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Degenerate Oligonucleotide Primed-PCR: Thermalcycling Modifications and Comparison Studies

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DEGENERATE OLIGONUCLEOTIDE PRIMED-PCR:

THERMALCYCLING MODIFICATIONS AND COMPARISON STUDIES

A Thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

by

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List of Abbreviations

5'CTCGAGNNNNNNNNNNATGTGG3' 10N

5'NNNNNNNNNNNNNNNNATGTGG3' 16N

Non-template addition ..... -A

Applied Biosystems, Inc. ..... ABI

Capillary electrophoresis ..... CE

Combined DNA Index System ..... CODIS

Distilled, deionized water ..... ddH_2O

Degenerate Oligonucleotide Primed-Polymerase Chain Reaction ..... DOP-PCR

Human telomerase reverse transcriptase gene ..... hTERT

Internal lane standard ..... ILS

Low Copy Number Polymerase Chain Reaction ..... LCN PCR

Multiple Displacement Amplification ..... MDA

New York City Office of the Chief Medical Examiner ..... NYC-OCME

Polymerase Chain Reaction ..... PCR

Primer Extension Preamplification ..... PEP

Restriction Fragment Length Polymorphism ..... RFLP

Relative fluorescence unit ..... rfu

Variable Numbers of Tandem Repeats ..... VNTR

Whole Genome Amplification ..... WGA

Abstract

DEGENERATE OLIGONUCLEOTIDE PRIMED-PCR: THERMALCYCLING MODIFICATIONS AND COMPARISON STUDIES

By Denise N. Rodier, B.S.

A Thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

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Degenerate Oligonucleotide Primed-PCR (DOP-PCR) can potentially enhance analysis of low copy number DNA samples. Theoretically, this procedure replicates fragments of the genome that can then be used for downstream multiplex STR analysis. The objective of this study is to optimize DOP-PCR by examining ramp/elongation times and cycle numbers in the non-specific amplification portion of DOP-PCR, and by modifying the degenerate primer. Additionally, other methods such as Multiple Displacement Amplification (MDA) and Low Copy Number PCR (LCN PCR) were

examined for their ability to create accurate DNA profiles from low DNA input amounts. Increasing the ramp/elongation times showed no effect on downstream STR amplification success. An increase of cycle number increased DNA yield, but STR amplification success was undetermined. Although modifying the degenerate primer to one with a higher degeneracy decreased DNA yield, it ultimately improved STR amplification success. In comparison studies, LCN PCR produced higher STR amplification success than MDA.

CHAPTER 1 INTRODUCTION

History of DNA use in forensic DNA analysis

Forensic DNA typing has been in use for nearly twenty years. The need for identification by DNA was great; serological methods of the past limited identification only to blood type, which allowed for inclusion of a minimum of 4% and a maximum of 47% of the United States population (1). The advent of DNA analysis allowed for greater certainty in identification based exclusively on serological evidence. Dr. Alec Jeffreys applied a technique known as restriction fragment length polymorphism (RFLP) to VNTR analysis (2). Using this technique, DNA is digested by a restriction enzyme around regions containing variable numbers of tandem repeats (VNTR) and the resulting product was size-separated by gel electrophoresis and analyzed by traditional Southern blot procedures (2). This procedure required a large amount of input DNA, was time consuming, and often was very expensive to complete.

Since the time of RFLP, a progression has been made to the use of the polymerase chain reaction (PCR), a technique first described by Dr. Kary Mullis (3, 4). In PCR, complementary strands of DNA are denatured (separated) by the addition of heat. Once the strands of DNA are separated, small sequences of nucleotides called primers are annealed to the complementary strands through the use of lower temperatures optimized

for the specific sequence of that primer. A forward primer will anneal to one strand of DNA, and a reverse primer will anneal to the opposite strand. At a slightly higher temperature, a thermalstable DNA polymerase such as *Taq* polymerase (from *Thermus aquaticus*) will add nucleotides to the 3'end of the primer, in a complementary fashion to the DNA sequence of the original strand. This results in a doubling of the number of DNA strands. On the next cycle, primers will not only anneal to the original strand, but to the replicated strand as well. A large number of repetitions of this denaturing, annealing, and extending process will allow for exponential doubling of a target region, bounded by the forward and reverse primer. This technique is much faster than RFLP, and can be performed with less DNA at a reduced cost, which is ideal for the forensic community.

In forensic DNA analyses, PCR is used to amplify short tandem repeat (STR) regions of DNA. The STR repeats selected are polymorphic, and vary among individuals. The number of repeats can be the same for each chromosome in an individual (homozygous alleles), or can vary for each chromosome (heterozygote alleles) (5). The selected STR loci also have low mutation rates, which allow for stability in identification after long periods of time, and the alleles are also distributed evenly across the population. While amplifying and analyzing these STR repeats individually can have some informative use, the greatest power lies in using multiplex reactions, which allows for the amplification of multiple STR repeat regions simultaneously. Simultaneous amplification of these multiple regions decreases the time required for PCR reactions compared to amplifying each region individually. This also reduces costs of reagents used, since only one reaction mixture is made for a multiplex amplification. Most importantly, multiplex amplifications

save DNA, which is often available in limited amounts from forensic casework samples. Using PCR to amplify STR repeats from multiple chromosomes in a multiplex reaction can lead to distinguishing one individual from all others in the earth's population (5). The only exception to this distinguishing profile is in the instance of monozygotic twins, who would be expected to have identical STR allele profiles at the commonly tested loci (6).

The Amp*Fl*STR® Profiler Plus™ PCR Amplification Kit produced by Applied Biosystems, Inc. (ABI) is one of several DNA amplification kits validated for use in forensic DNA analysis. This kit contains primers for nine of the thirteen core STR loci found in the Combined DNA Index System (CODIS) and Amelogenin, for gender identification (7) (Table 1). This kit is optimized for 1-2.5ng input of sample DNA, although successful typing below 1ng has been reported (7, 8). This input DNA amount is also reported for other standard DNA amplification kits such as the Promega PowerPlex kits (9-11). Input is highly dependent upon the quality of the DNA sample. Samples which have higher quality (less degraded) DNA may allow for a lower input amount. Alternatively, many samples may be high quality, but at much lower DNA concentrations, or some samples may be highly degraded altogether. Each of these conditions result in a low copy number DNA sample available for STR analysis.

Biological evidence from crime scenes often provides lower quality DNA or may even require analysis from a single cell. For example, evidence of a crime or mass disaster may be limited to a single hair without root bulb; evidence from a rape case may yield only a few sperm. Also, contact (touch) DNA sources from crime scenes may be of high quality, but of low copy number. For example, sweat or shed epithelial cells from skin

surfaces (i.e. hands or feet) can be transferred to secondary surfaces through handling or other contact, at a crime scene. These types of trace DNA samples often contain less than 0.16ng of DNA (12). Successful DNA extraction could occur using these types of samples, but the amount would likely be below threshold for multiplex STR analysis. Thus, amplification using the standard multiplex STR amplification kits may not be successful for these low yield sample types.

Alternatively, DNA may be present in large amounts, but the quality may be very low, making analysis difficult. In vivo, DNA is a fairly stable molecule. However, when the cells begin to break down, DNA will start to degrade. Time, temperature, humidity, and ultraviolet radiation are all environmental conditions that can contribute to the breakdown of DNA. Once DNA is degraded, the success rate of obtaining a complete STR profile becomes severely reduced using standard DNA typing techniques.

Whole Genome Amplification

Due to the limitations discussed above, researchers in forensic DNA are actively pursuing alternative methods to amplify low copy number DNA samples for human identification. One type of amplification method that has become an option for synthesizing larger amounts of DNA from low concentration/low quality samples is a technique called Whole Genome Amplification (WGA). WGA methods work to amplify the entire genome, rather than specific STR loci. By amplifying the entire genome, the amount of original template is increased, allowing for increased success of downstream primer binding and multiplex STR analysis. Ideally, an optimal form of WGA for use in amplifying low copy number forensic samples will have techniques and reagents similar to

those already in use in forensic DNA laboratories. This will then reduce or eliminate the need for additional training of personnel or purchasing of new equipment, which will then keep operating costs low while maintaining the high quality assurance levels for forensic laboratories.

Degenerate Oligonucleotide Primed-PCR

One such method of WGA is Degenerate Oligonucleotide Primed (DOP)-PCR. This technique has been successfully used to amplify low concentration DNA samples for downstream cancer genetics and fetal diagnostics applications (13, 14). Unlike other PCR reactions, there is only a single primer used in the reaction (instead of a forward *and* reverse primer). This primer most often described in the literature is usually a 22 base long oligomer with a six nucleotide degenerate region in the center (Figure 1). This degenerate region is a random sequence composed of any of the four DNA nucleotides. The first five steps of the DOP-PCR procedure is a non-specific amplification step. The degenerate primer along with low annealing temperatures will cause random annealing at locations across the entire genome (Table 2). During PCR, extension will occur from these primers and create long fragments that, in theory, together represent copies of the entire genome (15). Even though the template is not a single, continuous molecule, the fragments produced from high molecular weight DNA should contain the STR regions intact. These DNA fragments produced by DOP-PCR can be subsequently used for other standard DNA analyses, including multiplex STR amplification. In the final 35 cycles of DOP-PCR, the annealing temperature is increased, and the degenerate primer will have a higher affinity

for these non-specifically amplified fragments (Table 2). This should help to create an increase in overall yield of the representative genomic fragments.

There are several advantages to bringing this particular technique into forensic DNA laboratories. First, the technique of DOP-PCR is similar enough to that of standard PCR that no new equipment would be needed, and minimal training of personnel would be required. In addition, it is possible for this technique to be automated, using liquid handling robotics that are commonly found in forensic laboratories. Lastly, because the reagents used in this procedure are common, no kit is needed and thus the cost to perform this procedure is very low.

While preliminary data has shown that partial STR profiles can be obtained by DOP-PCR from as low as 7.5 pg of DNA (~1-2 cells), there are still significant incidences of stochastic effects being seen after DOP-PCR. The most common seen undesired effect is allelic drop-out (Figure 2) (17). This occurs when an STR region is only partially amplified or completely fails to be amplified. Other stochastic effects that can be seen are preferential amplification of smaller alleles, allele shifting, stutter, and non-template addition (-A) (Figure 3). However, it is important to note that allelic "drop-in" or other spurious peaks are not seen after DOP-PCR (16). This is good because extraneous alleles may prohibit matching a sample to a known profile, or cause a profile to be interpreted as a mixture, or as contaminated. While DOP-PCR produced partial profiles can have some informative value, to be truly useful in the forensic setting, profiles must be near to complete and free of stochastic effects in order to ensure statistical profile uniqueness. The effects seen in preliminary DOP-PCR experiments could potentially be due to incomplete

coverage of the genome or insufficient genome-wide product, caused by DNA fragment sizes being of insufficient length. Fragment sizes in preliminary results never exceed 3kb, and are commonly only 500bp-1kb.

There are several potential reasons that DOP-PCR fragment sizes could be of insufficient length, thus causing allele drop-out in downstream multiplex PCR reactions. If the extension times during thermalcycling are not long enough, the polymerase may truncate or fail to generate long DNA fragments during DOP-PCR. Therefore, increasing extension times may increase the amount of time for nucleotide addition that occurs during PCR, causing larger product fragments. Increasing extension times in an effort to increase the size of PCR products has already been successfully used in a PCR method called long-range PCR. Long-range PCR approaches typically use a combination of increased thermalcycling times along with a *Taq*/proofreading enzyme combination to produce fragments of high molecular weight (17, 18).

Likewise, if the number of cycles in the initial non-specific amplification step of DOP-PCR (Table 2) are too few, this may fail to produce enough genome-wide fragments to be exponentially amplified in the specific amplification step of DOP-PCR. Downstream multiplex STR amplification would then be expected to fail due to lack of high molecular weight product with incomplete genomic coverage. Increasing the number of cycles in the non-specific amplification step could produce more genome-wide fragments for further exponential amplification.

Lastly, the degenerate primer could be a contributor to incomplete genomic coverage. While the degenerate portions of the primers and very low annealing

temperatures in the non-specific amplification cycles will aid to increase in the number of binding sites for the primers, the sequences at the 3' and 5' ends of the primers do contribute to some annealing specificity. These sequences may not be statistically found across the genome, which would then inhibit genome-wide coverage. Changing the primer sequence could potentially increase the coverage sites, with more template DNA available for STR amplification in downstream reactions. Potentially useful modifications will include partially removing the 5' specific portions of the original primer or completely removing the 5' specific portions of the original primer. Removed nucleotides could be replaced by the same number of degenerate nucleotides. This combination will increase degeneracy of the primer, thus increasing the number of potential binding sites throughout the genome. Ultimately, this may result in improved coverage of the desired STR regions.

By changing thermalcycling parameters, such as increasing extension times, increasing the number of cycles, and making changes in the primer used, longer fragment sizes and better genomic coverage should be obtained for samples whose initial DNA yields are too low for traditional STR analysis (<1 ng).

Multiple Displacement Amplification

Multiple Displacement Amplification (MDA) is a second method of WGA. With this technique, φ-29 polymerase is used in place of *Taq* or other more common DNA polymerases (19-21). Like the DOP-PCR reaction, MDA uses a random hexamer primer to achieve primer binding across the genome (19-21). However, there is no PCR-type reaction containing repeating cycles of denaturing, annealing, and extension. Instead, the MDA reaction occurs at a constant temperature of 30°C. At this temperature, random

hexamer primers anneal to the original template material. The φ-29 polymerase will elongate from these primers, and will also eventually displace already made strands from their template, and continue on until the sequence terminates. This is unlike *Taq*, which does not have this type of displacement capacity (19-21). Primers will then subsequently anneal to these newly-created displaced strands, and elongation will begin again without any change in temperature. This reaction occurs continuously during an incubation period of 16-18 hours, continuing until the dNTPs have been consumed (19-21). This procedure should be able to provide whole genome coverage similar to DOP-PCR, but the recommended input DNA amount is 10 ng, which is greater than that recommended for even the standard multiplex STR amplification kits and greater than is often obtained from compromised biological evidence (19-21). As noted earlier, trace DNA samples, a common type of forensic evidence produced primarily by contact of skin with an object, often yield less than 0.16 ng of DNA (12). While there is one published study involving the use of MDA for forensic DNA samples, the DNA concentration of the study samples was high (between 50 ng and 0.01 ng) (22). It is therefore expected that MDA will not be completely successful for very low template DNA samples, considering the comparatively high recommended input DNA amounts. However, some partial profiles may be achieved with this method.

Low Copy Number PCR

Other DNA amplification methods are currently being investigated for use in a forensic setting for low yield or highly degraded evidence samples. Low Copy Number (LCN) PCR is one such method. LCN PCR was described by Peter Gill, and is based on

increasing the total number of amplification cycles in the standard multiplex STR reaction from 28 to 34 (23). By increasing the number of cycles, one expects to increase the amount of exponential doubling seen in PCR reactions, leading to increased PCR product for lower quality samples (23). According to published studies, full STR profiles (thirteen loci) can be generated from as little as 25-59 pg of starting template DNA (24). However, it is reported that 40% of alleles contain high stutter peaks for 1ng inputs, and only 5% of alleles contain stutter for <100 pg inputs (24). It is also reported that an average of 38% of heterozygous loci will drop-out when five cells (approx. 30 pg of DNA) are extracted and amplified (23). In addition, 21 of 30 negative controls showed allele drop-in, attributed to background, potential contaminants, and, in the case of five alleles that occurred spuriously, an analyst's profile (24). These stochastic effects may be due to incomplete capture of full chromosomal sets for all cells in a sample (23). However, the authors claim that there can be some informativeness even with the stochastic effects. Gill *et al.* have developed specialized statistical analyses to account for these effects (24). However, the complicated nature of these statistical analyses would require either additional software capable of performing these analyses, or additional training of personnel, or both. Additionally, while the LCN PCR procedure has been accepted in courts in the United Kingdom, where use of the procedure originated, as of July of 2005, LCN PCR was not accepted in the United States courts. The New York City Office of the Chief Medical Examiner (NYC-OCME) has begun bringing a LCN PCR program for casework samples online as of March 2006, however this has not yet been tested in a courtroom setting (25). Based on our preliminary results, it is expected that DOP-PCR will have fewer stochastic

effects than is reported using LCN PCR (Figure 2). It is particularly important to note that no allele drop-ins were noted in data from preliminary DOP-PCR studies. Thus, use of an optimized DOP-PCR reaction would allow traditional STR amplification parameters and statistical analyses, thereby avoiding issues of admissibility.

Objectives

The objectives of this project are:

1. To optimize thermalcycling parameters in an effort to produce higher yield, higher molecular weight fragments using DOP-PCR. Thermalcycling extension times in the non-specific amplification step of DOP-PCR will be increased to potentially increase fragment sizes. Additionally, increasing the number of cycles for the non-specific amplification step, and/or modifying the sequence of the primers used in thermalcycling may be examined.
2. To perform comparative studies of other low copy number methods such as the traditional LCN PCR vs. the MDA method of WGA. These studies can later be compared to the optimized DOP-PCR results

CHAPTER 2 THERMALCYCLING MODIFICATIONS

Introduction

Current methods of forensic DNA analysis are not adequate for analysis of low copy number/low quality DNA from forensic evidence samples. Standard DNA STR amplification kits, including the Amp*Fl*STR® Profiler Plus™ PCR Amplification Kit produced by Applied Biosystems Inc. (ABI), are optimized for samples of at least 1ng input (7, 8), which is not always available from evidentiary samples such as touch DNA samples. Due to these limitations, researchers in forensic DNA are actively pursuing alternative methods to amplify low copy number DNA samples for human identification.

Whole Genome Amplification (WGA) is an amplification method that can produce larger amounts of DNA from low copy number samples. WGA methods work to amplify the entire genome, rather than specific STR loci. By amplifying the entire genome, the amount of original template is increased, allowing for increased success of downstream primer binding and multiplex STR analysis. One such method of WGA is Degenerate Oligonucleotide Primed (DOP)-PCR, which has been successfully used to amplify low concentration DNA samples for downstream cancer genetics and fetal diagnostics applications (13, 14). Unlike other PCR reactions, there is only a single primer used in the reaction (instead of a forward and reverse primer). This primer is a 22 base long oligomer

with a six nucleotide degenerate region in the center (Figure 1). Used in conjunction with low annealing temperatures, the primer will set down randomly at locations across the entire genome (15).

During PCR, extension will occur from these primers and create fragments that, in theory, together represent copies of the entire genome (15). Even though the template is not a single, continuous molecule, the fragments should contain the STR regions. These DNA fragments produced by DOP-PCR can be subsequently used for other standard DNA analyses, including multiplex STR amplification. This technique is of particular interest to forensic DNA laboratories as it is similar enough to that of standard PCR that no new equipment would be needed, and minimal training of personnel would be required. In addition, it is possible for this technique to be automated, using liquid handling robotics commonly found in forensic laboratories. Lastly, because the reagents used in this procedure are common and no kit is needed, the cost to perform this procedure is very low.

While preliminary data has shown that partial STR profiles can be obtained, there are still significant incidences of stochastic effects being seen after DOP-PCR. Allelic drop-out is the most common effect seen; in addition allele shifting, stutter, and non-template addition (-A) are observed from preliminary results (16) (Figure 3). These effects could potentially be due to incomplete coverage of the genome, caused by DNA fragment sizes being of insufficient length, or insufficient genome-wide product. Fragment sizes in preliminary results never exceed 3kb, and are commonly only 500bp-1kb. However, it is interesting to note that allelic "drop-in" or other spurious peaks are not typically seen after DOP-PCR in preliminary experiments (16).

There are several potential reasons that DOP-PCR fragment sizes could be of insufficient length, thus causing allele drop-out in downstream multiplex PCR reactions. If the extension times during thermalcycling are not long enough, the polymerase may truncate or fail to generate long DNA fragments during DOP-PCR. Therefore, increasing extension times may increase the amount of time for nucleotide addition that occurs during PCR, causing larger product fragments.

Alternatively, if there are not sufficient numbers of cycling in the DOP-PCR reaction, DNA fragments would only be produced in low concentrations, which would then cause downstream multiplex STR analysis to fail. Increasing cycling numbers could potentially increase the DNA yield, and therefore improve the profile success.

Lastly, the degenerate primer could be a contributor to incomplete genomic coverage. The sequences at the 3' and 5' ends of the primers do add some specificity. If these sequences are not statistically found across the genome, genome-wide coverage could be inhibited, in spite of the degeneracy of the primer. Changing the primer sequence could potentially increase the coverage sites, with more template DNA available for STR amplification in downstream reactions. Modifications could include partially removing or completely removing the 5' specific portions of the original primer. If removed portions are replaced by the same number of degenerate nucleotides this would be expected to result in an increase in the degeneracy of the primer, and thus an increase in the number of potential binding sites throughout the genome. Ultimately, this may result in improved coverage of the desired STR regions.

This study predicts that by increasing extension time and cycle numbers in the non-specific portion of the DOP-PCR protocol, and by modifying the primers used, longer fragments will be produced in a high enough quantity for successful downstream multiplex STR amplifications.

Methods

DNA preparation

DNA was extracted from buccal swabs using the Qiagen QiaAmp DNA Mini kit for DNA extraction (26, 27). Primate-specific DNA was quantified by real-time PCR using the ABI Quantifiler kit with the ABI 7000 Prism® for detection and ABI 7000 Prism® SDS Software version 1 for analysis (28, 29). DNA was diluted using distilled, de-ionized water (ddH_2O) to 0.125 ng and 0.062 ng concentrations, each per 5µL volume, for DOP-PCR reactions. Samples and dilutions were both stored at -20°C in a freezer designated for pre-PCR products. Post-amplification products were stored at -20°C in a freezer designated for post-PCR products.

Degenerate Oligonucleotide Primed (DOP)-PCR amplification and amended thermalcycling parameters

Samples amplified using DOP-PCR for all experiments included: a positive control consisting of 1ng of K562 DNA, a negative control consisting of 5uL of TE, 0.125 ng DNA, and 0.062 ng DNA. The DOP-PCR reaction mix was created based on the Roche DOP-PCR master kit (30) and consists of 10uL of BioRad *iTaq* 10X reaction buffer, 2.5U BioRad *iTaq* polymerase, 3uL of 50 mM $MgCl_2$, 5uL of 4mM each of dNTPs, and 5uL of 40uM degenerate primers. TE buffer (pH = 7.6) was added to bring the total reaction

volume up to 100μL. The sequence of the standard (control) degenerate primer is 5'-CCGACTCGAGNNNNNNATGTGG-3' (30).. All PCR analyses were performed on a GeneAmp PCR System 9600 thermalcycler. Four repetitions of the original (control) DOP-PCR cycling parameters were completed (see Table 2); this includes a 3 minute ramp and three minute extension at 72°C for 5 cycles. In the first optimization experiment (n=4), the three minute ramp from 30°C to 72°C and the three minute extension at 72°C that occur in step two (five cycle, non-specific amplification step, see Table 2) were simultaneously modified to each of the following six times: one minute, two minutes, five minutes, eight minutes, ten minutes, and twelve minutes. In the second optimization experiment (n=4), the cycle number of this non-specific amplification step was modified to each of the following six cycle numbers: 3 cycles, 4 cycles, 7 cycles, 9 cycles, 12 cycles, and 15 cycles. In the third optimization experiment, the primer was first modified to 5'-CTCGAGNNNNNNNNNNATGTGG-3' (10N) and tested using the control cycling conditions (n=4). A second primer was modified to 5'-NNNNNNNNNNNNNNNNATGTGG-3' (16N). However, with this near completely degenerate primer, the thermalcycling parameters were adjusted to 40 cycles of the non-specific amplification step of the DOP-PCR reaction (step 2, Table 4), with no specific cycling rounds being performed (n=4).

Agarose gel electrophoresis

All DOP-PCR products were analyzed for fragment size using agarose gel electrophoresis. Products were run on 1% PCR-grade agarose gels at 80V for five hours until adequate separation of the molecular weight markers had occurred. The gels were

stained using ethidium bromide and visualized on an ultra-violet transilluminator. Fragment sizes were compared to a high molecular weight marker for determining the size range of fragments produced.

Real-Time PCR quantification

DOP-PCR products resulting from elongation time modifications were re-quantitated using real-time PCR. Human specific DNA was quantified by real-time PCR using the ABI Quantifiler kit with the ABI 7000 Prism® for detection and ABI 7000 Prism® SDS Software version 1 for analysis as described by Green et al. and in the ABI Quantifiler User's Manual (28, 29).

Slot blot hybridization quantification

All DOP-PCR products were also re-quantitated using the slot blot hybridization method with the ABI Quantiblot® Human DNA Quantitation kit for probing of the D17Z1 locus as described by Walsh et al. (31). DNA samples for loading were prepared by adding 5μL of DNA sample to 150μL of spotting buffer. For detection, 5mL of ECL Reagent 1 (Amersham Biosciences, New Jersey) was mixed with 5mL of ECL Reagent 2 (Amersham Biosciences, New Jersey); the mixture was placed on the membrane and was shaken for 1 minute prior to exposure to autoradiograph film. DNA quantitation was determined by a visual comparison of each sample to the standards.

Profiler Plus™ STR amplification

All DOP-PCR products were amplified for nine STR loci using the Amp*Fl*STR® Profiler Plus™ PCR Amplification Kit (7, 8). The master mix is composed of 5.7μL of Amp*Fl*STR® PCR Reaction Mix, 2.0μL of Amp*Fl*STR® Profiler Plus™ Primer Set, 2.1μL

of PCR-TE, and 0.20μL of 5U/uL AmpliTaq Gold DNA polymerase. Approximately 2.5 ng of DNA for each sample at a volume of 5μL was input into the reaction mix. In cases where 2.5 ng of DNA was not available, the entire DNA sample was concentrated to a volume of 5uL using Microcon® YM-10 concentrators (Millipore Corporation, Bedford, MA) and used for the reaction (32). Thermalcycling parameters used were as follows: 95°C for 11 min; 28 cycles of 94°C for 1 min, 59°C for 1 min, 72°C for 1 min; 60°C for 90 min; hold at 4°C (7, 8).

Fragment analysis by capillary electrophoresis

Capillary electrophoresis was performed on an ABI-3100 *Avant* genetic analyzer, using 1.2uL of the STR product. Only the samples that perform the best after DOP-PCR (per agarose and quantitation results) were analyzed for STR success. One uL of Profiler Plus™ allelic ladders was included with each sample run for allele-calling. In addition, 0.5μL of ROX was added to each sample/ladder for use as an internal sizing standard and 12uL of Formamide was used in each sample as a denaturant and to maintain fluorescence. Samples were initially denatured at 95°C for five minutes, and then placed on ice for five minutes. All samples were injected using the default injection parameter for Amp*Fl*STR® analysis. This includes the default injection time (10 seconds), POP-4 polymer, and use of a 36cm capillary. Data was collected using ABI Prism® 3100-*Avant* Genetic Analyzer Data Collection Software version 2.0.

Data analyses

Post-DOP-PCR samples were analyzed for fragment size range (by gel electrophoresis) and total DNA yields (by real-time PCR and/or slot blot). After STR

amplification, DOP-PCR samples were analyzed using the ABI GeneMapper *ID* Software version 3.2 for concordance to the expected profiles (shown in Table 5) with a 75 relative fluorescence unit (rfu) threshold. The number of successful loci (including correct full loci and those with partial profiles), and heterozygote peak balance (where possible) were evaluated for each sample. Peak balance was calculated by dividing the height of the minor peak (in rfu) by the height of the major peak (in rfu). Additionally, each sample was evaluated for stochastic effects such as allele drop-in/drop-out, -A, and high stutter occurrence.

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 mean 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 is calculated to determine significance. An 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 Student's t-test using a 0.25 α value. *P* values less than 0.25 will be used to indicate significance.

Results

Elongation time titration using real-time PCR quantitation

For this experiment, agarose gels showed an increase in fragment size of approximately 1kb per additional minute of ramp/extension through the eight minute time point, at which time the fragment size peaked. The maximum average fragment size seen at the eight minute ramp/extension time point was 8.5kb for positive controls and 7kb for

both the 0.125 ng and 0.062 ng sample input amounts ($\rho = 0.9973$ for positive control and 0.8550 for samples). Above this time point, fragment sizes began to decrease, with the appearance of amplification product in the negative control at both the ten minute and twelve minute ramp/elongation time points (Figure 4).

For quantitation, the 1 ng input control sample was the only sample that yielded results using the real-time quantitation method. For this control (1 ng input) sample, total yields after DOP-PCR did not correlate to the ramp/elongation time. Average total yield for this sample ranged from 6.98 ng for eight minute extension, to 0.918 ng (less than original input) for ten minute extension, to 1543 ng for the twelve minute extension ($\rho = 0.6416$). Thus, from the control (1 ng input sample), total yields after DOP-PCR were determined not to be significantly affected by increasing or decreasing non-specific elongation times. However, it is impossible to draw conclusions using this quantitation method since the sample data fell below the lower limits of detection for the real-time quantitation method.

As stated above, quantitation using the real-time PCR method curiously showed very low (or no) yields for all samples especially when compared to what had been seen in preliminary results using the slot blot method for quantitation. For example, using the three minute ramp/elongation (control) in previous studies, DOP-PCR samples showed DNA yields increasing up to an average of 19.45 ng for 1 ng input amounts while real-time quantitation for this experimental control for our studies showed only an average yield of 1.17 ng after DOP-PCR. This was especially surprising given that it has now been reported that samples quantitated using slot blot quantitation methods routinely give values

that are approximately two times smaller than values obtained when the same samples are quantitated using the real-time PCR based methods (33). In fact, when our DOP-PCR samples were re-quantitated with the slot blot method, the average yield for the 1ng inputs at three minute ramp/elongation increased to 2.34 ng, approximately 2 fold higher than what was seen with real-time PCR quantitation. This trend continued with almost all other elongation time set data. From this data, it became clear that real-time PCR as a quantitation technique was not appropriate for post-DOP-PCR DNA samples. This is likely because the DOP primers are not annealing and amplifying the real-time PCR target locus, the human telomerase reverse transcriptase gene (hTERT).

Although the quantitation method and results may not be useful, the gel electrophoresis data showed a clear correlation between ramp/elongation time and fragment length so therefore, all samples were STR amplified. Although capillary electrophoresis was performed on all of these samples, the Amelogenin gender locus properly typed for only four out of 56 samples and only one sample produced an appreciable partial STR profile. At the one minute ramp/extension time, a sample whose initial input into the DOP-PCR reaction was 0.062 ng produced six properly typed alleles out of the eighteen in the expected profile and one additional non-profile allele that was attributed to stutter (Figure 5). The D5S818 locus for this sample gave a full heterozygote profile with a peak balance of 86.5%.

The initial dilutions, quantitation data, and STR results in this experimental study were all based solely on real-time PCR based quantitation values. As discussed above, this method is shown not to be a reliable technique for evaluation of DNA quantity post-DOP-

PCR amplification. Due to these combined difficulties that may have all attributed to the lack of STR data in this study, the study was repeated using DNA dilutions based upon the slot blot method of quantitation and using the slot blot method for evaluation of DNA yields.

Elongation time titration using slot blot quantitation

Two replicates of DOP-PCR amplifications were performed using fresh DNA dilutions that were based upon slot blot quantitation values. As shown in Figures 6 and 7, examination of DOP-PCR amplification products showed a strong correlation between the fragment size and elongation time ($\rho = 0.8391$ for positive controls, 0.8913 for 0.125 ng, and 0.8833 for 0.062 ng). Again, the low level DNA samples seemed to consistently produce the longest fragments at the eight minute ramp/elongation time point.

Total post-DOP-PCR yields were measured using the slot blot quantitation method. These slot blot quantitaion values were more consistent with those seen in preliminary DOP-PCR studies. The control thermalcycling parameters (three minute ramp/elongation time) gave average yields of 32.5 ng, 25 ng, and 17.5 ng for 1 ng (positive control), 0.125 ng, and 0.062 ng inputs, respectively, which is an average 167 fold increase in total DNA. Again, DNA yields for the various ramp/elongation times tested were highly variable and did not seem to correlate to the ramp/elongation time ($\rho = 0.0800$ for positive controls, 0.6180 for 0.125 ng, and 0.7283 for 0.062 ng inputs) (Figure 8). In fact, there was no significant difference in yield between time points tested for any sample input value when compared to the control conditions (three minute ramp/elongation time). The average fold increase in total DNA after DOP-PCR ranged from 145 to 218 fold for 0.125 ng inputs and

100 to 363 fold for 0.062 ng for all time points tested (Figure 8). At the ten and twelve minute ramp/extension time point, there was no detectable DNA for the 0.062 ng input samples.

Based on the gel and quantitation results, the three, five, and eight minute ramp/elongation time reactions were amplified for STR analysis. STR success was determined by counting loci (of ten possible) that were successful and accurately typed. Included in this count were those above threshold and complete as well as those that displayed only partial profiles (loci containing at least one of two expected alleles). Considering that there were a number of expected alleles that were below the 75rfu threshold, these alleles were also included in these calculations. A total of ten loci were examined: nine STR loci and the Amelogenin locus for gender determination. For 0.125 ng and 0.062 ng inputs, the three minute ramp/elongation time had the highest level of STR success, with an average of 65% of loci tested successful for 0.125 ng inputs, and an average of 40% of loci tested successful for 0.062 ng inputs (Figures 9 and 10). For the 0.125 ng input low-level sample, the difference seen between the three minute time point versus both the five minute and eight minute elongation time point is significant (P = 0.1198 and 0.1694, respectively). However, for the 0.062ng input low-level DNA sample, only the difference seen between the control (three) minute elongation time point and the eight minute time point is significant (P = 0.2195). It is interesting to note that the five minute extension did have a higher rate of success than the three minute extension in the positive control sample (only) with 65% of loci being successfully amplified.

Peak quality in terms of interlocus balance could not be determined because the majority of heterozygous loci only showed a partial profile (one of two expected alleles). However, it should be noted that while allele and locus drop out did occur with the larger loci, there were no instances of allelic drop-in.

Cycle number titration

All cycle number modifications resulted in similar fragment sizes as the five cycle control at approximately 3kb ($\rho = 0.7362$ for positive control, $\rho = 0.5791$ for 0.125 ng, and $\rho = 0.7046$ for 0.062 ng). For total DNA yields after DOP-PCR, while the positive control input (1 ng) only shows slight variation when increasing the number of non-specific amplification cycles in the reaction, both the 0.125ng and 0.062ng input amounts show an increase in yield that correlates with cycle number ($\rho = 0.6091$ for positive control, 0.9432 for 0.125 ng, and 0.9799 for 0.062 ng). The increase is more prominent for the 0.062 ng input amounts, producing a total yield of 65.63 ng with fifteen cycles. This is nearly a 1000-fold increase from the initial input amount (Figure 11). However, there was an increase in amplification product seen in the negative control beginning with a single sample at nine cycles, and increasing in frequency with twelve and fifteen cycle samples. Although the yield data for the highest cycle numbers was promising, only the five, seven, and nine cycle samples were multiplex STR amplified, as these amplification products appeared more frequently in the yield gel data. In addition, it should be noted that the sample dilutions used in this experiment were made based upon real-time PCR data. Therefore DNA yields observed may actually be lower than would be expected for these input amounts.

As was seen in the elongation time experiment using samples quantitated by real-time PCR, the samples the cycle number experiment also produced no appreciable STR profiles for any sample. There was however an interesting artifact at the vWA locus seen in seventeen samples (of 48 total samples tested). Additionally, a second artifact appeared alongside the vWA artifact that was observed. This artifact appeared as a higher baseline (background) with a high rfu value in D3S1358 locus. This artifact hindered evaluation of this locus for presence of below threshold alleles (Figure 12). Amelogenin typed correctly (above the 75 rfu threshold) in three samples. In addition, Amelogenin alleles were present but below threshold in another eight samples. D8S1179 had the correct 12,12 typing for one positive control, and had one of the heterozygous alleles below threshold (22-32 rfu) in four samples. Any other peak called as an allele or off-ladder allele in these samples was determined to be pull-up from the vWA artifact.

Investigation of additional primers

Visualization of DOP-PCR fragments on agarose gel showed no visible product for all samples tested with the modified primers (10N and 16N). Therefore fragment sizes of the DNA products could not be determined. Also, only the 10N primer produced any yield when quantitated by the slot blot method. An input value of 0.125 ng of DNA returned a quantitation value for a total yield of 20 ng, a 160-fold increase in amount. While the 16N primer did return quantitation values for positive controls, they were low (total yields of 4-6.25 ng), and none of the low-level DNA samples tested showed product. Due to this apparent lack of success with the modified primers, only two replicates of the 10N primer were amplified for multiplex STR analysis.

STR success was determined by counting loci (of ten possible) that were successful and accurately typed. Included in this count were those above threshold and complete as well as those that displayed only partial profiles (loci containing at least one of two expected alleles). Considering that there were a number of expected alleles that were below the 75rfu threshold, these alleles were also included in these calculations. The average STR success rates for the 0.125 ng and 0.062 ng low-level DNA samples significantly increased with the modified 10N primer ($P = 0.2039$ and $P = 0.1872$ respectively). STR success rates were 80% (for 0.125 ng sample) and 70% (for 0.062 ng sample) with the modified 10N primer, compared with only 65% and 40% success rate for the control primer for the same low-level samples (Figure 13). It is also important to note that the positive control for one of the two replicates of the 10N primer showed at least one allele per locus, a 100% loci success rate. However, for this sample, when partial loci are excluded from this calculation, the success rate drops slightly to 90% (Figure 14).

STR data quality was examined by evaluation of the heterozygote peak balance. Since many allele peaks fell slightly below our threshold, the average percent peak balance could only be effectively evaluated when including below threshold peaks for both control and 10N primer reactions. For the data obtained from the 10N primer reactions, the average heterozygote peak balance was 61.4%, which is above the desired minimum balance of 50%. The control reaction (with the unmodified primer), however, showed even better heterozygote peak balance (88.3%). Allele drop-in did appear to occur for one positive control sample. However, since this occurred in only a single sample at multiple loci in that sample, this is most likely due to contamination and could be attributed to user

error. It is important to note that this phenomenon did not occur for any other DNA samples tested.

Discussion and Conclusions

Quantitation of DOP-PCR amplified products became a big issue in all of the optimization experiments described. Real-time PCR is increasingly used in forensic DNA labs primarily because of its sensitivity and dynamic range, its high level of accuracy and its reduction in "hands-on" user time. However, this study found that this quantitation method may not work well for whole genome amplified DNA samples. Accurate quantitation using the Quantifiler method depends on the presence of the hTERT gene in levels equivalent to the STR regions in the DOP-PCR product DNA, if the quantitation data is to be useful for downstream STR multiplex amplification. DOP-PCR had been shown in preliminary studies to not necessarily provide a full, even genomic coverage, which could have been contributing to the downstream success of multiplex STR amplification, which showed preferential amplification of smaller loci. The necessary hTERT region was likely not amplifying well using the DOP primer, causing low or zero values upon quantitation with the Quantifiler kit even when procedures such as the slot blot method of human quantitation show higher quantitation values for the same samples. It would therefore be important for forensic DNA labs that choose to adopt this WGA technique to complete a thorough validation study using this method *in conjunction with* whichever method of quantitation they will use prior to using it with casework samples. In the future, it would be interesting to explore the success rate of DOP-PCR using other

available real-time PCR primers and probes for quantitation of whole genome amplified DNA samples.

While increasing the elongation time in the non-specific amplification cycling portion of the DOP-PCR did seem to increase the length of amplified fragments as stated in the hypothesis, there did not seem to be an overall corresponding increase in profile success in downstream multiplex STR amplification reactions. The three minute (control) ramp/elongation time continued to show the highest rate of STR success. This may actually be a indication of a lack of primer annealing sites throughout the entire genome. Instead of extending fragment lengths reaching through STR regions (that had failed to amplify previously), these extended fragment lengths are instead apparently continuing to extend through regions without STRs. Therefore, it is recommended that the three minute elongation time continue to be used with future DOP-PCR reactions.

Though the STR data was lacking for most samples tested in the cycle number titration study, these types of thermalcycling modifications should not be discounted for the potential improvement of the DOP-PCR reaction for forensic DNA analysis. There were several factors that could have inhibited the potential STR success of this experimental set. First, the dilutions used for this reaction were based on a quantitation value that had been obtained via a real-time PCR method. As stated earlier, this method of quantitation may not be suitable for DOP-PCR product DNA. For example, using this value, instead of 0.125 ng or 0.062 ng of DNA being added into the reaction mix, as little as 6-11 pg of DNA was likely added; this is approximately equivalent to the amount of

DNA found in one to two cells. Therefore the genomic compliment used likely had a much higher chance of being incomplete from the start.

Second, there were likely instrumental problems that occurred that led to the lack of STR success of these samples. Control samples in the elongation time titration experiment at least provided partial profiles, where none were seen for control samples in the cycle number titration experiment. One piece of evidence for this theory is the "pull-up" of peaks from one color into another that was seen in many samples. Typically, pull-up occurs between colors that are close to each other in the fluorescent matrix used by the computer to determine color separation. When fluorescence is high, the software is unable to separate colors well. In the capillary electrophoresis (CE) data for many of the samples evaluated, pull-up problems were observed often in association with one of the aforementioned vWA or D3S1358 artifacts. This problem can disrupt the internal lane standard (ILS), which can then inhibit both sizing and allele-calling of the data. At the time of this publication, additional instrumental troubleshooting was underway and a resolution is expected soon. Thus, it is recommended that these samples be re-run after all spectral problems are resolved.

Third, there could also or instead be a problem with the DOP-PCR reaction mixture. The artifacts observed in the cycle number samples only occurred in DOP-PCR samples, and never occurred in the STR amplification controls or DOP-PCR negative controls. The artifact also consistently occurred at a single locus – vWA – whereas if this was due to instrument problems, the artifacts would likely vary in location. Thus, there could be a component of the DOP-PCR reaction appearing as this artifact in the CE data.

It is therefore recommended that the STR amplification products obtained from the DOP-PCR DNA templates be purified after STR amplification and before CE analysis in the future.

Perhaps the most promising results were seen with the change in primers, using a primer with an increase in the degenerate region and fewer nucleotides on the 3' end of the primer. Although agarose gel electrophoresis data and quantitation data were below our levels of detection for both the 10N and 16N primer, the resulting STR success rate was increased with the new, more degenerate primers. This is especially true when below threshold peaks are taken into account, which could be easily corrected with longer injection times. This data indicates that the number of binding sites was likely increased as the primer degeneracy increased. Combining these primers with modifications to cycling number may bring about an increased yield, such that these samples could more easily be visualized for gels and detected upon quantitation, potentially bringing STR peaks up to or beyond the threshold level. Modifications to the capillary electrophoresis protocol, including an increase in injection time and/or an increase in the Formamide/amplification product ratio used could also be beneficial to bringing peak heights to the desired threshold levels. Further research to this portion of the experiment should involve STR amplification of the remaining 10N replicates, as well as amplification of the 16N replicates to determine if the same level of improvement in STR profiles is seen.

Additional areas of study for DOP-PCR in general should include the addition of proofreading enzymes, which could aid in allowing the *Taq* enzyme to extend from mismatch errors caused by low annealing temperatures used. Further, modifying the

buffering conditions, including the use of additives, may provide better functionality and stability of *Taq*. Also, combining modifications such as the seven and nine cycle non-specific amplification step with the 10N degenerate primer and testing these on reference DNA samples could be very informative as to the level of genomic coverage achieved. By increasing the sample number, the correlations and confidence intervals mentioned for statistical analysis should improve. In the future, studies should include using the optimized modified version of DOP-PCR for evaluation of highly degraded DNA samples and non-probative low-level DNA case samples.

CHAPTER 3 COMPARISON STUDIES

Introduction

DNA amplification kits used in forensic DNA analysis are optimized for input levels of 1-2.5 ng of DNA in order to obtain complete, balanced STR profiles. However, this amount of high quantity, high quality DNA is not always available for amplification. Biological evidence from a crime scene may be limited to only one or a few cells. Additionally, DNA may be present in higher concentrations, but may be severely degraded. Alternate methods of DNA amplification are being investigated in the forensic DNA community in order to obtain successful STR profiles from these types of less than optimal samples.

One type of DNA amplification that may be of benefit to improving the success rate of achieving DNA profiles is Whole Genome Amplification (WGA), which includes methods such as Degenerate Oligonucleotide Primed-PCR (DOP-PCR), Primer Extension Preamplification (PEP), Multiple Displacement Amplification (MDA), and OmniPlex. WGA methods amplify the entire genome, thereby creating DNA in amounts that are theoretically high enough for further downstream STR amplification. Alternatively, optimization of the STR amplification has also been examined to increase amplification of STR regions from low or degraded DNA inputs. Low Copy Number PCR (LCN PCR),

one type of PCR modification for STR amplification of low copy number samples, increases the number of cycles in traditional STR amplification to achieve partial or full profiles. This study is a comparative analysis that focuses on MDA and LCN PCR for use with low quantity DNA samples.

MDA uses random hexamers to achieve theoretical annealing across the entire genome. A φ-29 polymerase is used in place of *Taq* or other more common polymerases to then extend from these primers. This polymerase has the capability of displacing DNA fragments that have already been synthesized from the template and continuing extension to the end of the template. New primers will set down on strands as they are being synthesized, allowing for continuous amplification. The displacement reaction occurs at 30°C (without the typical thermalcycling steps) for 16-18 hours. This procedure should be able to provide whole genome coverage, but the recommended input DNA amount is 10 ng, which is greater than that recommended for even the standard multiplex STR amplification kits and greater than is often obtained from compromised biological evidence (19-21). Additionally, it has been reported that non-template amplification often occurs, which can cause large size product fragments in negative controls to be created in amounts great enough to be visualized on agarose gels. While there is one published study involving the use of MDA for forensic DNA samples, the DNA concentration of the study samples was high (between 50 ng and 0.01 ng) (22). There is currently no published literature on the downstream success of lower concentration MDA product DNA for multiplex STR amplification.

LCN PCR, as described by Peter Gill, involves the standard reaction mix for STR amplification, but modifies the thermalcycling protocol by increasing the total number of cycles from 28 to 34. This increase in cycle number would theoretically increase the amount of exponential doubling that occurs in PCR reactions, thereby increasing PCR product to a detectable range. According to published studies, full STR profiles (thirteen loci) can be generated from starting template DNA amounts down to 25-50 pg (24). However, it is reported that 40% of alleles contain high stutter peaks for 1ng inputs, 5% of alleles contain high stutter for <100 pg inputs (24). It is also reported that an average of 38% of heterozygous loci will drop-out when five cells (approx. 30 pg of DNA) are extracted and amplified (23). In addition, 21 of 30 negative controls showed allele drop-in, attributed to background, potential contaminants, and, in the case of five alleles that occurred spuriously, an analyst's profile (24). These stochastic effects may be due to incomplete capture of full chromosomal sets for all cells in a sample (23). Gill *et. al* have developed complex statistical analyses capable of accounting for the stochastic effects while retaining the informativeness of this procedure. However, the complicated nature of these statistical analyses would require either additional software capable of performing these analyses, additional training of personnel, or both. Additionally, while the LCN PCR procedure has been accepted in courts in the United Kingdom, where use of the procedure originated, LCN PCR has only been used for human identification of unidentified remains in the United States. The New York City Office of the Chief Medical Examiner (NYC-OCME) has begun bringing a LCN PCR program for casework samples online as of March 2006, however this has not yet been tested in a courtroom setting (25).

Both Multiple Displacement Amplification and Low Copy Number PCR are comparatively evaluated for the ability to achieve complete, balanced STR profiles with a minimal occurrence of stochastic effects.

Methods

DNA preparation

DNA was extracted from buccal swabs using the Qiagen QiaAmp DNA Mini kit for DNA extraction (26, 27). Primate-specific DNA was quantified by real-time PCR using the ABI Quantifiler kit with the ABI 7000 Prism® for detection and ABI 7000 Prism® SDS Software version 1 for analysis (28, 29). 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: 0.25 ng, 0.125 ng, 0.062 ng, 0.031 ng, 0.016 ng, and 0.0075 ng. Samples and dilutions were both stored at -20°C in a freezer designated for pre-PCR products. Post-amplification products were stored at -20°C in a freezer designated for post-PCR products.

Multiple Displacement Amplification (MDA)

Samples amplified using MDA included a positive control consisting of 1ng of K562 DNA, a negative control consisting of 5uL of TE, 0.25 ng, 0.125 ng, 0.062 ng, 0.031 ng, 0.016 ng, and 0.0075 ng of input DNA. MDA was performed according to the GenomiPhi kit protocols and published studies (19-21). A total of 6 replicates were completed for this experiment (n=6). Denaturation, incubation, and post-amplification heat inactivation steps will occur on the GeneAmp PCR System 9600 thermalcycler in order to maintain consistent temperatures during each step.

Agarose gel electrophoresis

All MDA products were analyzed for fragment size using agarose gel electrophoresis. Products were run on a 1% PCR-grade agarose gel at 80V for five hours until adequate separation of molecular weight markers had occurred. The gel was stained using ethidium bromide and visualized on a ultra-violet transilluminator. Fragment sizes were compared to the Invitrogen 1kb DNA Extension ladder (a high molecular weight marker) for determining the size range of fragments produced.

Real-Time PCR quantification

All MDA products were re-quantitated using real-time PCR. Human specific DNA was quantified by real-time PCR using the ABI Quantifiler kit with the ABI 7000 Prism® for detection and ABI 7000 Prism® SDS Software version 1 for analysis as described by Green et al. and in the ABI Quantifiler User's Manual (28, 29).

Profiler Plus™ STR amplification

All MDA products were amplified for nine specific STR loci using the Amp*Fl*STR® Profiler Plus™ PCR Amplification Kit (7, 8). The master mix used was composed of 5.7μL of Amp*Fl*STR® PCR Reaction Mix, 2.0μL of Amp*Fl*STR® Profiler Plus™ Primer Set, 2.1μL of PCR-TE, and 0.20μL of 5U/uL AmpliTaq Gold DNA polymerase. Approximately 1ng of DNA for each sample (at a volume of 5μL) was input into the reaction mix when available. In cases where 1 ng of DNA was not available, the entire DNA sample was concentrated to a volume of 5uL in TE using Microcon® YM-50 concentrators (Millipore Corporation, Bedford, MA) (32). Thermalcycling parameters are

as follows: 95°C for 11 min; 28 cycles of 94°C for 1 min, 59°C for 1 min, 72°C for 1 min; 60°C for 90 min; hold at 4°C (7, 8).

Low Copy Number (LCN) STR amplification

LCN PCR STR amplification was performed using the Amp*Fl*STR® Profiler Plus™ PCR Amplification Kit as described above with one modification to cycling number (7, 8) as described by Gill et al. (23, 24). Samples to be amplified using LCN PCR were a positive control consisting of 0.5ng of 9947A DNA, a negative control consisting of 5uL of TE, and the full range of dilutions described above. A total of four replicates were completed for this experiment (n=4). Thermalcycling parameters were as described for Amp*Fl*STR® Profiler Plus™ STR amplification, except for an increase in cycle number from 28 cycles to 34 cycles (23).

Fragment analysis by capillary electrophoresis

Capillary electrophoresis was performed on an ABI-3100 *Avant* genetic analyzer, using 1.2uL of the MDA or LCN PCR STR product. All MDA and LCN PCR products are analyzed. One uL of Profiler Plus™ allelic ladders was included with each sample run for allele-calling. In addition, 0.5µL of ROX was added to each sample/ladder for use as an internal sizing standard and 12uL of Formamide was used in each sample as a denaturant and to maintain fluorescence. Samples were initially denatured at 95°C for five minutes, and then placed on ice for five minutes. All samples were injected using the default injection parameter for Amp*Fl*STR® analysis. This includes the default injection time (five seconds for LCN PCR and ten seconds for MDA), POP-4 polymer, and use of a 36cm capillary. A second injection was also performed with a two second injection length

for LCN PCR samples. Data was collected using ABI Prism® 3100-*Avant* Genetic Analyzer Data Collection Software version 2.0.

Data analyses

Post-MDA samples were analyzed for fragment size range (by gel electrophoresis) and total DNA yields (by real-time PCR). All MDA and LCN PCR samples were analyzed after the STR multiplex reaction using the ABI Prism® GeneScan® Analysis Software version 3.7.1 (minimum relative fluorescence unit (rfu) threshold set at 75) and ABI Genotyper® Software version 3.7 or ABI GeneMapper *ID* Software version 3.2 for concordance to expected profiles (Table 4) with a 75rfu threshold. Number of alleles per sample and number of loci accurately typed were evaluated for each sample. Successful loci counted included those with either complete or partial loci. A locus was counted as "successful" a long as the expected profile was seen – even if additional alleles or stochastic effects were noted. Additionally, each sample was evaluated for heterozygote peak balance (where possible) and for stochastic effects such as allele drop-in/drop-out, -A, and high stutter occurrence. Peak balance was calculated by dividing the height of the minor peak (in rfu) by the height of the major peak (in rfu).

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. An 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.

Results

Multiple Displacement Amplification

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 (Figure 15). Total yields observed after MDA products were variable, with no specific trend noted. Seventeen out of forty samples that were expected to produce quantitation results were instead reported as "undetermined" when quantitated by real-time PCR. The lowest given average yield for low yield samples was 0.12 ng for the 0.016 ng input samples (a 7.5-fold increase); the highest was 1683.5 ng for the 0.25 ng input samples (a 6734-fold increase) (Figure 16).

When examining the STR success rate of samples after MDA, the trend seemed to show a decrease of locus success with decreasing input amounts, as expected. At 0.25 ng, the success rate was 88.3%; this gradually decreased to a low of 24% for 0.016 ng inputs ($\rho = 0.8729$) (Figure 17). An indication of sex was determined in 21 of 44 samples, although a Y allele did appear for Amelogenin in one sample, when the expected Amelogenin profile for the source DNA was X,X.

Data quality was observed by calculating average heterozygote peak balance. Complete heterozygous loci produced through MDA were highly imbalanced, with overall averages being below 50% for all inputs except 0.125 ng, which averaged a slightly higher 56.8%. Additionally, severe interlocus imbalance (within individual samples) was noted,

with imbalances between 2.8% and 96.5% being observed for individual loci within a single sample.

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. The number of alleles present per locus observed was increased for higher DNA input amounts (greater than 0.062 ng inputs) with averages up to four alleles per locus for 0.25 ng inputs. This phenomenon seemed to level off at approximately one allele per locus for lower input amounts (<0.062 ng) (Figure 18), suggesting that this procedure would be most useful for samples with input DNA of 0.031 ng or less. The highest number of STR alleles seen at a single locus was 22 alleles for a 0.25 ng input at the D5S818 locus (Figure 19). An increased number of alleles that typed as "Off-ladder" were the primary contributor to this increased average of alleles per locus. However, none of these were determined to be due to instrumental anomalies (such as spikes or spectral failure "pull-up") nor to stochastic effects such as –A or high stutter. Additionally, all negative control samples produced at least one allele. One of these negative control samples contained a complete heterozygote locus that typed to the stock solution sample and two had one partial locus typed to the stock solution sample. The remainder of alleles seen in the negative control samples were either off-ladder alleles or in-bin alleles that did *not* match with either the positive control profiles or the examiner profile.

Low Copy Number PCR

STR locus success for LCN PCR samples with inputs above 0.031 ng produced at least 80% success (including partial loci). However, below this input, success decreased to 45% for 0.016 ng inputs and 27.5% for 0.0075 ng inputs ($\rho = 0.6757$ for all). Results were comparable when the injection time was decreased to two seconds (Figure 20). In addition, all samples were expected to only have a female profile at the Amelogenin gender locus, however, with the five second injection, two samples contained a Y allele and with the two second injection, one additional sample contained a Y allele.

STR data quality after LCN PCR was examined by calculation of average heterozygote peak balance. Heterozygote peak balance was variable for all LCN PCR samples analyzed and seemed to have a moderate correlation with sample DNA input ($\rho = 0.7263$). 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 0.25 ng and 0.125 ng input samples. All other inputs had an average peak balance below 50% using the five second injection time (Figure 21). Average heterozygote peak balance did slightly increase when lower injection times were used for most samples tested; however, average peak balance even at lower injection times never exceeded 62% ($\rho = 0.0819$ for 2 second injection). It should be noted that although some partial loci was observed, no heterozygous loci were observed for 0.0075 ng sample inputs (Figure 21).

Analysis of LCN PCR products showed that the average number of alleles per locus for samples with inputs above 0.062 ng was greater than the expected 1.8 alleles per locus. 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. Pull-up alleles were discounted from calculations, however, the pull-up peaks were not always easily identifiable due to the fact that some slight shifting of these peaks was noted and thus, they were not likely completely eliminated. The number of typed alleles correlated with input amount, ranging from an average of 11.3 alleles per locus for 0.25 ng inputs to 0.4 alleles per locus with 0.0075 ng inputs ($\rho = 0.9990$), indicating allele drop-out with lower input samples (Figures 22 and 23). Using the default settings, the 0.062 ng input sample was closest to the expected number of alleles with an average of 2.1 alleles per locus (Figure 23). However, by reducing the injection time from five seconds to two seconds, a reduction in the number of extraneous alleles is observed for all samples, with a corresponding increase in expected allele drop-out in the lower input samples. Using a two second injection, the average for 0.25 ng input samples dropped to 5.8 alleles per locus and 0.325 alleles per locus for the 0.0075 ng input samples ($\rho = 0.9750$). At this lower injection time, the 0.062 ng input is still closest to expected with an average of 1.725 alleles per locus (Figure 23). This data indicates that this method may only be useful for samples with at least 0.062 ng of DNA available for input into the MDA reaction; beyond

this amount, the presence of extraneous, unaccounted for alleles would make analysis and interpretation extremely difficult.

Discussion and Conclusions

Although both MDA and LCN PCR do have potential value for low copy number DNA evidence samples, both would also need significant optimization to be useable in a forensic DNA laboratory setting and to be court-accepted. While LCN PCR has the maximum 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 it occurs in multiple amplifications from the same DNA source. While MDA did not produce the same random allele recurrence patterns, it did seem to produce more random alleles than LCN PCR altogether (Table 5). This, coupled with the decreased success rate of samples as compared to LCN PCR, could severely limit the ability of an examiner to use the MDA techniques to obtain high quality, accurate STR profiles.

In order to enhance the STR success rate and to reduce the amount of stochastic effects seen in both procedures it may be useful to modify the multiplex STR amplification thermalcycling procedures. A reduction in cycle number for LCN PCR may reduce the success of the very low input samples, but it could also reduce the height of stutter peaks to

be below threshold. Given that the STR success and quality seemed to be best at levels of 0.062 ng and above, it may be useful to further modify LCN PCR cycle numbers for samples whose template DNA is less than 0.062 ng. Perhaps LCN PCR would work best if the cycle number was variable depending on initial sample yield and quality. Both LCN PCR and MDA would need a further increase in the final extension time during thermalcycling – possibly increasing several hours or more. This would reduce both the occurrence of –A seen in LCN PCR samples as well as reducing some of the anomalies seen due to high DNA concentrations in the MDA samples.

Additionally, prior to use with forensic DNA evidence, capillary electrophoresis would need to be optimized for use with both MDA and LCN PCR samples. In our studies, a decrease in injection time brought a decrease in stochastic effects without causing large decreases in overall success rate of STR profiles for both methods. In their work with the LCN PCR procedure, the NYC-OCME has also modified the laser wavelength used, along with other CE parameters, when using the LCN PCR method with the Amp*Fl*STR® Identifiler™ amplification kit (ABI);(25).

Another important consideration for both methods is the laboratory's ability to accurately quantify human DNA. MDA seemed to show fewer extraneous alleles at DNA input values of ≤0.031 ng after STR amplification. However, LCN PCR samples showed fewer extraneous alleles at DNA input values of ≤0.062 ng. Perhaps if a lab is to choose a method or a lower limit of DNA that could be used for amplification, it will be necessary for the lab to have a validated quantitation method that can consistently and accurately quantify samples whose concentrations are in the appropriate range. In addition, the input

DNA levels appropriate for either method would need to be clearly defined by the user laboratory. For example, if a higher DNA concentration is used in multiplex STR amplification for either method, the obtained profiles might show increased incidence of extra alleles which could lead to questioning of the accuracy of the profile obtained. This could be especially problematic with MDA samples, as MDA tends to produce very large amounts of DNA. Lastly, real-time PCR may not be the best method for quantification of DNA samples after WGA, particularly MDA. For example, real-time PCR results for our MDA samples produced many "undetermined" values, which could be considered as a zero value in the forensic DNA laboratory setting. However, out of seventeen samples that gave an "undetermined" quantitation value, only two – both 0.0075 ng inputs – failed to produce alleles from the STR multiplex amplification. This could be likely due to either to the inability of the real-time method to detect very low DNA levels or due to the fact that genomic coverage is not guaranteed to be even upon WGA. There could be regions of DNA missing in the MDA amplification, leading to either an over-quantitation of sample DNA in cases where the hTERT target gene is in numbers excess to the rest of the genome, or under-quantitation, where the hTERT gene has failed to amplify. Using an alternate method of quantitation for MDA samples may bring about a better concentration value, and thus improve profile success while reducing stochastic effects.

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. 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.

CHAPTER 4 DISCUSSION

When introducing low copy number amplification procedures such as DOP-PCR, MDA, or LCN PCR into a forensic DNA laboratory setting, there are many considerations that need to be taken into account before choosing one specific procedure. While DOP-PCR does not yet produce complete multiplex STR profiles for all low DNA input concentrations, it does not produce drop-in of spurious alleles. Other stochastic effects such as –A and stutter are at a minimum and below threshold, and do not need to be distinguished from true alleles. This simplifies the determination of a profile for an analyst and allows for the use of common statistical analyses with these samples. For MDA and LCN PCR procedures, a full multiplex STR profile can be determined at certain DNA input concentrations, however a large number of other called alleles are also produced. While some of the LCN PCR effects can be attributed to instrument effects such as pull-up, in which the proper separation of the fluorescent matrix can not be clearly determined due to large amounts of DNA, often times stochastic effects such as –A and stutter appear in high amounts, and are then typed as alleles by the genotyping software. Determination of a clear profile then becomes difficult for an analyst because a single source sample after LCN PCR could begin to look like a mixture of two or more profiles. In this case, care must be taken to separate out true alleles from those occurring from stochastic effects,

unintended contamination, or spurious allele drop-in. With these types of issues, complex statistical analyses must then be employed to assist in distinguishing the original profile. Difficulties may be encountered when bringing results from these types of samples into the courtroom setting, as it may be difficult to explain to a lay jury why removing many extraneous alleles from a called profile is acceptable.

Using the most accurate quantitation methods possible would assist with all of these procedures. With DOP-PCR and MDA, quantitation with methods such as real-time PCR may be difficult, due to the typical uneven genomic amplification that is seen after WGA. DOP-PCR, for example, appeared to be under-quantitated with real-time PCR, and MDA was under-quantitated for some samples (while possibly being over-quantitated for others) with no correlation to DNA input concentration. A more accurate determination of DNA quantitation will lead to improved success with multiplex STR amplification after WGA. In addition, LCN PCR seemed to work best around a certain concentration (0.062 ng of DNA input). While sacrificing DNA for truly low concentration DNA samples may not be desired, for cases where expected concentration is towards the higher concentration range, using a technique like real-time PCR would be useful to determine if a low copy number DNA sample may actually need to be further diluted for this technique.

In terms of costs to laboratories, LCN PCR would have the lowest cost for the technique itself. It uses the exact same reaction setup as is used with the standard multiplex STR PCR reaction, with only a change in cycling parameters. While DOP-PCR does have the whole genome amplification step prior to the multiplex STR reaction, the materials used are inexpensive standard PCR reagents, making this method the next least

expensive. However, the MDA reaction would require purchasing of either a kit and/or additional reagents that are not typically encountered in a forensic DNA lab setting, including the φ-29 polymerase and new buffers – which would increase the cost significantly. For technique time, again LCN PCR has the benefit with the only extra time required for extra cycles. Though DOP-PCR has an additional step, it is relatively short, at approximately 6.5 hours for the basic cycling parameters. Alternatively, the MDA reaction takes 16-18 hours to complete and would take the most additional time of all methods tested. Although using these considerations may indicate that LCN PCR would have the lowest cost overall, the complexity of the analysis of LCN PCR would add a significant amount of cost in terms of purchasing either expensive software to perform the statistical analyses, by providing extensive amounts of training to personnel, and by requiring additional analyst hands-on time.

Overall sample quality, as discussed throughout, is the key to the success and acceptance of any of these procedures. While LCN PCR has seen some limited use for human identification and will be implement for low-level DNA casework soon in the United States (25), the biggest criticism from the scientific community has not only been the lack of data quality, but the lack of reproducibility. This is important to note, because rules of evidence such as *Daubert* (34) state that in order for a technique to be accepted for evidence, it must be reliable, reproducible, and generally accepted in the scientific community. MDA would likely have the same difficulties being accepted in court as LCN PCR, for similar reasons. DOP-PCR however, with the lack of occurrence of spurious

alleles, would be more likely to gain acceptance in the scientific community and in the courtroom.

DOP-PCR has great potential for the forensic DNA community. Further studies must be undertaken in order to make this technique a valid option for forensic DNA laboratories. Continued modification of the DOP-PCR procedure should include alternative polymerases such as the addition of proofreading enzymes, which could easily extend from base pair mismatches inherently caused in the annealing process of DOP-PCR and could increase product yield and STR success. Additional modifications to the thermalcycling procedure, such as those to elongation times and cycling number in the specific cycling portion of DOP-PCR, should be examined for effect as well. A combination of modifications should then be examined, such as increasing cycling number and changing the primer as seen in this study as well as any other optimal modifications that are found, for a full range of sample dilutions (0.25 ng to 7.5 pg) as has been tested with the LCN PCR and MDA procedures. The sample size tested (n value) for this completely optimized procedure should be increased as well, and should include DNA from several different sample sources. The optimized DOP-PCR reaction should be then tested with a variety of non-probative case samples, including source tissues, aged and degraded DNA sources, and samples that include the presence of inhibitors in order to determine the optimal performance of this technique.

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Literature Cited

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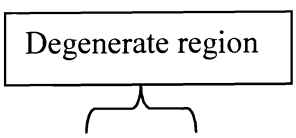
APPENDIX

FIGURES AND TABLES



5-CCGACTCGAGNNNNNNATGTGG-3

Figure 1: Sequence of Degenerate Oligonucleotide Primed-PCR primer. The degenerate region combined with low annealing temperatures provides for theoretical binding across the entire genome.

Locus	D3S1358	vWA	FGA	D8S1179	D21S11	D18S51	D5818	D13S317	D7S820
Chromosomal location	3p21	12p12	4q28	8q24.1-24.2	21q21.1	18q21.3	5q21-q31	13q22-q31	7q

Table 1: Table of Amp*Fl*STR® Profiler Plus™ PCR Amplification Kit STR loci[5,7,8]. The location of the STR loci on separate chromosomes necessitates DOP amplification to be genome wide. All of these loci need to be evenly amplified to obtain high yield DNA that can provide high quality STR results with few stochastic effects.

Original DOP-PCR Parameters[15,30]			
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	(+14s at each cycle)	

Table 2: Table of original thermalcycling parameters for DOP-PCR amplification. Included in this procedure are non-specific amplification cycles, where full-genome coverage is essential. It is expected that increasing the non-specific cycle length from the original will subsequently increase the size of fragments achieved.

Pages 59-60 Missing

Modified DOP-PCR Parameters for 16N Primer		
Step 1:	**Step 2: 40 cycles (non-specific amplification):**	**Step 3:**
5 minutes, 95°C	1 minute, 94°C	7 minutes, 72°C
	1.5 minutes, 30°C	Hold at 7°C
	3 minutes, 30→72°C	
	3 minutes, 72°C	

Table 3: Table of modified thermalcycling parameters for use with 16N primer for DOP-PCR amplification. This consists exclusively of non-specific amplification cycles, which differs from the original DOP-PCR reaction that has a combination of non-specific and specific cycles. This should assist in providing full genomic coverage for the primer.

	D3S1358	vWA	FGA	Amel	D8S1179	D21S11	D18S51	D5S818	D13S317	D7S820
Alleles	15,17	15,18	20,24	X,X	11,14	29,33.2	12,13	11,12	8,8	10,11

Table 4: Expected STR profile for sample DNA used for low-level DNA samples. All of these loci should be above a 75 rfu threshold, and heterozygous loci should display peak balance of at least 50%.

Pages 63-82 Missing

	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

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. (* $P = 0.0006$, ** $P = 0.0004$, † $P = 0.0055$, †† $P = 0.0010$)

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