Final report of NIJ Grant # 2005-DA-BX-K003

"Improving the efficiency of DNA casework analysis through simple, effective, PCR-based screening methods"

5/1/06 – 9/30/09

Principal Investigator –

Eric Buel, Ph.D.

Vermont Forensic Laboratory

Waterbury, VT 05671

Authors – Janice A. Nicklas, Ph.D. and Eric Buel, Ph.D.

ABSTRACT:

The forensic laboratory must continually meet the challenge of increased casework and the criminal justice community's demand for timely analysis of evidence. New assays that could assess sample quality (degraded versus intact) or easily yield preliminary source information (DNA screening to differentiate victim, suspect, male, female) would allow the laboratory to focus its energy on the most probative case samples.

To achieve these goals, this grant had two major Aims: 1) development of a fast, simple profiling method for sample screening to select samples which would be most probative and 2) development of a test for sample DNA degradation state.

To accomplish Aim #1, multiplex SNP assays were developed using a melting FRET technique. In this assay, two probes are present: a sensor with a perfect match to one allele (with fluorophore) but with one mismatch to the other allele and a second, anchor probe (with quencher). As PCR proceeds, fluorescence is quenched; in the melting phase, fluorescence is gained. Determination of which allele(s) is present depends on the melting temperature where the fluorescence is regained. Several assays were developed for the six color Corbett RG6000 and for other four color real-time instruments. This technique can discriminate between 95-99% of samples from different individuals. A paper describing these results is published in the Journal of Forensic Sciences.

The SNP FRET assay is quite complicated in terms of number of primers and it uses biallelic SNPs which are unfamiliar to most forensic analysts. We, therefore, sought a new assay which uses multi-allelic STRs familiar to forensic analysts by attempting to adapt the melting FRET technique to STRs. While some differences were detected, the technique did not work as hoped and was abandoned.

We have added an additional part to Aim #1 (AIM #1C) through a GAN: Development of a new assay for fast determination of stain donor using high resolution melting (HRM) of STRs. HRM goes beyond classical melt curve analysis by studying the melt in much finer detail using special DNA dyes such as Eva Green™. PCR products can be differentiated based on length, sequence or complementarity. In particular, it was hoped that HRM could distinguish alleles of STRs. Twenty-two forensic STRs were chosen for analysis and tested with 30-50 sample DNAs with different, known genotypes by performing real-time PCR and HRM melting in a Corbett Rotorgene RG6000. The three STRs that generated the greatest difference in melting profiles between genotypes were chosen for further study and development into an assay. The assay was then tested for reproducibility, uniformity for a known genotype, and melting profile constancy over dilution. The assay can discriminate between individuals, making it useful as a screening technique. We also performed some preliminary experiments using Plexor technology and melting of STRs which were very promising.

To accomplish Aim #2, we designed a multiplex PCR with two overlapping *Alu* amplicons using the Plexor technology. The Plexor technology relies on the use of two alternative basepairs, isoC and isoG, which pair only to each other. The forward primer is designed with a 5' isoC with attached fluorophore and during amplification, an isoG with attached quencher is incorporated and fluorescence is quenched due to proximity of the fluorophore and quencher. The decrease in fluorescence is monitored during PCR. Amplification of the longer *Alu* amplicon is only possible in non-degraded or slightly degraded DNA whereas the smaller amplicon will be amplifiable even in highly degraded DNA. The ratios of the concentrations of the two products give a quantitative measure of degradation state. The concentration of DNA measured by the long product can be used to determine input DNA for STR analysis and the ratio predicts the amount of ski-slope observed.

Table of Contents

EXECUTIVE SUMMARY:

This final report for grant #2005-DA-BX-K003 describes the results of the two main Aims. The thrust of the research was to develop forensic assays to streamline forensic laboratory analysis in order to save both time and money. The forensic laboratory must continually meet the challenge of increased casework and the criminal justice community's demand for timely analysis of evidence. New assays that could easily yield preliminary source information (DNA screening to differentiate victim, suspect, male, female) or an assay that would assess sample quality (degraded versus intact) would allow the laboratory to focus its energy on probative case samples and analyze them in the most appropriate manner.

The AIMs of this current research proposal were:

1) Development of a fast, simple profiling method for sample screening to permit the judicious selection of relevant samples in order to reduce the total number of samples taken through the entire DNA process to only those which would be most probative. STR profiling using conventional typing techniques, i.e. gel-based or capillary electrophoresis methods, is routinely performed for sample individualization. However, it is impractical to use such costly and labor-intensive methods to analyze each and every sample found at a crime scene. A simple analytical method, which could allow the analyst to select only those probative samples for further analysis, would save time and resources. A crime scene may have numerous stains; many of these may be from the same victim or the same suspect. Clearly, identity can be determined to near certainty using the usual 13 STR loci but often such a determination is of limited value from a criminal justice point of view; for example, determining that blood on the assailant's shirt is his/her own blood. Also, victim stains in the victim's house may not be as relevant as

the suspect's stains in the victim's house. Such a complete analysis often becomes a waste of time and money. A method is needed to quickly screen these stains to determine which are from the same person (duplicates) or an irrelevant person (to the crime) and, therefore, not a priority for STR analysis. Quick analysis of several SNPs or one or two STR loci, while certainly not by any means a definitive test of identity, will usually allow determination of whether a blood sample came from a certain individual. This is sufficient for a quick screen of crime scene stains to answer the question – Is this stain of possible victim or assailant origin? Development of such a screen would be of benefit to the forensic community.

2) Development of a test for sample DNA degradation state to rapidly determine which type of analysis would yield the best DNA profile (i.e., regular STRs, mini-STRs, mtDNA). Environmentally challenged forensic samples may often be degraded into small DNA fragments, resulting in a limited DNA profile using commercial STR profiling kits. For example, DNA degraded to an average size of 150bp will not allow amplification of a 320bp D18S51 allele. Because of this, forensic researchers have developed sets of primers to create smaller STR products (mini STRs) (Butler et al., 2003; Wiegand and Kleiber, 2001). Appropriate quantitation methods designed to examine the average base pair size of the sample, would better predict the amount of template required to optimize the performance of regular STR kits or mini STR kits, and would allow the examiner to decide which assay would be a more appropriate approach for analysis. A precious sample could be conserved for an appropriate "mini STR" analysis or time and resources saved if it is determined that a sample is too degraded to profile. Alternatively, if one DNA sample from a crime scene is less degraded, then testing that sample is preferable. If there is only one sample, then determining how degraded the sample is allows the

correct choice as to DNA input into STR analysis. When this research was begun, the current DNA quantitation kits would not accurately predict the DNA quantity needed for STR analysis when the DNA was degraded; thus, development of this test was important.

Completion of the Aim #1 first involved development of several multiplex assays using SNPs to differentiate between crime scene samples. SNPs were chosen because techniques for genotyping SNPs are easily multiplexed in a real-time instrument, equipment which most forensic laboratories currently utilize. The technique chosen involved the melting of FRET (fluorescence resonance energy transfer) probes. Briefly, this assay employs dual probes known as the sensor and anchor, one fluorescently labeled and the other labeled with a quencher. The sensor probe is a perfect match to one allele of the SNP but one mismatch to the other allele; the anchor binds adjacent to the sensor probe and has a 3' extra phosphate to prevent elongation. As the PCR proceeds, fluorescence is quenched because when both probes bind to the PCR product, the energy from the excited fluorophore is transferred to the quencher without the emission of a photon. In the melting phase, fluorescence is gained because the sensor and anchor probes are separated when they melt off of the PCR product. Determination of which allele(s) is present in a particular sample depends on the melting temperature where the fluorescence is regained. The DNA of a homozygote for the allele matching the probe melts at high temperature, whereas the DNA of a homozygote for the allele not matching the probe melts at low temperature and the DNA of a heterozygote melts half at high and half at low temperature. This assay uses only one fluorophore to type a SNP; thus, in a six-color instrument, six SNPs can be simultaneously typed by putting a different fluorescent reporter on each specific anchor probe. Other methods such as TaqMan require two reporters for each SNP.

Assays were designed using the software program Beacon Designer. The primers were ordered and tested to ensure they generated the correct size product. The probes were then tested to determine if sufficient melting temperature differences existed between the alleles (i.e., the peaks were clearly separate). The probes (and occasionally the primers) for several of the loci had to be re-designed to change the allele on the probe, change the orientation of the probes (sense strand versus antisense strand) or move the sensor and anchor on the PCR product. The assay for some loci could not be adjusted to get resolution of the peaks and were dropped in favor of other, more robust loci. An assay for gender was also developed using the differences between the X-linked ZFX gene and the Y-linked ZFY gene. This assay went through several modifications changing primers and probes to obtain the final assay. Once the final loci were chosen, they were multiplexed.

Several multiplex SNP assays using FRET chemistry were developed. The assays were optimized for probe and primer concentrations, mastermix, and PCR cycling parameters. The assay was also validated on ten samples using confirmation by DNA sequencing. Two alternate 6-plex assays (with and without gender determination) were developed for the six-color RG6000 real-time instrument (Corbett Robotics) and one seven SNP plus gender assay (performed as two 4-plex assays, one with gender the other without) has been developed for use in four/five color real-time instruments. The assay was recently checked for the effect of inhibitors and its ability to detect mixtures. The data presented also suggests that by running appropriate quantitation standards, this SNP assay could double as a means to quantitate the DNA in samples.

Because this assay is quite complicated in terms of the number of probes and primers involved and also because SNPs are not familiar to most forensic analysts, attempts were made to develop a discrimination assay based on STRs. STRs have much higher discrimination powers than SNPs because they have a larger number of alleles. Use of the same melting FRET

technique was attempted; here, however, the sensor probe would now be made long to cover the longest allele of the STR. Long STR alleles in a sample would theoretically melt at high temperatures while short alleles would melt at lower temperatures, therefore providing a way to discriminate alleles and, thus, discriminate individuals. This assay was attempted with several arrangements of sensor and anchor probes using the THO1 locus. This included sense and antisense orientations and moving the anchor to cover part of the STR repeat. While some discrimination was observed, it was not sufficient to develop a viable assay. Some experiments were also attempted using D6S2956 which has a high GC content in its repeat (CGTC) in the hopes that the higher GC content would yield higher melting temperatures and more discrimination between alleles. Amplification of this locus was poor and the STR FRET studies were abandoned for another technique involving high resolution melting (HRM).

HRM uses special dyes, controlled melting and software to type SNPs by slight melting temperature shifts. The technology can theoretically detect any difference between PCR products such as sequence (polymorphism) or length. Some real-time PCR instruments are now equipped to handle this technology. The goal was to try HRM on selected STRs instead of SNPs to see if HRM is capable of differentiating the various genotypes at a locus. This method only allows the evaluation of one STR at a time unless the multiplexing of two STRs with highly different melting temperatures is shown to be a viable option. However, if the differentiation of the myriad of genotypes within a polymorphic STR locus is possible with HRM, then the information content of one or two STRs will equal that of perhaps eight or more SNPs. It is not envisioned that HRM could replace the usual capillary or gel-based analysis of STRs, but the simplicity of this technique, a set of primers and a specific dye, could open the door to the widespread use of a facile screening method to speed the analysis of the most probative crime scene samples. Preliminary data using a demo loaner instrument from Corbett Research on two

individuals for each of ten different THO1 genotypes (20 total individuals) plus duplicate wells for eight individuals of genotype 8-8 was obtained. The results were very encouraging as the different genotypes had different melting patterns and were the same for individuals with the same genotype. Additional work with several other loci indicated that HRM effectively differentiated genotypes and that this approach could be successful.

A two color plus HRM RG6000 from Corbett was purchased to pursue these studies. We tested all of the 12 CODIS STRs plus a number of additional forensic STRs (22 total loci). Each locus was tested on 30-50 sample DNAs with different, known genotypes by performing real-time PCR and HRM melting in a Corbett Rotorgene RG6000. The three STRs that generated the greatest difference in melting profiles between genotypes were chosen for further study (THO1, vWA and D18S51) and development into an assay. The assay was then tested for reproducibility, uniformity for a known genotype, melting profile constancy over dilution effect of inhibitors, ability to detect mixtures and discrimination potential. The assay can discriminate between individuals, making it useful as a screening technique.

Some work was also performed testing the Plexor methodology's ability to detect STR genotypes by melting differences. The Plexor technology was chosen because it would be possible to multiplex several STRs. Preliminary results with THO1 using HRM and also regular melting are very promising.

For Aim #2's degradation detection assay, we initially started with a multiplex PCR with three overlapping *Alu* amplicons using a gel readout system. The amplification of the long and medium length amplicons will only be possible in non-degraded or slightly degraded DNA while the smaller amplicon will be amplifiable even in highly degraded DNA. Thus, ratios of the different products should give a qualitative (or maybe even quantitative) measure of degradation state. After this method was tested with a gel readout and shown to be feasible, we switched to

using a real-time system using Plexor™ technology. This technology uses fluorescently labeled primers (different fluorophores can be used for different multiplexed amplicons) to easily detect the different products in a real-time format. The key points of the technology are that the forward primer is designed with a 5' isoC with attached fluorophore, during the second round of amplification, an isoG with attached quencher is incorporated into the amplicon by base pairing with the isoC residue; thus, fluorescence is quenched due to the proximity of the isoC and isoG. The decrease in fluorescence is monitored during PCR in a real time instrument. For these experiments, we placed a different fluorophore on each of the specific reverse primers (FAM for long, CAL Fluor Orange 560 for medium and Cy5 for short).

During extensive experiments to optimize this new real-time version of the assay, it became clear that balancing the three reverse primers to make a robust assay was not possible. A necessary validation for the assay is that a simple dilution of a sample will not change the results for the long to short, long to medium or medium to long ratios. This is tested by plotting the ratio (difference in Cts) versus the log of the DNA concentration. The graph should be a flat line (slope between -0.1 and 0.1). It was not possible to obtain such a line despite numerous attempts at adjusting multiple variables in the assay (primer concentrations, temperatures, buffers, Mg++, fluorophores used). Because of this problem, the assay was reduced to a duplex assay with only the long and short primers. This new duplex assay could be validated with diluted DNA with lines having slopes in the proper range. The assay was optimized and then tested on degraded DNAs. Degraded DNAs were created by treating ten high concentration DNAs with DNase I for 0 to 128 minutes. Using these degraded DNAs, the ratio of the concentrations of the short to long products was shown to give a quantitative measure of degradation state. In order to determine if the calculated degradation ratio (ratio of the concentration of short to long product), was predictive of STR success, degraded DNAs were

diluted to 0.1ng/ul based on the quantitation determined by the long product. The DNAs were amped using the ABI COfiler kit. All amplifications were successful in producing a full seven locus profile if 1ng of input DNA could be added. Thus, the concentration of DNA measured by the long product can be used to determine input DNA for STR analysis. The short/long ratio for a DNA sample also predicted the amount of ski-slope (loss of RFUs for larger STR loci) observed.

After a several month-long hiatus, experiments resumed on the degradation assay to evaluate the reproducibility of the measurements on a variety of environmentally degraded samples. Samples that had been kept at room temperature, 37°C or taped to a window in sunlight were studied. The data from the degradation assay indicated that the samples kept at 37°C were the most degraded, as expected. Unfortunately, the results for the long amplification were not as consistent as hoped (although the results for the short amplification were good); this inconsistency in the long results clearly affects the short/long ratio. Another experiment used six samples (five from the above set and also the 9947 control DNA from an Applied Biosystems STR kit) and ran them three times in duplicate, twice in the RG6000 and once in the RG3000. The numbers are once again not as tight as one might like. There are outliers for several of the samples.

Experiments during the last three months of the grant performed a number of replicate experiments which worked well, tested the effects of inhibitors and tested some new fluorophore combinations.

This assay may not be applicable to routine casework analysis, but some samples which may have been exposed to conditions known to degrade DNA could be candidates for this assay. Samples of limited quantity and of a critical evidentiary nature may be screened prior to typing using this assay to determine the quality of the DNA. As many laboratories may not have the

expertise to do mini-STR typing, appropriate sample selection could allow labs to perform their in house analyses and send highly degraded samples to commercial laboratories that offer this service. As these commercial services are typically very expensive, the ability to select only those samples which truly need mini-STR analysis will save money.

In conclusion, the research performed under this grant developed assays to meet both of the AIMs. SNP FRET melting assays and a STR HRM assay were developed as screening assays to discriminate whether crime scene samples were from different individuals and thereby determine which were most probative. A Plexor™-based state of DNA degradation assay was developed to determine whether a DNA sample was suitable for STR, mini-STR or mtDNA analysis and to better assess the concentration of DNA needed for STR analysis.

MAIN BODY OF THE FINAL TECHNICAL REPORT:

This is the final report for grant #2005-DA-BX-K003. The project was successful in that assays were developed to meet both of the stated goals.

I. Introduction

1. Statement of the problem:

Because of the growing realization of the power of DNA analysis, forensic laboratories are faced with ever increasing numbers of samples to test. This results in increasing backlogs and increasing costs. New quick methods must be developed to streamline processes to test only the most probative samples. This will speed up the process and also eliminate testing duplicate samples or low quality samples not likely to yield court worthy or CODIS uploadable results. Crime scenes often have many samples from the victim or suspect, this must be reduced to a workable amount of samples that can be processed efficiently. Complete STR analysis of numerous nonprobative samples from the victim (e.g. victim samples in the victim's house) is not an efficient use of resources; one wants to profile samples that are not from the victim, i.e. possible suspects. If the samples can be quickly screened to remove the victim's samples then time and money are saved. Development of such screening assays is very important. It is also imperative to determine the quality of the sample; if one sample is less degraded, then testing of that sample is preferable. If there is only one sample, then determining how degraded the sample is allows the correct choice as to DNA input into STR analysis or change to a different assay type. Development of assays to determine the degradation state of the DNA in a sample is, thus, also very important.

2. Literature citations and review

Aim #1A+B - Development of a Fast, Simple Profiling Method For Sample Screening – STR FRET and SNP FRET

FRET technology is the basis for detection using the Roche LightCycler™. Hiratsuka et al. (2002) describe using melting FRET to detect five polymorphisms involved in drug sensitivity. The 5' probe was labeled with FITC and the 3' probe with LC Red 450 and detection was performed on a LightCycler™. Heterozygotes and the two homozygotes could easily be determined. Pont-Kingdon and Lyon (2003) also used melting FRET on a LightCycler™ to type six chromosome 21 SNPs as 3 duplexes. They used fluorescein label on the Reference probe and LC Red 705 or LC Red 640 on the two multiplexed anchor probes. They actually performed quantitation with this assay to determine trisomy 21 (i.e. two copies of one allele and one copy of the other). Lareu et al. (2001) typed Y chromosome SNPs on a LightCycler™. They created four singleplexes and two duplexes using fluorescein label on one probe and LC Red 705 or LC Red 640 on the two multiplexed probes. They were able to multiplex all 4 SNPs although some peaks overlapped making interpretation somewhat difficult. The assay was quite sensitive (down to 100pg of DNA) and they could detect the Y SNPs in a mixture of female DNA (up to 1:300). Martinez-Garcia et al. (2004) describe methods to even out peak heights in heterozygotes by reducing probe concentration. This should make mixtures or imbalance easier to detect. Most of these assays detect only a single SNP although several duplex assays (Lin et al., 2004; Faggioni et al., 2006), a triplex assay (Schutz et al., 2006), and a four-plex assay (Murani et al., 2005) are reported.

SNPs have become very important in gene mapping, pharmacogenetic and forensic applications. Reviews of SNP applications in forensics (Krawszak, 1999; Amorim and Pereira,

2004; Gill, 2001; Chakraborty et al., 1999) find advantages and disadvantages to using SNPs. Advantages include smaller PCR product size (more useful on degraded samples), higher throughput technologies and possibly lower cost/sample. Disadvantages are the lack of databases, mixture interpretation, and the requirement of 3-4 times more SNPs to obtain the same information content as STRs. Two recent reviews summarize technologies for typing SNPs (Budowle, 2004; Sobrino et al., 2005). The main technologies include: single nucleotide extension (ABI's SNaPshot kit; Orchid's SNPstream), allele specific hybridization (old DQ alpha kits), FRET hybridization probes, TaqMan™, molecular beacons, oligonucleotide ligation followed by hybridization (Illumina), invasive cleavage (Invader®), minisequencing (Pyrosequencing), genechip arrays, mini-sequencing followed by MALDI-TOF mass spectrometry (Sequenom), fluorescence polarization and allele specific PCR. Many of these technologies require an expensive piece of specialized equipment (MALDI-TOF, Pyrosequencer) and/or are not easily multiplexed (TaqMan, Invader). A very large number of SNPs are available for development of forensic assays. Many thousand are present in the NCBI SNP database (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?CMD=search&DB=snp). The ALFRED database (http://alfred.med.yale.edu/alfred/) contains the allele frequencies for a large number of SNPs in many populations around the world. SNPs which have low variation in allele frequencies between populations (low Fst) can be found. A low Fst makes the SNP useful in any population as the frequencies will be similar.

Aim #1C - Development of a Fast, Simple Profiling Method For Sample Screening – HRM assay

Melt curves are often used in real-time PCR to determine if the product generated is specific. This approach is applicable for SYBR Green or non-TaqMan based probe methods, with a single

sharp melt curve the expected outcome for a well-designed assay. High resolution melting (HRM) (Wittwer et al., 2003; Gundry et al., 2003) goes beyond the classical melt curve analysis by studying the melt in much finer detail. PCR products can be differentiated based on length, sequence or complementarity; single base changes (SNPs) can be genotyped (Grievink and Stowell, 2008; Kristensen and Dobrovic, 2008; Tedde et al., 2008) or single base mutations can be detected (Krypuy et al., 2006, 2007; Saitsu et al., 2008). The HRM method requires the two amplification primers, a PCR mastermix containing a specific dye suitable for the analysis [LCGreen™ I and LCGreen® Plus (Idaho Technology Inc., Salt Lake City, Utah), SYTO 9 (Invitrogen, Carlsbad, CA), Eva Green™ (Biotium, Hayward, CA), BEBO (Tataa Biocenter, San Francisco, CA)] and the proper instrument utilizing specific melting parameters and dedicated software. Initially HRM was performed on only a few dedicated instruments [HR-1 (Idaho Technology Inc.); Light Typer (Roche Applied Science, Indianapolis, IN), LightScanner® (Idaho Technology Inc.)] but now the technology is offered by other manufacturers [Rotorgene 6000 (Corbett Robotics, Inc, San Francisco, CA); ABI 7500 Fast (Applied Biosystems, Foster City, CA)].

Aim #2 - Development of a Test for Sample DNA Degradation State

For forensic samples, it is often important to determine the degradation state of the DNA to know if amplification of the larger autosomal STRs will be possible. Analysis of weathered samples or old bones will be compromised by degradation of the DNA. Niederstatter et al. (2007) developed a dual human total nuclear (*RB1* gene) and mtDNA quantitation assay. They used several sized products for each locus in order to assess DNA degradation. When DNA is degraded, amplification of the larger PCR products will be decreased or fail relative to the smaller products. Swango et al. (2006) have also developed an assay for degraded DNA using a

small (*CSF1PO*) and larger (*THO1*) genomic target and well as an IPC. The ratio of the amplification seen for the two loci can be used to determine a ratio to assign a degradation score. This assay has been validated for casework samples (Swango et al., 2007).

3. Statement of hypothesis or rationale for the research

The Aims of proposal were: 1) development of a fast, simple profiling method for sample screening to permit the judicious selection of relevant samples in order to reduce the total number of samples that must be taken through the entire DNA process to only those which would be most probative and 2) development of a test for sample DNA degradation state to determine quickly which type of analysis would yield the best DNA profile (i.e. regular STRs, mini-STRs, mtDNA).

II. Methods

Aim #1A - Development of a Fast, Simple Profiling Method For Sample Screening – SNP FRET assay

While STR profiling is completely definitive for sample identification, it is expensive and time-consuming and, for these reasons, cannot be performed on every single sample that may be found at a crime scene. A faster, cheaper method is needed to quickly screen crime scene samples to determine which samples require complete profiling. This new method need not be perfectly definitive nor give a complete profile; it simply must be able to differentiate between most victim and suspect and, perhaps resident, samples. While full profiling for CODIS requires 13 STRs, SNPs are also used for identification purposes and more commonly for disease association studies and mapping. SNPs have the advantage that many molecular methods have been developed for their rapid and inexpensive detection. Using an assay with four SNPs, each

with p=q=0.5, the chance of two random individuals having the same result (same genotype) is only 2% while for 8 SNPs it is only 1/2500 (see Insert 1).

Insert #1 - SNP identity percentage calculation
Assume p=q=0.5 for alleles A and B of a SNP.
Genotypes are: AA (freq 0.25), AB (freq 0.50), BB (freq 0.25) by Hardy-Weinberg
Determine freq of two people being identical: AA+AA (freq =0.25x0.25=0.0625) plus AB+AB (freq=0.5x0.5=0.25) plus BB+BB (freq =0.25x0.25=0.0625). Total = 0.375
For n independent SNPs = $(0.375)^n$; in particular for four SNPs = $(0.375)^4$ = 0.01978 or ~2% or for 8 SNPs = $(0.375)^8$ = 0.00039 or 0.04% or ~1 in 2500.

FRET hybridization probe melting technologies can be used to detect SNPs (Hiratsuka et al., 2002; Pont-Kingdon and Lyon, 2003; Lareu et al., 2001). Figure Method 1 demonstrates the FRET melting point assay method. In this assay, PCR is performed using two primers flanking the SNP. Two probes are also present: one 5' situated probe with a 3' fluorophore label which covers the SNP, referred to as the reference or sensor probe, and a second, 3' situated probe called the anchor probe, labeled with a 5' quencher and a 3' phosphate to prevent elongation. The reference/sensor probe is designed as a perfect match to one allele but has one mismatch with the other allele. As PCR proceeds, additional fluorescence is quenched because when both probes bind to the PCR product, the energy from the excited 3' fluorophore is transferred to the quencher and there is no emission of light (see Figure Method 1).

In the melting phase, fluorescence is gained because as the temperature increases, the probes melt off the PCR product and FRET between the fluorophore and the quencher is lost in solution. Determination of which allele(s) is present depends on the melting temperature where the fluorescence is regained. This method uses only one reporter fluorophore per SNP; thus, theoretically in a four-color real-time instrument, four SNPs can be multiplexed making a simple, one-tube profiling assay feasible. Currently, several five-color and one six-color real-time instruments (the Corbett RG6000 used here) are commercially available allowing even

greater multiplexing. Figure Method 2 shows a typical SNP melting curve for a fluorescence method (from Mutation Detection using FRET analysis application note, Corbett Research).

Figure Method 1 – FRET SNP melting assay. A) Lists the components used in the assay. i.e. two unlabeled PCR primers, a fluorescently tagged SNP probe complementary to alleles A with a 1 bp mismatch to C at the SNP position and an anchor probe which has a 5' quencher. B) Demonstrates that as PCR proceeds, additional probes anneal and thus, fluorescence is quenched. C) Presents the melting phase that takes place from 50°C to 80°C after 45 rounds of PCR is completed. The mismatched SNP probe will melt off first; thus, the DNA from an individual with this allele will regain fluorescence at a lower temperature. The perfect match probe melts off later; thus, this allele will regain fluorescence at a higher temperature. A heterozygote will gain half the fluorescence at the lower temperature and half at the higher temperature. D) Shows the plot generated by the Corbett software with the change in fluorescence over the change in temperature (dF/dT) plotted versus the temperature. This demonstrates the low (mismatch) and high (perfect match) peaks.

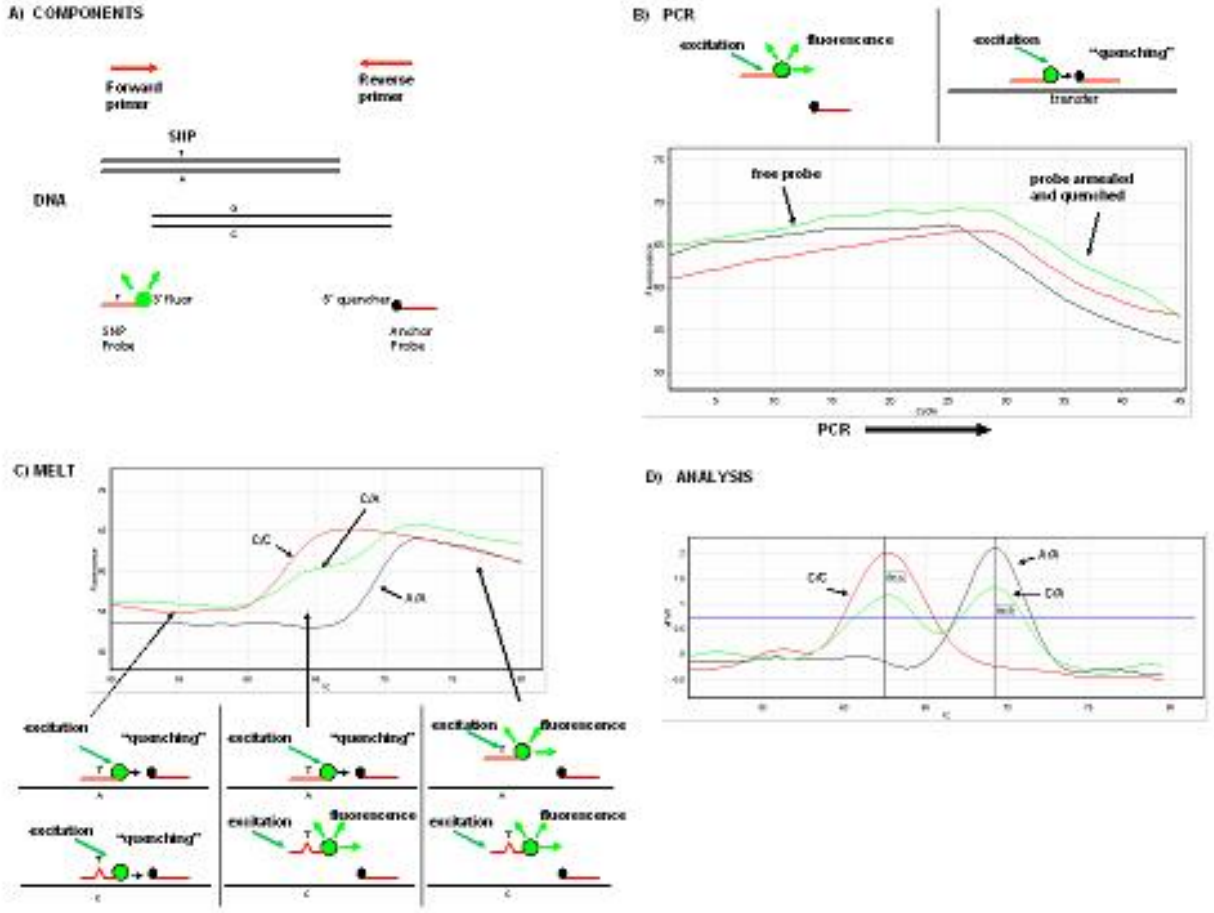
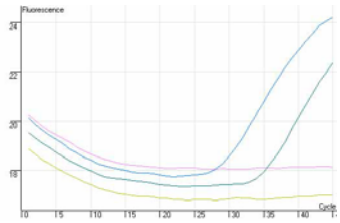
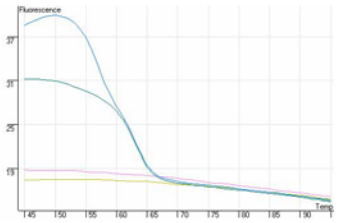


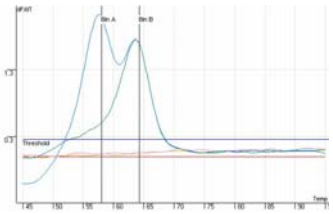
Figure Method 2 – Melt curve analysis. (From Corbett Research "Mutation detection using FRET analysis") While two fluorophores are used here, fluorescence of only 1 fluorophore is being detected (the 5' fluorophore on the second probe); A. Fluorescence increases during amplification. B. Raw data for melt. C. Analyzed data for melt showing homozygote and heterozygote curves.



A.



B.



C.

A main advantage to using this technology is that only one fluorescence channel is used per SNP; thus, four SNPs can be multiplexed in a 4-color real-time instrument, five SNPs in a 5-color instrument and six SNPs in a 6-color instrument.

Table Method 1 – SNP FRET Primers. Primers were purchased from Applied Biosystems and 200 µM stocks in TE buffer (10mM Tris, 0.1mm EDTA, pH 7.5) were created.

Locus (chromosome) SNP#	**Forward Primer (relative to the probes)**	**Reverse Primer (relative to the probes)**	**Size of product**
H63D	TCAGAGCAGGACCTTGGTCTTTC	GGCTTGAAATTCTACTGGAAACCC	133bp
A2BP1 (chr 16) rs7205345	CCTTGGGTCATCTCTATCATAG	GAACCAGGAACTCTTCTACATC	156bp
ATP13A4 (chr 3) rs6444724	AAAGGTTAGGGATTGGAATGG	CTATGGTTTCATAAAGGGAAGG	180bp
FLJ43720 (chr 5) rs315791	TTGTACCAGGGGTGTTTCC	GGCTAGTGGCTACCAAATTG	141bp
HSPA12A (chr 10) rs740598	GAAATGCCTTCTCAGGTAATGG	TTGTCCTCCTTGAGATGTGG	184bp
HSPA12A new (chr 10) rs740598	GAAATGCCTTCTCAGGTAATGG	TTGTCTCAGTTTGCATTGTCC	199bp
LOC650568 (chr 22) rs987640	TACTCCAGAATGAAGTGAAGG	GGCAATGAGCTGTTTAAGG	198bp
LY9 (chr 1) rs560681	CACTCTAAAGGGCTCTCACC	CAACCTCACATGCACAGC	192bp
PALLD (chr 4) rs6811238	ACTGTTCAGGTCCTCAAAGC	ATCCCAGGGAAGGAATAAGTAC	176bp
RAB31 (chr 18) rs9951171	ACGGTTCTGTCCTGTAGG	AAAGAAAGAATGAATCAAAGGG	184bp
THSD2 (chr 6) rs2503107	AATCCAAAAGGAGTGTTGTATC	AGTCAGTGTTTCTCATTTGTTC	175bp
ZFXY (X/Y)	AAGAGGTGGCGATTCAATAAC	GTACTGACTGTGATTACACTACC	244bp
ZFXY double (X/Y)	CATAACTTTGTTCCTATGACC	TTTCTGCTTCCACTACACTATC	341bp
ZFXY double NEW Rev (X/Y)	CATAACTTTGTTCCTATGACC	TGTTCTGGCATAGACATTGAGG	242bp
Below are loci for which primers were tested but no probes made			
KIAA1052 (chr11) rs2305826	CCGGCAGCATCTGAA	GCAGAACTCAGGCACTGG	185bp
EPM2A(chr6q24) rs453609	TTCTTTGTTCCTGACTTCAAGG	GGTTCCAATGAGTAGAGTTTGC	142bp
SSTR4 (chr20) rs2567608	CGTGACCAGCCTTGATGC	GAGCAGTGGCATAGTAGTCC	200bp

Table Method 2 - SNP FRET Probes. Yellow indicates SNP position. Sets in grey indicate those with position of fluorophore and quencher swapped on the sensor and anchor. Probes were purchased from Biosearch Technologies (Novato, CA) and kept as 200 µM stocks in TE. BHQ stands for Black Hole Quencher.

Probe	Sequence (yellow shows SNP)	5' modification	3' modification
H63D probe	CGACTCTCATCATCATAGAACACGAACAG	NONE	FAM
H63D anchor quencher	GGTCATCCACGTAGCCCAAAGC	BHQ-1	Phosphate
H63D anchor fluor	GGTCATCCACGTAGCCCAAAGC	Quasar 670	Phosphate
A2BP1-anchor	TCCACATCCTTAGTGCAGGTGCCC	BHQ2	3' phosphate
A2BP1-SNP	TCTGTGTCTGCCTCTCACACTAGA	NONE	3' Quasar 670
ATP13A4-anchor	ACTTGCTCTCATTTACTACGGAGTAGGAAG	BHQ2	3'phosphate
ATP13A4-SNP	GAACACTGGTTACCGTGCTAGGTATTTA	NONE	CAL Fluor RED
FLJ43720-anchor	TGGCAGACAGAAATTAACAAAGGAGCAAATAAGA	BHQ1	3' phosphate
FLJ43720-C	ACTAATGCATAGGCCAGTTTCATCCTTAT	NONE	CAL Fluor Orange 560
FLJ43720-SNP Gold	ACTAATGCATAGGCCAGTTTCATCCTTAT	NONE	CAL Fluor Gold
HSPA12A-anchor	CTGCTCAAACCCTGGCCCTGC	BHQ1	3' phosphate
HSPA12A-SNP- FAM	TGGTTAGTCTCACAGCCACATTCT	NONE	FAM
HSPA12A-anchor new	CTGCTCAAACCCTGGCCCTGCAAAG	BHQ2	3' phosphate
HSPA12A-A new	CTATGGTTAGTCTCACAGCCACATTCTCA	NONE	CAL Fluor Red 610
HSPA12A-anchor newest	CTGCTCAAACCCTGGCCCTGC	BHQ2	3' phosphate
HSPA12A-A newest	TGGTTAGTCTCACAGCCACATTCT	NONE	CAL Fluor Red 610
HSPA12A-anchor RED	TTCGTAAGTATTTCAAATAGCAATGGCTCGTC	NONE	CAL Fluor Red 610
HSPA12A-G	ATGGTTAGTCTCGCAGCCACATTCT	BHQ2	3' phosphate
LOC650-anchor	AAATAAGACTTAATACAGACGATGGCATGG	BHQ1	3' phosphate
LOC650-SNP	CTCTCTTTCCACCCTTGTAGAAATAC	NONE	3' FAM
Ly9-anchor	AACAGATGTTCTCAGAAAGAAACTGGTGGG	BHQ1	3' phosphate
Ly9 – C	GTGACCTGAGTAAACAGAGATGGAGAGAAA	NONE	3' CAL Orange 560
Ly9 – C	GTGACCTGAGTAAACAGAGATGGAGAGAAA	NONE	3' Biosearch Blue
LY9-anchor – FAM	TCCCACCAGTTTCTTTCTGAGAACATCTGT	NONE	FAM
LY9-G	TTTCTCTCCATCTCTGTTTACTCAGGTCAC	BH1	3' phosphate
PALLD-anchor	AAACACACAGTCTTCTCCTCTCAGTGACT	BHQ1	3' phosphate
PALLD-SNP	TACTATCATAACACTTTAAACAAACCTGGC	NONE	3' FAM
PALLD-SNP2	TACTATCATAACCCTTTAAACAAACCTGGC	NONE	3' FAM
RAB31-anchor	AGTGAACAGGTCCCAGCATGAAAGC	BHQ2	3' phosphate
RAB31-A	GCTTTATGGATTGCCCTGCC	NONE	3' CAL Fluor Red 610
RAB31-A	GCTTTATGGATTGCCCTGCC	NONE	3' Quasar 705
THSD2-anchor	TGCCTAAATGGTTGTGGAGACCTAGCC	BHQ2	3' phosphate
THSD2-C	TGTTCATATTTTGCTATCTATACCTAACTTTCTCA	NONE	Quasar 670
ZFXY-anchor	TTCTCTGCCTTGCTGGTCAGCTTGTGG	BHQ1	3' phosphate
ZFXY-T	TCCCACACTCATCACATTCAATGGC	NONE	FAM
ZFXY new#2 – anchor	TCTGCCTTGCTGGTCAGCTTGTGG	BHQ1	3'phosphate
ZFXY new#2 – SNP	CACACTCATCACATTCAATGGCCT	NONE	FAM
ZFXY double anchor	AGTTACATCTGAGTCCAGCACTTGCTCA	BHQ1	3' phosphate
ZFXY double SNP	ACTGTGCAATGTGCTAAAGAAACTTCT	NONE	3' FAM
ZFXY double SNP Y	ACTGTGCAGTGTGGTAAAGAAACTTCT	NONE	3' FAM

Mastermix

Quantitect mix from Qiagen (Valencia, CA) (204543).

Chemicals

All chemicals used for the inhibitor studies were purchased from Sigma (St. Louis, MO) and prepared as follows: Hematin (100 mM stock in 0.1 N NaOH), Indigo (100 mM stock in 2% Triton X), Humic Acid (1 mg/mL stock in dH_2O), Tannic Acid (1 mg/mL in dH_2O), Calcium Hydrogen Phosphate (100 mM stock in 0.5 N HCl), Melanin (1 mg/mL in 0.5 N Ammonium Hydroxide) and Collagen (1 mg/mL in 0.1 N Acetic Acid).

DNA Samples

The DNA samples utilized in this study were mostly in-house controls used for STR testing in the laboratory (lab personnel) or DNA samples from convicted offenders. These were isolated using an organic extraction method (Anonymous, 1989) as modified in Akane et al. (1993). The GM9947A DNA control DNA from AmpFlSTR® COfiler™ and Profiler Plus™ PCR Amplification Kits (Applied Biosytems) was also utilized as a DNA sample. Human Genomic DNA:Male [Catalog #G1471 Promega (Madison, WI)] was used for the standard curves.

PCR setup for FRET SNP assay

For a 20ul reaction (6-plex):

2.288ul deionized H_20
10ul Quantitect Mastermix
1.8ul Primer mix
3.4ul Probe mix
0.512ul BSA
=18ul total (add 2ul of sample)

For a 20ul reaction (4-plex):

3.688ul deionized H_20
10ul Quantitect Mastermix
1.4ul Primer mix
2.4ul Probe mix
0.512ul BSA
=18ul total (add 2ul of sample)

Table Method 3 – Concentrations of Mastermixes for FRET SNP

Primer mixes (for 100 reactions)

6plex-A
2ul FLJ Forward primer
20ul FLJ Reverse primer
5ul THSD2 Forward primer
40ul THSD2 Reverse Primer
2ul ATP Forward primer
20ul ATP Reverse primer
4ul RAB31 forward primer
20ul RAB31 Reverse primer
4ul LY9 Forward primer
20ul LY9 Reverse primer
2.5ul ZFXY Forward primer
40ul ZFXY Reverse primer
0.5ul ddH20

6plex-B
2ul FLJ Forward primer
20ul FLJ Reverse primer
5ul THSD2 Forward primer
40ul THSD2 Reverse Primer
2ul ATP Forward primer
20ul ATP Reverse primer
4ul RAB31 forward primer
20ul RAB31 Reverse primer
4ul LY9 Forward primer
20ul LY9 Reverse primer
4ul PALLD Forward primer
20ul PALLD Reverse primer
19ul ddH20

4plex#1
2ul FLJ Forward primer
20ul FLJ Reverse primer
5ul THSD2 Forward primer
40ul THSD2 Reverse Primer
2ul ATP Forward primer
20ul ATP Reverse primer
2.5ul ZFXY Forward primer
40ul ZFXY Reverse primer
8.5ul ddH20

4Plex#2
4ul A2BP1 Forward primer
20ul A2BP1 Reverse primer
4ul RAB31 forward primer
20ul RAB31 Reverse primer
4ul LY9 Forward primer
20ul LY9 Reverse primer
4ul PALLD Forward primer
20ul PALLD Reverse primer
44ul ddH20

Probe Mixes (for 100 reactions)

6plex-A
20ul FLJ Sensor probe (CAL Fluor Orange 560)
20ul FLJ Anchor probe
20ul THSD2 Sensor probe (Quasar 670)
40ul THSD2 Anchor Probe
20ul ATP Sensor probe (CAL Fluor Red 610)
40ul ATP Anchor probe
20ul RAB31 Sensor probe (Quasar 705)
40ul RAB31 Anchor probe
20ul LY9 Sensor probe (Biosearch Blue)
40ul LY9 Anchor probe
20ul ZFXY Sensor probe (FAM)
40ul ZFXY Anchor probe

6plex-B
20ul FLJ Sensor probe (CAL Fluor Orange 560)
20ul FLJ Anchor probe
20ul THSD2 Sensor probe (Quasar 670)
40ul THSD2 Anchor Probe
20ul ATP Sensor probe (CAL Fluor Red 610)
40ul ATP Anchor probe
20ul RAB31 Sensor probe (Quasar 705)
40ul RAB31 Anchor probe
20ul LY9 Sensor probe (Biosearch Blue)
40ul LY9 Anchor probe
20ul PALLD Sensor probe (FAM)
40ul PALLD Anchor probe

4plex#1
20ul FLJ Sensor probe (CAL Fluor Orange 560)
20ul FLJ Anchor probe
20ul THSD2 Sensor probe (Quasar 670)
40ul THSD2 Anchor Probe
20ul ATP Sensor probe (CAL Fluor Red 610)
40ul ATP Anchor probe
20ul ZFXY Sensor probe (FAM)
40ul ZFXY Anchor probe
20ul H20

4Plex#2
20ul A2BP1 Sensor probe (Quasar 670)
40ul A2BP1 Anchor probe
20ul RAB31 Sensor probe (CAL Fluor Red 610)
40ul RAB31 Anchor probe
20ul LY9 Sensor probe (CAL Fluor Orange 560)
40ul LY9 Anchor probe
20ul PALLD Sensor probe (FAM)
40ul PALLD Anchor probe

Amplification

PCR conditions for the RG6000 are: 95°C 15 min then 45 cycles of 94°C 15 sec, 56°C 30 sec, 72°C 30 sec (fluorescence is read during the 56°C step) then one cycle of 94°C 15 sec, 40°C 60 sec and 50°C for 60 sec. The melting phase is carried out from 50°C to 80°C rising by 1°C/step, waiting for 5 sec for each step. Gains were set: FAM (Green channel, Gain 5.67), CAL Fluor Orange 560 (Yellow channel, Gain 8.0), CAL Fluor Red 610 (Orange channel, Gain 8.67), Quasar 670 (Red channel, Gain 8.0), Quasar 705 (Crimson channel, Gain 5.33) and Biosearch Blue (Blue channel, Gain 1.0).

PCR conditions for the MX3005P are the same as the Corbett settings but with an increase of 15 sec for the denaturation step. The melt was carried out over a default ramp from 50°C to 80°C. For the MX3005P, FAM (FAM channel, Gain x2), CAL Fluor Orange 560 (Cy3 channel, Gain x2), CAL Fluor Red 610 (ROX channel, Gain x1) and Quasar 607 (Cy5 channel, Gain x1).

Data Interpretation

Experiments run on the RG3000 were analyzed using the Corbett Robotics Rotorgene software (currently the Rotorgene 6 version 6.0, Build 38 software). Experiments run in the RG6000 were performed and analyzed using the Corbett Robotics Rotorgene software (currently the RG6000 Series software, Version 1.7, Build 61). Bins need to be placed and then the genotypes defined before the Corbett instruments will call the alleles automatically in a report. Experiments performed in the MX3005P were run using the MxPro software Version 3.00, Build 311. Results were also analyzed using the Plexor Desktop Analysis software v1.1.4 from Promega as the MxPro software does not have the capability of analyzing quenching curves.

The Plexor technology is also a quenching system and in order for this technology to be used on all real-time instruments, Promega has developed the Plexor software to import data from many instruments including the MX3005P.

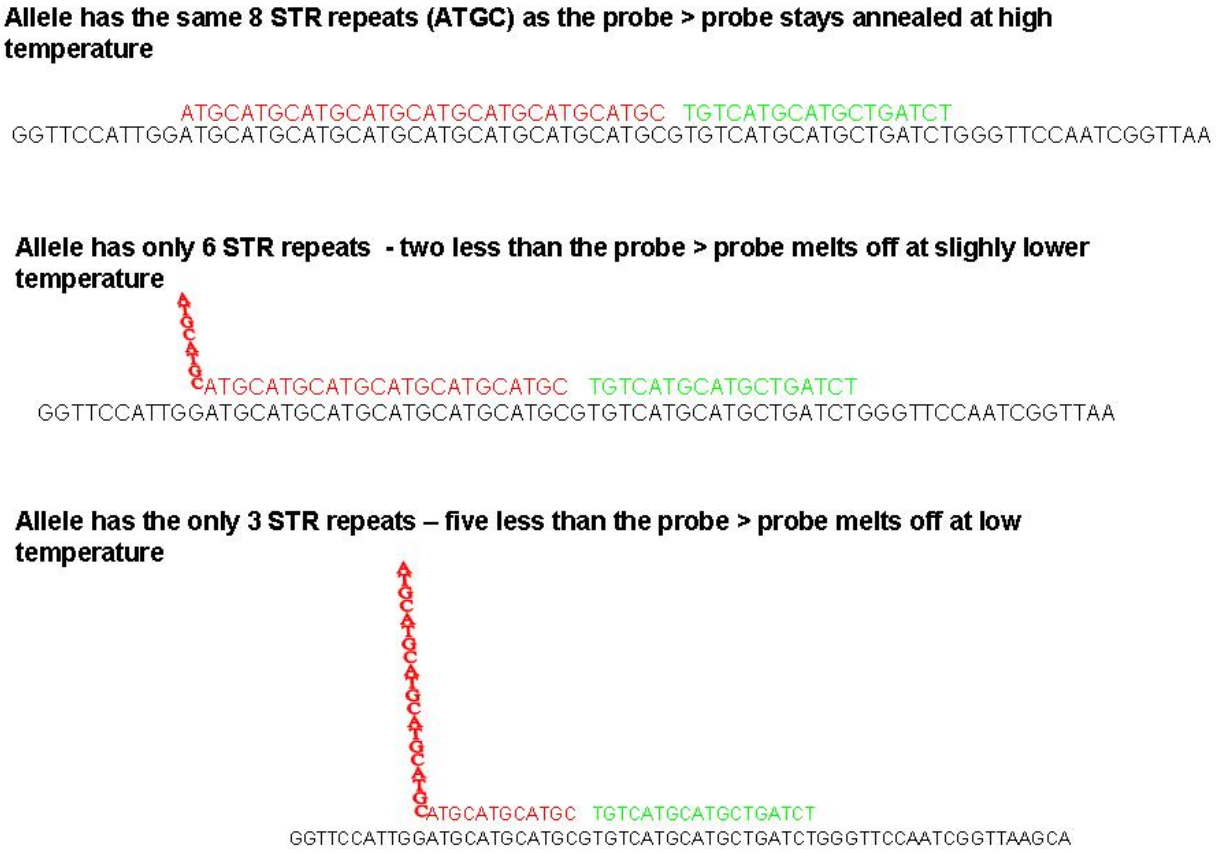
Aim #1B - Development of a Fast, Simple Profiling Method For Sample Screening – STR FRET assay

In our search for another assay to discriminate individuals that could be simpler and more "familiar" to forensic DNA analysts than the SNP FRET assay, we decided to try to determine if the melting FRET technique could be adapted to STRs. The basic idea is similar to the SNP FRET described above (Figure Method 1) except that the sensor probe that was made to cover the SNP is now longer to cover an extended set of the STR repeats. The anchor probe covers the adjoining unique sequence. A closeup of the binding of the probes to a long, intermediate and short STR allele is shown in Method Figure 3. If the STR allele is short, then not all of the probe can hybridize to the amplified DNA and it should melt off sooner than a longer allele where the hybridization is complete.

For initial experiments, the THO1 locus was chosen because it had a limited number of small alleles so a probe could be designed to cover the longest common allele (allele with 10 repeats). Several variations of the assay were designed using THO1 as the test locus (Table Method 4). These included putting the anchor on either side of the locus (therefore sense and antisense), adding some mismatches to the sequence (3* and 8* in the table) where variations are known to occur in the THO1 alleles in the hopes that this might improve resolution, and lastly moving the anchor to cover part of the STR repeat. A second STR, D6S2956, was used for some experiments. This STR was chosen because it has a GC rich repeat (CGTC). It was hoped

that the GC rich sequence would give more melting differences between the alleles. It was found by Dr. Mike Coble who gave us the sequence and primers.

Figure Method 3 – Diagram of the differences in annealing and melting of STR alleles in an STR FRET melting assay.



Experimental Design

The experiments were performed as described above for the SNP FRET assay.

Table Method 4 – Sequences of primers and probes use in the STR FRET experiments

Probe Name	**Probe sequence 5'-3'**	**5' quencher**	**3' modification**
THO1 sense 3' anchor	GCCTGTTCCTCCCTTATTTC	NONE	3' FAM
THO1 sense 5' STR	TCATTCATTCATTCATTCATTCATTCATTCATTCAT	BHQ1	3' phosphate
THO1 anti 3' STR	ATGAATGAATGAATGAATGAATGAATGAATGA	NONE	3' FAM
THO1 anti 5' anchor	GAAATAAGGGAGGAACAGGCCA	BHQ1	3' phosphate
THO1 sense STR *3	TCATTCATTAATTCATTCATTCATTCATTCATTCAC	BHQ1	3' phosphate
THO1 sense STR *3+*8	TCATTCATTAATTCATTCATTCATTAATTCATTCAC	BHQ1	3' phosphate
THO1 NEW sense anchor	CTTATTTCCCTCATTCATTCAT	NONE	3' FAM
THO1 NEW sense STR	ATTCATTCATTCATTCATTCAC	BHQ1	3' phosphate
THO1 NEW sense STR *3	ATTCATTAATTCATTCATTCAC	BHQ1	3' phosphate
D6S2956 sense	TGCTTGCTGTCCGTCCGTCC	BHQ1	3' phosphate
D6S2956 anchor	CCGTCCGTCCGTCCGTCCGTCCGTCCGTCCGTCCGTCCGTCC	NONE	3' FAM
Primer Name	**Primer sequence**		
THO1 forward	CCTGTTCCTCCCTTATTTCCC		
THO1 reverse	GGGAACACAGACTCCATGGTG		
D6S2956 forward	Provided by Dr. Michael Coble		
D6S2956 reverse	Provided by Dr. Michael Coble		

Aim #1C – Development of a Fast, Simple Profiling Method For Sample Screening – STR HRM assay

Melt curves are often used in real-time PCR to determine if the product generated is specific. High resolution melting (HRM) goes beyond classical melt curve analysis by studying the melt in much finer detail using special DNA dyes [LCGreenTM I and LCGreen® Plus (Idaho Technology Inc., Salt Lake City, Utah), SYTO 9 (Invitrogen, Carlsbad, USA), Eva Green™ (Biotium, Hayward, CA), BEBO (Tataa Biocenter, San Francisco, CA)]. PCR products can be differentiated based on length, sequence or complementarity; single base changes (SNPs) can be genotyped or single base mutations can be detected. The HRM method requires the two amplification primers, a PCR mastermix containing the specific dye suitable for the analysis and the proper instrument utilizing specific melting parameters and dedicated software.

Table Method 5 – Primers for STR HRM.

Locus	Forward primer (5'-3')	Reverse primer (5'-3')	Product size
Penta E	GGCGACTGAGCAAGACTC	GGTTATTAATTGAGAAAACTCCTTACA	79 - 474
Penta D	GAGCAAGACACCATCTCAAGAA	GAAATTTTACATTTATGTTTATGATTCTCT	76 - 449
D19S433	GCACCCATTACCCGAATAAA	CCTGGGCAACAGAATAAGATT	106 - 140
D2S1338	TGGAAACAGAAATGGCTTGG	GATTGCAGGAGGGAAGGAAG	289 - 341
D21S11	ATTCCCCAAGTGAATTGC	GGTAGATAGACTGGATAGATAGACGA	138 - 256
D18S51	TGAGTGACAAATTGAGACCTT	GTCTTACAATAACAGTTGCTACTATT	264 - 394
D16S539	ATACAGACAGACAGACAGGTG	GCATGTATCTATCATCCATCTCT	233 - 277
D13S317	TCTGACCCATCTAACGCCTA	CAGACAGAAAGATAGATAGATGATTGA	193 - 237
D8S1179	TTTGTATTTCATGTGTACATTCGTATC	ACCTATCCTGTAGATTATTTTCACTGTG	123 - 175
D7S820	GAACACTTGTCATAGTTTAGAACGAAC	TCATTGACAGAATTGCACCA	253 - 297
D5S818	GGGTGATTTTCCTCTTTGGT	AACATTTGTATCTTTATCTGTATCCTTATTTAT	134 - 178
D3S1358	CAGAGCAAGACCCTGTCTCAT	TCAACAGAGGCTTGCATGTAT	97 - 149
VWA	AATAATCAGTATGTGACTTGGATTGA	ATAGGATGGATGGATAGATGGA	52 - 212
TPOX	CTTAGGGAACCCTCACTGAATG	GTCCTTGTCAGCGTTTATTTGC	209 - 257
TH01	CCTGTTCCTCCCTTATTTCCC	GGGAACACAGACTCCATGGTG	160 - 204
FGA	AAATAAAATTAGGCATATTTACAAGC	GCTGAGTGATTTGTCTGTAATTG	196 - 348
CSF1PO	ACAGTAACTGCCTTCATAGATAG	GTGTCAGACCCTGTTCTAAGTA	276 - 320
D10S1248	TTAATGAATTGAACAAATGAGTGAG	CAACTCTGGTTGTATTGTCTTCAT	79-123
D14S1434	TGTAATAACTCTACGACTGTCTGTCTG	AATAGGAGGTGGATGGATGG	70-98
D1S1677	TTCTGTTGGTATAGAGCAGTGTTT	TGACAGGAAGGACGGAATG	81-117
D22S1045	ATTTTCCCCGATGATAGTAGTCT	GCGAATGTATGATTGGCAATATTTTT	82-115
D4S2364	CTAGGAGATCATGTGGGTATGATT	GCAGTGAATAAATGAACGAATGG	67-83

Mastermix

Amplification for HRM analysis was performed using the Quantace, Inc. (Norwood, MA) SensiMix HRM™ mastermix which contains the dye Eva Green.

Chemicals

All chemicals used for the inhibitor studies were purchased from Sigma (St. Louis, MO) and prepared as follows: Hematin (100 mM stock in 0.1 N NaOH), Indigo (100 mM stock in 2% Triton X), Humic Acid (1 mg/mL stock in dH_2O), Tannic Acid (1 mg/mL in dH_2O), Calcium

Hydrogen Phosphate (100 mM stock in 0.5 N HCl), Melanin (1 mg/mL in 0.5 N Ammonium Hydroxide) and Collagen (1 mg/mL in 0.1 N Acetic Acid).

DNA Samples

The DNA samples used were from laboratory personnel, convicted offender samples or samples obtained from NIST.

PCR Setup

Reactions were performed in a 15ul volume including 4.8ul ddH2O, 7.5ul mastermix, 0.6ul of Eva Green dye, 0.3ul of 20uM each primer, and 1.5ul of sample DNA.

Amplification

Amplification and HRM was performed in a two color + HRM Corbett Rotorgene 6000 (now Qiagen Rotorgene Q). Amplication – 95˚C -10 min, 95˚C – 5 sec, 56˚C – 20 sec, 65˚C – 30 sec – 45X, then 72˚C – 2 min, then 95˚C 20 sec, 55˚C – 20 sec then 56˚C – 2 min then HRM – melting was performed from ~3 degrees below the first melting peak to ~3 degrees above the highest melting peak with 0.1 degrees/step and a 90 sec wait before the first step and 2 sec thereafter.

Data Interpretation

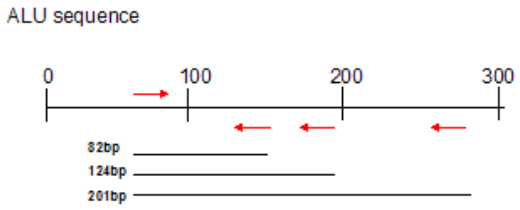
Data was interpreted by eye from the melt curves. Some data was initially interpreted using the HRM software of the Corbett Rotorgene. One example of each genotype was used as a standard and the software was allowed to call the genotypes for the rest of the samples. This

software method had difficulties (many samples did not have a genotype called) so calling by eye was necessary to give results for all samples.

Aim #2 - Development of a Test for Sample DNA Degradation State

Environmentally challenged forensic samples may often be degraded into small DNA fragments, resulting in a limited DNA profile using commercial STR profiling kits. For example, DNA degraded to an average size of 150bp will not allow amplification of a 320bp D18S51 allele. Because of this, forensic researchers have developed sets of primers to create smaller STR products (mini STRs) (Butler et al., 2003; Wiegand and Kleiber, 2001). Prior knowledge of the degradation state of a sample would allow the examiner to decide how best to proceed with the analysis of a sample. A precious sample could be conserved for an appropriate "mini STR" analysis or time and resources saved if it is determined that a sample is too degraded to profile. Current DNA quantitation kits will not accurately predict the DNA quantity needed for STR analysis when the DNA is degraded. The Applied Biosystems Quantifiler™ kit has a 62bp amplicon and, therefore, will not accurately predict if there is a sufficient quantity of large fragments to support a full amplification using commercial STR kits. [The new Applied Biosystems Duo kit has larger amplicons and should more accurately predict amplifiable DNA in a sample as will the Promega Plexor HY kit; however, these kits were not available when our studies began.] We therefore wished to develop an assay to detect the degradation state of the DNA. To achieve this, we initially started with a multiplex PCR with several overlapping *Alu* amplicons (Figure Method 4) using a gel readout system.

Figure Method 4 – *Alu* amplicons for degradation studies.



The longer amplicons will only be possible in non-degraded or slightly degraded DNA while the smaller amplicons will be amplifiable even in highly degraded DNA. Thus, ratios of the different products should give a qualitative (or maybe even quantitative) measure of degradation state.

After this method was tested and shown to be feasible, we switched to using a real-time system using Plexor™ technology. This technology uses fluorescently labeled primers (different fluorophores can be used for different multiplexed amplicons) to easily detect the different products in a real-time format. Figure Method 5 shows the Plexor technology (from Promega website). The key points of the technology are that the forward primer is designed with a 5' isoC with attached fluorophore, during the second round of amplification an isoG with attached quencher is incorporated into the amplicon by base pairing to the isoC residue, fluorescence is quenched due to the proximity of the isoC with fluorophore and isoG with quencher. The decrease in fluorescence is monitored during PCR in a real-time instrument. For these experiments, we placed a different fluorophore on each of the specific reverse primers (FAM for long, CAL Fluor Orange 560 for medium and Cy5 for short).

There was a great deal of difficulty in getting this triplex assay to work. Balancing the three reverse primers did not seem possible. We, therefore, switched to a simpler duplex assay

using only the long and short amplicons. The medium amplicon did not add much to the assay and the assay worked much better without it. It was now possible to balance the primers appropriately.

Figure Method 5 - Plexor™ Assay (based on EraGen).

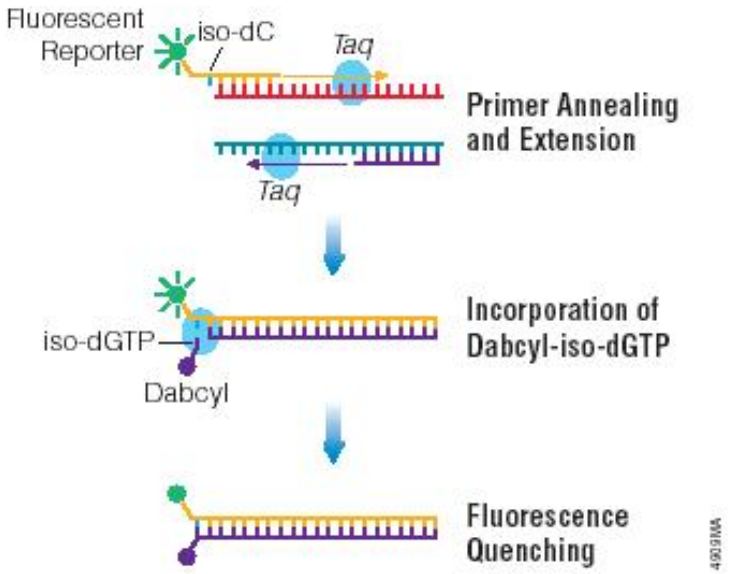


Figure 1. Schematic diagram illustrating the Plexor™ System real-time PCR process.

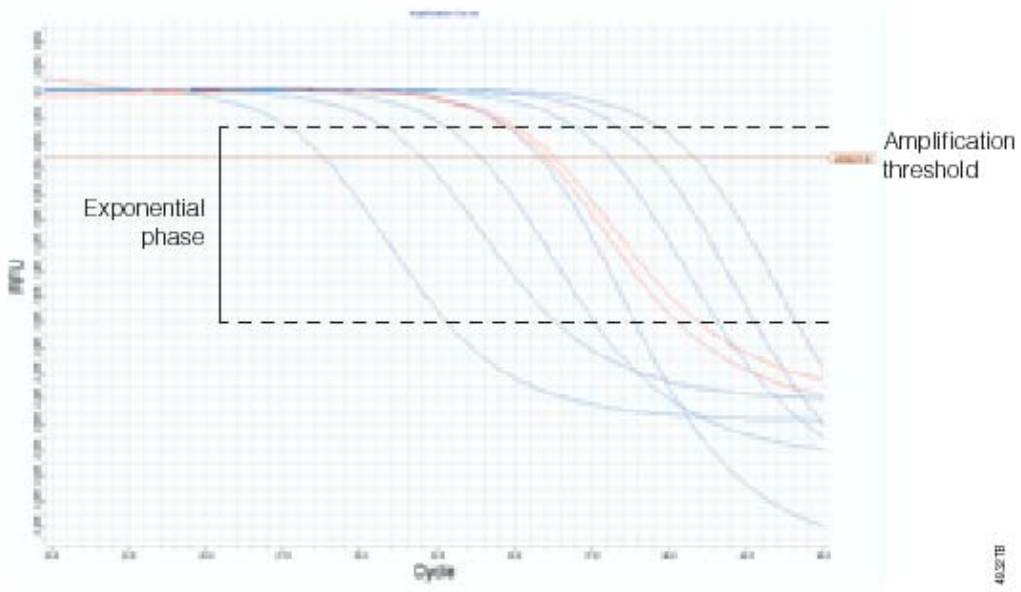


Figure 2. Representative Plexor™ qPCR System amplification curve. The amplification curves show the fluorescence (in relative fluorescence units, RFU) at each cycle of the reaction. The amplification threshold is indicated by a horizontal line across the graph. This line is used to determine the cycle threshold (C_t) for the samples.

PCR primers

Alu short reverse primer – 5'Quasar 670-isoC-CTCGTGATCCGCCCGCCTC 3'

Alu long reverse primer – 5'FAM-isoC-GAGTGCAGTGGCGGGATCTC 3'

Common forward primer – 5'GGGCGCGGTGGCTCAC3'

All primers are diluted to 200uM stock in the MOPS/EDTA buffer supplied with the Promega Plexor mastermix. Working concentrations of primers (20uM) are also diluted in MOPS/EDTA buffer.

Mastermix

Promega Plexor qPCR System (A4011) Mastermix, 2X (Note: the Plexor mastermix has 25ng/ul final BSA. We add BSA to make a final concentration of 180ng/ul.)

Chemicals

All chemicals used for the inhibitor studies were purchased from Sigma (St. Louis, MO) and prepared as follows: Hematin (100 mM stock in 0.1 N NaOH), Indigo (100 mM stock in 2% Triton X), Humic Acid (1 mg/mL stock in dH_2O), Tannic Acid (1 mg/mL in dH_2O), Calcium Hydrogen Phosphate (100 mM stock in 0.5 N HCl), Melanin (1 mg/mL in 0.5 N Ammonium Hydroxide) and Collagen (1 mg/mL in 0.1 N Acetic Acid).

Other Reagents

Primer/BSA mix (100 reactions)

43.2ul BSA stock
5ul of 200uM forward primer stock
1.5ul of 200uM short primer stock
2.0 ul of 200uM long primer stock
76.5ul deionized H20

PCR setup

For a 20ul reaction:

6.718ul deionized H20 (supplied with kit)

10ul mastermix

1.282ul Primer/BSA mix

=18ul total (add 2ul of sample)

Experimental Setup

A standard curve of 32 ng/ul down to 0.0078 ng/ul is made using the Promega DNA.

Amplification

PCR cycling conditions: 95°C, 2 min, then 32X of 95°C, 5 sec and 60°C, 25 sec.

Data Interpretation

Analyze the results using the Corbett software or the Plexor software supplied by Promega. Determine the concentrations for each sample based on the short and long results for the standard curve. Calculate the short/long ratio. For un-degraded DNA this ratio should be approximately 1.0. If the ratio is greater than 10, this indicates significant degradation.

III. Results

1. Statement of Results

Aim #1A - Development of a Fast, Simple Profiling Method For Sample Screening – SNP FRET assay

Assay development started with SNP selection and primer design. Review of the data provided from the ALFRED database (http://alfred.med.yale.edu/alfred/) permitted the selection of SNPs such that the variations in allele frequencies between populations were low (low Fst). Additional criteria for the chosen SNPs were: non-coding, not medically relevant, allele frequencies (p and q) close to 0.5, located on different chromosomes and not patented. Probes with G:T mismatches were always avoided. In total, the 73 low Fst SNPs in ALFRED were reviewed to select 27 p=q=0.5 SNPs.

Primers and probes for the assays were designed using Beacon Designer 4.0 (Premier Biosoft International, Palo Alto, CA) which has a module for designing FRET primers and probes. For the first several SNPs, the default design parameters were utilized; however, later the probe lengths were shortened from the 35 bp default to 30 bp in an attempt to increase the T_m difference between probes. Also, Beacon Designer 4.0 was often not successful in finding a primer/probe set (approximately 60% of the SNPs yielded sets). In these cases, several parameters individually or in combination were adjusted as follows: the T_m difference between probe and primer was decreased to 6ºC (from the recommended 8ºC), the primers were made longer (up to 25 bp) or the PCR product size was increased (up to 250 bp). Beacon Designer found probe/primer sets for ~16 of the 27 SNPs selected from ALFRED. Probes and primers were made for 12 of these to select the 7 SNPs used in the designed assays.

In order for the assay to determine the gender of the individual from whom a sample derived, detection of a sequence difference (SD) between the ZFX and ZFY genes was incorporated. While this is not technically a SNP, it is still a nucleotide difference and it can be treated as if it were a SNP in assay design. For the gender SD, two different positions in the ZFXY genes were selected with one additional swap of strand to obtain the chosen probe/primer set. The chosen probe contains two differences between the X and Y sequences which leads to greater discrimination in melting temperature (T_m) between the selected sequences.

Seven different fluorophores and associated quenchers (in parentheses) were utilized on the probes: FAM (BHQ-1), Biosearch Blue (BHQ-1), CAL Fluor Orange 560 (BHQ-1), CAL Fluor Red 610 (BHQ-2), Quasar 607 (BHQ-2), Quasar 705 (BHQ-2) and CAL Fluor Gold 540 (BHQ-1). Each SNP assay was amplified separately with its probes in the Corbett RG3000 or RG6000 real-time instrument and amplification and melting monitored. Individual SNP assays up to 4-plex experiments were performed initially in a Corbett RG3000 (Corbett Robotics) - until a RG6000 (Corbett Robotics) was purchased to perform the 6-plex experiments. Some 4-plex experiments were also performed in the Stratagene MX3005P (Stratagene, La Jolla, CA) to develop a seven SNP plus gender assay as two 4-plexes.

Initial experiments tested that the primers gave the correct size product on an agarose gel (Figure 1). Initial testing of the first SNP assay with probes gave poor results (Figure 2). While amplification clearly occurred because the RFUs decreased as PCR progressed, the melting "curves" were flat and chaotic.

Consultations with Corbett Research suggested that the ratio of forward to reverse primer was critical (the concentration of the forward primer needed to be ~1/10 of the reverse primer). This has been published by Szilvási et al. (2005). Optimal concentrations for the forward and