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GENOMIC DNA ISOLATION FROM AMPLIFIED PRODUCT FOR RECURSIVE GENOTYPING OF LOW-TEMPLATE DNA SAMPLES

by

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GENOMIC DNA ISOLATION FROM AMPLIFIED PRODUCT FOR RECURSIVE GENOTYPING OF LOW-TEMPLATE DNA SAMPLES

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ABSTRACT

Biological evidence may contain any number of cells in any proportion. Extreme low-template DNA samples are often very difficult to interpret due to complex signal or peaks which may be indistinguishable from baseline noise. Current solutions focus on increasing the amount of amplicon detected by adjusting PCR cycle number or capillary electrophoresis injection parameters. Consensus profiling is an additional option. However, the aforementioned solutions are often not helpful for extreme low-template samples due to the high occurrence of allelic drop-out. Additionally, PCR is a destructive technique that causes one amplification to completely exhaust this type of sample, making further typing and analysis impossible. Therefore, a technique that allows for the re-generation of a DNA template in order to amplify it multiple times would be an extremely useful tool.

This study outlines the development of a method that allows for the recursive amplification of a DNA sample. Amplification was performed using biotinylated

primers for an STR locus and the resulting product was cleaned using streptavidin-coated magnetic beads to sequester the amplicons. Subsequent centrifugal filtration was used to remove the remaining PCR components, thus isolating the original genomic DNA. Re-amplification was then successfully performed at a different STR locus.

Though successful, multiple run-throughs of the method indicated retention of signal from the original amplification as well as significant genomic DNA loss during the process. This study outlines experiments seeking to characterize the cause(s) of these imperfections in order to effectively direct method optimization. A computer generated dynamic model was also created and used to simulate the recursive amplification process to assist in development. When optimized, it is expected that recursive amplification can significantly reduce the difficulties associated with low-template DNA analysis and eradicate the concept of an 'exhaustive' DNA sample.

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ABBREVIATIONS

bp	Base Pairs
CE	Capillary Electrophoresis
df	Degrees of Freedom
dNTP	Deoxyribonucleotide Triphosphate
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic Acid
ELT	Extreme Low-Template
mM	Millimolar
ng	Nanogram
PCR	Polymerase Chain Reaction
pg	Picogram
qPCR	Quantitative PCR
rcf	Relative Centrifugal Force
RFU	Relative Fluorescence Units
TE	Tris-EDTA
µL	Microliter
µM	Micromolar
UV	Ultraviolet

1. Introduction

1.1. Low-Template DNA Analysis

Many biological samples that are left at and collected from crime scenes contain mixtures of DNA from two or more individuals. These samples are referred to as low-template, because they contain very few copies of DNA. The difficulties associated with the interpretation of complex mixtures are well documented [1-7] and many attempts to compose general guidelines for mixture interpretation have been published [5, 8-11]. However, even with these attempts and newly published guidelines, complex low-template DNA samples are not interpreted in many laboratories. Low-level profile interpretation contains inherent complexities, even when the sample is single source. These complexities may also prevent the analyst from appropriately interpreting the evidence profile(s) without consulting the profile of the alleged contributor, which is contrary to the recommendations set forth by the SWGDAM Mixture Interpretation Guidelines [10,12].

In response to the issues associated with low-template analysis, several state-of-the-art interpretation methods to determine the number of contributors to a mixture, probability of allelic drop-out (Pr(DO)), and probability of allelic drop-in (Pr(DI)) have recently been published. Despite these efforts, high probabilities of DO, DI, peak height imbalance, etc. still render the possibility of forming meaningful conclusions regarding the inclusion or exclusion of a suspect improbable. A number of methods aiming to improve complex DNA

interpretation have been developed and include, but are not limited to, using probabilistic modeling [13,14], Linear Mixture Analysis [6,15], and Least Squares Deconvolution [16]. However, many of these methods are limited by their ability to infer genotypes from mixtures containing two contributors. Additionally, many make assumptions that may not apply to extreme low-template (ELT) samples [13,14,16].

1.2. Laboratory Improvements for Low-Template DNA Analysis

The aforementioned methods for low-template DNA interpretation all focus on algorithmic solutions, but research centering on improving laboratory methods which aid in analysis has also been conducted. Recent work in the area of next generation sequencing (NGS) has been promising, but error rates and the mass of DNA required both need to be decreased before widespread application to forensics can ensue [17,18].

In the interim, forensic DNA laboratories will continue to struggle with low-template sample processing and interpretation. Because of this, other studies have focused on methods that seek to increase the signal-to-noise ratio of traditional capillary electrophoresis techniques. These include removing excess primers and salts from amplicons using post-PCR purification methods [19], adjusting the PCR master mix and/or number of cycles [20,21], increasing the injection time/voltage, and decreasing the analytical threshold [22]. Although these methods have been shown to increase allele detection, they all require the use of specialized interpretation guidelines.

Another suggested laboratory method for improved low-template sample processing is consensus profiling [23-25]. In general, this method utilizes one extract/sample that is split between three (or more) amplifications. If a peak is observed at least twice, then it is designated as a true allele. This method may be used in conjunction with probabilistic genotyping [25].

Recent publications have suggested that consensus profiling is the preferred method over single-amplification for low-template samples, while others suggest consensus profiling has a limited usefulness, particularly for ELT samples (i.e. < 100 pg) [26-28]. Consequently, *recursive* amplifications (as opposed to consensus) would theoretically lead to profiles that can be used for comparison purposes. Data with high levels of information, regardless of the amount of DNA present in the original sample, would be able to be obtained. In this context, 'recursive' means that the amplification procedure can be applied repeatedly to the same DNA sample.

1.3. Re-Amplification

Re-amplification, the act of amplifying an aliquot of the PCR work-product, has previously been studied. Fresh reagents were added to these aliquots, which were then placed back into the thermal cycler to be run through additional amplification cycles [29-32]. This method seemed promising, but problems soon became clear. For example, one study found that re-amplification of an aliquot of PCR product resulted in smeared gel electrophoresis bands. This smearing was still observed even after post-PCR purification methods were used prior to re-

amplification. In addition, amplification would stop spontaneously. In particular, longer targets stopped copying at lower cycles than shorter ones. It was suggested that partial length strands were accumulating and annealing to one another, causing the amplification to abort [29].

Others have suggested re-amplification of this type can also lead to primer dimer formation, which inhibits efficient amplification of the DNA [33]. Fang et al encountered primer dimer formation when working on the identification of single nucleotide polymorphisms in fruiting mei (aka Plum Blossom) from amplified fragment length polymorphisms (AFLP) [31]. Initial amplification was performed using *Eco*RI primers and the product was run on a 1.5% agarose gel. The bands were excised and purified using several heated incubations, centrifugations, and washes with glycogen and ethanol as outlined by Or et al [34]. A portion of the final supernatant was used directly for the next PCR. This re-amplification was performed using the "selective" *Eco*RI primers, which were the same as the original primers except for two additional nucleotides on the 3' end. Primer dimers were generated when re-amplifying with the selective primers.

Fang et al hypothesized the primer dimer problem was because there was a low quantity of the purified fragments (what was being re-amplified) compared to a relatively high concentration of primers (2.5 ng of primer in a 20 µL reaction). They attempted to reduce this effect by decreasing the amount of selective primer added during re-amplification setup, which successfully allowed for the generation of target fragments without primer dimers. While this re-amplification

setup was effective for this particular research, low primer concentrations may lead to non-optimal PCR conditions especially for ELT samples.

1.4. Recursive Amplification

Many of the problems that occur during the re-amplification of the PCR work-product are likely due to the presence of excess amplicons, primers, and other PCR components leftover from the original reaction. These reagents can interfere with the amplification process. Additionally, the original genomic/template DNA is still remaining in the solution. However, the amount present is exponentially less than the number of newly synthesized amplicons. In response to this, a novel method has been proposed in which PCR components and amplicons are sequestered from the genomic DNA [35]. This isolation would permit successful re-amplification of the original template. This method, dubbed 'recursive amplification,' would improve profiling success of ELT samples. Isolation of the genomic DNA from PCR products and reagents would allow for the re-amplification of a template of any length if an error occurred during PCR processing, such as contamination, a failed positive control, or a laboratory power failure, without having to re-extract from the item of evidence.

PCR is currently a destructive technique: Once the genomic DNA is placed in a PCR tube, additional typing cannot be performed. For ELT samples, one amplification completely exhausts the original sample. Therefore, this technique would allow for multiple amplifications such that the concept of an 'exhaustive'

sample could theoretically become obsolete. This would even allow for long term storage of DNA samples that could later be tested with new technologies.

The recursive amplification method discussed here uses biotinylated primers. During amplification, these primers are incorporated into amplicons, resulting in amplicons that are biotinylated. After the amplification, the samples undergo a 'cleanup process,' which is depicted in Figure 1.

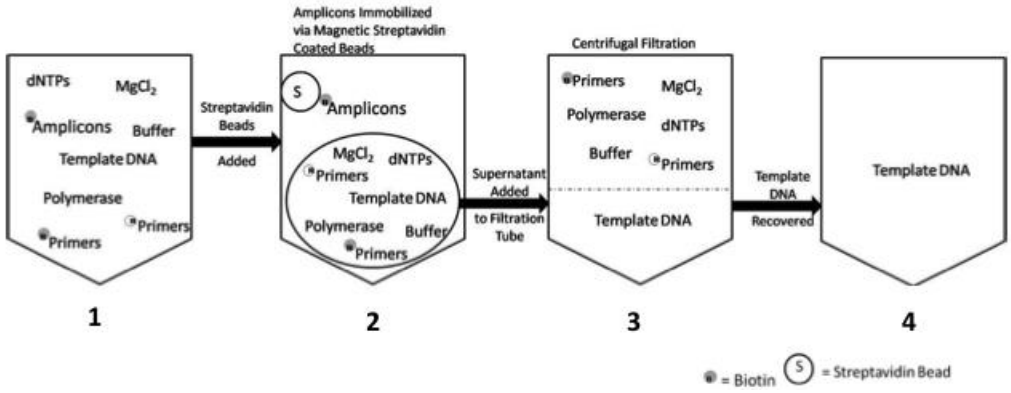

Figure 1: Schematic of Cleanup Process for Recursive Amplification [35].

Streptavidin-coated beads are used to remove the amplicons out of solution, by exploiting the strong affinity between streptavidin and biotin molecules. In addition, the beads are magnetic, so placing the tubes near a magnet would force the beads to the side of the tube, thus removing the beads from the solution and separating the amplicons from the other PCR components and template DNA (i.e. Figure 1, tube 2). The streptavidin-biotin interaction is independent of what the biotin is attached to, meaning any biotinylated molecule will be attracted to the beads. Figure 2 shows the possibilities for this.

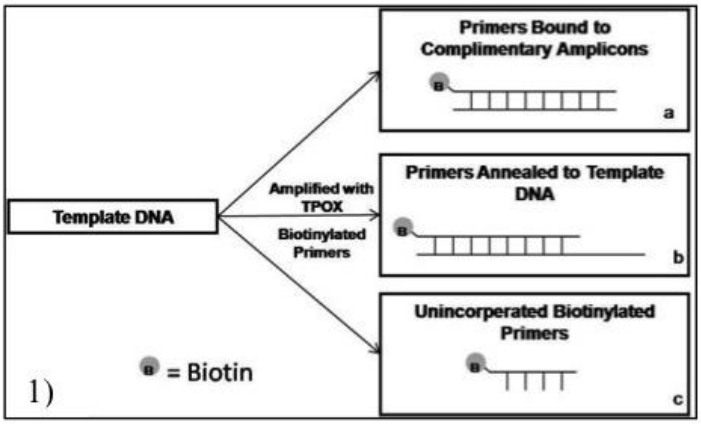

Figure 2: Schematic of Possible Biotinylated Primer Interactions with Template DNA and Complimentary Amplicon DNA [35].

As seen in Figure 2, both the unincorporated biotinylated primers and the primers annealed to template DNA can bind to the beads. If there is a significant amount of primers annealed to the template, then a substantial amount of genomic DNA may be lost during post-PCR cleanup. Alternatively, if a significant portion of the streptavidin sites is taken up by unincorporated primers, then a large number of amplicons may remain in solution and complicate subsequent amplifications.

After bead cleanup, the remaining solution is run through centrifugal filter units to remove additional PCR components (i.e. dNTPs, buffer, excess primers, etc.). This step results in a final solution containing only the original genomic DNA (i.e. Figure 1, tube 4). Theoretically, this DNA can then be re-amplified with new PCR reagents without interference.

1.5. Goal of Study

The main goal of this study is to continue development of the recursive amplification technique. Previous work has experimentally established an amplification protocol using biotinylated primers, and has also shown preliminary results which demonstrate proof of principle [35]. However, these initial studies showed that there is a significant loss of genomic DNA during the cleanup process. Re-amplified samples have also exhibited retained signal from the original amplification. This study seeks to characterize the cause(s) of these observed imperfections in order to evaluate the best manner to proceed with method development and optimization.

1.6. Dynamic Modeling

System dynamics (or systems thinking) is a methodology of representing an event, process, etc. as a complex, constantly changing system composed of factors with distinct cause-and-effect relationships between them. After using input data to establish some of these relationships, algorithms can be utilized to simulate interactions between the system components and quantitatively analyze properties of interest [36,37]. The final composition is considered a 'dynamic model.'

In general, the system represented in a dynamic model is composed of three main components: elements, rules, and background. Elements are the items that are directly moving/changing within the system. The rules are the specific

relationships between the elements. Finally, the background is anything within the system that remains unchanged.

When the dynamic model design has been completed, simulation runs can be performed. Parameters can be changed for each run and compared to output. Dynamic models can therefore be used to aid in method optimization such that a number of comparisons can be made in a short time. This in turn is highly efficient when attempting to establish optimal method parameters for complex processes, such as the one described herein.

Systems dynamics can therefore be applied to this recursive amplification method and a dynamic model can be created of the method from start to finish. Specifically in this case, the elements in the system are genomic DNA, TPOX biotinylated primers, D5S818 primers, TPOX amplicons, D5S818 amplicons, and the capillary electrophoresis signal in RFU. The rules outlining the relationships between these elements are represented as constants and equations, which are detailed below (Section 2.1.2). The background for the system is the laboratory, which includes the reaction tubes, instruments, etc. The properties of interest are the TPOX and D5S818 capillary electrophoresis peak heights.

1.7. Implications of Recursive Amplification for Criminal Justice Policy and Practice

The purpose of this work is to develop a recursive amplification method that would allow for the amplification of a DNA template that has already gone through the PCR process. This is accomplished through a ‘cleanup’ technique

that sequesters the amplicons and filters out other PCR components such as dNTPs, primers, etc. The original genomic DNA would therefore be isolated and available for re-amplification without interference.

This method would allow forensic DNA laboratories to genotype low-template DNA samples multiple times and, since it does not split the sample, is expected to be a more successful alternative to consensus profiling. These samples could also be amplified using different kits/chemistries in order to obtain the highest level of genetic information possible.

Recursive amplification is expected to have a significant impact on forensic DNA analysis, as it would allow for:

- testing of both autosomal- and Y-STRs for limited sexual assault samples,
- the ability to re-amplify a low-template, degraded, and/or inhibited DNA sample with a mini-STR kit or an enhanced amplification setup,
- recursive profiling of low-level DNA samples without the need to split into three or more amplifications (i.e. consensus profiling) to improve identification reliability,
- the re-amplification of a sample after an amplification failure or unforeseen laboratory event,
- the re-amplification of exhaustive/low-template samples multiple times in order to obtain a complete profile,
- independent defense counsel testing of samples thus minimizing crime laboratory disruption,

- and the ability to test long term stored DNA samples with new or emerging technologies.

2. Methods

All aspects of the study were conducted in compliance with ethical standards set out by the Institutional Review Board of Boston University School of Medicine – Protocol H – 26187.

2.1. Dynamic Model

2.1.1. Overview and Purpose of Model

Representing this recursive amplification method as a dynamic systems model is a useful way to visualize the movement of genomic DNA and amplicons during the amplification and cleanup processes. Understanding these relationships, specifically by focusing on their impact on observed capillary electrophoresis peak heights, can help define which areas of the method require optimization in order to maximize recursive amplification success and effectiveness as a DNA analysis technique.

2.1.2. Model Development and Structure

The completed model structure represents the method from initial amplification to analysis using capillary electrophoresis. To develop this dynamic model, STELLA® version 9.1.4 (ISEE Systems, Lebanon NH) was used.

The first portion of the model represents the amplification of DNA with TPOX biotinylated primers and the Dynabead® cleanup of the resulting amplicons (i.e.

Steps 1-2, Figure 1). The following is a description of the input parameters for each stock, flow, or converter shown in Figure 3.

- ***Available DNA***: A mass of 2 ng of DNA was always the initial input amount available for amplification and was chosen based on the use of 2 ng in the laboratory portion of the work. The available DNA is converted in terms of copy number, where it was assumed that 1 ng of DNA contained ~333 copies of DNA [45].
- ***PCR Efficiency***: The following equation was used to describe the PCR efficiency and was based on previous work performed in this laboratory:

  $$\left(\text{NORMAL}(0.96, 0.096)\right) - \left(1.94714 \times 10^{-13} \times Available\_DNA\right) \quad \text{(Equation 1)},$$

  where the PCR efficiency is modeled to be 0.96 ± 0.096 and decreases at a rate which is dependent upon the number of amplicons (*Available DNA* in the model) produced from the cycle before. The amplification occurs when the number of unincorporated TPOX primers is greater than the number of amplicons (i.e. *Available DNA*) at a given cycle, otherwise the equation is 0 and the amplification stops.
- ***TPOX Amplicons Bound to Beads***: A certain percentage of TPOX amplicons are removed by the Dynabeads®. This was one of the input parameters varied during simulation. The remaining are *Retained TPOX Amplicons*.

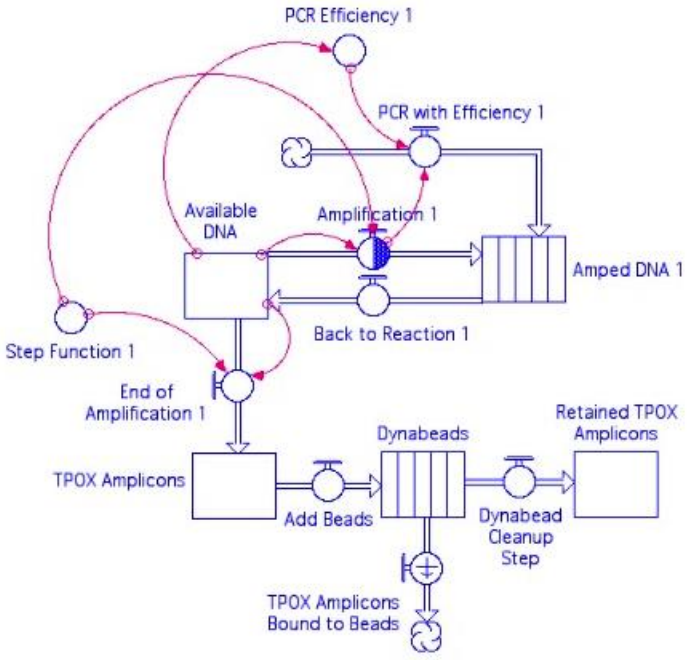

Figure 3: Stock-Flow Diagram of "Initial Amplification" Portion of Recursive Amplification Model.

The next portion of the model represents further processing of the TPOX biotinylated primers through the system. The following is a description of the input parameters for each stock, flow, or converter shown in Figure 4.

- ***TPOX Primers***: 6.022×10^{12} biotinylated TPOX primers is always the input amount. This was calculated based on the optimized concentration of primer in the reaction and was the number of primers used in the laboratory portion of this work.
- ***Primer Usage***: The number of primers incorporated into amplicons is calculated as follows,

$$Unincorporated\_TPOX\_Primers = TPOX\_Primers - TPOX\_Amplicons \quad \text{(Equation 2)},$$

where the remaining primers are *Unincorporated TPOX Primers*.

- ***Primers on Beads***: A certain percentage of unincorporated TPOX primers bind to the Dynabeads®. This input parameter can be varied during simulations. The remaining are *Primers After Beads*.
- ***Filtered TPOX Primers***: A certain percentage of the primers that are remaining after bead cleanup is filtered out by Amicon®. This input parameter can be varied during simulations. The remaining are *Retained TPOX Primers*.

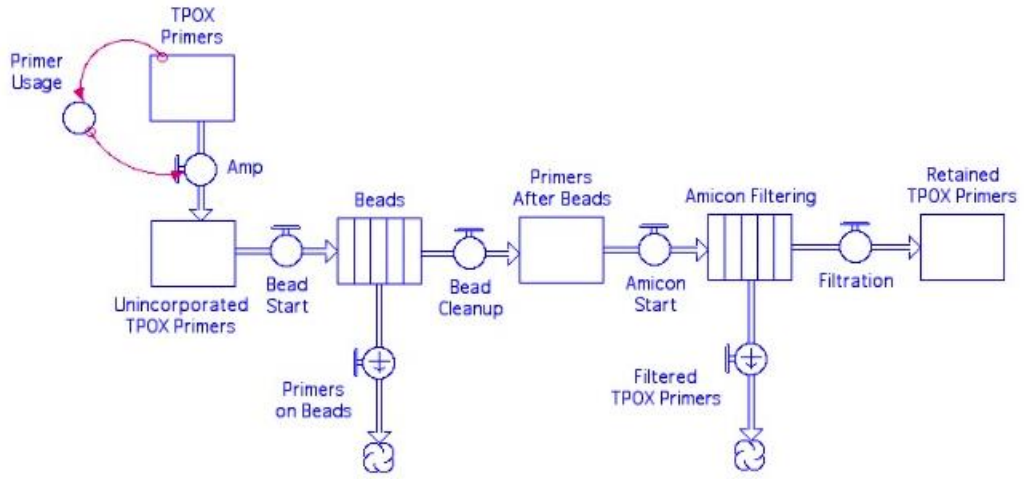

Figure 4: Stock-Flow Diagram of "Movement of TPOX Biotinylated Primers" Portion of Recursive Amplification Model.

The next portion of the model represents the movement of genomic DNA through the system. The following is a description of the input parameters for each stock, flow, or converter shown in Figure 5.

- ***Input DNA***: An input amount of 2 ng is always the initial mass of DNA.

- ***DNA Loss on Beads***: A certain percentage of the input DNA has biotinylated primer annealed to it and is removed by the Dynabeads®. This was one of the input parameters varied during simulations. The remaining is *DNA After Beads*.
- ***DNA Loss on Amicon®***: A certain percentage of the DNA remaining after bead cleanup is lost during the filtration. This was one of the input parameters varied during simulations. The remaining is *DNA After Amicon*.

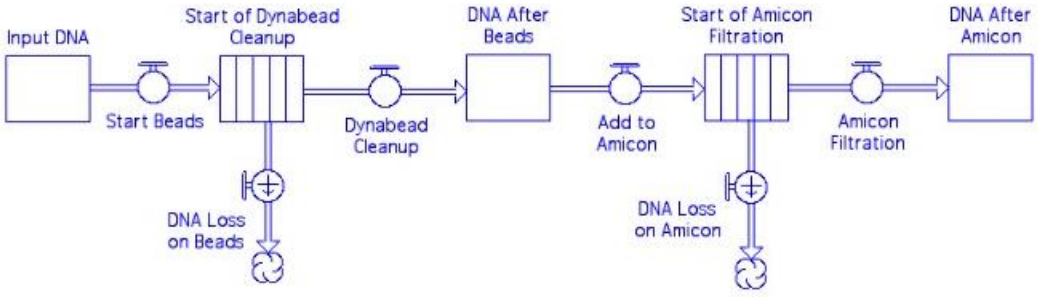

Figure 5: Stock-Flow Diagram of "Movement of Genomic DNA" Portion of Recursive Amplification Model.

The final portion of the model (Figure 6) represents the second amplification and capillary electrophoresis. This PCR is represented twice in the model, once for TPOX and once for D5S818, though the two loci amplify simultaneously in a single reaction. The second amplification is constructed similarly to the first one. The differences are stated below:

- ***DNA Copies Conversion***: The genomic DNA remaining after cleanup is converted into number of copies, which is then doubled, so that half may be pulsed into *Available DNA TPOX* while the other half is pulsed into

Available DNA D5. Since TPOX and D5S818 are on separate chromosomes, the doubling and subsequent splitting of the number of copies represents the primers for both loci being equally accessible to the DNA during the second amplification. In essence, the two loci amplify independently of one another.

- ***Input D5 Primers***: 6.022 x 10^{12} D5S818 primers is always the input amount and is the number of primers used during the laboratory portion of this work.
- ***Retained TPOX Primers***: The number of TPOX primers available for the second amplification is the number remaining after the bead and filter post-PCR cleanup steps.
- ***D5 Peak Height***: The amount of D5S818 amplicons from re-amplification is converted into a capillary electrophoresis peak height in RFU based upon the following experimentally determined sensitivity equation (see Section 3.1.1),

$$D5\_Peak\_Height = 1.0 \times 10^{-8} \left(D5\_Amplicons \right) \qquad \text{(Equation 3)}$$

- ***TPOX Peak Height***: The amount of TPOX amplicons retained after cleanup and the amount of TPOX amplicons created during re-amplification are converted into a capillary electrophoresis peak height in RFU based upon the following experimentally determined sensitivity equation (see Section 3.1.1),

$$TPOX\_Peak\_Height = 8.0 \times 10^{-9} \left(\left(TPOX\_Amplicons\_2 \right) + \left(Retained\_TPOX\_Amplicons \right) \right)$$

(Equation 4)

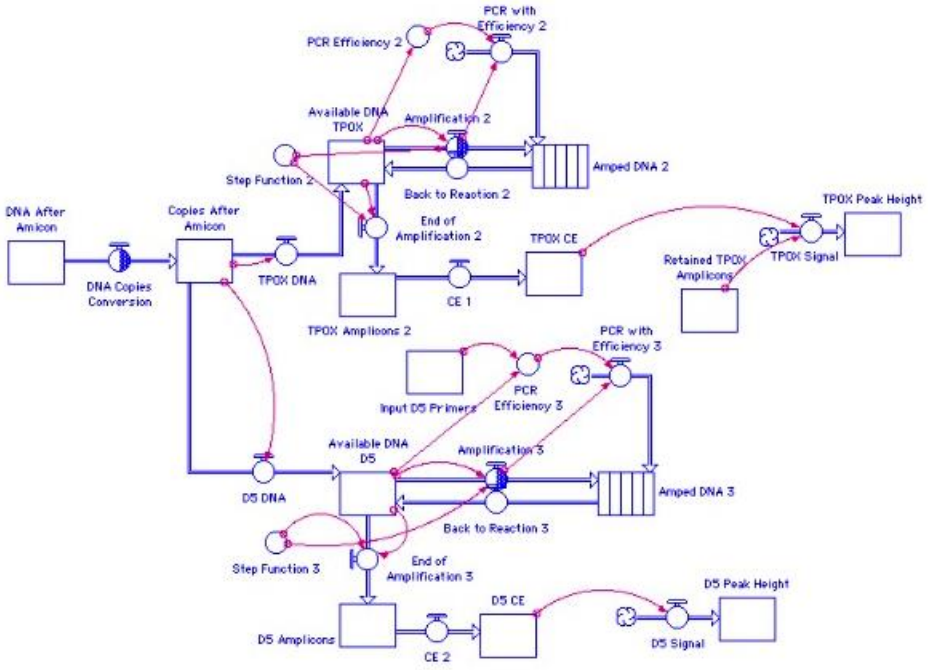

Figure 6: Stock-Flow Diagram of "Second Amplification and Capillary Electrophoresis" Portion of Recursive Amplification Model.

2.1.3. Validation of the Model

Before running simulations, it was necessary to validate that the model was designed and working properly. To do this, the model was run once with *DNA Loss on Beads* set to 100% and once with *DNA Loss on Amicon* set to 100%. These settings represent scenarios in which all of the input template DNA is lost during the cleanup process. Therefore, the resultant D5S818 peak height is 0 for both runs whether *DNA Loss on Beads* is 100% or *DNA Loss on Amicon* is 100% because in either case there is complete removal of genomic DNA from the tube,

leaving none available for re-amplification. Next, the model was run with both *TPOX Amplicons Bound to Beads* and *Filtered TPOX Primers* set to 100%, representing the scenario where all TPOX amplicons are removed by the beads and all unincorporated TPOX primers are filtered out of the solution. The resultant TPOX peak height is expected to be 0 if the model was working properly. It was also necessary to check that the DNA masses used in the dilution series for the sensitivity equations (referred to in Section 2.4.4) resulted in the expected peak heights when the model was run.

2.1.4. Model Simulations and Analysis

The completed and validated model was simulated with the following being varied: *DNA Loss on Beads*, *DNA Loss on Amicon*, and *TPOX Amplicons Bound to Beads*. The effects of the first two of these variables were evaluated by noting the resulting D5S818 peak height, and the third variable was evaluated by noting the resulting TPOX peak height. Table 1 shows the parameters that were varied for modeling the effect of genomic DNA loss on the resulting D5S818 peak height. Table 2 shows the parameter that was varied for modeling the effect of retained TPOX amplicons on the resulting TPOX peak height in re-amplified samples.

Table 1: Parameters Used for Modeling the Effect of Genomic DNA Loss on D5S818 Peak Height.

DNA Loss on Beads	*DNA Loss on Amicon*
0%	0%, 100%
10%	10%, 90%
20%	20%, 80%
30%	30%, 70%
40%	40%, 60%
50%	50%
60%	60%, 40%
70%	70%, 30%
80%	80%, 20%
90%	90%, 10%
100%	100%, 0%

Table 2: Parameter Used for Modeling the Effect of Retained TPOX Amplicons on TPOX Peak Height in Re-amplified Samples.

TPOX Amplicons Bound to Beads
0%
10%
20%
30%
40%
50%
60%
70%
80%
90%
100%

For each parameter variation, the model was run five times and the peak height was recorded. The average peak height value was calculated for each variation. A graph of peak height versus total genomic DNA loss was made for the D5S818 peaks and a graph of peak height versus amplicon retention was

made for the TPOX peaks. Points on the graph represent average values, and the error bars represent ± two standard deviations.

2.2. Description of Process

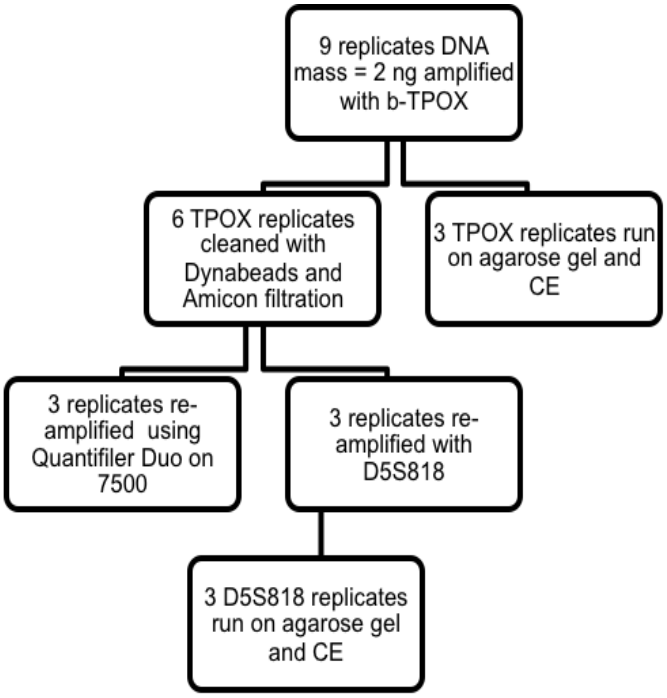

Figure 7: Schematic of Overall Recursive Amplification Process. b-TPOX = biotinylated TPOX primers.

2.3. Assessment of Method

The preliminary method design was established experimentally and published previously [35].

2.3.1. DNA Amplification

The TPOX locus was chosen to begin method development. The amplification process was performed using AmpliTaq Gold® kit reagents (Life Technologies, Carlsbad CA). Reagent concentrations were optimized

experimentally previously [35], and were 200 μM dNTPs, 2.25 mM $MgCl_2$, 0.5 μM forward and reverse primers, and 0.025 U/μL AmpliTaq Gold® DNA Polymerase. Primers for the TPOX locus were used, with the forward primer being functionalized with biotin on the 5' end and the reverse primer containing a 5' ROX fluorophore for downstream detection purposes. Nine replicates were amplified and then divided into groups of three (designated "sets A, B, and C"). Each set also had a negative control (no DNA added). Amplifications were performed using a 2 ng DNA target. The cycling conditions for the PCR were 95°C for 10 minutes, followed by 32 cycles of 94°C for 1 minute, 58°C for 1 minute, and 72°C for 1 minute, then one 90 minute period at 60°C, and then held at 15°C.

2.3.2. Dynabead® Cleanup

After amplification, set C was stored for later analysis. Sets A and B were cleaned using the Dynabeads® M-270 Streptavidin magnetic beads (Life Technologies, Carlsbad CA). For each sample, 20 μL of beads at a concentration of 6 x 10^5 beads/μL were used based on the bead binding capacity for double stranded DNA described in the manual [38]. The bead preparation process was conducted as per the manufacturer's recommended protocol. Additionally, the binding and washing buffer was prepared following the instructions provided in the manual. Each amplified sample was added to the beads in a microcentrifuge tube and incubated at room temperature for 15 minutes on a rotating plate.

2.3.3. Amicon® Filtration

After incubation, the samples were placed on a magnet for 3 minutes in order to isolate the beads on the sides of the microcentrifuge tubes. Amicon® Ultra-0.5 50K filtration units (Millipore, Billerica MA) were used to remove the unincorporated primers, salts, etc. from the samples. This procedure was performed according to the manufacturer's recommended protocol [39]. TE buffer was first added to the filters. Then, the supernatant from the sample tubes on the magnet was added to the filters. The samples were spun at 14,000 rcf for 20 minutes, with the flow-through being discarded. TE buffer was again added to the filters, which were then spun for 50 minutes at 14,000 rcf. For sample collection, the filters were inverted and placed into clean collection tubes and spun at 1,000 rcf for 2 minutes.

2.3.4. Re-amplification

The isolated genomic DNA from the cleaned samples was then re-amplified. The D5S818 locus was chosen for this step. The same reagents were used as in the first amplification, with the concentrations being 200 µM dNTPs, 2 mM $MgCl_2$, 0.6 µM forward and reverse primers, and 0.025 U/µL AmpliTaq Gold® DNA Polymerase. Briefly, a PCR master mix was made using D5S818 primers, and the cleaned set A served as the template DNA. These samples were amplified with the same cycling conditions previously described.

2.3.5. Agarose Gel Electrophoresis

The D5S818 re-amplified samples (set A) and the original TPOX amplified samples (set C) were electrophoresed on a 2% agarose gel and stained with GelStar® Nucleic Acid Gel Stain (Lonza Inc, Walkersville MD). A 100 base pair DNA ladder (Promega, Madison WI) was used as a size reference. TPOX amplicons are expected to be ~230 bp in length and D5S818 amplicons are expected to be ~136 bp in length for the template DNA used. The gel was photographed on an ultra violet light box using a Canon PowerShot A630 camera with a UV filter lens and the fluorescent photography setting. Gel images were analyzed using ImageJ – a public domain image processing software [40]. After importing the gel image into the software, the background was subtracted. Using the software's gel analysis tool, a plot was generated in the form of signal intensity versus location on the gel. This method was used to establish an intensity value for each band in set C (TPOX amplicons) and set A (D5S818 amplicons from re-amp). Signal intensities were compared between sets A and C.

2.3.6. Quantitative PCR

Quantitative PCR (qPCR) was performed on set B using a 7500 Real-Time PCR system (Life Technologies, Carlsbad CA) with the Quantifiler® Duo kit (Life Technologies, Carlsbad CA) using the manufacturer's recommended protocol [41] which included use of a single validated standard curve as per recommendations set forth by Grgicak et al [42]. The slope and y-intercept used

to quantify all samples were -3.311 and 28.561, respectively. Additionally, the template DNA stock used in the initial amplification was quantified and determined to be 2.2 ng/µL. The qPCR results were used to calculate percent recovery. The yield of DNA recovered was calculated by multiplying the DNA concentration by 11 µL (the final sample volume after cleaning). This value was divided by the input DNA mass and multiplied by 100% to calculate percent recovery.

2.3.7. Capillary Electrophoresis

Fragment analysis was performed on sets A and C using the 3130 Genetic Analyzer (Life Technologies, Carlsbad CA) and a mixture of HiDi (highly-deionized) formamide (8.3 µL/sample) and GeneScan™-600 LIZ® Size Standard (0.7 µL/sample) (Life Technologies, Carlsbad CA). A volume of 9 µL of the mixture and 1 µL of the sample was added to appropriate wells of a 96 well-plate. The plate was placed on a heating block at 95°C for 3 minutes and then snap-cooled at -20°C for 3 minutes. 5-second injections at 3 kV were performed on the samples and run according to the manufacturer's recommended protocol [43]. The results were analyzed using GeneMarker® HID (Softgenetics, State College PA) software using an analytical threshold of 50 RFU.

2.4. Identification of Sources of Genomic DNA Loss and TPOX Signal Retention

2.4.1. Dynabead® Removal of Genomic DNA

In order to assess the amount of genomic DNA removed by the Dynabeads®, the DNA bound to the beads was eluted by following the protocol outlined in the manual [38]. The eluents underwent further cleaning with Amicon® filtration to remove the formamide used during the elution since it is a known PCR inhibitor [44]. The samples were run through qPCR as described previously, and any quantified human DNA was attributed to genomic DNA that was eluted from the beads. Since preliminary results indicated that Amicon® filtration is responsible for up to 50-60% loss, the calculated mass of eluted genomic DNA was doubled in order to account for loss incurred during the filtration step.

2.4.2. Amicon® Filter Membrane Contribution to DNA Loss

The amount of genomic DNA removed by the Dynabeads® can be used to calculate DNA loss to Amicon® filtration. For a given sample, the mass of DNA lost in the filter can be approximated by subtracting both the final DNA mass after re-amplification (recovery) and the mass of DNA removed by the beads from the input DNA mass.

In order to assess the amount of DNA loss due to Amicon® filtration experimentally, two samples of template DNA (2.2 and 1.3 ng, not amplified) was added with TE buffer to an Amicon® filter and centrifuged as described previously. Once the purified solution of DNA was obtained, the final volume for

each sample was noted. Quantification was performed as previously described in order to determine the amount of DNA in each concentrate. The concentration given by qPCR (in ng/µL) was multiplied by the concentrate volume (µL) to calculate the total mass of DNA present (ng). This mass was subtracted from the input DNA mass to determine the quantity of DNA lost during filtration. The input DNA mass used for this calculation was confirmed by quantifying several aliquots of the DNA stock that was used and then calculating the average concentration.

2.4.3. Leftover TPOX Amplicons

To determine the efficacy of TPOX primer removal during post-PCR processing, DNA from two different individuals was obtained (designated "DNA 1" and "DNA 2"). DNA 1 (2 ng) was amplified with TPOX biotinylated primers, cleaned with Dynabeads®, and filtered with Amicon® as described previously. When preparing the master mix for the second PCR, no primers were added. DNA 2 (2 ng) was added to each of the cleaned samples. Then, the samples were re-amplified and fragment analysis using a 3130 Genetic Analyzer was completed as described previously. At the TPOX locus, DNA 1 has an 8,8 genotype and DNA 2 has an 11,11 genotype. When examining the capillary electrophoresis results, it was noted what peak(s) was/were observed at TPOX. If an 8 peak (~232 bp) and an 11 peak (~244 bp) were both present, then DNA 1 and DNA 2 both amplified. Due to the re-amplification setup, DNA 2 would only amplify if TPOX primers were still present in the cleaned solution. Observing only an 11,11 type would therefore imply there was a significant level of leftover

TPOX primers. Observing only an 8,8 type would imply TPOX amplicons are not completely removed during post-PCR processing. If both an 11,11 and an 8,8 are observed, then a mixture of the two factors is occurring.

2.4.4. TPOX and D5S818 Locus Sensitivity

A sensitivity study was conducted in order to establish a relationship between the peak height (i.e. RFU) and the number of amplicons for each locus (TPOX and D5S818). This was accomplished by creating a five point dilution series of DNA from ~2 ng/µL down to ~0.125 ng/µL. Each dilution was amplified in triplicate using both TPOX and D5S818 primers using the amplification protocol previously described. Fragment analysis was performed using a 3130 Genetic Analyzer as detailed above. A plot was generated of RFU versus amplicon number after 32 PCR cycles for both TPOX and D5S818.

2.5. Preliminary Method Optimization

In order to assess the effect of centrifugal force (spin speed) on DNA recovery, 2 ng of genomic DNA (not amplified) was added with TE buffer to Amicon® filters as described previously. During the 50 minute spin period, three replicates were centrifuged at each of the following speeds: 14,000, 4,000, 3,500, 3,000, and 2,500 rcf, as suggested by Garvin et al [46]. Once the purified solution of DNA was obtained, the final volume for each sample was noted. The qPCR was performed as previously described in order to quantify the amount of DNA in each concentrate. Percent recoveries were calculated for each of the five spin speeds. A *t*-test statistical analysis was performed on the results.

Similarly, an assessment was made on the effect of the centrifugal force of the DNA concentration spin. The process was performed in the same manner as previously described, except the 50 minute spin was kept at 14,000 rcf and three replicates were spun for 2 minutes at each of the following speeds for the purified DNA concentration step: 1,000, 5,000, 10,000, and 14,000 rcf. A *t*-test statistical analysis was performed on the results.

3. Results and Discussion

3.1. Dynamic Model

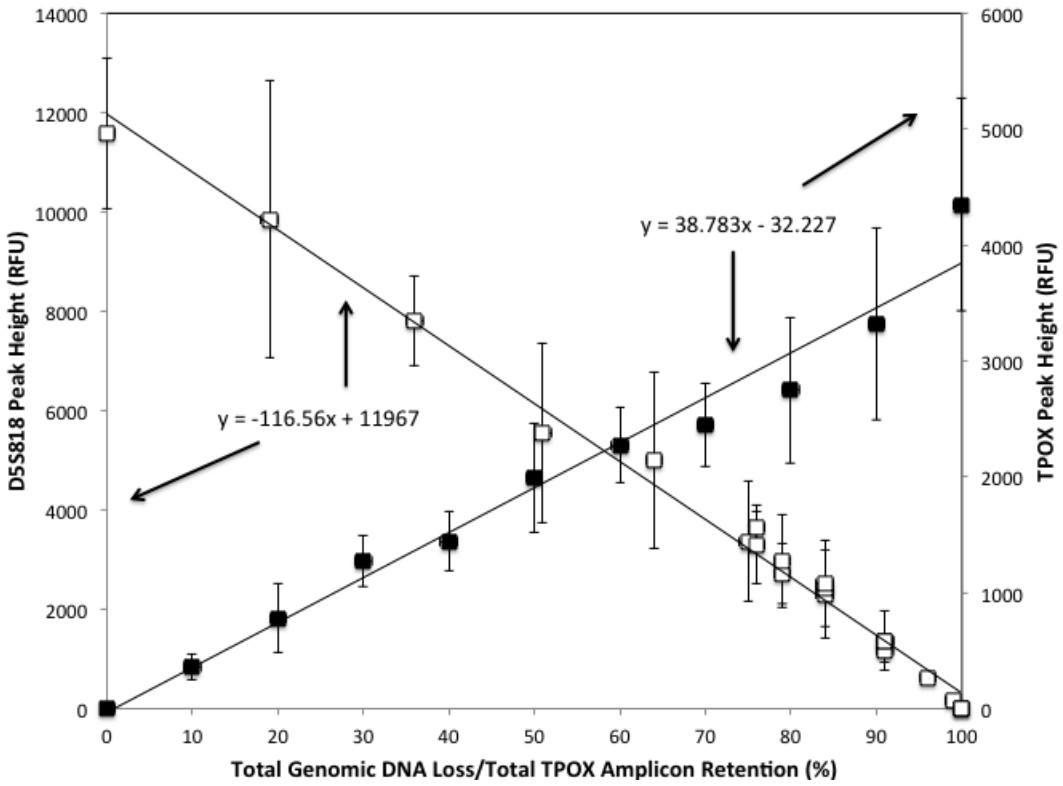

Figure 8: Recursive Amplification Dynamic Model Data. (□) D5S818 Peak Height (RFU) versus Total Genomic DNA Loss (%). (■) TPOX Peak Height (RFU) versus Total TPOX Amplicon Retention (%). Error bars represent ± two standard deviations.

3.1.1. Expected D5S818 and TPOX Peak Heights via Dynamic Modeling

The left side of Figure 8 contains a graph of the simulated D5S818 peak height in RFU versus total genomic DNA loss (%). As previously described, the model was engineered to estimate the resultant TPOX and D5S818 signal as the parameters varied. When the proportion of DNA lost was set to 1, a resultant D5S818 peak height of 0 was obtained, as expected, indicating the model was designed properly.

However, to estimate the peak height after amplification, a relationship between signal and copy number must be established. To do this, a sensitivity study which assessed the TPOX and D5S818 peak heights with respect to amplicon copy number was conducted and is exhibited in Figure 9.

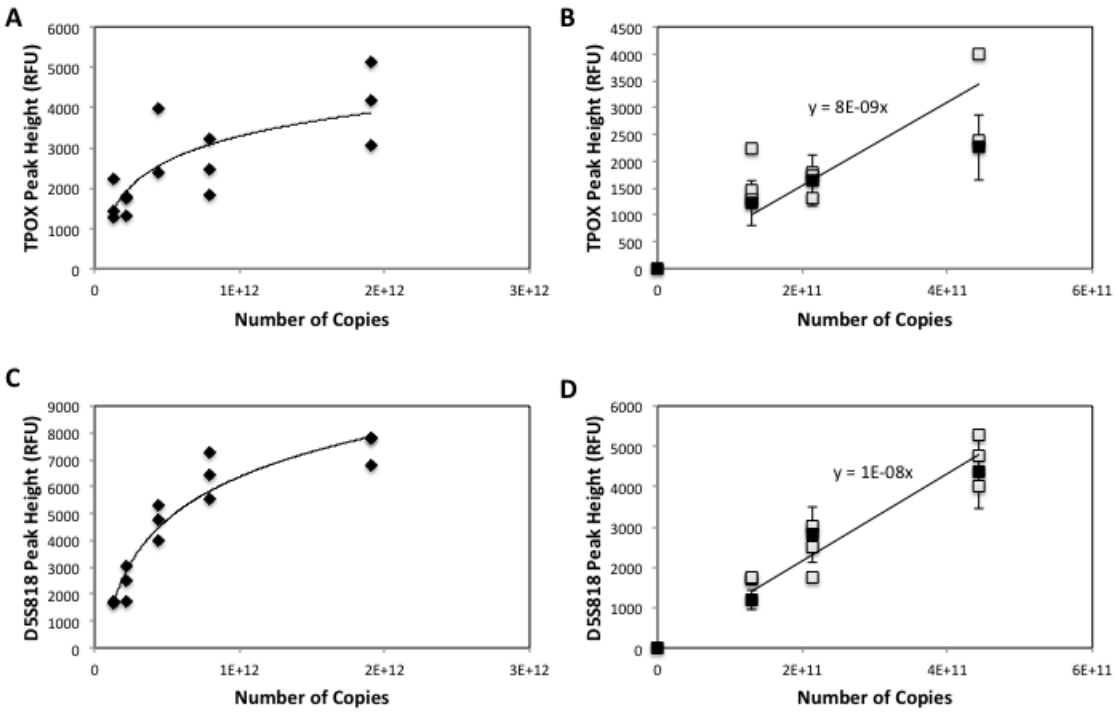

Figure 9: Capillary Electrophoresis Sensitivity Analysis for TPOX and D5S818 Loci Using a Five-Point Dilution Series. A) TPOX peak height (in RFU) versus number of copies. B) Linear regression analysis of three lowest dilutions for TPOX. C) D5S818 peak height (in RFU) versus number of copies. D) Linear regression analysis of three lowest dilutions for D5S818. (□) Laboratory data. (■) Data generated with model for validation purposes. Error bars represent ± two standard deviations.

Figure 9A and 9C show the respective RFU of the TPOX and D5S818 signal after 32 cycles of amplification versus the number of amplicons, which was determined via the following equation,

$$N_{32} = \left(333\frac{copies}{ng} \cdot M_o\right) \cdot 2^{32} \qquad \text{(Equation 5)},$$

where N_{32} is the expected copy number of amplicons at cycle 32 given the PCR efficiency is 1 at every cycle, and M_o is the target mass of DNA in ng.

Qualitatively, it is observed that as the target mass increases, so does the peak height; however, it does not increase at a constant rate. The signal seems to plateau between 8 x 10^{11} and 2 x 10^{12} copies (~0.6 – 1.5 ng target), suggesting the PCR efficiency decreases with increasing levels of DNA. Since plateauing effects are already modeled via the change in efficiency with the number of amplicons (Equation 1, Section 2.1.2), only the signal with respect to amplicon number needs to be established. This relationship can therfore be approximated by examining the linear portion of the curve, which is represented in a clearer manner in Figures 9B and 9D for TPOX and D5S818, respectively. That is, if the PCR efficiency is approximately 1 – which is expected at the endpoint cycle of 32 when template levels are low – and it is assumed the RFU is directly proportional to the concentration of the amplified product (number of amplicons), then the following linear relationship is obtained:

$$RFU = qN_i 2^{32} + A \qquad \text{(Equation 6)},$$

where A is the y-intercept (and is expected to be 0 if the proportionality is unbiased), N_i is the number of copies at cycle i, and the slope is 2^{32} multiplied by a proportionality constant q. If optimal conditions are met, the samples contain accurate DNA concentrations, and the approximation that PCR efficiency is 1 is valid, then a plot of RFU versus C_i should result in a straight line where the slope is a reflection of the expected change in RFU with amplicon. As the dynamic model predicts the number of amplicons via Equation 1, then this slope obtained from ordinary least squares linear regression of the low-template samples can be

utilized within the model to predict the expected peak height when a specified target mass is available for amplification. Specifically, the slope generated via the sensitivity analysis using 0.09 to 0.3 ng was 1 x 10^{-8} and 8 x 10^{-9} for D5S818 and TPOX, respectively.

Once the model data was generated (Figure 8), an ordinary least squares regression line ensued for the D5S818 data and resulted in a coefficient of determination (R^2) of 0.992. The equation of the line is:

$$RFU_{D5S818} = -116.56\left(\%Loss_{total}\right) + 11967 \qquad \text{(Equation 7)},$$

where RFU_{D5S818} is the expected peak height of the D5S818 peak after 32 amplification cycles. Therefore, if a sample has been through the recursive amplification process, then this equation can be used to determine the total genomic DNA loss by using the observed D5S818 peak height value. Further, the model can be validated by comparing the RFU_{D5S818} at 0% and 100% loss at all DNA targets, and these values can be compared back to the observed values obtained during the sensitivity study. Figures 9B and 9D shows this simulated data for the TPOX and D5S818 experimental peak heights, which show both the sensitivity and the PCR efficiency equations are good predictors of amplification outcomes.

It is important to note that the dynamic model results shown in Figure 8 can only be used for samples run through the method in the specified way described in Section 2.1 of the Methods. The input DNA mass in Figure 8 was always 2 ng, so Equation 7 is only applicable to 2 ng samples. Additionally, the model's

structure, constants, and equations would need to be altered if any of the method's current reagents, equipment, etc. were modified.

The right y-axis of Figure 8 shows the TPOX RFU versus total TPOX amplicon retention (%). All data were obtained through dynamic model simulations as described previously. All simulations where TPOX amplicon removal was set to 100% produced a TPOX peak of 0, validating the model.

An ordinary least squares regression line ensued for the TPOX data and resulted in a coefficient of determination (R^2) of 0.973. The equation of the line is:

$$RFU_{TPOX} = 38.783(\%retention_{total}) - 32.227 \quad \text{(Equation 8)},$$

where RFU_{TPOX} is the expected peak height of the TPOX peak after 32 amplification cycles. Therefore, if a sample has been through the recursive amplification process and there are no retained TPOX primers (as shown below in Section 3.3.3), then Equation 8 can be used to determine the total amount of TPOX retention by using the observed TPOX peak height value.

3.2. Assessment of Method

Figure 10A shows an agarose gel electrophoresis image of samples after amplification at the TPOX locus (C-1 to C-3) and samples that have been cleaned and re-amplified with D5S818 primers (A-1 to A-3). Figure 10B displays the signal of each band on the gel in units as determined by ImageJ software.

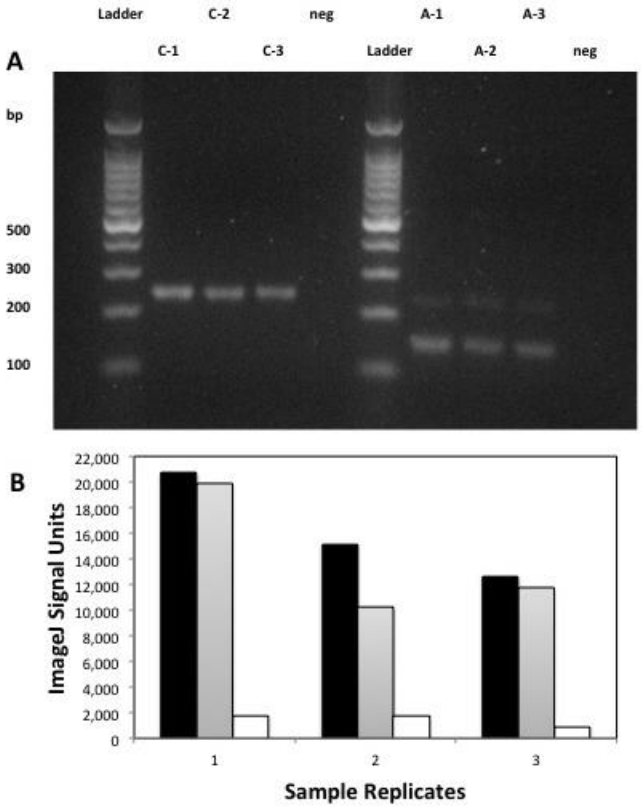

Figure 10: Re-amplification after Dynabeads® M-270 Streptavidin and Amicon® Ultra-0.5 Cleaning. A) Image of samples on agarose gel after amplification with TPOX biotinylated primers (C-1 – C-3) and re-amplified with D5S818 primers after cleaning with Dynabeads® and Amicon® filters (A-1 – A-3). B) Graph representing of the results generated by ImageJ. (■) Signal from C-1 to C-3. (■) D5S818 signal in A-1 to A-3. (□) Signal from leftover TPOX product in A-1 to A-3.

Figure 10 depicts that all of the original TPOX amplifications showed positive results for TPOX amplicons between 200 – 300 bp, which is the expected range for this locus. All samples that were re-amplified at the D5S818 locus also showed positive results for D5S818 amplicons in the expected size range. Qualitatively, Figure 10B shows that the D5S818 signal is slightly lower than the TPOX signal in the original amplification samples. Additionally, all three re-

amplified samples showed the presence of TPOX amplicons, indicating the cleanup procedure was not 100% efficient. On this gel, the average leftover TPOX amplicon signal was 9 ± 4% of the signal intensity displayed by the original TPOX amplicon bands.

Moreover, all of these signal values were similar between this gel and several other gels of the same experiment, whereby the average signal of the original TPOX product was 15,792 ± 5400 units and the average signal of the D5S818 re-amplified product was 12,527 ± 8400 units. The leftover TPOX amplicon signal averaged 1,400 ± 650 units. These data suggest that the Dynabead®-Amicon® cleaning method effectively isolates the amplicons such that the original genomic template left in solution can be used for downstream PCR processing at a significant and detectable level. It is also important to note that these successful results were reproducible each time the method was run. However, the residual TPOX amplicon band was also observed. It was present in all re-amplified samples with an overall average signal intensity of 8.95 ± 4.12% of the average signal intensity of the original TPOX amplicon bands.

Capillary electrophoresis (CE) is a second technique that can be used to visualize the results. For these experiments, CE is used for the same purpose as the gel but it is more sensitive. The same samples run on the agarose gel (Figure 10A) were analyzed using CE and compared to the gel results. The corresponding electropherograms for the samples in Figure 10 can be seen in Figure 11.

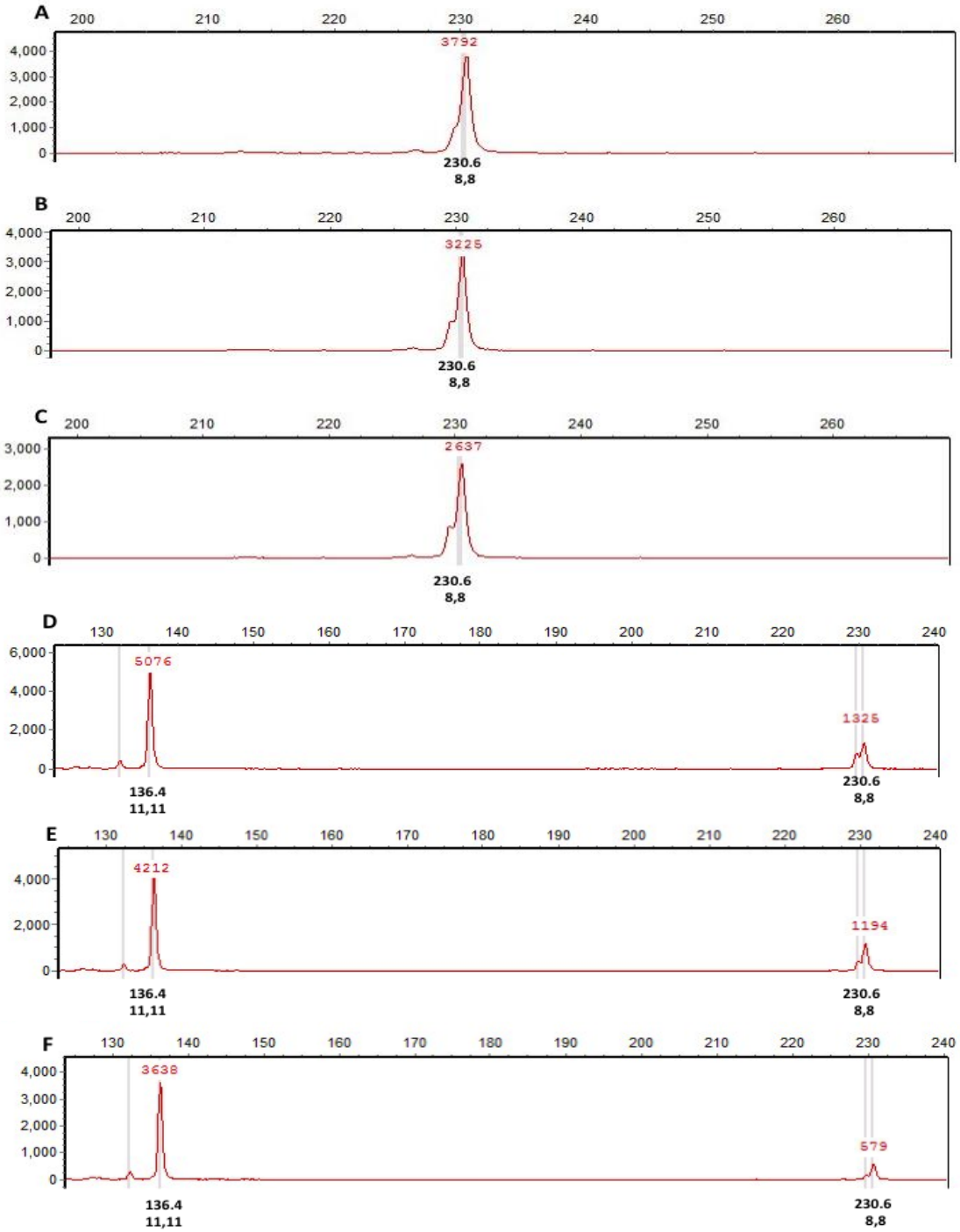

Figure 11: Representative Electropherograms of Sets C and A. A) TPOX amplification replicate 1. B) TPOX amplification replicate 2. C) TPOX amplification replicate 3. D) D5S818 re-amplification replicate 1. E) D5S818 re-amplification replicate 2. F) D5S818 re-amplification replicate 3. Red number = peak height in RFU; Below peak = size in bp and allele repeat numbers.

The samples from set C, which contain the original TPOX amplified products, showed a peak in the 216 – 264 bp range, as expected. The template DNA used is homozygous at TPOX for allele 8, which corresponds to the ~230.6 bp peak seen on the electropherograms (Figure 11). The samples from set A, which were amplified using TPOX primers, cleaned, and then re-amplified using D5S818 primers, showed a peak in the 115 – 163 bp range, as expected. The template DNA is homozygous at D5S818 for allele 11, which corresponds to the ~136.4 bp peak seen on the electropherograms (Figure 11). All of these samples showed one smaller peak at ~230.6 bp, which is indicative of unwanted TPOX retention or amplification. Similar results were observed for CE analyses of sample sets from the replicate runs (data not shown).

Overall, the re-amplified samples of all capillary electrophoresis runs exhibited an average D5S818 peak height of 4073 ± 2500 RFU. The original TPOX amplicons averaged 3586 ± 1066 RFU, while the retained TPOX peak averaged 1122 ± 1050 RFU. The CE results imply there is a higher degree of TPOX retention than the gel results indicated (Figure 10B), where the peak height of the retained TPOX fragment was ~27% of the peak height of the original TPOX fragment.

Although the agarose gel and capillary electrophoresis data are able to show that the method is working qualitatively and that there is TPOX carryover, it is not useful for quantitative information regarding percent recovery of genomic DNA due to plateau effects associated with PCR. Therefore, to evaluate the level of

genomic DNA recovery, a second set of cleaned sample replicates was re-amplified using the Quantifiler® Duo kit for the purpose of analyzing the RPPH1 locus. Direct comparison of genomic DNA quantities pre- and post-cleaning using qPCR is an effective quantitative evaluation of percent recovery. This method detects the signal after every cycle (real-time PCR), so plateau effects associated with end-point PCR analysis do no impact accurate quantification as they do with the samples on the gel/CE.

The significance of the qPCR data is twofold: Firstly, it supplements the proof of principle displayed by the gel/CE data by showing the successful re-amplification of a second locus. Secondly, it allows for more accurate calculations of percent recovery. Percent recovery is one of the most important values for determining the success of this recursive amplification method. It is understood that this method is most effective when recovery is approaching 100%. A recovery percentage significantly less than 100% will cause the number of re-amplifications that can successively be performed on one DNA sample to be finite. Further, if the loss between recursive amplifications is significant, then stochastic effects will become more prominent, complicating DNA interpretation. Assuming the loss of genomic DNA is relatively constant between re-amplifications, lower percent recoveries will reach this limit in fewer re-amplifications than higher ones. From a forensics standpoint, maximizing the number of re-amplifications is optimal. More recursive amplifications can lead to

stronger DNA results and the ability to gain as much data/information as possible.

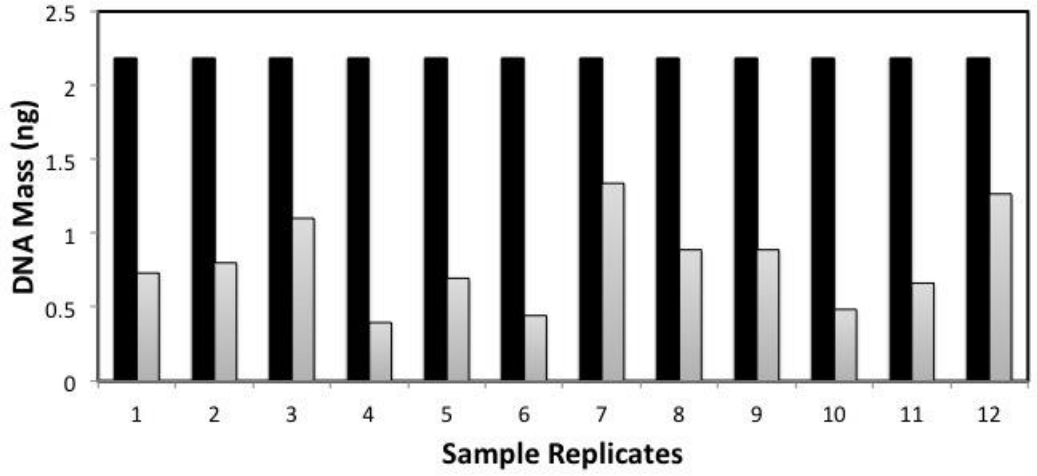

Figure 12: DNA Mass Recovery (in ng) Determined via qPCR for all Samples Run Through Full Method. (■) Input DNA mass. (■) DNA mass after running recursive DNA cleanup method on sample.

Table 3: Percent Recovery Calculations using qPCR.

Sample	Initial DNA Mass (ng)	Post-cleaning DNA Mass (ng)	Percent Recovery
Amp1–1		0.731	33.36 %
Amp1–2		0.800	36.51 %
Amp1–3		1.101	50.25 %
Amp2–1		0.396	18.07 %
Amp2–2		0.696	31.77 %
Amp2–3	2.191	0.443	20.22 %
Amp3–1		1.338	61.07 %
Amp3–2		0.888	40.53 %
Amp3–3		0.888	40.53 %
Amp4–1		0.485	22.14 %
Amp4–2		0.663	30.26 %
Amp4–3		1.266	57.78 %
Average		**0.8 ± 0.6**	**37 ± 28%**

Figure 12 shows a side-by-side comparison of input DNA mass (pre-cleaning) and post-cleaning DNA mass for each sample. These masses can be seen in Table 3 along with the corresponding percent recovery calculations. The average amount of original template DNA recovered was approximately 0.8 ± 0.6 ng. This corresponds to an average percent recovery of 37 ± 28%, implying that approximately 63% of the input DNA mass is lost during the recursive post-PCR cleanup process of the method.

As seen in Figure 11D-F, laboratory testing of the post-PCR recursive genotyping purification method produced an average D5S818 peak height of ~4000 RFU. Specifically, the average D5S818 peak height was 4072 over 12 replicates. By substituting 4072 RFU into Equation 7 and solving for ($\%Loss_{total}$), a total genomic DNA loss of 68% is the result, and is insignificantly different from the 63 ± 28 % average loss established via qPCR (Table 3).

Figure 8 and the resultant equation will therefore prove to be very useful during method optimization. The D5S818 peak heights for re-amplified samples and Equation 7 can be used to establish an approximate total genomic DNA loss percentage for those samples. If method optimization is successful, then D5 peak heights will progressively increase in tandem with a decrease in total genomic DNA loss.

Although the method was shown to be successful, this loss of genomic DNA may be considered too high to be applied to low-template forensic DNA samples and for effective recursive amplification. For example, if DNA was extracted from

a stain on an item of evidence, and quantification determined it to contain 20 pg of DNA, then after only two recursive amplifications 2.7 pg of DNA would remain. A DNA mass this low is unlikely to produce genotyping results that can be easily interpreted, thereby resulting in limited value. Such a high genomic DNA loss rate would prevent this method from being applied to extreme low-template DNA samples. Since one of the goals of recursive amplification is to improve interpretation success of this kind of sample, it is clear that minimizing this loss must be at the forefront of method optimization.

3.3. Identification of Sources of Genomic DNA Loss and TPOX Signal Retention

3.3.1. Dynabead® Removal of Genomic DNA

One potential cause of template DNA loss is the binding of biotinylated primers to the original DNA. If the biotin molecules on these primers become bound to the streptavidin-coated beads, the template DNA would be removed from the solution and would not be available during recursive amplification. As discussed previously, minimization of template loss would have significant implications to criminal justice policy and practice. Therefore, to assess whether or not this was occurring, the DNA bound to the used beads was eluted, collected, and quantified using qPCR.

Figure 13 and Table 4 show the amount of genomic DNA present in the bead eluents. All of the signal obtained during qPCR can be attributed to genomic DNA. The beads may have genomic DNA, unincorporated biotinylated primers,

and TPOX amplicons all bound in various amounts. However, since Quantifiler® Duo amplifies the RPPH1 locus, only the genomic DNA can contribute to the signal used to calculate the amount of DNA present in the eluent.

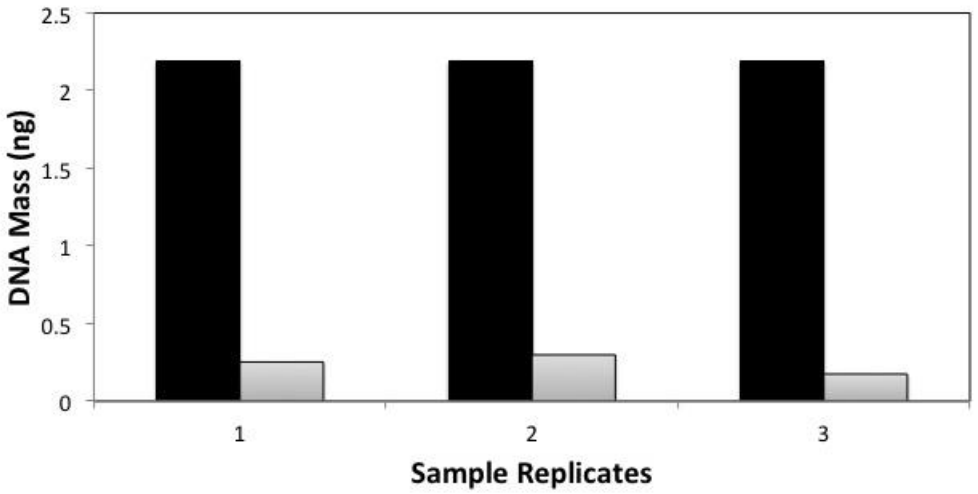

Figure 13: DNA Mass (in ng) Eluted from Dynabeads® Used to Clean Samples. (■) Input DNA mass. (■) DNA mass eluted from Dynabeads®, calculated via qPCR. Estimated 50% DNA loss during Amicon filtration taken into account by doubling the mass detected by qPCR.

Table 4: Genomic DNA Removed During Dynabead® Post-PCR Processing Due to Biotinylated Primer Hybridization to Genomic DNA.

Sample	DNA input (ng)	DNA eluted from beads (ng)	Percent Loss due to primer binding to genomic DNA
1-1		0.2526	11.53%
1-2	2.191	0.2985	13.63%
1-3		0.1747	7.98%
Average		**0.24 ± 0.12**	**11 ± 6%**

The results show that all samples tested indicated the presence of genomic DNA on the beads, confirming the hypothesis that this step is responsible for a

portion of the overall loss of DNA. The samples showed an average loss of 11 ± 6% of the input DNA mass due to the biotinylated primers hybridizing to the genomic DNA, which is then captured by the streptavidin-coated beads. Since the overall average DNA mass lost is 1.4 ng and the average DNA mass bound to the beads is only 0.24 ng, the streptavidin-biotin removal of genomic DNA represents about 1/5 of the total DNA loss observed.

3.3.2. Amicon® Filter Membrane Contribution to DNA Loss

The second potential contributor to the genomic DNA loss is the Amicon® filtration step. Previous studies suggest that filtration procedures may be responsible for significant levels of DNA loss. It was hypothesized that the DNA was being retained within the filter membrane [46], resulting in a decrease in DNA yield. The Amicon® filtration step of the recursive PCR cleanup method was initially designed following the manufacturer's recommended spin times and angular velocity (i.e. 50 minutes, 14,000 rcf). However, previous studies have suggested that this angular velocity is not ideal for forensic purposes, with the high forces causing the genomic DNA to become "stuck" within the cellulose filter membrane. Recent studies have shown genomic DNA recovery rates of ~50% when centrifuging samples at 14,000 rcf [46]. Assuming the observed genomic DNA loss is attributed to only the two factors previously mentioned, the amount of DNA lost in the filter can be calculated for a given sample using the following equation:

DNA lost in Amicon = Input DNA Mass - Recoverd DNA Mass - DNA lost on beads

(Equation 9)

The input DNA mass is the amount of DNA that was placed into the original PCR tube and the final DNA mass is the amount remaining after the cleanup process. These values can be seen in Figure 14.

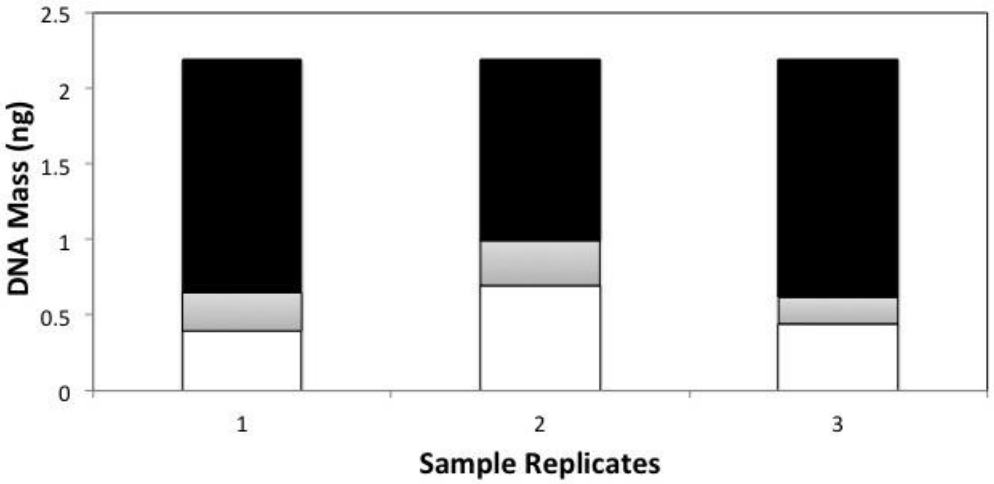

Figure 14: DNA Mass (in ng) Lost on Dynabeads®, Lost in Amicon®, and Recovered for Samples Run Through Recursive Amplification. All samples had a DNA input mass of 2.191 ng. (□) DNA mass recovered. (■) DNA mass lost on Dynabeads® as per qPCR of DNA eluted off beads. (■) DNA mass lost in Amicon filter as per Equation 9.

It is important to note that the values used for the amount of DNA lost on the beads were double what the qPCR data read. This doubling was necessary because the solution that was eluted from the beads was cleaned with Amicon® filters in order to remove the formamide (a known PCR inhibitor). It was assumed that 50% of the eluted DNA was lost during this step.

Calculating the amount of DNA lost during filtration with Equation 8 results in an average loss of 66 ± 10% of the input DNA mass. Since ~20% of the genomic DNA is removed by the Dynabeads® as seen in Figure 13, this suggests approximately 75% of the DNA which reaches the filtering process is lost to the cellulose filter. This significant finding was confirmed by additional experiments. In these experiments, ~2 ng of DNA was directly added to the Amicon® filter and spun and retrieved as per the manufacturer's recommended protocol at 14,000 rcf for 50 minutes [39]. Figure 15 shows a side-by-side comparison of the DNA mass pre- and post-filtration.

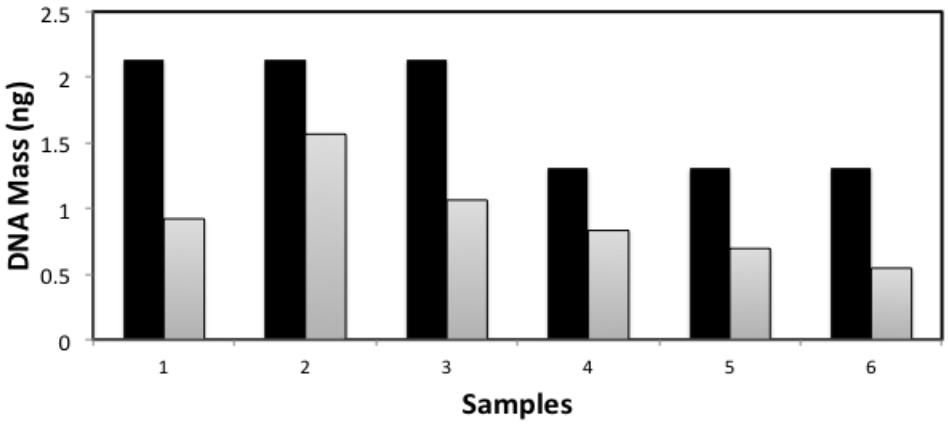

Figure 15: DNA Mass (in ng) Recovered After Being Run Through Amicon® Filtration Devices at 14,000 rcf for 50 Minutes. (■) Input DNA mass. (■) DNA mass after Amicon® filtration.

The results demonstrate that at least 25% of the input DNA was lost during the filtration for each sample, with the average being 46 ± 11%. This average is similar to the values shown in the literature, but is slightly lower than the 75% average loss calculated previously. One possible reason for this is that the

experiments differ slightly in that the filtration protocol within the recursive amplification technique involves an additional TE buffer wash with a 20 minute spin at 14,000 rcf. Therefore, to take the full recursive method into account, the experiment was repeated with the additional TE wash and spin added in.

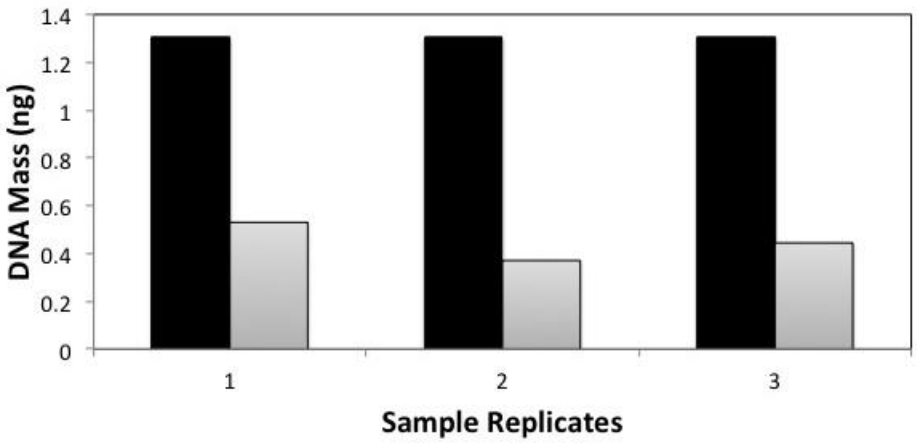

Figure 16: DNA Mass (in ng) Recovered After Being Run Through Amicon® Filtration Devices at 14,000 rcf for 20 Minutes, Followed By 50 Minutes at 14,000 rcf. (■) Input DNA mass. (■) DNA mass after Amicon® filtration.

Figure 16 shows that the genomic DNA loss was 66 ± 12% and is insignificantly different from the ~75% loss calculated using Equation 9, suggesting the additional TE wash and 20 minute filtration spin contribute to the decrease in DNA yield. All of these experimental results confirm that a significant amount of DNA is lost during Amicon® filtration, and is consistently seen in all samples. Additionally, these diagnostic experiments suggest that the filtration step is the major contributor to the low DNA yield.

3.3.3. Leftover TPOX Amplicons

Similar to the genomic DNA loss, the source(s) of the TPOX amplicon retention need to be identified and corrected. It is important for all of these amplicons to be removed, not only because their presence can interfere with further amplification, but the resulting peak on the electropherogram will also complicate downstream recursive profile interpretation. If a significant level of TPOX amplicon signal remains, and multiple recursive amplifications are desired, it is expected that the retained signal will become more prevalent as the number of recursive amplifications increases. In the extreme case, the profile will become so complicated that it will not be able to garner any new or additional information.

Figure 17 shows the capillary electrophoresis results of a sample amplified with the biotinylated TPOX primers, as described in Methods Section 2.4.3.

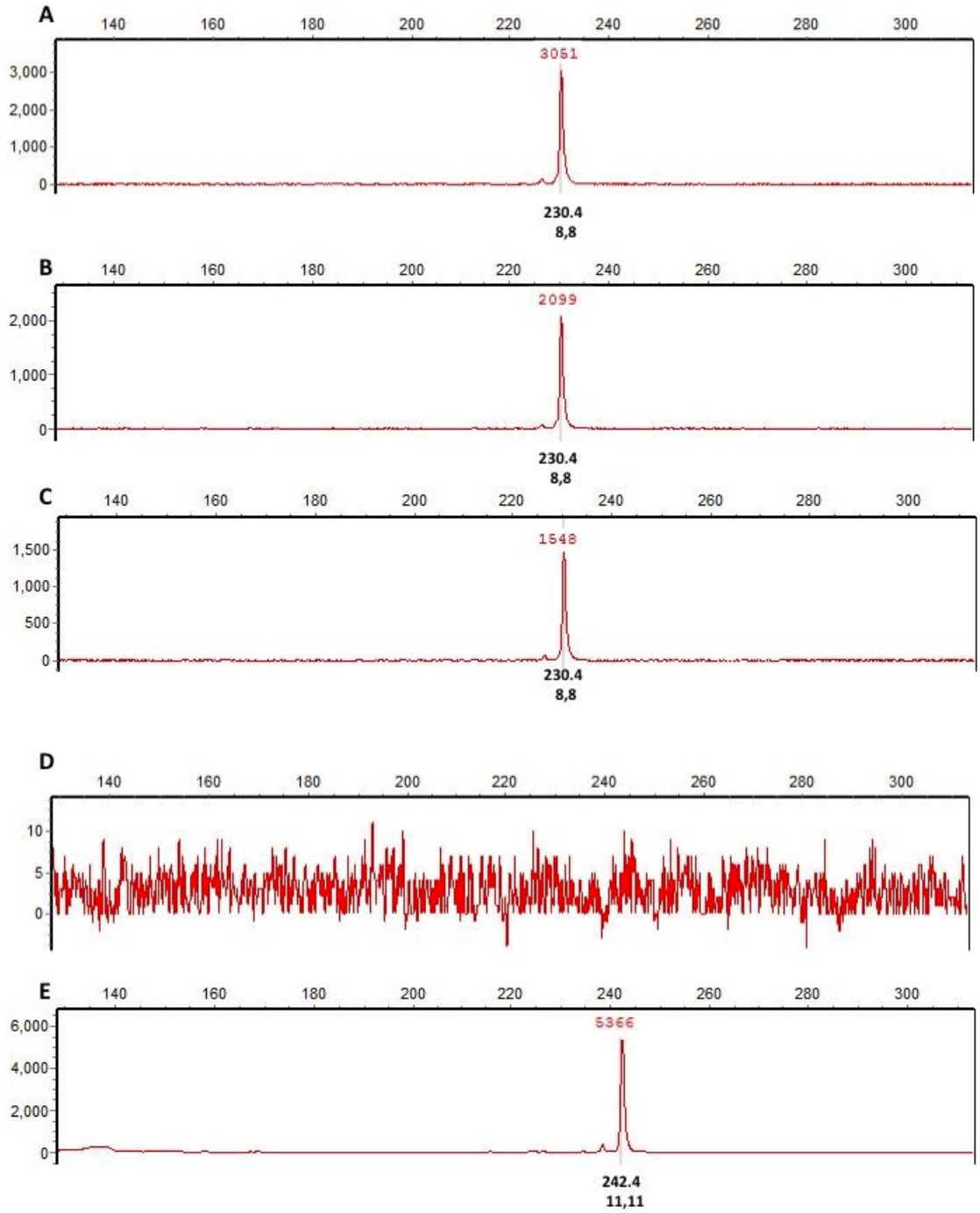

Figure 17: Representative Electropherograms of TPOX Leftover Assessment. A-C) DNA 1 amplified with TPOX biotinylated primers, cleaned, re-amplified with DNA 2 with no primers added to master mix. D) Negative control – re-amplified with TPOX primers with no DNA added. E) Positive control – DNA 2 amplified with TPOX primers. Red number = peak height in RFU; Below peak = size in bp and allele repeat numbers.

Three replicates (Figure 17A-C) showed amplified product at 230.4 bp (8,8 genotype). The 11,11 (i.e. 242 bp) amplicon of DNA 2 is not observed, despite containing 2 ng of DNA 2 in the amplification setup. Further, Figure 17D shows the negative control, which was amplified with biotinylated TPOX primers without the presence of DNA. It showed no amplicon signal, as expected. Figure 17E shows the result of the positive control experiment where 2 ng of DNA 2 was amplified using TPOX biotinylated primers. The 11,11 peak observed at 242.2 bp of DNA 2 indicates that DNA 2 was present and able to successfully amplify.

Since only an 8 peak is observed in the sample electropherograms, it can be inferred that the biotinylated TPOX primers were successfully removed during the post-PCR cleanup strategy. These results also suggest that the observed TPOX signal in the re-amplified samples (Figures 10 and 11D-F) may be due to the presence of retained TPOX amplicons not completely removed by the Dynabeads®. That is, the TPOX retained signal is due to TPOX amplicon from a previous amplification remaining in solution and not due to insufficient TPOX primer removal. This is a significant finding since it allows for targeted optimization of the procedure such that an increase in TPOX amplicon removal is pursued during method development.

Laboratory testing of the method produced an average retained TPOX peak height of 1122 ± 1050 RFU. Substituting 1122 RFU into Equation 7 and solving for ($\%retention_{total}$) results in a TPOX amplicon retention of 30%. No values for

primer retention were simulated since the experimental results exhibited in Figure 17 show the biotinylated TPOX primers are efficiently removed.

Using Equation 8 will also prove to be useful for guiding method optimization. A large percentage of TPOX amplicon retention is expected to interfere with re-amplification. Laboratory work has shown D5S818 re-amplification to be successful despite the ~30% signal retention of the TPOX locus. However, for this method to be considered useful for forensic testing, the procedure will need to be multiplexed in which case incomplete amplicon removal is expected to have a significant impact on recursive amplification.

Prior to profile interpretation, DNA analysts set an “analytical threshold” for peak heights. This means that any peak with a height below this pre-determined RFU value will not be considered a “true allele” and will thus not be included during interpretation. A leftover TPOX peak would prove to be problematic for profile interpretation if its height is above the threshold. A given analytical threshold value can be substituted in for the peak height in Equation 8. The amplicon retention percentage can then be calculated. Experimentally observed TPOX amplicon retention must be below this percentage to not cross the analytical threshold and interfere with profile interpretation. Therefore, for a 30 RFU threshold, the amplicon retention must be below 1.60%. A 50 RFU threshold would equate to a 2.12% retention rate. A lab using a 100 RFU threshold would need retention below 3.41%. It is clear that these percentages

are quite low. In terms of method optimization, this suggests that essentially all amplicons need to be removed during the streptavidin-biotin purification step.

In conclusion, it has been shown that recursive amplification of template DNA is possible. However, two areas require optimization if this process is to be deemed useful for forensic purposes. 1) The level of genomic DNA lost during the post-PCR purification processing must be decreased and 2) the retained TPOX signal must be decreased. Characterization of the sources of genomic DNA loss has been completed and the results suggest that 20% of the input template is lost during the Dynabead® cleanup step, with an additional 45% of the DNA (i.e. 75% of the remaining DNA) being inadvertently removed during the filtration step. Further, identification of the sources of the retained TPOX signal was completed and results indicate it stems from incomplete amplicon removal as opposed to preserved primer.

3.4. Preliminary Method Optimization

Similar to decreasing TPOX amplicon retention, genomic DNA loss must also be minimized. Data obtained herein suggests a significant level of template is removed during both the streptavidin-biotin and filtration processes. It has previously been suggested that using a centrifugal force during Amicon® filtration lower than that recommended by the manufacturer can lead to an increased recovery of genomic DNA [46]. It was hypothesized that high speeds potentially cause DNA to become caught up within the filter membrane. As a result, the following experiment tested whether the template loss would be negated by

decreasing the centrifugal force during the filtration step. This was assessed by filtering ~2 ng of genomic DNA for 50 minutes at 14,000 (recommended protocol), 4,000, 3,500, 3,000, and 2,500 rcf. An analysis via qPCR was used to determine the recovered DNA mass.

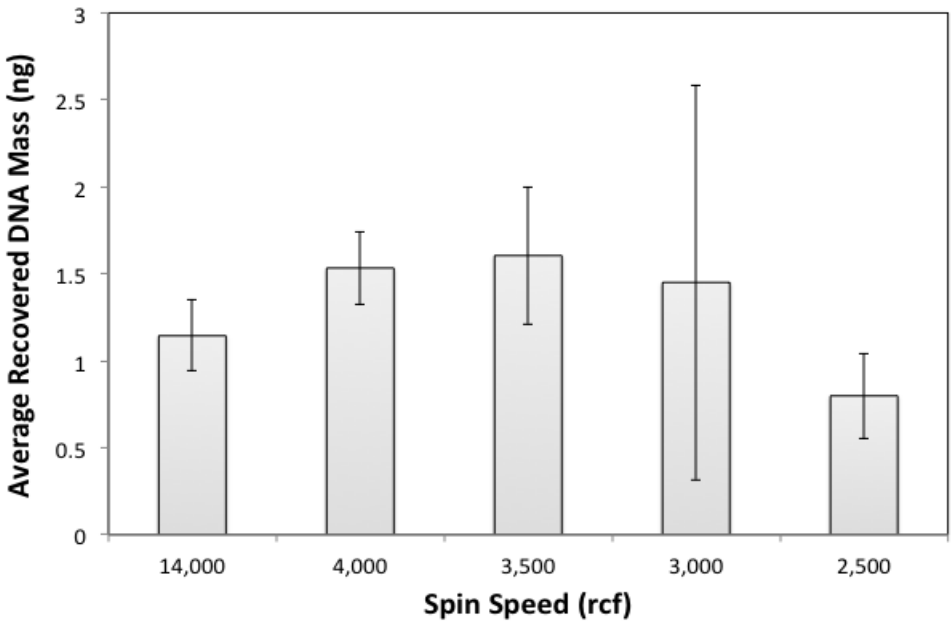

Figure 18: Average DNA Mass Recovered (in ng) for Samples Filtered with Amicon® at Several Spin Speeds. All samples had an input DNA mass of ~2 ng. Error bars represent ± two standard deviations.

Table 5: Amicon® Spin Speed Percent Recovery Assessment.

Centrifugal Force used for 50 minutes during Amicon® Filtration	Average Recovered DNA Mass (ng)	Average Percent Recovery	Average Volume of Concentrated Sample (µL)
14,000 rcf	1.1 ± 0.2	57 %	10
4,000 rcf	1.5 ± 0.2	76 %	27
3,500 rcf	1.6 ± 0.4	80 %	28
3,000 rcf	1.5 ± 1.2	72 %	30
2,500 rcf	0.8 ± 0.2	39 %	27

Figure 18 and Table 5 show that there is an average genomic DNA recovery increase for three of the four speeds tested against the current protocol. The 2,500 rcf spins produced an average recovery less than that of the current spin speed. This speed was expected to have the highest recovery, though the experiment generally showed the hypothesized trend overall for the other spin speeds.

A two-tailed unpaired *t*-test was performed on the data set to determine if the observed difference in average genomic DNA recoveries was in fact statistically significantly different between the spin speeds (Table 6).

Table 6: *t*-test Results of Amicon® Spin Speed Percent Recovery Assessment.

Comparing:	*p value*	*t value*	*df*	*Significant?*
14,000 and 4,000 rcf	0.0101	4.5945	4	Yes
14,000 and 3,500 rcf	0.0228	3.5984	4	Yes
14,000 and 3,000 rcf	0.4094	0.9204	4	No
14,000 and 2,500 rcf	0.0199	3.7535	4	Yes
4,000 and 3,500 rcf	0.6085	0.555	4	No

Table 6 shows that the average genomic DNA recovery values for the 4,000 and 3,500 rcf spins were significantly different than the average for the currently used spin speed. The 2,500 rcf spin was significantly different as well, but this average recovery was lower than the 14,000 rcf average. The data suggest there is an optimal range of spin speeds to be used in conjunction with Amicon® filtration to increase genomic DNA recovery, and ranges between 4000 – 3500 rcf. Experiments conducted by Garvin et al showed optimal DNA yield at a spin speed of 3,000 rcf [46], corroborating the results obtained in this laboratory. An additional *t*-test comparing the 4,000 and 3,500 rcf results showed that the two are not statistically significantly different. Since the lower speeds did improve overall recovery, then the current spin time of 50 minutes would potentially need to be increased. Table 5 shows that a slower speed would need a longer time period to concentrate the DNA into the 11 µL volume needed for re-amplification.

In addition to the filtration spin, the method involves another spin where the filter cup is inverted and used to elute the purified DNA out of the Amicon® device and into a clean collection tube. Currently, this spin is 2 minutes at 1,000 rcf. It was hypothesized that increasing the spin speed from 1,000 rcf will cause any genomic DNA trapped within the membrane to be released and able to elute into the solution. This was assessed by filtering ~2 ng of genomic DNA for 50 minutes at 14,000 rcf, and then eluting it at 1,000, 5,000, 10,000, and 14,000 rcf for 2 minutes. An analysis via qPCR was used to determine recovered DNA mass.

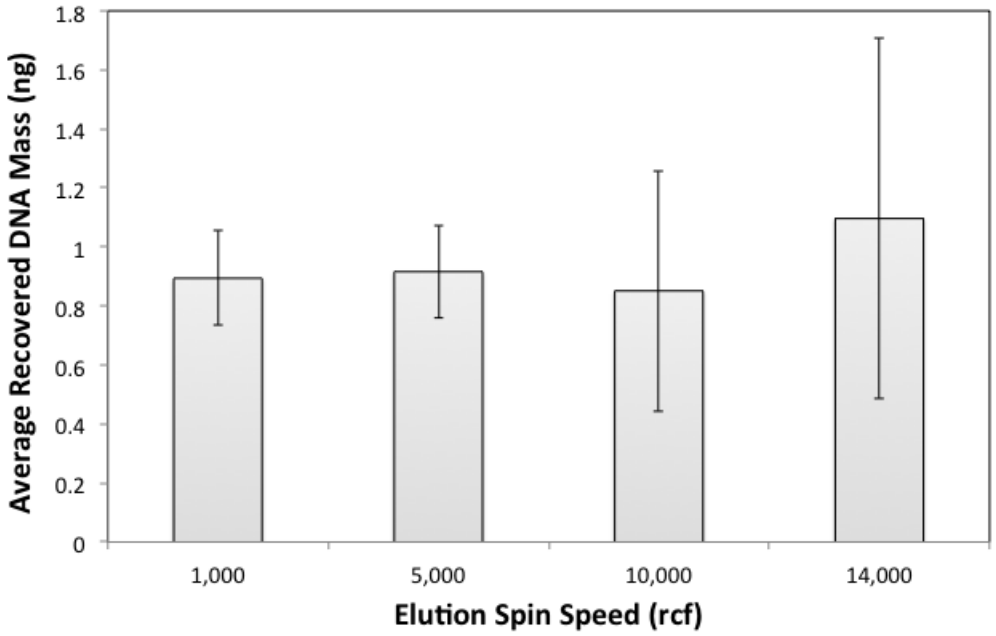

Figure 19: Average DNA Mass Recovered (in ng) for Samples Filtered with Amicon® then Eluted at Several Spin Speeds. All samples had an input DNA mass of ~2 ng and were initially spun at 14,000 rcf for 50 minutes. Error bars represent ± two standard deviations.

Table 7: Amicon® Elution Spin Speed Percent Recovery Assessment.

Sample	Average Recovered DNA Mass (ng)	Average Percent Recovery
1,000 rcf	0.89 ± 0.2	44 %
5,000 rcf	0.92 ± 0.1	45 %
10,000 rcf	0.85 ± 0.4	42 %
14,000 rcf	1.1 ± 0.6	54 %

Figure 19 and Table 7 show the mass and percent recovered after an initial spin of 14,000 rcf for 50 minutes and varying the elution speeds. All of the elution speeds showed similar average percent recovery values and were comparable to the 1.1 ± 0.2 ng recovery shown in Table 5, indicating reproducibility. A two-tailed unpaired *t*-test was performed on the data set to determine if the averages were statistically significantly different between the speeds (Table 8).

Table 8: *t*-test Results of Amicon® Elution Spin Speed Percent Recovery Assessment.

Comparing:	*p value*	*t value*	*df*	*Significant?*
1,000 and 5,000 rcf	0.7467	0.3461	4	No
1,000 and 10,000 rcf	0.7529	0.3372	4	No
1,000 and 14,000 rcf	0.3281	1.113	4	No

The results in Table 8 show that none of the average recoveries for the new speeds tested were significantly different from the average recovery of the

current method. Therefore, the elution speed can remain at 1,000 rcf without affecting percent recoveries of the template DNA.

4. Future Studies

Future experiments will focus on continuing method optimization. Work will continue to characterize the role of Amicon® filtration in genomic DNA loss. Before integrating a slower spin speed into the filtration step of the post-PCR purification method (i.e. 4000 – 3500 rcf; Table 5), the time to spin the volume down to 11 µL (or other approximate volume) must be determined. Then, the method can be tested using the new speed(s) and an assessment will be made on overall percent recovery.

Additional experiments will attempt to reduce the amount of DNA removed by the Dynabeads®. This can be performed by determining a temperature that will cause the primers to denature from the template DNA without causing the amplicons to denature [35]. Ideally, this change in temperature will prevent streptavidin interactions with biotinylated primers annealed to the template DNA.

Another area for future study would be to attempt to decrease/eliminate the retained TPOX peak. Preliminary experimental results have indicated that this peak is due to the presence of TPOX amplicons not removed during the streptavidin-biotin binding step. It would be useful to determine whether this peak would decrease with multiple streptavidin-biotin washes. This would allow for more streptavidin-biotin interactions, increasing the chance that binding will

occur for the remaining amplicons. An alternative way of testing this is by increasing the volume of Dynabeads® used per sample.

Additionally, it would be beneficial to perform more runs of the dynamic model. The parameters could be varied in order to better understand the relationship between the different aspects of the recursive amplification technique with the observed capillary electrophoresis peak heights.

5. Conclusions

A novel method that allows for re-amplification of DNA that has already been amplified was developed and tested in the laboratory. This recursive amplification method could provide additional processing options for extreme low-template and/or exhaustive DNA samples that traditionally have been considered of insufficient quantity/quality for multiple amplifications.

Initial amplification was performed at the TPOX locus. The PCR product was then cleaned with streptavidin beads followed by centrifugal filtration. Results indicated this cleanup is adequate enough to allow re-amplification of the template DNA at the D5S818 locus. Analysis via qPCR showed successful re-amplification of cleaned samples at the RPPH1 locus. However, the qPCR data also indicated a significant loss of genomic DNA during the cleanup process. In addition, capillary electrophoresis of samples re-amplified with D5S818 primers showed a retained TPOX signal.

Eluting DNA off of the beads indicated that approximately 20% of the overall loss of template can be attributed to the removal of genomic DNA due to the annealing of biotinylated primers to the streptavidin-coated beads. The additional loss of the remaining DNA occurs during the centrifugal filtration step, potentially from genomic DNA becoming “stuck” within the cellulose membrane of the filter device.

Assessment of the cause(s) of the retained TPOX signal indicated that this signal is due to the presence of TPOX amplicons from the original amplification that were not removed by the beads. The data also suggest that the TPOX primers are being removed from the solution to a sufficient level as to not cause re-amplification of this locus during the second PCR.

A dynamic model of the recursive amplification method was designed to aid in understanding the overall effect of genomic DNA loss on the observed D5S818 peak height on CE. This model was also used to correlate the percentage of retained TPOX amplicons with the observed TPOX signal in re-amplified samples. Model data was used to create regression lines for the peak heights of both loci. Utilizing both the average peak heights and the line equations during method optimization allows for estimation of the overall genomic DNA loss percentage as well as the overall TPOX retention percentage.

Preliminary experiments assessed the contribution the filtration spin speed had on the level of genomic DNA loss. Results indicated that lowering the speeds of the initial filtration spin has the potential to increase DNA recovery by

decreasing the amount of DNA trapped within the cellulose filter. In addition, a spin is used in the recursive amplification cleanup protocol in which the filter is inverted and spun in order to elute the purified DNA. The results suggested that increasing the speed during this spin has little to no impact on DNA recovery and is not expected to impact the ability to recursively amplify extreme low-template forensic DNA samples.

LIST OF JOURNAL ABBREVIATIONS

Forensic Sci. Int.	Forensic Science International
Int. Congr. Ser.	International Congress Series
Int. J. Legal Med.	International Journal of Legal Medicine
J. Forensic Sci.	Journal of Forensic Science
Plant Mol. Bio.	Plant Molecular Biology
Plant Sci.	Plant Science
Sci. and Justice	Science and Justice
Water Air Soil Pollut.	Water Air and Soil Pollution

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EDUCATION

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Anticipated Degree: M.S. Biomedical Forensic Sciences
Forensic Biology – DNA Analysis Track

Thesis: Genomic DNA Isolation from Amplified Product for Recursive Genotyping of Low-Template DNA Samples

Boston College September 2007 – May 2011

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Undergraduate Research: Investigation of the nutritional necessities and cellular response to environmental stressors in the budding yeast *Saccharomyces cerevisiae*

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Research Assistant BU Biomedical Forensic Sciences, Boston, MA
January 2013 – May 2013

- Thesis Laboratory Research Work on Recursive Amplification of Low-Template DNA Samples

Laboratory Technician Pervasis Therapeutics, Cambridge, MA
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AWARDS AND HONORS

2012 Northeastern Association of Forensic Scientists Carol DeForest Student Research Grant Awardee; $2,500