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Thesis

STREPTAVIDIN-BIOTIN BINDING OF DNA AMPLICONS: METHODS FOR THE TYPING AND RE-TYPING OF FORENSICALLY RELEVANT SHORT TANDEM REPEATS

By

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STREPTAVIDIN-BIOTIN BINDING OF DNA AMPLICONS: METHODS FOR THE TYPING AND RE-TYPING OF FORENSICALLY RELEVANT SHORT TANDEM REPEATS

JESSICA ANNA-MARIE SHEA

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ABSTRACT

Submission of evidentiary samples to DNA units for exhaustive testing is becoming commonplace. For these samples, only one attempt at amplification is possible. However, more than one amplification may be necessary if the condition of the DNA causes poor amplification, more than one type of STR kit testing is required, or if there is an instrument malfunction during amplification. Current research into the re-amplification of already amplified samples focuses on placing the PCR product back into the thermal cycler with new reagents for additional cycles. These methods typically result in outcomes which are unsatisfactory for forensic purposes. As a result, there is a need for a forensic method capable of recovering the original template DNA for purposes of re-amplification.

This study outlines the development of a novel method to recover the original template DNA in a condition that allows for re-amplification using new

STR loci. A dynamic model was designed to assist in the experimental optimization. Amplification was performed using biotinylated primers and the post PCR ‘work product’ was subsequently cleaned using streptavidin coated magnetic beads to remove the STR amplicons. Centrifugal filtration followed in order to remove any remaining primers and salts that may interfere with re-amplification. Re-amplification was then performed with non-biotinylated primers. Re-amplification of the template DNA using a new STR locus was successful, making the amplification of limited DNA samples non-destructive and the notion of ‘exhaustive DNA typing’ obsolete.

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LIST OF ABREVIATIONS

CE	Capillary Electrophoresis
DNA	Deoxyribonucleic Acid
dNTPs	Deoxynucleotide Triphosphates
EDTA	Ethylenediaminetetraacetic Acid
IPC	Internal PCR control
$MgCl_2$	Magnesium Chloride
NaCl	Sodium Chloride
PCR	Polymerase Chain Reaction
qPCR	Quantitative PCR
STR	Short Tandem Repeats
T_m	Melting Temperature
VNTR	Variable Number Tandem repeats

1. Introduction

In forensic DNA analysis, short tandem repeat (STR) testing is used for identification purposes. This technology stems from two important developments in 1985. The first development was that of “DNA fingerprinting” presented by Alec Jeffreys. Using hypervariable minisatellite regions (i.e. VNTRs) and restriction enzymes, Jeffreys determined that each individual possessed a different combination of repeat lengths within those regions. He also noted that while close relations have more alleles in common, the only individuals with the exact same number of repeats were identical twins[1].

The second important development was the polymerase chain reaction (PCR)[2]. The amplification of small amounts of DNA template using PCR analyzed directly without the need for restriction enzyme digestion resulted in a sensitive technique. By amplifying regions containing tandem repeats, human identification from biological samples took significantly less time and required less template[3]. In addition, multiple regions of the DNA could be amplified at the same time[4]. This allowed for human identity testing with high powers of discrimination with low limits of detection, whereby only tens of cells are required to obtain an unambiguous STR profile.

Since VNTRs are too long for efficient amplification, the polymorphisms currently used in forensic DNA laboratories are microsatellite regions known as short tandem repeats (STRs). Like VNTRs, these regions are differentiated by the number of repeats present. However, STR repeat units are significantly

shorter in length and typically range from two to six repeats versus the ten to one hundred repeats typically seen within VNTR regions. Although STRs are less hypervariable within the population, they are easily amplified using PCR and can be multiplexed allowing one amplification reaction to generate information from multiple DNA locations[5]. While there are many STRs within the human genome, a small subset has been chosen for forensic and human identification purposes.

Since the discovery of DNA fingerprinting and PCR amplification, there have been many improvements to the technique, including the addition of a thermostable polymerase. This Taq polymerase, which stands for Thermus aquaticus, allows the PCR to be performed at higher temperatures. Higher temperatures means that the specificity, sensitivity, and yield are improved[6]. Further advancing the specificity of PCR, this Taq polymerase has been modified to remain inactive until heated to 95ºC before the PCR cycling begins. Using this modified Taq increases the product yield and decreases the production of non-specified product during cycling[7].

1.1. Polymerase Chain Reaction (PCR)

Along with modifying the polymerase, there are a variety of factors that can affect the PCR amplification reaction. These factors include reagent concentrations and cycling conditions. Typically, the reagents used in PCR are buffer, $MgCl_2$, deoxyribonucleotide triphosphates (dNTPs), polymerase, primers, and template DNA. Commercial kits are most often used, which specify the

optimal reagent concentrations; however, when using primers that are not part of a commercial kit, it is important to optimize the concentrations to the specific reaction. Primers need to be in excess in order to allow amplification to continue throughout all cycles. Additionally, the concentration of $MgCl_2$ and dNTPs need to be balanced. Like the primers, the concentration of dNTPs should also be in excess to allow DNA polymerization to continue unhindered through each cycle[8-10].

Cycling conditions and the number of cycles used are as important as reagent concentrations. The temperatures and length of time spent on each step - denaturation, annealing, and elongation - are critical factors and specific recommendations are typically outlined for the commercially available kits. The annealing temperature is the one that is dependent on the primer set. This is because the annealing temperature is dependent upon the melting temperature of the primers. Higher annealing temperatures decrease non-specific amplification as higher annealing temperatures lead to an increase in binding specificity. The number of PCR cycles is also an important factor to consider since amplification leads to an exponential growth in PCR product. As a result, this parameter is important to take into consideration when the sample contains a minimal amount of DNA template. Recent work on characterizing the effects increasing cycle numbers have on STR profiles is available in the literature and suggest care must be taken during interpretation of profiles generated with increased cycle numbers[9,10].

1.2. Low Template PCR

As technology advances and forensic DNA typing methods become more sensitive, PCR is applied to smaller and smaller amounts of DNA. This type of sample is termed low template DNA from the small number of DNA copies in the sample. With these samples, more amplification cycles than are normally used may be necessary to reach the method's minimum distinguishable signal. Often, these samples are amplified using the traditional PCR protocols, yet there is still too little DNA to generate a usable profile. Numerous techniques aimed at increasing the sensitivity of human identification via STR analysis have been published. Most notably, the work of Gill et al. focuses on increasing the amplification cycles from 28 to 34. Although this increases the sensitivity of the test, it also increases the rate of Type I error or drop-in[11-13].

Other studies have focused on re-amplifying aliquots of the amplified product which are placed back into the thermal cycler for more amplification cycles with fresh reagents[14-17]. This method is also fraught with problems. For example, one study found that re-amplification of an aliquot of the amplified 'work product' resulted in a smeared band during gel electrophoresis. To eliminate this effect, a post-PCR purification method was used before re-amplification; however, significant band smearing still resulted. Furthermore, it was noted that in all cases, amplification would stop occurring spontaneously. Longer targets stopped copying at lower cycle numbers than shorter ones, and it was suggested that the

accumulation of partial length strands was causing the abortions to occur due to the partial strands annealing to each other [15].

It has also been noted that primer dimers can form during re-amplification causing interference with the actual amplification process[18]. Attempts to decrease the effect primer dimers have on re-amplification have been made by decreasing the amount of primer added before the re-amplification. While this eliminated some of the effects seen by primer dimer formation, decreasing the amount of primer may lead to non-optimized PCR conditions – conditions not recommended for limited forensic samples [16].

Regardless of the method to increase sensitivity, PCR is considered a destructive technique. For these low template samples, one amplification completely exhausts the original sample, leaving re-amplification impossible. Therefore, a technique which allows for the re-generation of DNA template, such that ‘re-amplification’ of DNA can be accomplished multiple times, with multiple kits, is desirable and would be expected to have significant impact on criminal justice policy and practice.

1.3. Streptavidin-Biotin Bond

One purification method that may be used for this purpose is streptavidin-coupled magnetic beads that capture biotin. These beads work by exploiting the strong non-covalent bond, consisting of hydrogen bonds and Van der Waals forces, between streptavidin and biotin[19].

Streptavidin is a tetrameric protein which can bind up to four biotin molecules per one streptavidin molecule. As with many proteins, when this binding occurs, streptavidin undergoes changes to its structure. Without any substrate bound, streptavidin consists of four β barrels that are bound together. There is a pocket at the end of each of these β barrels. Within this pocket, the residues are polar, so a water molecule normally fills this space. For the biotin molecule to bind to the streptavidin, it must displace the water molecule. Once the water is displaced, the biotin interacts with various residues within the β barrel forming a series of hydrogen bonds. These interactions then cause ordering of a surface loop that covers the biotin, increasing the strength of the bond [20].

In order for the bonds between streptavidin and biotin to be broken, the streptavidin must unfold or denature. The amount of energy required to cause denaturation is proportional to the number of β barrels contained in a biotin molecule [20]. When the streptavidin is saturated with four biotin molecules the denaturation is monophasic, yet when there are less than four but more than one biotin molecule bound, the denaturation of streptavidin is biphasic. The melting temperature of streptavidin is the point at which half of the streptavidin in a solution is denatured. When there are biotin molecules bound in all four of the streptavidin's β barrels, the melting temperature of the protein increases from 75°C to 112°C[21,22].

1.4. Streptavidin-Biotin Interaction: Current Uses

The streptavidin-biotin interaction has been used within numerous laboratory protocols which include, but are not limited to, cell, protein, and nucleic acid isolation. In one study, the commercially available Dynabeads® M-270 Streptavidin which utilizes the streptavidin-biotin interaction was used for immune-precipitation in order to find a transcription factor[23]. In this work, the biotin was attached to labeled oligonucleotide sequences which were mixed with denatured DNA to specifically capture the target sequence on the streptavidin coated beads. In another study, Dynabeads® M-270 Streptavidin were pre-coated with an antibody and used to bind monocytogenes[24].

Dynabeads® M-270 Streptavidin are designed to be used for the capture of sequence specific DNA or RNA fragments, where the primers used to amplify the specific sequence are functionalized with biotin. The streptavidin on the beads binds to the biotin, capturing the double stranded DNA. This DNA can then be eluted from the beads for further use[23,25,26]. Two common applications of these beads are in microarray technologies[26,27] and pathogen identification via DNA presence[24,28].

1.5. Goal of Study

In forensic DNA analysis, a DNA sample containing genomic DNA (i.e. template DNA) is amplified via PCR. The amplified 'work product' from this reaction contains the original template DNA along with the components added for PCR. Figure 1 shows the components typically found in the amplified 'work

product' - dNTPs, primers, $MgCl_2$, buffer, polymerase, and amplicons, as well as the template DNA. The amount of template DNA is exponentially less than the amount of amplicons present. Previous studies have shown that simply re-amplifying this 'work product' in any scenario does not work. The goal of this study was to remove all of the components added for PCR (i.e. dNTPs, primers, $MgCl_2$, buffer, polymerase, and amplicons) to regain only the original template DNA (Figure 1).

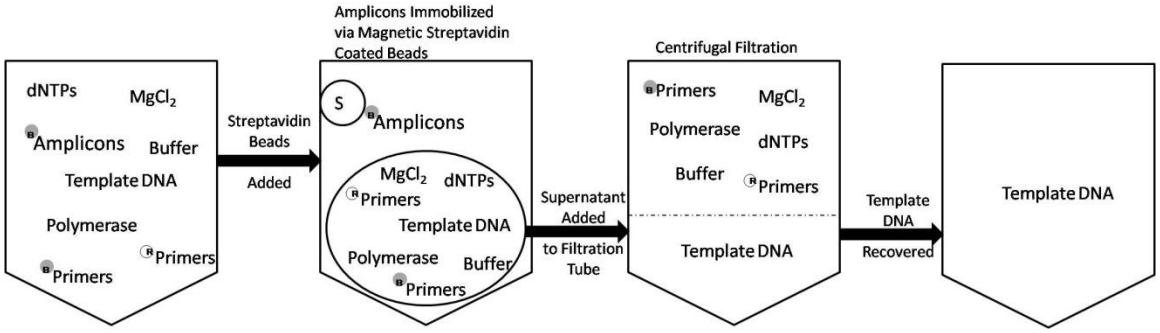


Figure 1: Schematic of Clean-Up Process B = Biotin S = Streptavidin Bead

In order to achieve this goal, the primers used during amplification are biotinylated. As a result, the biotin molecules are incorporated into the amplicons. Streptavidin coated beads can then be used as a purification method to remove these amplicons. The intent is to utilize the strong bond between streptavidin and biotin to 'pull' the amplicons out of the solution thereby regaining the original template DNA. The other components (i.e. dNTPs, $MgCl_2$, buffer, polymerase, and unincorporated primers) will be removed from the solution using centrifugal

filtration. When these two cleaning methods are employed, all of the PCR components should be removed from the solution with only the template DNA remaining. This template DNA could then be re-amplified with new PCR reagents such that more or different STR information can be procured.

1.6. Dynamic Modeling

Dynamic modeling is the creation of a computer model that shows events occurring over time and can be used to model situations that can also be tested in the laboratory. These models can provide useful information to guide experimentation as well as provide additional information regarding the experimental results[29].

In general, a dynamic model can be defined in three categories: elements, rules, and background. Elements are the items involved in the system. For example, in a model of a river that flows down hill into a lake, the elements are the river and the lake. Rules are the relationships between the elements. Using the same example, a rule would be the rate at which the river flows into the lake. The background is anything that is present in the system that does not change during the simulation. Again using the river example, a piece of the background would be that the water always flows down the hill (i.e. follows the laws of physics).

Figure 2 shows a simple dynamic model of the example explained above. The elements (i.e. river and lake) are represented as squares while the rule (i.e. *rate of water flowing*) is represented as an arrow which implies the movement of an

item (in this case water) from one element (the river) to another element (the lake). If the relationship between the two elements was more complex (i.e. the *rate of water flowing* was based on the amount of water currently in the lake) then another parameter would be added to the model (Figure 3).

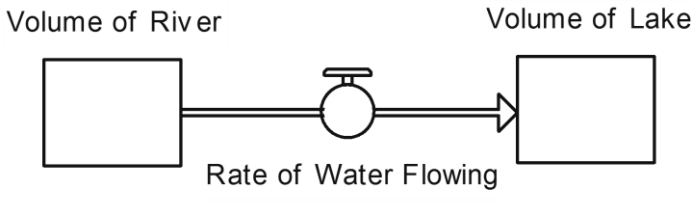


Figure 2: Dynamic Model of a River Flowing into a Lake.

The representative equation for the elements in Figure 2 is

$$V_{lake}(t) = V_{lake}(t - dt) + \mu(t) \qquad \text{Equation 1}$$

where V_{lake}(t) is the volume of water in the lake at time t, $V_{lake}(t - dt)$ is the volume of water in the lake at $t - dt$, dt is the time unit used during simulation, and μ is the rate of water flowing.

Figure 3 shows the same model with the additional variable of the *amount of water in lake*. In this model, the amount of water in the lake is represented by a circle and is connected to the *rate of water flowing*. Now, when the amount of water in the lake is changed, the *rate of water flowing* is automatically adjusted.

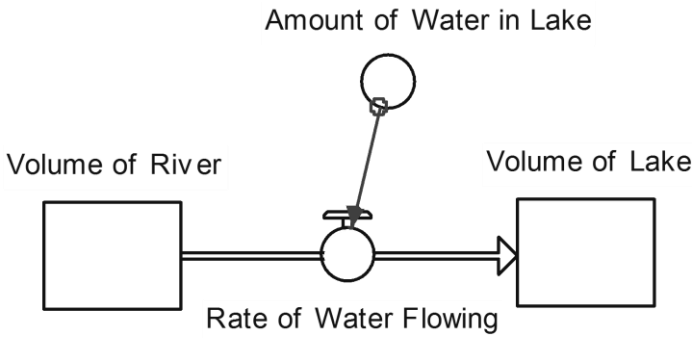


Figure 3: Dynamic Model of a River Flowing into a Lake with the Amount of Water in the Lake Affecting the Rate of Water Flowing.

Once the dynamic model is complete, a simulation run can be performed. The results from the model are termed properties of the system. For example, a property of the model in Figure 3 could be the amount of water that flows from the river to the lake in 1 hour. These properties should answer the question for which the model was built. Since the model is a representation of natural phenomena, it is important to be aware of any assumptions being made during the modeling process. For example, in the model in Figure 3, it was assumed that the water flowed at a constant rate ignoring any variables that may change that assumption.

1.7. Dynamic Modeling of Streptavidin-Biotin Interaction

Using the same methods as those outlined in the previous section, a dynamic model of the streptavidin-biotin interaction can be created. The elements for this interaction are the streptavidin and biotin molecules both in their unbound and bound forms. The unbound streptavidin and biotin molecules ‘flow’ into bound

streptavidin-biotin molecules. For the purposes of this study, it is necessary to break the biotin into the different ways it is incorporated into the DNA during PCR (i.e. bound to *unincorporated primers*, bound to *primers annealed to template DNA*, and bound to *primers incorporated into complimentary amplicons*).

There are a few rules for this model that outline the relationship between streptavidin and biotin. Streptavidin is a tetrameric protein meaning that for each streptavidin molecule, four biotin molecules bind to create one bound streptavidin-biotin molecule. The dissociation constant (K_D) for streptavidin is $1x10^{-16}$ M and the binding rate for the streptavidin-biotin bond is $3x10^{6}$ $M^{-1}s^{-1}$.

The background for this system is the tube the reaction is occurring in as well as the other components in the solution. These components are the binding and washing buffer (10mM Tris-HCl pH 7.5, 1mM EDTA, 2M NaCl), distilled water, and PCR components (dNTPs, $MgCl_2$, buffer, water, polymerase). Other important aspects of the background are the length of incubation (15 minutes) and the rotating plate on which the tubes are being incubated. All of the background is based on the experimental procedure used in this study.

The assumptions made for this dynamic model are the efficiency of the PCR amplification (Equations 2 & 3) and that the total amount of biotin in the system is kept constant.

The properties resulting from this dynamic model are the number of bound streptavidin and biotin molecules. Here the biotin will still be broken down into the same three categories (i.e. *complimentary amplicons bound to streptavidin*,

biotinylated primers annealed to template DNA bound to streptavidin, and *unincorporated biotinylated primers bound to streptavidin*).

1.8. Dynamic Modeling of Streptavidin-Biotin Bond and Oligonucleotide Melting

The second step of the streptavidin-biotin model is the melting of the streptavidin-biotin bond and the oligonucleotides. For this step of the model, the elements are the *streptavidin bound to biotin*, *complimentary amplicons bound to streptavidin*, and *biotinylated primers annealed to template DNA bound to streptavidin*. The melting temperature of each of these molecules and the reaction temperature are also elements.

The rule for this portion of the model is that when the reaction temperature is greater than the melting temperature of the molecule, melting will occur. If the reaction temperature is not greater than the melting temperature of the molecule, melting will not occur.

The background for this step of the model is the same as for the previous step. There are no new assumptions made for this step of the model; however, the assumptions made in the previous step carry over into this step as the number of each molecule present in the solution.

The properties resulting from this step of the dynamic model will be the amount of *melted streptavidin-biotin bonds*, melted oligonucleotides (i.e. *melted biotinylated primers annealed to template DNA, and melted complimentary amplicons*), and the amount of time for melting to occur.

1.9. Purpose of Study

The purpose of this work is to develop a method which would allow for the amplification of a DNA target which has already been amplified and is now a constituent of the amplified 'work product'. This was accomplished by removing the amplicons and other PCR components such as primers, dNTP's, etc. to sequester the original target DNA such that it was available for re-amplification with additional human identification chemistries. This allows DNA crime laboratories to genotype limited or exhaustive samples using a variety of kits/chemistries without the need for additional extraction or evidence processing. The implications to forensic DNA identity testing would be substantial as this approach would allow for,

- testing of both autosomal- and Y- STRs for limited sexual assault samples,
- the ability of the analyst to re-amplify with mini-STR's or an enhanced amplification scheme (i.e. more Taq Polymerase, repair enzymes, more BSA) if it is realized that the DNA was degraded, damaged and/or inhibited,
- the re-amplification of the sample due to an amplification failure and,
- re-amplification of exhaustive samples multiple times such that composite profiles may be interpreted.

The novel method combines well characterized methodologies, such as streptavidin-biotin interactions and centrifugal filters, to clean the amplified product such that re-amplification of the original target DNA is possible.

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-26415.

2.1. Overview

A dynamic model of streptavidin-biotin binding and the subsequent melting step of the amplicon clean-up method was created. The method was also optimized and verified experimentally.

2.2. Model

This model was generated using STELLA® version 9.1.4 (isee systems, Lebanon NH).

2.3. Model of Steptavidin-Biotin Binding and DNA Melting

A model containing two separate steps was created to determine the necessary number of streptavidin coated magnetic beads to ensure all of the amplicons are bound to the beads (Figure 1). This is based on the input mass of DNA into the PCR amplification. Figure 4 is a schematic of the possible biotinylated primer interactions with template DNA and complimentary amplicon DNA that occur during amplification. The schematic starts with template DNA being amplified via PCR with biotinylated primers. There are three possible ways

for the biotinylated primers to be incorporated into the DNA through this process. Figure 4a shows the biotinylated primers which are incorporated into the complimentary amplicons as they are amplified. Figure 4b shows the primers annealing to the template DNA without extension of the amplicon occurring. Figure 4c shows unincorporated biotinylated primers remaining in the solution.

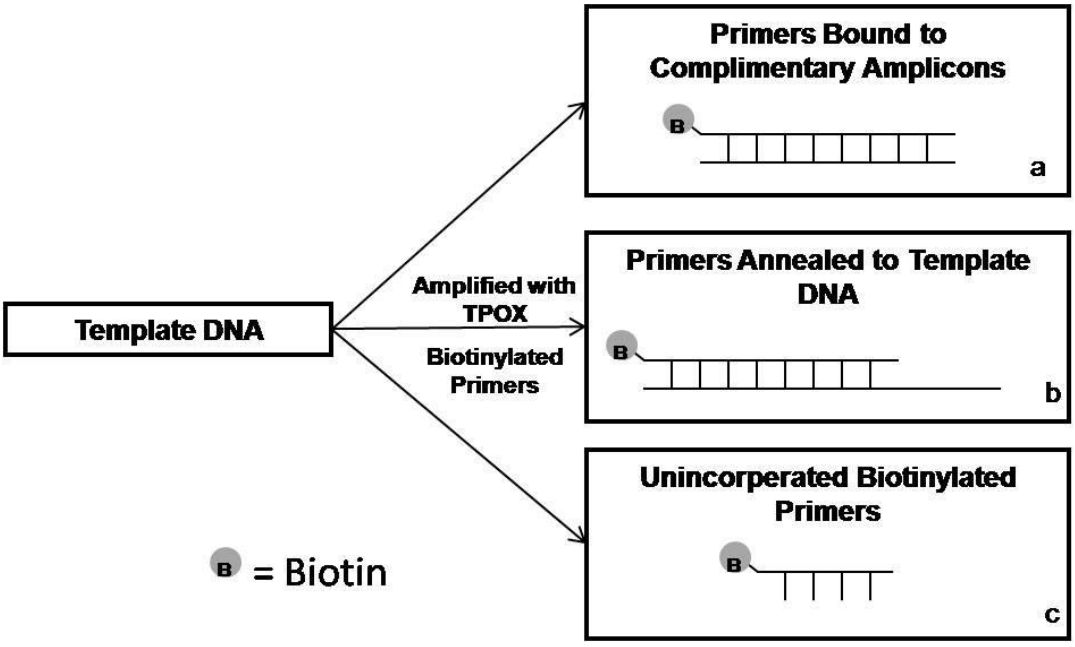


Figure 4: Schematic of Possible Biotinylated Primer interactions with Template DNA and Complimentary Amplicon DNA

Figure 5 shows a schematic of the two steps which occur during binding. The first step is the possible streptavidin-biotin interactions. During this step, streptavidin coated beads are added to the amplified 'work product'. The biotin, now incorporated into the DNA or remaining unincorporated primers, binds to the streptavidin coated beads. The beads are then immobilized with a magnet to the

side of the tube and the supernatant containing the template DNA and other PCR components is removed for further testing (Figure 1).

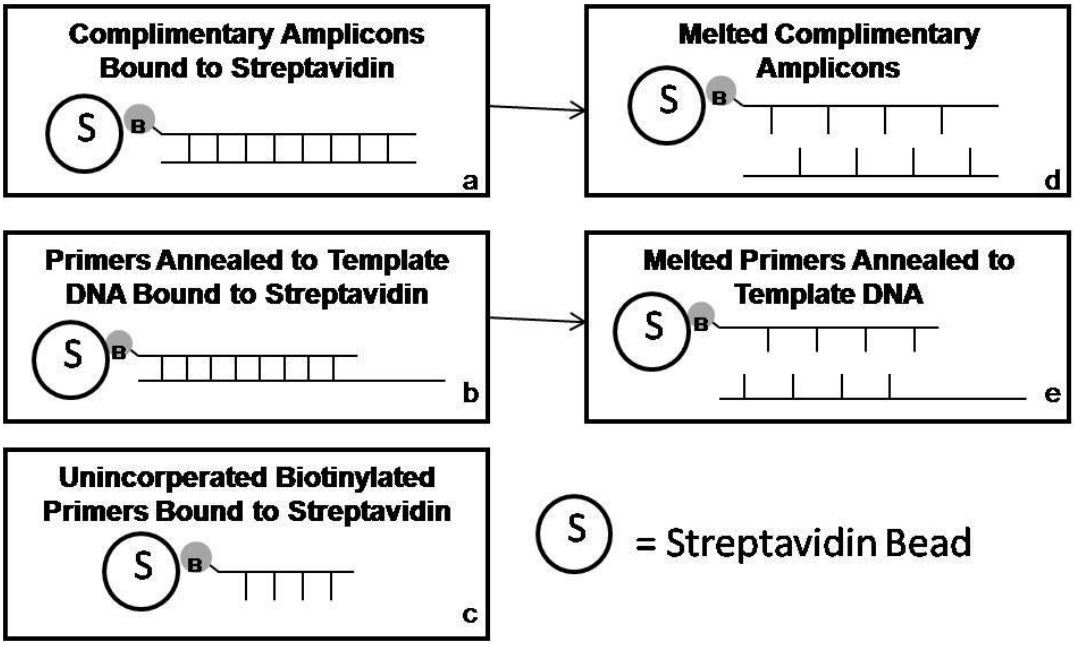


Figure 5: Schematic of Possible Streptavidin-Biotin Interactions and DNA Melting.

The second step is the subsequent melting of the DNA. Since the purpose of this work was to develop an efficient amplicon cleaning/separation procedure, and the T_m of the amplicons is expected to be different from the T_m of primer annealed to template DNA, it was of interest to determine the melting temperature that would maximize denaturation of *primers annealed to template DNA* (Figure 5e) while minimizing the denaturation of *complimentary amplicons* (Figure 5d).This would ensure that *primers bound to template DNA* and attached to the streptavidin coated beads would not erroneously be removed from solution

and remain for further amplification. Denaturation/melting is achieved by increasing the reaction temperature above the T_m of the primers. If the reaction temperature is too high (i.e. above the T_m of the amplicons), the amplicons will also melt (Figure 5d). This will cause some amplicons to be retained alongside the template DNA which may be detrimental to the re-amplification process.

2.3.1 Binding Step

The first step of this dynamic model is the binding of the streptavidin coated beads to the biotinylated oligonucleotides. There are three categories of biotinylated olioguclеotides included in the model: *biotinylated primers incorporated into complimentary amplicons*, *biotinylated primers annealed to template DNA*, and *unincorporated biotinylated primers* (Figure 5). The binding occurs for 15 minutes, which is the incubation time used in the experimental section of this work.

2.3.1.1. Input Parameters

Table 1 shows the constants used for this step of the model. Either the input number of streptavidin beads or the input mass of template DNA for PCR was changed for each simulation. Table 2 shows the values used for both of these variables as well as the number of *biotinylated primers incorporated into complimentary amplicons* which is based on the input mass of template DNA for PCR and assumes 100% of the biotinylated primers are incorporated into the amplicons produced after 32 cycles.

Table 1: Constants Used for Binding Step of Model.

Binding Rate	Initial No. of Biotinylated Primers	Initial No. of Streptavidin Beads	Input Mass of Template DNA for PCR
$1.5x10^{-4}s^{-1}$	0.04 µM	10,000,000	1 ng

Table 2: Independent Variables Used for Initial Values in the Binding Step of Model.

Input Number of Streptavidin Beads	
12, 000, 000 10,000,000 1,000,000 100,000 10,000 1,000	
OR	
Input Mass of Template DNA for PCR (ng)	**Number of Biotinylated Primers Incorporated into Complimentary Amplicons (i.e. No. PCR Amplicons after 32 Cycles)**
1 0.5 0.25 0.125 0.0625 0.03125	$6.4x10^{11}$ $3.4x10^{11}$ $1.7x10^{11}$ $8.8x10^{10}$ $4.4x10^{10}$ $2.2x10^{10}$

- ***Number of Unbound Biotinylated Primers Annealed to Template DNA***: A mass of 1 ng of DNA contains ~333 copies of each locus[30]. If it is assumed that 100% of the template hybridizes with primer (as opposed to its own complimentary strand), 1 ng of DNA resulted in 333 primers annealing to an equivalent number of template molecules.
- ***Number of Unbound Complimentary Amplicons***: An equation for PCR and PCR efficiency was used and are as follows,

$$C_n = C_o\,(1+E)_n \qquad \text{Equation 2}$$

$$E = 1 - (1.0456X10^{-10}x[DNA]_n) \qquad \text{Equation 3}$$

where C_n is concentration of DNA at cycle *n*, C_o is the initial concentration of DNA, *E* is PCR Efficiency, and *n* is cycle number.

Equation 3 is used to take into account the decrease in amplification efficiency with an increase in cycle number and is estimated from qPCR data of the amplification of RPPH1 locus [31]. Using these equations, 1 ng of DNA input to a 32 cycle PCR amplification generates 1,923,461,080 ng of product DNA. Multiplying this by the 333 copies per 1 ng of DNA, assuming primers are in excess, results in $6.4x10^{11}$ *biotinylated primers incorporated into complimentary amplicons* for 1 ng of input DNA. Other input values and the corresponding *biotinylated primers incorporated into complimentary amplicons* are given in Table 2.

- ***Number of Unbound Streptavidin***: The number of streptavidin coated beads can be controlled within the laboratory and when added to the reaction, where there are 6×10^8 beads per 1 ml of stock when using the commercially available Dynabeads® M-270 Streptavidin kit [25]. The number of streptavidin coated beads originally entered into the model parameters (i.e. 12,000,000 beads) was calculated using the binding capacity of the Dynabeads® M-270 Streptavidin. For double stranded oligonucleotides, the binding capacity is approximately 10 µg of oligonucleotides per 1 mg of streptavidin beads [25]. Based on the experimental optimization of the PCR amplification, 0.04 µM of

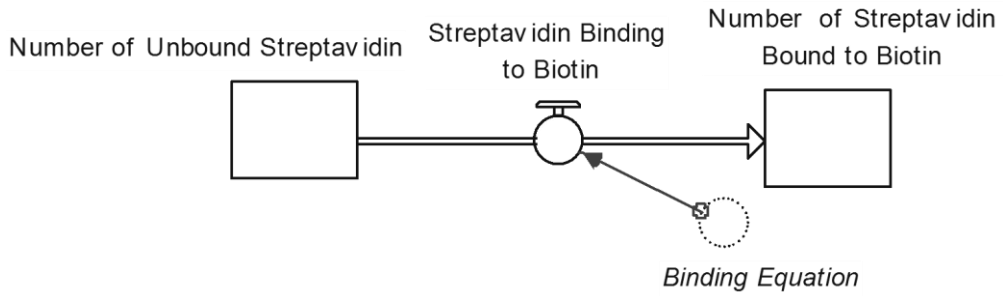
biotinylated primers are used per sample. From these values, the number of streptavidin needed to bind all of the biotin was determined to be 12,000,000 beads.

- **Binding rate of streptavidin and biotin**: The reported binding rate for these molecules is $3x10^{6}$ $M^{-1}s^{-1}$[19]. There are $5x10^{-11}$ M biotinylated primers per sample which makes the binding rate $1.5x10^{-4}s^{-1}$.

2.3.1.2. Output Parameters

- ***Number of Unbound Streptavidin***: The number of streptavidin beads that are not bound to biotinylated oligonucleotides.
- ***Number of Unbound Biotinylated Primers Annealed to Template DNA***: The number of *biotinylated primers annealed to template DNA* that are not bound to streptavidin coated beads.
- ***Number of Unbound Complimentary Amplicons***: The number of *biotinylated complimentary amplicons* that are not bound to streptavidin coated beads.
- ***Number of Unbound Unincorporated Biotinylated Primers***: The number of *unincorporated biotinylated primers* that are not bound to streptavidin coated beads.

Figures 6-9 show the binding step of the model broken down by the type of molecule (i.e. streptavidin beads, complimentary amplicons, primers annealed to template DNA, and unincorporated primers). Below each figure is the corresponding binding equation for the streptavidin beads and biotin molecules.



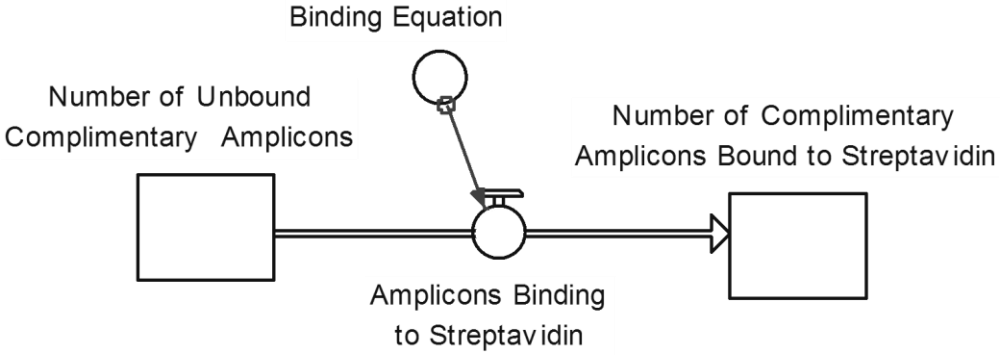
$= Binding\ Rate\ x$

Binding Equation

$$\left(\frac{Number\ of\ Complimentary\ Stranded\ Amplicons + Number\ of\ Unincorperated\ Biptinylated\ Primers + Number\ of\ Template\ DNA\ Annealed\ to\ Primers}{4}\right)$$

$x\ Number\ of\ Streptavidin$

Figure 6: Schematic of Streptavidin Beads Binding to Total Biotin in Solution

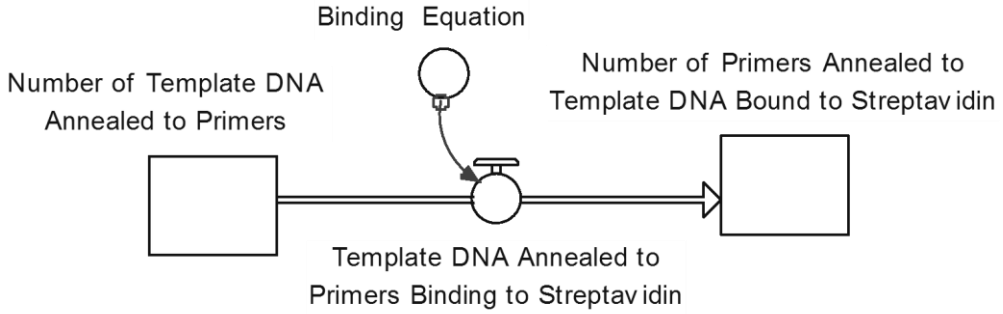


Binding Equation

$= \boldsymbol{Binding\ Rate\ x}$

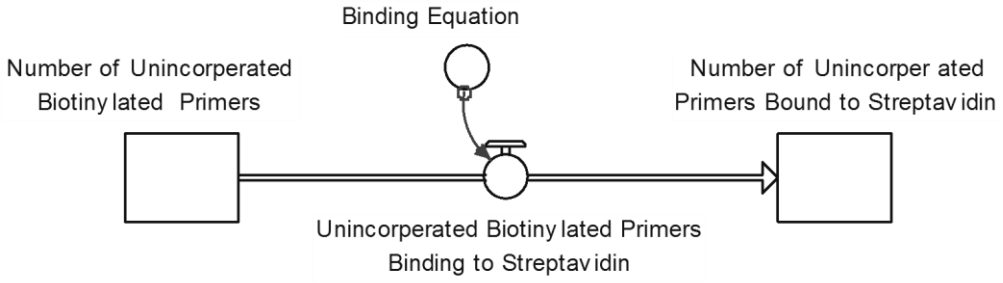
$$\left(\frac{\boldsymbol{Number\ of\ Double\ Stranded\ Amplicons}}{\boldsymbol{4}}\right) \boldsymbol{x\ Number\ of\ Streptavidin}$$

Figure 7: Schematic of Biotinylated Primers Incorporated into *Complimentary Amplicons* Binding to Streptavidin Beads.



$$\text{Binding Equation} = \boldsymbol{Binding\ Rate\ x} \left(\frac{\boldsymbol{Number\ of\ Primers\ Annealed\ to\ Template\ DNA}}{\boldsymbol{4}}\right) \boldsymbol{x\ Number\ of\ Streptavidin}$$

Figure 8: Schematic of *Biotinylated Primers Annealed to Template DNA* Binding to Streptavidin Beads.



$$\text{Binding Equation} = \boldsymbol{Binding\ Rate\ x} \left(\frac{\boldsymbol{Number\ of\ Unincorperated\ Biotinylated\ Primers}}{\boldsymbol{4}}\right) \boldsymbol{x\ Number\ of\ Streptavidin}$$

Figure 9: Schematic of *Unincorporated Biotinylated Primers* Binding to Streptavidin Beads.

2.3.2 Melting Step

The second step of the model focuses on determining the effects melting temperatures have on denaturing the *biotinylated primers annealed to template DNA.* Optimal T_m's are expected to decrease the amount of template DNA lost through binding to the streptavidin coated beads due to annealing of the biotinylated primers to the template DNA. The more template DNA remaining after cleaning, the more accurate re-amplification is expected to be. During this step, the sample is heated to a chosen reaction temperature and allowed to incubate for a specific amount of time.

2.3.2.1 Input Parameters

Table 3 shows the constants used for this step of the model. Table 4 shows the reaction temperatures used for each simulation.

Table 3: Constants Used for Melting Step of Model

Constants	Values
Number of Guanine in TPOX Amplicons	52
Number of Cytocine in TPOX Amplicons	31
Number of Adenine in TPOX Amplicons	63
Number of Thymine in TPOX Amplicons	42
Number of Guanine in TPOX Primers	7
Number of Cytocine in TPOX Primers	6
Number of Adenine in TPOX Primers	8
Number of Thymine in TPOX Primers	3
Salt Concentration	1M
Transition Temperature in the Absence of Biotin (T°)	112.2°C
Gas Constant (R)	$1.987x10^{-3}$ Kcal/M
Enthalpy in the Absence of Biotin (ΔH)	209.8 Kcal/M
Association Constant (Ka)	$1x10^{15}$ M^{-1}
Amount of Total Biotin	$2.4x10^{22}$ molecules

Table 4: Variables Used for Melting Step of Model

Variables	Values
Reaction Temperature	80, 85, 90, 95, 100, 105°C

- **Melting Temperatures (T_m's) for Primers and Amplicons**: T_m's were calculated by taking into account the length of the oligonucleotide, GC content of the sequences, and the salt concentration of the sample (Equation 4).

$$T_m = 100.5 + \left(41\ x\ \frac{G+C}{A+T+C+G} - \frac{820}{A+T+C+G}\right) + 16.6\ x\ \log_{10}[Na] \qquad \text{Equation 4}$$

where G is the number of guanine molecules present in one oliognucleotide strand, C is the number of cytocine molecules present in one oliognucleotide strand , A is the number of adenine molecules present in one oliognucleotide strand, T is the number of thymine molecules present in one oliognucleotide strand, and [Na] is the salt concentration in the solution.

- **Standard Deviation of Oligonucleotide T_m's**: Because the T_m calculated via Equation 5 is in actuality the point at which 50% of the DNA in the solution is denatured, it represents the average temperature of denaturation. Therefore, in this model, T_m is entered as average ± 2 standard deviations and assumed a normal variation. Here, the standard deviation was 5°C Celsius and was chosen based on a review of melting curves published in other studies. The melting curves published were examined to determine the range of temperatures where the oligonucleotide melted. The range was identified as the space between the points of inflection in the melting curves (10°C). The T_m of these curves was used as the average[32-35].
- **T_m of Streptavidin-Biotin Bond**: The T_m of the streptavidin-biotin bond was also considered since it was expected that high temperatures will have an

effect on the streptavidin-biotin bond[25,36]. As work by Weber et al. and Shrake et al. show, the streptavidin biotin bond breakage can be described by Equation 5 [21,22].

$$T_m = [\frac{1}{T^\circ} - \frac{R}{\Delta H}\ x\ \ln(K_a\ x\ L) + \ 1]^{-1} \qquad \text{Equation 5}$$

where $T°$ is the transition temperature in the absence of biotin, R is the gas constant, ΔH is the enthalpy in the absence of biotin, K_a is the association constant, and L is the amount of total biotin in the solution.

- **Standard Deviation of Streptavidin-Biotin Bond T_m**: Because the T_m calculated is in actuality the point at which 50% of the streptavidin-biotin bonds are denatured, it represents the average temperature of denaturation. Therefore, in this model, T_m is entered as average ± 2 standard deviations and assumed a normal variation. Here, the standard deviation was held constant at 5°C and was chosen based on a review of a denaturation curve published in Gonzalez et al.[22]. The denaturation curve was examined to determine the range of temperatures where the streptavidin molecule denatured, releasing the biotin. The range was identified as the space between the points of inflection (10°C). The T_m of this curve was used as the average[22, 32-35].

As a result, the dynamic process takes into account the effect the reaction temperature has on the proportion of biotin-streptavidin bonds and the proportion of unbound streptavidin and biotin in solution. If the reaction temperature is greater than the T_m of the streptavidin-biotin bond, the hydrogen bonds will break

and melting will occur. It is expected that at this temperature any amplicons or template DNA bound to the beads will be ‘released’ and return to solution, which may cause deleterious effects during downstream re-amplification with new reagents [15,16]. If the reaction temperature is not greater than the melting temperature then no melting will occur, and all the amplicons will theoretically be segregated from solution.

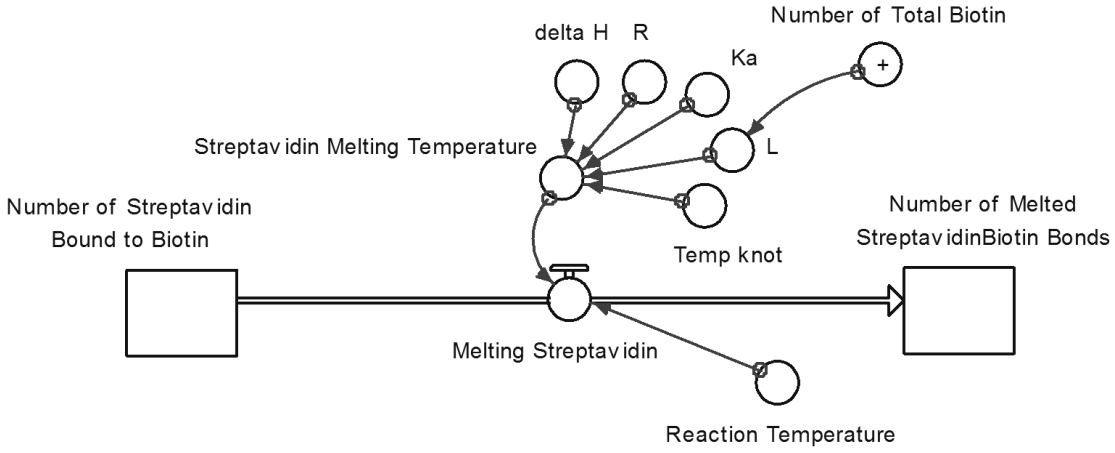
For the purposes of this study, only the TPOX primers and amplicons being used in the experimental section were modeled. The TPOX primers have an average melting point of 89ºC while the amplicons have an average melting point of approximately 117ºC and the streptavidin-biotin bond’s melting point is approximately 115ºC. The model is designed to allow for other melting temperatures to be calculated based on the variables listed in Table 3 and subsequently entered as the average for the melting equations.

2.3.2.2 Output Parameters

- ***Number of Melted Streptavidin-Biotin Bonds***: The number of streptavidin beads that are no longer bound to biotinylated oligonucleotides as the bonds have been ruptured.
- ***Number of Melted Biotinylated Primers Annealed to Template DNA*****:** The number of *biotinylated primers annealed to template DNA* that have separated leaving the template DNA free in solution.

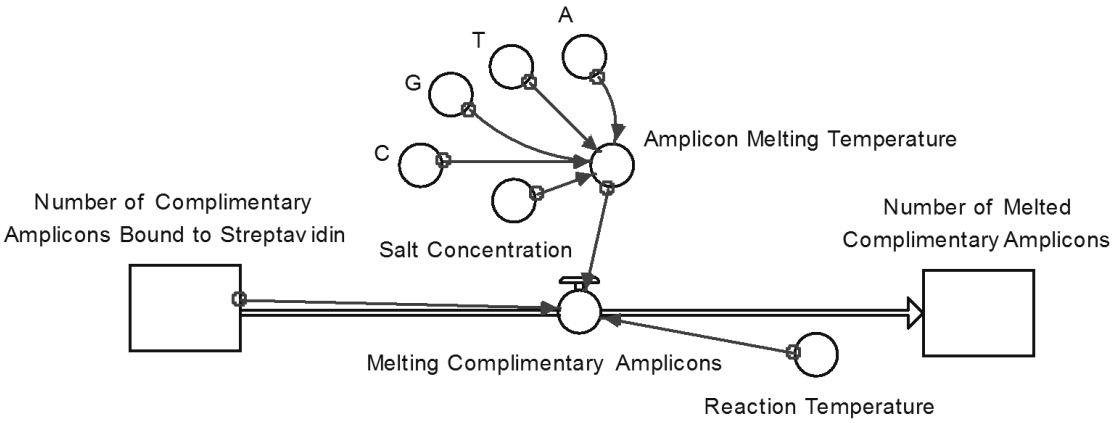
- ***Number of Melted Complimentary Amplicons***: The number of complimentary amplicons that have separated leaving the non-biotinylated strand free in solution.
- **Amount of Time for Melting to Occur**: The amount of time in minutes for complete melting of the oligonucleotides or the streptavidin-biotin bond. This value is then multiplied by 2 in order to calculate the total amount of time for melting to occur. This is done because T_m represents only 50% melting.

Figures 10-12 show the melting step of the model broken down by the type of molecule (i.e. streptavidin beads, *complimentary amplicons*, and *primers annealed to template DNA*). Below each figure is the corresponding condition for melting of the molecules to occur.



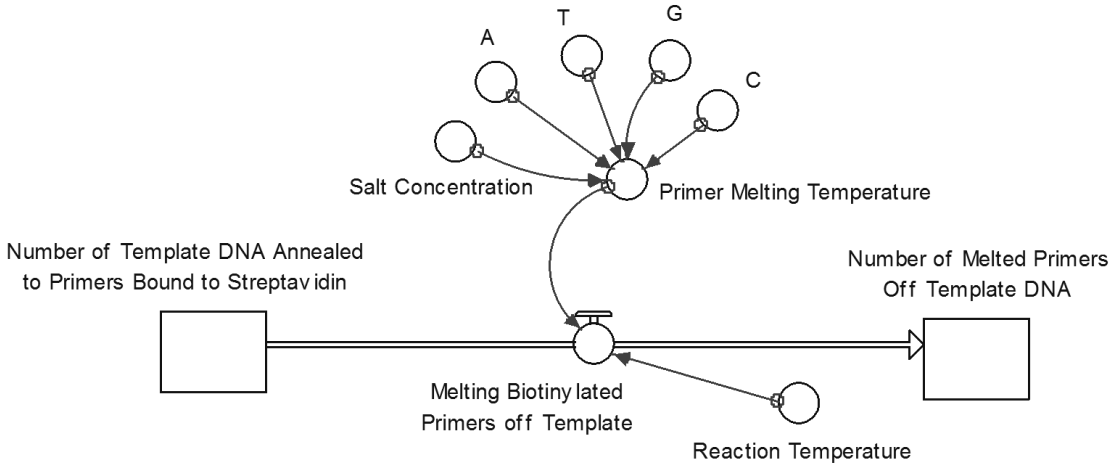
Condition; IF Reaction Temp > Streptavidin Melting Temp THEN Melting Occurs

Figure 10: Model of Streptavidin-Biotin Bond Melting.



Condition; IF Reaction Temp > Amplicon Melting Temp THEN Melting Occurs

Figure 11: Model of Complimentary Amplicons Melting.



Condition; IF Reaction Temp > Primer Melting Temp THEN Melting Occurs

Figure 12: Model of Primer Melting - Release of Template DNA.

2.4 *Amplification Optimization*

To begin development, the TPOX locus was chosen. The primers that were used for this locus were published previously. Working with one locus (TPOX) instead of a commercial kit, the amplification process needed to be optimized.

The template DNA used was the 9947a female standard[37]. Optimization was achieved by amplifying samples with varying magnesium chloride and primer concentrations, starting with the recommended concentrations for PowerPlex 16® kits (Life Technologies, Carlsbad CA) (Table 5). The cycling conditions used were 95ºC for 10 minutes followed by 32 cycles of 94ºC for 1 minute, 58ºC for 1 minute, and 72ºC for 1 minute followed by 90 minutes at 60ºC and held at 15ºC. The amplified products were electrophoresed on agarose gels and stained with GelStar® Nucleic Acid Gel Stain (Lonza Inc, Walkersville, MD). The gels were photographed on an ultra violet light box using a UV filter lens and the fluorescent setting on a Canon PowerShot A630 camera.

Table 5: Concentration of reagents used for PCR optimization with non-biotinylated primers

Sample Name:	**3-1**	**3-2**	**3-3**	**3-4**	**3-5**	**Neg**
dNTPs (µM)	200	200	200	200	200	200
$MgCl_2$ (mM)	2	1.75	2.25	2	2.25	2.25
Forward Primer (µM)	0.05	0.05	0.05	0.04	0.06	0.06
Reverse Primer (µM)	0.05	0.05	0.05	0.04	0.06	0.06
Template DNA (ng)	1	1	1	1	1	0
AmpliTaq Gold DNA Polymerase (U/µL)	0.025	0.025	0.025	0.025	0.025	0.025

The images were then analyzed using ImageJ - a public domain open source image processing software[38]. For this analysis, the gel image was imported into the software. The background was subtracted, creating an image with the gel bands distinctly visible on a black background. A plot was generated using the gel analysis function in ImageJ. The results are in the form of intensity (in pixels) versus location on the gel and can be used as a semi-quantitative method to

determine amplification success and base pair size[38]. This information was then exported and graphed for comparison between samples.

Once the optimal concentration was determined, the forward primer was functionalized with biotin on the 5' end. Using the now biotinylated primer, the samples were amplified using the same PCR component concentrations and conditions as previously described (Table 5). This set of samples also included two additional samples that were amplified with higher concentrations of primers and $MgCl_2$ than those in the first five samples (Table 6). The same analysis process was followed for these samples to determine the optimal concentration of the PCR components with biotin functionalization.

Table 6: Concentration of reagents used for PCR optimization with biotinylated primers

Sample Name:	**4-1**	**4-2**	**4-3**	**4-4**	**4-5**	**4-6**	**4-7**	**neg**
dNTPs (µM)	200	200	200	200	200	200	200	200
$MgCl_2$ (mM)	2	1.75	2.25	2	2.25	2.25	2.5	2.25
Forward Primer (µM)	0.05	0.05	0.05	0.04	0.06	0.06	0.07	0.06
Reverse Primer (µM)	0.05	0.05	0.05	0.04	0.06	0.06	0.07	0.06
Template DNA (ng)	1	1	1	1	1	1	1	0
AmpliTaq Gold DNA Polymerase (U/µL)	0.025	0.025	0.025	0.025	0.025	0.025	0.025	0.025

2.5. Post-PCR Clean Up Method Design

Figure 13 shows a schematic of the overall clean-up process starting with the amplification of the TPOX locus with the biotinylated primers. The TPOX amplicons are expected to be 216-264 bp in length. After amplification, a portion of the 'work product' is saved for analysis. The remainder of the 'work product' is cleaned with Dynabeads® M-270 Streptavidin and Amicon® Ultra-0.5 filtration.

The cleaned product is re-amplified using non-biotinylated D5S818 primers. D5S818 amplicons are expected to be 115-163 bp in length. The original amplified ‘work product’ and the re-amplified product are analyzed via agarose gel or capillary electrophoresis.

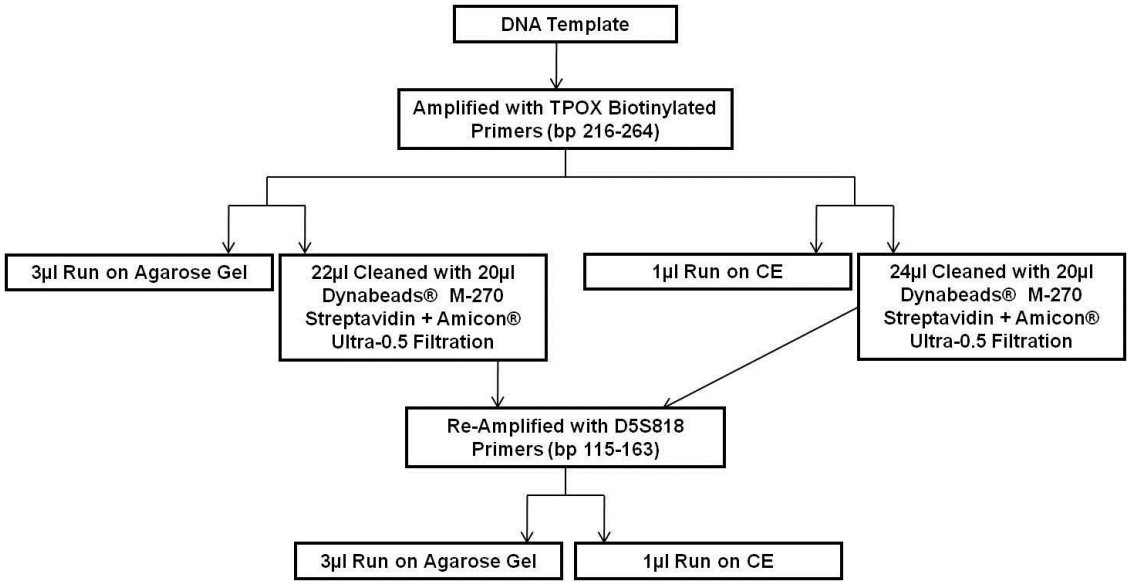


Figure 13: Schematic of Overall "Clean-up" Process

Using the optimal PCR component concentrations and conditions, samples were amplified with 2 ng of DNA. These samples were split in half (1 ng DNA each), and one half was cleaned with the Dynabeads® M-270 Streptavidin magnetic beads. For each sample (1 ng of DNA), 20 µl of beads at a concentration of $6x10^5$ beads/µL were used. This amount was selected based on the typical binding capacity for double stranded DNA reported in the Dynabeads® M-270 Streptavidin manual and the post-PCR clean-up process was conducted

as per the manufacturer's recommended protocol. The beads were washed using the recommended buffer. The amplified 'work product' was added to the beads and incubated at room temperature for 15 minutes on a rotating plate. The samples were then placed on a magnet to sequester the beads on the sides of the microcentrifuge tubes and the supernatant was transferred to a clean microcentrifuge tube. The buffer used consisted of 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, and 2 M NaCl[25].

The elution of the DNA was tested with 95% formamide at 90ºC for 2 minutes, 65ºC for 5 minutes, and 90ºC for 10 minutes[36]. In order to verify the effectiveness of the Dynabeads® M-270 Streptavidin method, the supernatant, and the eluent were prepared for electrophoresis on an agarose gel. If all of the amplicons are removed, the supernatant is expected to contain the original template DNA and no 216 - 264 bp amplicons. Some unincorporated primers and salts may also remain in the supernatant.

In order to remove the unincorporated primers and salts from the supernatant, Amicon® Ultra-0.5 (Millipore, Billerica MA) filtration was used. This purification was performed according to the manufacturers recommendations [39, 40, 44, 45] using TE buffer. TE buffer was added to the filter followed by the sample. The samples were spun at 14,000 rcf for 20 minutes and the eluent was discarded. TE buffer was added to the filter again and the samples were spun for 45-50 minutes at 14,000 rcf. The filter was then inverted into a clean collection tube and spun at 1,000 rcf for 2 minutes and the samples were collected.

2.6. Method Verification

Samples containing 2 ng of DNA were amplified using the optimal concentrations for the biotinylated primer and split in half (1 ng of DNA each). One set of samples was set aside while the second set was purified first with the Amicon® Ultra-0.5 filtration and then with Dynabeads® M-270 Streptavidin. After purification, the supernatants were re-amplified using the non-biotinylated TPOX forward primer at the optimal conditions determined previously (Table 5). These re-amplified samples were then electrophoresed along with the original amplification products that had been set aside.

Two samples were amplified using the biotinylated TPOX primers. These samples were cleaned using the Dynabeads® M-270 Streptavidin. A portion of each was set aside and the remainder cleaned/concentrated using Amicon® Ultra-0.5 filters. The supernatant from the Dynabeads® M-270 Streptavidin cleaning only and the concentrated sample after Amicon® Ultra-0.5 filtration were then run on a 7500 Real time PCR system (Life Technologies, Carlsbad CA) using the Quantifiler® *Duo* kit (Life Technologies, Carlsbad CA). This experiment illustrated the importance of changing the order of post-PCR cleaning methods.

Again, samples containing 2 ng of DNA were amplified using the optimal concentrations for the biotinylated primer and split in half (1 ng of DNA each). One set of samples was set aside while the second set was cleaned first with the Dynabeads® M-270 Streptavidin and then with the Amicon® Ultra-0.5 filtration. After cleaning, the supernatants were re-amplified using the non-biotinylated

forward TPOX primer. These re-amplified samples were then electrophoresed along with the original amplification products that had been set aside.

A second locus was chosen based on the amplicon size and chromosome location to allow for differentiation between the two amplicons via agarose gel electrophoresis. The TPOX amplicons range between 216 - 264 bp, so the second locus was chosen to have an amplicon size easily differentiated from TPOX amplicons on the agarose gel. Also, the second locus was chosen from a chromosome other than chromosome 2 where TPOX is found. This was performed in order to verify that the re-amplification signal originated from the genomic template DNA and not re-amplification of remaining amplicons. Based on these requirements, the D5S818 locus was chosen. This locus's amplicon size ranges from 115-163 bp and is located on chromosome 5. The primers for this locus were based on sequences previously published.

A new set of samples were amplified with TPOX biotinylated primers, cleaned with Dynabeads® M-270 Streptavidin, filtered with Amicon® Ultra-0.5 filters, and re-amplified using non-biotinylated D5S818 primers. These samples (D5S818) and the original amplified samples (TPOX) were electrophoresed. This gel was imaged and analyzed as described previously.

2.7. Capillary Electrophoresis

Capillary electrophoresis was performed with the 3130 Genetic Analyzer (Life Technologies, Carlsbad CA) for original amplification products of the TPOX, D5S818, and a duplex of the two STR regions. The results were analyzed using

GeneMarker® HID (Softgenetics, State College PA) software. A ladder was created in the software based on the theoretical sizes of the amplicons[40]. Samples that were successfully re-amplified on the agarose gel were also analyzed via capillary electrophoresis for comparison.

3. Results and Discussion

3.1. Part I – Model

3.1.1. Binding Step of Model

Figure 14 shows the number of unbound molecules versus the input number of streptavidin beads when the input mass of template DNA for PCR is constant at 1 ng. It shows that as the number of streptavidin beads in solution is increased, less unbound biotinylated oligonucleotides remain. This is expected since an increase in the number of streptavidin beads means an increase in the number of binding locations for biotin and therefore less biotin will remain unbound in solution. When the input number of streptavidin beads reaches $1.1x10^5$ beads, no unbound biotinylated oligonucleotides remain, meaning that all of the biotinylated oligonucleotides are bound to streptavidin beads. This is consistent with the recommended number of streptavidin beads used for the experimental portion of this work, which was $1.2x10^7$ beads. These results suggest that this number of streptavidin coated beads used is sufficient to remove all of the amplicons from the solution which is the goal of this post-PCR step.

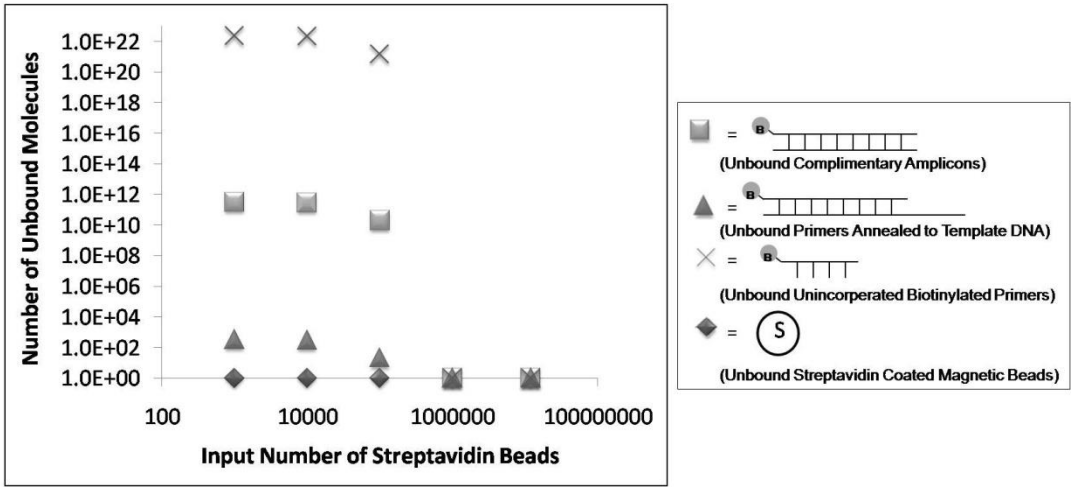


Figure 14: Graph of the Results from the Streptavidin-Biotin Model when the Input Number of Streptavidin Coated Beads is Varied.

Figure 15 shows the number of unbound molecules versus the input mass of template DNA (ng) to PCR amplification when the input number of streptavidin beads is kept at $1x10^5$. It shows that as the input mass of template DNA is increased, the number of *unbound unincorporated biotinylated primers* does not vary. This is because the number of biotinylated primers added to the reaction is kept constant and is in excess, so the number of *biotinylated unincorporated primers* is too large for minimal variation to be visible. In contrast, the number *of unbound complimentary amplicons* and *unbound biotinylated primers annealed to template DNA* can be seen to increase along with the increasing input mass of template DNA. This is also expected because when the input mass of template DNA for PCR is increased, more amplicons are present in the amplified 'work

product'. There is also an increased number of template DNA strands for biotinylated primers to bind.

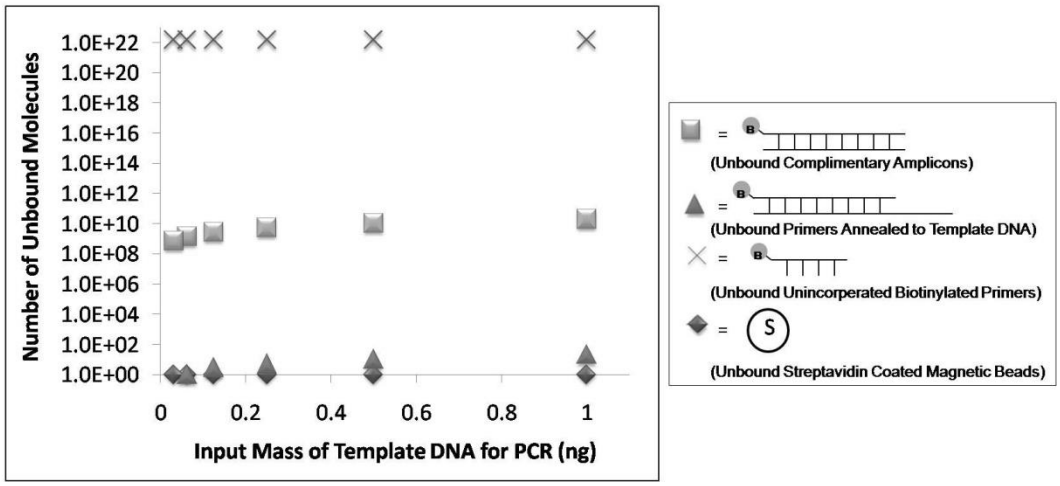


Figure 15: Graph of the Results from the Streptavidin-Biotin Model when the Input Mass of Template DNA for PCR is Varied.

These results show that when the input mass of template DNA for PCR is decreased, less streptavidin coated beads are needed because less biotinylated oligonucleotides are present in solution. This would decrease the cost of the method. However, there is an excess of *unincorporated biotinylated primers* remaining which may compete for binding to the streptavidin coated beads. The model does not take this into consideration; therefore, experimentation would need to be performed to determine if decreasing the amount of streptavidin coated beads with decreases in input mass of template DNA would be viable.

3.1.2. *Melting Step of Model*

Figure 16 shows the number of melted molecules versus the reaction temperature. The purpose of these results is to identify a reaction temperature where only the *biotinylated primers annealed to template DNA* melt (i.e. the primers and template DNA separate). From Figure 16, it can be seen that a reaction temperature between 85°C and 95°C would melt only the *biotinylated primers annealed to template DNA* leaving both the complimentary amplicons and the streptavidin-biotin bond intact. A temperature below this range would not melt anything in solution. A temperature above this range would melt the *complimentary amplicons* and the streptavidin-biotin bond as well.

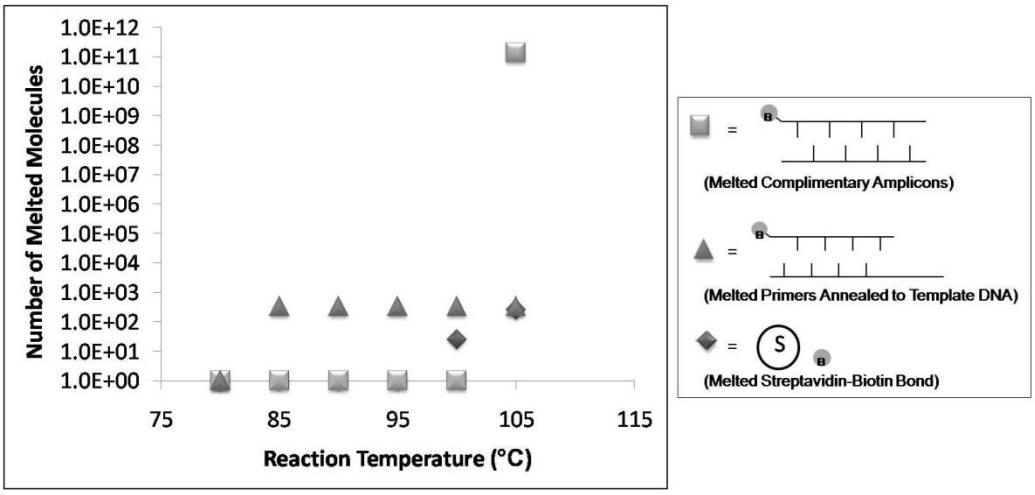


Figure 16: Results from the Melting Step of the Streptavidin-Biotin Model where Reaction Temperature is Varied.

Another aspect to consider is the amount of time to incubate the samples at the chosen reaction temperature. Figure 17 shows the approximate amount of

time for complete melting of the *biotinylated primers annealed to template DNA* to occur versus the reaction temperature. As a result, the incubation time could range from 32 minutes for 85°C to ~19 minutes for 95°C. Based on these results, the most efficient reaction temperature is 95°C because the reaction incubation time is the shortest at ~19 minutes and only the *biotinylated primers annealed to template DNA* will melt. Using the results from Figures 16 and 17 as a guide, optimized reaction temperatures and incubation times can be identified experimentally.

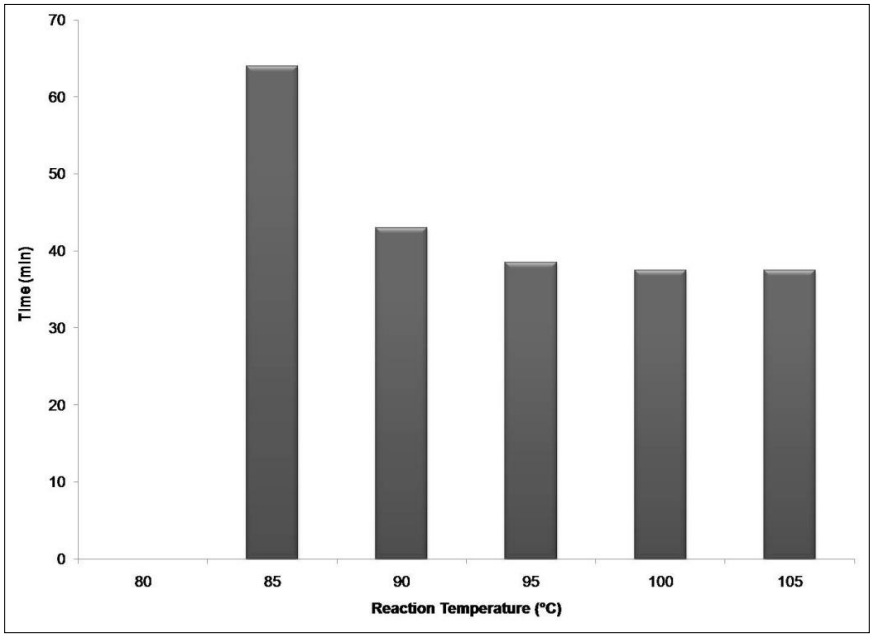


Figure 17: Time for Complete Melting of *Biotinylated Primers Annealed to Template DNA from* the Melting Step of the Streptavidin-Biotin Model where Reaction Temperature is Varied.

In summary, the dynamic model predicts that an experimental protocol which utilizes 0.04 µM primers, 1 ng template DNA, and 12,000,000 streptavidin coated beads would result in complete binding of all biotin to streptavidin coated

beads. The following section shows the experimental results obtained when the aforementioned conditions were applied.

3.2. Part II - Experimental

3.2.1. Amplification Optimization

Figure 18 shows the signal of five samples, amplified with TPOX primers using concentrations of PCR components listed in Table 5. All five samples showed positive results for the presence of TPOX amplicons. ImageJ image processing was performed on this gel and the outputs graphed (Figure 18). Sample 3-5, which contained 2.25 mM $MgCl_2$ and 0.06 µM primers (Table 5), resulted in the highest number of pixels. As a result, these concentrations of $MgCl_2$ and primers were used henceforth.

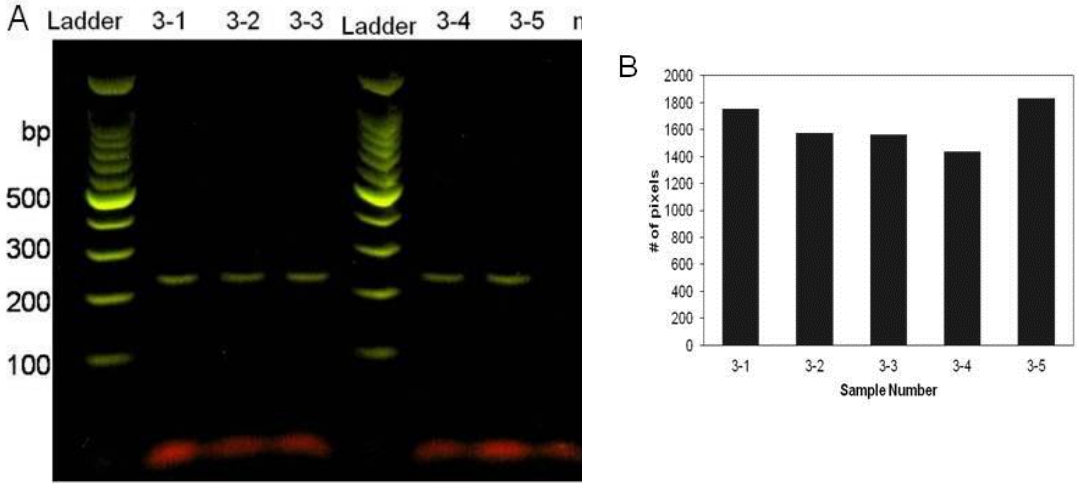


Figure 18: Amplification Optimization with Non-biotinylated Primers A) Image of gel with non-biotinylated primers varying the MgCl2 and primer concentrations. B) A representation of the results generated by ImageJ.

Figure 19 shows seven amplicons after agarose gel electrophoresis using biotinylated TPOX primers. All seven samples showed positive results for the presence of TPOX amplicons. ImageJ image processing was performed on this gel and the outputs graphed (Figure 19B). Sample 4-4, which contained 2 mM $MgCl_2$ and 0.04 µM primers (Table 6), resulted in the highest number of pixels. As a result these concentrations of $MgCl_2$ and primers were used. These concentrations are both lower than those identified when the primers were not functionalized with biotin.

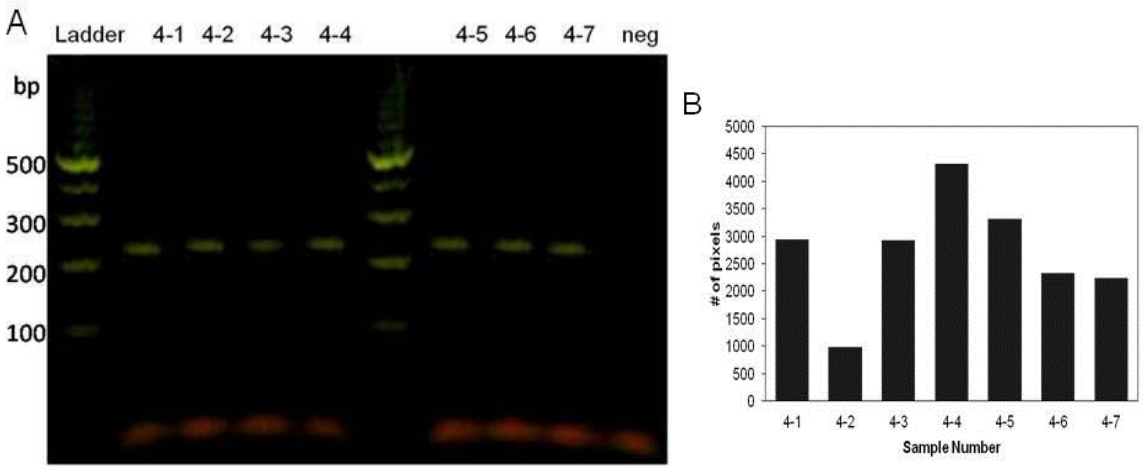


Figure 19: Amplification Optimization with Biotinylated Primers A) Image of gel with biotinylated primer varying the MgCl2 and primer concentrations. B) A representation of the results generated by ImageJ. Sample 4-4 contains the most optimal reagent concentrations.

A decrease in $MgCl_2$ concentration is expected when there is a decrease in primer concentration due to the stabilizing effect of Mg^{2+} on the newly forming amplicons. It has been shown that too much Mg^{2+} can cause a decrease in the

fidelity of the reaction by stabilizing mismatched amplicons[41,42]. If there is a decrease in primer concentration, fewer amplicons are generated at any one time; therefore, there is more Mg^{2+} in the solution to stabilize the mismatched amplicons. Decreasing the Mg^{2+} concentration along with the decrease in primer concentration controls for this effect, keeping the amount of free Mg^{2+} consistent with the non-biotinylated primer reaction.

3.2.2. Post-PCR Purification Method Design

After cleaning the amplified work product with Amicon® Ultra-0.5 filters followed by Dynabeads® M-270 Streptavidin, the remaining supernatant and the eluent were electrophoresed on an agarose gel (Figure 20). The original samples were positive for TPOX amplicons. The supernatant showed no discernible signal in the TPOX region (216 - 264 base pairs). However, the eluents were positive for TPOX amplicons when elution was performed at 90ºC for 10 minutes in 95% formamide. Two other temperature and time combinations were tested but neither showed positive elution of the TPOX amplicons. The first was 90°C for 2 minutes and the second was 65°C for 5 minutes, both in 95% formamide.

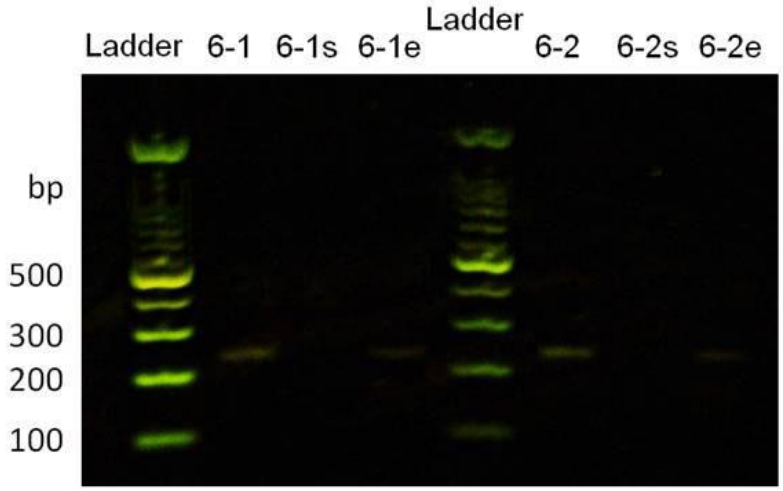


Figure 20: Dynabeads® M-270 Streptavidin Cleaning Stages. The image of the gel after amplification with biotinylated primers (Samples 6-1 and 6-2) and the supernatant (Samples 6-1s and 6-2s) and eluent (Samples 6-1e and 6-2e) of the same samples after cleaning with Dynabeads® M-270 Streptavidin beads.

These samples and two others were then re-amplified using TPOX non-biotinylated primers (Figure 21). All of the original amplifications were positive for TPOX amplicons and showed bands in the correct location (200-300 bp region). All of the re-amplified samples showed no bands indicating that there is not enough original template DNA remaining in the supernatant to allow for amplification. Since no bands remained from the original amplification, it can be inferred that the clean-up method is sufficient to remove the original amplified products.

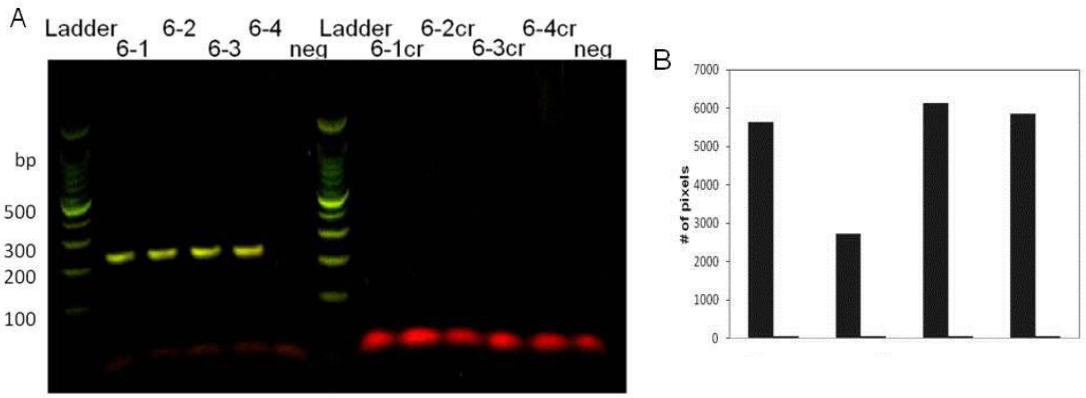


Figure 21: Re-amplification After Dynabeads® M-270 Streptavidin. A) The image of the gel after amplification with biotinylated primers and the same samples after cleaning with Streptavidin coated magnetic beads and re-amplification. B) The graph is a representation of the results in number of pixels generated by ImageJ. (■)Signal from original amplification product. (■)Signal from Amicon/Biotin/ Streptavidin cleaning procedure with re-amplification using TPOX locus.

Because only a small portion of the supernatant is added to the re-amplification reaction, the clean up steps were re-ordered so the filtration step followed the streptavidin coated bead cleaning. This was expected to lead to the entire sample being added to the re-amplification reaction. A second reason the order of the cleaning methods was reversed is based on qPCR results that showed inhibition of the supernatant.

Figure 22 shows two samples cleaned using only Dynabeads® M-270 Streptavidin. As the cycle number increases, the fluorescent signal of the Internal PCR Control (IPC) is expected to increase at an exponential rate, and reach a ΔRn value of 0.2 at the same cycle number for each reaction. If the reaction is inhibited, the cycle number at which the fluorescent signal crosses 0.2 ΔRn will

increase[31]. When the supernatant from these samples was amplified using qPCR, both showed no amplification of the sample or IPC. This is most likely due to inhibition from a leftover component of the Dynabeads® M-270 Streptavidin cleaning process. Specifically, the buffer of the streptavidin-biotin binding contains 1 mM EDTA - a chelator. A chelator is a compound that binds metal such as magnesium. This is expected to be detrimental to post-PCR cleaning processes since magnesium is a cofactor for Taq polymerase, meaning that it is necessary that magnesium be free in solution for amplification to occur[43].

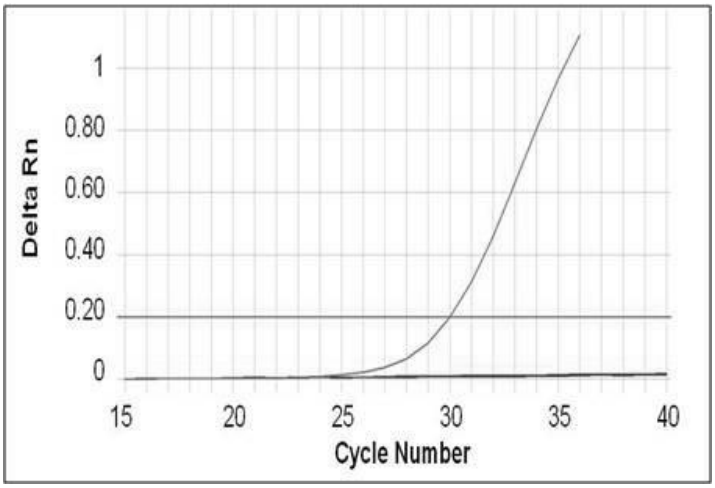


Figure 22: qPCR Amplification Plot with Dynabeads® M-270 Streptavidin Cleaning Only. (-) IPC from Quantification Negative and the IPCs from (-) Sample 1 and (-) Sample 2 after cleaning with Streptavidin coated magnetic beads, indicating significant inhibition of amplification.

An additional amplification where the samples were amplified with TPOX biotinylated primers, cleaned with Dynabeads® M-270 Streptavidin, then cleaned with Amicon® Ultra-0.5 filters, and re-amplified using non-biotinylated TPOX primers was completed. The re-amplification using the non-biotinylated TPOX

primers showed positive results for the original (TPOX amplicons) and re-amplified (TPOX amplicons) samples (Figure 23).

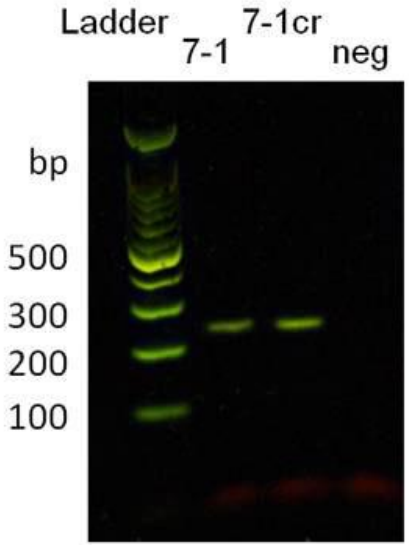


Figure 23: Re-amplification After Dynabeads® M-270 Streptavidin and Amicon® Ultra-0.5 Cleaning. Image of gel after amplification with biotinylated primers (7-1) and the same sample after cleaning with Streptavidin coated magnetic beads and re-amplification with TPOX non-biotinylated primers (7-1cr).

The same samples from Figure 22 were then cleaned using Amicon® Ultra-0.5 filters and run using qPCR again (Figure 24). Both the samples and the IPCs amplified. This result, as well as the re-amplification succeeding (Figure 23) suggests that the inhibitors are removed by the filtration method and the original template DNA is retained for re-amplification.

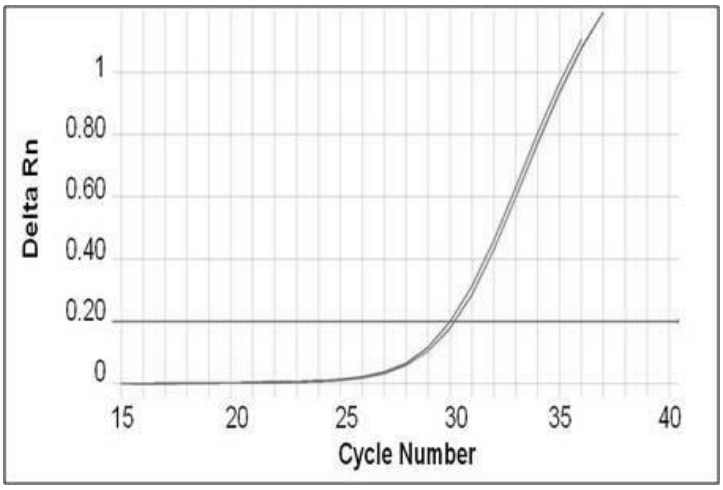


Figure 24: qPCR Amplification Plot. (-) IPC from Quantification Negative and the IPCs from (-) from Sample 3 and replicate (-) Sample 4 after cleaning with Streptavidin coated magnetic beads and centrifugal filters, indicating an additional post-PCR clean up is required to negate inhibition.

3.3. Method Verification

To verify that the positive re-amplification results were not caused by the amplification of remaining TPOX amplicons, a new sample set was amplified with TPOX biotinylated primers, cleaned with Dynabeads® M-270 Streptavidin followed by Amicon® Ultra-0.5 filters and re-amplified using non-biotinylated D5S818 primers. All of the original TPOX amplifications showed positive results for TPOX amplicons. The samples that were re-amplified using the D5S818 non-biotinylated primers also showed positive results for D5S818 amplicons in all samples (Figure 25).

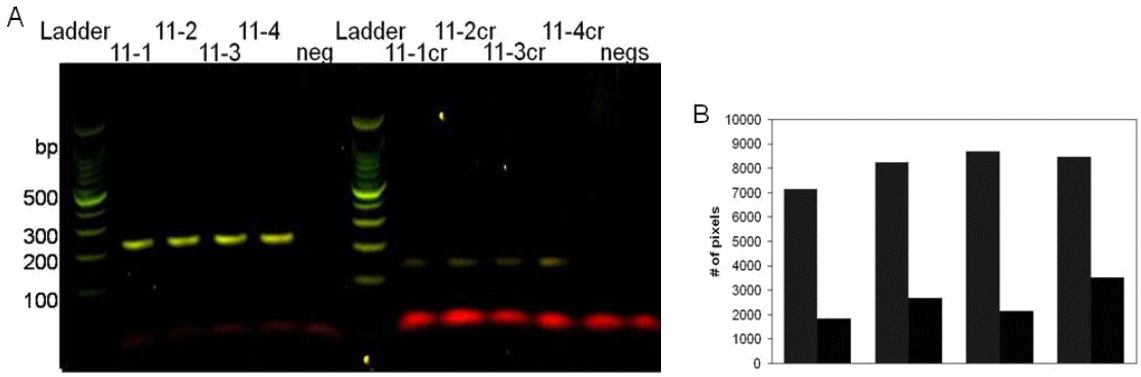


Figure 25: Re-amplification After Dynabeads® M-270 Streptavidin and Amicon® Ultra-0.5 Cleaning. A) Image of gel after amplification with biotinylated primers and the same samples after cleaning with Streptavidin coated magnetic beads and centrifugal filters and re-amplification. B) The graph is a representation of the results generated by ImageJ. (■) Signal from original amplification product. (■) Signal from Amicon/Biotin/Streptavidin cleaning procedure with re-amplification using TPOX locus.

Table 7: Table of DNA recovered based on 1ng original input.

Sample #	% Recovery	DNA Input Mass (ng)	Approximate Mass of DNA Recovered (ng)
11-1	25.6	1	0.256
11-2	32.6	1	0.326
11-3	24.7	1	0.247
11-4	41.6	1	0.416
Average	31.1	1	0.311

On this gel, it is also possible to see that the resultant bands from the D5S818 primers were in the correct size range (115-163 bp) compared to the TPOX amplicons (216-264 bp). The results from this gel were analyzed via ImageJ and the amount of original template DNA recovered was approximated (Figure 25B & Table 7). If the product is taken as a direct indication of the mass of original template then the average amount of original template DNA recovered was

approximately 0.31 ng ± 0.16 ng, which represents ~31% ± 16% of the original 1 ng input. While this can be extremely useful in cases where there would be no original template DNA remaining, a loss of almost 70% of DNA input mass may be considered too high for forensic purposes. The determination of the origin of the loss is of importance since the post-PCR process may need to be modified to ensure optimal recovery. Previous studies suggest some loss occurs during the post streptavidin filtering process whereby some DNA may adhere to the filter membranes. However, this loss is expected to be less than 10%[39,44].

In an effort to identify the amount of loss occurring from the Amicon® Ultra-0.5 filters, two samples were electrophoresed on a gel pre-and post-filtration. All of these samples showed bands for TPOX amplicons (Figure 26). The results were analyzed via ImageJ and the proportion of amplicons recovered was calculated to be approximately 96%. By assuming the original template DNA behaves in the same manner as the amplicons, an approximate loss of 4% ± 5% of the DNA from the Amicon® Ultra-0.5 filtration is the result, suggesting the majority of the loss associated with the clean-up method may be occurring during the streptavidin coated bead step.

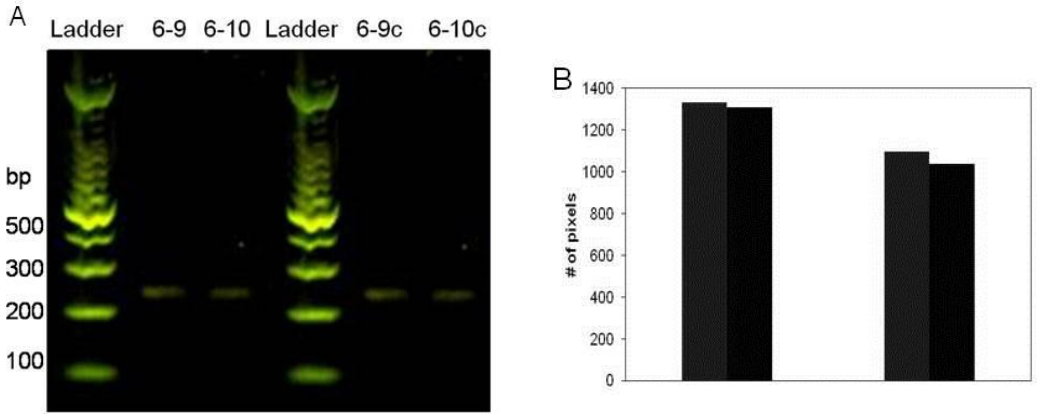


Figure 26: Amicon® Ultra-0.5 Filtration Testing. A) Image of the gel after amplification with TPOX biotinylated primers and the same samples after cleaning with centrifugal filters only. B) The graph is a representation of the results generated by ImageJ. (■) Signal from original amplification product. (■) Signal from Amicon/Biotin/Streptavidin cleaning procedure with re-amplification using TPOX locus.

One cause of this loss could be the binding of biotinylated primers to the original template DNA. The biotinylated primers are then bound to the streptavidin coated beads and removed from the solution taking the original template DNA along. The second step of the streptavidin-biotin dynamic model deals with one possible solution to this problem - to denature the primers after streptavidin coated bead binding in order to release the original template DNA without releasing any amplicons. Further research can be done using the information from the model to perform experiments to verify the effectiveness of this solution.

3.4. Capillary Electrophoresis

Capillary electrophoresis (CE) is commonly used in forensic STR typing. For this reason, the now verified method was tested using capillary electrophoresis

for analysis. The biotinylated primers for both TPOX and D5S818 and a duplex of the two primers were used to amplify the samples. These samples were then analyzed using CE. These samples containing the original amplified products showed peaks at 216 - 264 bp or 115-163 bp as expected (Figure 27). The correct number of peaks was also observed. The 9947a sample is homozygous at both the TPOX and D5S818 for alleles 8 and 11 respectively. The duplex also showed sufficient peak height balance between the two STR loci. These results indicate that the biotinylation does not affect the analysis of the amplicons via capillary electrophoresis, suggesting that the original amplification product can be analyzed for forensic or human identification purposes without the need to change analysis methods. Because the original amplicons can be recovered from the streptavidin coated beads, this method can be used to analyze the results of each amplification or re-amplification using the same analysis methods and parameters, which could be important for forensic case work when comparing evidence samples to standards as the standards will generally not need re-amplification.

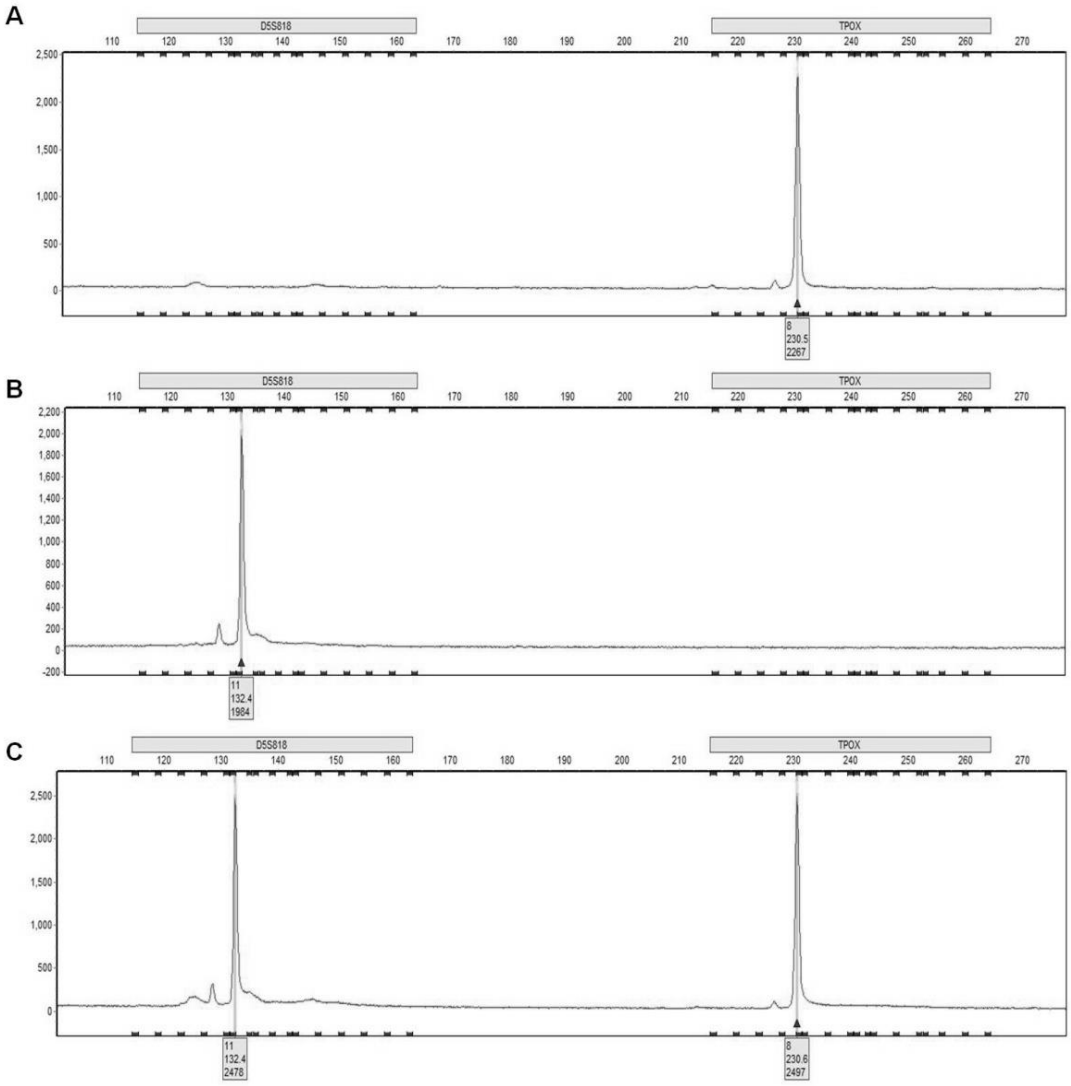


Figure 27: Electropherograms of Biotinylated Primer Amplicons.
A) TPOX primers only. B) D5S818 primers only. C) Duplex using both TPOX and D5S818 primers.

The same samples run on the agarose gel (Figure 25) were analyzed using capillary electrophoresis to compare to the gel results (Figure 28). These results showed that the original TPOX amplicons were greatly reduced from ~2,000 RFU to ~400 RFU but not completely removed from the solution. During re-

amplification the electropherogram shows that the D5S818 locus amplified and was visible in the expected base pair region of 115-163 bps. The D5S818 locus exhibited RFU values of ~4,800 RFU.

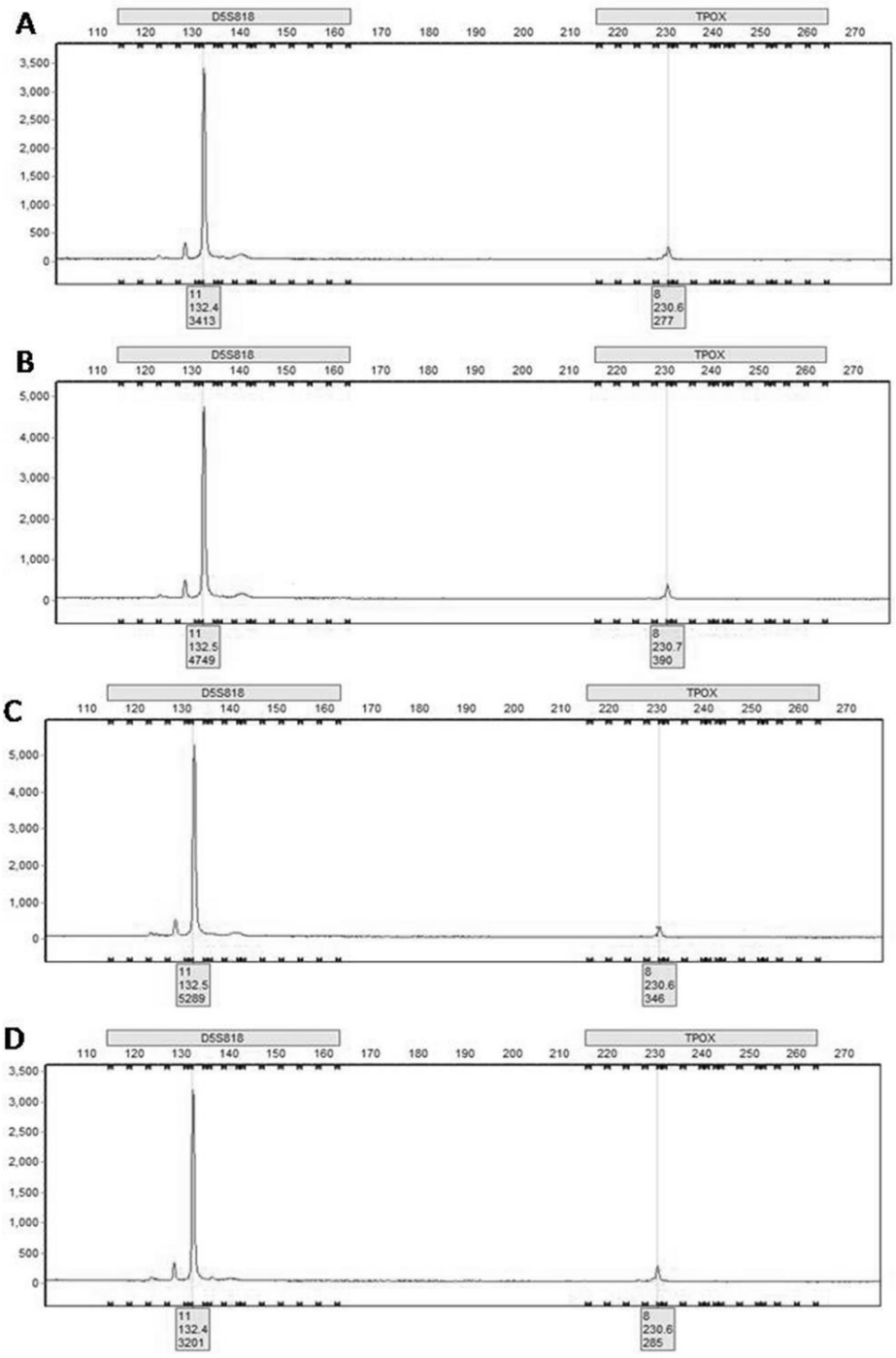


Figure 28: Electropherograms of Re-amplification After Dynabeads® M-270 Streptavidin and Amicon® Ultra-0.5 Cleaning. Electropherograms for amplification products using biotinylated TPOX primers and re-amplified using D5S818 non-biotinylated primers.

The D5S818 peaks had significantly higher RFUs than the peaks corresponding to the remaining TPOX amplicons (~3,890 and ~350 RFU respectively). From these results, it does not appear that a significant amount of TPOX primers remain after the cleaning method. This can be inferred from the very small RFU values of the TPOX peaks (352 ± 99 RFU) and the lack of any amplicons seen in the gel. If a large number of primers were remaining, the TPOX locus would be re-amplifying along with the D5S818 locus, which would result in a larger TPOX peak than is seen in Figure 28.

4. Future Studies

4.1. Part I - Model

The streptavidin-biotin model can be expanded in the future to incorporate multiple primers for multiplexing reactions. This would mean that multiple melting temperatures (one for each primer and amplicon) can be added into the model making the model even more beneficial since the more locations amplified, the more variables that need to be taken into account. By adding these additional layers to the model, the most efficient input number of streptavidin beads and the melting temperature range can be generated.

Another area for expansion of the streptavidin-biotin model would be to include the loss of DNA template incurred at each stage. This would make the output numbers more accurate to what was seen experimentally. The information for this would need to be determined via more experimentation before being incorporated into the model. It is also important to note that either the loss rate

would need to vary based on the variables in the reaction or, if the loss rate is kept constant, it would need to be listed as an assumption for the model.

A second dynamic model could be constructed in order to answer the question of whether the *primers annealed to template DNA* actually cause the expected template DNA loss. The melting step of the current streptavidin-biotin model could then be incorporated into the new model to determine if this step is beneficial in decreasing the loss of the original template DNA assuming primer binding is found to be the cause.

4.2. Part II - Experimental

One experimental area of future study would be to decrease the loss incurred through the cleaning method. This can be attempted by using heat to denature the primers from the original template DNA. To test this, the model can be used to generate temperature and incubation times to begin experimental testing. The denatured samples can be added to the supernatant for filtration and re-amplification. The re-amplified products can be compared to the original samples using capillary electrophoresis or qPCR to determine if more template DNA is recovered.

Another area for future study would be to attempt to decrease the remaining original amplification peak. A few methods can be attempted for this purpose. One method would be to increase the number of streptavidin coated beads used to bind the biotinylated complimentary amplicons. It is unlikely that this will have a significant effect as the number of streptavidin coated beads currently used is

sufficient according to the streptavidin-biotin model. The second method would be to perform the streptavidin bead cleaning step twice. This will allow more streptavidin-biotin interaction increasing the likelihood that binding will occur for any remaining biotinylated complimentary amplicons.

A third future study would be to determine the limit of detection based on the initial input mass of DNA for PCR. This can be accomplished by creating a dilution series ranging from the ideal 1 ng input to low template inputs such as 32 pg. These dilutions can be amplified with TPOX primers, cleaned using the optimized method, and re-amplified using D5S818 primers. From these results an initial input limit of detection can be determined. This could also be tested when changing the primer sets or using a multiplex reaction.

It will also be beneficial to determine the number of times this method can be applied to one sample. A situation when this can be useful is if the initial amplification is performed with a standard STR kit, but it is seen that there is degradation, so the sample is cleaned and amplified using a mini STR kit. The sample may then need to be amplified again if information from a Y STR kit is desired. The number of times one sample can be amplified and cleaned is based on the amount of loss incurred. By taking one sample at an initial input of 1 ng and performing this method repeatedly, the point at which there is too much loss to generate reliable results from re-amplification can be determined. At each stage, before re-amplification, a portion of the sample would need to be removed and tested via qPCR. These results can be used to determine the number of

times the method can be used on a 1 ng sample. The same process could also be repeated for other initial inputs of template DNA.

5. Conclusions

The streptavidin-biotin dynamic model shows that the amount of streptavidin beads added to the reaction is sufficient to bind the ~0.04 µM biotin present in the solution. For the TPOX locus, a reaction temperature of 95°C for ~19 minutes maximizes the primer-template melting while minimizing the amplicon melting and minimizing the reaction time. This temperature would ensure the highest recovery of the original template DNA while removing all amplicons from the solution. The results from the streptavidin-biotin model do not correlate exactly to the experimental data. In the experimental data some TPOX amplicons remain; however, the streptavidin-biotin model shows all amplicons bind to the streptavidin coated beads. This suggests that the streptavidin-biotin model could be used to predict initial optimal numbers of streptavidin coated beads, reaction temperature, and incubation time for this method.

Further, a novel method which allows for re-amplification of DNA that has already been amplified and is now a constituent of the amplified ‘work product’ was developed in the laboratory. This method could be utilized with, and provide additional processing options for exhaustive samples that may benefit from multiple amplifications (i.e. traditional- and Y-STR) and have traditionally been deemed of sufficient quantity/quality for only one amplification (i.e. DNA yields <

1 ng) or where there was a technical issue with the amplification (i.e. contamination of negative control or thermal cycler deficiency).

The optimal PCR component concentrations for amplification with non-biotinylated primers were not the same for the biotinylated primers, suggesting optimization of amplification conditions with biotinylated primers may be required prior to use. Additionally, centrifugal filtration is necessary to prevent significant levels of PCR inhibition. Hence additional processing, following streptavidin-biotin binding, is necessary. Centrifugal filtration such as Amicon® Ultra-0.5 may be used in this capacity to remove remaining excess primers, salts, and additional inhibitors that may be present in the supernatant.

Results indicate that when the TPOX locus was amplified, signal at ~230 bases was observed. After method clean-up the D5S818 locus (~130 bases) was amplified and electrophoresed on an agarose gel. These lanes showed no TPOX bands suggesting that the original genomic DNA remains in the supernatant for future DNA processing.

Further, there was no indication of remaining TPOX amplicons from the original amplification demonstrating that clean-up was sufficient and that the streptavidin-biotin post-PCR clean-up process was sufficient and ensured amplicons were not present to complicate down-stream amplifications. However, there was a significant loss of genomic DNA incurred during the clean-up process. As a result, re-amplification may be able to be accomplished only a finite number of times.

Capillary electrophoresis results were similar to the results from the agarose gel electrophoresis. These results did show that some TPOX amplicons from the original amplification remained, indicating the need for optimization to bind all of the amplicons. Both the original amplification peaks and the re-amplification peaks were seen at the correct base pairs. This suggests that the biotin does not affect the analysis of the STRs via capillary electrophoresis, and that biotin-streptavidin interactions result in a viable post-PCR purification method that could allow for re-amplification of the genomic DNA template.

LIST OF JOURNAL ABREVIATIONS

Am J Hum Genet	American Journal of Human Genetics
Anal. Chem.	Analytical Chemistry
BMC Biotechnology	BioMed Central Biotechnology
Forensic Sci. Int.	Forensic Science International
Int.Congr.Ser.	International Congress Series
Int.J.Food Microbio	International Journal of Food Microbiology
J. Forensic Sci.	Journal of Forensic Science
Mol. Diagn.	Molecular Diagnosis
Plant Mol. Bio.	Plant Molecular Biology

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Vita

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EDUCATION

Boston University Aug 2010 – Sept 2012

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

Thesis: Streptavidin-Biotin binding of DNA Amplicons for the Typing and Re-typing of Forensically Relevant Short Tandem Repeats

University of Connecticut Aug 2006 – May 2010

Degree Earned: B.S. Pathobiology
Minors: Molecular and Cellular Biology; Psychology

RELEVANT WORK HISTORY

Intern - OpenArray R&D Life Technologies, Beverly, MA
May 2012-August 2012

- Development of novel applications involved in qPCR and Taqman chemistry on nanofluidic platform, Data analysis, Comparing the performance of different qPCR platforms

DNA Lab Assistant BU Biomedical Forensic Sciences Department, Boston, MA
January 2011-May 2012

- Instrument Calibrations, Laboratory/Instrument Cleaning, Solution Preparation, Inventory

Undergraduate Lab Assistant UConn Psychology Department, Storrs, CT
January 2009-May 2010

- Operant Conditioning, Slide Staining and Preparation, Animal Care and Handling