Identification and Separation of Same Gender Mixtures of Various Cell Types Using Interphase FISH Techniques and Laser Microdissection

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Abstract

The generation of clean, single source genetic profiles from sexual assault and touch evidence cellular mixtures is an enduring challenge in the field of forensics. Evidence of this nature can contain low copy amounts of DNA from mixtures of cell types of various morphologies. The goal of this research was to improve the methods of DNA mixture resolution in the forensic laboratory by improving laser microdissection (LM) techniques. Laser microdissection (LM) has proven to be an effective method for cell mixture separations in the forensic laboratory. While sperm and epithelial cell sexual assault mixtures can easily be separated based upon morphological differences, mixtures of the same cell type are more difficult to separate. The key objective of these studies was to separate cellular mixtures of similar morphology and same gender by using sequence specific fluorescence *in situ* hybridization (FISH) probes, which are based on the genetic polymorphisms associated with the Duffy and ABO blood groups. The Duffy and ABO blood grouping systems were chosen as the basis for these assays because of the existence of their prevalent polymorphisms throughout multiple populations. Each system has several alleles that can be targeted through a multicolor probe assay. The genetic sequences associated with the variations of each system are conserved and manageable in length. Thirty to three hundred base pair long FISH probes were designed to detect the single nucleotide polymorphisms observed between individuals of blood type A and blood type B. Tyramide signal amplification (TSA) was utilized to increase the sensitivity of FISH when detecting the short FISH probes. Before TSA-FISH techniques were employed, it was necessary to optimize individual steps for standard FISH detection as TSA cannot compensate for poor signals resulting

from suboptimal pretreatment and hybridization conditions. For this research, epithelial cells from donors with blood groups A and B were used to examine the feasibility of TSA-FISH combined with LM as a method for separating cellular mixtures of similar morphology and same gender. Findings indicated that the FISH techniques utilized in this research were unsuitable for differentiation of the ABO blood groups; however, this could be achieved in the future pending research of other FISH methods or by pursuing other genetic marker systems that consist of larger genetic differences. The completed results will be disseminated through the law enforcement and scientific communities via seminars, journal articles, and poster presentations.

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Executive Summary

The generation of clean, single source genetic profiles from sexual assault and touch evidence cellular mixtures is a consistent and difficult challenge in the field of forensics. Evidence of this nature can contain low copy amounts of DNA from mixtures of cell types of various morphologies. The goal of this research was to improve the methods of DNA mixture resolution in the forensic laboratory by improving laser microdissection (LM) techniques. Laser microdissection (LM) has proven to be an effective method for cell mixture separations in the forensic laboratory. While sperm and epithelial cell sexual assault mixtures can easily be separated based upon morphological differences, mixtures of the same cell type are more difficult to separate. The key objective of this study was to separate cellular mixtures of similar morphology and same gender using sequence specific fluorescence *in situ* hybridization (FISH) probes, which are based upon the genetic polymorphisms associated with the Duffy and ABO blood groups. The Duffy and ABO blood grouping systems were chosen as the basis for these assays because of the existence of their prevalent polymorphisms throughout multiple populations. Each system has several alleles that can be targeted through a multicolor probe assay. The genetic sequences associated with the variations of each system are conserved and manageable in length. To accomplish the goal outlined above, the research performed can be divided into five distinct sections:

- Optimization of standard FISH protocol to ensure that signal amplification techniques are not hindered by suboptimal pretreatment or hybridization conditions
- Bioinformatic analysis of the Duffy and ABO markers

- ABO probe design
- Biotin labeling of DNA probes
- Tyramide signal amplification (TSA) FISH detection of ABO probes

Optimization of Standard FISH Protocol

Before TSA-FISH techniques were employed, it was necessary to optimize individual steps for standard FISH detection. Although TSA is a powerful technique, it cannot compensate for poor signals resulting from suboptimal pretreatment and hybridization conditions. Multiple areas of the standard FISH protocol were examined and optimized prior to performing any TSA-FISH experiments.

Membrane Slide Evaluations

Membrane slides are glass slides covered with a thin membrane that prevents the cells from coming into direct contact with the glass slide. This biochemically inert membrane enables the area surrounding a cell of interest to be cut with the high energy UV laser of the PALM® Microbeam prior to catapulting the cell into a collection vessel. Membrane slides enable the user to decrease the energy levels and focal settings of the laser to the lowest but most effective level in order to limit the damage that might be caused to a DNA sequence when a nucleus is pressure catapulted from a slide. Two different types of membrane slides, polyethylene naphthalate (PEN) and polyethylene teraphthalate (PET), were investigated during this phase of testing. PEN and PET slides were spotted with known numbers of cells and LM was performed using the Zeiss PALM® Microbeam system. Six collections of 30 cells were performed with each slide

type. Cells were extracted and amplified. Samples collected from both slide types generated full (32 alleles) or high partial (30-31 alleles) profiles. When samples fixed to PEN and PET slides were subjected to standard FISH testing with Vysis CEP X® and Y® alpha satellite probes, it was observed that PEN slides are highly auto fluorescent whereas the PET slides demonstrate little to no auto fluorescence.

Increased background signal caused by auto fluorescence may interfere with fluorescent probe signal interpretation. These results indicated that PET slides should be incorporated into fluorescent LM procedures.

Pepsin Pretreatment Protocol Implementation

The resulting quality of a FISH assay is often dependent on the pretreatment of the slide prior to hybridization of the DNA probes. A pretreatment of the slides with a buffer containing pepsin, a protease, followed by post-fixation of the cells in buffered formalin can reduce the background fluorescence that is often observed using this technique. Pepsin digests the cell wall and cytoplasm, isolating the nucleus. This technique increases the fluorescence of the probes by reducing interfering cytoplasmic background fluorescence. The results from buccal cells that were not pretreated prior to hybridization were compared to those that were pretreated prior to hybridization. A decrease in background fluorescence was observed in the cells that were subjected to the pretreatment procedure. Based on these results, a pretreatment protocol was incorporated into the standard FISH procedure prior to denaturation and hybridization of the DNA probes in order to decrease the background fluorescence observed.

Optimization of FISH Conditions

Prior to implementation of TSA, it was necessary to optimize the standard FISH processing procedure due to inconsistent and weak probe signals that were observed following standard FISH testing with Vysis CEP X® and Y® alpha satellite probes. Poor probe signals are indicative of suboptimal pretreatment and hybridization conditions. The first step toward resolving the signal intensity issues was to improve the consistency and reproducibility of the temperatures used for incubation steps, denaturation and hybridization by purchasing a StatSpin® ThermoBrite™ Slide Hybridization/Denaturation System. After the instrument was incorporated into the FISH procedure, probe signals were still found to be weak and inconsistent. Various pepsin incubations, hybridization conditions, denaturation times and temperatures, as well as post-hybridization stringency conditions were investigated. Results indicated that a two minute incubation in pepsin can dramatically improve the quality of the probe signals. The hybridization times and post-hybridization stringency conditions demonstrated the greatest impact on probe signal intensity. Therefore, it was determined that reducing the hybridization time to 45 minutes and decreasing the stringency of the first post-hybridization wash step greatly improved the signal intensity of both the X and Y probes.

Bioinformatic Analysis of the Duffy and ABO Markers

In an attempt to elucidate the single nucleotide polymorphisms (SNPs) underpinning the differentiation between ABO markers, a bioinformatic study of the gene family was performed. The basis of the bioinformatic study was a phylogenetic tree reconstruction, which was performed using a data matrix containing the entire gene

sequence for each individual gene type and subtype. This study was performed to identify the relatedness within the ABO alleles to identify the existence of key SNPs that were intended to be used as lynchpins for assigning the A, B, or O phenotype to an unknown individual.

Based on the phylogenetic reconstruction of the ABO gene, there is an extremely low level of structure within the gene family. The bioinformatic analysis was performed by first aligning the sequences from the data matrix with ClustalX software. This alignment was then imported into PAUP* 4.0b phylogenetic software where the gene trees were reconstructed using a parsimony based neighbor joining algorithm. All tree topologies created with this algorithm were bootstrapped for 10,000 replicates to determine the "robustness" of the final tree topology. The resulting tree showed a gene group with large polytomies, or areas of the tree, where the information used was of insufficient informativeness to recreate evolutionary relationship. Thus, it was determined early that the ABO blood group would be a difficult candidate for a SNP assay of this nature.

ABO Probe Design

Phenotypically, the ABO gene is a simple gene comprised of only four blood phenotypes: A, B, AB, and O. Despite this apparent simplicity, there is extensive sequence heterogeneity underlying each of the major alleles. Blood groups A, B, and O contain 69, 47, and 63 different allele variants, respectively. These allele variants can be identified by analyzing 180 SNPs present across the gene. Allele variants may differ from each other by as few as one SNP. Although the gene is incredibly diverse, the most

common alleles are A101, A201, B101, O01, and O02 (1,2,3). Two SNP locations were selected for probe design: nucleotide position 796 on exon 7 and nucleotide position 261 on exon 6. These SNPs can be used to differentiate the majority of the alleles in the ABO system, including the five most common.

Sequence information for the desired locations was obtained from the UCSC Genome Browser, and then Primer3 software was employed to design the primers necessary for probe manufacture (4,5). Strict guidelines regarding length, GC content, and melting temperature were followed for primer design. Primers were successfully designed within the desired parameters. The resulting amplicons (30-300 bp) were hapten labeled with biotin to create ABO probes. Biotin labeling was necessary because probes of this size are below the detection limits of traditional FISH, and a signal amplification technique must be employed to increase the sensitivity of FISH when detecting probes as small as 319 bp. Following hapten labeling of the amplicons, it was intended that the probes be used to differentiate cellular mixtures comprised of different blood types.

Biotin Labeling of DNA Probes

A key step toward successful detection of the targeted DNA sequences is efficient biotin labeling of the DNA probes during manufacture. Before beginning probe design, several methods of probe manufacture and labeling were examined using approximately 300 base pair long fragments of DNA. After determining which method achieved both a sufficient yield of probe and efficient biotin labeling, probes for the ABO screening assay were designed and manufactured.

Nick Translation

One common method of incorporating biotin into a DNA probe is nick translation. Nick translation is a DNA tagging technique where DNA Polymerase I is used to replace some of the nucleotides of a DNA sequence with labeled analogues. Invitrogen's BioNick™ Labeling System was used to biotin label a 310 base pair (bp) long DNA fragment. This created a biotin tagged DNA sequence that was purified for use as a probe in fluorescence *in situ* hybridization. The effectiveness of nick translation as a labeling method was evaluated by examining the final yield of the biotin labeled probe, the time associated with generating the probe, and the success of the probe when used in a TSA-FISH reaction. Nick translation resulted in a very low yield of purified biotin labeled probe, and the procedure was very lengthy, taking a minimum of seven to eight hours to complete. Use of these probes for TSA-FISH staining of epithelial cells resulted in non-specific binding of the probes. The results associated with this procedure indicated that it would be beneficial to examine the Polymerase Chain Reaction (PCR) as an alternative method of labeling DNA with biotin.

Polymerase Chain Reaction

Biotin may also be incorporated into DNA probes using the polymerase chain reaction (PCR). Several benefits of PCR manufacturing of biotin labeled probes include: reduced time to produce a purified probe, enhanced control over reaction conditions, and increased yield of biotinylated product. Various thermal cycling parameters were examined to determine which would successfully incorporate biotin into the PCR product. After determination of the optimum thermalcycling parameters, varying amounts

of biotin-11-dUTP were incorporated into the PCR product. AmpliTaq Gold® DNA polymerase generated PCR products in which 25%, 50%, and 75% of dTTP was replaced with biotin-11-dUTP. As the volume of biotin-11-dUTP used in the reaction increased, it was necessary to make adjustments to the thermalcycling parameters to account for the additional biotin-11-dUTP. The resulting yields of biotin labeled product were significantly higher than those observed following nick translation; however, assessment of the labeling efficiency indicated that the labeling reaction was not efficient.

To address the labeling efficiency issues, $Vent_R$ (exo-) DNA polymerase was tested as an alternative to AmpliTaq Gold. Various amplification conditions, such as thermal cycling parameters and biotin-11dUTP concentration, were tested to determine the optimum conditions for the biotin labeling reaction. The labeling efficiency of the reaction was then determined using biotin labeled PCR product generated by the optimum conditions.

Following evaluation of the probe labeling methods, the optimized PCR procedure was utilized to prepare ABO probe 1. Specificity of the probe was evaluated with biotin chromogenic detection procedures. ABO probe 1 was hybridized to the DNA from two donors. Donor A represented blood group A with a C allele at position 796. Donor B represented blood group B with an A allele at position 796. Manipulation of the hybridization stringency suggested that a single SNP is insufficient for differentiating between blood groups A and B.

Tyramide Signal Amplification Fluorescence *In Situ* Hybridization (TSA-FISH)

Tyramide signal amplification fluorescence *in situ* hybridization (TSA-FISH) utilizes the activity of horseradish peroxidase to generate increased signal amplification of the target DNA sequence of interest *in situ*. TSA-FISH is a multistep process that includes: *in situ* hybridization of biotin labeled probes to target DNA, detection of the biotin labeled probe with streptavidin-horseradish peroxidase (SA-HRP), activation of multiple copies of signal enhancing fluorescent dye-labeled tyramide molecules by SA-HRP, and imaging. Prior to hybridization of the biotin labeled DNA probes to the target DNA sequences, endogenous biotin blocking was used to reduce background signals caused by endogenous biotinylated proteins present in the mitochondria of mammalian cells. All experiments were performed on epithelial cells.

Initial Evaluation

Initial TSA-FISH experiments utilized varying amounts of biotin labeled 310 bp long probes generated by both nick translation and PCR. Initial TSA-FISH experiments produced poor results. Despite the use of endogenous biotin blocking procedures, TSA-FISH results demonstrated high background signals resulting from nonspecific staining. This background fluorescence obscured the appearance of true FISH signals. As the probe concentration used in the hybridization decreased, nonspecific staining was

observed to decrease as well. Reduced probe concentration is recommended to minimize the adverse effects of nonspecific staining.

ABO Probe 1

Although specificity results indicated that a single base pair difference is insufficient for blood group differentiation, it was also necessary to assess the sensitivity of ABO probe 1. This assessment was designed to indicate whether the number of biotin molecules incorporated into the probe was sufficient for detection. TSA-FISH was performed with 50 ng of ABO probe 1 on slides spotted with: epithelial cells from donor A (blood group A), epithelial cells from donor B (blood group B), and a 1:1 mixture of epithelial cells from donors A and B. Specific probe signals were not detected subsequent to the procedure. Failure to detect probe signals may have resulted from an insufficient number of biotin molecules present in the probe, excessive background fluorescence caused by nonspecific biotin detection, or a combination of the two factors.

Conclusions

Findings indicated that the FISH techniques utilized in this research were unsuitable for differentiation of the ABO blood groups. Traditional double stranded DNA probes are unable to discriminate between sequences that differ by one base pair. Specific probe signals were not observed following sample hybridization with ABO probe 1. This was most likely caused by insufficient biotinylation of the probes. Nonspecific staining is another factor that may contribute to probe detection issues. The presence of even minimal nonspecific staining can obscure true probe signals. The research performed has

indicated that there are FISH techniques, such as tyramide signal amplification, that previously have not been explored for forensic applications. Consideration of alternative FISH methods could expand forensic FISH capabilities beyond standard X and Y chromosome FISH probing, thus expanding the forensic uses of laser microdissection.

Introduction

Statement of the Problem and Literature Review

The generation of clean, single source genetic profiles from sexual assault and touch evidence cellular mixtures continually proves to be a difficult challenge in the field of forensics. Evidence of this nature can contain low copy amounts of DNA from mixtures of cell types of various morphologies. While evidence containing mixtures of spermatozoa and epithelial cells can more easily be separated by preferential lysis methods or laser microdissection techniques using morphological differences, those mixtures containing only combinations of epithelial or blood cells require further processing in attempt to discriminate the evidentiary components by gender and other genetic polymorphisms.

It has been shown through previous work on NIJ Grant# 2006-DN-BX-K032, and in published literature, that multiple donors of morphologically similar cell mixtures can be visually identified by gender with fluorescence *in situ* hybridization (FISH) X chromosome and Y chromosome sequence probes and physically separated by utilizing laser microdissection (LM) instruments (6,7,8,9). FISH is a traditional cytogenetic

technique used to detect the presence or absence of specific chromosomes and/or sequences of an individual's genome. This method utilizes fluorescent probes that are designed to bind to the targeted conserved sequences of individual chromosomes. Fluorescence compatible microscopes are typically employed to visualize the multicolor probes used in these hybridizations. In general, FISH examinations can be divided into two categories: metaphase and interphase analysis. Metaphase FISH analysis normally involves the culturing of various cell and tissue types, fracturing cellular membranes for the purposes of DNA release, and the systematic spreading of chromosomes for visual interpretation. Interphase FISH (I-FISH) techniques incorporate probes which pass through cellular membranes and into the nucleus, eliminating the need to lyse cells during processing. The absence of membrane rupture during I-FISH techniques represents a distinct functional advantage over metaphase methods for the purposes of forensic operations. Several types of probes are commonly used in I-FISH to hybridize to specific targeted DNA sequences of interest. Among these probe types are chromosome enumeration probes (CEP) and locus-specific probes (LSP). CEP probes hybridize to repetitive DNA sequences, referred to as α-satellite DNA, found near the centromeres of chromosomes. The repetitive sequences are typically 171 base pairs in length and are repeated thousands of times to span 250,000-5,000,000 bases (10). This large target area allows for hybridization to occur many times and generates a bright signal within the nuclei. CEP probes are used to enumerate the number of copies of a chromosome in a cell as seen in X and Y chromosome FISH probing. LSP probes hybridize to unique, nonrepetitive DNA sequences. These probes typically hybridize to DNA regions ranging

from hundreds of thousands kilobases (kb) to 1 kb, with an ideal size of approximately 40 kb (11).

FISH techniques have allowed for the visual identification of male and female cells from sexual assault evidence using X chromosome and Y chromosome probes (8,9). Following fluorescence processing, intact sample cells can be removed from the slides via LM methods and extracted for further STR interpretation. In studies to separate male/female mixtures using interphase FISH techniques, Vysis CEP X® alpha satellite and CEP Y® satellite III probes were employed to visually identify the sex origin of each cell. Initially, only male and female epithelial cells were tested. A working protocol for the hybridization of these probes was developed by merging the manufacturer's recommended procedure, those procedures published in scientific literature, and those techniques learned through personal scientific experience into one complete protocol encompassing all aspects of this type of analysis. Interphase FISH processing was tested with success on both epithelial and white blood cell sample types. Processing samples with this technique has allowed for the ability to visually identify the male and female contribution to each sample mixture. Sex chromosomes were easily identified from epithelial/epithelial, white blood cell/white blood cell, and epithelial/white blood cell mixtures. An X chromosome is visually identified by the presence of a green fluorescent marker, while a Y chromosome can be readily detected by the presence of an orange fluorescent marker. Figures 1, 2, and 3 demonstrate the results that have previously been observed when using this technique to identify the male and female contributors in sample mixtures of various cell types.

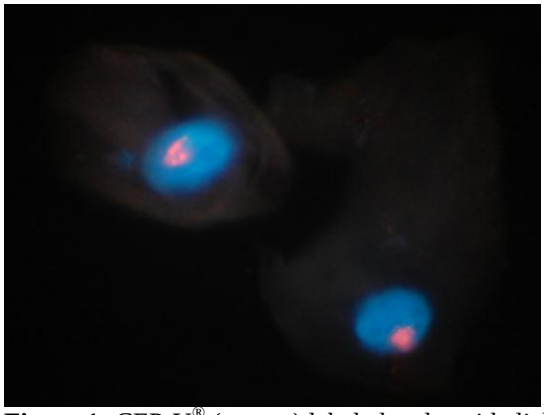


Figure 1: CEP-Y® (orange) labeled male epithelial cells with DAPI counterstain visualized at 630X magnification as viewed through DAPI/FITC/TRITC filter.

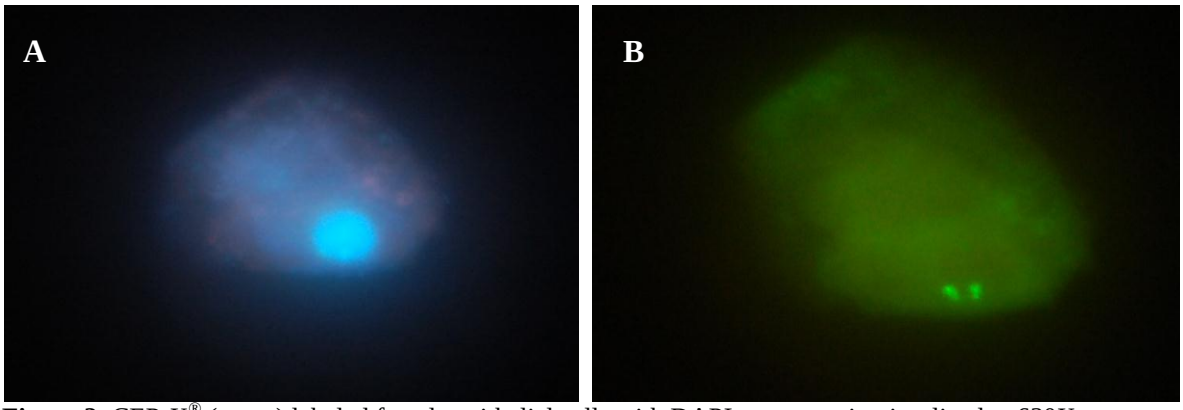


Figure 2: CEP-X® (green) labeled female epithelial cells with DAPI counterstain visualized at 630X magnification as viewed through (A) DAPI/FITC/TRITC filter and (B) FITC filter.

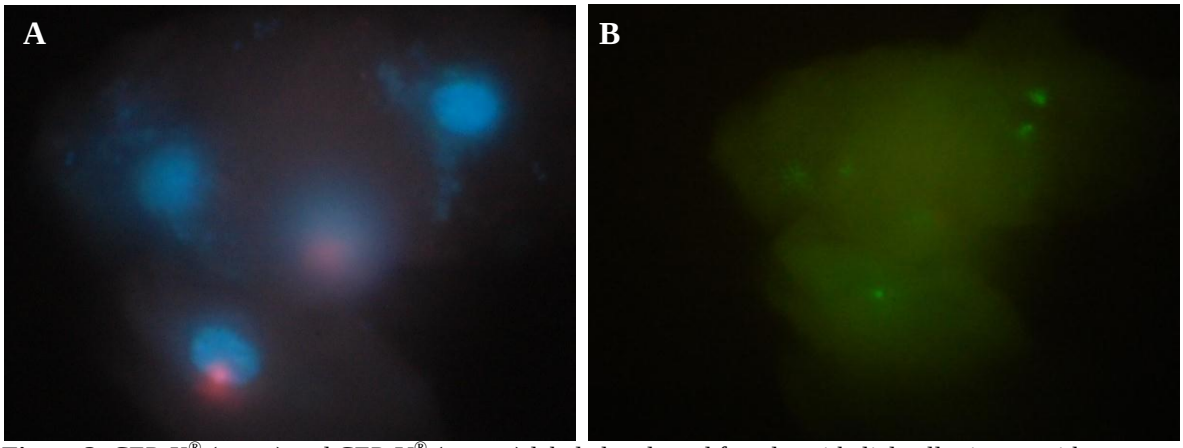


Figure 3: CEP-X® (green) and CEP-Y® (orange) labeled male and female epithelial cell mixture with DAPI counterstain visualized at 630X magnification as viewed through (A) DAPI/TRITC/FITC filter and (B) FITC filter

The results generated during these previous studies demonstrated that low copy male/female cellular mixtures of similar morphology can be successfully separated and

profiled using FISH and LM techniques. FISH probes can be hybridized to the nuclear DNA of various cell types without inhibiting further downstream genetic analysis. Using this method, as few as 10 cells have been collected with the Arcturus Pixcell® II and Zeiss PALM® MIcrobeam instruments and processed to generate full DNA profiles using the ABI AmpF*l*STR® MiniFiler™ amplification kit, and 20 to 30 cells have been processed to generate full profiles using the ABI AmpF*l*STR® Identifiler® kit. This technique has been used to successfully generate STR profiles for the male and female contributors in sample mixtures of various cell types (Figures 4 and 5).

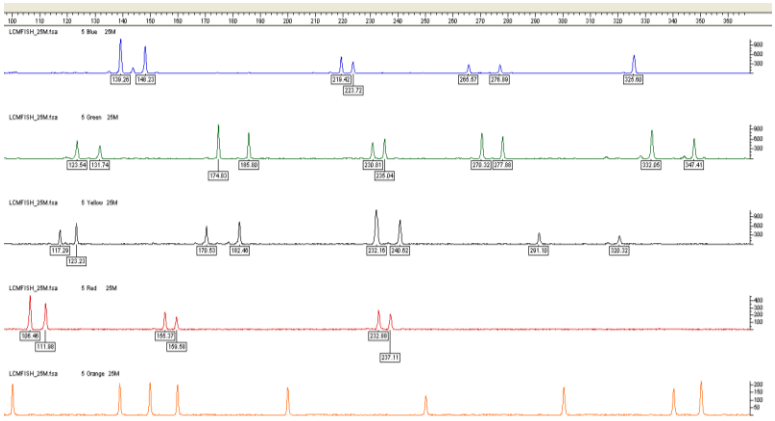


Figure 4: Electropherogram displaying a full STR profile generated from the extraction of FISH processed male epithelial cells from a male/female mixture.

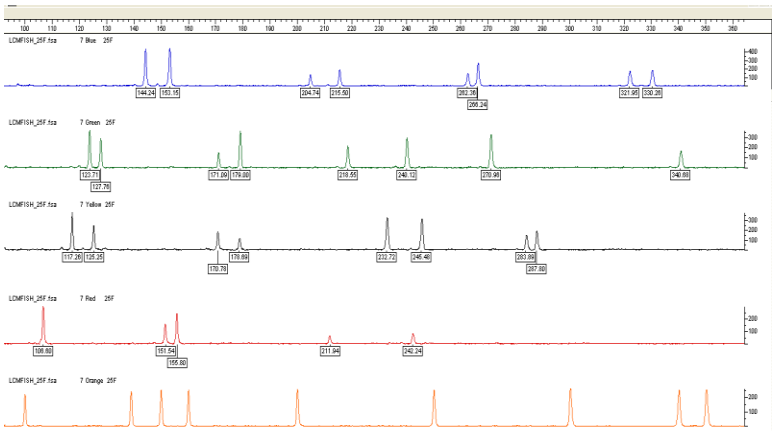


Figure 5: Electropherogram displaying a full STR profile generated from the extraction of FISH processed female epithelial cells from a male/female mixture.

Building on previous research results from NIJ Grant# 2006-DN-BX-K032, this research proposed to separate cellular mixtures of similar morphology and same gender by using sequence specific FISH probes based upon the genetic polymorphisms associated with the Duffy and ABO blood groups. The Duffy and ABO blood grouping systems were chosen as the basis for these assays because of the existence of their prevalent polymorphisms throughout multiple populations. Each system only has several alleles that can be easily targeted through a multicolor probe assay. The genetic sequences associated with the variations of each system are conserved and manageable in length. This research proposed that probes could be designed to target the genetic loci associated with each blood grouping system and visually identify individuals in forensic mixtures.

The Duffy blood group genetic polymorphisms were selected to try to differentiate cellular mixtures of the same gender. The human Duffy blood group (*FY*) consists of antigens that are transmembrane glycoproteins that function as receptors for chemicals secreted by blood cells during inflammation. Most Duffy blood group variation is determined by the presence of two common alleles, *FY*A* and *FY*B*, or the absence of protein production from allele *FY*O*. These sequences are located on chromosome 1. DNA sequence characterization of these alleles has shown that *FY*A* and *FY*O* are derived variants, each resulting from a single mutation in an ancestral *FY*B* background (12). The sequences for these alleles have been published and used for genotyping assays (13). The Duffy null phenotype FY (a-b-) is very rare in Caucasians and Asians, but is found in 68% of African Americans (14). The frequencies of the three Duffy phenotypes vary among the major populations in North America (Table 1).

Table 1: Percent Duffy phenotypes in major North American populations

Phenotype	Caucasian	African American	Asian
FY (a+b+)	49%	1%	9%
FY (a-b+)	34%	22%	<1%
FY (a+b-)	17%	9%	91%

Another blood group polymorphism that can be examined for the purposes of cellular differentiation is the ABO system. This blood grouping method is based upon three different alleles (A, B, and O) located on chromosome 9, position 9q34.1-q34.2. The genes of this blood group code for glycoproteins that reside on the outer membrane of red blood cells (15). The sequences for these alleles have also been published and used for genotyping assays (16). The labeling of cellular mixtures based on ABO genetic polymorphisms was intended to separate same gender cell mixtures of similar morphology. In the U.S., approximately 46% of the population is blood type O (3 major subtypes), 40% type A (2 major subtypes), 10% type B (1 major subtype), and 4% type AB (14).

This research proposed to develop short probes (30-300 base pairs in length) to detect the single nucleotide polymorphisms observed between individuals of A and B blood groups; however, probes of this size were below the detection limits of traditional FISH methods. Signal amplification techniques have been utilized to increase the sensitivity of FISH when detecting probes as small as 319 bp. Tyramide signal amplification (TSA) utilizes the activity of horseradish peroxidase to generate increased signal amplification of the target DNA sequence of interest *in situ*. In TSA-FISH, a biotin labeled probe is hybridized to the target DNA sequence. The hybridized biotin labeled

probe is detected with a streptavidin-horseradish peroxidase conjugate (SA-HRP). Streptavidin, a tetrameric protein derived from *Streptomyces avidinii*, has an extremely high affinity for biotin. The streptavidin-biotin bond is one of the strongest known noncovalent biological interactions. In the presence of peroxide, multiple copies of signal enhancing fluorescent labeled tyramide are activated by the HRP. HRP converts the labeled tyramide into short-lived tyramide radicals that covalently bind to electron rich regions of adjacent tyrosine residues found in the proteins in cellular preparations. The activated tyramide radicals only bind in the vicinity of the activated HRP (Figure 6) (11,17). A second round of TSA signal generation may be performed using anti-fluorescein/Oregon green antibody conjugates labeled with HRP. Imaging is performed on a fluorescence capable microscope.

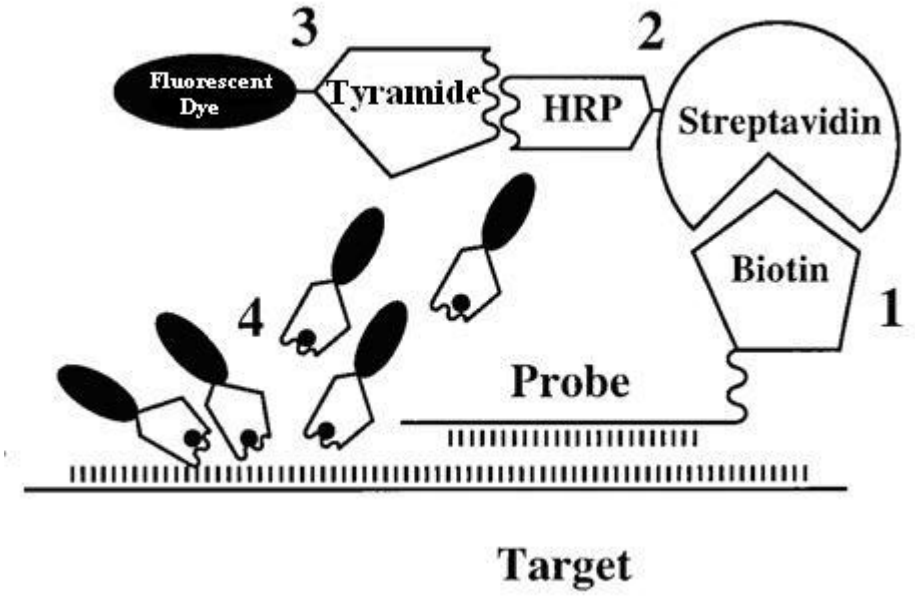


Figure 6: Schematic representation of TSA-FISH. [1] The targeted DNA is detected with a biotin labeled probe. [2] HRP-labeled streptavidin binds to the biotin labeled probe. [3] The fluorescent dye labeled tyramide is activated by HRP. [4] Activated tyramide radicals bind near the activated HRP (18).

Successful tyramide signal amplification is highly dependant on successful biotin labeling of the FISH probes. Biotin is a small B-complex vitamin molecule, vitamin B_7, with a high affinity for streptavidin and avidin. It is linked to nucleotides, deoxynucleotides, or dideoxynucleotides with a linker arm that ranges in length from 7-20 C/N atoms (Figure 7) (19). The linker arm can be attached to the number five position of pyrimidines or the number seven position of purines (20). The length of the linker arm affects the labeling efficiency of the reaction, the final yield of labeled product, and the streptavidin-biotin bond (Table 2). Biotinylated deoxynucleotides with longer linkers are more commonly used due to the improved formation of the streptavidin-biotin complex.

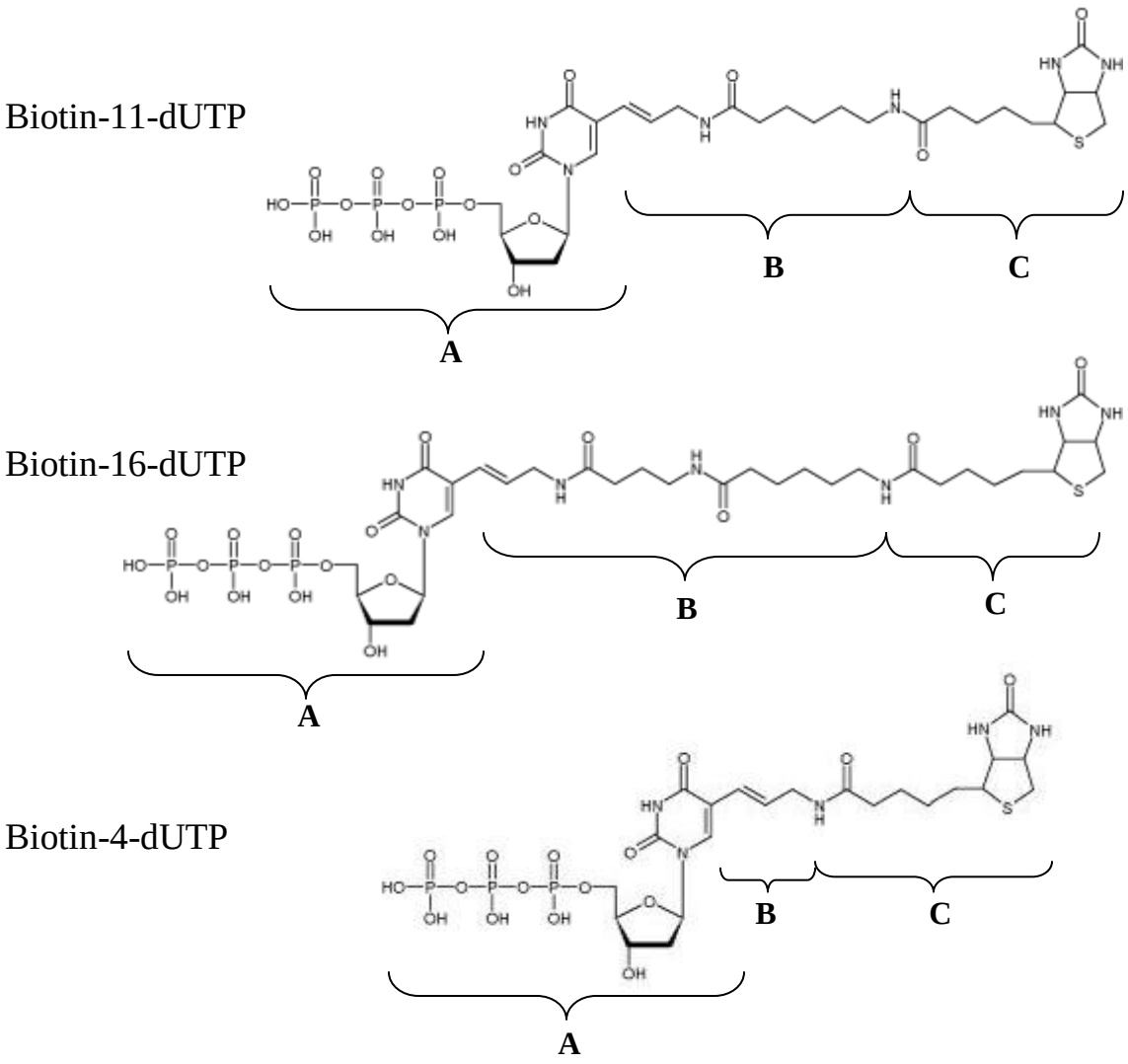


Figure 7: Biotin-11, -16, and -4-deoxyuridine triphosphate. (C) Biotin is linked through a (B) linker arm to (A) deoxyuridine (http://www.enzolifesciences.com).

Table 2: Effects of linker arm length on final labeled product (14,16,17)

Linker arm length	Yield of Labeled Product	Labeling Efficiency	Streptavidin Binding
Short (biotin-4-dUTP)	Increased	Decreased	Decreased
Long (biotin-11-dUTP, biotin-16-dUTP)	Decreased	Increased	Increased

Biotin labeling is typically accomplished through nick translation, random primed labeling, or the polymerase chain reaction (PCR). Nick translation is a DNA tagging technique where DNA Polymerase I is used to replace some of the nucleotides of a DNA sequence with labeled analogues. Double-stranded DNA is treated with Dnase I, which is used to introduce randomly distributed scissions, or nicks, into DNA in the presence of magnesium ions. The resulting nicks provide 3' hydroxyl groups that serve as primers for DNA synthesis catalyzed by the 5' → 3' polymerase activity of the Klenow fragment of *E. coli* DNA polymerase I. The 5' → 3' exonuclease activity of the polymerase simultaneously removes nucleotides from the 5'-PO_4 terminus and adds nucleotides to the 3'-OH terminus. The polymerase activity replaces the removed nucleotides with deoxyribonucleotide triphosphates (dNTPs), one of which is replaced with a labeled nucleotide (Figure 8). For example, during synthesis, dTTP, dCTP, dGTP, and biotin-14-dATP are incorporated in the growing chain of DNA while the nick is translated along the DNA by virtue of the 5' → 3' exonuclease activity carried by the enzyme. The resulting product may be used as a FISH probe (23). Nick translation is typically recommended for labeling double stranded DNA fragments larger than 1 kb. Random primed labeling is similar to nick translation; however, it does not involve the formation of nicks in the DNA strands. In a random primed labeling reaction, random short oligonucleotide primers are annealed to a denatured DNA template and complementary strands are synthesized with the Klenow Fragment, exo- in the presence of biotin-dUTP. Starting from the 3'-OH end of the annealed primer, Klenow fragment synthesizes new DNA along the single-stranded substrate, incorporating the biotin-dUTP into the final product. PCR can also be used to create biotin labeled DNA probes. Natural

deoxynucleotides are replaced with biotinylated deoxynucleotides when using PCR to label probes with biotin. For instance, dTTP is replaced with biotin-11-dUTP. As the PCR reaction takes place, biotin-11-dUTP is incorporated into the amplicons in the place of dTTP. Following purification to remove excess primers and unincorporated nucleotides, the resulting biotinylated amplicons can be used as FISH probes. The PCR may be modified to aid the incorporation of biotinylated deoxynucleotides. Taq DNA polymerase, a family A-type DNA polymerase, is commonly used in PCR, but it has been shown that some family B-type polymerases, such as $Vent_R$ (exo-) DNA polymerase, are more successful at integrating modified dNTPs into PCR products (21,24).

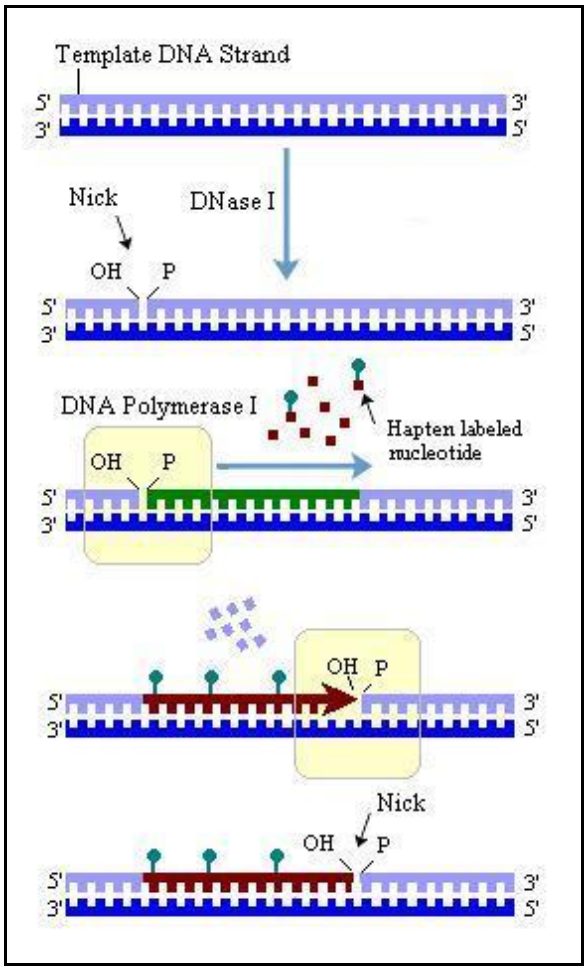


Figure 8: Schematic representation of nick translation

Statement of Hypothesis

The proposed research included the creation of sequence specific probes for FISH analysis based upon the Duffy and ABO sequences. The fluorescent labeling of these cells based upon Duffy genotypes were thought to be discriminating in the identification of cellular mixture contributors. A screening FISH system based upon the Duffy system could effectively separate mixtures containing cells with the same morphology and originating from contributors of the same gender. Positive probe controls would also be included in assays to ensure the accuracy and validity of phenotypes based upon the absence of alleles (e.g. FY(a-b-)). These positive control probes would target a non-

polymorphic conserved sequence prevalent in all human genomes. Processed cells could then be physically separated from mixtures by means of laser microdissection applications. A multicolor probe screening system, similar to those designed for X/Y and Duffy variations, could be designed based upon ABO allele polymorphisms for the effective separation of mixture samples. When used in conjunction with laser microdissection, these FISH probe screening systems could be used to successfully separate cellular mixtures originating from contributors of the same gender

It was the goal of this research to improve the methods of DNA mixture resolution in the forensic laboratory by using FISH based laser microdissection techniques. Grant# 2006-DN-BX-K032 from the NIJ was initiated in 2006 to study the use of laser microdissection instruments for the isolation of sperm and epithelial cells from sexual assault and touch mixtures. While that grant did focus on separating male and female cells of similar morphology using chromosome X/Y hybridization probes, it did not address separating cells of similar morphology and same gender. The proposed research was intended to expand upon the field's ability to resolve mixtures by meeting the following two research objectives:

1. Design and test sequence specific hybridization probes based upon Duffy blood group genetic variations.
2. Design and test sequence specific hybridization probes based upon ABO blood group genetic variations.

Methods

In order to accomplish the targeted goal of identifying and separating cellular mixtures of the same gender and cell type, the research was subdivided into five distinct sections. First, bioinformatic analyses were performed on the ABO and Duffy markers to determine the minimum number of SNPs necessary to identify inclusion in a specific category. Analysis of the Duffy blood group revealed one SNP difference between the groups, providing limited opportunities for probe design. Therefore, due to the increased number of SNPs present in the ABO blood group gene, research efforts focused on this group for the remainder of the project. Techniques and methodologies generated during the ABO FISH research could later be applied to Duffy following successful development of a FISH panel for ABO blood group differentiation. Probes for specific SNPs differentiating the A and B blood groups were designed. These probes were labeled with multiple biotin molecules via either nick translation or a polymerase chain reaction (PCR). The probes were hybridized to target DNA sequences *in situ*, and tyramide signal amplification was used to detect the hybridized biotin labeled probes (Figure 9). Prior to carrying out signal amplification, the standard FISH protocol was optimized to ensure the pretreatment, hybridization, and post-hybridization conditions would produce the most favorable results.

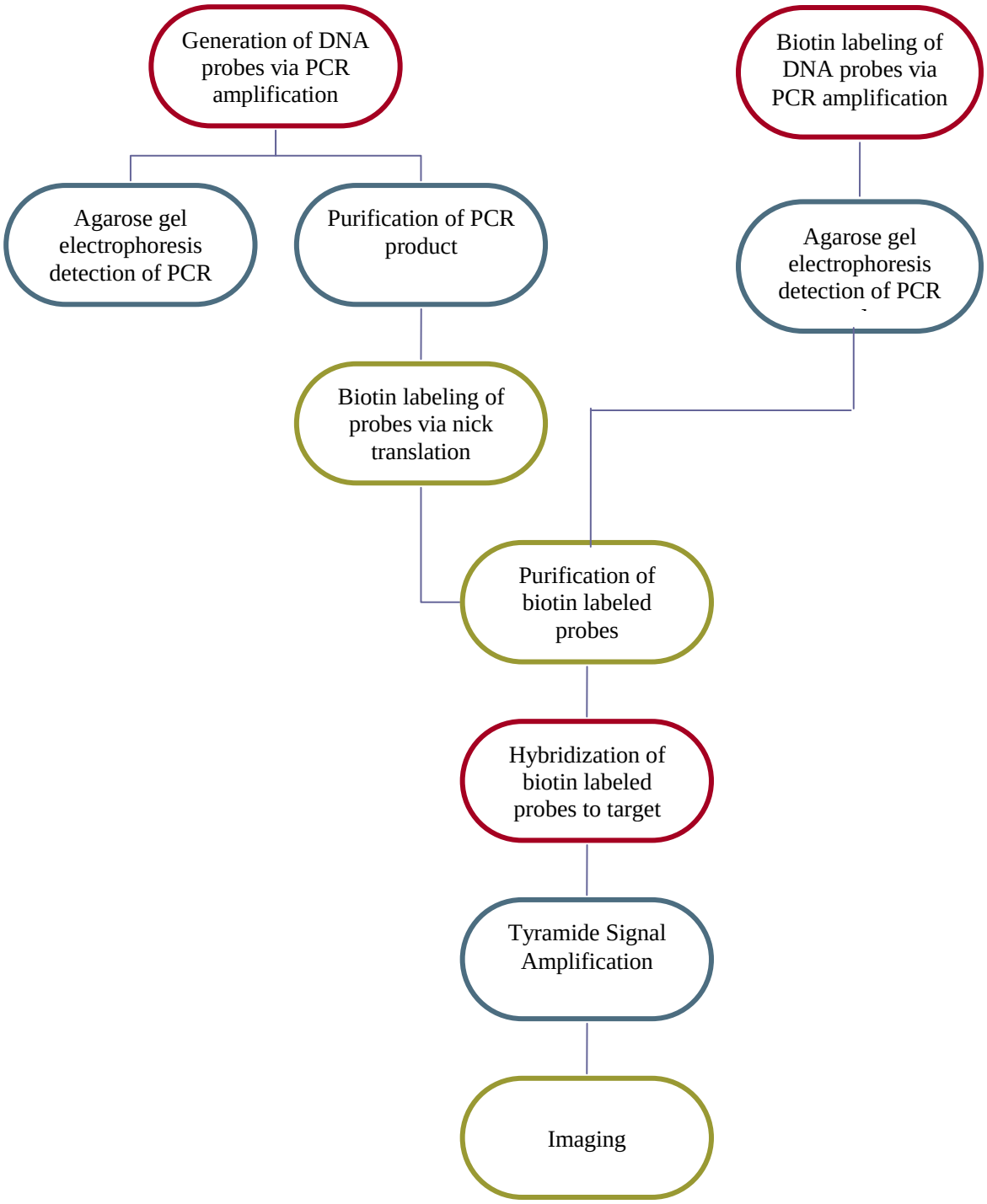


Figure 9: Diagram of overall procedure from probe generation to imaging.

Optimization of Standard FISH Protocol

Membrane Slide Evaluations

PEN and PET membrane slides were spotted with 200 counts of purified buccal cells. Test samples were collected from one individual not associated with these tests using cotton swabs. Cells were eluted from the swabs into 1X phosphate buffered saline (PBS) by vortex agitation and centrifugation. Buccal samples were counted prior to spotting on the slides using C-Chip Disposable Hemocytometers (InCyto). These samples were allowed to air dry for 60 minutes prior to processing. Six separate collections of 30 cells were performed using the Zeiss PALM® Microbeam system.

For the purposes of this study, the QIAGEN QIAamp® DNA Micro Kit ("Laser Microdissected Samples" protocol) was utilized for all extractions. Extracts were amplified with the PowerPlex® 16 System (Promega) at a reaction volume of 13 µl for 30 cycles. The GeneAmp® PCR System 9700 (Applied Biosystems) was used for thermal cycling and the ABI Prism® 3100 Genetic Analyzer (Applied Biosystems) was used for gene fragment analysis.

PEN and PET membrane slides were also evaluated for compatibility with the FISH protocols currently utilized at Bode Technology.

Pepsin Pretreatment Protocol Implementation

Slides spotted with epithelial cells were prepared as described in the "Optimization of FISH Conditions" protocol. Protease pretreatment, hybridization, post hybridization stringency washes, and detection proceeded as specified in the original

standard FISH testing procedure below. Results from buccal cells that were not pretreated prior to hybridization were compared to those that were pretreated prior to hybridization.

Optimization of FISH Conditions

Prior to implementation of tyramide signal amplification, it was necessary to optimize the standard FISH processing procedure due to inconsistent and weak probe signals that were observed following standard FISH testing with Vysis CEP X® and Y® alpha satellite probes. Poor probe signals are indicative of suboptimal pretreatment and hybridization conditions. The first step toward resolving the signal intensity issues was to purchase a StatSpin® ThermoBrite™ Slide Hybridization/Denaturation System to improve the consistency and reproducibility of the temperatures used for the incubation steps, denaturation, and hybridization. A series of optimization tests were performed to improve the consistency and quality of FISH results. Various pepsin pretreatment times, hybridization times and temperatures, denaturation times and temperatures, as well as post-hybridization stringency condition were investigated.

Sample slides for each test were prepared using the following method. Buccal swabs were collected from donors not associated with the processing of any samples related to this project. Cells were eluted from the swabs by agitation in 1X phosphate PBS at room temperature for two hours with shaking at 900 rpm. A cell pellet was generated by centrifugation at 3000 rpm for two minutes. The supernatant was removed and the pellet was resuspended in Carnoy's Fixative (3:1 methanol:glacial acetic acid). Twenty µl of the resuspended cell pellet was applied to a glass or PET slide. PET slides were used when subsequent laser microdissection of the detected cells was anticipated. The slides were briefly steamed over a water bath prior to drying on a heat block at 56°C

for two minutes. The cells were heat fixed to the slides at 60°C for two to three hours. Before pretreatment, the slides were aged in a desiccator for a minimum of eight hours.

During the original standard FISH testing procedure, the slides were immersed in 2X SSC for two minutes at 73°C, 1.27 U/µl pepsin buffer for 10 minutes at 37°C, 1X PBS/50mM $MgCl_2$ solution for five minutes at room temperature, 2.5% formalin buffer for five minutes at room temperature, and 1X PBS for five minutes at room temperature. The slides were then dehydrated through a series of ethanol washes (70%, 85%, and 100%) for one minute each at room temperature. Sample slides were denatured in a denaturant solution (70% formamide and 2X SSC) for five minutes at 80°C. Slides were then dehydrated in a series of ethanol washes (70%, 85%, and 100%) for one minute each at room temperature. Probes were prepared by combining 7 µl of CEP Hybridization Buffer®, 1 µl of CEP X® probe, 1 µl of CEP Y® probe, and 1 µl of dH2O in a 0.5 µl microcentrifuge tube. The probe solution was denatured in a water bath for five minutes at 80°C. Ten µl of the probe mixture was applied to the slides, covered with glass cover slips, and sealed with rubber cement. Samples were then hybridized in a humidified container overnight at 37°C. Following hybridization, the cover slips were removed and the slides were incubated in a 0.4X SSC/0.05% Tween 20 solution for two minutes at 73°C. The slides were then immersed in 2X SSC for one minute at room temperature. Two to three drops of Vectashield Mounting Medium with DAPI (Vector Labs) were applied directly to the slides. The samples were then visualized using fluorescence microscopy.

Modifications were made to the original FISH processing protocol to improve the signals intensities in preparation for TSA. The StatSpin® ThermoBrite™ Slide

Hybridization/Denaturation System was incorporated into the heated wash steps and the denaturation and hybridization steps. All heated wash steps were executed on the ThermoBrite system with the use of reusable perfusion chambers (Electron Microscopy Sciences). Probes were prepared by combining 7 µl of CEP Hybridization Buffer®, 1 µl of CEP X® probe, 1 µl of CEP Y® probe, and 1 µl of dH2O in a 0.5 µl microcentrifuge tube. Ten µl of the probe solution was applied directly to the slides. The probe solution was covered with a glass cover slip and sealed with rubber cement. Various denaturation and hybridization conditions were tested at this time. Denaturation occurred at 73˚C or 80˚C for five or nine minutes. This was immediately followed by hybridization at 37˚C or 42˚C for 45 or 360 minutes. Further modifications included elimination of the initial 2X SSC wash in favor of aging the slides in a desiccator for at least 24 hours before beginning the FISH procedure, reduction of the length of the pepsin pretreatment incubation to one, two, or five minutes, and reduction of the stringency of the first post hybridization wash by increasing the salt concentration from 0.4X SSC to 1X SSX.

Bioinformatic Analysis of the Duffy and ABO Markers

In the case of the ABO blood markers, in-depth research into the specific apomorphies underpinning each of the different individual ABO blood groups and alleles was performed. Before bioinformatics of probe development could begin, it was imperative that the overall relatedness within the ABO alleles be elucidated and therefore identify key lynchpin SNPs, which could be used to denote association within each of the major haplogroups (A,B,O) as well as the multitudinous sub-haplogroups (alleles) within each major haplogroup. For SNP markers to exhibit the informativeness necessary to be

effective in this level of differentiation, it must be assumed that based on nucleotide sequence, the individual members of each of the three major groups are more closely related to each other than they are to those in other groups. In the absence of any information to the contrary, monophyly of each of the three major groups was assumed.

In order to determine if the underlying assumption of monophyly within the ABO group was correct, a phylogenetic analysis of all known ABO alleles was performed in-house. The SNPs identified to date that appear to have the most direct effect in determining allele state within ABO come from exons 2 through 7 of the Fy Glycoprotein (Duffy) gene. Although research focused on this central portion, the bioinformatic analysis was performed using full gene sequence. A gene alignment consisting of 180 nucleotide positions from each of the 161 known ABO haplotypes was used as the basis of for the bioinformatic analyses (25). The majority of the categorized apomorphies are nucleotide substitutions in the form of transitions and transversions, although a number of indel events are also present. For this study, the use of nucleotide substitutions was prioritized rather than indels to create the final selection of probes. However, to recreate the most exact phylogenetic model of the gene family, all indel positions were included in the bioinformatic analyses.

Briefly, the bioinformatic analysis began with the alignment of all sequences represented in the data matrix using ClustalX software. The fully aligned files were exported in a # NEXUS format, which was then imported into PAUP* 4.0b phylogenetic software suite. In this software, a parsimony based neighbor joining algorithm was used to reconstruct the evolutionary relationship between all of the ABO groups and alleles. The results were then bootstrapped for 10,000 replicates using the fast stepwise addition

option to determine the “robustness” of the tree node reconstruction. Through recovering the correct gene tree topology, or branching pattern, of the underlying sequence based relationship between all of the alleles we can identify the synapomorphies responsible for each most recent common ancestor node can be identified. These shared derived characters are SNPs that are categorical for all of the alleles contained within. It is these shared synapomorphies, in SNP form, which were intended to be exploited to create FISH probes which would categorize a sample with any ABO allele into its specific ABO group.

ABO Probe Design

The ABO gene is located on chromosome 9 and consists of 6 introns and 7 exons. Phenotypically, the ABO gene is a simple gene comprised of only four blood groups: A, B, AB, and O. Despite this apparent simplicity, there is extensive sequence heterogeneity underlying each of the major alleles. Blood groups A, B, and O contain 69, 47, and 63 different allele variants, respectively, for a total of 179 variants. These allele variants can be identified by analyzing 180 SNPs present across the gene (25). Allele variants may differ from each other by as few as one SNP. Although the gene is incredibly diverse, the most common alleles are A101, A201, B101, O01, and O02 (1,2,3).

Two SNP locations were selected for probe design: nucleotide position 796 on exon 7 and nucleotide position 261 on exon 6. These SNPs can be used to differentiate the most common alleles in the ABO system as well as 156 other allele variants (1). Of the remaining 18 alleles, 16 non-A type alleles are indistinguishable from an A allele and two non-B type alleles are indistinguishable from a B allele (Table 3).

Sequence information for the desired locations was obtained from the UCSC Genome Browser, and Primer3 software was employed to design the primers necessary for probe manufacture (4,5). Strict guidelines were followed for primer design. Primers were designed to be 18 to 22 bases in length, contain a GC content of 45 to 60%, and have a melting temperature (T_m) between 57.0°C and 63.0°C. The targeted SNP was positioned at the 3' end of the resulting amplicons. Acceptable primers were restricted to those that would produce amplicons between 30 and 60 bases in length with a GC content of 45 to 60% (Table 4). Amplicons were generated with DNA extracted from a donor representing blood group A. The donor possessed a C allele at SNP position 796 and a G allele at SNP position 261. The resulting amplicons were hapten labeled with biotin to create ABO probes for use in a FISH assay.

Table 3: Allele variants covered by the ABO FISH assay (1)

Alleles for ABO Grouping	Allele Variants Covered	SNP Position 796	SNP Position 261
A allele	A101-109, A201-215, Aban, A301-307, Ax01-11, Aw01-13, Ael01-107, Am01-02	C	G
B allele	B101-103, B107-114, B101vr, B301-306, B(A)01-06, Bx01, Bel01-05, Bw02-12, Bw15-19	A	G
O allele	O01-02, O011, O04-07, O09-13, O16, O18, O21-23, O25-36, O39-40, O43-47, O54-59, O61-66	C	deletion
O allele	O24, O41-42, O70, O71	A	deletion
Identical to A allele	B115, O03, O08, O14-15, O19-20, O48-O53, O60, cis-AB01, cis-AB02	C	G
Identical to B allele	cis-AB03, cis-AB04	A	G

Table 4: Sequences of the primers used to generate the SNP specific FISH probes

ABO Probe #	Exon	SNP Location	Polymorphism	Forward Primer	Reverse Primer
1	7	796	C/A	caggcctacatccccaag	aggtagtagaaatcgccctcgt
2	6	261	G/deletion	acgcctctctccatgtgc	ccacgaggacatccttccta

Biotin Labeling of DNA Probes

Different techniques of labeling DNA probes were investigated to determine the best method for incorporating biotin into the DNA probes. The two methods evaluated include nick translation and a polymerase chain reaction. Nick translation was used to replace dATP with biotin-14-dATP. PCR was used to replace dTTP with biotin-11-dUTP.

Nick Translation

The 310 base pair DNA fragments used for nick translation were generated using PCR. The reaction mixture contained 1X PCR buffer (ABI), 0.2 mM of each dNTP, 0.4 µM forward primer, 0.4 µM reverse primer, 10 units AmpliTaq Gold (ABI), and 30 ng DNA template in a total volume of 50 µl. The following primers were used in the reaction: forward 5'–AGGGTAGAGACCCAGGCAGT-3' and reverse 5'-GAGCAGGAAACATCTGGAGC-3'. The PCR was performed in an ABI 9700 thermal cycler. The incubation step consisted of a 10 minute denaturation at 96°C, followed by 42 cycle thermalcycling reaction (94°C, 20 sec; 60°C, 20 sec; and 72°C, 55 sec), and an indefinite 4°C stop of the reaction. The PCR products were purified using Centri-Sep columns (Princeton Separations) or Qiagen's MinElute PCR Purification kit. For Centri-Sep purification, up to 30 µl of PCR product was applied to the column, followed by centrifugation at 750 x *g* for two minutes. For MinElute purification, five volumes of

Buffer PB were added to one volume of PCR product and the sample was applied to the MinElute column. The sample was spun at 17,900 x *g* for one minute. 750 µl Buffer PE was applied to the column and the sample was spun at 17,900 x *g* for one minute. The MinElute column was transferred to a clean tube. 30 µl ddH_2O was added to the column and was incubated at room temperature for five minutes. The sample was spun at 17,900 x *g* for one minute. Quantification of the purified PCR products was performed via UV spectrophotometry at OD260. Invitrogen's BioNick Labeling System kit was used to perform the nick translation reaction. 1000 ng of purified PCR product was added to 1 µl 10X dNTP Mix and 5 µl 10X Enzyme Mix for a final volume of 50 µl. The sample was incubated at 16°C for two hours. At the completion of the incubation step, 5 µl Stop Buffer were added. Using the above protocol, a second Centri-Sep purification was performed on the resulting product to separate the unincorporated nucleotides from the labeled DNA probe. Quantification of the purified biotin labeled product was performed via UV spectrophotometry. Probes were then used for TSA-FISH staining.

Polymerase Chain Reaction

A second method for incorporating biotin labeled dNTPs into a DNA probe is using PCR. After amplification, both strands of each resulting DNA fragment include biotin-labeled DNA product, with the exception of the primers at each 5' end of the DNA duplex. Various thermalcycling parameters were utilized to incorporate varying amounts of biotin-11-dUTP into the PCR product. Two polymerases, AmpliTaq Gold® DNA polymerase and $Vent_R$ (exo-) DNA polymerase, were examined to determine which would maximize the labeling efficiency of the reaction.

AmpliTaq Gold® DNA polymerase

In PCRs utilizing AmpliTaq Gold® DNA polymerase (Applied Biosystems), unmodified dTTP (Fermentas) was replaced by biotin-11-dUTP (Fermentas) in a stepwise manner, with biotin-11-dUTP replacing 25, 50, and 75% of dTTP. For 25% replacement of dTTP with biotin-11-dUTP, the following reaction mixture was used: 1X PCR Buffer, 0.2 mM dATP, dCTP, dGTP (Fermentas), 0.15 mM dTTP, 0.05 mM biotin-11-dUTP, 0.4 µM forward primer, 0.4 µM reverse primer, 10U taqGold, 30 ng template DNA, and water up to a final reaction volume of 50 µl. The PCR was performed in an ABI 9700 thermal cycler. The incubation step consisted of a 10 minute denaturation at 96°C, followed by 12X (94°C, 12 sec; 60°C, 20 sec; and 72°C, 55 sec), 30X (94°C, 12 sec; 60°C, 20 sec; and 72°C, 55 sec), a 10 minute hold at 72°C , and a hold at 4°C indefinitely. For 50% replacement of dTTP with biotin-11-dUTP, the following reaction mixture was used: 1X PCR Buffer, 0.2 mM dATP, dCTP, dGTP, 0.1 mM dTTP, 0.1 mM biotin-11-dUTP, 0.4 µM forward primer, 0.4 µM reverse primer, 10U taqGold, 30 ng template DNA, and water up to a final reaction volume of 50 µl. The PCR was performed in an ABI 9700 thermal cycler. The incubation step consisted of a 10 minute denaturation at 96°C, followed by 42X (95°C, 45 sec; 60°C, 1 min; and 72°C, 2 min), a seven minute hold at 72°C , and a hold at 4°C indefinitely. For 75% replacement of dTTP with biotin-11-dUTP, the following reaction mixture was used: 1X PCR Buffer, 0.2 mM dATP, dCTP, dGTP, 0.05 mM dTTP, 0.15 mM biotin-11-dUTP, 0.4 µM forward primer, 0.4 µM reverse primer, 10U taqGold, 30 ng template DNA, and water up to a final reaction volume of 50 µl. The PCR was performed in an ABI 9700 thermal cycler. The incubation step consisted of a 10 minute denaturation at 96°C, followed by 42X (95°C, 45 sec; 60°C,

1 min; and 72°C, 3 min), a seven minute hold at 72°C, and a hold at 4°C indefinitely. A control containing no biotin-11-dUTP was amplified alongside each PCR. The reaction mix for every control contained: 1X PCR Buffer, 0.2 mM each dNTP, 0.4 μM forward primer, 0.4 μM reverse primer, 10U TaqGold, 30 ng template DNA, and water up to a final reaction volume of 50 μl.

Centri-Sep or MinElute purification of the PCR products was performed according to the aforementioned protocols. Quantification of the purified biotin labeled probe was performed via UV spectrophotometry at OD260.

Evaluation of biotin labeling efficiency was performed using a Biotin Chromogenic Detection Kit (Fermentas). Biotin was detected using a dot blot assay which takes advantage of streptavidin's affinity for biotin. In this assay, the biotin labeled probe was bound to a nylon membrane. Streptavidin conjugated to alkaline phosphatase (AP) was applied to the membrane and bound to the biotin labeled probe. Alkaline phosphatase cleaved the substrate, BCIP-T (5-bromo-4-chloro-3-indolyl phosphate, p-toluidine salt) which contained the enhancer chromogen NBT (nitro blue tetrazolium). As a result, a well defined blue spot appeared on the membrane where the probe was applied (26). A dilution series ranging from 300 pg to 0.01 pg was prepared from the biotin labeled probe. One μl of each dilution was spotted onto a nylon membrane (GE). The spots were air dried for 45 minutes. The membrane was placed spotted side down on a UV trans-illuminator for five minutes to immobilize the probe on the membrane. The membrane was washed in Blocking/Washing Buffer for five minutes. Next, the membrane was blocked in Blocking Solution for 30 minutes. The membrane was then incubated in Streptavidin-AP conjugate for 30 minutes. Following incubation, the

membrane was washed two times in Blocking/Washing Buffer for 15 minutes. The membrane was incubated in Detection Buffer for 10 minutes, followed by incubation in Substrate Solution overnight in the dark. All incubation and washing steps were performed at room temperature with moderate shaking. All buffers and solutions were discarded immediately after completion of the step. The reaction was stopped by discarding the Substrate Solution and rinsing the membrane with ddH_2O. Results were recorded immediately after the rinse.

$Vent_R$ (exo-) DNA polymerase

For amplification reactions utilizing $Vent_R$ (exo-) DNA polymerase (New England Biolabs), unmodified dTTP was replaced by Biotin-11-dUTP in a stepwise manner, with biotin-11-dUTP replacing 20, 40, 60, 80, and 100% of dTTP. The DNA template used for $Vent_R$ (exo-) DNA polymerase reactions was a previously generated, unlabeled 310 bp PCR product. For 20% replacement of dTTP with biotin-11-dUTP, the following reaction mixture was used: 1X ThermoPol Reaction Buffer, 0.2 mM dATP, dCTP, dGTP, 0.16 mM dTTP, 0.04 mM biotin-11-dUTP, 0.4 μM forward primer, 0.4 μM reverse primer, 2U $Vent_R$ (exo-) DNA Polymerase, 20 ng template DNA (310 bp PCR product), and water up to a final reaction volume of 50 μl . For 40% replacement of dTTP with biotin-11-dUTP, the following reaction mixture was used: 1X ThermoPol Reaction Buffer, 0.2 mM dATP, dCTP, dGTP, 0.12 mM dTTP, 0.08 mM biotin-11-dUTP, 0.4 μM forward primer, 0.4 μM reverse primer, 2U $Vent_R$ (exo-) DNA Polymerase, 20 ng template DNA (310 bp PCR product), and water up to a final reaction volume of 50 μl. For 60% replacement of dTTP with biotin-11-dUTP, the following reaction mixture was

used: 1X ThermoPol Reaction Buffer, 0.2 mM dATP, dCTP, dGTP, 0.08 mM dTTP, 0.12 mM biotin-11-dUTP, 0.4 μM forward primer, 0.4 μM reverse primer, 2U $Vent_R$ (exo-) DNA Polymerase, 20 ng template DNA (310 bp PCR product), and water up to a final reaction volume of 50 μl. For 80% replacement of dTTP with biotin-11-dUTP, the following reaction mixture was used: 1X ThermoPol Reaction Buffer, 0.2 mM dATP, dCTP, dGTP, 0.04 mM dTTP, 1.6 mM biotin-11-dUTP, 0.4 μM forward primer, 0.4 μM reverse primer, 2U $Vent_R$ (exo-) DNA Polymerase, 20 ng template DNA (310 bp PCR product), and water up to a final reaction volume of 50 μl. For 100% replacement of dTTP with biotin-11-dUTP, the following reaction mixture was used: 1X ThermoPol Reaction Buffer, 0.2 mM dATP, dCTP, dGTP, 0 mM dTTP, 2.0 mM biotin-11-dUTP, 0.4 μM forward primer, 0.4 μM reverse primer, 2U $Vent_R$ (exo-) DNA Polymerase, 20 ng template DNA (310 bp PCR product), and water up to a final reaction volume of 50 μl. The PCR was run with a control without biotin-11-dUTP that contained: 1X ThermoPol Reaction Buffer, 0.2 mM each dATP, dTTP, dCTP, dGTP, 0.4 μM forward primer, 0.4 μM reverse primer, 2U $Vent_R$ (exo-) DNA Polymerase, 20 ng template DNA (310 bp PCR product), and water up to a final reaction volume of 50 μl. The PCR was performed in an ABI 9700 thermal cycler. The incubation step consisted of a two minute denaturation at 95°C, followed by 30X (95°C, 30 sec; 69°C, 30 sec; and 72°C, 5 min), and a hold at 4°C indefinitely.

Centri-Sep or MinElute purification of the PCR products was performed according to the aforementioned protocols. Quantification of the purified biotin labeled probe was performed via UV spectrophotometry at OD260. Evaluation of labeling efficiency was performed according to the aforementioned protocol.

Following evaluation of the probe labeling methods, the $Vent_R$ (exo-) DNA Polymerase PCR procedure with 100% biotin-11-dUTP was utilized to prepare ABO probe 1. The template DNA for the probe labeling reaction was 20 ng of 41 bp PCR product generated with the aforementioned primers designed for ABO probe 1. The specificity of ABO probe 1 was examined via chromogenic detection. 3000-10 pg of DNA from donors A and B was spotted on a nylon membrane. Donor A represents blood group A with a C allele at position 796. Donor B represents blood group B with an A allele at position 796. The membrane was placed spotted side down on a UV trans-illuminator for five minutes to immobilize the DNA on the membrane. The membrane was incubated in prehybridization solution (6X SSC, 5X Denhardt's solution, 0.5% SDS, and 50% deionized formamide) and 50 µg/ml denatured sonicated salmon sperm DNA for four hours at 37°C to 42°C with shaking. The prehybridization solution was discarded, and the membrane was incubated in 1 ml prehybridization solution containing 100 ng/ml denatured ABO probe 1 overnight at 37°C to 42°C with shaking. Following hybridization, the membrane was washed twice with 2X SSC, 0.1% SDS wash buffer for 10 minutes at room temperature. Next, the membrane was washed twice with 0.1X SSC, 0.1% SDS wash buffer for 20 minutes at 65°C. Hybridization of ABO probe 1 to the DNA targets was performed with the Biotin Chromogenic Detection Kit as previously described.

Tyramide Signal Amplification Fluorescence *In Situ* Hybridization (TSA-FISH)

Buccal swabs were collected from two donors not associated with the processing of any samples related to this project. Donor A represented blood group A with a C allele at position 796. Donor B represented blood group B with an A allele at position 796. Cells were eluted from the swabs by agitation in 1X PBS at room temperature for two hours with shaking at 900 rpm. A cell pellet was generated by centrifugation at 3000 rpm for two minutes. The supernatant was removed and the pellet was resuspended in Carnoy's Fixative (3:1 methanol:glacial acetic acid). Twenty µl of the resuspended cell pellet was applied to a glass or PET slide. PET slides were used when subsequent laser microdissection of the detected cells was anticipated. Slides consisted of approximately 10,000 epithelial cells from donor A; 10,000 epithelial cells from donor B; and a 1:1 mixture of 5,000 epithelial cells from donor A and 5,000 epithelial cells from donor B. The slides were briefly steamed over a water bath prior to drying on a heat block at 56°C for two minutes. The cells were heat fixed to the slides at 60°C for two to three hours. Before pretreating the samples, the slides were aged in a desiccator for a minimum of eight hours.

For removal of excess cytoplasm, the slides were pretreated with 1.27 U/µl pepsin buffer for three minutes at 37°C. The slides were then treated with 1X PBS/50mM $MgCl_2$ solution, 2.5% formalin buffer, and 1X PBS for five minutes each at room temperature. Slides were then dehydrated in an ethanol series, 70%, 85% and 100% for one minute each.

Probes were prepared for FISH hybridization by combining biotin labeled probe (50, 100, or 500 ng), 10µl Human COT-1 DNA (500µg, 1mg/ml), and 1µl salmon sperm DNA (10mg/ml). Precipitation of probes was performed by adding 1/10 volume 3M Na-acetate and 2.5-3.0X volume of cold 100% EtOH, vortex mixing the solution, and storing the solution overnight at -20°C. Precipitated probes were centrifuged at 14,000 rpm at 4°C for 20 minutes. The supernatant was removed, and the pellet was dried in a vacuum centrifuge for 10 minutes.

Initial Evaluation

Initial evaluation of the TSA-FISH procedure proceeded with the 310 bp probe (50, 100, or 500 ng) labeled with biotin via a PCR with $Vent_R$ (exo-) DNA polymerase. Five µl of deionized formamide (pH 7.0-7.5) were added to each probe. The sample was mixed and incubated at 37°C for 30 minutes with shaking at 900 rpm. Five µl of 20% dextran sulfate in 2X SSC, pH 7.0 were added to the probe mixture. Slides were dipped in freshly prepared ice cold 70% ethanol for three minutes, followed by 90% and 100% ethanol dips for three minutes each. The probe mixture was applied to the desired sample area, covered with a glass cover slip, and sealed with rubber cement. Codenaturation and hybridization of the probe mixture and sample took place on the StatSpin® ThermoBrite™ Slide Hybridization/Denaturation System. Codenaturation occurred at 80°C for five minutes followed immediately by hybridization at 40°C for 24 hours.

Following hybridization, the slides were washed in 1X SSC/0.05% Tween 20 solution at 73°C for two minutes, followed by a wash in 2X SSC at room temperature for one minute. Tyramide signal amplification was performed using TSA™ Kit #22 (Invitrogen). The slides were blocked with 100 µl 1% blocking reagent in PBS and

incubated at room temperature for 30 minutes. One hundred µl of streptavidin-HRP working solution was applied to the slides and they were incubated at room temperature for 30 minutes. The slides were then washed three times in 1X PBS buffer at 37°C for five minutes each. One hundred µl of tyramide working solution containing 0.0015% H_2O_2 was applied to the slides and they were incubated at room temperature for 10 minutes. The slides were then washed three times in 1X PBS buffer at 37°C for five minutes each. Vectashield Mounting Medium with DAPI (Vector Labs) was applied to the slides. The slides were visualized on the Zeiss PALM Microbeam system.

ABO Probe 1

The sensitivity of ABO probe 1 was assessed to determine whether the number of biotin molecules incorporated into the probe was sufficient for detection. Five µl of deionized formamide (pH 7.0-7.5) were added to the 50 ng pellet of ABO probe 1. The sample was mixed and incubated at 37°C for 30 minutes with shaking at 900 rpm. Five µl of 20% dextran sulfate in 2X SSC, pH 7.0 were added to the probe mixture. Endogenous biotin blocking was performed as previously described. Slides were dipped in freshly prepared ice cold 70% ethanol for three minutes, followed by 90% and 100% ethanol dips for three minutes each. The probe mixture was applied to the desired sample area, covered with a glass cover slip, and sealed with rubber cement. Codenaturation and hybridization of the probe mixture and sample took place on the StatSpin® ThermoBrite™ Slide Hybridization/Denaturation System. Codenaturation occurred at 80°C for five minutes followed immediately by hybridization at 40°C for 24 hours.

Following hybridization, the slides were washed in 1X SSC/0.05% Tween 20 solution at 73°C for two minutes, followed by a wash in 2X SSC at room temperature for

one minute. Tyramide signal amplification was performed as previously described. Vectashield Mounting Medium with DAPI was applied to the slides. The slides were visualized on the Zeiss PALM Microbeam system.

Results

Optimization of Standard FISH Protocol

Membrane Slide Evaluations

STR Results:

PEN membrane slides did not interfere with the downstream analysis of captured cells. Each of the six amplifications generated full (32 alleles) or high partial (30-31 alleles) profiles. The electropherogram below displays a profile generated during these PEN membrane studies (Figure 10).

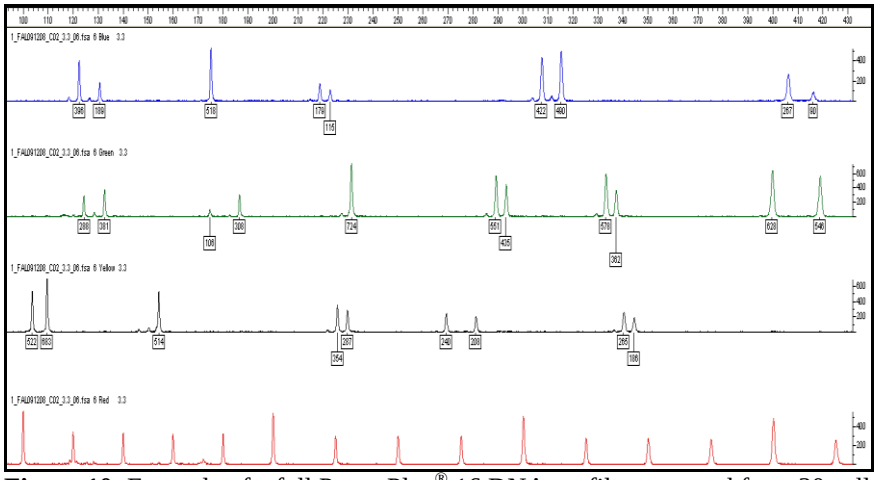


Figure 10: Example of a full PowerPlex® 16 DNA profile generated from 30 cells laser microdissected from a PEN membrane slide.

FISH results:

Hybridizations of Vysis CEP X® alpha satellite probes were successfully accomplished with epithelial cells adhered to PEN membrane slides (Figures 11 and 12).

Incorporation of membrane slides into the FISH protocol drastically reduced both the need for multiple firings of the laser and the catapulting energy necessary to successfully capture the target area. PEN membrane slides were found to require less cutting and catapulting energy than that PET membrane slides; however, PEN membrane slides displayed significant background fluorescence caused by autofluorescence of the PEN membrane. Background fluorescence obscured the DAPI stained epithelial cell nuclei and hindered efficient identification of cellular material. Improved results were achieved with PET membrane slides, which do not exhibit strong background fluorescence. This decrease in PET membrane slide autofluorescence corresponded with increased visibility of the stained nuclei (Figure 13).

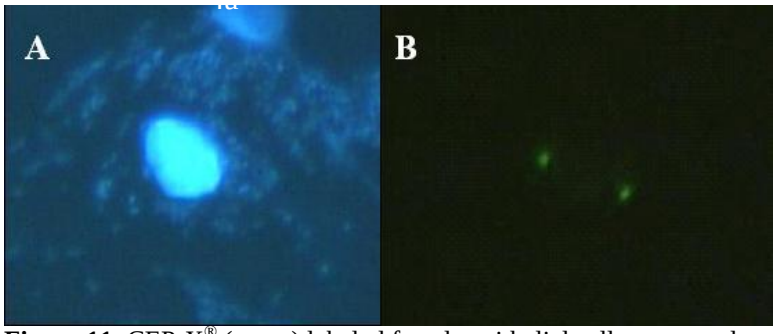


Figure 11: CEP-X® (green) labeled female epithelial cells processed on a PEN membrane slide visualized at 400X magnification through (A) DAPI and (B) FITC filters.

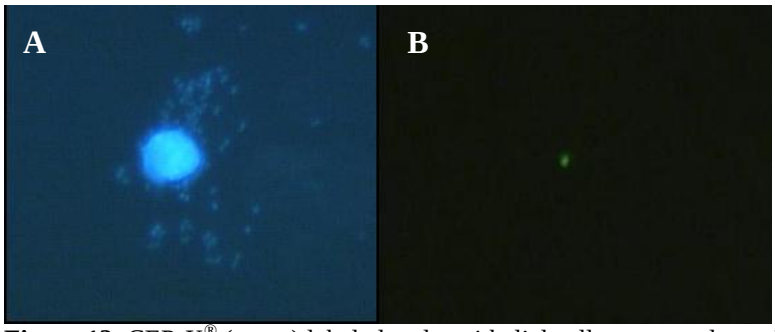


Figure 12: CEP-X® (green) labeled male epithelial cells processed on a PEN membrane slide visualized at 400X magnification through (A) DAPI and (B) FITC filters.

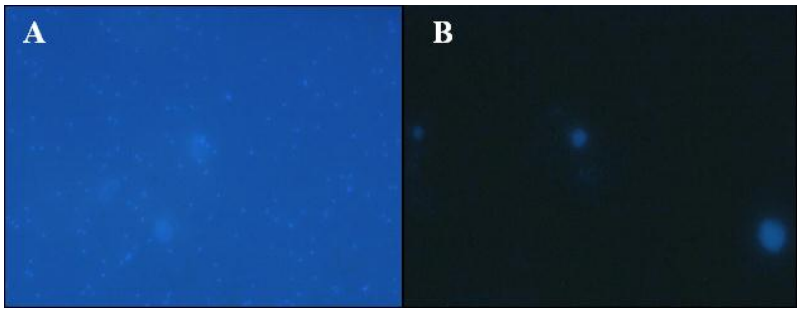


Figure 13: Epithelial cells with a DAPI counterstain visualized at 400X magnification through a DAPI filter. (A) Epithelial cell nuclei on a *PEN* membrane slide. (B) Epithelial cell nuclei on a *PET* membrane slide.

Pepsin Pretreatment Protocol Implementation

The results from buccal cells that were not pretreated prior to hybridization were compared to those that were pretreated prior to hybridization. Figures 14 and 15 display images that were captured using the Zeiss PALM® Microbeam following interphase FISH with the Vysis CEP X® alpha satellite probes and counterstained with DAPI.

As observed in figures 14 and 15, a decrease in background fluorescence was generated from the cells that were subject to the pretreatment procedure. Therefore, based on these results, a pretreatment step was incorporated into the FISH procedure prior to denaturation and hybridization of the DNA probes in order to decrease the background fluorescence observed.

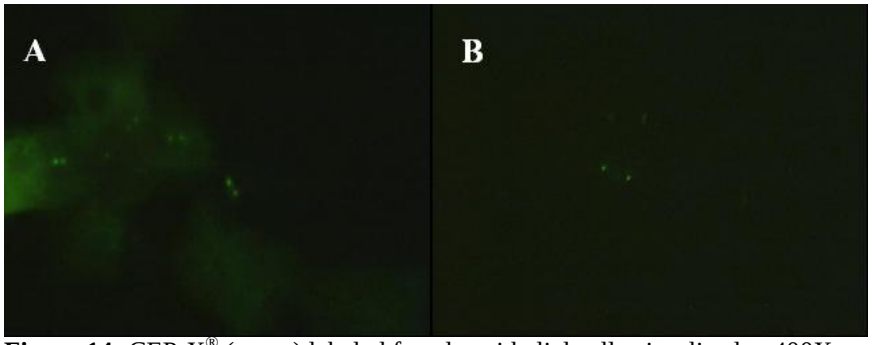


Figure 14: CEP-X® (green) labeled female epithelial cells visualized at 400X magnification through a FITC filter. (A) Epithelial cells that were not subjected to the pretreatment procedure. (B) Epithelial cells that were subject to the pretreatment procedure.

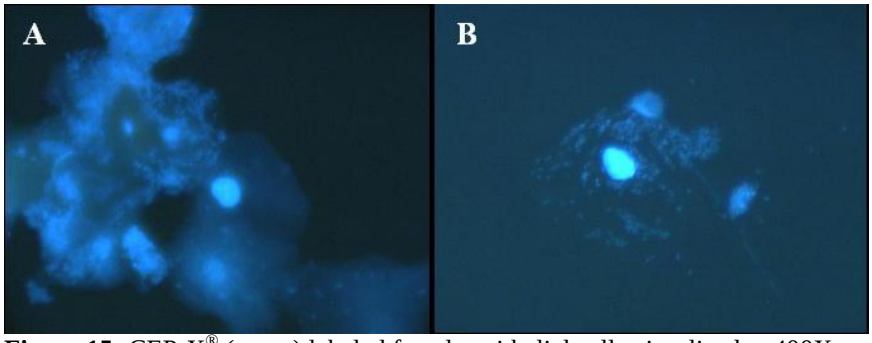


Figure 15: CEP-X® (green) labeled female epithelial cells visualized at 400X magnification through a DAPI filter. (A) Epithelial cells that were not subjected to the pretreatment procedure. (B) Epithelial cells that were subject to the pretreatment procedure to remove excess cytoplasm.

Optimization of FISH Conditions

Initial integration of the ThermoBrite System into the original FISH procedure produced poor results (Figures 16 and 17). While nuclear morphology was acceptable, the FITC and TRITC FISH signals were weak. Altering the denaturation and hybridization temperatures and times produced varied results (Figures 18 and 19). Close examination of the pepsin pretreatment procedure indicated that decreasing the pepsin incubation time may improve the probe signals (Figures 20 and 21). Improved FISH signals were seen using the following conditions: elimination of the initial 2X SSC incubation, reduction of the pepsin incubation to two minutes, codenaturation of probes and sample at 73˚C for five minutes, hybridization at 42˚C for 45 minutes, and reduction of the stringency of first post-hybridization to 1X SSC (Table 5).

Figure 16: Weak FISH results after initial incorporation of ThermoBrite System with (A) DAPI, (B) FITC, and (C) TRITC filters.

Figure 17: Weak FISH results after initial incorporation of ThermoBrite System with (A) DAPI, (B) FITC, and (C) TRITC filters.

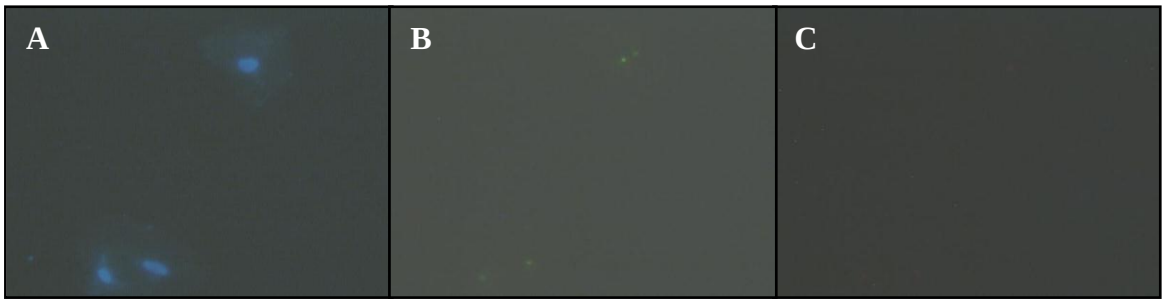


Figure 18: Lower hybridization temperature: inconsistent hybridization viewed in the same area of a slide. Three nuclei were visible under the (A) DAPI filter. One cell demonstrated visible (B) FITC and (C) TRITC signals. Weak signals were viewed in the other cells.

Figure 19: Nine minute denature, 6 hour 37°C hybridization, elimination of all heated wash steps. One cell was visible under the (A) DAPI filter. The cell displayed a strong (B) FITC signal and weak (C) TRITC signal.

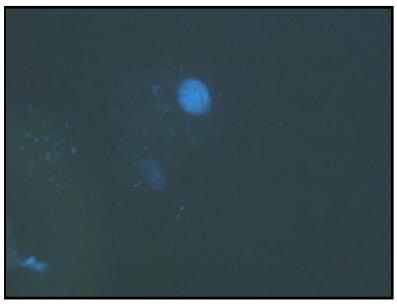


Figure 20: Integration of 10 minute pepsin step resulted in possible damage to nuclear material. The probe signals appeared weak and diffuse.

Figure 21: Integration of a two minute pepsin step resulted in consistent hybridization and improved signal intensities under (A) DAPI, (B) FITC, and (C) TRITC filters.

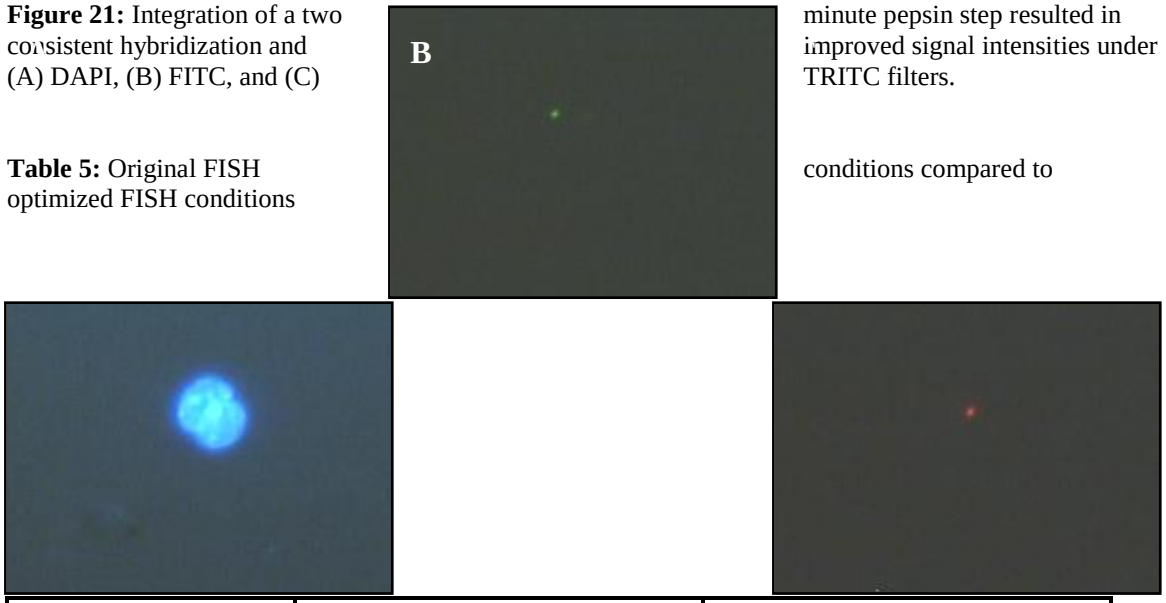


Table 5: Original FISH conditions compared to optimized FISH conditions

Step	Original FISH Procedure		Optimized FISH Procedure	
	Time	Temperature	Time	Temperature
2X SSC incubation	2 minutes	room temperature	N/A	N/A
Pepsin treatment	10 minutes	room temperature	2 minutes	room temperature
Probe denaturation	5 minutes	80°C	5 minutes	73°C
Slide denaturation	5 minutes	80°C		
Hybridization	overnight	37°C	45 minutes	42°C
0.4X SSC / 0.05% Tween 20 Wash	2 minutes	73°C	N/A	N/A
1X SSC / 0.05% Tween 20 Wash	N/A	N/A	2 minutes	73°C

Bioinformatic Analysis of the Duffy and ABO Markers

Following bioinformatic analysis, the resolution of the data set was not at the level that allows for the leveraging of findings for the identification of ABO categorical SNPs. The reconstructed phylogenetic gene tree consisted of a large polytomy of unresolved relationships. It was expected to see nested clades of A, B, and O alleles grouping together into distinct and separate groupings, coalescing into single tree nodes

or bifurcation points. However, analysis revealed a few nested clades, but the vast majority of the haplotypes were intermixed with, for example, some of the A alleles more closely related to O alleles than the other A's. It was possible to identify a few small groups of similar alleles and the SNPs that identify them. None of these were very useful for unknown cell origin differentiation as the contained alleles comprised a small percentage of the overall ABO gene types. The bootstrapped ABO gene family trees are available upon request, as their large size makes them clumsy candidates for inclusion into a report of this nature. Data suggested that there are extremely low levels of genetic structure within the ABO gene group based on sequence data. This led to the determination that the ABO blood group is a challenging candidate for LM separation, as an extremely large number of probes (more than a dozen per group) would be needed to categorize all subgroups into their respective ABO blood groups, making the final probe panel unusable on forensic samples. Despite this, it was possible to design a probe panel that differentiated a majority of the subgroups, with some exceptions, into their respective blood groups.

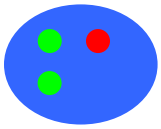
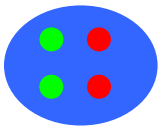
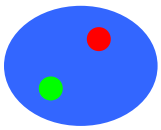
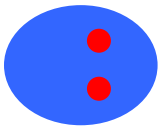
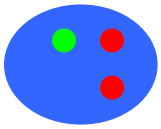
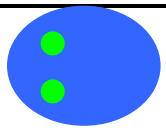
ABO Probe Design

Primers were used to successfully produce probes within the desired parameters (Table 6). ABO probe 1 is 41 bp in length with a 56.1% GC content. ABO probe 2 is 40 bp in length with a 57.5% GC content. It was proposed that following hapten labeling of the amplicons, the resulting probes could be used to differentiate cellular mixtures comprised of different blood types (Table 7).

Table 6: Double stranded DNA probe sequences

ABO Probe #	SNP	Allele	Color	Length (bp)	Final Probe Sequence
1	796	C	●	41	caggcctacatccccaaggacgagggcgatttctactacct gtccggatgtaggggttcctgctcccgctaaagatgatgga
2	261	G	●	40	acgcctctctccatgtgcagtaggaaggatgtcctcgtgg tgcggagagaggtacacgtcatccttcctagaggagcacc

Table 7: Expected ABO blood group specific FISH probe signals for the most common ABO alleles

Phenotype	Genotype	796 Allele	261 Allele	Probes
Blood Group A	A	C ●	G ●	
	O	C ●	deletion	
Blood Group A	A	C ●	G ●	
	A	C ●	G ●	
Blood Group B	B	A	G ●	
	O	C ●	deletion	
Blood Group B	B	A	G ●	
	B	A	G ●	
Blood Group AB	A	C ●	G ●	
	B	A	G ●	
Blood Group O	O	C ●	deletion	
	O	C ●	deletion	

Biotin Labeling of DNA Probes

Nick Translation

Nick translation produced a total yield of 150-400 ng of biotin labeled probe (Table 8). Yields of biotinylated DNA probes were considerably lower than the 1000 ng of input DNA. To generate significant amounts of probes, many reactions must be performed using large amounts of input DNA. The time associated with the labeling procedure, including PCR, purification of the PCR product, quantification of the purified PCR product, nick translation, purification of the biotin labeled product, and quantification of the purified biotin labeled product, is approximately seven to eight hours. Nick translation labeling of PCR products with biotin was abandoned due to low yields and lack of ability to control reaction conditions and biotin incorporation.

Table 8: Yield of biotin labeled 310 bp probes generated via nick translation

310 bp Probe	Total Input DNA (ng)	Total Labeled Product Output (ng)	DNA Loss After Labeling (%)
1	1000	150	85
2	1000	185	81.5
3	1000	400	60
4	1000	310	69

Polymerase Chain Reaction

When biotin labeled PCR products were visualized on 2% agarose gels with ethidium bromide, products produced bands approximately 300 bp in size. A 100 bp ladder was run with each gel for comparison purposes. As the concentration of biotin-11-dUTP in the reaction mixtures increased, an upward shifted product band was detected (Figures 22 and 23). This change in amplicon mobility was due to the large bulk introduced by the incorporated biotin molecules. Controls containing only natural dNTPs produced the expected results of approximately 300 bp. Quantification of the purified

PCR products showed that as the concentration of biotin-11-dUTP in the reaction mixtures increased, the yields of the PCR products decreased (Table 9). This result was anticipated as other research suggested that increased incorporation of biotin is achieved at the expense of yield (21). However, direct comparison of the AmpliTaq Gold DNA polymerase yield results was difficult as each reaction was subject to different thermal cycling parameters. The labeling efficiency of the PCR reaction is acceptable if 0.1 pg-0.03 pg is easily detected. Evaluation of labeling efficiencies indicated that all PCR products regardless of biotin concentrations are inefficiently labeled (Table 10). Little to no change in labeling efficiency was observed between the AmpliTaq Gold polymerase chain reactions. $Vent_R$ (exo-) DNA Polymerase allowed for 100% of dTTP to be replaced with biotin-11-dUTP. All subsequent reactions were performed with $Vent_R$ (exo-) DNA Polymerase to allow for maximum biotin incorporation into the resulting double stranded DNA probes.

The 41 bp long ABO probe 1 was successfully labeled using the $Vent_R$ (exo-) DNA Polymerase PCR procedure (Figure 24). The sequence of the biotinylated probe is seen in Figure 25. Six biotin molecules were incorporated into the sense strand of the probe, and five biotin molecules were incorporated into the antisense strand. Chromogenic detection results indicated that increasing the stringency of the hybridization would not allow the probe to discriminate between the two donors. As the hybridization temperature was raised from 37°C to 42°C, ABO probe 1 continued to bind to DNA from both donors (Table 11). Evaluation of the probe specificity revealed that one base pair difference was insufficient for probe differentiation of the two donors.

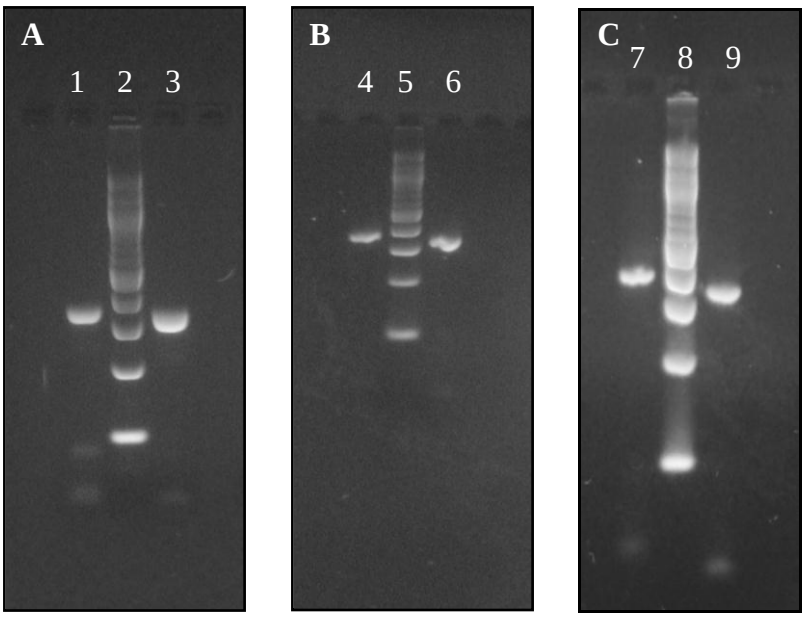


Figure 22: PCR products from AmpliTaq Gold® DNA polymerase amplifications containing: (A) 0.05 mM (25%) biotin-11-dUTP. Lane 1 contains biotin labeled PCR product. Lane 2 contains 100 bp ladder. Lane 3 contains the 0mM biotin-11-dUTP control; (B) 0.10 mM (50%) biotin-11-dUTP. Lane 4 contains biotin labeled PCR product. Lane 5 contains 100 bp ladder. Lane 6 contains the 0mM biotin-11-dUTP control; (C) 0.15 mM (75%) biotin-11-dUTP. Lane 7 contains biotin labeled PCR product. Lane 8 contains 100 bp ladder. Lane 9 contains the 0mM biotin-11-dUTP control.

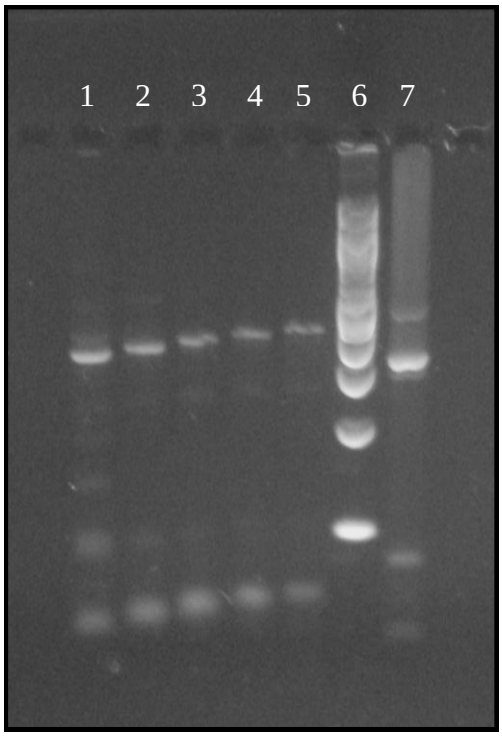


Figure 23: PCR products from AmpliTaq Gold® DNA polymerase amplifications containing: lane 1, 0.04mM (20%) biotin-11-dUTP; lane 2, 0.08mM (40%) biotin-11-dUTP; lane 3, 0.12mM (60%) biotin-11-dUTP; lane 4, 0.16mM (80%) biotin-11-dUTP; lane 5, 0.20mM (100%) biotin-11-dUTP; lane 6, 100 bp ladder; and lane 7, 0mM biotin-11-dUTP control.

Table 9: Comparison of the yields of the AmpliTaq Gold and $Vent_R$ (exo-) DNA polymerase purified biotin labeled PCR products

Percent dTTP replaced by biotin-11-dUTP	Biotin-11-dUTP Concentration (mM)	AmpliTaq Gold® DNA Polymerase Total Yield Labeled PCR Product (ng)	$Vent_R$ (exo-) DNA Polymerase Total Yield Labeled PCR Product (ng)
20%	0.04	N/A	1300
25%	0.05	1950	N/A
40%	0.08	N/A	950
50%	0.10	1900	N/A
60%	0.12	N/A	1100
75%	0.15	1450	N/A
80%	0.16	N/A	650
100%	0.20	N/A	750

Table 10: Comparison of the labeling efficiencies of the AmpliTaq Gold and $Vent_R$ (exo-) DNA polymerase biotin labeling PCRs

Percent dTTP replaced by biotin-11-dUTP	Biotin-11-dUTP Concentration (mM)	AmpliTaq Gold® DNA Polymerase Labeling Efficiency Detection Limit (pg)	$Vent_R$ (exo-) DNA Polymerase Labeling Efficiency Detection Limit (pg)
20%	0.04	N/A	3
25%	0.05	3	N/A
40%	0.08	N/A	1
50%	0.10	1	N/A
60%	0.12	N/A	3
75%	0.15	3	N/A
80%	0.16	N/A	3
100%	0.20	N/A	1

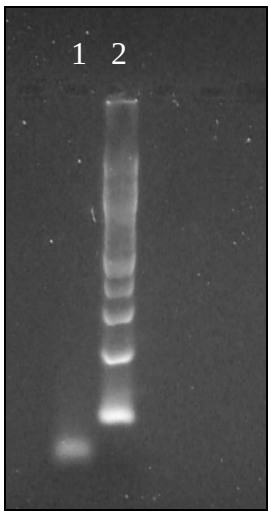


Figure 24: Yield gel results for biotin labeled ABO probe 1. Lane 1 contains the 41 bp long probe produced via PCR with $Vent_R$ (exo-) DNA Polymerase and 100% incorporation of biotin-11-dUTP. Lane 2 contains a 100 bp ladder.

Figure 25: Sequence of biotin labeled double stranded DNA probe. During PCR, 100% of dTTP was replaced with biotin-11-dUTP.

Table 11: Probe specificity to donors from blood groups A and B. Probe continues to hybridize to DNA from both donors despite increases in hybridization stringency

Temperature (°C)	Donor A Detection (pg)	Donor B Detection (pg)
37	300	300
38	300	300
39	300	300
40	300	300
41	300	300
42	3000-1000	3000-1000

Tyramide Signal Amplification Fluorescence *In Situ* Hybridization (TSA-FISH)

Initial Evaluation

Results from the initial evaluation were examined for the presence of probe signals and high background fluorescence. Potential probe signals were observed in the samples for all three probe concentrations. All samples displayed regions of concentrated fluorescence that could result from successful hybridization. Due to the presence of background fluorescence caused by nonspecific staining, it was difficult to determine whether detected signals were the result of specific hybridization. Samples hybridized with 500 ng of ABO probe 1 displayed significant nonspecific staining under the FITC filter. Although true probe signals may be present, they are completely masked by the

background fluorescence (Figure 26). Samples hybridized with 100 ng of ABO probe 1 exhibited less background fluorescence than was seen in the 500 ng samples. Potential probe signals are visible but may still be obscured by nonspecific staining (Figure 27). Samples hybridized with 50 ng of ABO probe 1 express minimal nonspecific staining. Nonspecific staining is least likely to interfere with probe signal detection at this concentration (Figure 28). Results indicated that background fluorescence decreases with probe concentration. These findings corroborated with those of Yang et al (27). ABO probe 1 testing proceeded with 50 ng of probe. Reduced probe concentration was expected to reduce or eliminate nonspecific signals while maintaining observable specific signals.

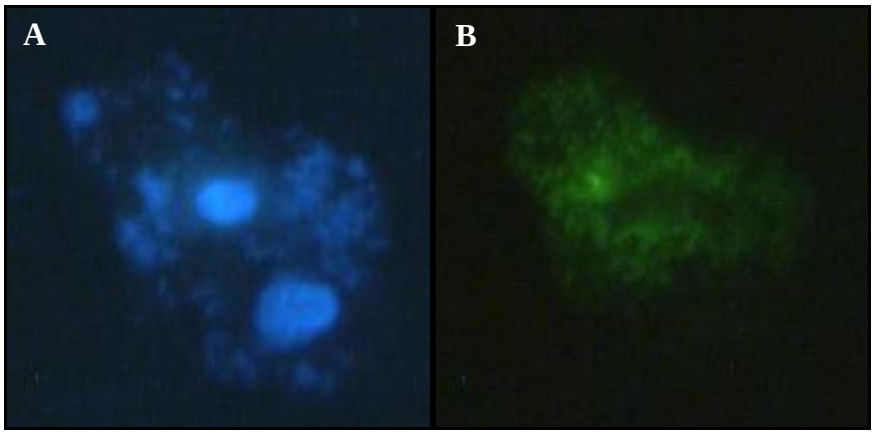


Figure 26: Epithelial cells hybridized with 500 ng of ABO probe 1 visualized at 400X magnification with (A) DAPI and (B) FITC filters.

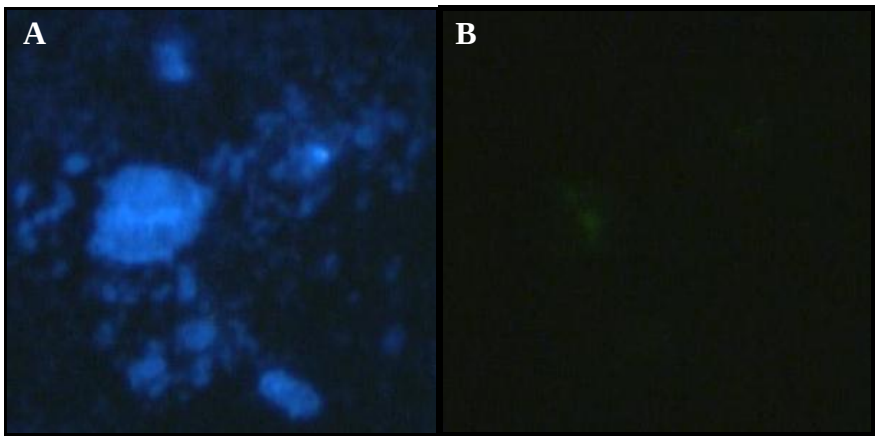


Figure 27: Epithelial cells hybridized with 100 ng of ABO probe 1 visualized at 400X magnification with (A) DAPI and (B) FITC filters.

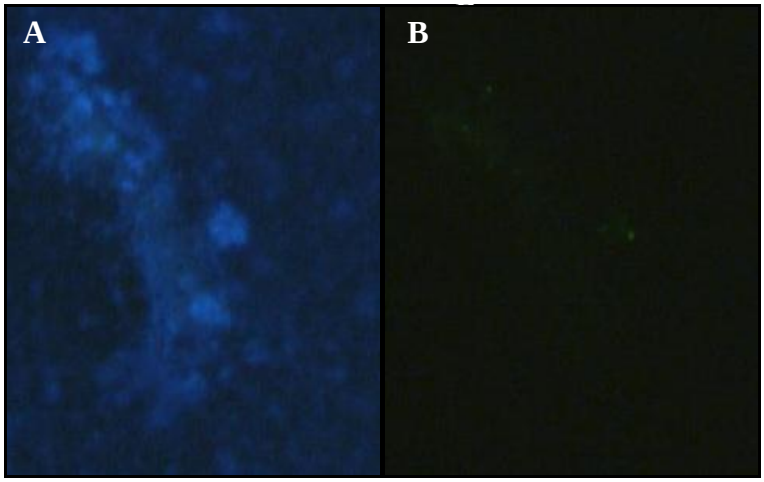


Figure 28: Epithelial cells hybridized with 50 ng of ABO probe 1 visualized at 400X magnification with (A) DAPI and (B) FITC filters.

ABO Probe 1

Results from ABO probe 1 were examined for the presence of probe signals and high background fluorescence resulting from nonspecific staining. Specific probe signals were not observed in any of the samples. Although minimal nonspecific staining was present, it was observed in some areas of the slides. Hybridization of ABO probe 1 to epithelial cells from donor A resulted in no detectable probe signals and some minor nonspecific hybridization under the FITC filter (Figure 29). Hybridization of ABO probe 1 to epithelial cells from donor B produced similar results to those seen with donor A

(Figure 30). Hybridization of ABO probe 1 to a mixture of epithelial cells from donors 1 and 2 resulted in no detectable probe signals and minimal to no nonspecific staining under the FITC filter (Figure 31).

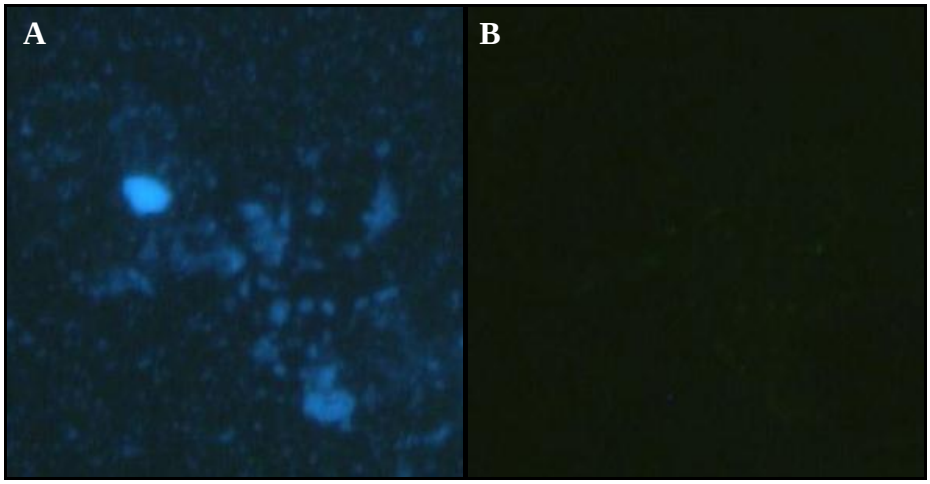


Figure 29: Epithelial cells from donor A hybridized with ABO probe 1 visualized at 400X magnification with (A) DAPI and (B) FITC filters.

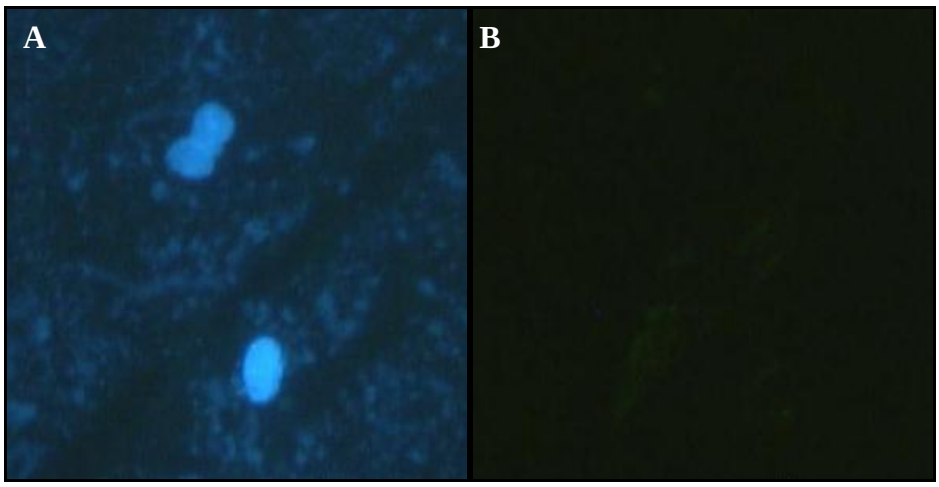


Figure 30: Epithelial cells from donor B hybridized with ABO probe 1 visualized at 400X magnification with (A) DAPI and (B) FITC filters.

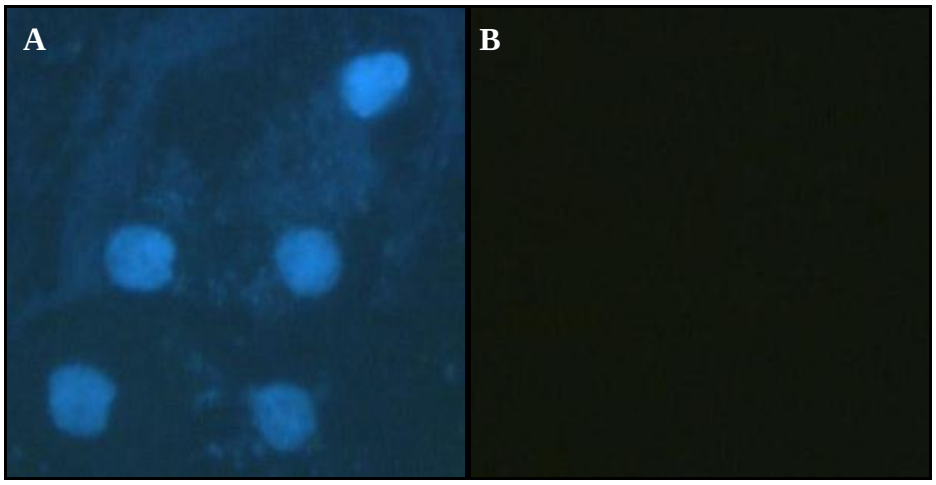


Figure 31: A mixture of epithelial cells from donors A and B hybridized with ABO probe 1 visualized at 400X magnification with (A) DAPI and (B) FITC filters.

Conclusions

Discussion of Findings

The key objective of this study was to separate cellular mixtures of similar morphology and same gender using laser microdissection and sequence specific fluorescence *in situ* hybridization (FISH) probes. The Duffy and ABO blood grouping systems were chosen as the basis for the FISH probes because of the existence of their prevalent polymorphisms throughout multiple populations and the known DNA sequence associated with each blood group. The following five research tasks were performed to develop FISH probes associated with ABO blood groups to separate cell mixtures: i. Optimization of standard FISH protocol to enhance signal amplification, ii. Phylogenic analysis of the Duffy and ABO blood group, iii. ABO probe design, iv. Biotin labeling of the DNA probes and v. Tyramide signal amplification (TSA) of the FISH ABO probes.

The results from this study indicate that FISH probes could not be developed to reliably differentiate between cells with different ABO blood groups. Therefore this project was not successful in developing a FISH LM technique to differentiate cellular

mixtures consisting of the same morphology and same gender. However, the project did develop and optimize methods that improved the signal intensity and reduced background fluorescence associated with FISH. These optimized methods can be used in forensic laboratories to separate mixtures containing male and female cells with the same cell morphology.

A phylogenetic analysis of the ABO blood group including all known subgroups was performed to identify key SNPs responsible for inclusion into each of the three main blood groups. Bioinformatic analysis of the ABO group based on sequence data showed extremely low resolution. Rather than the terminal branches of subgroups coalescing back into three major branches, the resultant tree contained limited branching overall due to the unresolved relationships among the genes. A phylogenetic assessment of ABO, based solely on sequence data, suggests little if any relationship or structure among the ABO subgroups. This is most likely due to an interaction between the retention of amino acid structure for specific antigen production and the degeneracy of the amino acid code. A further study on the breakdown between base pair position and transitions and transversions responsible for recreation of each of the ABO subgroups would help to further elucidate this issue. It is unlikely that a SNP FISH panel can be devised to differentiate all the ABO alleles into the groups in which they are expressed phenotypically. However, a SNP FISH panel was devised that could differentiate a majority of the alleles in the ABO system, including the five most common alleles: A101, A201, B101, O01 and O02 (1, 2, and 3).

The findings of this study indicated that the FISH techniques utilized are unsuitable for differentiation of the ABO blood groups. Manipulation of the hybridization

stringency suggested that with these techniques a single base pair difference is insufficient for differentiation between the ABO blood groups. Traditional double stranded DNA probes are not suitable for discriminating between sequences that differ by one base pair.

An additional area of concern was the sensitivity of the ABO probes. Specific probe signals were not observed following sample hybridization with ABO probe 1. Failure to detect probe signals may have resulted from an insufficient number of biotin molecules present in the probe, excessive nonspecific staining, or a combination of the two factors. Insufficient biotinylation is the most probable cause given that potential probe signals were observed in samples hybridized to longer probes but not those hybridized to shorter probes. The longer probes were expected to contain more biotin molecules than the shorter probes. This indicated that amplification is not an optimum method of biotin incorporation for probes of this length (approximately 40 bp). Labeling of the probe with biotin by amplification is dependent on a sufficient amount of thymine present in the sequence. It may be difficult to design probes that meet all the desired criteria as well as contain a sufficient number of thymine nucleotides. Chemical synthesis may be a more suitable method of probe labeling than by amplification or nick translation.

Nonspecific staining is another factor that may contribute to probe detection issues. Although nonspecific staining may be mitigated by endogenous biotin blocking and reduction of probe concentration, it cannot be completely eliminated. The presence of even minimal nonspecific staining can obscure true probe signals. This type of background fluorescence can be caused by nonspecific biotin detection or nonspecific

hybridization of the probe to DNA other than the target. Endogenous biotin may be detected in cells even with the use of endogenous biotin blocking procedures. A simple solution to nonspecific staining caused by nonspecific biotin detection is to replace biotin with a hapten that does not occur naturally within human cells. Nonendogenous haptens are compatible with TSA-FISH techniques and should not result in nonspecific detection events. Nonspecific hybridization of the probe to DNA other than the target sequence can be alleviated through a variety of approaches. It is important to ensure that the stringency of the hybridization is high enough to reduce nonspecific hybridization events, but low enough to allow the probe to hybridize to the target sequence. Similarly, the post hybridization wash conditions should be stringent enough to remove loosely bound or unbound probes, but not so stringent that specifically hybridized probes are removed. Optimum stringency conditions should be determined empirically. Typically, nonspecific hybridization should not be addressed by increasing the length of the probes. First, a minimum of 18 bp should be sufficient to create a probe specific to a desired DNA target under optimum hybridization and wash conditions. Second, FISH probes are subject to rigid size parameters dependent on the type and purpose of the probe. It is not recommended to increase the probe length in most instances.

Although this research was not able to produce a successful FISH assay for discriminating the ABO blood groups, important techniques were developed and optimized for FISH LM, which can be applied to the separation of mixtures of male and female cells that have the same cell morphology. Methods were optimized to reduce the amount of background fluorescence. When samples fixed to polyethylene naphthalate (PEN) and polyethylene teraphthalate (PET) membrane slides were subjected to standard

FISH testing with Vysis CEP X® and Y® alpha satellite probes, it was observed that PEN slides are highly auto fluorescent whereas the PET slides demonstrate little to no auto fluorescence. Increased background signal caused by auto fluorescence may interfere with fluorescent probe signal interpretation. These results indicated that PET slides, not PEN slides, should be incorporated into fluorescent LM procedures. Furthermore, the research demonstrated that pretreatment of PET slides with pepsin buffer, followed by post-fixation of the cells in buffered formalin can significantly reduce the amount of background fluorescence thereby increasing the sensitivity of the probe system.

Working protocols for FISH LM were developed for techniques not currently used in the forensic field. The techniques that were developed for the hapten labeling of FISH probes using biotin may prove beneficial in future LM research. With further optimization, tyramide signal amplification (TSA) may be a valuable technique in forensic applications of fluorescent microscopy. While the first attempt to adapt these techniques for forensic use was unsuccessful to differentiate ABO blood groups, the basic techniques of TSA were developed and can be used to increase the signal intensity and sensitivity of FISH probes associated with genetic marker systems being developed for future forensic research.

Implications for Policy and Practice

At this time, these findings have no direct impact on policy or practice. The research indicated that the FISH probe method could not be used to separate mixtures of cells from individuals with different ABO blood groups. Based on this research, FISH laser microdissection cannot be applied to separating cellular mixtures that contain the

same cell morphology and same gender. However, previous research has shown that laser microdissection techniques can be used to separate forensic mixtures consisting of cells that differ in morphology, gender, and the separation of biological cells from soil or other particulate matter (6,7,8,9,27,28).

The research performed has indicated that there are FISH techniques, such as tyramide signal amplification, that previously have not been explored for forensic applications. Many of these techniques have been utilized for years in clinical and diagnostic applications. Consideration of alternative FISH methods could expand forensic LM-FISH capabilities beyond standard X and Y chromosome FISH probing, thus expanding the forensic uses of laser microdissection to separate mixtures of cells from evidentiary samples.

Implications for Further Research

This research has laid the groundwork for NIJ award number 2009-DN-BX-K250, "Identification and Separation of Evidence Mixtures Using SNP-Based FISH Techniques and Laser Microdissection." NIJ award number 2009-DN-BX-K250 proposed the development of a fluorescent probe screening system based upon human genetic single nucleotide polymorphisms (SNPs) that could provide a basis for the separation of mixture samples with LM technology. Based on ABO probe research, alternative types of FISH probes will be examined to determine if any might be suitable for this level of discrimination. Also, the probes will be labeled with haptens other than biotin to eliminate the effects of nonspecific biotin detection. Other haptens that are commonly used with TSA-FISH procedures include digoxigenin and dinitrophenol. To address issues related to insufficient biotinylation of the probes, probes for NIJ award number

2009-DN-BX-K250 will be chemically synthesized with a specified amount of digoxigenin or dinitrophenol incorporated by a commercial manufacturer.

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Dissemination of Research Findings

The NIJ Conference 2009, Washington, D.C., July, 2009

- Poster Presentation: "Physical Separation and STR Analysis of Forensic Mixtures Using Laser Microdissection and Fluorescence In Situ Hybridization" R. Driscoll, H. Cunningham and R. Bever

19th International Symposium on Human Identification, Madison, WI., October, 2008.

- Poster Presentation: "Physical Separation and STR Analysis of Forensic Mixtures Using Laser Microdissection and Fluorescence In Situ Hybridization" R. Driscoll, H. Cunningham and R. Bever