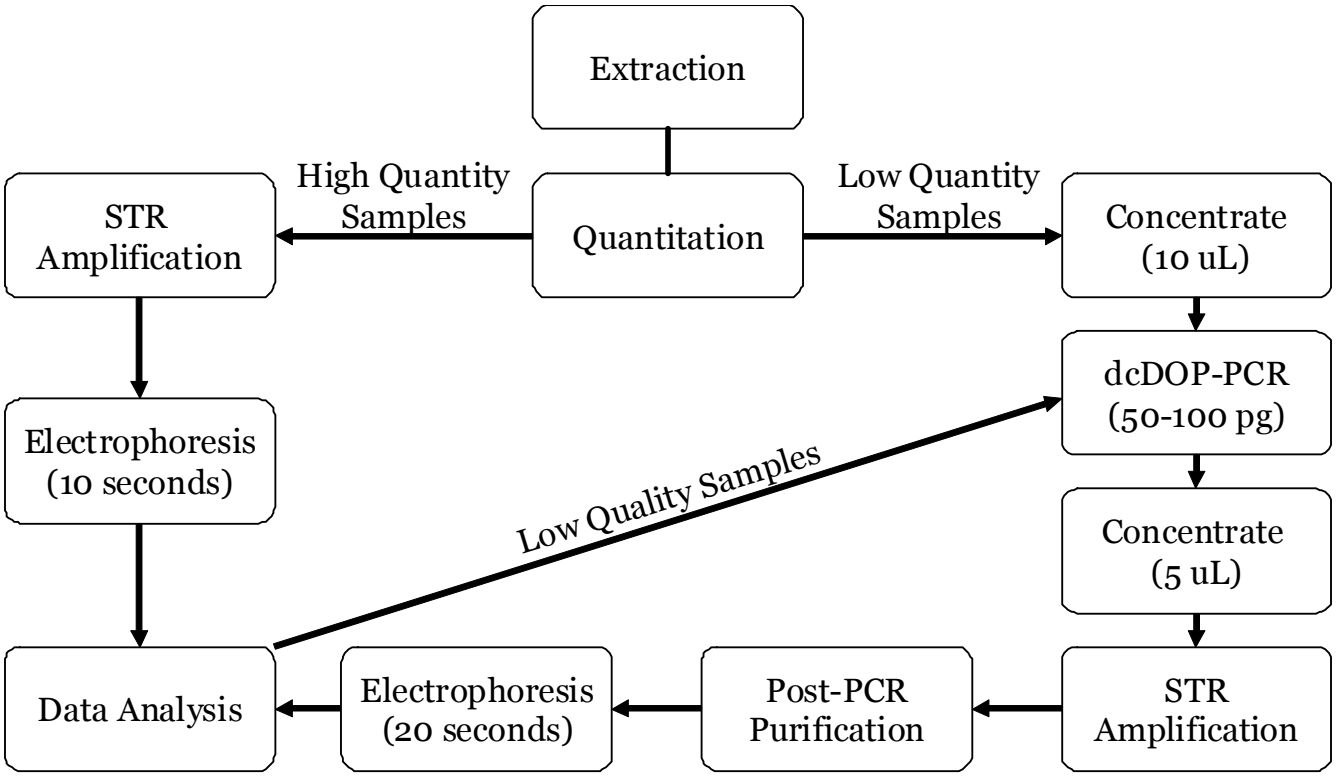
CE Analysis:
Refer to the Dawson Cruz Lab protocol files for these procedures – "MULTIPLEX STR SAMPLE PREPARATION FOR ANALYSIS ON ABI PRISM 3100-*AVANT* INSTRUMENT USING ABI DATA COLLECTION SOFTWARE, VERSION 2.0, AND GENEMAPPER *ID*, VERSION 3.2"
OR

"SEQUENCING DATA ANALYSIS USING GENEMAPPER ID, VERSION 3.2 AND SEQUENCHER™ DATA ANALYSIS SOFTWARE VERSION 4.1 F BY GENE CODES CORPORATION"

Upon completing these next steps, please make note of the following:

- For Profiler Plus:
  - Use 0.25 µL of GS 500 – ROX and 12.25 µL of Hi-Di Formamide for a TOTAL = 12.5 µL /sample of standard mixture
- For Identifiler
  - Use 0.15 µL of GS 500 – LIZ and 8.85 µL of Hi-Di Formamide for a TOTAL = 9.0 µL /sample of standard mixture
- For STR CE analysis, in the "Instrument Protocol 1" column of the run plate record, select a protocol from the drop-down list that corresponds to the run parameters and STR kit type to be used. Please note there are different injection times:
  - For ALL dcDOP-PCR samples & STR amp/dcDOP-PCR negative controls
    - Select **F_set_20SecInj** for Profiler Plus or COfiler
    - Select **G5_set_20SecInj** for Identifiler
  - STR amp and dcDOP-PCR positive controls
    - Select **F_set_Default** (5 seconds) for Profiler Plus or COfiler
    - Select **G5_set_Default** (5 seconds) for Identifiler
  - Ladder
    - Select **F_set_10SecInj** for Profiler Plus or COfiler
    - Select **G5_set_10SecInj** for Identifiler

Figure 1: dcDOP-PCR workflow (8)



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8. Brown L. Kelly, "An evaluation of dcDOP-PCR for the analysis of mock and non-probative casework samples." (MS internship, Virginia Commonwealth University, 2008)