Identification and Separation of Evidence Mixtures Using SNP-Based FISH Techniques and Laser Microdissection
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Final Technical Report

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Abstract

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 be easily separated based upon morphological differences, mixtures of the same cell type are more difficult to separate. Past research has demonstrated that male/female cellular mixtures of similar morphology can be successfully separated using X/Y chromosome fluorescence *in situ* hybridization (FISH) probing. The goal of this research was to separate cellular mixtures of the same morphology and gender by developing FISH probes based on human genetic single nucleotide polymorphisms (SNPs). Screening panels of SNP FISH probes were intended to visually detect the individual contributors of sample mixtures, while laser microdissection would physically separate the cells for further STR processing. Padlock probes paired with tyramide signal amplification and rolling circle amplification were examined as methods of achieving this goal. Findings indicated that the FISH techniques utilized in this research were unsuitable for the detection of single SNP differences between individuals. Differentiation may be achieved in the future through research of other rolling circle amplification methods or by pursuing genetic marker systems that contain larger genetic differences. Multiple improvements in LM sample processing techniques were also examined. The use of cytogenetic on-slide lysis techniques and direct placement of LM collected cells into amplification reactions were evaluated as methods that would allow for the direct collection of nuclei and eliminate the need for DNA extraction prior to amplification. In addition, various amplification systems, amplification additives, and extraction techniques were investigated as alternative methods for processing samples in labs utilizing LM technologies. Although the on-slide lysis and direct amplification techniques were

incompatible with this type of sample, the ZyGEM *forensic*GEM™ Saliva extraction and Promega's PowerPlex® 16 HS System showed promise for work with LM collected samples. These techniques would also be ideal for labs attempting to process difficult evidence containing low copy number (LCN) cellular mixtures. The completed results obtained from this research will be disseminated through the law enforcement and scientific communities via seminars, journal articles, and poster presentations.

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Executive Summary

Laser microdissection (LM) is an effective method for the separation of cellular mixtures in the forensic laboratory. LM uses laser energy to isolate and collect cells of interest from biological samples and mixtures. A laser is used to cut target cells, which are transferred to a collection tube with a pulse of laser energy. In the last ten years, this technique has been used to separate individual cells from forensic cellular mixtures. 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. It has been demonstrated that male/female cellular mixtures of similar morphology can be successfully separated using X/Y chromosome fluorescence *in situ* hybridization (FISH) probing. Although some progress has been made in separating cellular mixtures, the generation of clean, single source genetic profiles from cellular mixtures of the same morphology and gender remains a challenge in the field of forensics. The goal of this research was to develop FISH probes based on human genetic single nucleotide polymorphisms (SNPs) that would provide a basis for LM separation of samples of the same morphology and gender. Screening panels of SNP based FISH probes were intended to visually detect the individual contributors in sample mixtures, and LM technologies would physically separate the cells for further STR processing. Multiple improvements in LM sample processing techniques were also examined as alternative options to commercial DNA kits. Cytogenetic on-slide lysis techniques and the direct placement of LM collected cells or nuclei into amplification reactions were evaluated. The goal of this research was to use these methods to reduce the number of cells required for DNA profiling and eliminate the need for a DNA extraction step. Alternative extraction and amplification techniques were also examined. Incorporation of all of the aforementioned LM and FISH procedures was intended to provide alternative methods for

processing samples to labs interested in utilizing LM technologies. The research performed can be divided into three distinct sections:

- Development and evaluation of SNP specific hybridization probes
- Improvement of front end cellular preparations via cytogenetic membrane lysis
- Optimization of post collection processing techniques for use with LM collected samples

Development and Evaluation of SNP Specific Hybridization Probes

SNP Loci Evaluation

Candidate SNP sites were verified by sequencing a panel of individuals representing diverse ethnic and cultural backgrounds to assess and avoid any potential ethnic bias or binding site mutations in the final probe set. It was necessary for the FISH assay to contain SNPs that were both stable and informative. The three main criteria for SNP selection were low Fst values, high heterozygosity, and functional non-linkage.

Nineteen potential SNP sites were identified for possible inclusion in the final SNP FISH assay. Donors representing Caribbean American, Egyptian, Caucasian, Chinese, Polish, German, African American, and Filipino ancestries were genotyped at each of the 19 SNP sites using the ABI PRISM® SNaPshot™ SNP genotyping kit. Once genotyped, the random match probability was calculated for each sample using each of the three available population statistics. No evidence of ethnic bias was seen in any of the samples, meaning that the difference between the statistics using each of the allele frequency databases was not statistically significant. Based on these results, two SNP locations were selected for probe design.

Probe Design and Manufacture

Traditional FISH probes are not typically designed to distinguish between DNA sequences that vary by only one SNP difference. Accordingly, they were deemed unsuitable for this research. In the early 1990s, padlock probes were developed to improve both the specificity and sensitivity of traditional FISH probes. As the proposed assay required a high degree of specificity, padlock probes were explored as a method of identifying SNPs *in situ*. Probes were designed for use with two methods of probe detection: tyramide signal amplification and rolling circle amplification.

Due to the cost of probe manufacture, probes were initially designed and manufactured for only one SNP location. Padlock probes were designed using ProbeMaker software, which allows the user to input a target sequence and specific parameters for probe design.

Strict parameters regarding GC content, length, and melting temperature (T_m) were applied to the probe design. The probes were chemically synthesized by a commercial manufacturer (Eurofins MWG Operon). Although expensive, chemical oligonucleotide synthesis ensured both accurate probe construction and adequate hapten incorporation into the probe.

Probe Evaluation

Tyramide signal amplification and rolling circle amplification padlock probes were applied to slides containing cells from two donors. Donor A possessed a T allele at SNP position 15. Donor B possessed a C allele at SNP position 15. A variety of hybridization and detection conditions were evaluated for both probe types. Following optimization to reduce background fluorescence, neither set of probes produced probe signals specific to the desired SNP.

Improvement of Front End Cellular Preparation via Cytogenetic Membrane Lysis

Cytogenetic on-slide lysis techniques were intended to eliminate the need for commercial DNA extraction kits when using LM processed samples. Cytogenetic on-slide lysis is a method of isolating nuclei through osmotic manipulation and rupture of cellular membranes. Elimination of standard DNA extractions would allow for fast and efficient processing of low copy samples while avoiding the sample loss that occurs during extraction. This process would also save valuable time and money when compared to the commercial DNA extraction kits that are employed in forensic laboratories today.

Cytogenetic membrane lysis techniques were used to lyse buccal and white blood cells as they were deposited on a PEN membrane slide. The majority of the buccal cells and white blood cells were successfully lysed, resulting in isolation of the nuclei. Free nuclear material from the buccal cell and white blood cell slides was visualized and collected with the Zeiss PALM Microbeam System. These LM collected samples were then subjected to a brief protease digestion before direct input into a PowerPlex® 16 HS amplification reaction.

No genetic profiles were obtained from any of the samples. Along with poor results, the necessity of "dropping" samples onto the slide caused this technique to be unsuitable for forensic use. The dropping technique is difficult to control with precision and may result in loss of the sample.

Optimization of Laser Microdissected Sample Processing Techniques

This research proposed to eliminate the DNA extraction stage of processing LM collected samples in favor of using direct amplification to improve the speed and sensitivity of low copy

number (LCN) sample analysis. Critical amounts of LCN sample template DNA would be conserved by eliminating standard DNA extraction procedures. Direct amplification of cells was evaluated with several STR amplification kits that include hot start *Taq* DNA polymerases in their reaction buffers. It was hypothesized that the initial high temperature step required to activate the polymerase would aid in cell membrane lysis and release the target DNA for amplification. Further evaluations were performed to examine the use of a brief protease digestion to help facilitate the release of DNA prior to direct amplification. Alternative extraction and amplification methods that can be used to improve the speed and sensitivity of DNA recovery were also examined. All experiments were performed on buccal cells collected from donors with known profiles.

Direct Amplification of Cellular Suspensions

Experiments were first performed to determine if direct amplification of cells was a valid proposition. To accomplish this, varying amounts of buccal cells suspended in 1X PBS were directly input into AmpFℓSTR® MiniFiler™ PCR Amplification Kit, PowerPlex® 16 HS System , and PowerPlex® S5 System amplification reactions. All samples produced the expected profiles. Some dropout events were seen in the 50 cell samples. These results indicated that no further lysis steps were necessary for successful amplification. Although it produced satisfactory results, the PowerPlex® S5 System was eliminated from future tests. The profiles generated by the system were of nominal statistical value because only five loci are examined.

Direct Amplification of Laser Microdissected Cells

Once the direct amplification of cellular suspensions was determined to be viable, laser microdissected cells were prepared for direct amplification. The number of cells to be collected

and amplified was determined based on the previously obtained direct amplification data. Varying amounts of laser microdissected cells were amplified with the AmpFℓSTR® MiniFiler™ PCR Amplification Kit and PowerPlex® 16 HS System. The majority of the samples did not produce profiles. The cause of this was indeterminate, although it is possible that the PEN membranes interfered with amplification either by preventing the DNA from being released into the reaction or by inhibiting PCR.

To address the challenges posed by direct amplification of laser microdissected cells, a study was performed to investigate the effectiveness of a proteinase K incubation step. Prior to amplification, LM collected cells were incubated in a proteinase K solution. Following amplification with the PowerPlex® 16 HS System, no profiles were produced by any of the samples. Although proteinase K should have aided the release of DNA into the reaction, no purification step was performed to remove inhibitors from the sample. These results indicated that the PEN membranes may have interfered with the amplification. As the use of membrane slides greatly improves the ease with which laser microdissection is performed, direct amplification of laser microdissected cells was abandoned at this time.

Laser Microdissection Extraction Compatibility Examination

Based on the results from the direct amplification studies, both with and without proteinase K, further attempts were made to optimize the methods for processing LM collected samples by evaluating extraction methods with minimal processing times. The Qiagen EZ1 DNA Investigator robotic extraction method and the ZyGEM *forensic*GEM™ extraction kit were examined. These kits provide a purified DNA template in 20 minutes with minimal to no tube transfers. The effectiveness of these extraction methods was evaluated on low copy number laser microdissected cells. The aforementioned extraction methods and the QIAamp DNA micro

extraction were also investigated as methods for extracting LM collected cells that had undergone FISH processing.

Both the Qiagen EZ1 DNA Investigator extraction and ZyGEM *forensic*GEM Saliva extraction were performed on LM collected buccal cells. All eluates were concentrated and amplified with the PowerPlex® 16 HS System. LM collected cells extracted with both the ZyGEM and EZ1 kits were successfully and consistently amplified. Overall, the ZyGEM extracted samples displayed better balanced profiles; however, these samples also had more occurrences of dropout. The EZ1 extraction resulted in less balanced profiles but did exhibit less occurrences of dropout. When extracting FISH treated cells, both the ZyGEM and EZ1 extraction methods produced poor profiles. Due to this, it is strongly recommended to use the QIAamp DNA micro extraction method.

Laser Microdissection Amplification Compatibility Examination

To further investigate effective methods of processing laser microdissected samples, various amplification systems were examined for compatibility with laser microdissection. The systems selected were highly informative, contained at least 15 loci (not counting amelogenin), and could be used to produce significant statistical data. Prior to amplification, LM collected cells were extracted with the Qiagen EZ1 DNA Investigator kit. The extracts were concentrated and amplified with three commercially available amplification systems. While all three systems performed adequately, the PowerPlex® 16 HS system produced the most favorable results.

Next, amplification additives were examined as a means of improving the results obtained from amplifications using the PowerPlex® 16 HS System. The effectiveness of Biomatrica's STRboost® and PCRboost® on low copy number DNA from LM collected buccal

cells was evaluated. STRboost® and PCRboost® are amplification additives that purport to enhance the results obtained from low copy number, degraded and inhibited samples. LM collected cells were extracted with both the Qiagen EZ1 DNA Investigator and ZyGEM *forensic*GEM extraction kits. The extracts were concentrated and amplified with PowerPlex 16 HS. STRboost® and PCRboost® reagents were added to the amplification reactions. Comparisons of ZyGEM extracted samples amplified with the PCRboost® and STRboost® reagents with control samples indicated that neither PCRboost® nor STRboost® is necessary to improve results in the 50 cell range. However, results indicated that PCR Boost may improve RFU values and peak height balances for EZ1 extracted samples. Overall, the ZyGEM extracted samples continued to display better balanced profiles without the need for any additional steps during amplification.

Conclusions

The findings of this research indicate that SNPs are not ideal targets for forensic FISH probing. No probe signals specific to the desired SNP were detected. Based on these results, it would be advantageous to explore genetic marker systems that consist of larger genetic differences than SNPs. To this end, large insertion and deletion (INDEL) polymorphisms may be excellent targets for forensic FISH probing.

Various amplification systems, amplification additives, and extraction techniques were also examined as alternative methods for sample processing LM collected samples. The ZyGEM extraction and PowerPlex® 16 HS amplification were found to be the best suited to processing low numbers of LM collected cells that have not undergone FISH. Even without LM, both ZyGEM and PowerPlex® 16 HS could be beneficial to processing suspected LCN samples.

Introduction

Statement of the Problem and Literature Review

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. Mixtures containing spermatozoa and epithelial cells can be separated using preferential lysis methods or laser microdissection techniques; however, mixtures containing combinations of epithelial or blood cells require further processing to discriminate evidentiary components by gender and other genetic polymorphisms (Figure 1).

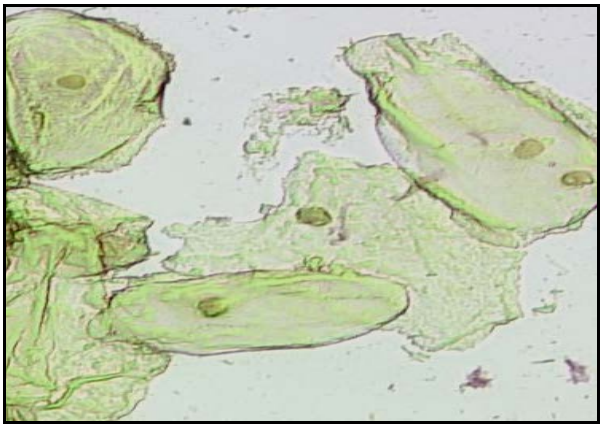

Figure 1: Evidence mixture containing epithelial cells from multiple males.

It was shown in previous work on NIJ award number 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, followed by physical separation via laser microdissection (LM) (1-4). 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. FISH examinations can typically be divided into two categories: metaphase and interphase analysis. Metaphase FISH analysis 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 that pass through cellular membranes and into the nucleus, eliminating the need to lyse cells during processing. This technique works on all cell types, living or dead, regardless of mitotic phase. 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 I-FISH probes are commonly used 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 (bp) in length and are repeated thousands of times to span 250,000-5,000,000 bases (5). This large target area allows for hybridization of the probe to occur many times, generating 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 1 kilobase (kb) to hundreds of thousands of kb, with an ideal size of approximately 40 kb (6).

FISH techniques have allowed for the visual identification of male and female cells from sexual assault evidence using X chromosome and Y chromosome probes (3, 4). Following fluorescence processing, intact sample cells can be removed from the slides via laser

microdissection methods and extracted for further STR interpretation. In studies that separated male/female mixtures using I-FISH techniques, Vysis CEP X® alpha satellite and CEP Y® satellite III probes were employed to visually identify the gender 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, procedures published in scientific literature, and techniques learned through firsthand scientific experience into one complete protocol encompassing all aspects of this type of analysis. I-FISH processing was tested with success on both epithelial and white blood cell sample types. Processing samples with this technique allowed for the visual identification of male and female contributors from sample mixtures. X chromosomes were visually identified by the presence of green fluorescent markers while Y chromosomes were detected by the presence of orange fluorescent markers. Sex chromosomes were easily identified from epithelial/epithelial, white blood cell/white blood cell, and epithelial/white blood cell mixtures. Figures 2, 3 and 4 demonstrate the results observed when using this technique to identify the male and female contributors to sample mixtures of various cell types.

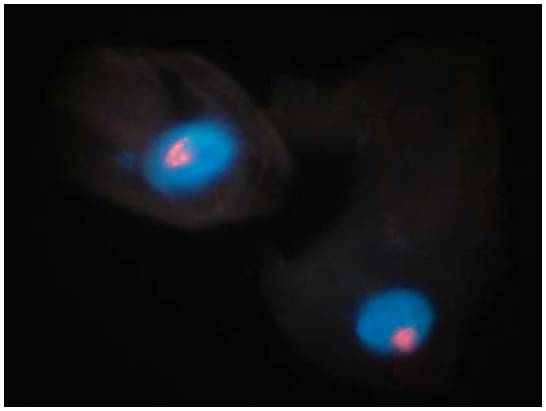

Figure 2: CEP-Y (orange) labeled male epithelial cells with DAPI counterstain visualized at 630X magnification as viewed through a DAPI/FITC/TRITC filter.

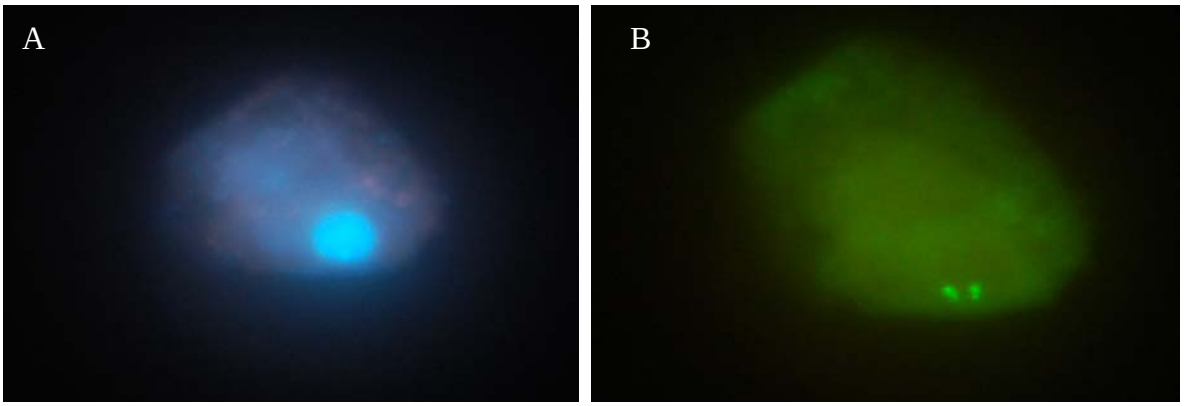

Figure 3: CEP-X (green) labeled female epithelial cells with DAPI counterstain visualized at 630X magnification as viewed through a (A) DAPI/FITC/TRITC and (B) FITC filters.

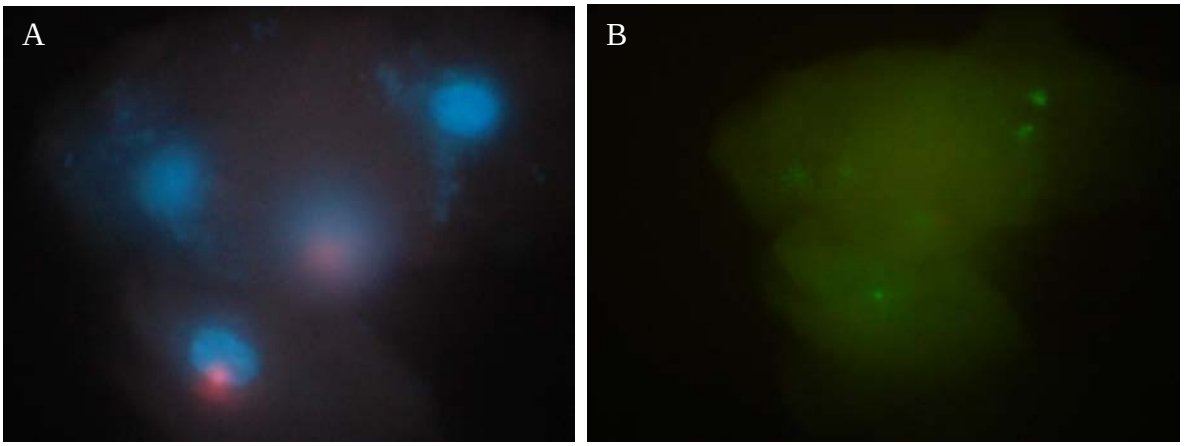

Figure 4: CEP-X (green) and CEP-Y (orange) labeled male and female epithelial cell mixture with DAPI counterstain visualized at 630X magnification as viewed through a (A) DAPI/FITC/TRITC and (B) FITC filters.

These 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 have generated full STR DNA profiles using the ABI AMPF*l*STR® MiniFiler™ amplification kit, and 20 to 30 cells have generated full profiles using the ABI AMPF*l*STR® Identifiler® kit. Mock evidence mixtures placed on 100% cotton and stainless steel substrates were also successfully collected, identified, separated (30 to 40 cells), and STR profiled. This technique has been used to successfully

generate STR profiles for the male and female contributors in sample mixtures of various cell types (Figures 5 and 6).

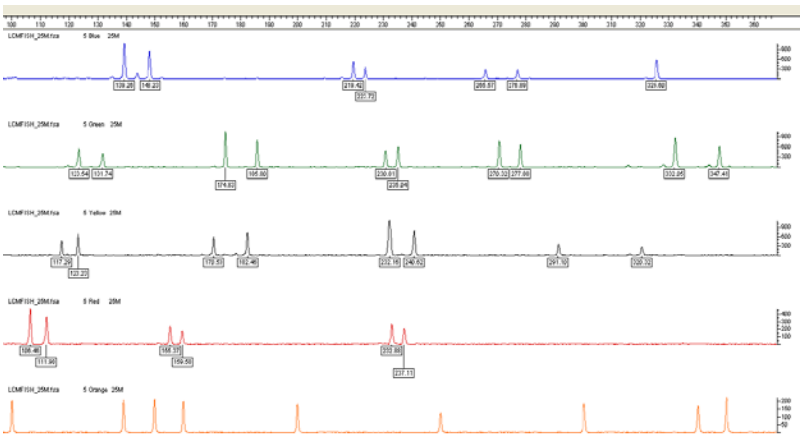

Figure 5: Electropherogram displaying a full STR profile generated from the extraction of FISH processed male epithelial cells from a male/female mixture.

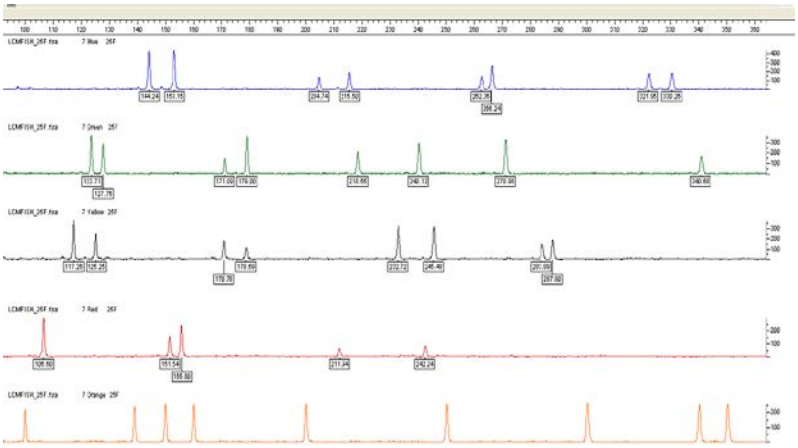

Figure 6: Electropherogram displaying a full STR profile generated from the extraction of FISH processed female epithelial cells from a male/female mixture.

Building on the results from NIJ award numbers 2006-DN-BX-K032 and 2008-IJ-CX-K016, this research proposed to use LM/FISH methodologies to separate cellular mixtures of similar morphology and same gender. Currently, there are no scientific methods or techniques available to forensic scientists for the clean separation of difficult mixture samples. For every 100,000 DNA samples handled a year, approximately 10 percent are deemed unusable because they contain DNA from more than one person (7). Over 15% of the sexual assault and property

crime evidence received at Bode Technology contains a mixture sample of some form. These mixture samples may reveal a critical connection between victim(s) and suspects(s) or between the suspects themselves. Unresolved same gender/same morphology cellular mixtures have been reported in numerous sexual assault (multiple offender and genital/digital penetration), murder, burglary, theft, and product of conception cases (7-11). If resolved, these mixture samples could be extremely informative to the forensic scientist and may ultimately lead to the successful conclusion of many pending and incomplete investigations.

This research proposed to separate cellular mixtures of similar morphology and same gender by using sequence specific FISH probes based upon genetic single nucleotide polymorphisms (SNPs). SNPs are polymorphic variations that occur at a single nucleotide at a particular point in the genome. Most SNPs are biallelic with two possible alleles and three possible genotypes. The minor allele must have a frequency of 1% or more (12). They possess low mutation rates and are the most frequent type of polymorphism with one to ten SNPs present for every 1,000 nucleotides (13). Millions of SNPs exist per individual, and they are starting to play an important role in differentiating individuals from one another (14). SNPs have played a very informative role in paternity investigations, mixture studies, and the processing of degraded samples (11,13,15,16). Using these polymorphisms for human identification represents a highly successful alternative to standard STR profiling assays. In this research, SNP polymorphisms were taken advantage of through the design of a FISH probe screening panel for the visual identification and separation of forensic mixture cellular components of the same gender and morphology. By coupling SNPs with FISH and LM techniques, it was the goal of this research to develop a tool for the separation of complex forensic mixtures.

The proposed research intended to create sequence specific probes for FISH analysis based upon highly distinguishable SNP loci. SNPs have been well characterized in panels created by the SNP*for*ID consortium, NIJ award number 2004-DN-BX-K025 (*IISNP*), and Kidd, et al. (17-19). It has been demonstrated that the 52 SNP*for*ID SNPs are well suited for forensic genetic investigations in crime, paternity, and immigration cases (20). Nineteen of the most polymorphic autosomal SNPs from the aforementioned sources were evaluated for inclusion in the FISH panel. Several issues were considered when selecting the SNPs for inclusion in the final panel. It was necessary to select a SNP that was unique, easy to type, unlinked, and intronic but not subject to drift (18). Generally, all the SNPs listed in the previously mentioned panels have met these criteria. For the purposes of this assay, as long as the SNPs listed in the SNP*for*ID and IISNP compendiums met a population criteria of high heterozygosity (>45%), low genetic variance among populations (Fst <6%), and were unlinked, they were considered for the mixture interpretation panel. The SNPs selected for use in the FISH probe screening panel did not target any specific population or convey any ethnicity or medical information related to the samples screened.

Due to the challenges associated with signal amplification techniques, the assay was ultimately narrowed down to two SNPs. Each SNP was to be associated with one of the fluorophore colors available for FISH paneling: red, aqua, green, magenta, orange, and far red. Following identification with FISH, the cells were to be physically separated from mixtures via LM and STR profiled using standard forensic procedures. The goal of this was to produce a SNP FISH screening system that would effectively separate many of the problematic mixture samples that are currently unexploited in forensic laboratories across the country.

Traditional FISH probes are not typically designed to distinguish between DNA sequences that vary by only one SNP difference, so these probes were deemed unsuitable for the level of discrimination required by this research. In the early 1990s, padlock probes were developed to improve both the specificity and sensitivity of traditional FISH probes. These probes are composed of two target-complementary sequences that are connected by a linker of variable length, typically approximately 50 bp long. Fluorophores or haptens may be incorporated into the linker for detection purposes. The two target-complementary ends of the padlock probe are hybridized to the target sequence so that the 5' and 3' ends meet (Figure 7).

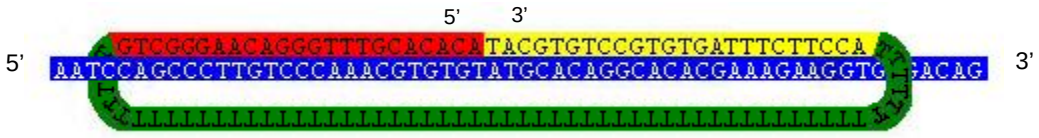

Figure 7: Structure of a padlock probe hybridized to its target sequence. At the 5' end, a 23 target-complementary nucleotide positions (red) is followed by a linker segment of 57 T residues (green). Finally, the 24 nucleotides at the 3' end of the probe are yellow. The target sequence is shown in blue (21).

The 5' end contains a phosphate group that allows a DNA ligase to join the two probe segments. The probe is designed with the desired SNP positioned at the final base location of the 3' end of the probe (21,22). Padlock probe specificity is increased through the action of the DNA ligase. When the 3' terminal end of the padlock probe fails to hybridize to the target SNP, the two probe ends cannot be joined through ligation (Figure 8). Hence, circularization does not occur and the mismatched probe can be washed away under high stringency wash conditions. Because the circularization of the probes allows for the use of extremely stringent wash conditions, the sensitivity of the assay is also increased through the reduction of nonspecific signals (21). While not ubiquitous, padlock probes have appeared in the literature steadily since their development and may be suited for forensic purposes.

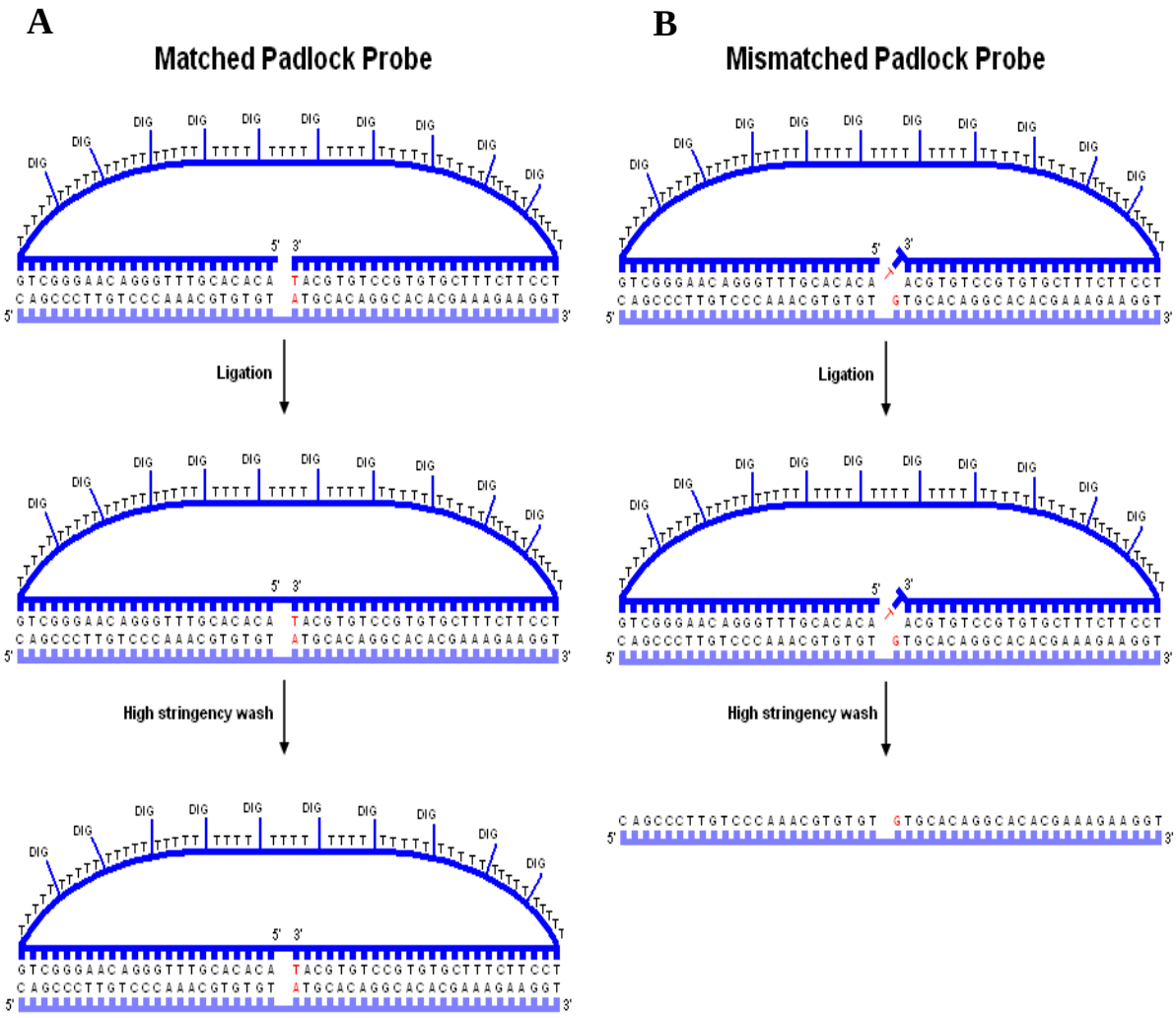

Figure 8: [A] Matched and [B] mismatched padlock probes hybridized to target DNA sequence. The final base position at the 3' end of the matched padlock probe hybridizes to the desired SNP location of the target DNA. The probe is successfully ligated and remains bound to the target sequence following a high stringency wash. The final base position at the 3' end of the mismatched padlock probe does not hybridize to the desired SNP location of the target DNA. The probe is not ligated and is washed away following a high stringency wash. The linker sequences of the depicted probes contain hapten molecules for subsequent tyramide signal amplification.

Although padlock probes are highly discriminatory, additional signal amplification techniques are required for visualization of a single SNP difference. Signal amplification can be achieved through the processes of either rolling circle amplification (RCA) or tyramide signal amplification (TSA).

RCA utilizes the circularized padlock probe as the DNA template for a DNA synthesis reaction, followed by detection and visualization of the replicated DNA. Since its development, rolling circle amplification has been proven successful over a variety of sample types including mitochondrial DNA, interphase nuclei, and metaphase chromosomes (23-25).

Several methods of performing RCA exist; however, only target primed rolling circle amplification was examined in this research. In target primed rolling circle amplification, the DNA of the target sequence is cleaved 3' of the padlock probe to provide a free 3'-end on which the synthesis reaction will occur (Figure 9) (23,24). This method requires the use of unique restriction enzymes, exonucleases, and polymerases. Restriction enzymes are used to cut double-stranded DNA at specific recognition nucleotide sequences called restriction sites. When choosing a restriction enzyme for RCA, the restriction enzyme should correspond to a restriction site 3' to the probe's target sequence; however, restriction enzymes must be carefully selected to ensure that the target sequence does not contain other restriction sites specific to the enzyme. For this work, the restriction enzymes BseYI and HphI were selected (Table 1). Exonucleases are used to produce the single stranded DNA necessary for RCA. Lambda (λ) exonuclease is commonly used for rolling circle amplification single stranded target DNA preparation due to its 5' to 3' activity. Polymerase is the key component in a successful RCA reaction. DNA polymerases with low processivity were initially used for RCA and were shown to be inefficient (26). In 1998, phi (Φ) 29 polymerase, a highly processive polymerase with strong strand

displacement and 3’ to 5’ exonuclease activity, was introduced to the procedure (27-29). It can produce up to 90kb DNA from each circularized probe per hour, which corresponds to approximately 1,000 copies of a typical padlock probe, depending on probe length (30). For these reasons, Φ29 polymerase is ideal for RCA.

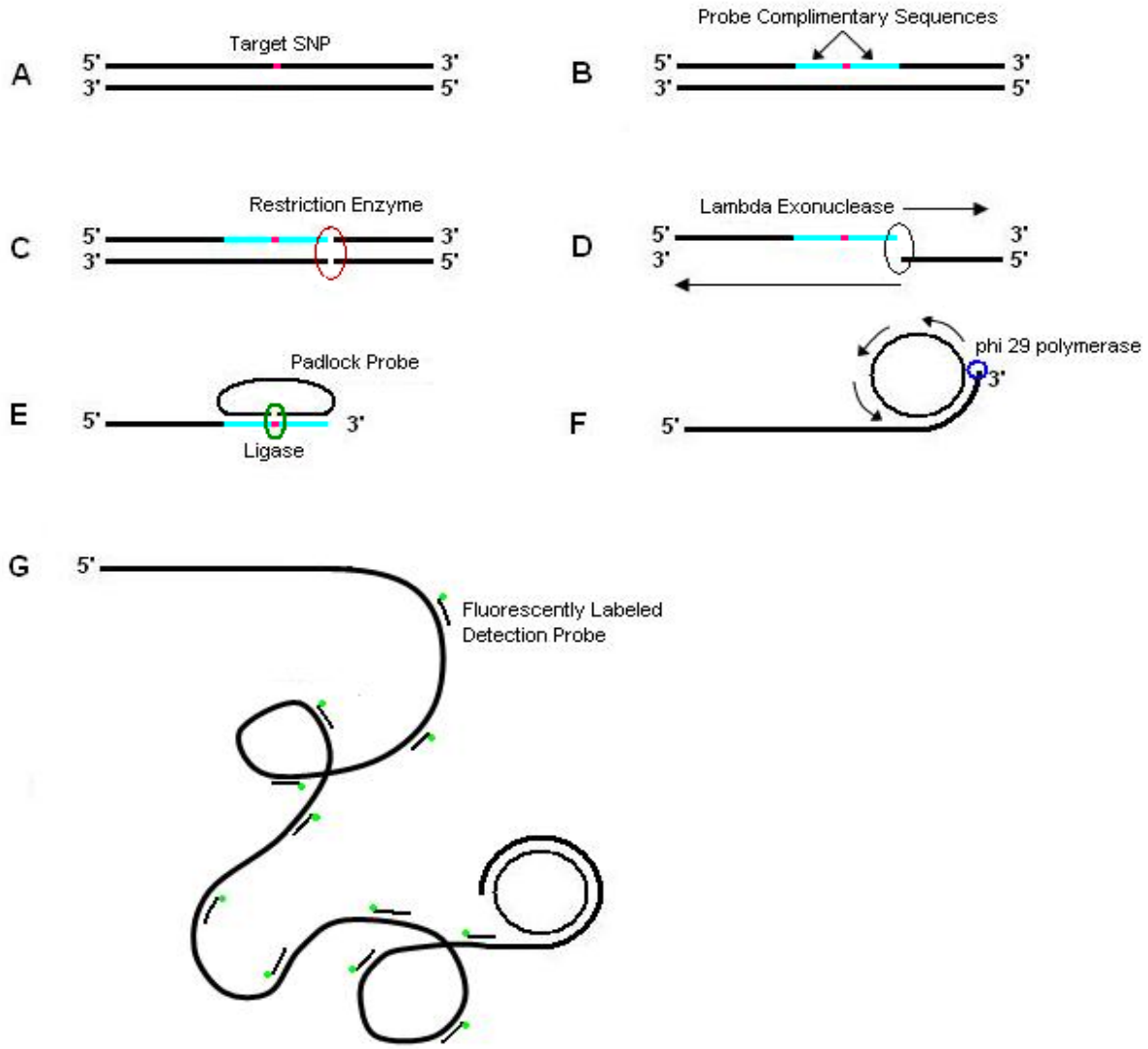

Figure 9: Detection of a SNP using padlock probes and target primed rolling circle amplification. [A] Target SNP is identified. [B] Probe is designed to be complementary to regions directly flanking SNP, with the most 3’ nucleotide complementary to the target SNP. [C] A restriction enzyme cleaves the DNA 3’ to the probe binding site. [D] The DNA is made single stranded through the 5’→3’ exonuclease activity of lambda exonuclease. [E] The padlock probe hybridizes to the target region. The probe is circularized through a ligation reaction. Ligation will only occur if hybridization between the probe and targeted SNP is successful. [F] Rolling circle amplification proceeds with the addition of phi 29 polymerase. The 3’ end of the target DNA serves as the reaction primer and the circularized probe functions as the DNA template. RCA causes the padlock probe DNA sequence to be replicated hundreds of times. [G] The RCA product is visualized by hybridization of a fluorescently labeled detection probe to a segment of the replicated padlock probe’s linker sequence (24).

Table 1: Restriction enzymes (New England Biolabs)

Restriction Enzyme	Source	Restriction Site
BseYI	*E. coli* strain that carries the cloned BseYI gene from *Bacillus* species 2521	5′...C▼CCAGC...3′ 3′...GGGTC▲G...5′
HphI	*E. coli* strain that carries the cloned HphI gene from *Haemophilus parahaemolyticus*	5′...GGTGA $(N)_8$▼...3′ 3′...CCACT $(N)_7$▲...5′

Tyramide signal amplification utilizes the activity of horseradish peroxidase to generate increased signal amplification of the target DNA sequence of interest *in situ*. TSA-FISH techniques have been utilized to increase the sensitivity of FISH when detecting probes as small as 319 bp (31). In TSA-FISH, a hapten labeled probe is hybridized to the target DNA sequence. Haptens used for this purpose include digoxigenin (DIG), dinitrophenol (DNP), and biotin. The hybridized hapten labeled probe is detected with an anti-hapten or streptavidin-horseradish peroxidase conjugate (anti-DIG-HRP, anti-DNP-HRP, or SA-HRP). In the presence of peroxide, multiple copies of signal enhancing fluorescent labeled tyramide are activated by 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, allowing the concentrated signal to be visualized with fluorescence microscopy (Figure 10) (32, 33). A second round of TSA signal generation may be performed using anti-fluorescein/Oregon green antibody conjugates labeled with HRP.

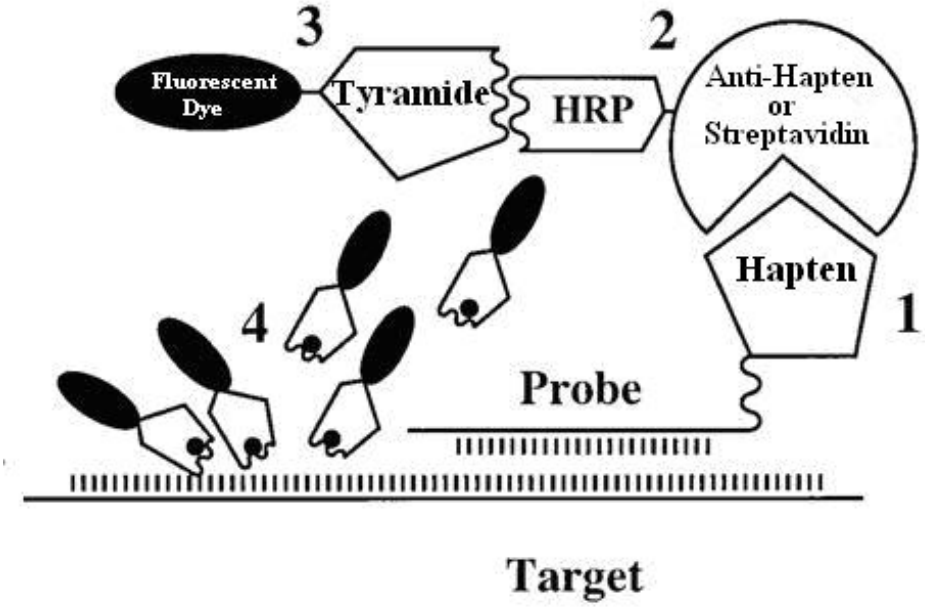

Figure 10: Schematic representation of TSA-FISH. [1] The targeted DNA is detected with a hapten labeled probe. [2] HRP-labeled anti-hapten or streptavidin binds to the hapten labeled probe. [3] The fluorescent dye labeled tyramide is activated by HRP. [4] Activated tyramide radicals bind near the activated HRP (34).

The findings from NIJ award number 2008-IJ-CX-K016 indicated that successful tyramide signal amplification is highly dependent on hapten selection. Nonspecific background fluorescence that interferes with probe signal detection can be reduced by labeling probes with the appropriate haptens. Each of the three most common haptens, biotin, DIG, and DNP, has properties that affect its suitability for SNP FISH probing. Biotin is a small B-complex vitamin molecule, vitamin B_7, with a high affinity for streptavidin, a tetrameric protein derived from *Streptomyces avidinii*. The streptavidin-biotin bond is one of the strongest known noncovalent biological interactions. However, biotin is found in the cytoplasm of nearly every cell type, as well as the mitochondrial matrix (35, 36). Visualization of specific FISH probe signals can be obscured when streptavidin nonspecifically hybridizes to the endogenous biotin found in cells. Due to this, both DIG and DNP are more suitable for sensitive assays. Neither DIG nor DNP is endogenous to human cells; therefore, antibodies to these haptens are unlikely to bind nonspecifically to cellular material.

In addition to FISH screening assays, cytogenetic on-slide membrane lysis techniques were examined as a means of improving LM analysis of low copy number (LCN) forensic mixtures. On-slide membrane lysis techniques are traditionally employed to prepare chromosome spreads for metaphase FISH analysis. A chromosome spread is a slide preparation method in which cellular suspensions are fixed to a slide in a manner that results in a flattened nucleus containing metaphase chromosomes spread out on the same plane with no overlap. Sufficient chromosomal spreading can be influenced by variables including dropping height, humidity, and slide drying time (37). Cellular preparations of this type involve inflating the inner cellular area of sample cells with a hypotonic solution. The osmotically manipulated samples are then dropped onto glass slides, causing cellular membranes to rupture; and the resulting isolated nuclei are fixed to the slide surface (38). This work aimed to create a novel method of obtaining genetic information by merging this established cytogenetic method with LM. Following fixation, the isolated nuclei were removed from the slide via LM and were placed directly into an amplification reaction. The cytogenetic on-slide membrane lysis step was intended to eliminate the need for commercial DNA extraction kits when processing LM collected samples. The high percentage of lost sample template typically encountered with traditional DNA extraction methods would be significantly reduced, thereby increasing the likelihood of successfully generating STR profiles from low copy number (LCN) samples. Cellular preparations of this type would be integrated easily into LM and FISH processing protocols. While this technique would be most beneficial for processing LCN samples, it would provide labs equipped with LM instruments with another tool for forensic sample processing. This process would also be less time consuming and expensive than commercial extraction kits.

An additional approach to minimizing LCN sample loss during extraction was to eliminate the extraction step altogether. Omission of the DNA extraction stage of processing cellular material by directly inputting laser microdissected samples into amplification reactions was anticipated to improve the speed and sensitivity of LCN sample analysis. Critical amounts of LCN sample template DNA would be conserved by eliminating standard DNA extraction procedures. Several commercially available STR amplification kits (PowerPlex 16 HS, PowerPlex S5, and MiniFiler) include hot start *Taq* DNA polymerases in their reaction buffers. The inclusion of hot start *Taq* in the buffers has allowed these kits to generate more informative profiles from challenging samples (39-41). A combination of proprietary buffers, hot start *Taq* polymerases, and optimized thermalcycling parameters allows these systems to overcome inhibitors commonly encountered in forensic samples (39-41). Of further benefit to LCN sample processing is the assertion that these kits are able to fully amplify less than 100 pg of template DNA. It was hypothesized that the initial high temperature step required for hot start *Taq* polymerase activation would lyse the cell membranes and release the target DNA, allowing the laser microdissected LCN sample cells to be directly amplified without an extraction step. The only inhibitors present for the polymerase and associated reagents to overcome would be those liberated from inside the cellular matrix. A distinct functional advantage of processing evidence samples with a LM instrument is that sample cells collected via LM are theoretically pure of any outside contaminants. Whole cells are removed from an evidence sample, leaving behind many detrimental environmental elements. If feasible, this technique would increase the opportunity for successfully processing low copy number samples. Effective direct amplification of LM samples would be an extremely advantageous tool for labs equipped with LM instruments.

Additionally, alternative extraction and amplification methods were examined to improve the processing of LM collected LCN samples. Since the introduction of LM to forensic sample processing, a major goal has been to obtain the maximum amount of genetic information possible from a small number cells. To this end, a variety of extraction methods have been examined for compatibility with LM (Table 2). Due to the range of variables that have been studied, it is difficult to directly compare the efficacy of these methods; however, in general, the most successful have been shown to be the Qiagen® QIAamp® DNA Micro extraction and single tube Proteinase K based methods (1,3,41-50). Typical methods of processing extracted LCN samples involve increasing PCR cycle numbers and increasing the injection time of the run on the 3100 instrument. LCN samples are subject to several issues including stochastic effects, such as heterozygote peak imbalance, allelic drop out and contamination. To reduce the negative effects seen when processing LCN samples, extraction methods with minimal tube transfers and various amplification systems were examined. The Qiagen EZ1 DNA Investigator extraction method is a robotic extraction in which DNA binds to silica coated magnetic beads in the presence of chaotropic salt. The DNA is washed and eluted from the beads. This method is commonly used for forensic DNA extractions. The ZyGEM *forensic*GEM™ extraction kit is a single tube extraction method which utilizes a thermophilic proteinase to degrade proteins and release DNA. Both of these kits provide a purified DNA template in 20 minutes with minimal to no tube transfers. Reducing the number of tube transfers maximizes the amount of DNA that can be obtained from a LCN sample; however, many of the stochastic effects associated with LCN processing are the result of increased cycle numbers during PCR. This research aimed to produce easily interpretable genetic profiles from LCN samples without exceeding the amplification systems' recommended number of cycles during PCR.

Table 2: Summary of the extraction methods and variables that have been examined in conjunction with LM. The staining methods include haematoxylin/eosin (H&E), nuclear fast red (NFR), picroindigocarmine (PI), Christmas tree stain (CTS), FISH, suspension-FISH (S-FISH), Papanicolaou (PAP), methyl green (MG), Wright's stain (WRT), acridine orange (AO), and *in situ* hybridization (ISH).

Extraction Method	Extraction Type	LM Instrument	Staining Method	Number of Cells Collected	Cell Type	Amplification Kit and Number of Cycles	Citation
Lyse-and-Go™ (Thermo Scientific Pierce)	Single Tube	Leica	None	75-300	Epithelial	Profiler Plus® 34 cycles	(41)
					Sperm		
			H&E, NFR, SYBR14/PI	150	Epithelial	Profiler Plus 28 cycles	
				300	Sperm		
Proteinase K	Single Tube	Leica	CTS	25-50	Epithelial	Identifiler® 28 cycles	(42)
				30-50	Sperm		
		Zeiss	H&E, None	50	Epithelial	*gen*RES® MPX-2 35 cycles	(43)
					Sperm		
			FISH	5-80	Epithelial	SGM Plus® 34 cycles	(3,44,45)
Arcturus® PicoPure® (Life Technologies)	Single Tube	Zeiss	None	25-200	Peripheral blood mononuclear (PBMC)	4 loci multiplex 33 cycles	(46,47)
				10-50	Sperm		
			None	1-20	PBMC	Profiler Plus 33 cycles	
				10-50	Sperm		
			S-FISH	2-10	Epithelial	Profiler Plus 33 cycles	(48)
MicroLYSIS® (Microzone)	Single Tube	Leica	None	75-300	Epithelial	Profiler Plus 34 cycles	(41)
					Sperm		
Alkaline DNA Extraction	Single Tube	Zeiss	None	25-200	PBMC	4 loci multiplex 33 cycles	(46)
DNA IQ™ (Promega)	Magnetic Resin	Leica	PAP, CTS	10-30	Sperm	Identifiler 28 cycles	(51)
		Zeiss	None	25-200	PBMC	4 loci multiplex 33 cycles	(46)
QIAamp DNA Micro (Qiagen)	Silica Column	Leica	H&E, CTS, MG, WRT, AO	150	Epithelial	Profiler Plus 28 cycles	(41)
				300	Sperm		
			None	75-300	Epithelial	Profiler Plus 34 cycles	
					Sperm		
		Pixcell II	H&E	6-326	Sperm	SGM Plus 28 cycles	(49)
		MMI	FISH	10-40	White blood cell (WBC)	SGM Plus 34 cycles	(50)
			ISH	10-50	Epithelial	SGM Plus 34 cycles	(1)
		Leica	PAP, CTS	10-30	Sperm	Identifiler 28 cycles	(51)
Chelex® 100 (Bio-Rad)	Resin	Zeiss	H&E	200	WBC	Profiler Plus 28 cycles	(52)
					Buccal		
			H&E, None	50	Epithelial	*gen*RES MPX-2 35 cycles	(43)
					Sperm		
		Leica	PAP, CTS	10-30	Sperm	Identifiler 28 cycles	(51)

When envisioning the role of LM and FISH within the forensic laboratory, it is important to note that the instrument and its associated processes are not intended to be high-throughput techniques. There is little need to utilize LM for standard high yield/non-mixture evidence. Normal laboratory procedures are already well-suited and optimized for the handling of these samples. LM should be employed when there is a known mixture sample or a low quantity of cells located on items of interest. LM and FISH are effective techniques for the resolution of previously uninterpretable mixtures and LCN samples (1,3,4,10,53,54). These tools should be considered a functional option when difficult and imperative evidence arrives in the laboratory. Figure 11 demonstrates the suggested workflow for FISH/LM processing of forensic evidence.

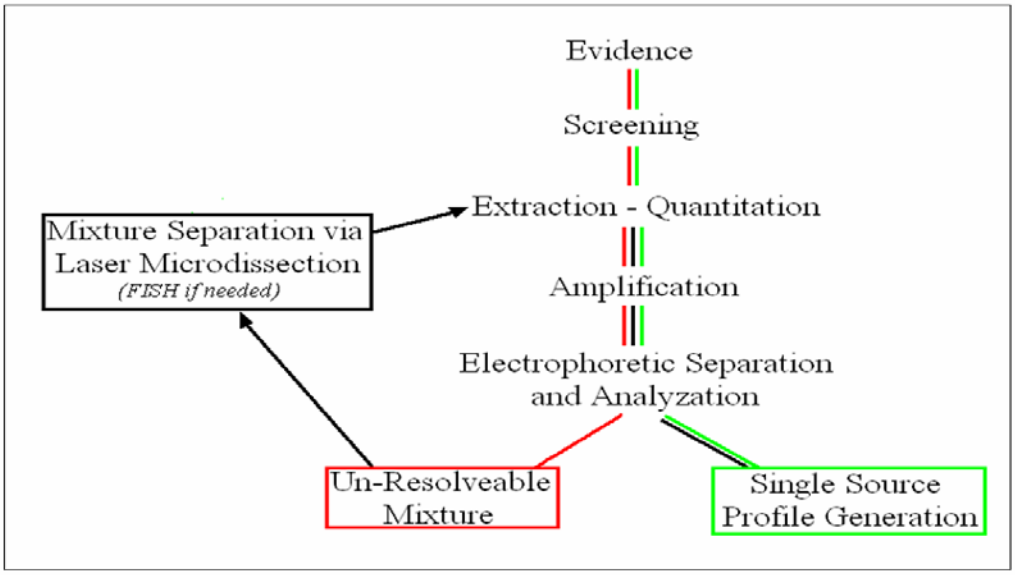

Figure 11: Flow chart illustrating the implementation of LM techniques in the forensic laboratory. The green pathway represents the handling of standard high yield/non-mixture evidence for which LM is not needed. The red pathway exhibits an instance in which an item of evidence contains an unknown mixture. At the conclusion of normal processing, a mixture profile is generated. The remaining original sample is then successfully processed using LM/FISH. The black pathway depicts evidence that is known to contain a mixture. The sample is immediately processed using LM/FISH techniques. Each pathway theoretically results in clean, interpretable profile generation.

Statement of Hypothesis

It was the goal of this research to use laser microdissection techniques to develop improved methods for resolving DNA mixtures in the forensic laboratory. NIJ award number 2006-DN-BX-K032 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. The results indicated that mixtures of male/female cells of similar morphology collected from various items of mock forensic evidence could be resolved to produce single source profiles. While that grant focused 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. At the end of 2008, NIJ award number 2008-IJ-CX-K016 was awarded to resolve cellular mixtures of same morphology and gender using FISH probes based upon the ABO and Duffy blood grouping systems. The following research served as an extension of the previous grant and expanded on the examination of new tools for genetic differentiation of these mixture types. The proposed research was intended to expand upon the ability to resolve forensic mixtures by meeting the following research objectives:

1. Design and test sequence specific hybridization probe screening panels based upon SNP genetic variations.
2. Improve upon front-end cellular preparations by using cytogenetic on-slide membrane lysis techniques.
3. Improve upon LCN evidence processing by optimizing the post collection processing techniques used with LM collected samples

It was the goal of these objectives to improve the methods of resolving forensic DNA mixtures containing cells of the same gender and morphology.

Methods

Development and Evaluation of SNP Specific Hybridization Probes

SNP Loci Evaluation

The following experiment was conducted to verify candidate SNP sites for inclusion in the final probe set. Sequencing was performed on a panel of individuals of diverse ethnic and cultural backgrounds to assess and avoid any potential ethnic bias or binding site mutations.

With the SNP FISH panel only able to identify a fraction of the number of SNPs utilized by other sequencing methodologies, it was essential to employ rigid selection criteria for inclusion of SNP sites into the final panel and to empirically test each of the individual SNPs prior to probe development. The SNPs chosen for the final FISH panel had to be both extremely stable and informative.

The main criteria for SNP selection were a low Fst value, high heterozygosity and functional non-linkage. The Fst value is a measure of the dissimilarity between populations; therefore a low value (<6%) signifies that there is no significant genetic distance between populations. This is important in a human identification SNP panel because loci with high Fst values could discriminate between different ethnicities extremely well, but they would be functionally useless within the individual populations themselves. High heterozygosity (>45%), combined with essentially identical allele frequencies in all populations, was ideal because the probability of unrelated individuals having the same genotype would be nearly constant irrespective of population. Finally, non-linkage refers to the fact that the loci are either located on different chromosomes or are so far apart in map linkage units on the same chromosome that

they are considered functionally unlinked with respect to random assortment. This allows the use of the product rule in determining the probability of exclusion from the individual SNPs. Nineteen potential SNP sites were identified for possible inclusion in the final SNP FISH assay (Tables 3 and 4).

The nineteen SNPs shown in tables 3 and 4 are based on the findings of Kidd *et* al. (19). Each of the SNPs listed were located in the National Center for Biotechnology Information (NCBI) SNP database (55). This national database centrally locates all extant information for each SNP, including web links to genome position maps, scientific publications, and population specific allele frequency estimates. The data found in the tables 3 and 4 were compiled from NCBI and ALlele FREquency Database (ALFRED).

Table 3: Potential SNP sites

SNP #	NCBI rsID	Polymorphism	Forward Amplification Primer	Reverse Amplification Primer
1	rs279844	A/**T**	TTGCCATGTTTGTCACAGGT	CTGCAAAAACACAGAGTGCAT
2	rs1058083	**A**/G	TGCACTGGTTCAAGGTTCTG	GGGATCGTTTCTCCTCTTCC
3	rs13182883	A/**G**	GGGTCCCTTCTGGCCTAGT	CTGTGCACCTCGATTGAAGA
4	rs560681	**A**/G	AAAATCACCCCAACCTCACA	CTAAAGGGCTCTCACCCACA
5	rs740598	A/**G**	AGCCACTCTTTCAGGCAAAA	CGGGATGTCCCGTCTTATTA
6	rs1358856	**A**/C	GGCTGTTTTATCCCATTAGCA	TGCTGGCAGTGTTATTTCTTTC
7	rs9951171	A/**G**	GTTCCTCTGGGATGCAACAT	CAAGGGAAGCCTGTTGGTTA
8	rs7520386	**A**/G	CATAGACCTCTGTGGCAGCA	TACCTGCGGTACCCAAGAAG
9	rs13218440	A/**G**	GTTCTCCTCGCCTACTGTGC	GGGAGCTGTACCTCAAGCAG
10	rs2272998	C/**G**	CCCGTTAAACTGGCATCTGT	TTTCCACTTCTGGGGTTGAC
11	rs12997453	**A**/G	GAGAGACAGGGGGAAGGAAG	CAGCTCTGATGATGTGCAAGA
12	rs214955	**A**/G	GGTGAGCATGAACCTTATTGG	TTGGATGCTTGCAAACAAAG
13	rs13134862	A/G	CAATTTTTCAAGCCCACACC	TTTGGAGCTGCACATTCTTG
14	rs1410059	**C**/T	GATGCTTGAACTCCCCAAAA	ACACATCAAAGCTGGGAACC
15	rs338882	**C**/T	TCCTGCTCTCTGGCTTCATT	GGGTTCTCTACCAGCTGTGC
16	rs2503107	A/**C**	TTTCAAAAATGGCAGGGTCT	ATGGCTAGGTCTCCACAACC
17	rs315791	A/**C**	TGGGATTCAGGAGTGAACAA	TAGAGCCCTCGCACCTCTTA
18	rs6591147	C/**T**	TCCAGCCACTGGATTTGAGT	GTCACTCCTCAGCCCTGGT
19	rs985492	C/**T**	TCCACCAAACAGTGTTGGAA	TGCCACAACCTGCTTCACTA

Table 4: SNP population frequencies

SNP #	NCBI rsID	Polymorphism	Caucasian	African American	Asian (Chinese)	Hispanic
1	rs279844	A/**T**	.460/.540	.440/.560	.456/.544	NA
2	rs1058082	**A**/G	.380/.620	.380/.620	.370/.630	NA
3	rs13182883	A/**G**	.390/.610	.390/.610	.390/.610	NA
4	rs560681	**A**/G	.650/.350	.650/.350	.670/.330	NA
5	rs740598	A/**G**	.630/.370	.610/.390	.480/.520	NA
6	rs1358856	**A**/C	.572/.428	.528/.473	.561/.439	NA
7	rs9951171	A/**G**	.430/.570	.440/.560	.400/.600	NA
8	rs7520386	**A**/G	.475/.525	.474/.526	.472/.528	NA
9	rs13218440	A/**G**	.347/.653	.440/.560	.422/.578	NA
10	rs2272998	C/**G**	.380/.620	.400/600	.410/.590	NA
11	rs12997453	**A**/G	.370/.630	.390/.610	.390/.610	NA
12	rs214955	**A**/G	.510/.490	.524/.476	.470/.530	NA
13	rs13134862	A/G	.380/.620	.370/.630	.350/.650	NA
14	rs1410059	**C**/T	.479/.521	.478/.522	.625/.375	NA
15	rs338882	**C**/T	.417/.583	.543/.457	.438/.562	NA
16	rs2503107	A/**C**	.600/.400	.600/.400	.524/.476	NA
17	rs315791	A/**C**	.417/.583	.522/.478	.521/.479	NA
18	rs6591147	C/**T**	.667/.333	.652/.348	.604/.396	NA
19	rs985492	C/**T**	.625/.375	.5/.5	.396/.604	NA

Each SNP was tested using the ABI PRISM® SNaPshot™ SNP genotyping kit. This method of SNP genotyping combines the use of a polymerase chain reaction (PCR) with single base extension (SBE) to determine SNP genotype. First, the flanking regions for each of the SNPs were imported into Primer3 software (56). Utilizing an eighteen to twenty base pair region directly upstream (5') to the SNP of interest as a SBE probe, primers suitable for producing (>250) base pair amplicons were identified for use in the SNaPshot reaction.

Buccal swabs were taken from eight donors encompassing Caribbean American, Egyptian, Caucasian, Chinese, Polish, German, African American, and Filipino ancestries. Each swab was extracted using Qiagen QIAamp® DNA Micro kits according to manufacturer's recommended protocols for swabs. The extracts were quantified using Quantifiler® Human DNA Quantification Kit and normalized to one ng/µl.

Samples were then amplified in a PCR reaction with an annealing temperature of 50°C for 40 cycles using an ABI 9700 GeneAmp® PCR system. The PCR mixture components and volumes are specified in Table 5.

All samples were then cleaned via ExoSAP-IT® and used as template for an SBE reaction using the ABI SNaPshot™ Multiplex Kit according to the manufacturer's standard recommendations (Table 6). The reactions were then cleaned again using a shrimp alkaline phosphate (SAP) reaction. LIZ 120 size standard and HI-DI formamide were added to each sample, and they were run on an ABI 3100 Genetic Analyzer using a 36 cm capillary array. Sample files were exported into GeneMapper v3.2 and analyzed. Once genotyped, the random match probability was calculated for each sample using each of the three available population statistics.

Table 5: Reagent component volumes for both amplification and sequencing reactions

Amplification Component	Volume (µl)
ddH_2O	11.91
Forward Primer	0.75
Reverse Primer	0.75
Gold ST★R 10X Buffer (Promega)	1.5
AmpliTaq Gold® DNA Polymerase (ABI)	0.09
DNA Template	2
Total Volume	17

Table 6: SNaPshot reaction components and volumes for SBE reactions

SNaPshot Component	Volume (µl)
SNaPshot Multiplex Ready Reaction Mix	5
0.2 µM SBE Primer	1
ddH_2O	1
PCR Product	3
Total Volume	10

Probe Design and Manufacture

Traditional FISH probes are not typically designed to distinguish between DNA sequences that vary by only one SNP difference. Accordingly, they were deemed unsuitable for the level of discrimination required by this research. Padlock probes were developed to improve upon both the specificity and sensitivity of traditional FISH probes. As the proposed assay required a high degree of specificity, padlock probes were explored as a method of identifying SNPs *in situ*.

SNP locations 10 and 15 were selected for padlock probes design. Due to the cost of probe manufacture, it was intended for the probes for SNP 10 to be designed following successful evaluation of the SNP 15 probes. Padlock probes were designed for SNP 15 using ProbeMaker software (57). ProbeMaker software allows the user to input a target sequence, mark the desired SNP location, and specify the linker sequence. The user then stipulates parameters such as melting temperature (T_m) and % GC content. Melting temperatures of the 3' and 5' ends are designed to be as similar as possible. Finally, padlock probes that meet the design requirements are generated by the software.

It was necessary to design two discrete sets of padlock probes for use with different signal amplification techniques. One set of padlock probes was designed for use with tyramide signal amplification. Multiple DIG molecules were incorporated into the linker sequences of these probes during synthesis. A second set of padlock probes was designed for use with rolling circle amplification. A detection molecule labeled with fluorescein was designed to hybridize to a sequence in the linker segment of the padlock probe following RCA.

Tyramide Signal Amplification Padlock Probes

The following target sequence was input into the software: 5' TCTGAGAGACA TCCCAGCCGAAGAATCCAGCCCTTGTCCCAAACGTGTGT[A/G]TGCACAGGCACACG AAAGAAGGTGAGACAGAAGGAGAGAAAAAT 3'. SNP 15 is indicated by brackets. The design parameters that were applied to the TSA SNP 15 padlock probe design are outlined in Table 7.

Several methods have been developed to facilitate hapten labeling of FISH probes. Based on the findings of NIJ award number 2008-IJ-CX-K016 and the complex nature of padlock probe design, the probes were chemically synthesized by a commercial manufacturer (Eurofins MWG Operon). Although expensive, chemical oligonucleotide synthesis ensured both accurate probe construction and adequate hapten incorporation into the probe.

Table 7: SNP 15 padlock probe design parameters

Melting temperature (°C)	GC content (%)	Target specific block length (bp)	Sodium ion concentration (M)	Probe concentration (nM)
55 ±5	45-60	18-25	0.2	1.0

Rolling Circle Amplification Padlock Probes

The following target sequences were input into the software: 5' TCTGAGAGACA TCCCAGCCGAAGAATCCAGCCCTTGTCCCAAACGTGTGT[A/G]TGCACAGGCACACG AAAGAAGGTGAGACAGAAGGAGAGAAAAAT 3' (antisense strand) and 5' GGCTTCATTTTTCTCTCCTTCTGTCTCACCTTCTTTCGTGTGCCTGTGCA[C/T]ACACA CGTTTGGGACAAGGGCTGGATTCTTCGGCTGGGATGTCTCTCAGA 3' (sense strand). SNP 15 is indicated by brackets. The design parameters applied to rolling circle amplification padlock probe design are identical to those depicted in Table 7. Due to the complex nature of padlock probe structure, these probes were also chemically synthesized by a commercial

manufacturer (Eurofins MWG Operon) to ensure accurate probe construction. The detection molecule was also commercially manufactured to ensure appropriate fluorescein labeling.

Probe Evaluation

Slide Preparation

Buccal swabs were collected from two donors not associated with the processing of any samples related to this project. Donor A possessed a T allele at SNP position 15. Donor B possessed a C allele at SNP position 15. Cells were eluted from the swabs by agitation in 1X phosphate buffered saline (PBS) at room temperature (RT) for two hours with shaking at 900 rpm. A cell pellet was generated by centrifugation at 3,000 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 membrane slide. PET slides were used when subsequent laser microdissection of the detected cells was anticipated. Slides consisted of approximately 10,000 buccal cells from donor A; 10,000 buccal cells from donor B; or a 1:1 mixture of 5,000 buccal cells from donor A and 5,000 buccal 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/50 mM $MgCl_2$ solution, 2.5% formalin buffer, and 1X PBS for five minutes each at RT. Endogenous peroxidase activity was blocked by incubating the slides in 0.3% hydrogen peroxide (H_2O_2) for 10 minutes at RT.

The slides were then washed in 1X PBS for five minutes at RT. Finally, the slides were dehydrated in an ethanol series (70%, 85% and 100%) for one minute each.

Reagent Preparation

Reagents were prepared for use with the hybridization, ligation, and detection procedures (Table 8). All hybridization, ligation, amplification, and detection reagents were prepared directly prior to use.

Table 8: Reagents prepared for hybridization, ligation, and detection of padlock probes

Reagent	Components (final concentration)
10 X Ligase Buffer	1 M KCl 100 mM $MgCl_2$ 200 mM Tris-Cl, pH 7.9 10 mM EDTA 10 mM Dithiothreitol 1% (v/v) Triton X-100
Buffer A	0.1 M Tris-HCl, pH 7.5 0.15 M NaCl 0.05% Tween 20
Buffer B	2X SSC 0.05% Tween 20
Detection Solution	250 nM Fluorescence-labeled detection probe 2X SSC 20% Formamide 0.5 µg/µl Salmon sperm DNA
Fluorophore Amplification Reagent Stock Solution	Reconstitute fluorophore amplification reagent (Perkin Elmer) in 150 µl HPLC grade dimethyl sulfoxide
Fluorophore Amplification Reagent Working Solution	Dilute the fluorophore amplification reagent stock solution 1:50 in 1X Plus Amplification Diluent
Lambda (λ) Exonuclease Reagent	0.2 U/µl λ exonuclease reagent (New England Biolabs) 1X λ exonuclease reagent reaction buffer (New England Biolabs) 0.2 µg/µl BSA 10% glycerol
RCA Padlock Probe Hybridization Solution	100 nM sense or antisense RCA padlock probe 2X SSC 20% formamide 0.5 µg/µl sonicated salmon sperm DNA
RCA Mixture A	50 mM Tris-HCl, pH 7.5 10mM $MgCl_2$ 20 mM $(NH_4)_2SO_4$ 0.2 µg/µl BSA 1 mM DTT 0.25 mM dNTP 10% Glycerol

	1 U/µl phi (Φ) 29 polymerase (Thermo Fisher)
RCA Mixture B	1X Φ 29 polymerase reaction buffer 0.2 µg/µl BSA 0.25 mM dNTP 1 U/µl phi (Φ) 29 polymerase (Thermo Fisher)
RCA T4 Ligation Mix A	10 mM Tris-HCl, pH 7.5 10 mM $MgCl_2$ 250 mM NaCl 1 mM ATP 0.2 µg/µl BSA 0.1 U/µl T4 DNA ligase (New England Biolabs)
RCA T4 Ligation Mix B	1X T4 DNA Ligase Reaction Buffer 0.2 µg/µl BSA 0.1 U/µl T4 DNA ligase (New England Biolabs)
RCA *Tth* Ligation Mix	1X *Tth* ligase buffer 1 mM nicotinamide adenine dinucleotide (NAD) (New England Biolabs) 8.7% (w/v) glycerol 0.1 µg/µl denatured sonicated salmon sperm DNA (Sigma) 0.1 µg/µl BSA 0.25 U/µl *Tth* DNA ligase (Affymetrix)
Restriction Enzyme Reagent	0.5 U/µl BseYI or HphI Restriction Enzyme Reagent (New England Biolabs) 1X NEBuffer 3 or 4 (New England Biolabs) 0.2 µg/µl bovine serum albumin (BSA) (Roche)
Stop Buffer	3.3X SSC 50 mM EDTA
TNB Buffer	0.1 M Tris-HCl, pH 7.5 0.15 M NaCl 0.5% Blocking Reagent (Perkin Elmer)
TNT Wash Buffer	0.1 M Tris-HCl, pH 7.5 0.15 M NaCl 0.05% Tween 20
TSA Overnight hybridization mix	25 µl 20% (v/v) deionized formamide in 2X SSC 3 µl 5 mg/ml denatured sonicated salmon sperm DNA 1 µl 30 fmol labeled padlock probe
TSA *Tth* Ligation Mix	1X *Tth* ligase buffer 1 mM nicotinamide adenine dinucleotide (NAD) (New England Biolabs) 8.7% (w/v) glycerol 0.1 µg/µl denatured sonicated salmon sperm DNA (Sigma) 0.1 µg/µl BSA 75 nM labeled padlock probe (Eurofins MWG Operon) 0.25 U/µl *Tth* DNA ligase (Affymetrix)

Tyramide Signal Amplification Padlock Probes Evaluation

Hybridization and Ligation Protocol - Standard Probe Concentration

Slides were preheated on the StatSpin® ThermoBrite™ Slide Hybridization/Denaturation System at 55°C. To each slide, 55.0 µl of TSA *Tth* ligation mix was added and covered with a cover slip. The ThermoBrite System was run for two minutes at 92°C to denature the chromosomes, followed by ligation for 15 minutes at 55°C. Following hybridization, slides were transferred to stop buffer preheated to 55°C for two minutes. The slides were washed in 2X SSC containing 30% deionized formamide for 10 minutes at 42°C. Then, the slides were washed in 2X SSC for five minutes at 55°C. The slides were transferred to wash buffer containing 2X SSC/0.05% (v/v) Tween 20 at RT before proceeding with detection

Alternate Hybridization and Ligation Protocol - Low Probe Concentration

The slides were denatured in 2X SSC containing 70% (v/v) deionized formamide for two minutes at 70°C, followed by a two minute incubation in ice cold 2X SSC. Next, the slides were dehydrated through serial ethanol washes (70%, 85%, and 100%) for two minutes each and were allowed to air dry. To each slide, 30.0 µl TSA overnight hybridization mix was added, covered with a cover slip and sealed with rubber cement. An overnight incubation was performed on the ThermoBrite at 37°C. Following hybridization, the rubber cement was removed and the slides were incubated in 2X SSC for five minutes at 37°C to remove excess probe. The slides were dehydrated through serial ethanol washes (70%, 85%, and 100%) for two minutes each, air dried, and preheated to 55°C on the ThermoBrite System. To each slide, 55.0 µl of TSA *Tth* ligation mix (with the probes omitted from the mix) was added and covered with a cover slip. Ligation was performed on the ThermoBrite for 15 minutes at 55°C, and then the slides were transferred

to stop buffer preheated to 55°C for two minutes. The slides were washed in 2X SSC containing 30% deionized formamide for 10 minutes at 42°C, followed by a wash in 2X SSC for five minutes at 55°C. Before proceeding with detection, the slides were transferred to wash buffer containing 2X SSC/0.05% (v/v) Tween 20 at RT.

Detection

TSA detection was performed using the Perkin Elmer TSA™ DNP (HRP) System. The slides were blocked in TNB buffer for 30 minutes at RT before incubating for 30 minutes at RT in 100 - 300 µl of anti-digoxigenin-HRP (Perkin Elmer) diluted 1:2,000 to 1:5,000 in TNB buffer. The slides were then washed three times in TNT buffer for five minutes at RT with agitation. After washing, the slides were incubated in 100 - 300 µl fluorophore amplification reagent working solution for five to ten minutes at RT, followed by three washes in TNT buffer for five minutes at RT with agitation. The slides were counterstained with Vectashield Mounting Medium with DAPI (Vector Labs) and visualized on the Zeiss PALM Microbeam system with fluorescence microscopy.

Rolling Circle Amplification Padlock Probes Evaluation

Enzymatic Target Preparation

To each slide, 20.0 µl of the BseYI (sense) or HphI (antisense) restriction enzyme was added and covered with a cover slip. The slides were incubated at 37°C for 30 minutes. Following incubation, they were rinsed in Buffer A. The restriction fragments were rendered single stranded through the addition of 20 µl λ exonuclease reagent to the slides. The slides were

covered with a cover slip and incubated at 37°C for 15 or 30 minutes. Following incubation, the slides were rinsed in Buffer A.

Rolling Circle Amplification Padlock Probe Hybridization and Ligation

To each slide, 20.0 µl of the sense or antisense RCA padlock probe hybridization solution was added. Hybridization occurred at 37°C for 15 minutes. The experiment was repeated with an incubation time of two hours. Excess probe solution was removed by washing the slides in Buffer B at 37°C for five minutes, followed by a rinse in Buffer A. Padlock probes were circularized in 55.0 µl RCA T4 ligation mix A, RCA T4 ligation mix B, or RCA *Tth* ligation mix. Probes circularized with T4 ligation mix were incubated at 37°C for 15 minutes or RT for two hours. Probes circularized with *Tth* ligation mix were incubated at 55°C for 15 minutes. The slides were washed in Buffer B for five minutes at 37°C, rinsed in Buffer A, and dehydrated in 70%, 85%, and 100% ethanol washes for one minute each at RT.

Rolling Circle Amplification

The RCA reaction was performed by applying 40.0 µl of RCA mixture A or B to the slides. The slides were covered with a cover slip and incubated at 37°C for 15 or 30 minutes, followed by a rinse in Buffer A. To each slide, 50.0 µl of detection solution was applied and covered with a cover slip. Then, the slides were incubated at 37°C for 15 or 30 minutes, rinsed with Buffer A, and dehydrated in 70%, 85%, and 100% ethanol washes for one minute each at RT. The dehydrated slides were counterstained with Vectashield Mounting Medium with DAPI and visualized on the Zeiss PALM Microbeam system with fluorescence microscopy.

Improvement of Front End Cellular Preparation via Cytogenetic Membrane Lysis

Classic cytogenetic on-slide membrane lysis techniques were examined as a method of improving front-end cellular preparations. The released free nuclear material resulting from this method was stained with DAPI fluorescent solution for visualization and collected via LM. Collected DNA was subjected to a brief protease digestion before direct input into an amplification reaction. The elimination of standard DNA extractions was intended to allow for fast and efficient processing of low copy samples without experiencing template loss during extraction.

To obtain buccal cells, six buccal swabs were collected from a donor with a known profile. The donor was not associated with the processing of any samples related to the project. The swabs were incubated in 500 µl of 1X PBS for two hours at RT (22°C) with shaking at 900 rpm. The samples, including swab, were transferred to centrifuge filter baskets in a 2.0 ml tubes and were centrifuged for 15 minutes at 3,000 rpm. After removing the supernatant, the pellets were combined and resuspended in 800 µl of 1X PBS. In order to obtain white blood cells 2 ml of whole blood was used directly.

The buccal cell samples and whole blood samples were transferred to separate 15.0 ml conical tube and then 10.0 ml of 0.075M KCl, prewarmed to 37°C, was added drop by drop to the samples with gentle agitation. The samples were mixed and followed by incubation in a 37°C water bath for 20 minutes. Four to five drops of freshly prepared Carnoy's fixative (3:1 methanol/acetic acid) were added to the samples. The samples were centrifuged for five minutes at 1,200 rpm. The supernatant was removed, leaving approximately 0.5 ml of solution in the tube. Five ml of fixative was added down the side of the tube. The samples were centrifuged for

five minutes at 1,200 rpm. These steps, beginning with the removal of the supernatant and addition of 5.0 ml of fixative, were performed a total of three times. The resulting pellets were resuspended in 1 ml freshly prepared Carnoy's fixative.

Approximately 30.0 µl of each sample type were dropped onto PEN membrane slides from a distance of approximately 12 inches. The slides were dried on a heat block at 56°C for one to two minutes. To each slide, 20.0 µl of DAPI Vectashield diluted 1:100 in water was applied and was covered with a cover slip. Following a 15 minute incubation, the samples were visualized on the Zeiss PALM Microbeam Laser Microdissection System using fluorescence microscopy.

Fifty nuclei were collected in triplicate from both the buccal cell and white blood cell slides. Each sample was collected in the cap of a 0.5 ml tube in 60.0 µl ddH_2O. Following collection, samples were spun at full speed for five minutes. To each sample, 10.0 µl of Qiagen 20 mg/ml proteinase K was added followed by incubation at 56°C for 20 minutes with shaking at 900 rpm.

All samples were concentrated to a volume of 17.5 µl using Microcon® YM-100 Columns. The nuclei were amplified on a 9700 thermal cycler with the PowerPlex® 16 HS System in a 25.0 µl reaction volume (Tables 9-11). The samples were prepared for capillary electrophoresis and data collection using a standard 3100 protocol. A master mix was created by combining 9.5 µl of Hi-Di formamide with 0.5 µl of ILS 600 for each well. Ten µl of the mixture was dispensed into the wells of a 96-well MicroAmp plate. Amplified product (1.0 µl) was added to each designated well containing master mix. Each tray also included two wells consisting of 10.0 µl of formamide + ILS master mix and 1.0 µl of PowerPlex® 16 HS ladder. The samples and allelic ladders were injected on an ABI 3100 Genetic Analyzer at 3 kV for 10 seconds and

run at 15 kV at 60°C. The data for the amplified product separation was collected using data collection software version 1.1. All results were analyzed using GeneMapper v3.2.1 software.

Table 9: PowerPlex® 16 HS master mix for 25 µl amplification volume

Master Mix	Volume per Reaction (µl)
PowerPlex HS 5X Master Mix	5.0
PowerPlex 16 HS 10X Primer Pair Mix	2.5
µl added to each reaction	7.5
Template DNA	17.5
Total Volume	25.0

Table 10: PowerPlex® 16 HS amplification controls

Control	Reagent	Volume per Reaction (µl)
Positive	Promega 9947A DNA 0.05 ng/µl	17.5 µl
Negative	ddH_2O	17.5 µl

Table 11: PowerPlex® 16 HS thermalcycling parameters

Hold at:	96°C for 2 minutes
10 cycles at:	ramp 100% to 94°C for 30 seconds
	ramp 29% to 60°C for 30 seconds
	ramp 23% to 70°C for 45 seconds
22 cycles at:	ramp 100% to 90°C for 30 seconds
	ramp 29% to 60°C for 30 seconds
	ramp 23% to 70°C for 45 seconds
Hold at:	60°C for 30 minutes
Hold at:	4°C forever

Optimization of Laser Microdissected Sample Processing Techniques

To improve the speed and sensitivity of LCN sample processing, DNA extraction was omitted and LM collected cells were directly added to various amplification reactions. Further experiments facilitated the release of DNA by subjecting the collected cells to a brief protease

digestion prior to amplification. Based on the results from these experiments, alternative extraction and amplification methods were examined to determine the optimum processing techniques for use in conjunction with laser microdissected samples.

Direct Amplification of Cellular Suspensions

Two buccal swabs were collected from a donor with a known profile. The donor was not associated with the processing of any samples related to the project. The following standard cellular elution protocol was used for all experiments. The swabs were incubated in 500 µl of 1X PBS for two hours at RT (22°C) with shaking at 900 rpm. The samples, including swabs, were transferred to centrifuge filter baskets in 1.5 ml tubes and were centrifuged for 10 minutes at full speed. After discarding the supernatant, the pellets were combined and resuspended in 250 µl of 1X PBS. A cell count was performed using a hemocytometer and light microscope.

AmpFℓSTR® MiniFiler™ PCR Amplification Kit

Cells were amplified on a 9700 thermal cycler with the AmpFℓSTR® MiniFiler™ PCR Amplification Kit in a 25.0 µl reaction volume (Tables 12-14). Triplicate amplifications of 1,000, 500, 100, and 50 cells were performed by adding 10.0 µl of 100, 50, 10, and 5 cells/µl solutions, respectively. The samples were prepared for injection and data collection using the standard 3100 protocol, above, with the following deviations: the master mix was created by combining 10.0 µl of Hi-Di Formamide with 0.12 µl of GeneScan-500 LIZ, and 0.7 µl amplified product or 1.0 µl of MiniFiler™ ladder was added to the tray as appropriate.

Table 12: MiniFiler™ master mix for 25 µl amplification volume

Master Mix	Volume per Reaction (µl)
MiniFiler Master Mix Primer	10.0
MiniFiler Primer Set	5.0
µl added to each reaction	15.0
Template DNA	10.0
Total Volume	25.0

Table 13: MiniFiler™ amplification controls

Control	Reagent	Volume per Reaction (µl)
Positive	AmpFℓSTR Control DNA 007 0.05 ng/µl	10.0 µl
Negative	ddH_2O	10.0 µl

Table 14: MiniFiler™ thermalcycling parameters

Hold at:	95°C for 11 minutes
30 cycles at:	94°C for 20 seconds
	59°C for 2 minutes
	72°C for 1 minute
Hold at:	60°C for 45 minutes
Hold at:	4°C forever

PowerPlex® 16 HS System

Cells were amplified on a 9700 thermal cycler with the PowerPlex® 16 HS System in a 25.0 µl reaction volume as recommended by the manufacturer (Table 9-11). Triplicate amplifications of 1,000, 500, 100, and 50 cells were performed by adding 17.5 µl of 57, 29, 6, and 3 cells/µl solutions, respectively. Samples were prepared for injection and data collection using the standard 3100 protocol.

PowerPlex® S5 System

Cells were amplified on a 9700 thermal cycler with the PowerPlex® S5 System in a 25.0 µl reaction volume (Tables 15-17). Triplicate amplifications of 1,000, 500, 100, and 50 cells were performed by adding 17.5 µl of 57, 29, 6, and 3 cells/µl solutions, respectively. Samples were prepared for injection and data collection using the aforementioned 3100 protocol with the following deviation: 1.0 µl of PowerPlex® S5 ladder was added to the tray as appropriate.

Table 15: PowerPlex® S5 master mix for 25 µl amplification volume

Master Mix	Volume per Reaction (µl)
PowerPlex S5 5X Master Mix	5.0
PowerPlex S5 10X Primer Pair Mix	2.5
µl added to each reaction	7.5
Template DNA	17.5
Total Volume	25.0

Table 16: PowerPlex® S5 amplification controls

Control	Reagent	Volume per Reaction (µl)
Positive	Promega 9947A DNA 0.05 ng/µl	17.5 µl
Negative	ddH_2O	17.5 µl

Table 17: PowerPlex® S5 thermalcycling parameters

Hold at:	96°C for 2 minutes
30 cycles at:	94°C for 30 seconds
	60°C for 2 minutes
	70°C for 90 seconds
Hold at:	60°C for 45 minutes
Hold at:	4°C forever

Direct Amplification of Laser Microdissected Cells

Two buccal swabs were collected from a donor with a known profile. The donor was not associated with the processing of any samples related to the project. Cells were eluted from the swabs using the standard cellular elution protocol, above; however, the combined pellets were resuspended in 500 µl of 1X PBS. The following constitutes the standard slide preparation protocol used to prepare all slides. Following resuspension, the sample was centrifuged for 10 minutes at full speed. After discarding the supernatant, the pellet was fully resuspended in 100 µl of Carnoy's Fixative. On a PEN membrane slide, 20.0 µl of the resuspended cells were spotted and steamed over a water bath for approximately five seconds. Then, the slide was dried on a 56°C heat block for one to two minutes. The cells were fixed to the slide in 70% ethanol for one minute. Excess salts were removed by a quick submergence in ddH_2O.

The samples were visualized via brightfield microscopy using the Zeiss PALM Microbeam System. Twenty-five to one hundred cells were collected in triplicate in 20 µl of water in the cap of a 0.5 ml microcentrifuge tube.

To test the effectiveness of a proteinase K digestion, 100 cells were collected in triplicate in 20.0 µl of water in the cap of a 0.5 ml microcentrifuge tube. These cells were incubated in Qiagen 20 mg/ml proteinase K at 56°C for 20 minutes.

AmpFℓSTR® MiniFiler™ PCR Amplification Kit

Untreated cells were amplified on a 9700 thermal cycler with the AmpFℓSTR® MiniFiler™ PCR Amplification Kit using a 25.0 µl reaction volume (Tables 12-14). Amplifications of 100, 75, 50, and 25 cells were performed in triplicate and 10.0 µl of each cell solution was added to each amplification reaction. Samples were prepared for injection and data collection using the above 3100 protocol with the following deviations: the master mix was

created by combining 10.0 µl of Hi-Di Formamide with 0.12 µl of GeneScan-500 LIZ, and 0.7 µl amplified product or 1.0 µl of MiniFiler™ ladder was added to the tray as appropriate.

PowerPlex® 16 HS System

Untreated and proteinase K treated cells were amplified on a 9700 thermal cycler with the PowerPlex® 16 HS System in a 25.0 µl reaction volume (Tables 9-11). For untreated cells, triplicate amplifications of 100, 75, 50, and 25 cells were performed by adding 17.5 µl of each cell solution to each amplification reaction. For proteinase K treated cells, 17.5 µl of each solution was amplified. Samples were prepared for injection and data collection using the standard 3100 protocol.

Laser Microdissection Extraction Compatibility Examination

Qiagen EZ1 DNA Investigator and ZyGEM *forensic*GEM

The following work evaluates the effectiveness of the Qiagen EZ1 and ZyGEM *forensic*GEM extractions on low copy number laser microdissected buccal cells. Two buccal swabs were collected from a donor with a known profile. The donor was not associated with the processing of any samples related to the project. Samples were visualized via brightfield microscopy using the Zeiss PALM Microbeam System. Twenty-five to fifty cells were collected via LM in triplicate in 20.0 µl of water in the cap of a 0.5 ml microcentrifuge tube.

The Qiagen EZ1 DNA Investigator Extraction was performed as follows in triplicate on laser microdissected samples of 25 and 50 buccal cells. To each sample, 95.0 µl Buffer G2, 75.0 µl ddH_2O, and 20.0 µl Qiagen proteinase K were added. Samples were incubated on a thermomixer for one hour at 56°C with shaking at 900rpm. Carrier RNA, 1.0 µl, was added to

each sample. Samples were extracted on an EZ1 Advanced Robot with the DNA Investigator card trace protocol and an appropriate Qiagen EZ1 Investigator reagent cartridge.

The ZyGEM *forensic*GEM Saliva Extraction was performed in triplicate on laser microdissection collected samples of 25 and 50 buccal cells. To each sample, 69.0 µl ddH_2O, 1.0 µl *forensic*GEM, and 10.0 µl 10X Buffer were added. Samples were incubated at 75°C for 15 minutes followed by five minutes at 95°C.

All eluates were concentrated to a volume of 17.5 µl using Microcon YM-100 Columns. Samples were amplified on a 9700 thermal cycler with the PowerPlex® 16 HS System using manufacturer's recommended primer concentrations in a 25.0 µl reaction volume (Tables 9-11). Samples were prepared for injection and data collection using the standard 3100 protocol.

Extraction of FISH processed LM collected Cells

A series of experiments was performed to test the ZyGEM *forensic*GEM and Qiagen EZ1 extraction techniques on cells that had undergone FISH processing. A mixture of male and female cells was deposited on PET membrane slides using standard cell elution and slide preparation techniques. The slides were processed with CEP probes specific for the X and Y chromosomes. All heated steps were performed on the StatSpin ThermoBrite Slide Hybridization/Denaturation System. The slides were incubated in 1.27 U/µl pepsin buffer for 3 minutes at 37°C. Next, the slides were incubated in 1X PBS/50 mM $MgCl_2$ solution for five minutes at RT, 2.5% formalin buffer for five minutes at RT, and 1X PBS for five minutes at RT. The slides were then dehydrated through a series of ethanol washes (70%, 85%, and 100%) for one minute each at RT. Probes were prepared by combining 7.0 µl of CEP Hybridization Buffer®, 1.0 µl of CEP X® probe, 1.0 µl of CEP Y® probe, and 1.0 µl of ddH_2O 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. Denaturation occurred at 73°C for five minutes. This was immediately followed by hybridization at 42°C for 45 minutes. After hybridization, the cover slips were removed and the slides were incubated in a 1X SSC/0.05% Tween 20 solution for two minutes at 73°C. The slides were then immersed in 2X SSC for one minute at RT. Ten µl of Vectashield Mounting Medium with DAPI (Vector Labs) diluted 1:100 in ddH_2O were applied directly to the slides. The samples were then visualized using fluorescence microscopy.

Laser microdissection was used to collect 100 or 150 cells in 60.0 to 80.0 µl of ddH_2O in the cap of a 0.5 ml tube. Cells were extracted with the ZyGEM or EZ1 extraction kits using the aforementioned protocols. A modified version of the QIAamp DNA Micro protocol for isolation of genomic DNA from laser-microdissected tissues was also examined. Centrifugation was used to collect the sample in the bottom of the 0.5 ml tube. Sixty µl of Buffer ATL and 10.0 µl Qiagen 20 mg/ml proteinase K were added to the laser microdissected samples. The samples were incubated on a thermomixer 56°C for three hours with shaking at 900 rpm. To each sample, 60.0 µl of Buffer ATL, 1 µl carrier RNA, and 60 µl 100% ethanol was added, followed by a five minute incubation at RT. The samples were transferred to QIAamp MinElute column and spun at 8,000 rpm for one minute. After discarding the flow-through, the columns were washed with 500 µl Buffer AW1 and spun at 8,000 rpm for one minute. After discarding the flow-through, the columns were washed with 500 µl Buffer AW2 and spun at 8,000 rpm for one minute. To elute the DNA, 25.0 µl ddH_2O was incubated on the columns for five minutes and centrifuged at full speed for one minute. The elution step was performed two times.

All eluates were concentrated using Microcon YM-100 Columns to the maximum input template DNA volume for each amplification system. Samples were amplified on a 9700 thermal

cycler with the PowerPlex® 16 HS, PowerPlex® 16, or AmpFℓSTR® Identifiler™ amplification systems (Tables 9-11, 18-23). The PowerPlex® 16 HS samples were prepared for injection and data collection using the standard 3100 protocol. For the PowerPlex® 16 samples, the following deviations were made to the 3100 protocol: the master mix was created by combining 10.0 µl of Hi-Di Formamide with 0.6 µl of ILS 600, and 0.6 µl amplified product and 1.0 µl of PowerPlex® 16 ladder were added to the tray as appropriate. For the Identifiler™ samples, the following deviations were made to the 3100 protocol: the master mix was created by combining 10.0 µl of Hi-Di Formamide with 0.12 µl of GS-500 LIZ, and 0.7 µl amplified product or 1.0 µl of Identifiler™ ladder were added to the tray as appropriate.

Table 18: PowerPlex® 16 master mix for 25 µl amplification volume

Master Mix	Volume per Reaction (µl)
Gold ST★R 10X Buffer	2.5
PowerPlex 16 10X Primer Pair Mix	2.5
AmpliTaq Gold DNA Polymerase	0.8
µl added to each reaction	5.8
Template DNA	19.2
Total Volume	25.0

Table 19: PowerPlex® 16 amplification controls

Control	Reagent	Volume per Reaction (µl)
Positive	Promega 9947A DNA 0.5 ng/µl	2.0 µl
Negative	ddH_2O	19.2 µl

Table 20: PowerPlex® 16 thermalcycling parameters

Hold at:	95°C for 11 minutes 96°C for 1 minute
10 cycles at:	94°C for 0.5 minute Ramp: 0 seconds, 100% 60°C for 1 minute Ramp: 68 seconds, 33% 70°C for 1.5 minute Ramp: 50 seconds, 13%
20 cycles at:	90°C for 0.5 minute Ramp: 0 seconds, 100% 60°C for 1 minute Ramp: 60 seconds, 33% 70°C for 1.5 minute Ramp: 50 seconds, 13%
Hold at:	60°C for 30 minutes
Hold at:	4°C forever

Table 21: Identifiler™ master mix for 25 µl amplification volume

Master Mix	Volume per Reaction (µl)
AmpF*l*STR PCR Reaction Mix	10.5
AmpF*l*STR Identifiler Primer Set	5.5
AmpliTaq Gold DNA Polymerase	0.5
µl added to each reaction	15.0
Template DNA	10.0
Total Volume	25.0

Table 22: Identifiler™ amplification controls

Control	Reagent	Volume per Reaction (µl)
Positive	Promega 9947A DNA 1.0 ng/µl	1.5 µl
Negative	ddH_2O	10.0 µl

Table 23: Identifiler™ thermalcycling parameters

Hold at:	95°C for 11 minutes
28 cycles at:	94°C for 1 minute
	59°C for 1 minute
	72°C for 1 minute
Hold at:	60°C for 60 minutes
Hold at:	4°C forever

Laser Microdissection Amplification Compatibility Examination

The following work evaluated the effectiveness of Promega's PowerPlex® 16 HS, Promega's PowerPlex® 16 System, and Applied Biosystems' AmpFℓSTR® Identifiler™ amplification kit on low copy number LM collected buccal cells. No modifications were made to the manufacturer's recommended protocols for the aforementioned amplification kits.

The effectiveness of Biomatrica's STRboost® and PCRboost® on low copy number laser microdissection collected buccal cells was evaluated. STRboost and PCRboost are reagents that purport to enhance the amplification results obtained from low copy number and degraded samples as well as samples containing inhibitors.

Three buccal swabs were collected from a donor with a known profile. Standard cellular elution and slide preparation protocols were followed. Samples were visualized via brightfield microscopy using the Zeiss PALM Microbeam System. Fifty cells were collected in triplicate in approximately 20.0 µl of water in the cap of a 0.5 ml microcentrifuge tube.

LM collected samples to be amplified with the PowerPlex® 16 HS System, PowerPlex® 16 System, and AmpFℓSTR® Identifiler™ systems were extracted using the Qiagen EZ1 DNA Investigator kit using the aforementioned extraction protocol.

LM collected samples to be used for the STRboost® and PCRboost® evaluations were extracted using the Qiagen EZ1 DNA Investigator kit and the ZyGEM *forensic*GEM extraction kit using the previously mentioned protocols.

PowerPlex® 16 HS System

Three EZ1 eluates were concentrated to 17.5 µl using Microcon YM-100 Columns. Samples were amplified on a 9700 thermal cycler with the PowerPlex® 16 HS System in a 25.0 µl reaction volume (Tables 9-11). Samples were prepared for injection and data collection using the standard 3100 protocol.

PowerPlex® 16 System

Three EZ1 eluates were concentrated to 17.5 µl using Microcon YM-100 Columns. Samples were amplified on a 9700 thermal cycler with the PowerPlex® 16 System in a 25.0 µl reaction volume (Tables 18-20). Samples were prepared for injection and data collection using the standard 3100 protocol with the following deviations: the master mix was created by combining 10.0 µl of Hi-Di Formamide with 0.6 µl of ILS 600, and 0.6 µl amplified product and 1.0 µl of PowerPlex® 16 ladder were added to the tray as appropriate.

AmpFℓSTR® Identifiler™

Three EZ1 eluates were concentrated to 10.0 µl using Microcon YM-100 Columns. Samples were amplified on a 9700 thermal cycler with the AmpFℓSTR® Identifiler™ PCR Amplification Kit in a 25.0 µl reaction volume (Tables 21-23). Samples were prepared for injection and data collection using the standard 3100 protocol with the following deviations: the master mix was created by combining 10.0 µl of Hi-Di Formamide with 0.12 µl of GS-500 LIZ, and 0.7 µl amplified product or 1.0 µl of Identifiler ladder were added to the tray as appropriate.

STRboost® and PCRboost® Evaluation

Three ZyGEM eluates and three EZ1 eluates were concentrated to 15.0 µl using Microcon YM-100 Columns. Concentrated ZyGEM and EZ1 eluates were amplified on a 9700 thermal cycler with the PowerPlex® 16 HS System in a 25.0 µl reaction volume (Tables 9-11). All 15.0 µl of concentrated eluate was used in each individual reaction and 2.0 µl of either STRboost, PCRboost, or ddH_2O (negative control) was added. Samples were prepared for injection and data collection using the standard 3100 protocol.

Results

Development and Evaluation of SNP Specific Hybridization Probes

SNP Loci Evaluation

SNaPshot profiles were assessed according to the following guidelines. A peak at the proper location was scored as a positive result. Any other peaks that were not considered pull-up or artifacts and were within the examined range were recorded. Occasionally, when SNaPshot products are visualized, their sizes are slightly different than expected based on a primer length plus one model. Thus, clear peaks above 150 RFU within six base pairs of the expected primer location were scored as a positive result. Allele calls were recorded for each donor (Table 24). Once genotyped, the random match probability was calculated for each sample using each of the three available population statistics (Table 25). No evidence of ethnic bias was seen in any of the samples, meaning that the difference between the statistics using each of the allele frequency databases was not statistically significant. Based on these results, SNP locations 10 and 15 were selected for padlock probe design.

Table 24: Allelic breakdown by SNP number and sample showing differentiation of loci

SNP #	Caribbean	Caucasian	Egyptian	Chinese	Polish	German	African American	Filipino
1	A	T	A/T	A	A	A/T	A	A
2	A/G	G	A	A/G	A/G	G	G	A/G
3	A/G	A/G	A/G	A/G	G	G	A/G	A
4*	T	T	T	T	T	T	T	T
5	A/G	A	A/G	A	A	G	A/G	A/G
6	A	A/C	A	A/C	A	A/C	A	A/C
7	A/G	A/G	A/G	G	A/G	A	A	A/G
8	A	A/G	A/G	A/G	G	A/G	A/G	A/G
9	G	G	G	A	A/G	A/G	A	G
10	C	G/C	G	G	G	G	G	G
11	G	A/G	A/G	G	G	G	G	A/G
12	A/G	A	A	A/G	A/G	A/G	G	A/G
13	G	A/G	A/G	G	G	G	G	G
14	C	T/C	T	C	C/T	C/T	C	T
15	C	T	T	T	C/T	T	T	T
16	A/C	A	A	A	A	A/C	A	A
17	A/C	A	C	A/C	A	A	C	A
18	C	T	C	C	C/T	C/T	C	C
19	T/C	T	C/T	T	C/T	C/T	C/T	C/T

* There was an issue with the SBE primer of SNP 4, thus it was discarded from further use.

Table 25: Random match probabilities for each of the samples sorted by the three major populations

Ancestry	Caucasian	African American	Asian
Caribbean	2.85002E-11	3.15694E-11	4.33239E-11
Caucasian	1.99643E-11	3.10547E-11	3.1658E-11
Egyptian	1.16303E-10	3.15529E-11	1.41051E-11
Chinese	7.03306E-11	9.21547E-11	1.60266E-10
Caucasian (Polish)	1.06876E-10	1.34566E-10	7.15256E-11
Caucasian (German)	3.36147E-10	4.07937E-11	9.2743E-11
African American	1.14807E-10	5.5137E-11	1.1413E-10
Filipino	5.84641E-11	3.99239E-11	2.27873E-11

Probe Design and Manufacture

Tyramide Signal Amplification Padlock Probes

TSA probes targeting SNP 15 were successfully designed within the desired parameters (Table 26). Two TSA probes were designed for SNP 15: one incorporating 10 DIG molecules

and one incorporating 20 DIG molecules (Table 27). The probe labeled with 10 DIG molecules was successfully manufactured. Maximizing the number of DIG molecules incorporated into the probe was expected to enhance the sensitivity of the assay; however, the probe incorporating 20 DIG molecules could not be successfully synthesized and was abandoned accordingly.

Table 26: Sequences of the 5' and 3' target specific ends of SNP 15 TSA padlock probe

End	Melting temperature (°C)	GC content (%)	Length (bp)	Sequence
5'	55.1	56.5	23	ACACACGTTTGGGACAAGGGCTG
3'	54.8	50.0	24	ACCTTCTTTCGTGTGCCTGTGCA**T**

The targeted polymorphism is indicated in red.

Table 27: Final sequences of SNP 15 TSA padlock probes

SNP 15 TSA Probe	Length (bp)	Final Sequence
Unlabeled	99	5'-P-ACACACGTTTGGGACAAGGGCTG**(T)$_{52}$**ACCTTCTTTCGTGTGCCTGTGCA**T** 3'
Labeled with 10 DIG molecules	99	5'-P-ACACACGTTTGGGACAAGGGCTG**(TTTTT-DIG)$_4$-(TTTT-DIG)$_3$-(TTTTT-DIG)$_3$-TTTTT**ACCTTCTTTCGTGTGCCTGTGCA**T** 3'
Labeled with 20 DIG molecules	99	5'-P-ACACACGTTTGGGACAAGGGCTG**(TTT-DIG)$_5$-(TT-DIG)$_{11}$-(TTT-DIG)$_4$-TTT**ACCTTCTTTCGTGTGCCTGTGCA**T** 3'

The targeted polymorphism is indicated in red. The linker sequence is shown in bold.

Rolling Circle Amplification Padlock Probes

RCA probes targeting SNP 15 were successfully designed within the desired parameters (Table 28). Two RCA probes were designed for SNP 15: one complementary to the sense strand and one complementary to the antisense strand (Table 29). Both probes were synthesized successfully. Along with the padlock probes, a detection probe labeled with fluorescein was designed to hybridize to the replicated detection sequences following RCA DNA synthesis.

Table 28: Sequences of the 5' and 3' target specific ends of SNP 15 RCA padlock probes

End	Melting temperature (°C)	GC content (%)	Length (bp)	Sequence
Sense Probe				
5'	54.6	52.2	23	TGCACAGGCACACGAAAGAAGGT
3'	54.8	54.2	24	CAGCCCTTGTCCCAAACGTGTGT**A**
Antisense Probe				
5'	55.1	56.5	23	ACACACGTTTGGGACAAGGGCTG
3'	54.8	50.0	24	ACCTTCTTTCGTGTGCCTGTGCA**T**

The targeted polymorphism is indicated in red.

Table 29: Final sequences of SNP 15 RCA padlock probes

SNP 15 RCA Probe	Length (bp)	Final Sequence
Sense	87	5'-P- TGCACAGGCACACGAAAGAAGGT**CTTTCACGA *CTCAATGCACATGTTTGGCTCC*GCGACTAT**CAGCCCT TGTCCCAAACGTGTGT**A** 3'
Antisense	86	5'-P- ACACACGTTTGGGACAAGGGCTG**CCTTTCCTAC GA*CCTCAATGCACATGTTTGGCTCC*TCTT**ACCTTCTTT CGTGTGCCTGTGCA**T** 3'
Detection	23	5'-fluorescein-CCTCAATGCACATGTTTGGCTCC

The targeted polymorphism is indicated in red. The linker sequence is shown in bold with the detection sequence italicized.

Probe Evaluation

Tyramide Signal Amplification Padlock Probes

Results from the SNP 15 TSA padlock probes were examined for the presence of probe signals. No probe signals specific to SNP 15 were observed in any of the samples. Initial experiments exhibited high background fluorescence with many nonspecific signals (Figures 12 and 13). By incrementally increasing the dilution of anti-digoxigenin-HRP from 1:2,000 to 1:5,000, background fluorescence and the presence of nonspecific signals was reduced to minimal levels. The optimum dilution was found to be approximately 1:4,000 ± 500. Background fluorescence and nonspecific signals were further reduced through optimization of the fluorophore amplification reagent working solution incubation time, resulting in an optimum incubation time of seven minutes. The SNP 15 TSA padlock probe was expected to hybridize to

the cells from Donor A, while hybridization to the cells from Donor B should not occur. Hybridization of the SNP 15 TSA padlock probe to buccal cells from Donor A resulted in no detectable probe signals and minimal to no background fluorescence (Figure 14). Hybridization of the SNP 15 TSA padlock probe to buccal cells from Donor B produced similar results to those seen with donor A (Figure 15).

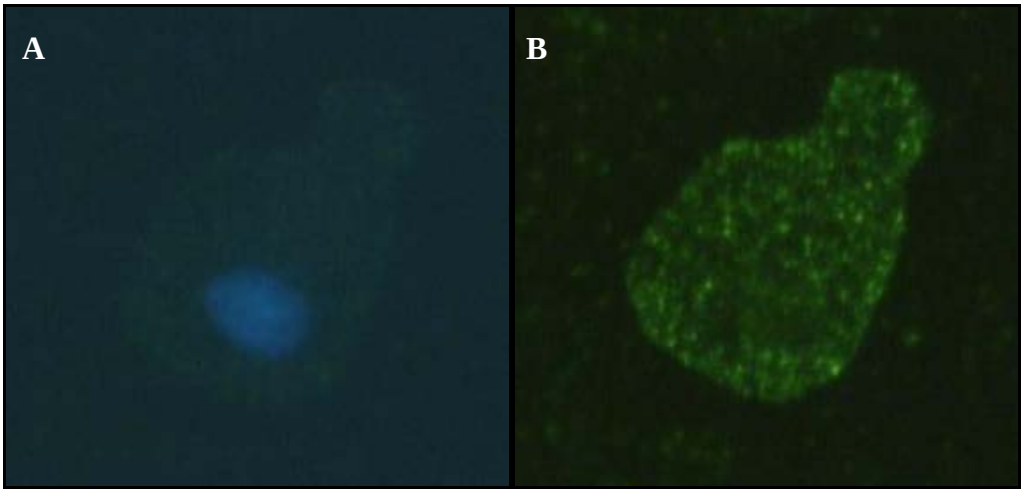

Figure 12: Buccal cells from Donor A hybridized with SNP 15 TSA padlock probe visualized at 400X magnification with (A) DAPI and (B) FITC filters.

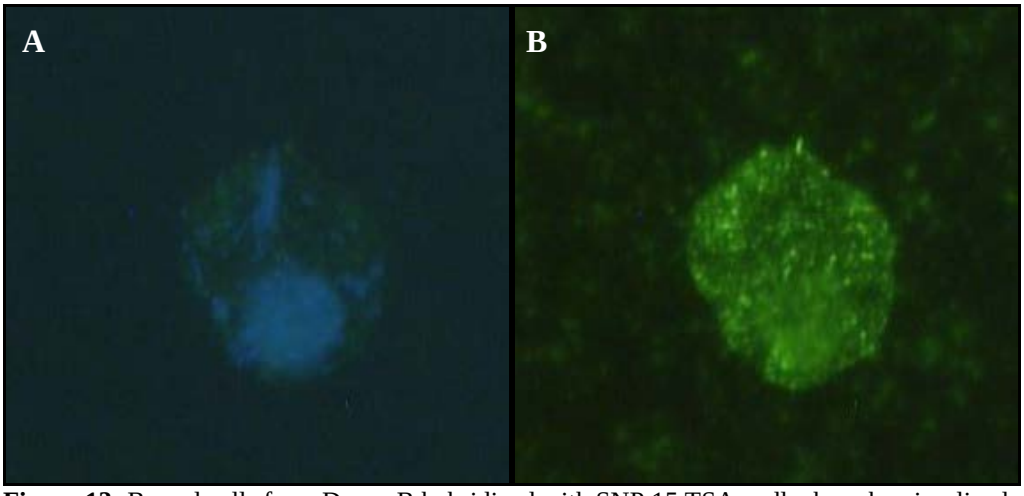

Figure 13: Buccal cells from Donor B hybridized with SNP 15 TSA padlock probe visualized at 400X magnification with (A) DAPI and (B) FITC filters.

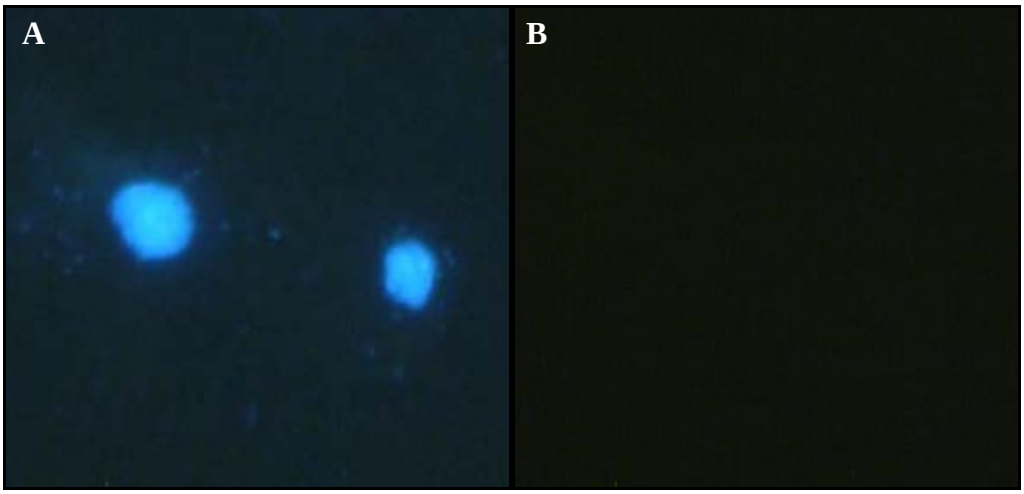

Figure 14: Buccal cells from Donor A hybridized with SNP 15 TSA padlock probe visualized at 400X magnification with (A) DAPI and (B) FITC filters.

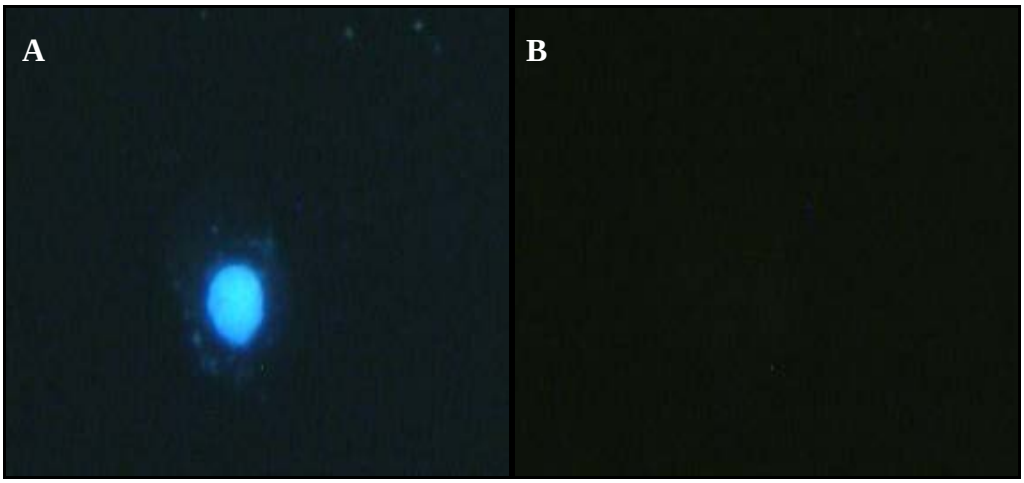

Figure 15: Buccal cells from Donor B hybridized with SNP 15 TSA padlock probe visualized at 400X magnification with (A) DAPI and (B) FITC filters.

Rolling Circle Amplification Padlock Probes

Results from the SNP 15 RCA padlock probes were examined for the presence of probe signals. No probe signals specific to SNP 15 were observed in any of the samples. No differences were observed between the sense and antisense probes. All samples displayed good nuclear morphology, minimal to no background fluorescence, and minimal to no nonspecific probe signals. The SNP 15 RCA padlock probe was expected to hybridize to the cells from Donor A, while hybridization to the cells from Donor B should not occur. Hybridization of the SNP 15 RCA padlock probe to the cells from Donor A resulted in no detectable probe signals and

minimal to no background fluorescence (Figure 16). Hybridization of the SNP 15 RCA padlock probe to buccal cells from Donor B produced similar results to those seen with Donor A (Figure 17). Variations in incubation and hybridization times and reagent mixtures produced similar results (Table 30).

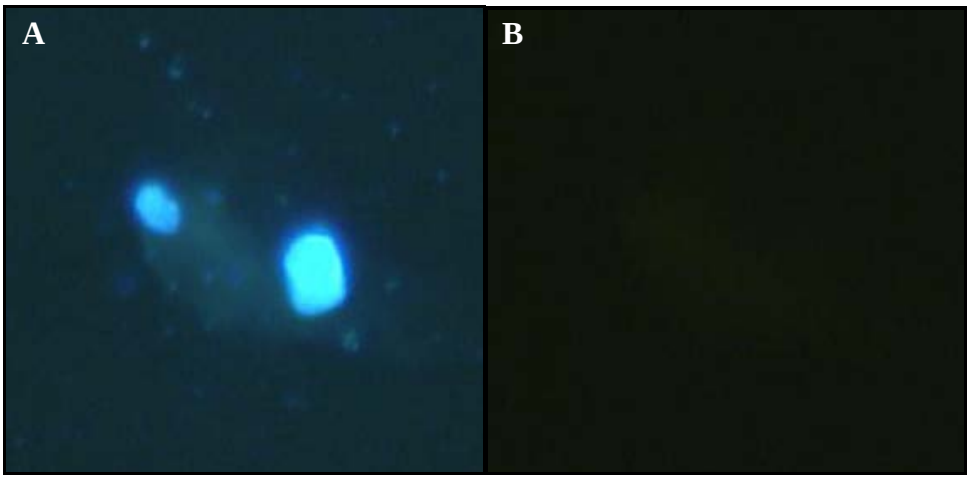

Figure 16: Buccal cells from Donor A hybridized with SNP 15 RCA padlock probe visualized at 630X magnification with (A) DAPI and (B) FITC filters.

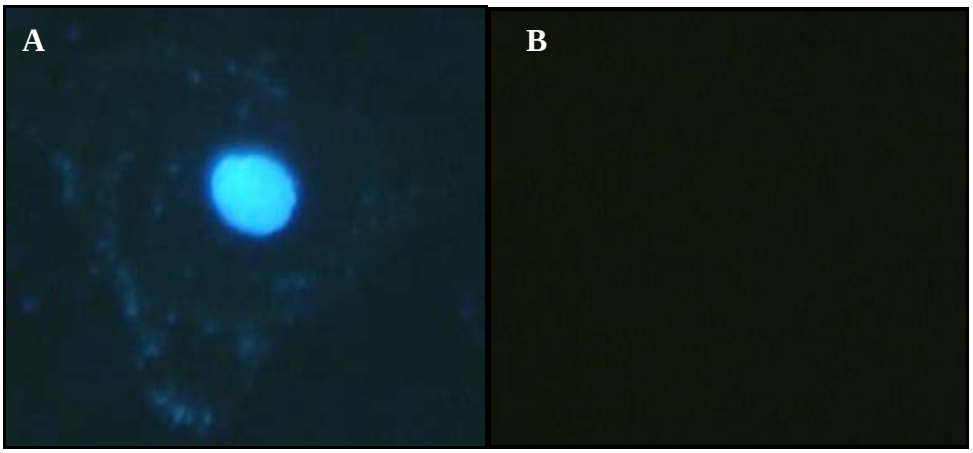

Figure 17: Buccal cells from Donor b hybridized with SNP 15 RCA padlock probe visualized at 630X magnification with (A) DAPI and (B) FITC filters.

Table 30: Summary of results produced by varying the RCA reaction conditions

Reaction Condition	Variation	Result
Duration of λ exonuclease incubation	15 minutes	No signal
	30 minutes	No signal
Duration of hybridization	15 minutes	No signal
	2 hours	No signal
Ligation mixture used	RCA Ligation Mix A	No signal
	RCA Ligation Mix B	No signal
	RCA *Tth* Ligation Mix	No signal
RCA mixture used	RCA Mixture A	No signal
	RCA Mixture B	No signal
Duration of RCA incubation	15 minutes	No signal
	30 minutes	No signal
Duration of detection incubation	15 minutes	No signal
	30 minutes	No signal

Improvement of Front End Cellular Preparation via Cytogenetic Membrane Lysis

The majority of the buccal cells and white blood cells were successfully lysed, resulting in isolation of the nuclei. Successful lysis was determined via visual confirmation (Figure 18). No genetic profiles were obtained from any of the samples (Figures 19 and 20).

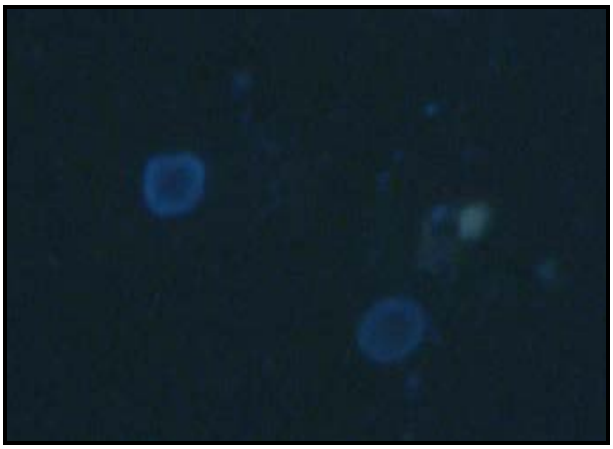

Figure 18: Nuclei successfully isolated from buccal cells following cytogenetic on-slide lysis visualized at 400X magnification with the DAPI filter.

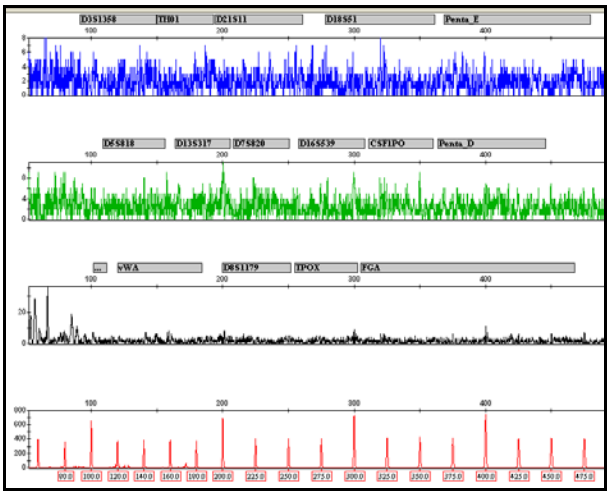

Figure 19: No profile was obtained following amplification of the nuclei from 50 epithelial cells.

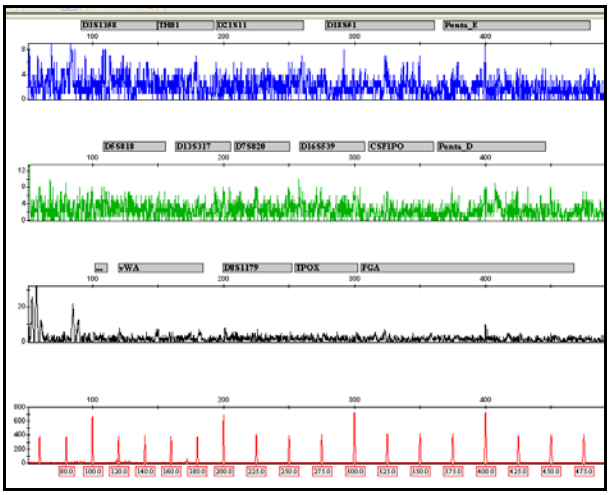

Figure 20: No profile was obtained following amplification of the nuclei from 50 white blood cells.

Optimization of Laser Microdissected Sample Processing Techniques

Direct Amplification of Cellular Suspensions

All samples produced the expected profiles with minimal dropout seen in the 50 cell samples amplified with the MiniFiler™ and PowerPlex® 16 HS kits (Table 31). Direct amplification of as few as 50 cells was performed successfully using the MiniFiler™, PowerPlex® 16 HS, and PowerPlex® S5 kits. At this time, no further lysis steps were deemed necessary for successful amplification of cellular suspensions. Although it produced satisfactory results, the PowerPlex® S5 System was eliminated from future tests. The profiles generated by the system are of nominal statistical value because only five loci are examined. Due to the limited genetic data produced by the kit, it may be more useful as a screening tool. Further testing was conducted with the MiniFiler™ and PowerPlex® 16 HS kits to determine if direct amplification was a viable approach for cells collected via laser microdissection.

Table 31: Summary of results for direct amplification of eluted buccal cells

System	Cells	Alleles Called	Minimum RFU	Maximum RFU
MiniFiler™	50-1	18/18	110	461
	50-2	16/18	53	286
	50-3	16/18	104	691
	100-1	18/18	127	1029
	100-2	18/18	185	715
	100-3	18/18	240	795
	500-1	18/18	791	2007
	500-2	18/18	742	2056
	500-3	18/18	849	1876
	1000-1	18/18	3442	7510
	1000-2	18/18	1603	5528
	1000-3	18/18	1896	5072
PowerPlex® 16 HS	50-1	28/32	101	923
	50-2	30/32	116	1147
	50-3	31/32	142	999
	100-1	32/32	198	2397
	100-2	32/32	136	2294
	100-3	32/32	192	1928
	500-1	32/32	1929	6865
	500-2	32/32	1138	6187
	500-3	32/32	1817	7819
	1000-1	32/32	2042	7632
	1000-2	32/32	1764	7798
	1000-3	32/32	1971	7649
PowerPlex® S5	50-1	10/10	197	716
	50-2	10/10	203	777
	50-3	10/10	227	805
	100-1	10/10	588	1609
	100-2	10/10	238	810
	100-3	10/10	219	1082
	500-1	10/10	1967	6050
	500-2	10/10	1453	4572
	500-3	10/10	1214	4634
	1000-1	10/10	2588	8138
	1000-2	10/10	2566	7654
	1000-3	10/10	2470	7594

Direct Amplification of Laser Microdissected Cells

Direct amplification of laser microdissected cells was not successful. A range of 25 to 100 laser microdissected cells were amplified with MiniFiler™ and PowerPlex® 16 HS, and the majority of the samples did not successfully amplify (Table 32). No profiles were produced from any of the MiniFiler™ samples. Peaks were observed in two of the 100 cell samples and one of the 50 cell samples amplified with PowerPlex® 16 HS.

A proteinase K digestion step was examined as a means of releasing DNA from the laser microdissected cells into the direct amplification reactions. Direct amplification of buccal cells following a brief incubation in proteinase K was not effective (Table 33). Triplicate samples that were amplified with PowerPlex® 16 HS did not produce profiles.

Direct amplification studies, both with and without proteinase K, did not result in the successful generation of STR profiles. Therefore, no further examinations of this technique were performed. However, further testing employing various extraction and amplification kits was explored.

Table 32: Summary of results for direct amplification of laser microdissection collected cells

System	Cells	Alleles Called	Low RFU	High RFU
MiniFiler™	100-1	0/18		
	100-2	0/18		
	100-3	0/18		
	75-1	0/18		
	75-2	0/18		
	75-3	0/18		
	50-1	0/18		
	50-2	0/18		
	50-3	0/18		
	25-1	0/18		
	25-2	0/18		
	25-3	0/18		
PowerPlex® 16 HS	100-1	23/32	76	210
	100-2	3/32	94	75
	100-3	0/32		
	75-1	0/32		
	75-2	0/32		
	75-3	0/32		
	50-1	0/32		
	50-2	0/32		
	50-3	1/32	138	138
	25-1	0/32		
	25-2	0/32		
	25-3	0/32		

Table 33: Summary of results for direct amplification with proteinase K treatment

System	Cells	Alleles Called	Low RFU	High RFU
PowerPlex 16 HS	100-1	0/32		
	100-2	0/32		
	100-3	0/32		

Laser Microdissection Extraction Compatibility Examination

LM collections of 25 and 50 cells extracted with both the ZyGEM *forensic*GEM and Qiagen EZ1 kits were successfully and consistently amplified with PowerPlex 16 HS (Table 34). Results in the 25 cell range were most promising with the ZyGEM extraction when compared to

the EZ1 extraction. Overall, more balanced profiles were observed from samples extracted with the ZyGEM method; however, these samples also had more occurrences of dropout. The EZ1 extraction resulted in less balanced profiles but exhibited fewer occurrences of dropout.

FISH treated cells that were extracted with ZyGEM consistently produced partial profiles with low RFU values and extensive dropout (Figure 21). Peaks with heights above 50 RFU were visible at approximately one third of the available loci. The EZ1 extraction also performed poorly on FISH treated cells. These samples produced results of partial profiles with peaks of very low RFU values or no peaks at all. In general, the majority of the loci demonstrated peak height values less than 50 RFU (Figure 22). The samples that were extracted with the QIAamp DNA Micro protocol for isolation of genomic DNA from laser-microdissected tissues produced the most promising results. Fewer instances of dropout were observed and the peak heights were demonstrably higher than those achieved with ZyGEM and EZ1 (Figures 23 and 24).

Table 34: Summarized ZyGEM and EZ1 extraction comparison data

	ZyGEM *forensic*GEM	Qiagen EZ1
Extraction Duration	20 minutes	1 hour 16 minutes
Extraction Type	single tube	robotic
50 Cells		
Average Height	338 RFU	435 RFU
Average Balance	80.0%	72.5%
Allele Dropout	5.2% of alleles*	0 alleles
Balance <50%	4.2% of loci	6.3% of loci
25 Cells		
Average Height	338 RFU	250 RFU
Average Balance	76.7%	63.7%
Allele Dropout	4.2% of alleles*	2.1% of alleles
Balance <50%	2.1% of loci	16.7% of loci

* Dropout in ZyGEM samples seen at larger loci: D18, Penta E, Penta D

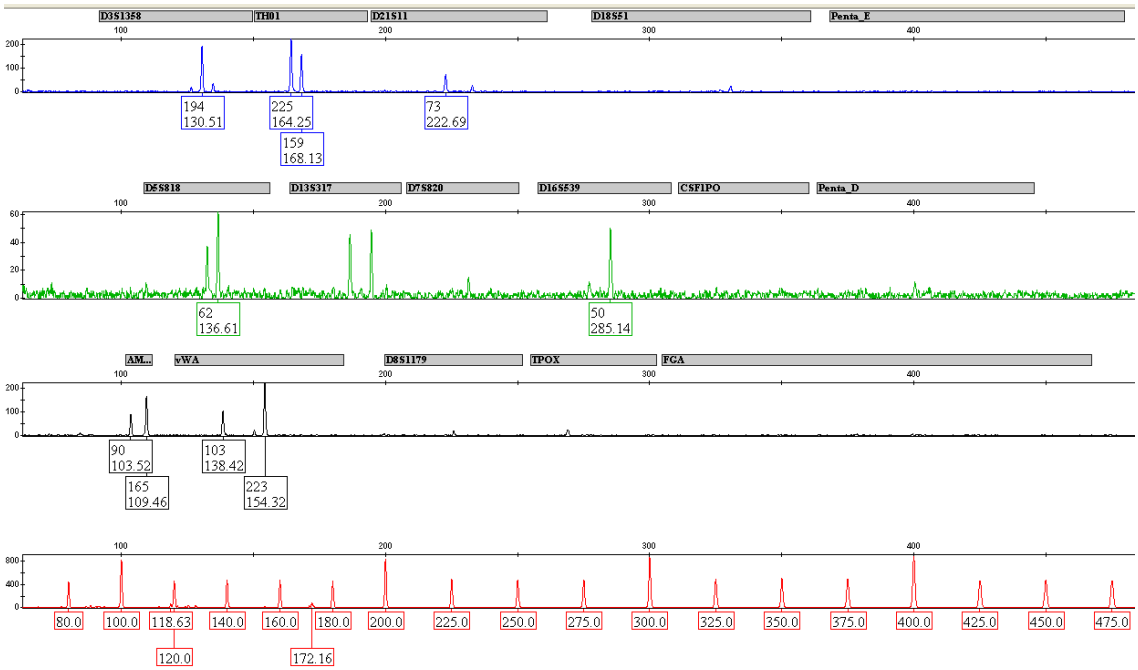

Figure 21: Partial profiles were obtained from FISH treated LM collected cells that were extracted with ZyGEM. One hundred FISH treated buccal cells were collected via LM, extracted with ZyGEM, and amplified with PowerPlex® 16 HS.

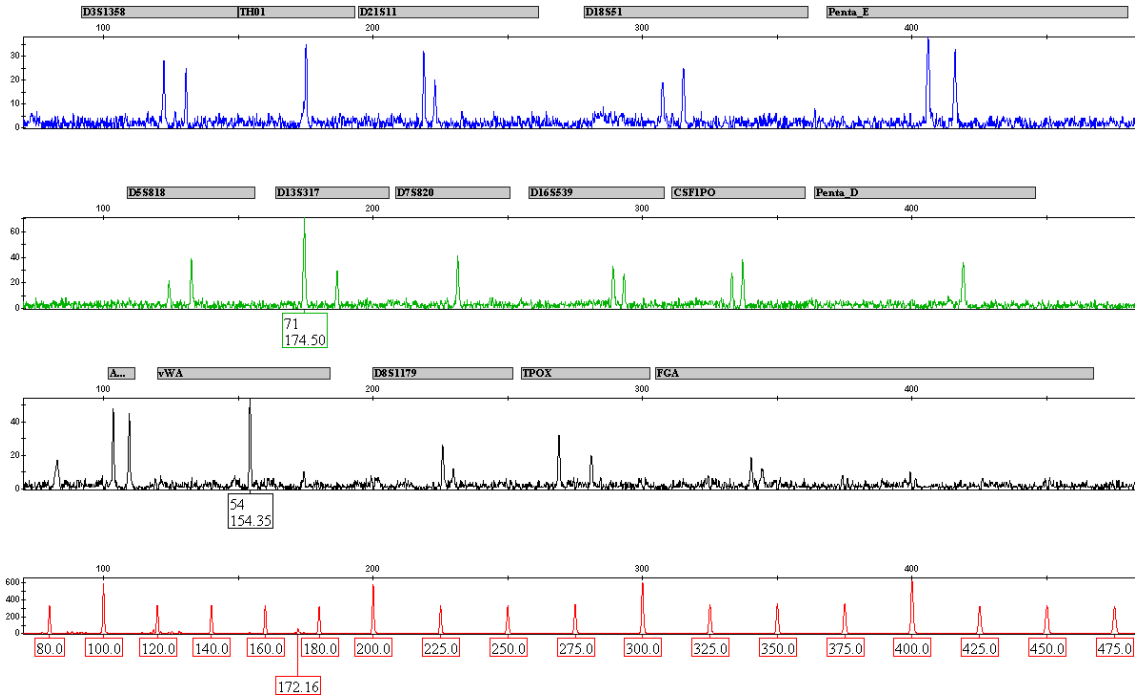

Figure 22: Very low partial profiles or no profiles were obtained from FISH treated LM collected cells that were extracted with EZ1. One hundred fifty FISH treated buccal cells were collected via LM, extracted with EZ1, and amplified with PowerPlex® 16.

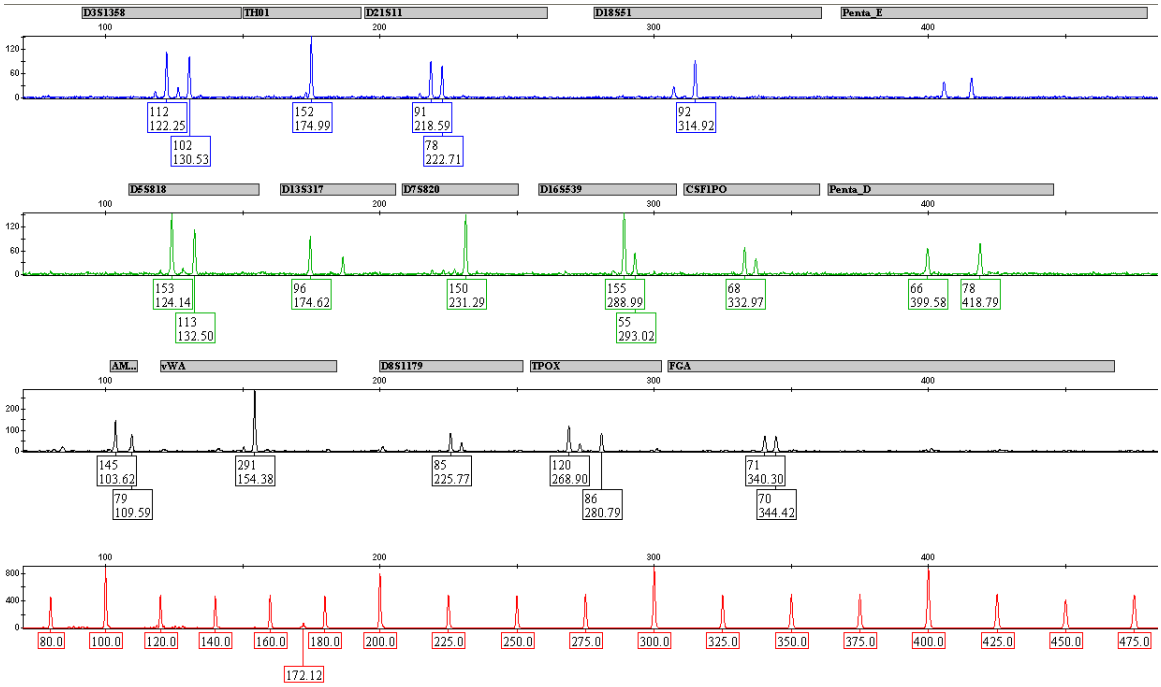

Figure 23: High partial profiles were achieved with the QIAamp micro extraction. One hundred fifty FISH treated buccal cells were collected via LM, extracted with QIAamp micro, and amplified with PowerPlex® 16.

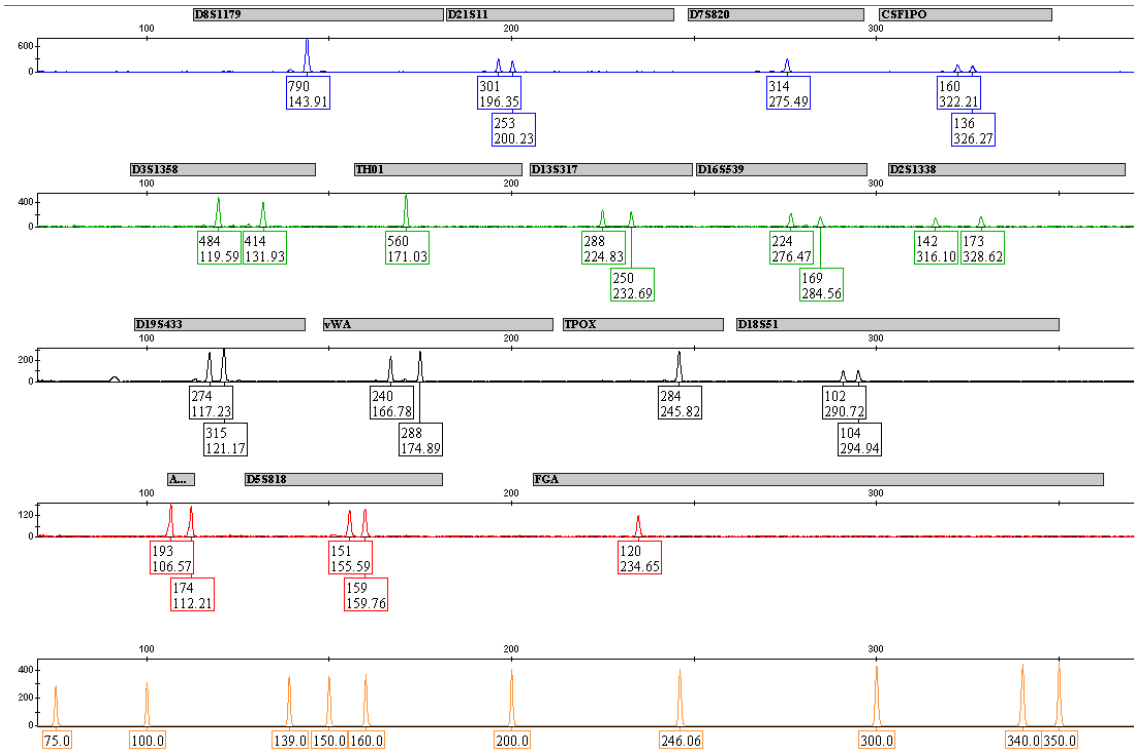

Figure 24: A full profile obtained with the QIAamp micro extraction. One hundred fifty FISH treated buccal cells were collected via LM, extracted with QIAamp micro, and amplified with Identifiler™.

Laser Microdissection Amplification Compatibility Examination

Amplification System Evaluation

All amplification kits produced high partial to full profiles from 50 cells collected via LM. PowerPlex® 16 HS amplified samples generated the highest RFU values with some allelic dropout (Table 35). PowerPlex® 16 amplified samples produced the most balanced profiles with some allelic dropout. Identifiler™ amplified samples demonstrated the lowest RFU values, consistent dropout and the most allelic imbalance.

Table 35: Summarized PowerPlex® 16 HS, PowerPlex® 16, and Identifiler™ amplification comparison data

	PowerPlex 16 HS	PowerPlex 16	Identifiler
50 cells			
Average Height	554 RFU	221 RFU	142 RFU
Average Balance	80.6%	86.3%	74.2%
Allele Dropout	8.3% of alleles	4.2% of alleles	16.7% of alleles
Balance <50%	0 loci	2.1% of loci	4.2% of loci
Cycles	32 cycles	30 cycles	28 cycles

STRboost® and PCRboost® Evaluation

Collections of 25 and 50 cells were extracted with the ZyGEM *forensic*GEM and Qiagen EZ1 extraction kits. Following amplification with STRboost® and PCRboost®, the ZyGEM extracted control samples generally performed better than the ZyGEM extracted samples amplified with STRboost® and PCRboost® (Table 36). Samples extracted with ZyGEM and amplified with STRboost® and PCRboost® demonstrated lower RFU values, more instances of allelic dropout, and less balanced profiles than the control samples. However, samples extracted with EZ1 and amplified with PCRboost® performed slightly better than the control samples. These samples demonstrated higher RFU values and better peak height balances than the controls.

Table 36: Summary of STRboost® and PCRboost® results

	Control	STRboost®	PCRboost®
ZyGEM *forensic*GEM Extraction – 50 Cells			
Average Height	680 RFU	571 RFU	435 RFU
Average Balance	85.5%	78.7%	75.6%
Allele Dropout	0% of alleles	0% of alleles	3.1% of alleles
Balance < 50%	0% of loci	2.1% of loci	9.4% of loci
Qiagen EZ1 Extraction – 50 Cells			
Average Height	366 RFU	295 RFU	666 RFU
Average Balance	75.8%	85.3%	81.3%
Allele Dropout	1.0% of alleles	8.3% of alleles	1.0% of alleles
Balance < 50%	8.3% of loci	2.1% of loci	2.1% of loci

Conclusions

Discussion of Findings

The findings of this research indicated that SNPs are not ideal targets for forensic FISH probing. Probe signals specific to the desired SNP were not detected. It is unknown if this was caused by the specificity of the probe hybridization or the sensitivity of the detection protocols; either is a plausible explanation for the lack of signals. Failure to successfully hybridize to the target sequence and successful hybridization with insufficient detection sensitivity would both produce negative results. Unsuccessful hybridization can be addressed by redesigning the probe or optimizing the hybridization conditions; however, it can be challenging and time consuming to identify the cause of the issue. Examination of alternative RCA techniques may improve the detection sensitivity; however, the alternative RCA techniques may not possess the efficiency necessary for forensic use. It has been reported that RCA is successful in only 20% to 55% of interphase nuclei targets (24, 28). For example, if 200 cells are present on a slide, as few as 40 would generate signals. Cells that are not successfully identified with FISH probing cannot be collected for further analysis and key evidence material may be left on the slide. Based on these

results, it is recommended to focus future efforts on genetic marker systems that consist of larger genetic differences.

Cytogenetic on-slide membrane lysis was evaluated as an alternative to extraction following LM collection. Following successful lysis of cellular membranes, STR profiles from the collected nuclear material could not be generated. While the cause is unknown, it is possible that the PEN membranes interfered with amplification. To catapult nuclei directly off of a glass slide, it is necessary to use higher laser energy and target the laser pulse directly on the nuclei. This may result in loss of nuclear material or ablation of nucleus. Membrane slides, which allow the user to cut around the nucleus and target an area outside the nucleus for catapulting, were used to prevent ablation of the nuclei during catapulting. While the membrane aids in collection, it is possible that it remains fixed to the nuclei during amplification, preventing the DNA from being fully released into the reaction. Other sources have indicated that although residual membranes may interfere with the amplification of unextracted laser microdissected samples, increasing the initial denaturation time to at least 15 minutes may allow for successful amplification (58). However, the work performed on this grant focused solely on performing amplifications according to the manufacturers' recommended protocols. The purpose of this was to minimize the processing time for laser microdissected samples and to allow for easy integration of the methods into forensic laboratories. Along with poor amplification results, the necessity of "dropping" samples onto the slide caused this technique to be unsuitable for forensic use. The dropping technique is difficult to control with precision and may result in loss of the sample. No further examinations of on-slide lysis techniques were performed because of the poor STR results obtained following amplification.

Although direct amplification of cellular suspensions resulted in interpretable profiles, direct amplification of laser microdissected cells was not successful. While the exact cause was not determined, it is possible that the PEN membrane interfered with amplification as mentioned above. As the use of membrane slides greatly improves the ease with which laser microdissection is performed, other methods for improving LM collected sample processing were investigated. To this purpose, various amplification kits and extraction methods were assessed. The results from this study demonstrated that the PowerPlex® 16 HS amplification kit shows promise for work with laser microdissected samples. Untreated LM collected cells extracted with both the ZyGEM and EZ1 kits were successfully and consistently amplified with PowerPlex® 16 HS. Results in the 25 cell range were most promising with the ZyGEM extraction when compared to the EZ1 extraction. Overall, the ZyGEM extracted samples displayed better balanced profiles; however, these samples also had more occurrences of dropout. Comparisons of PCR Boost and STR Boost with control samples for the ZyGEM extracted samples indicated that neither PCR Boost nor STR Boost is necessary to improve results in the 50 cell range. Results indicated that PCR Boost may improve RFU values and peak height balances for EZ1 extracted samples. Overall, the ZyGEM extracted samples continued to display better balanced profiles without the need for any additional steps during amplification.

Although the ZyGEM and EZ1 extraction methods performed well on untreated laser microdissected cells, neither extraction method was effective on cells that had undergone FISH processing. It was found that the QIAamp DNA micro extraction consistently outperformed both the ZyGEM and EZ1 extractions. Due to the fact that the ZyGEM extraction is a single tube extraction method that does not remove inhibitors, it is unsurprising that it performed poorly on FISH cells. If any reagents remained on the cells following FISH, these potential inhibitors

would not be removed from the DNA solution prior to input into the amplification reaction. At present, it is unclear why the EZ1 extracted samples did not produce more robust profiles. Both the EZ1 extraction and QIAamp DNA micro extraction methods are silica based, so it was assumed they would produce similar results. It is possible that the silica coated beads used in the EZ1 extraction do not perform as well as silica membranes on this type of sample. Another possibility is that one or more of the EZ1 extraction buffers adversely affects FISH treated DNA. Without further tests, a determination regarding the cause of the issues cannot be made. Based on the results of this research, the QIAamp DNA Micro extraction method produced the most consistent and robust results for the extraction of FISH treated LM collected cells.

The results from this study demonstrated that the ZyGEM single tube extraction shows promise for work with laser microdissected samples. The well balanced profiles seen in samples extracted with ZyGEM are of particular interest for low copy number work. When processing LM collected cells that have not undergone FISH, the following workflow is recommended: LM collected cells → ZyGEM extraction → Microcon concentration → PowerPlex 16 HS amplification. This process has the added benefit of not requiring any PCR additives or modifications to the manufacturer's recommendations for amplification. The use of ZyGEM and PowerPlex 16 HS may prove useful in LCN work when samples are processed without the use of LM. These procedures can be easily integrated into a workflow that utilizes standard sampling techniques such as swabbing, cutting, and scraping.

Implications for Policy and Practice

Laser microdissection provides a method for physically separating mixtures of cells that have similar and/or different morphologies as well as enhancing the ability to analyze LCN

samples. It has been demonstrated that LM can be used to separate cellular mixtures of different morphology as well as mixtures of the same morphology and different gender. Although separation of mixtures of the same morphology and same gender was not successful, it is important to continue to assess new techniques that can be applied to the forensic use of laser microdissection. The research performed has indicated that there are existing FISH techniques that have not been explored for forensic applications. Many of these techniques have been utilized for decades in clinical and diagnostic 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. Several forensic laboratories have invested in LM technology but are not actively using it to process casework samples. Development of new forensic uses for LM could encourage validation of LM techniques and more widespread implementation of the technology. LM can provide a tool for the law enforcement community to process difficult samples.

Concerning touch and assault evidence, although cells can be found on handled objects, they are often low in number, presenting problems for sampling, extraction, and amplification. Fast and reliable methods for processing low numbers of laser microdissected cells were examined. By using LM collection with a ZyGEM extraction and PowerPlex 16 HS amplification, full STR profiles from low numbers of cells were consistently generated while following the manufacturer's recommended protocols for all kits. Even without LM, both ZyGEM and PowerPlex 16 HS could be beneficial to processing suspected LCN samples.

Implications for Further Research

When developing a FISH panel for differentiating cells of the same gender and morphology, it would be advantageous to explore genetic marker systems that consist of larger genetic differences than SNPs. To this end, large insertion and deletion (INDEL) polymorphisms may be excellent targets for forensic FISH probing.

Most human DNA polymorphisms can be classified as either SNPs or INDELs. INDELs are highly prevalent and are thought to represent 16% to 25% of all human polymorphisms (59). The two types of INDELs, multiallelic and diallelic, can range in length from one bp to hundreds of kb (59,60). STRs, the predominant type polymorphism used in forensic science, are multiallelic INDELs. Diallelic INDELs comprise approximately 8% of all human polymorphisms but have received very little attention until recently (60).

Over the past few years, several forensic INDEL panels have been developed for use with capillary electrophoresis (61-68). These panels are typically comprised of diallelic INDELS ranging 2-30 bp in length. Short INDELs allow for multiplexing in a single PCR. With the size of the amplicons typically around 160 bp, multiplexed INDEL panels also improve the ability to amplify degraded DNA. Following amplification, the INDELs are analyzed with capillary electrophoresis. Although these specific INDELs are too short for FISH applications, they set a precedent for the use of INDELs in forensic genetic identification.

As previously mentioned, interphase FISH is typically performed on DNA sequences that are one kb to hundreds of thousands kb in length (6). FISH has already been used to detect large deletions and duplications of chromosomal regions (69-71). INDELS greater than 700 bp in length are of particular interest to forensic INDEL FISH panel development. Approximately 1,300 INDELs between 1,000 bp and 10,000 bp long have been identified (59). Based on length

alone, these INDELs may be suitable for FISH probing, but a more detailed literature review must be performed to determine if they are suitable for forensic use. Forensic INDEL selection should follow the same criteria that were used for SNP selection: a low Fst value, high heterozygosity, and functional non-linkage. Additionally, the INDELs should not convey any medical information. After appropriate INDELs are selected, a set of probes complementary to each INDEL will be associated with a specific fluorophore. For example, for a 1,000 bp long INDEL, approximately fifty 20 bp long probes labeled with many green fluorophores will be designed to hybridize to the desired INDEL. When hybridization occurs, the multiple green fluorophores will be visualized as one strong fluorescent signal, eliminating the need for time consuming signal amplification techniques. INDELs would allow for the creation of a less complicated forensic FISH identification assay.

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Dissemination of Research Findings

The NIJ Conference 2010, Arlington, VA., June 2010.

- Poster Presentation: "Optimization of Front-End Touch Sample Processing Techniques"
  R. Driscoll, A. Bathrick, and R. Bever

NFSTC Technology Transition Workshop, Lorton, VA., September 2010.

- Workshop: "Physical Separation of Forensic Mixtures using Laser Microdissection Techniques"
  R. Driscoll, H. Cunningham, A. Bathrick, and R. Bever

21st International Symposium on Human Identification, San Antonio, TX., October 2010.

- Poster Presentation: "Optimization of Front-End Touch Sample Processing Techniques"
  A. Bathrick, R. Driscoll, and R. Bever

The NIJ Conference 2011, Arlington, VA., June 2011.

- Poster Presentation: "FISH Probe Design and Development for a SNP Based Screening System for Cellular Mixtures of the Same Gender and Morphology"
  A. Bathrick and R. Bever

Mid-Atlantic Association of Forensic Scientists (MAAFS) Annual Meeting, Virginia Beach, VA., May 2011.

- Workshop: "Laser Microdissection Methods and Techniques"
  H. Cunningham and A. Bathrick

FBI Laboratory Nuclear DNA Unit Training Session, Quantico, VA., June 2012.

- Workshop: "Zeiss PALM Microbeam System Training Session"
  A. Bathrick