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

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

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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.

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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.

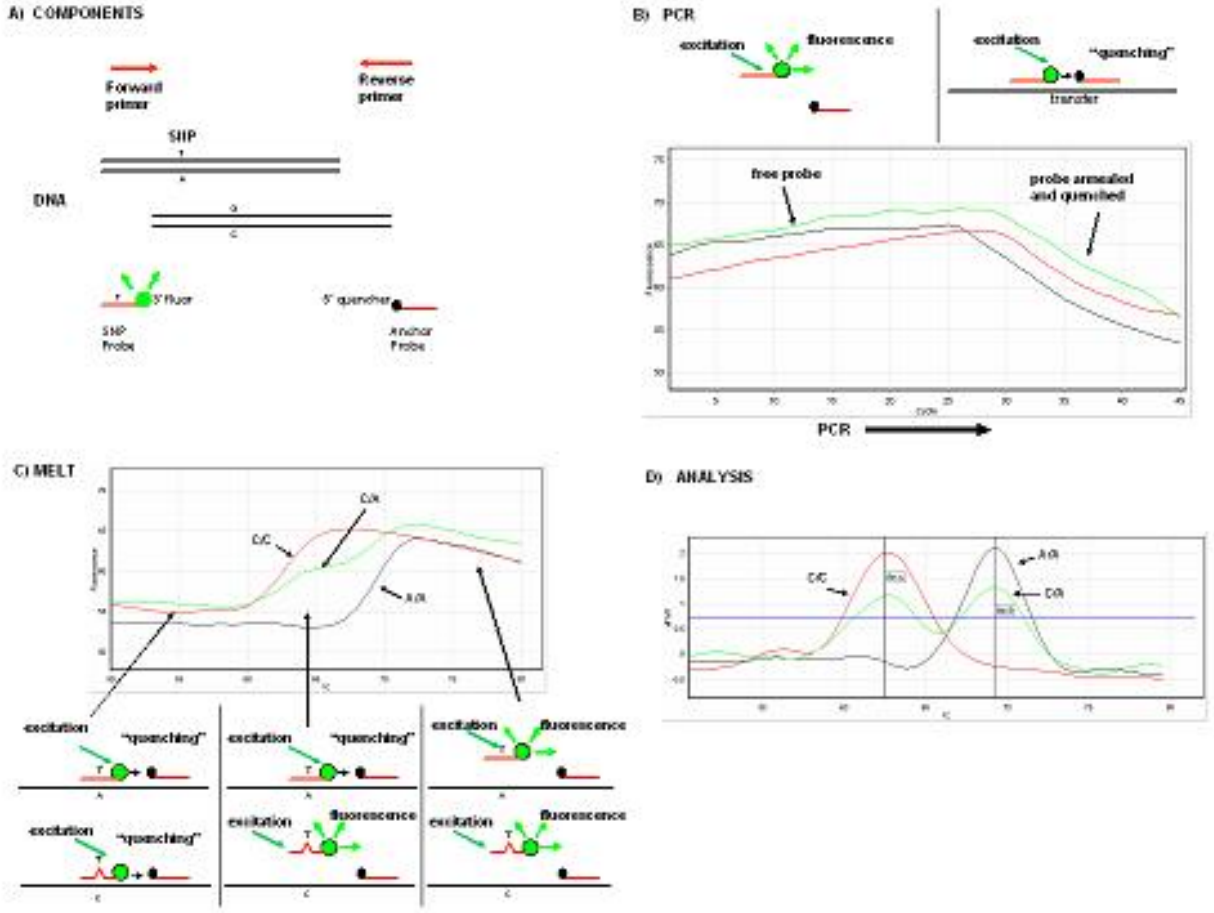
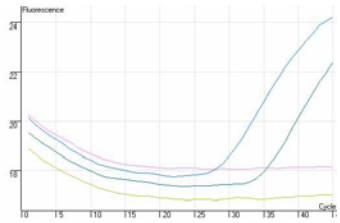
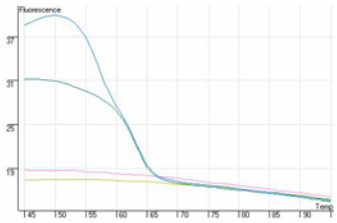


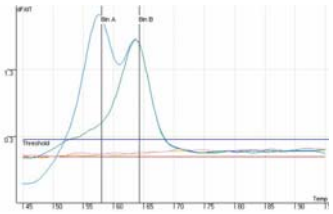
Figure Method 2 – Melt curve analysis. (From Corbett Research "Mutation detection using FRET analysis") While two fluorophores are used here, fluorescence of only 1 fluorophore is being detected (the 5' fluorophore on the second probe); A. Fluorescence increases during amplification. B. Raw data for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

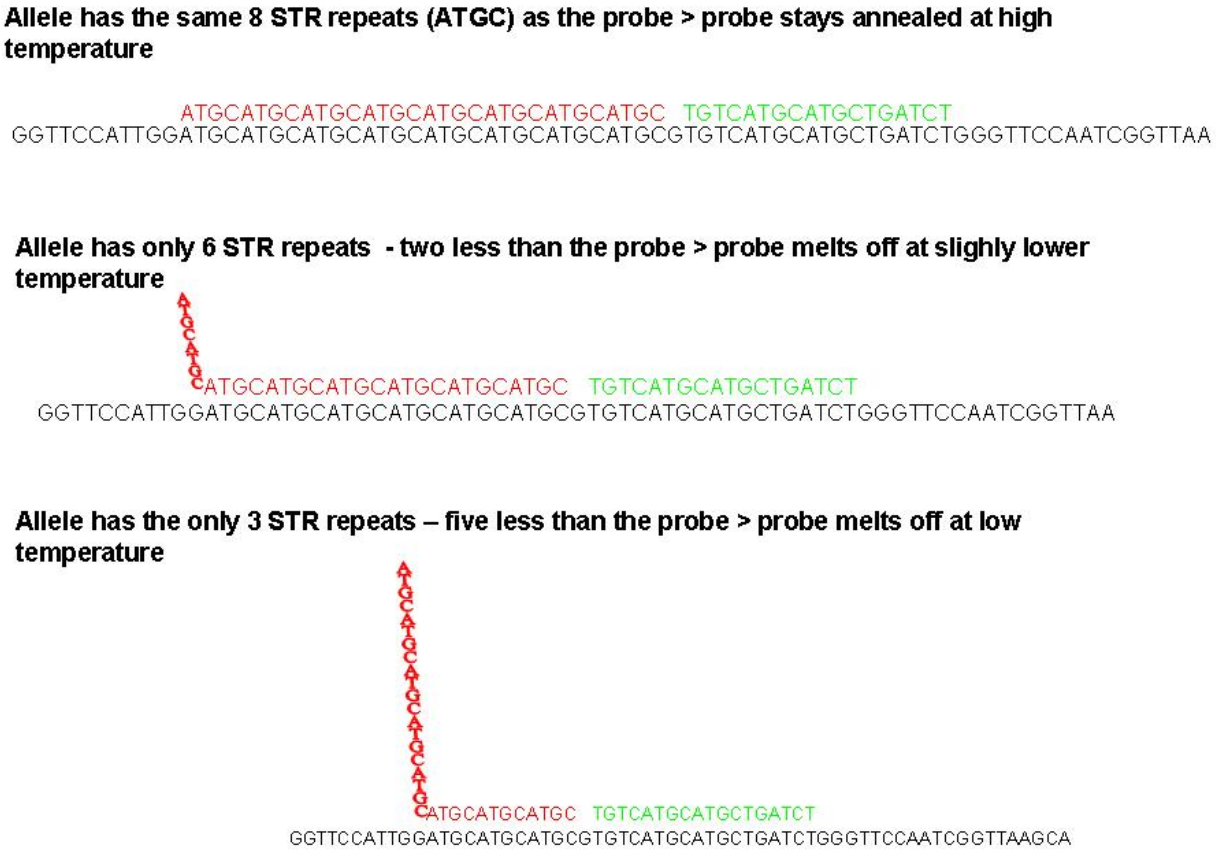
Aim #1B - Development of a Fast, Simple Profiling Method For Sample Screening – STR FRET assay

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

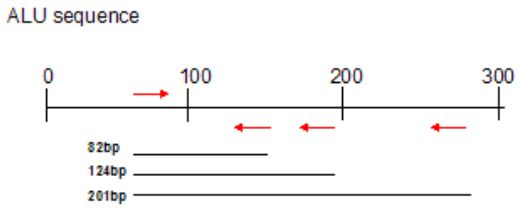
Data was interpreted by eye from the melt curves. Some data was initially interpreted using the HRM software of the Corbett Rotorgene. One example of each genotype was used as a standard and the software was allowed to call the genotypes for the rest of the samples. This

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).

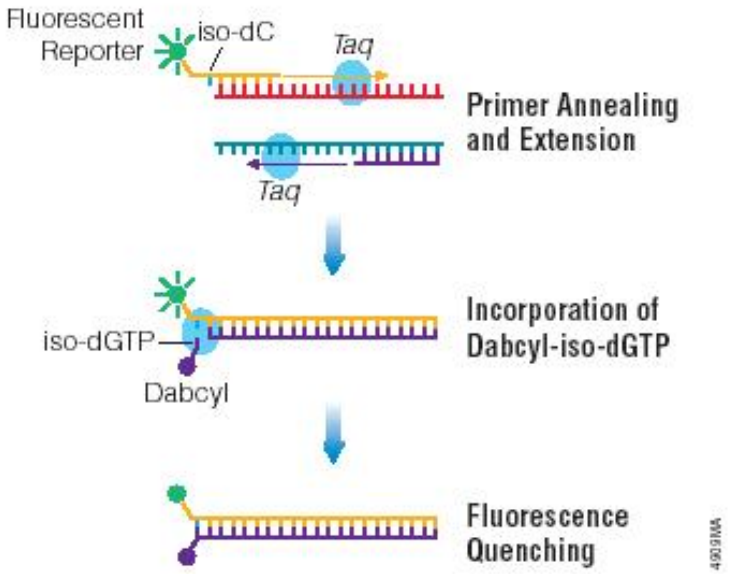


Figure 1. Schematic diagram illustrating the Plexor™ System real-time PCR process.

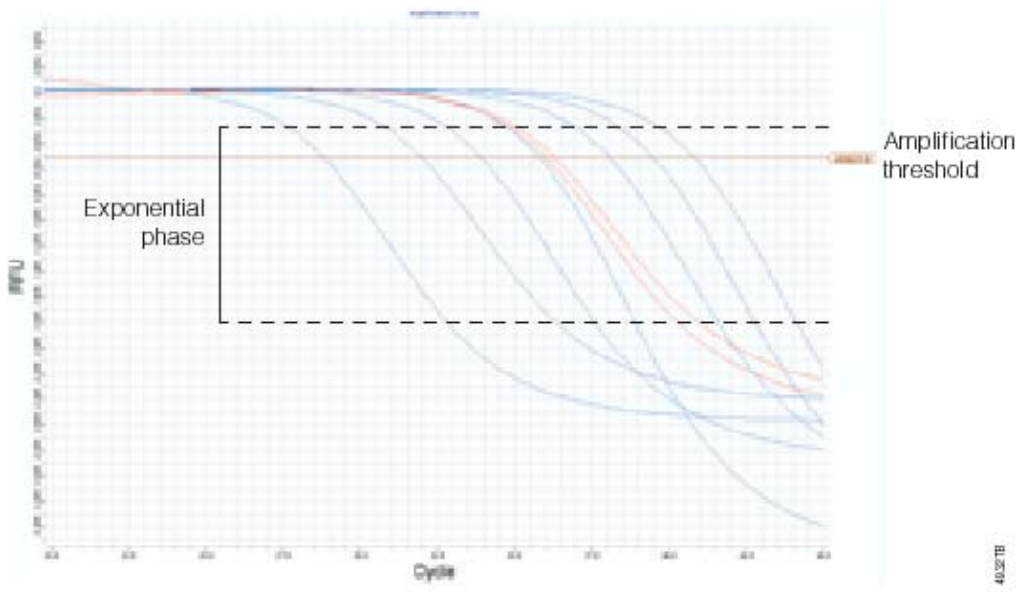


Figure 2. Representative Plexor™ qPCR System amplification curve. The amplification curves show the fluorescence (in relative fluorescence units, RFU) at each cycle of the reaction. The amplification threshold is indicated by a horizontal line across the graph. This line is used to determine the cycle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

reverse primer should be in the range of 20-60 nM and 200-400 nM, respectively. Primer and probe concentrations were optimized for each assay.

The assays were then tested in singleplex with DNA from ten individuals to obtain examples of both homozygotes and a heterozygote. In initial testing, some SNPs did not work well as there was little discrimination in melting temperature between the alleles (Figure 3).

Occasionally this could be remedied by making the probes to the other allele. These switched probes often had very different melting characteristics than the original set (Figure 4).

After making probes to a number of SNPs, it appeared that the SNP assays worked best with the match of C:G and a mismatch of C:T, C:C or C:A although some probes with an A:T match and a mismatch of A:C were used. The C:G SNPs were chosen when possible for the SNP assays.

Once a promising SNP was selected, the genotype results obtained in the FRET assay were confirmed by sequencing of the PCR product. PCR products were treated with ExoSAP-IT (USB Scientific, Cleveland, OH) according to the manufacturer's directions. Sequencing was performed by the Vermont Cancer Center DNA Analysis Facility on an Applied Biosystems 3130XL. Electropherograms were viewed and printed using Chromas Lite freeware (http://www.technelysium.com.au/chromas_lite.html, Technelysium Pty Ltd, Tewantin QLD, Australia). Table 1 gives the genotype results for all ten individuals for the seven SNPs eventually chosen as well as gender. Figure 5 shows an example of sequencing results for the PALLD SNP for four individuals. There was complete concordance in genotyping results with the two methods. The results for the ZFXY locus confirmed the identified gender for the ten individuals.

Several different primer/probe sets were designed for the ZFXY locus. The original set designed in intron 7 with the perfect probe match to the ZFY gene did not work as well as hoped in that the RFUs for the male peak were low (Figure 6). A new set was designed three basepairs shifted from the first set (Figure 7). Another set was designed to a different region of the loci. The sensor covered two basepair differences that were five basepairs apart. The initial probe was designed to match the ZFX gene. This had extremely different melting points for the perfect and imperfect matches (Figure 8). The probe was swapped to be a perfect match with the ZFY gene and the Tm of the perfect and imperfect match were closer but still well separated (Figure 9). This last set was used for the final multiplex.

Individual SNP assays were then multiplexed to determine how they worked together. Amazingly, no real difficulties were encountered in multiplexing the individual SNPs. Six commercial master mixes were evaluated using a 4-plex assay to determine compatibility with multiplexing [ABgene Absolute QPCR Mix (Rochester, NY), Qiagen QuantiTect Multiplex PCR NoROX Master Mix (Valencia, CA), SIGMA JumpStart Taq Ready Mix (St. Louis, MO), Stratagene Brilliant Multiplex QPCR Master Mix (La Jolla, CA), Eurogentec qPCR Mastermix – no ROX (San Diego, CA), Roche Fast Start TaqMan™ Master Mix (Indianapolis, IN)]. The Qiagen QuantiTect master mix performed the best, generating sharp peaks in the melting results (data not shown); thus, it was chosen for all future experiments.

Once the SNPs were tested individually, the primer ratios optimized, and the assay appeared robust, several multiplex assays were developed (Table 2). These were: two 6-plexes for the RG6000, one including five SNPs and the SD gender marker (6-plex A) and the other with six SNPs, i.e. an autosomal SNP replacing the SD (6-plex B) and a two well (4-plex #1 and #2) seven total SNP plus gender assay for four/five color real-time instruments.

Figure 10 shows melting curves of five individuals (picked from the ten test individuals to give an example of both homozygotes and a heterozygote at each SNP and include both male and female) with 6-plex A on the RG6000. Data was interpreted using the Corbett software (plot graphs the change in fluorescence vs. temperature (dF/dT)). The Corbett software automatically calls the genotypes once the bins are assigned and the genotypes are defined based on the bins. Both types of homozygotes and the heterozygotes are clearly differentiated.

Results using the 4-plex #1 on the Stratagene MX3005P for three of the four individuals represented are shown in Figure 11. Results are interpreted manually on the Stratagene MxPro software. Data for the other assays are not shown.

Once the assays were developed, they were tested with several parameters including with and without the addition of BSA to a final BSA concentration of 160 µg/ml and in 10 µL vs. 20 µL volumes on the RG6000 to determine instrument sensitivity. The 10 µL reaction was exactly half the 20 µL reaction [5 µL of 2X master mix, half the primers of the 20 µL reaction (still the same final concentrations), 1 µL of input DNA and H_2O to 10 µL]. The 20 µL reaction was used for all other experiments in the RG6000. All assays in the MX3005P in the 96-well plates were performed in 20 µL volumes as signal intensity was not sufficient with 10 µL samples. In addition, selected samples or the standard DNAs were serially diluted to test both dropout of alleles at low concentrations (to determine a minimal input DNA) and the potential of the assay for human DNA quantitation.

The 6-plex A results with and without the addition of BSA (160 µg/ml) were essentially identical except that the background for the FAM/Green channel was higher with BSA (data not shown). Thus, BSA can be added to the assay if needed to reduce or counteract the adverse effect of potential PCR inhibitors. To evaluate instrument performance, the 6-plex A assay was

tested on eight samples at both 20 µL and 10 µL volumes in the RG6000 in the 0.1 ml tubes; both results were generally comparable although some heterozygotes were slightly more difficult to detect at 10 µL (data not shown). Obviously at lower concentrations, the 20 µL reaction will fare better as it has twice as much input DNA (2 µL of DNA vs. the 1 µL input of the same DNA in the 10 µL reaction).

Additional experiments were performed to determine the sensitivity of the 6-plex A assay. Four single source samples (individuals #3, #5, #6 and #7) were diluted to ~72 pg, 18 pg and 4.5 pg total input DNA in a 20 µL reaction (based on quantitation performed using a TaqMan™ duplex (*Alu* plus gender) assay (34)) and eight replicates of the 6-plex A assay performed. Four samples were required to guarantee at least one heterozygote at each locus. The homozygote assay started to fail at 18 pg (10% failed) and failed 46% of the time at 4.5 pg. Both heterozygote alleles were observed approximately 67% of the time at 18 pg while at 4.5 pg both alleles were observed only 9.5% of the time.

Of interest, the assays also appear able to quantitate the amount of DNA present when run with a standard curve. While the melting curve determines genotype, the real-time amplification can be used to determine the amount of DNA. Although for this technique, the curves are inverted (because fluorescence decreases during amplification), the Cts are still proportional to the amount of DNA present. Figure 12 shows the analyzed data (inverted curves shown) for a set of standard curve samples (concentration of 2 µL of input DNA of 64 ng/µL to 0.0039 ng/µL) of the RAB31 locus of a 6-plex A assay performed on the RG6000 analyzed by the Corbett software.

Table 3 lists the R^2 values, reaction efficiencies and the calculated concentration for a known sample for each locus of the 6-plex A assay. The R^2 values are all close to 1.0 and the

reaction efficiencies are all very close to the expected 100%. For comparison, the last column of the table gives the concentration of each sample as determined by a TaqMan™ *Alu* quantitation method published previously (Nicklas and Buel, 2006). While the SNP assay is not as sensitive as *Alu*-based quantitation methods (single copy detection versus multiple-copy marker detection), it still provides a good indication of the DNA concentration of a sample. Values measured by the two assays did vary by up to 2.5 fold (sample #1) however, most values were similar and sufficiently close to allow a dilution which would generate an acceptable DNA concentration within the input range for STR typings. [Note: For studies performed in the MX3005P, quantitation of DNA concentration requires download of the results into the Plexor (Promega, Madison,WI) software which can handle the inverse curves.]

Recent experiments were performed using the SNP FRET assay to determine the effect of inhibitors on the assay, its ability to work on degraded DNA and its ability to detect mixtures.

Four inhibitors (hematin, indigo, calcium phosphate, tannic acid) were tested for their effects of the FRET melting assay. Concentrations were chosen based on previous results on the HRM and degraded assays (see next sections). In general, the inhibitors had less effect on the FRET assay than on the other assays, and essentially had little effect on the melt curves except where the concentrations were so high that PCR amplification was inhibited. The mismatch peak was lower than matched peak for Ly9. Ly9 in the blue channel was the most inhibited by the inhibitors while RAB31 in the crimson channel tended to be the least inhibited (see Table 4). Figure 13 shows the melting results for Ly9 in the blue channel.

The FRET assay was also tested on DNase I degraded DNA. Aliquots of a DNA sample were degraded for 15 sec to 128 minutes (Figure 14 right) and tested with the FRET assay. Even highly degraded DNA gave good melting profiles (Figure 14 left).

Experiments were performed in order to determine if the FRET melting assay would be useful for mixtures. Expectations were not too high for these experiments because there are only three possibilities for melting profiles at any locus. DNAs were mixed in combinations of 100% DNA#1:0% DNA#2, 75% DNA#1:25% DNA#2, 50% DNA#1:50% DNA#2, 25% DNA#1:75%DNA#2 and 0% DNA#1:100% DNA#2 (total concentration 0.1ng/ul). Four different combinations of two DNAs were created to get several examples of each combination of genotypes. Figure 15A and B show a common occurrence, namely that both DNAs have the same genotype (both homozygote - above and both heterozygote – below), thus no information is gained on a possible mixture. Figure 15C shows an example where both DNAs are different homozygotes. Here if the DNAs are not in equal amounts, then uneven peak height can suggest a mixture; however, if the ratio is 50% to 50% then the DNA looks like a heterozygote rather than a mixture of two homozygotes. In the last case (Figure 15D), a homozygote is mixed with a heterozygote, here a mixture can be fairly obvious because of peak height imbalance. However, peaks are not always perfectly balanced in heterozygotes and as shown above, inhibitors can affect the two peaks differently so while unbalanced peaks suggest a mixture, they do not prove one. If the two DNAs in a mixture have several loci where one DNA is homozygous and the other heterozygous, then a mixture will become more obvious.

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

The STR FRET experiments were another method designed to discriminate between individuals. It utilized the melting FRET assay described above for the SNP FRET assays but

used long probes covering the length of the STR repeat. It was hoped that shorter alleles would melt sooner than longer alleles, thus, providing a way to discriminate between the STR alleles.

The first experiments used the THO1 sense probes. Figure 16 shows the results with eight individuals of different genotypes. The melt curves are fairly close together but a graph of the combined allele size for each individual (the melt curve for an individual will be a combination of the melt curves of the two alleles, this was hopefully a way to reflect that) shows some relationship with melt temperature (Figure 17). The next experiment used sets of individuals with the same genotype to determine if a consistent melt temperature was obtained for a single genotype across individuals (Figure 18). The results showed some differences between the genotypes but it was not very consistent from individuals; there was a great deal of overlap in melting temperatures. The THO1 antisense probe set was also tried (Figure 19). The melt curves were very odd (negative values) and this probe set was not pursued further.

Three different mastermixes were tried to determine if a greater difference in melting temperature could be obtained with a different mastermix. (Initial experiments used the Quantitect mix from Qiagen.) The ABgene mastermix and the Sigma mastermix with both high (3.5mM) and low Mg++ (1.5mM) were utilized. Figure 20 shows results for THO1 genotype 6/6 and 9.3/9.3 (genotypes that should show the most difference in melting). The mix and especially the Mg++ concentration did change the melting temperatures but did not increase the differential between the 6/6 and 9.3/9.3 genotypes.

New probes, some with mismatches (either one mismatch or two), were then tried with eight individuals of different genotypes. Figures 21A-D show the results. The mismatches in A and B do not seem to affect the melting discrimination. In C and D, use of the new probe set

where the anchor contains STR repeats, clearly discriminates 6/6 from the other genotypes but they all appear identical.

One experiment was attempted using a probe designed to D6S2956 which has a CGTC (high G/C content). It was thought that the higher G/C content might make for higher melting temperatures and greater discrimination between alleles of different sizes. There was very little amplification in this experiment and the melt was thus very poor (Figure 22).

No further experiments were performed with this technique as it was not felt that it was going to be a viable technique.

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

. A simple analytical method, which could allow the analyst to select only those probative samples for further analysis, would save time and resources. As discussed above, we previously developed a SNP assay to determine if two samples are from the same individual; we now wish to try other simpler technologies including a FRET-based STR assay and high resolution melting (HRM). HRM uses special dyes, controlled melting and software to type SNPs by slight melting temperature shifts. Some real-time PCR instruments are now equipped to handle this technology. Our goal is to try HRM on selected STRs to see if HRM is capable of differentiating the various genotypes at a locus. This method may only allow 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.

Initially, a Corbett RG6000 with HRM capability was obtained as a loaner for several weeks. To test out the feasibility of using HRM to differentiate STR alleles, the THO1 STR (one of the thirteen CODIS loci) was chosen as a test case. Two individuals of each of 10 different THO1 genotypes (total twenty individuals) plus eight individuals of another genotype were tested with THO1 PCR using a SensiMix HRM kit (Quantace, Inc., Norwood, MA) on the RG6000. (6-6 indicates an individual homozygous for the 6 repeat allele while 6-9 indicates a heterozygous individual with one allele with 6 repeats and one allele with 9 repeats.) The data was analyzed by the Corbett software.

Figure 23 shows the simple melting results for one individual for each of the genotypes. As can be seen, most of the genotypes give a distinctive profile. Figure 24 shows the results for duplicate wells for eight individuals all of genotype 8-8. Thus, as expected, the pattern for persons of the same genotype are very reproducible. Figure 25 shows the actual HRM results. The melting differences are clearly not easily interpreted by eye but the software can pull genotype information from the shape of the curves.

To test the genotyping ability of the software, one individual of each genotype was used to define that genotype to the Corbett HRM software and the genotypes of the other individuals were called automatically. The results are shown in Table 5. Amazingly all the calls are correct except for one 8-9 being called a 7-10 (highlighted in yellow). This is close. It may not be possible to call every genotype correctly with this method; some may have to be grouped. Also,

it is possible that this individual may have some minor internal sequence variation which changes the melting slightly.

One preliminary experiment was also performed using the STR TPOX. Fourteen individuals representing eight different genotypes were tested. Figure 26 shows the simple melt results for one individual of each genotype. Once again there is a range of melting conditions (although not as distinctive as that obtained with THO1) and the genotype calls made with HRM were good for the six individuals tested (5 of 6 correct, data not shown).

Although the initial grant to develop this assay was turned down, a GAN was written to continue development of the HRM assay on this grant. A two color plus HRM RG6000 was purchased on the grant funds and studies were restarted in January of 2009.

Twenty-two forensic STRs were chosen for the initial analysis (Table 6). For each locus, primers were chosen to generate small PCR products (mini-STR primers of Hill et al., 2008 or Butler et al., 2003). Each STR was tested with 30-50 sample DNAs with different, known genotypes by performing real-time PCR and HRM melting in a Corbett Rotorgene RG6000 (now Qiagen Rotorgene Q). Initial studies showed a great deal of difference in the efficiency of amplification of some of the STRs (Figure 27). In order to remedy this, a number of amplification parameters were adjusted. This included Mg++ concentration, anneal time and temperature, primer concentration and cycle number. Increasing the Mg++ concentration lead to poor amplifications (data not shown). A number of experiments testing the other parameters were performed and it was determined that a combination of increasing the anneal time to 20 sec (from 10 sec), increasing the extension time to 30 sec (from 10 sec), decreasing the extension temperature to 65˚C (from 72˚C) and increasing the number of cycles to 45 (from 40) (data not shown) worked the best.

The STRs that generated the greatest difference in melting profiles between genotypes (i.e. had the most genotypes that could be differentiated) were chosen for further study (Table 6). The assay was then tested for reproducibility, uniformity for a known genotype, and melting profile constancy over dilution.

Figure 28 (top and bottom) shows the melting profiles of 12 different genotypes for THO1. Two samples (same color) are shown for most patterns. This STR has the potential to discriminate individuals with this method. Figure 29 shows the melting profiles of six different genotypes for vWA. Two samples (same color) are shown for most patterns. This STR also has the potential to discriminate individuals. Figure 30 shows the melting profile for eight genotypes for a third STR (D14). Note that this STR shows no difference in melting profile for any of the genotypes and therefore, has zero potential to discriminate individuals. Figure 31 shows the profiles of sets of individuals with the same genotypes for vWA. There is very good concordance in the melting profiles of DNAs from individuals with the same genotype.

The results are very consistent and stable. Figure 32 shows that melting profiles are unchanged after the tubes have sat for six days at room temperature in the instrument. Figure 33 shows results for five individuals performed on two different days for the first STR. The assay also gives consistent melting profiles across dilutions of a sample. Figure 34 shows two samples diluted serially from neat to 1/512 giving identical melting curves for THO1.

The assay can not definitively call all genotypes (for example, the melt curve of an 11/12 genotype can look the same as a 12/13 genotype) (Figure 35). However, HRM can also discriminate between individuals with the same STR genotypes. Figure 36 shows that some samples with the same genotype had different melting profiles (vWA). These differences were reproducible and undoubtedly due to the fact that some alleles, although the same length (thus,

have the same allele name by capillary electrophoresis and CODIS) have difference sequences and, thus, different melting characteristics. That these differences are due to minor sequence differences between alleles is being confirmed by DNA sequence analysis.

The amplification part of the assay is quite robust and should be possible for quantitation, similar to the use of the SNP FRET assay amplification for quantitation. Figure 37 shows results of a standard curve using this assay.

A test using HRM with THO1 and vWA was made using 64 different DNAs for 72 samples (some DNAs were used more than once). The genotypes of 48 of the samples were given to the reviewer but she was kept blind to the identity of the other 24 samples which were unique or replicates. Results: The reviewer correctly identified all of the duplicates. If one looked at the 64 samples, then 89% of the samples (57) were unique (able to be differentiated from the other samples according to combined THO1 and vWA melt results),

We felt that an assay with just two STRs would not give sufficient differentiation of victim and suspect so we took the three most variable STRs in our initial tests (vWA, D18S51 and THO1) and developed them as a test assay. Figure 38 shows how the data for all three loci can be displayed simultaneously so that comparisons of two samples can be made easily. Figure 38A displays all three loci for 9947 DNA while Figure 38B shows what duplicates of a single sample look like while Figure 38C shows how two different samples can be differentiated. Theoretical calculations would give a match at about 1/5000 for these three loci. Since we cannot discriminate all alleles, (although we can detect sequence differences that STR analysis cannot), our discrimination rate would be less than this.

We performed the following tests on this assay: test of whether added BSA affected the assay, a large test of 72 samples with duplicates to see if the duplicates could be identified and

that the non-duplicates could be differentiated as different, a test on samples isolated in different ways (simple sucrose method, organic method, Promega Maxwell robot), a test of DNase I treated DNA, a test of seven different inhibitors commonly seen in forensic samples (hematin, indigo, melanin, calcium, collagen, tannic acid and humic acid), a test of 4 samples against 60 others to see if all samples could be differentiated, testing on mixtures, testing with a second mastermix (Qiagen), sequencing samples with the same STR genotype but different melting profiles, and running the THO1 and vWA part of the assay as a duplex.

Five BSA concentrations (0ng/ul, 166ng/ul, 250ng/ul, 333ng/ul, 417ng/ul) were tested to see their effects on amplification and melting. Figure 39 shows the results for all three loci. There was little, if any, effect. A concentration of 250ng/ul was chosen for future experiments as this approximated the amount in other assays.

We tested whether DNA degradation would affect the assay. Figure 40 shows that degradation did not affect the melting profiles. The right panel shows the degraded DNA (DNase I treated) and the left panel shows the melting of the same DNA samples. We also tested if different DNA extraction methods would affect the HRM profiles. Two different DNAs extracted separately in replicate with the Promega Maxwell 16, the organic method and a simple sucrose extraction were tested. No difference was seen in melting profiles between DNAs isolated by the different methods (Figure 41).

The assay was tested with seven different inhibitors (hematin, indigo, melanin, collagen, calcium phosphate, tannic acid and humic acid). Although many of the inhibitors do affect the amplification at high concentrations (data not shown), most do not affect the melting characteristics (Figure 42). Collagen and calcium phosphate do shift the profile slightly to higher melting temperatures; however, this may be due to the dilution buffers rather than the

agent. Figure 43 shows that the collagen buffer alone (0.1N acetic acid) shifts the melting curve to higher temperatures. The calcium phosphate buffer itself (0.5N HCl) causes total loss of amplification.

A test of 20 DNA samples with replicates (0-6 replicates for a total of 72 tubes assayed) to ascertain accuracy of determining whether two samples were alike or different was made. Results for one sample (5 duplicates/tubes) were excluded because of poor amplification. For the remaining 19 samples/67 tubes, 53 were correctly identified with their duplicates. Two sets of two samples were found, i.e. the two samples and their duplicates could not be told apart but were correctly called together (another 12 samples). There were four wrong calls where the sample was put into the wrong set, one had only one tube (no replicate).

A second test was run with four samples as the test samples (DNA1, DNA2, DNA3 and 9947) against three different sets of 20 DNA samples each. (This was done so that all three loci could be tested against the four test samples and the 20 DNA samples on one run which made for easier analysis.) For all the 240 comparisons (4 x 60), only one melting profile match was found. When this was checked against the actual genotypes, the two individuals had the exact same genotypes for the three loci. The assay performed very well in this test, performing excellent discrimination and a perfect detection of the one accidental match. The difficulties in the previous test were undoubtedly partly due to the difficulties of comparing the large amount of data spread over three runs. In reality, when this test would be performed in the laboratory on casework, the data would be comparing several melting profiles for all three loci in the same run.

Mixture experiments for the HRM assay were quite interesting. Four combinations of different DNAs were made at five mixture combinations as for the FRET experiments. Melting profiles of the mixtures were often not the average of the two melt profiles of the individual

DNAs. This is not actually surprising as now having two DNAs in the sample allows for new unique hybrid molecules to be formed and melt. For example, if you mix a 7/7 homozygote with a 9/9.3 heterozygote, the 7/7 homozygote has only the 7-7 molecule to melt and the 9/9.3 heterozygote has 9-9, 9.3-9.3 and 9-9.3 molecules but the mixture has all of those plus 7-9 and 7-9.3 which could give an entirely new dimension to the melt curve. It can get even more complex if both DNAs were from heterozygotes. In general, the 75/25 mixes were reasonably close to the 100/0 patterns but the 50/50 mixes were often quite different. However, there were exceptions. If both patterns were initially similar then the mixture was usually similar as well. Figure 44 shows several examples of mixtures with the three loci. Table 7 summarizes the results.

Because the melting temperature of THO1 is so much higher than either vWA or D18S51, we thought that is might be possible to amplify either THO1 with vWA or D18S51 as a duplex. As a proof of concept, we tried THO1 with vWA on 24 DNA samples, both with separate amplifications and melts and a duplex amplification and melt. The results for the duplex were surprisingly good, perhaps even better than for the separate amplifications and melts. Figure 45 shows the results for 5 of the 24 samples. In the top panel, the separate amplification melts are displayed on the same graph while the bottom panel shows the duplex. The results for most samples look pretty similar except for the red sample which looks more defined in the duplex. The peaks for THO1 are also higher in the duplex which helps with differentiation of the profiles. This is definitely area for further research; if THO1 and vWA can be duplexed then perhaps a second high melting STR such as D2 could be multiplexed with D18S51 creating a much more powerful assay using only two wells.

We recently tested a new HRM mastermix available from Qiagen. Figure 46 shows the results with several vWA and THO1 genotypes. The results are slightly different, those with the Quantace buffer are bumpier but it is unclear whether either allows differentiation of more genotypes.

The Plexor technology has been used in the degradation assays and other assays in the laboratory. It was suggested that perhaps Plexor could be used for the STR HRM instead of an intercalating dye. The thought was that if it worked then several STRs could be multiplexed with different dyes on the Plexor primers. Primers were designed for THO1 using the Promega Plexor webpage software and ordered from BioSearch Technologies (For 5'AATTCAAAGGGTATCTGGGCTCTG3', Rev-5'FAM-isoC-TCACAGGGAACACAGACT CCAT3'). The Promega Plexor mastermix was used as well as Plexor amplification conditions but the default HRM conditions were used for melting. The results were very promising. All the THO1 genotypes tested (6/6, 6/7, 6/9.3, 5/9.3, 7/9.3, 8/9.3, 9/9.3, 9.3/9.3) could be resolved (Figure 47).

While this seemed very promising, it was realized that the Corbett RG6000 is only set up to perform HRM in the Green channel, thus, multiplexing using Plexor for HRM would not be possible. We decided to see if the actual HRM melting conditions were needed – could a sufficiently robust melt be performed in the regular (non-HRM) RG6000? The exact same experiment was repeated but in our 6-color (no HRM) RG6000 using a regular melt set to parameters similar to those of an HRM melt (0.1 degrees/step, 2 sec wait/step). The results were again surprisingly good. A blind analysis of the data grouped the genotypes almost perfectly (one could not be called and another was put into a group with a similar melt profile and a similar genotype) even though the melting range was too broadly set and the gain was set too

high. Figure 48 shows the results for the melt which look quite similar to those for the HRM melt. Future work needs to be performed to see if this approach will work for other forensic STRs.

DNA sequencing of six pairs of products (6 pairs for vWA) which had the same STR genotyping (by size) but different melting patterns are underway but were not completed in time for this final report.

A manuscript on this assay is being written and will be submitted.

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

Many forensic samples contain degraded DNA. 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 STR kits, and would allow the examiner to decide which assay, miniSTRs or conventional STRs or mtDNA analysis, would be a more appropriate approach for analysis. We started development of such a degradation state detection assay using different sized *Alu* amplicons with a simple gel-based readout. Figure 49A shows a quick demonstration of the three *Alu* amplicons on high-quality commercial DNA (giving 82bp, 124bp and 201bp products) while Figure 49B shows the results with DNase I degraded DNA where the larger amplicon is lost with degradation and the smaller amplicon increases.

While a gel-based assay would work, we decided that a real-time assay would be faster, more sensitive and less hands on. We therefore moved the assay to the Plexor™ System. Experiments found it very hard to balance the three reverse primers. When DNA is diluted the ratio of the RFUs (measured by the difference in the Cts) of the large to medium, large to small, or medium to small amplification should not change. This is tested by plotting the ΔCt versus

DNA concentration. The line should be flat (slope between -0.1 and 0.1); however, with this assay, the lines did not approach such values (Figure 50) even with numerous attempts to limit the concentration of the small specific reverse primer and changing numerous reaction conditions such as anneal and extend time, cycle number and temperature. We also tried swapping the fluorophores on the primers and using a new fluorophore (CAL Flour Red 610), but these did not help either. After 9 months of futile attempts to optimize this assay, we finally reduced the assay to a duplex (Figure 51). With this adjustment, it was possible to keep the slope of the line within the proper range for the ratio of the large to small RFUs (Figure 52). This new assay was optimized for volume (20ul), cycle number (32 cycles), anneal/extend time (30 sec) and temperature (60°C).

Degraded DNAs were created by treating 10 high concentration DNAs with DNase I for 0 to 128 minutes. Aliquots were taken at intervals to create DNAs with various levels of degradation (Figure 53). These samples were run in the assay and a graph was made of the ratio of short to long concentration versus time of degradation. Figure 54 and Table 8 show the results for 9 of the 10 degraded DNAs. As can be seen, the ratio increases with degradation. The final ratio is variable for the different DNAs which may reflect their initial quality or concentration. While an attempt was made to have the initial concentration the same for all, the DNAs were so stringy (i.e., the long high molecular weight strands strung out from the pipette tip when an aliquot was taken) that determination of an exact concentration was very difficult. The ratio also decreases for many of the samples as degradation time becomes very long; this is due to the fact that amplification of even the short product decreases as the degraded DNA size falls below that of the size of the short amplicon.

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. Figure 55 shows examples of the ski slope effect.

However, considerable ski slope was observed with the highly degraded samples. The ski slope was proportional to the degradation ratio; i.e. if the ratio of short to long product was high (DNA degraded) then the CSF (larger locus, ~300bp) RFU was much lower than the D3 (smaller locus, ~120bp) RFU (Figure 56).

Experiments on the degradation assay were halted for a number of months to work on other projects. Experiments resumed testing the reproducibility of the measurements on a variety of environmentally degraded samples (time, sunlight, heat). Table 9 shows duplicate results for a number of degraded samples. The samples have been kept either at room temperature, at 37˚C or taped to a window in sunlight. The date indicates the age of the sample. The samples are blood, saliva or semen. There are also a number of control samples which have been kept refrigerated or frozen. The data from the degradation assay indicates that the samples kept at 37˚C are the most degraded, as would be expected. The controls show the lowest level of degradation. Unfortunately, the results for the long amplification are not as consistent as could be hoped (although the results for the short amplification are good); this inconsistency in the long results clearly affects the short/long ratio. Another experiment took 6 samples (5 from the above set and 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 (Table 10). The numbers are

not as tight as one might like but they are not too bad. There are outliers for several of the samples.

Since previous data had been inconsistent, during the last three months of the grant, a set of 6 degraded samples and a control were chosen to run in 5-10 replicates on four occasions to test the reproducibility of the assay. The results are shown in Table 11. The data is quite consistent with the control having an expected ratio of about 1 and the degraded samples had generally consistent values above 1.0 to about 10 for the most degraded sample.

We also tried putting different fluorophores on the primers (from BioSearch Technologies). We tried the combinations of Long-FAM/short-CAL Fluor Orange 560, Long-CAL-Fluor Orange 560/short-FAM as compared to the current Long-FAM/short-Cy5. The 5 samples (from above) and three controls were used in duplicate. The results are shown in Table 12. The degraded ratios for the Long- 560/short-FAM are much higher than for the current assay while the ratios for the Long-FAM/short-560 are about the same. However, for sample #4 the replicate values are widely different for Long-560/short-FAM suggesting this combination may be subject to quite a bit of variability.

This assay was also tested with seven different inhibitors commonly seen in forensic samples; hematin, indigo, melanin, calcium, collagen, tannic acid and humic acid. These samples were diluted as suggested by Bruce McCord and tested at five concentrations believed to span from no inhibition to severe inhibition (plus a no inhibitor control). Figure 57 shows some results from inhibitors. Most inhibitors do not affect the degradation ratio when the assay is not inhibited to such an extent that PCR does not occur at all (Table 13). The exception is calcium phosphate which does increase the degradation ratio.

2. Tables

Table 1—Genotype results for the ten test individuals.

Sample	ZFXY	THSD2*	FLJ43720*	RAB31*	LY9*	ATP13A4*	PALLD*	A2BP1*
Individual #1	female	CC	CA	AA	TT	CC	AC	GC
Individual #2	female	AA	AA	GG	CT	CC	AA	CC
Individual #3	male	CA	CA	GG	TT	TT	AA	CC
Individual #4	male	CA	CA	GG	CT	CC	AC	GG
Individual #5	male	AA	CC	GA	TT	CC	AC	GC
Individual #6 (9947)	female	CC	CC	AA	CT	CC	AA	CC
Individual #7	female	AA	AA	AA	TT	CT	CC	GC
Individual #8	male	CA	AA	AA	CC	CC	AA	CC
Individual #9	male	AA	CA	GG	CT	TT	AA	GC
Individual #10	male	CC	CA	AA	CT	CC	AA	CC

*Genotype results from SNP assay and sequencing data

Table 2 - Description of loci and fluorophores used in each multiplex SNP FRET assay.

SNP/SD	6-plex A	6-plex B	4-plex#1	4-plex #2
A2BP1				Quasar 670
ATP13A4	CAL Fluor Red 610	CAL Fluor Red 610	CAL Fluor Red 610	
FLJ43720	CAL Fluor Orange 560	CAL Fluor Orange 560	CAL Fluor Orange 560	
LY9	Biosearch Blue	Biosearch Blue		CAL Fluor Orange 560
PALLD, SNP2		FAM		FAM
RAB31	Quasar 705	Quasar 705		CAL Fluor Red 610
THSD2	Quasar 670	Quasar 670	Quasar 670	
ZFXY, double Y	FAM		FAM	

Table 3—Quantitation results for 6-plex A assay in RG6000.

Sample	**ZFXY**	**ATP13A4**	**THSD2**	**FLJ43720**	**Ly9**	**RAB31**	**Mean**
R^2	0.986	0.977	0.998	0.989	0.974	0.976	0.983
Efficiency	97.7%	109.2%	98.6%	104.0%	95.2%	107.2%	102.0%

Individual	**ZFXY**	**ATP13A4**	**THSD2**	**FLJ43720**	**Ly9**	**RAB31**	**Mean ±SD**	**TaqMan™ *Alu* assay**
#2	0.0014	1.16	1.06	0.98	1.71	0.64	0.93±0.52	2.48
#3	0.68	0.88	0.99	0.85	0.79	1.07	0.88±0.13	2.08
#4	0.12	0.12	0.19	0.18	0.12	0.13	0.14±0.03	0.34
#5	5.37	5.33	3.95	5.63	20.22	7.12	7.94±5.57	5.89
#6	--	0.05	0.06	0.05	0.13	0.11	0.08±0.03	0.11
#7	0.01	1.79	1.33	1.60	1.62	2.92	1.55±0.85	1.67
#8	33.60	45.21	88.11	89.22	76.96	144.45	79.6±35.8	94.8
#9	32.07	42.55	31.09	47.70	65.83	26.47	40.9±13.2	38.2

Table 4 – Effects of Inhibitors on the FRET melting assay.

Locus (channel)	**Inhibitor**			
	Hematin	**Indigo**	**Calcium phosphate**	**Tannic acid**
Ly9 (blue)	Amp - some inhibition at 50 and 12.5mM Melt – no effects	Amp - total inhibition at 2.5mM and above, some at 0.63mM Melt – no effects	Amp - total inhibition at 5mM, some at 0.63mM Melt – no effects	Amp - some inhibition at 10 and 5 ng/ul Melt – peaks uneven
PALLD (green)	Amp - some inhibition at 3.12uM or above Melt – no effects	Amp – total inhibition at 10uM, some at 2.5uM Melt – no effects	Amp – total inhibition at 5uM some at 2.5uM and 0.63uM Melt – shift to higher temp melt ~1.0C	Amp – a little inhibition at 10uM Melt – no effects
FLJ43720 (yellow)	Amp – a little inhibition at 50uM Melt – no effects	Amp – total inhibition at 10uM, significant at 2.5uM, some at 0.63uM Melt – no effects	Amp – total inhibition at 5uM, some at 2.5uM Melt – shift of 0.3C	Amp - no effects Melt – no effects
ATP13A4 (orange)	Amp – no effects Melt – no effects	Amp – total inhibition at 10uM, significant at 2.5uM, some at 0.63uM Melt – no effects	Amp – total inhibition at 5uM, significant at 2.5uM Melt – shift of 0.3C	Amp - no effects Melt – no effects
THSD2 (red)	Amp – a little inhibition at 50uM Melt – no effects	Amp - total inhibition at 10uM, significant at 2.5uM, some at 0.63uM Melt – no effects	Amp – total inhibition at 5uM, some at 2.5uM Melt – not much shift	Amp - no effects Melt – no effects
RAB31 (crimson)	Amp – no effects Melt – no effects	Amp - total inhibition at 10uM, significant at 2.5uM, some at 0.63uM Melt – no effects	Amp – total inhibition at 5uM, some at 2.5uM Melt – not much shift	Amp - no effects Melt – no effects

Table 5 – THO1 HRM data (Confidence % is generated by the software).

Actual Genotype	Genotype called by HRM	Confidence %
6-6	6-6	98.43
7-7	7-7	94.97
9-9	9-9	98.54
9.3-9.3	9.3-9.3	99.28
6-9	6-9	98.7
6-9.3	6-9.3	99.37
7-9.3	7-9.3	99.38
7-10	7-10	98.62
8-9	7-10	95.41
9.3-10	9.3-10	98.27
8-8	8-8	96.64
8-8	8-8	95.74
8-8	8-8	92.7
8-8	8-8	99.07
8-8	8-8	98.71
8-8	8-8	99.71
8-8	8-8	97.32
8-8	8-8	99.81
8-8	8-8	95.91
8-8	8-8	99.36
8-8	8-8	98
8-8	8-8	99.08
8-8	8-8	99.77
8-8	8-8	93.91
8-8	8-8	94.74

Table 6 - The approximate number of genotypes that could be easily differentiated for the 22 STRs studied.

Locus tested	STR	# distinct melting patterns among common genotypes		Locus tested	STR	# distinct melting patterns
#1	THO1	8		#12	D4	3
#2	vWA	10		#13	D22	2
#3	D16	6		#14	D14	2
#4	D3	6		#15	D1	(noise only)
#5	D21	3		#16	D19	4
#6	D7	1		#17	D2	1
#7	CSF	1		#18	D5	3
#8	PentaD	7		#19	D10	2
#9	D18	7		#20	D13	4
#10	FGA	3		#21	D8	7
#11	PentaE	3		#22	TPOX	8

Table 7 – HRM DNA mixture experiments.

	vWA (genotypes)	D18S51 (genotypes)	THO1 (genotypes)
DNA1/DNA2	Not a simple combination	Not a simple combination	Not a simple combination
DNA2/DNA3	Not a simple combination	Patterns of two DNAs similar – mixtures are somewhat different	Mixtures all like DNA2 not DNA3
DNA3/9947	Patterns of two DNAs similar – mixtures most like DNA3	Mixtures mostly different than original DNAs	50/50 like DNA3
DNA1/9947	50/50 like 9947	Mixtures a combo of two DNAs	50/50 different

Table 8 – The ratio of short to long concentration for nine degraded DNA samples.

Degradation time (min)	Degraded DNA (short/long concentration ratio)								
	#1	#3	#4	#5	#6	#7	#8	#9	#10
0	0.76	0.81	0.56	0.85	0.88	0.74	0.82	0.82	1.43
0.25	1.73	1.03	0.64	0.78	0.98	1.38	1.21	1.44	1.04
0.5	1.59	1.42	1.22	0.95	1.11	1.26	1.53	1.53	1.08
1	3.58	1.91	2.08	2.19	1.30	2.55	2.35	1.78	1.26

2	8.21	6.12	4.29	18.5	2.82	14.4	6.44	2.06	7.33
4	203	93.9	44.1	216	20.6	144	12.8	5.74	132
8	672	134	130	574	82.7	1075	47.2	16.9	948
26	649	483	171	523	329	317	119	52.6	834
32	123	498	157	881	442	482	123	49.9	574
64	25.4	288	62	884	332	227	79.2	13.1	187
128	24.0	249	40.3	0.85	326	31.1	141	41.3	162

Table 9 – Degradation results for environmentally treated DNA samples and controls.

Item #	Sample Description	long	long	short	short	short/long	short/long
1-1	blood on cloth (7/2/03) Room Temp	0.02	0.00	0.03	0.03	2.14	8.01
1-2	blood on FTA (7/11/03) Room Temp	0.29	0.36	0.51	0.97	1.76	2.67
1-3	blood on cloth (6/11/04) Room Temp	0.18	0.09	0.22	0.29	1.24	3.25
1-4	blood on cloth (6/11/04) 37˚C	0.11	0.02	0.78	0.59	7.21	25.36
1-5	blood on cloth (10/31/05) Room Temp	0.16	0.16	0.28	0.24	1.77	1.49
1-6	blood on cloth (11/9/05) sunlight	0.11	0.03	0.29	0.23	2.67	8.52
1-7	blood on cloth (11/9/05) 37˚C	0.06	0.02	0.30	0.24	4.87	13.20
1-8	blood on denim (2/23/06) Room Temp	0.10	0.05	0.11	0.07	1.15	1.42
1-9	blood on denim (3/14/06) Room Temp	0.70	0.89	0.90	1.26	1.27	1.41
1-10	blood on cloth (9/17/07) Room Temp	0.29	0.24	0.28	0.22	0.97	0.90
1-11	blood/semen on cloth (12/15/05) Room Temp	0.10	0.04	0.11	0.09	1.12	2.44
1-12	blood/semen on cloth (12/15/05) sun	0.06	0.02	0.19	0.14	3.45	8.16
1-13	blood/semen on cloth (12/15/05) 37˚C	0.00	0.00	0.00	0.00	3.25	3.99
1-14	control blood on cloth frozen	0.16	0.10	0.11	0.10	0.73	0.99
1-15	Reagent Blank	0.02	no Ct	0.00	0.00	0.03	--
2-1	blood on cloth (7/26/04) sunlight	0.00	no Ct	0.00	0.00	16.68	--
2-2	buccal on FTA (2/13/06) Room Temp	0.07	0.03	0.04	0.03	0.54	1.08
2-3	saliva on cloth (6/11/04) Room Temp	0.02	0.01	0.22	0.21	11.43	22.07
2-4	saliva on cloth (6/14/04) 37˚C	0.00	no Ct	0.03	0.01	13.46	--
2-5	saliva on cloth (10/30/06) Room Temp	0.19	0.02	0.55	0.32	2.94	19.03
2-6	semen on cloth (6/14/04) Room Temp	3.17	0.07	6.14	3.12	1.93	45.87
2-7	semen on cloth (6/14/04) 37˚C	2.26	0.12	14.29	7.43	6.31	63.88
2-8	semen on carpet (11/7/05) Room Temp	0.40	0.36	0.65	0.92	1.62	2.59
2-9	semen on cloth (11/7/05) sunlight	0.64	0.49	2.40	2.89	3.76	5.95
2-10	semen on cloth (12/15/05) Room Temp	0.73	0.41	0.51	0.65	0.69	1.58
2-11	control blood on cloth frozen	0.31	0.44	0.29	0.55	0.96	1.25
2-12	Reagent Blank	0.00	no Ct	0.00	0.00	0.30	--
88	in house control, unknown	0.03	0.01	0.02	0.02	0.76	1.91
87	in house control, unknown	23.55	10.89	22.07	13.08	0.94	1.20
47	in house control, unknown	21.39	12.95	17.73	12.31	0.83	0.95
JLA	in house control, organic	0.15	0.16	0.23	0.20	1.55	1.27
1518	in house control, sucrose isolated	2.36	2.32	3.82	3.79	1.62	1.63
1414	in house control, sucrose isolated	14.85	8.86	16.81	12.57	1.13	1.42

Table 10 – Degradation assay triplicates for 6 samples over two days on two real-time instruments.

Sample	Date	Real-time machine	long	long	long	short	short	short	Short/ Long	Short/ Long	Short/ long
1-4	12-10-08	RG6000	0.07	0.06	0.06	0.36	0.46	0.41	4.90	8.06	6.78
1-4	12-26-08	RG3000	0.04	0.05	0.03	0.40	0.45	0.32	9.43	9.04	9.23
1-4	12-26-08	RG6000	0.02	0.02	0.01	0.38	0.42	0.28	20.99	17.50	34.29
1-6	12-10-08	RG6000	0.03	0.03	0.02	0.15	0.11	0.11	4.29	3.81	4.54
1-6	12-26-08	RG3000	0.02	0.04	0.02	0.09	0.12	0.08	3.59	3.31	4.22
1-6	12-26-08	RG6000	0.02	0.02	0.02	0.11	0.15	0.12	4.89	7.20	6.51
2-10	12-10-08	RG6000	0.37	0.35	0.29	0.63	0.47	0.48	1.71	1.37	1.67
2-10	12-26-08	RG3000	0.31	0.25	0.39	0.36	0.32	0.58	1.15	1.27	1.49
2-10	12-26-08	RG6000	0.41	0.27	0.25	0.40	0.40	0.38	0.99	1.52	1.50
2-5	12-10-08	RG6000	0.11	0.10	0.10	0.32	0.36	0.27	2.86	3.70	2.81
2-5	12-26-08	RG3000	0.10	0.09	0.11	0.40	0.27	0.35	3.97	3.00	3.28
2-5	12-26-08	RG6000	0.05	0.07	0.05	0.24	0.29	0.23	4.36	4.11	4.70
2-6	12-10-08	RG6000	1.77	2.02	1.43	6.53	4.23	9.69	3.69	2.10	6.77
2-6	12-26-08	RG3000	4.34	2.63	2.81	9.86	7.18	8.29	2.27	2.73	2.95
2-6	12-26-08	RG6000	2.21	0.06	0.14	5.96	6.40	8.45	2.69	99.09	61.27
9947	12-10-08	RG6000	0.07	0.04	0.08	0.05	0.06	0.04	0.71	1.58	0.50
9947	12-26-08	RG3000	0.10	0.09	0.11	0.08	0.06	0.07	0.80	0.67	0.60
9947	12-26-08	RG6000	0.08	0.08	0.05	0.06	0.06	0.06	0.70	0.81	1.26

Table 11 – Degradation ratios for five degraded samples and a control performed in 2-10 replicates on four occasions.

Repeat1	Replicates											
Sample	1	2	3	4	5	6	7	8	9	10	average	stdev
1	10.10	6.89	7.55	5.89	10.71	5.98	5.73	8.42	5.62	5.06	7.19	1.97
2	4.58	4.64	1.83	4.62	7.12	6.57	4.85	4.15	4.29	5.80	4.84	1.46
3	5.92	4.96	1.94	3.72	6.77	4.48	4.20	3.60	5.20	5.65	4.64	1.38
4	7.09	6.39	5.01	6.14	5.46	7.44	8.55	8.00	6.40	7.68	6.82	1.13
5	2.67	1.33	1.63	0.97	1.18	2.96	1.29	1.40	1.21	1.91	1.65	0.67
control	1.52	0.73	0.67	0.76	1.12	1.36	0.99	0.90	0.83	0.93	0.98	0.28
Repeat 2	Replicates											
Sample	1	2	3	4	5						average	stdev
1	3.37	4.37	5.79	2.40	2.18						3.62	1.49
2	2.15	2.85	4.29	3.21	2.28						2.96	0.86
3	4.05	2.76	2.26	2.87	3.66						3.12	0.72
4	6.23	4.28	4.51	5.96	15.18						7.23	4.52
5	1.69	1.23	0.97	0.93	1.43						1.25	0.32
control	0.74	0.86	1.03	0.93	1.02						0.92	0.12
Repeat 3	Replicates											
Sample	1	2	3	4	5						average	stdev
1	8.29	4.90	7.43	9.53	7.02						7.43	1.53
2	4.68	2.39	4.57	5.60	8.59						5.17	2.01
3	13.83	6.47	7.88	6.56	11.25						9.20	2.89
4	6.29	8.06	5.88	6.59	12.30						7.82	2.36
5	0.89	1.45	1.13	1.18	1.66						1.26	0.27
control	1.17	0.70	0.88	0.95	0.78						0.90	0.16
Repeat 4	Replicates											
Sample	1	2									average	stdev
1	5.58	5.14									5.36	0.31
2	4.32	4.87									4.60	0.38
3	6.98	6.37									6.67	0.43
4	10.30	12.23									11.26	1.37
5	0.96	1.34									1.15	0.27
control	0.96	1.47									1.21	0.36

Table 12 – Test of new dye combinations for the Degradation assay.

	Long - FAM Short Cy5			Long - 560 Short - FAM			Long – FAM Short - 560		
Sample	1	2	average	1	2	Average	1	2	average
1	5.58	5.14	5.36	22.72	21.11	21.92	4.74	7.46	6.10
2	4.32	4.87	4.60	11.52	26.96	19.24	3.85	13.04	8.45
3	6.98	6.37	6.67	27.34	15.27	21.31	3.16	4.49	3.83
4	10.30	12.23	11.26	247.58	5.22	126.40	4.32	4.42	4.37
5	0.96	1.34	1.15	2.43	2.31	2.37	2.04	2.60	2.32
control 1	0.96	1.47	1.21	1.85	1.56	1.71	1.49	0.92	1.20
control 2	0.99	0.71	0.85	1.42	1.14	1.28	1.21	1.82	1.51
control 3	0.85	0.96	0.90	1.82	0.31	1.07	1.42	0.33	0.88

Table 13 – Effects of Inhibitors on the DNA degradation assay.

Inhibitor	**Effects on Long (green)**	**Effects on Short (red)**	**Effects on ratio**
Hematin	50uM total inhibition	50uM minor inhibition	None
Indigo	False positive readings for 10mM and 2.5mM	False positive readings for 10mM and 2.5mM	Decreases ratio below 1.0 (due to dye interference)
Melanin	Partial inhibition for 20uM and 5uM	Total inhibition for 20uM	Only small increase in ratio above at 5Um
Collagen	Total inhibition for 100ng/ul and 50ng/ul	Significant inhibition 100ng/ul	None
Calcium phosphate	Total inhibition above 0.63mM	False positives 5mM and 2.5mM, slight inhibition 1.25mM	Large increase in ratio
Tannic acid	Partial inhibition for 10ng/ul	Partial inhibition for 10ng/ul	None
Humic acid	Partial inhibition for 5ng/ul and 10ng/ul	Slight inhibition for 5ng/ul and 10ng/ul	Slight increase at high concentrations
0.1N acetic acid	Total inhibition	Total inhibition	---
0.5N HCl	Total inhibition	False positive	---
0.5N ammonium hydroxide	No effect	No effect	Ratio low
2% triton X	No effect	No effect	None
0.1N NaOH	Total inhibition	Total inhibition	---

3. Figures

Figure 1 – Agarose gel of SNP products for FRET experiments.

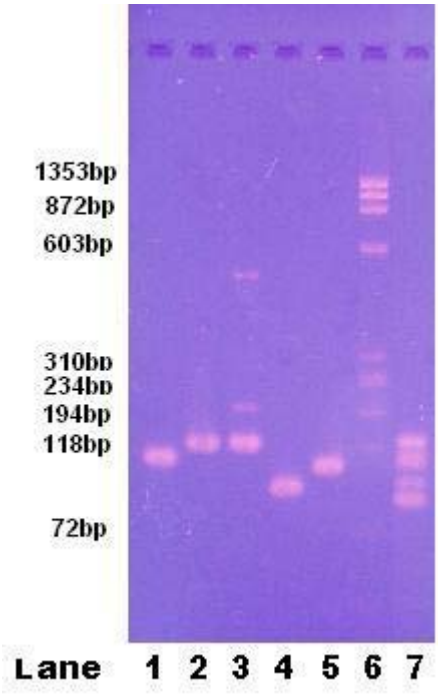


Figure 2 – First results (top – good amplification results as there was a lowering of RFUs; bottom – horrible melting results consisting of just noise).

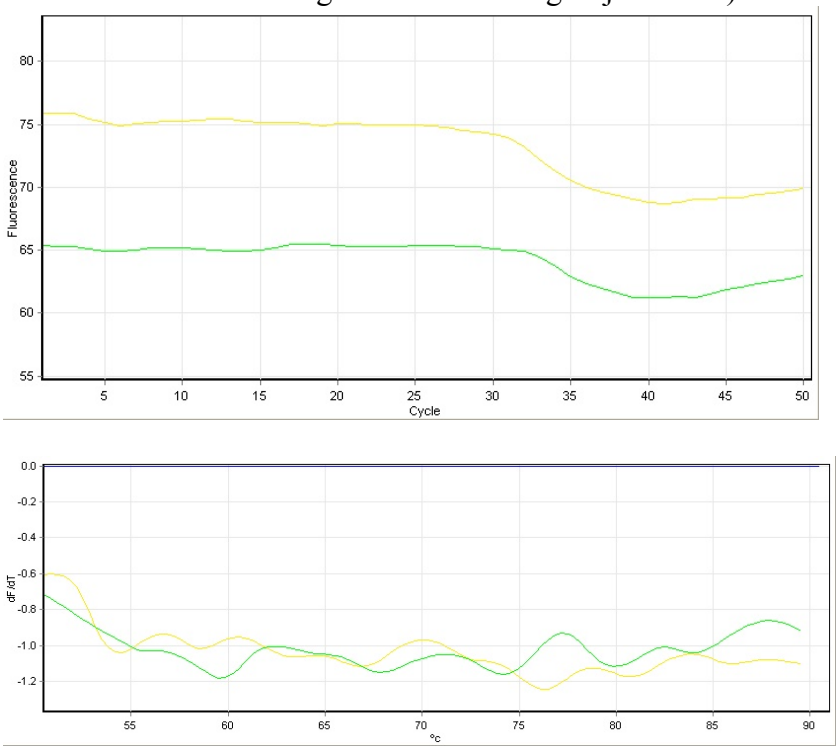


Figure 3 – HSPA12A SNP which did not show good melting temperature resolution between alleles.

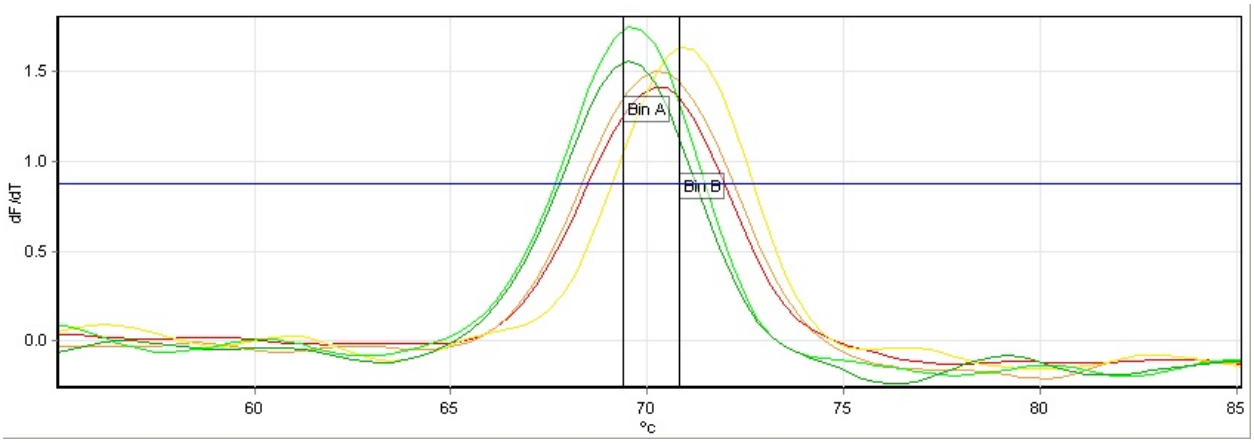
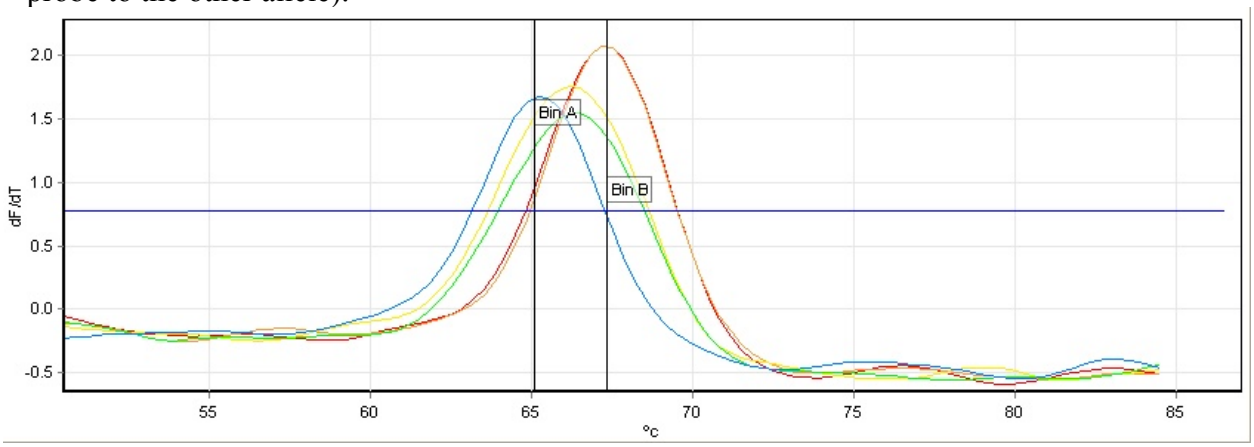


Figure 4 – PALLD SNP which showed poor resolution with the probes designed to one strand but good resolution when probes were designed to the other strand. (top – original probe, bottom – probe to the other allele).



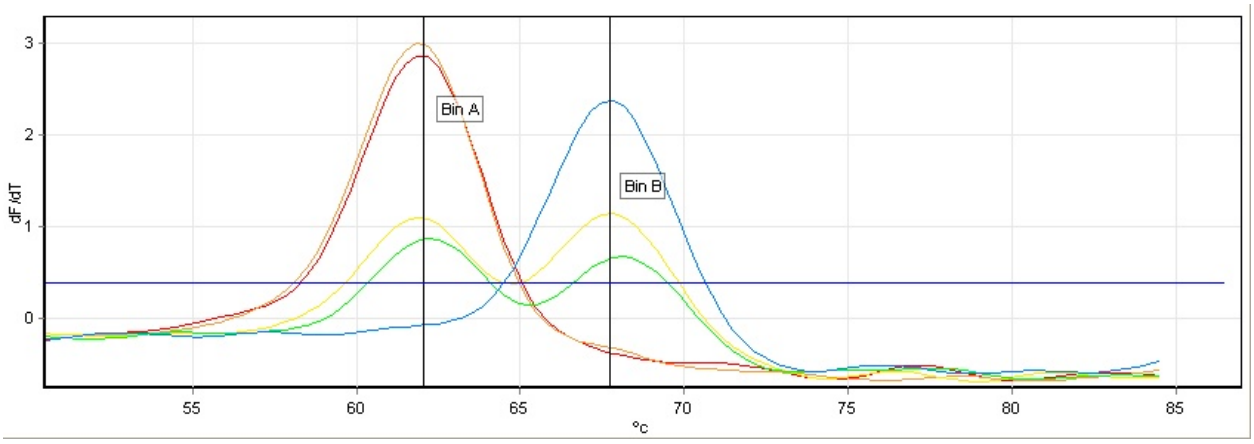


Figure 5 – DNA sequencing results for PALLD (2 heterozygotes and 1 of each homozygote).

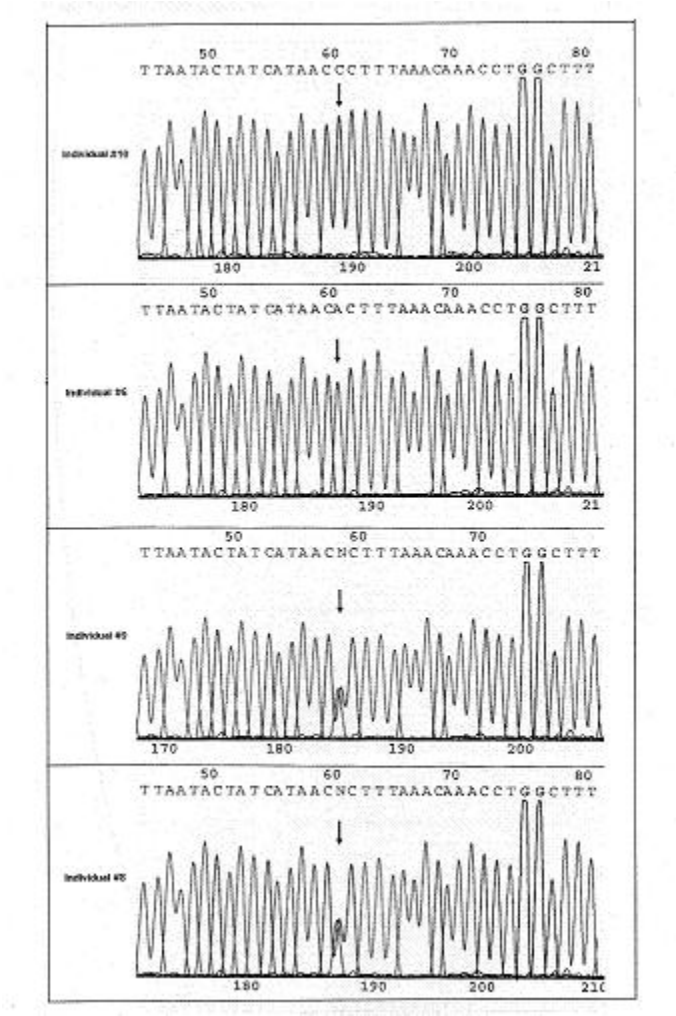


Figure 6 - The original ZFXY set designed in intron 7 with the perfect probe match to the ZFY gene.

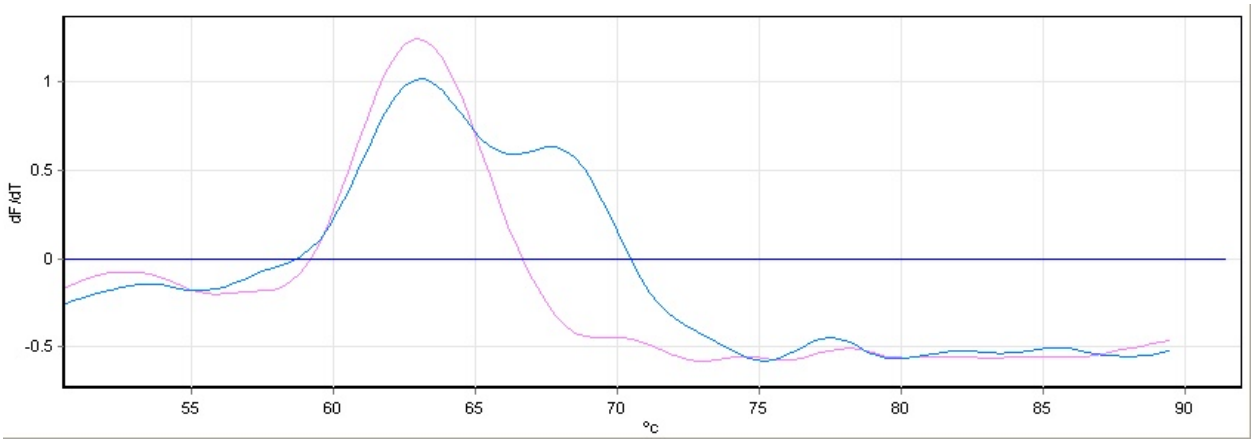


Figure 7 - A new ZFXY set designed three basepairs shifted from the first set.

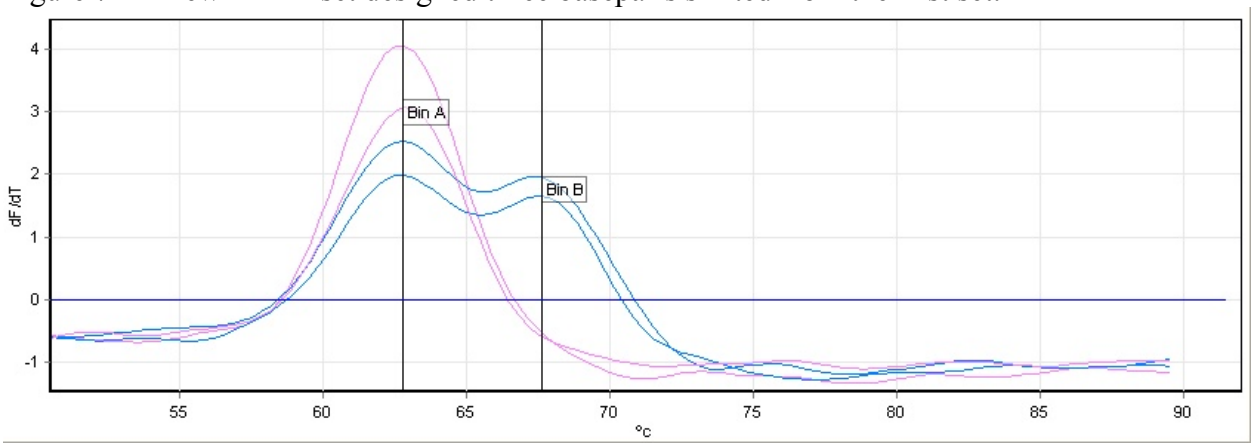


Figure 8 – Newest ZFXY set with the probe matched to the ZFX gene.

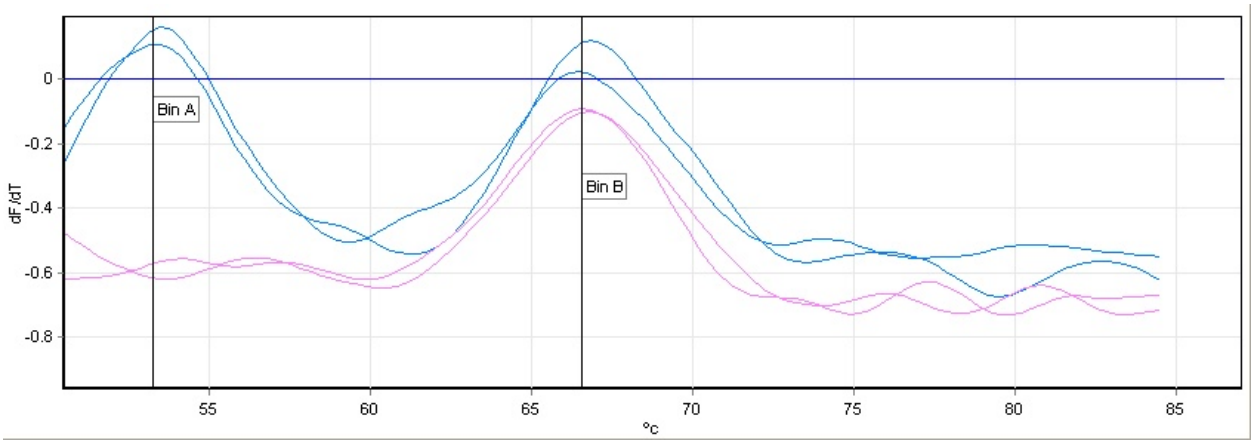


Figure 9 – Last ZFXY set with the probe a perfect match to the ZFY gene.

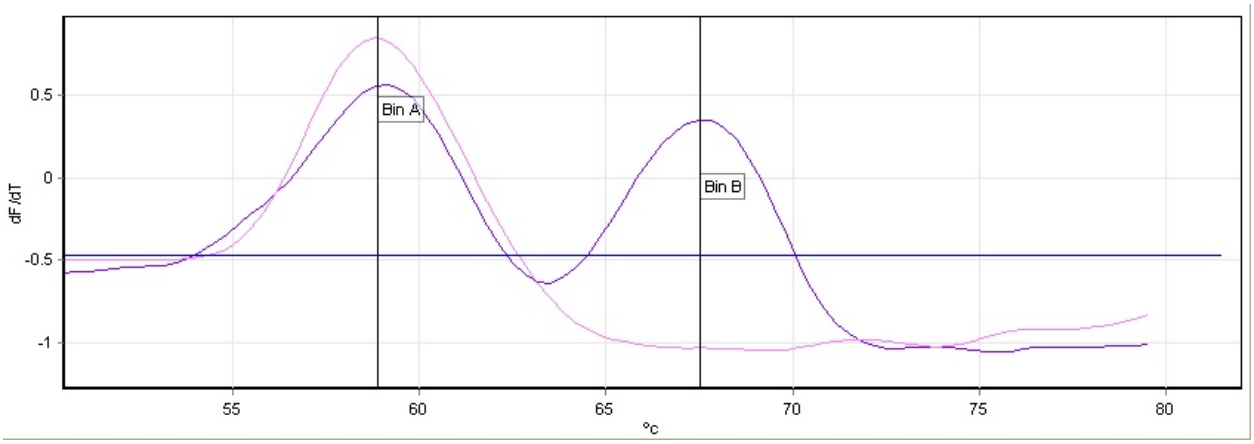


Figure 10 – 6-plex FRET results on the Corbett RG6000.

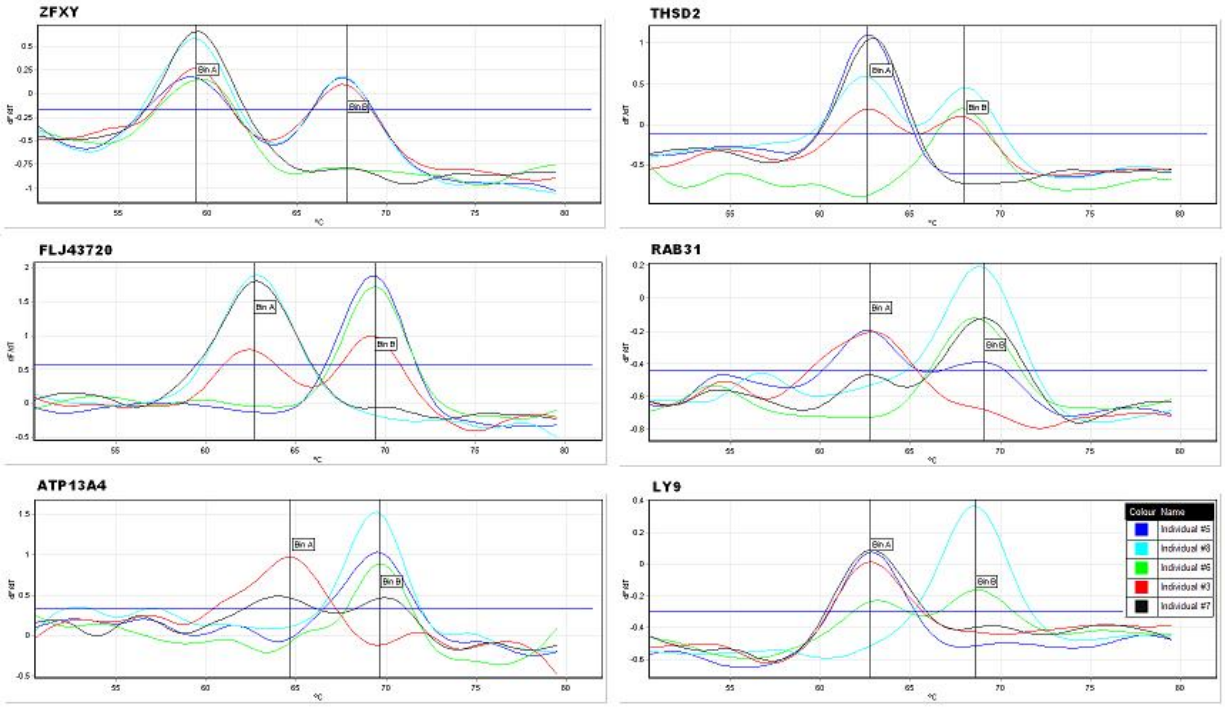


Figure 11 – Results from the Stratagene real-time instrument.

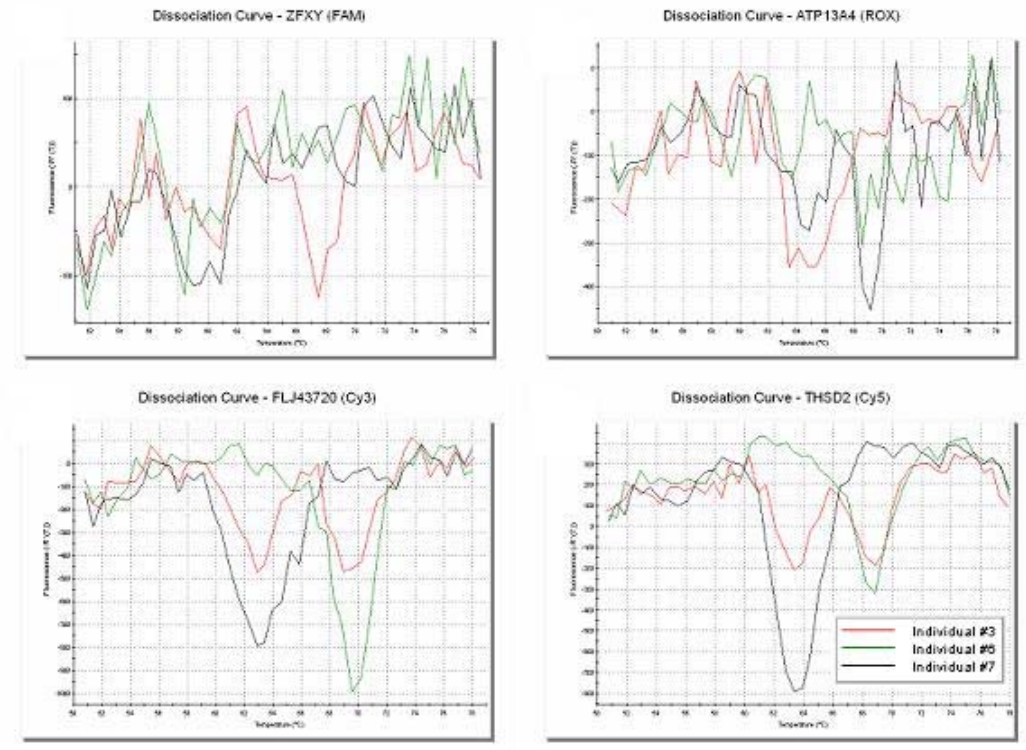


Figure 12 – FRET assay can be used for determination of DNA concentration.

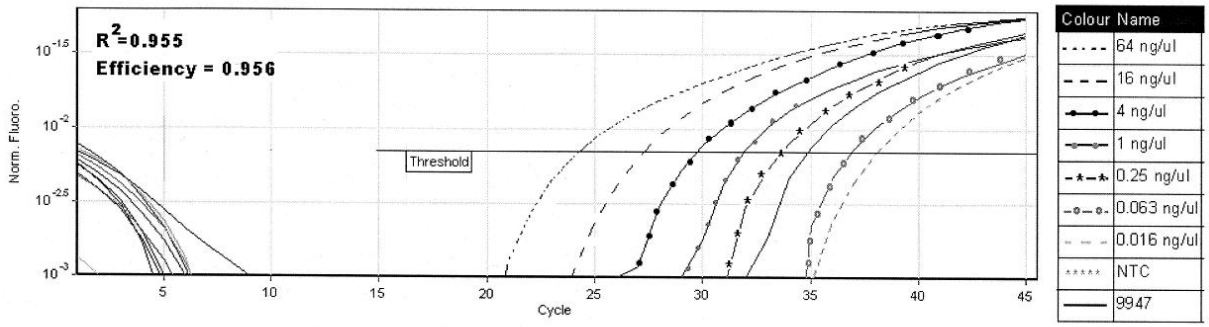
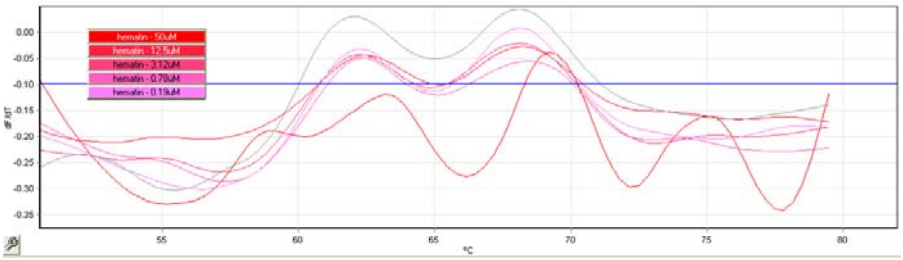
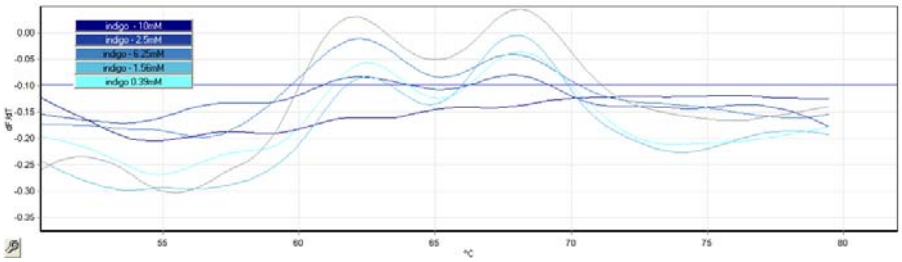


Figure 13 – Effects of four inhibitors (A. hematin, B. indigo, C. calcium phosphate, D. tannic acid) on the FRET melting assay (Ly locus shown).

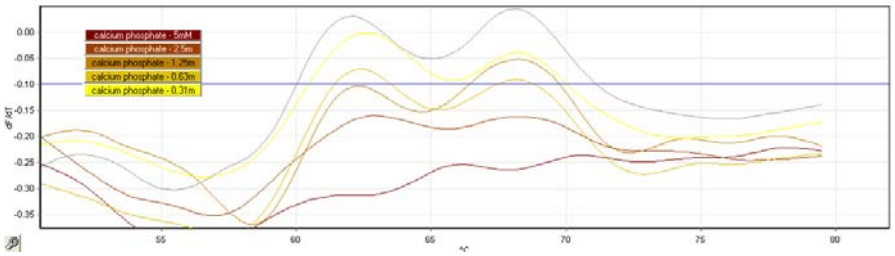
A.



B.



C.



D.

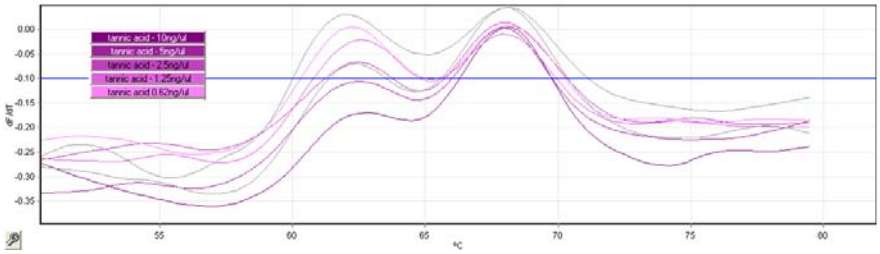
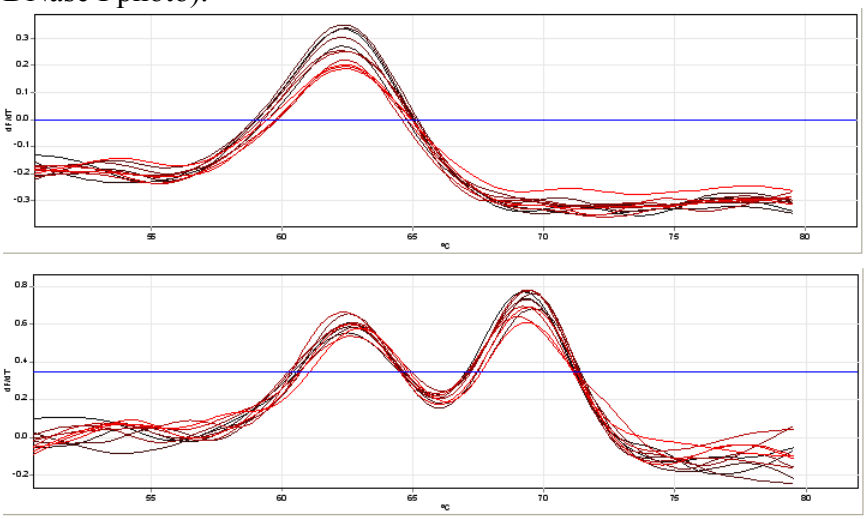


Figure 14 – Effects of DNA degradation on the FRET assay. Top panel Ly9. Lower panel RAB31. Right panel shows the DNase I degraded DNA samples used. Graphs go from undigested (brown – far right on DNase I photo) to digested for 128 minutes (red – far right on DNase I photo).



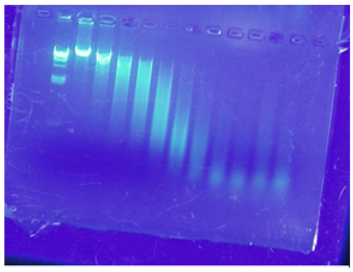
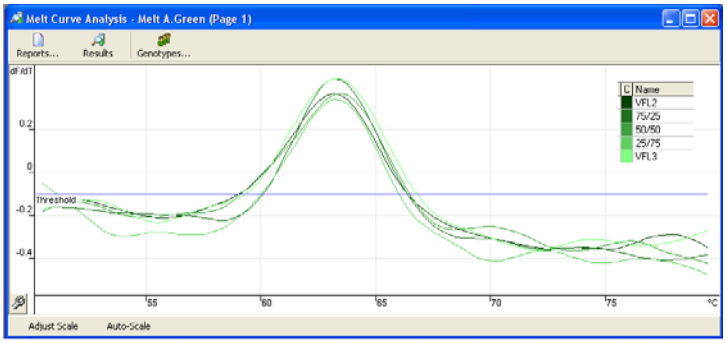
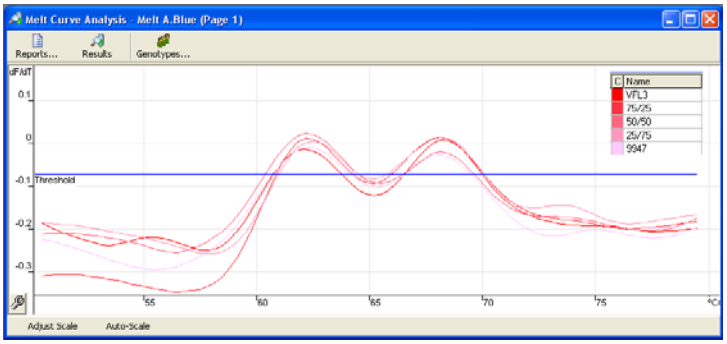


Figure 15 – Use of Mixtures on the FRET melting assay. A. Both DNAs from same homozygous individuals (PALLD). B. Both DNAs from heterozygous individuals (RAB31). C. One DNA from each homozygote. D. One homozygote, one heterozygote. Mixtures made in five ratios 100%/0%, 75%/25%, 50%/50%, 25%/75% and 0%/100%, all at 0.1ng/ul total.

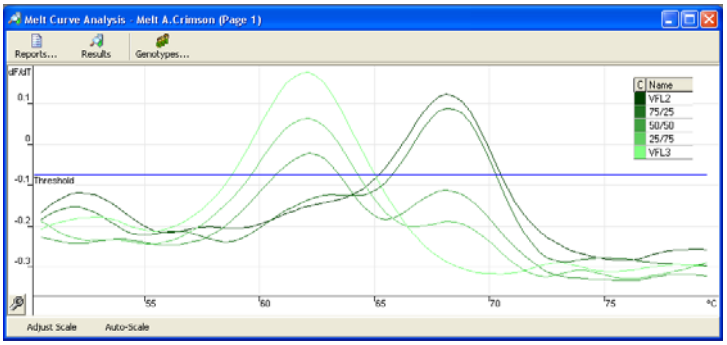
A.



B.



C.



D.

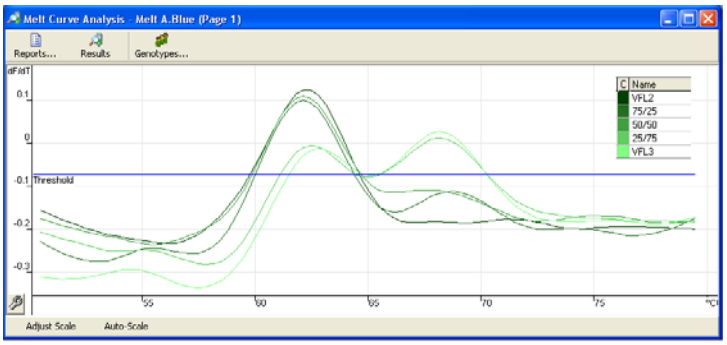
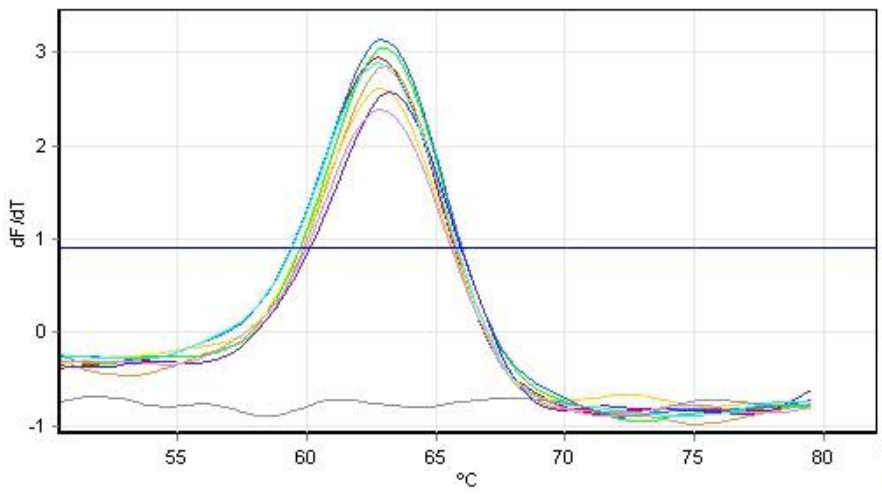


Figure 16 – THO1 melt curves for eight individuals with different THO1 genotypes.



No.	Colour	Name	Genotype	Peak 1
A1		6/7		62.8
A2		6/9.3		63.0
A3		6/9		63.0
A4		9/9		63.0
A5		8/9		63.0
A6		9.3/9.3		63.3
A7		7/9		63.0
A8		7/7		62.8
B1		NTC		

Figure 17 – The sum of the two THO1 allele sizes for an individual plotted against the melt temperature in the STR FRET assay.

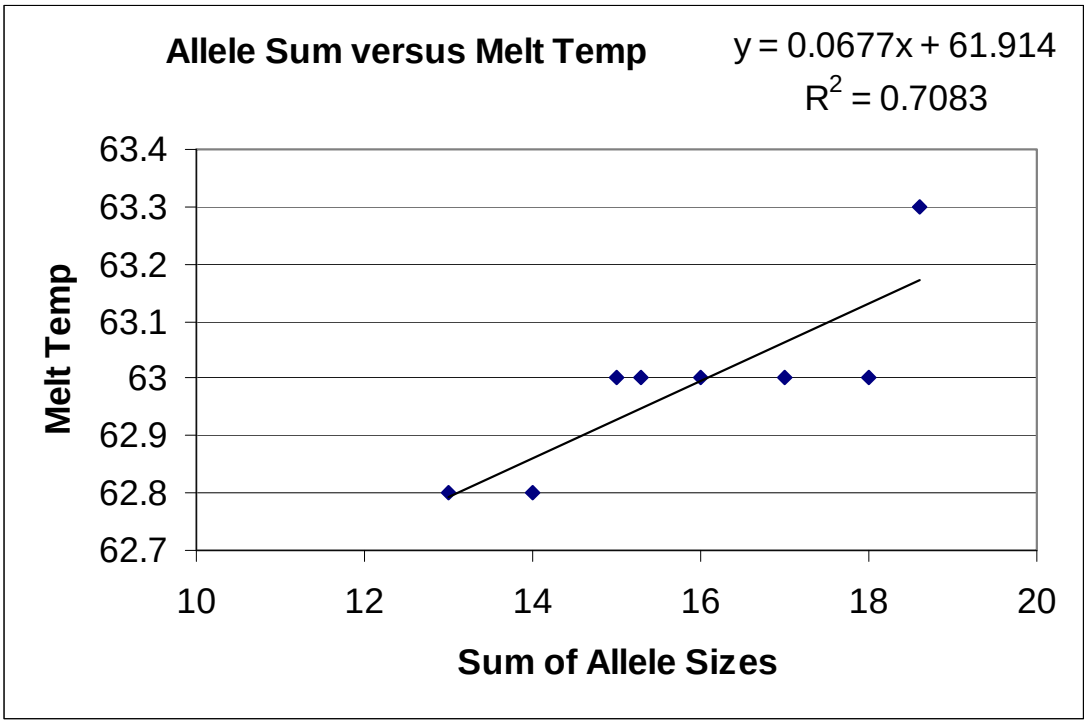


Figure 18 – Graph of melt temperatures versus genotype for the THO1 STR FRET assay on individuals with the same genotype.

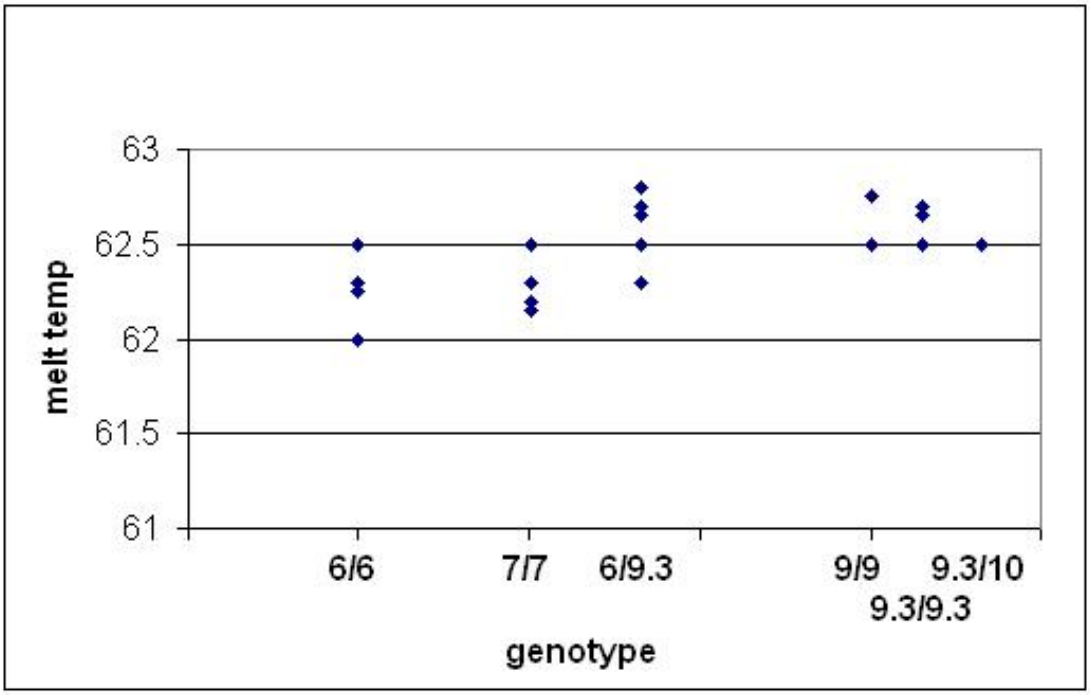
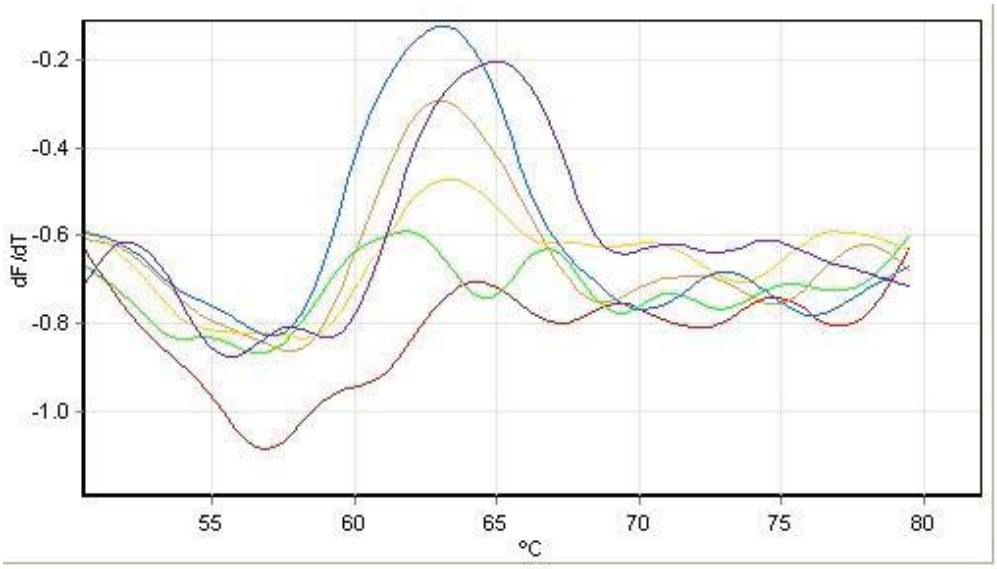
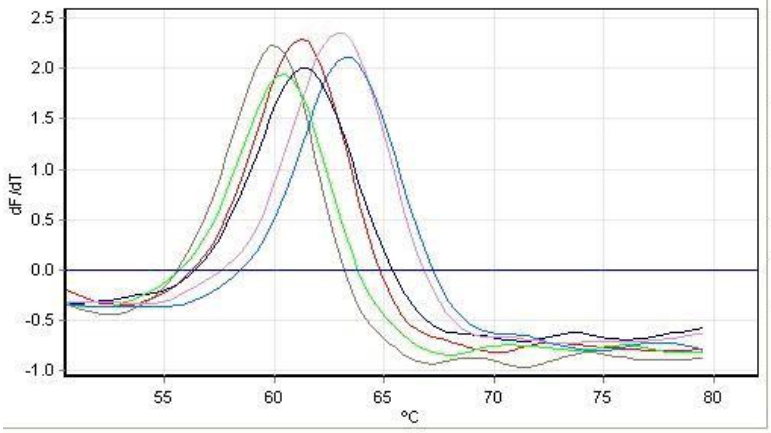


Figure 19 – Melting profile of six individuals using the THO1 antisense STR FRET probe set.



No.	Colour	Name
C1		6/9.3
C2		6/9.3
C3		6/7
C4		6/6
C5		9.3/9.3
C6		9.3/10

Figure 20 – Effect of three different mastermixes on melting temperature.



No.	Colour	Name	Peak 1
A1		6/6 - ABgene mix	61.2
A7		9.3/9.3 - ABgene mix	61.3
B1		6/6 - Sigma low Mg++	60.0
B7		9.3/9.3 - Sigma low Mg++	60.5
C1		6/6 - Sigma high Mg++	63.0
C7		9.3/9.3 - Sigma high Mg++	63.3

Figure 21 – Results with new THO1 Probes. A. old anchor primer with sense *3 primer, B. old anchor with sense 3*+8* primer, C. new sense primer and new sense anchor primer, D. new sense anchor primer with new sense 3* primer.

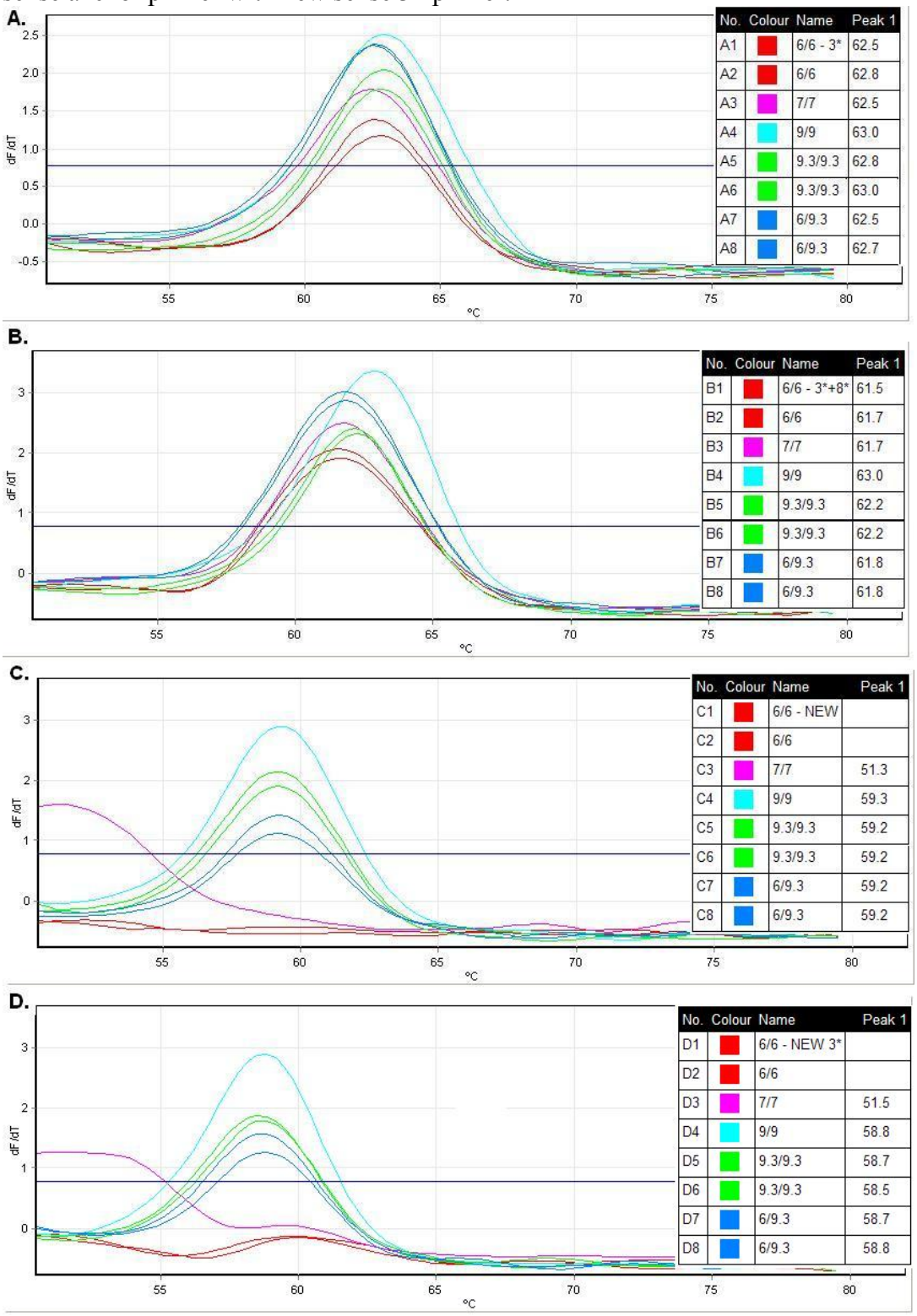
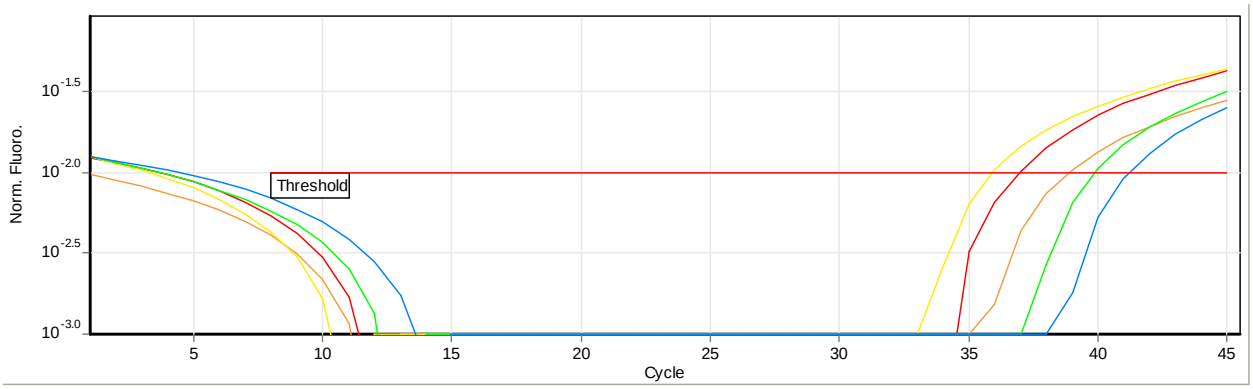


Figure 22 – STR FRET with five individuals for D6S2956. A. amplification B. melt

A.



B.

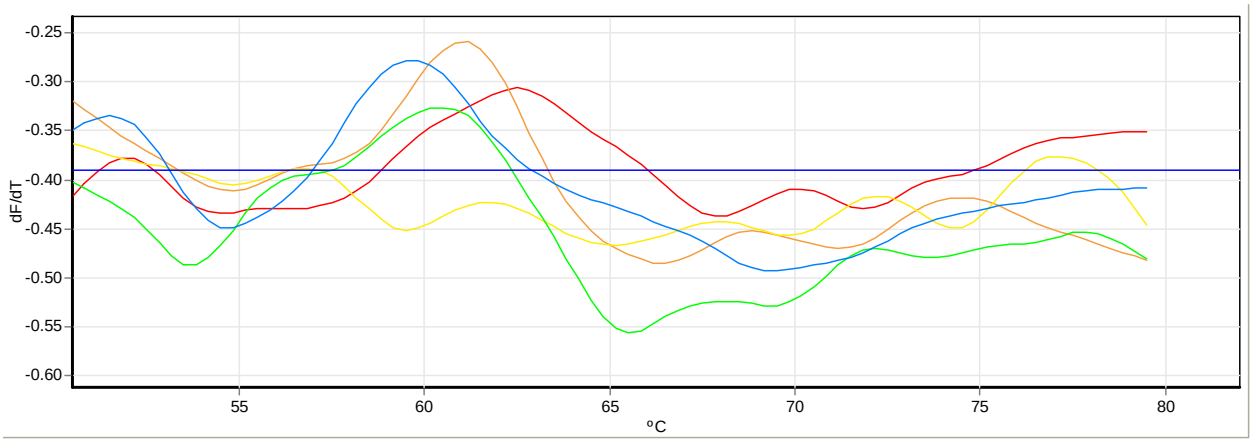


Figure 23 – Initial results with the HRM demo instrument – melting results for one individual for each of the genotypes for THO1.

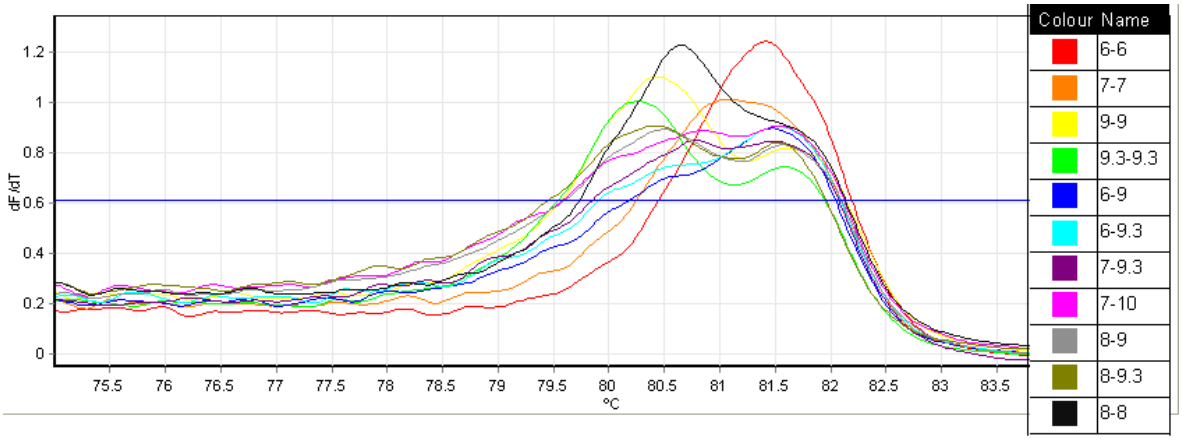


Figure 24 – Melting results for duplicate wells for eight individuals all of THO1 genotype 8-8.

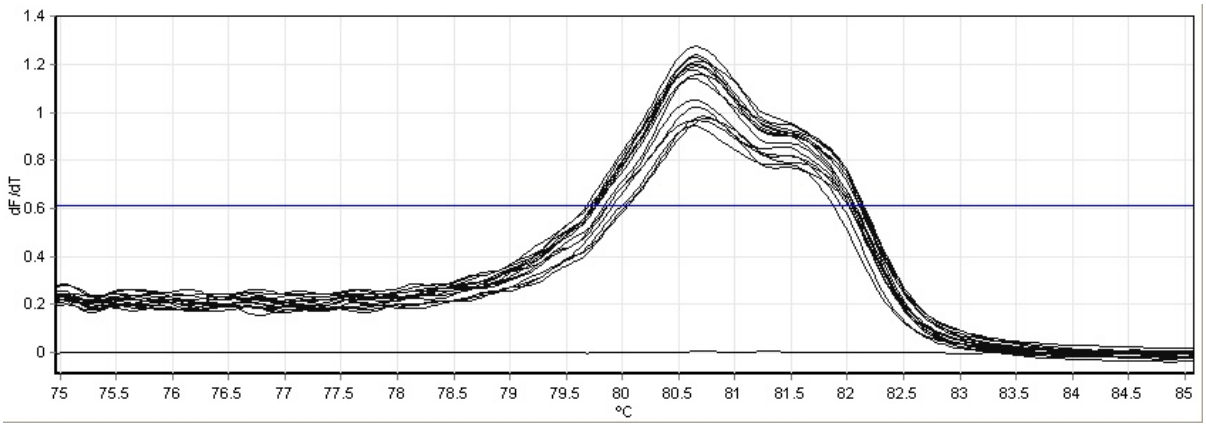


Figure 25 – Actual curve of HRM melt.

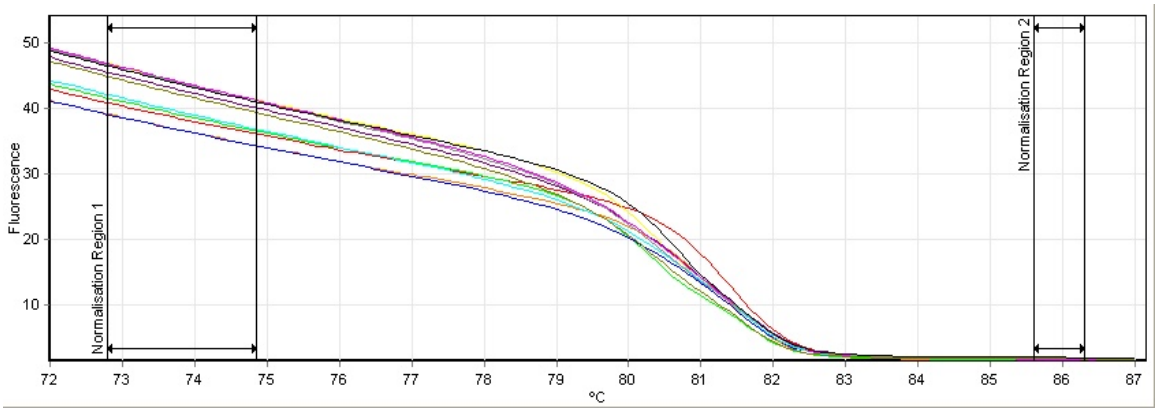


Figure 26 – Preliminary experiment with TPOX using individuals with 8 genotypes.

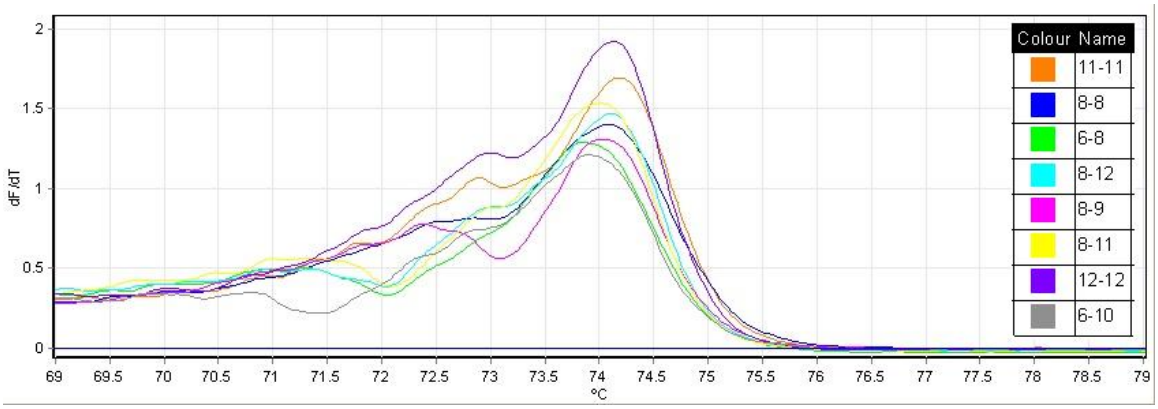


Figure 27 – Good amplification (THO1) versus poor amplification (D7).

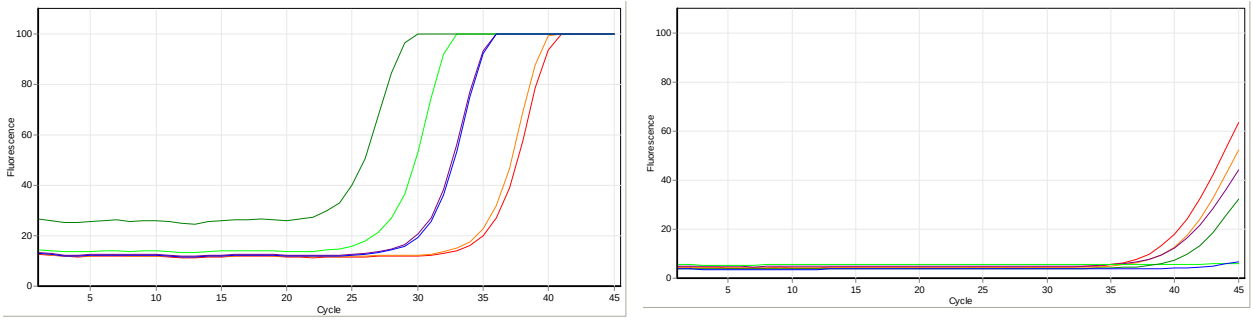


Figure 28 (top and bottom) shows the melting profiles of 12 different genotypes for THO1. Two samples (same color) are shown for most patterns.

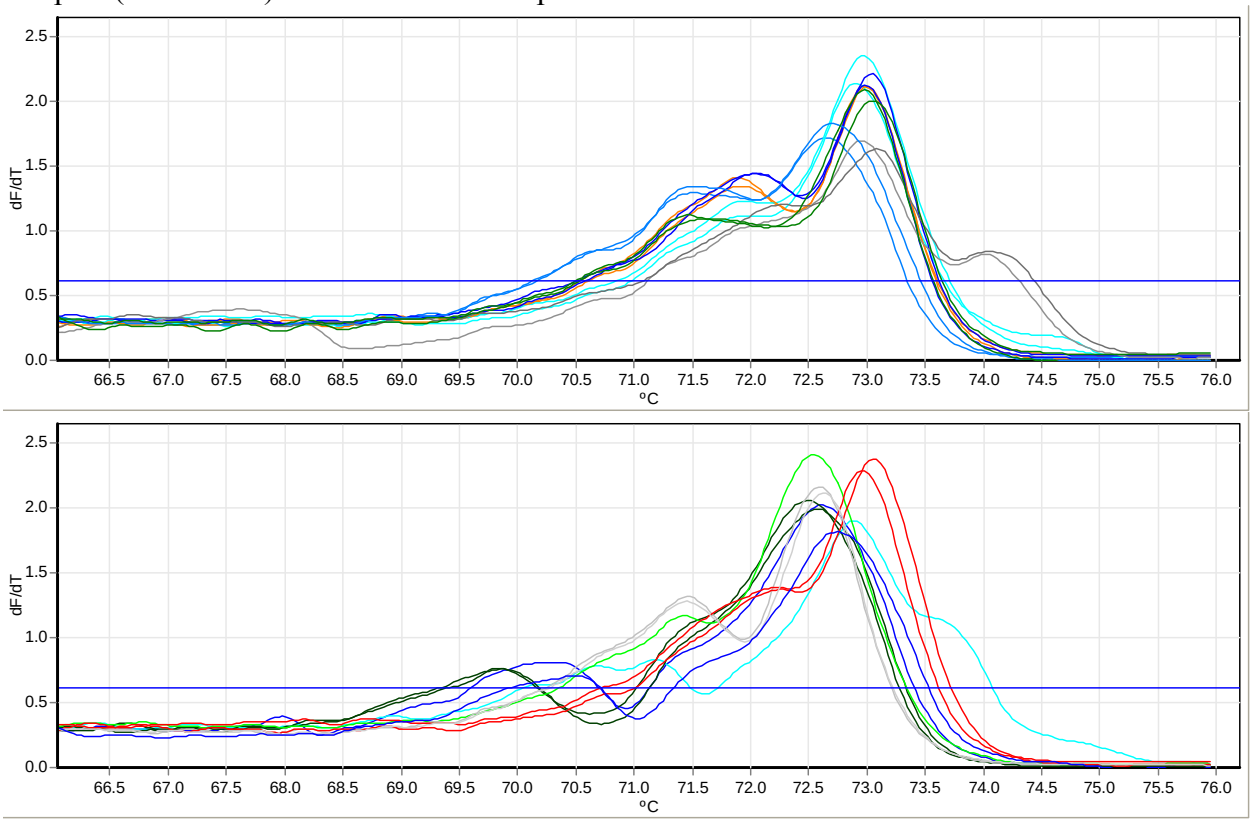


Figure 29 shows the melting profiles of six different genotypes for vWA. Two samples (same color) are shown for most patterns.

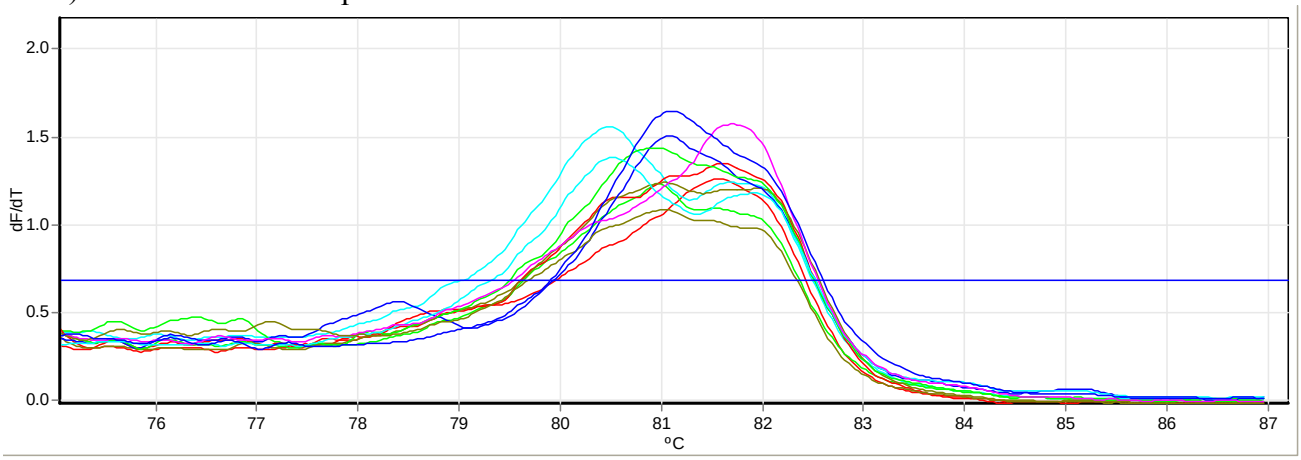


Figure 30 - Melting profile for eight genotypes for a third STR (D14) with no discrimination power.

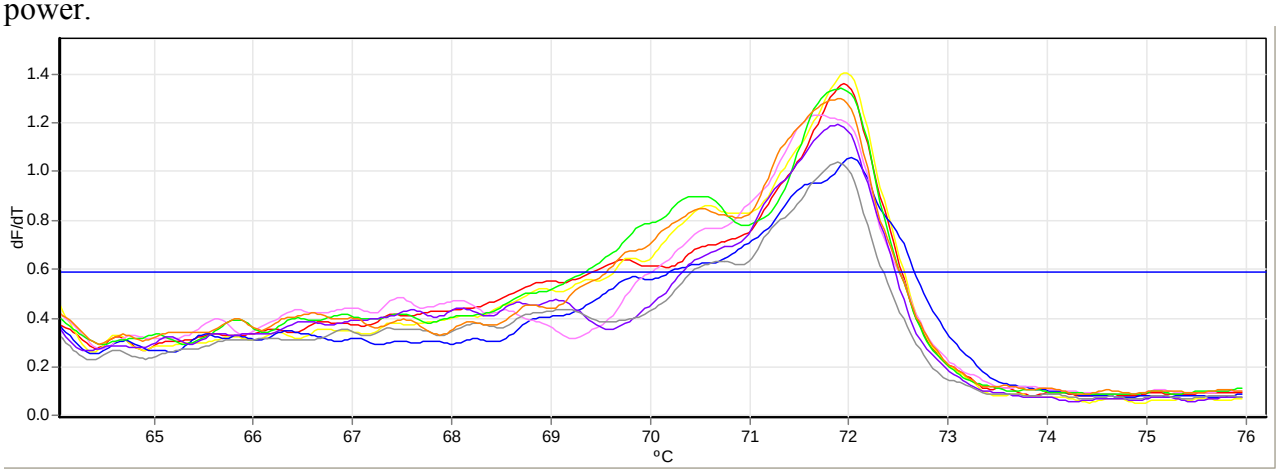
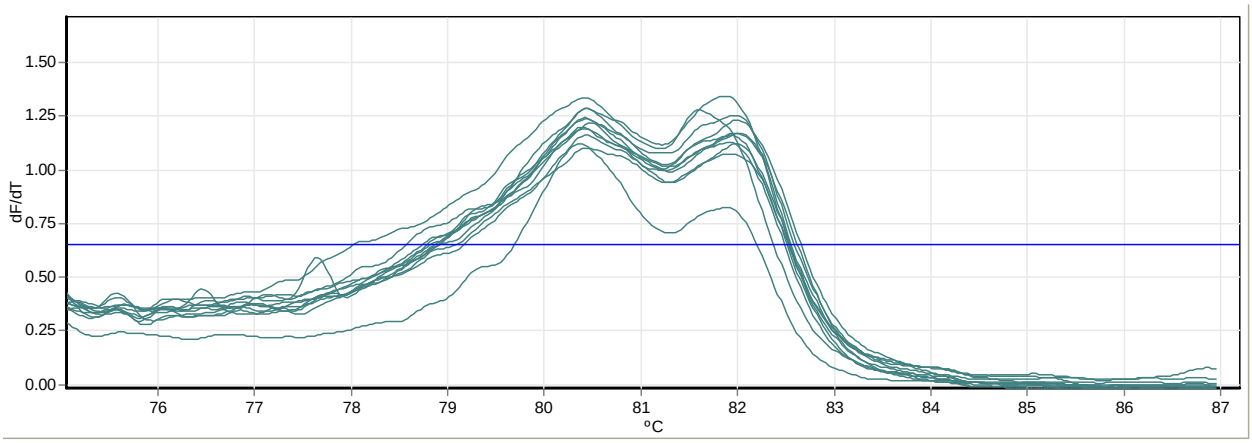
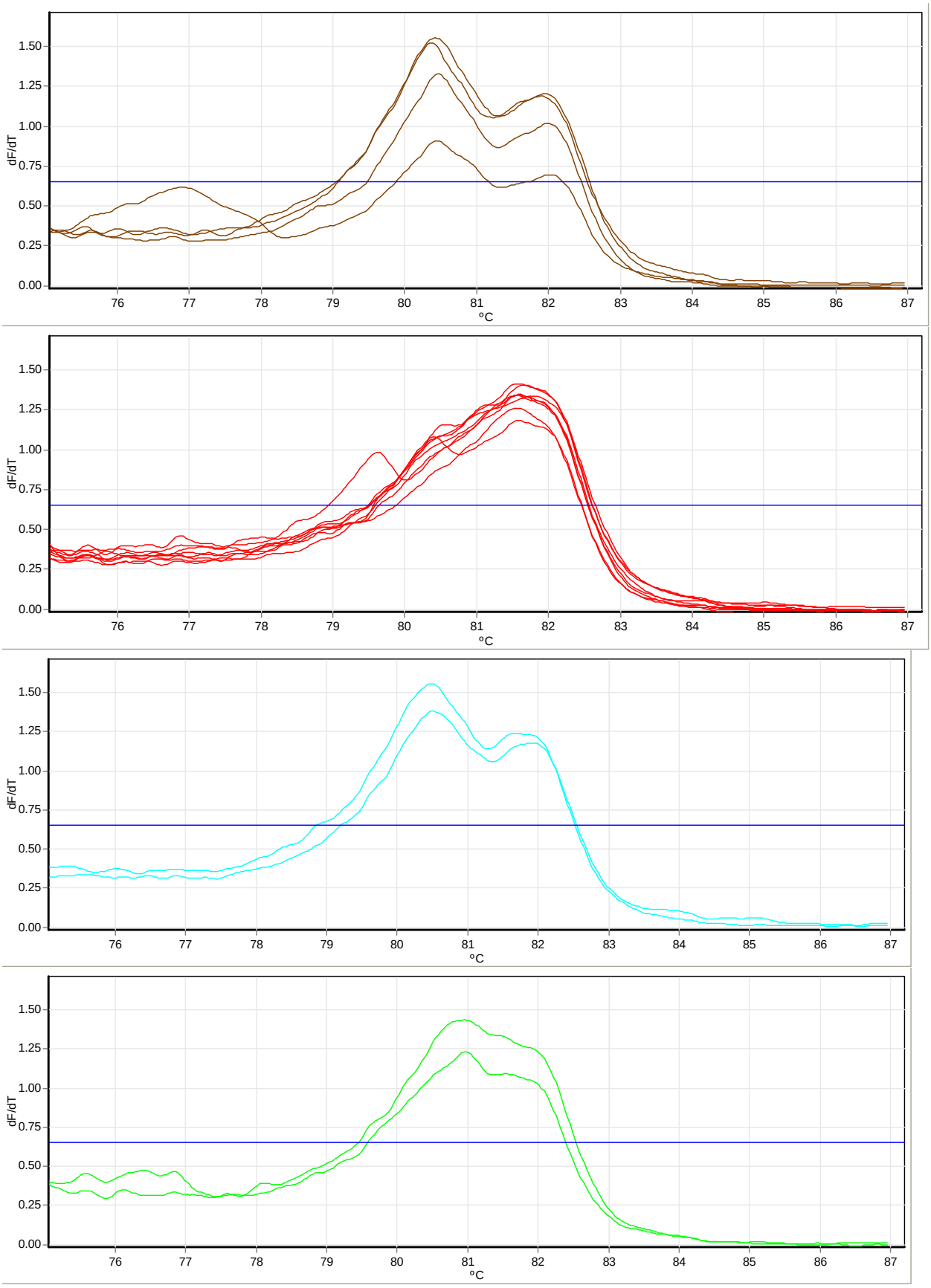
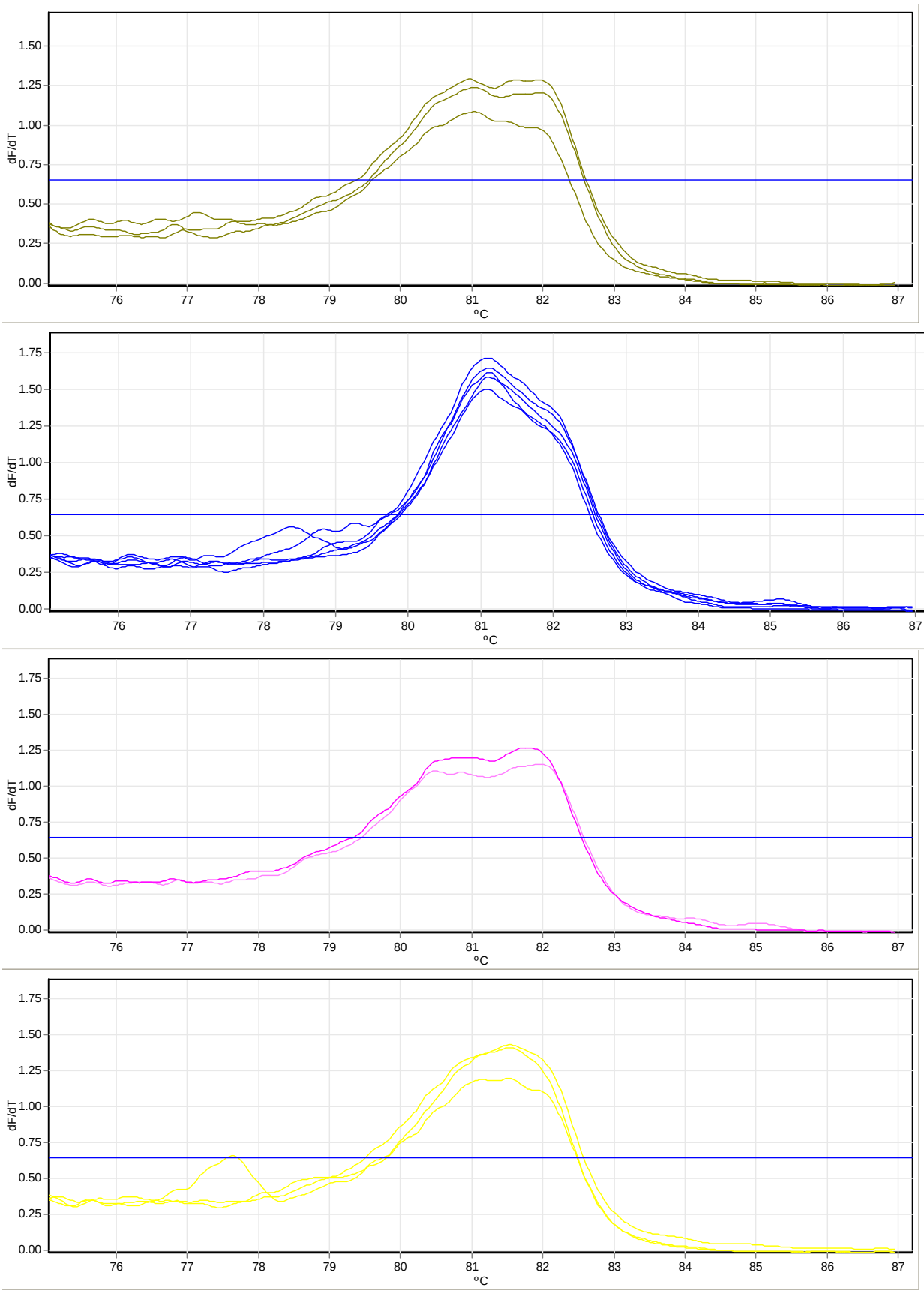


Figure 31- The profiles of sets of individuals with the same genotypes for vWA (each graph and color represents a different genotype).







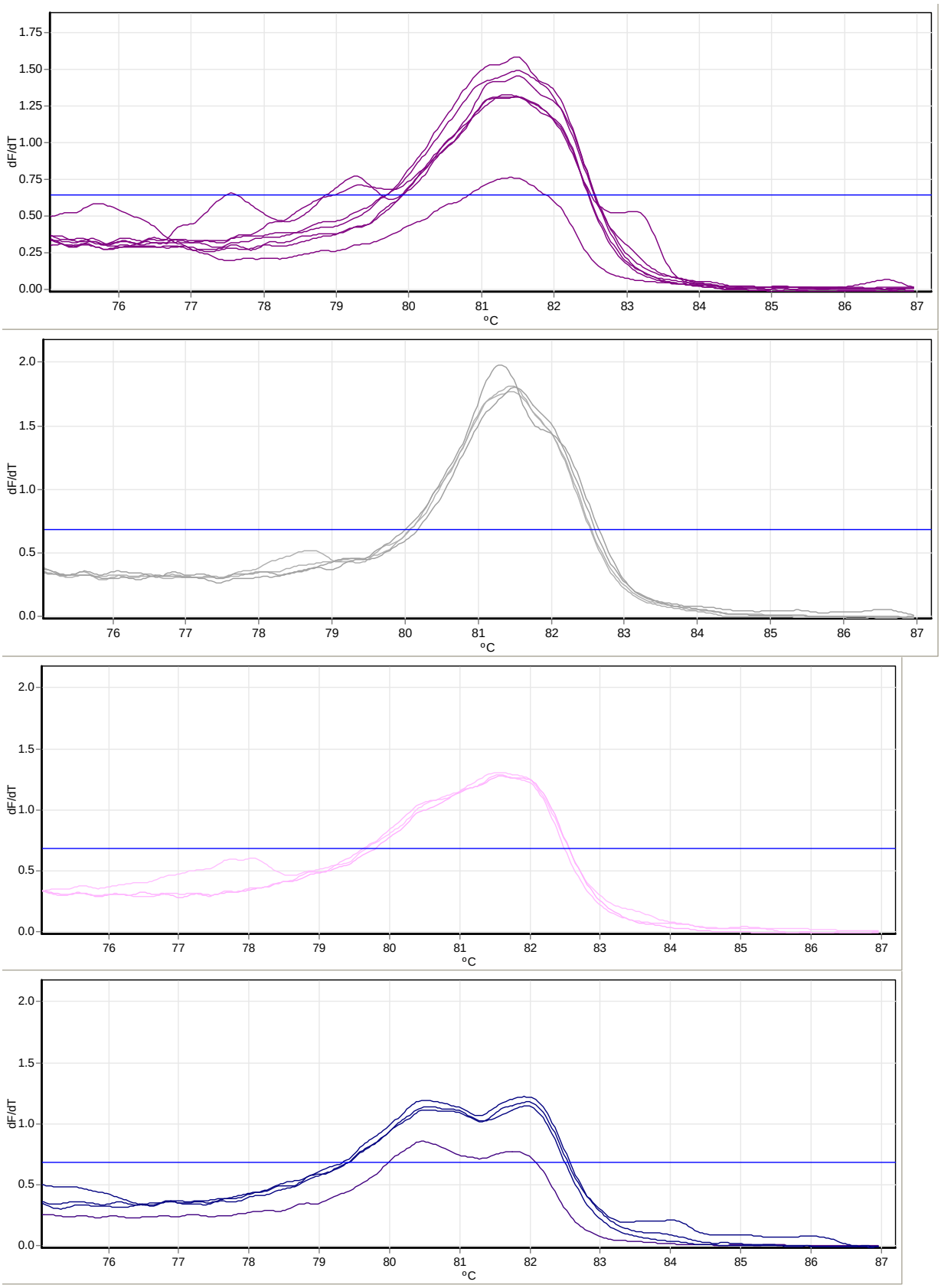


Figure 32 - The melting profiles are steady even when the tubes have set for six days at room temperature in the instrument (vWA).

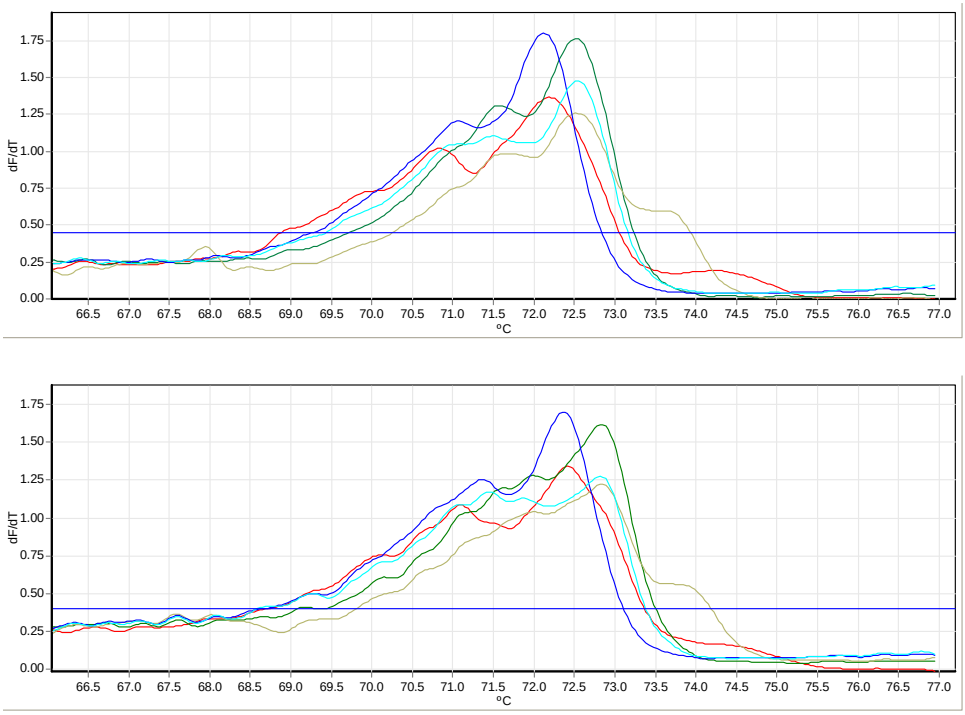


Figure 33 - Results for five individuals performed on two different days for THO1.

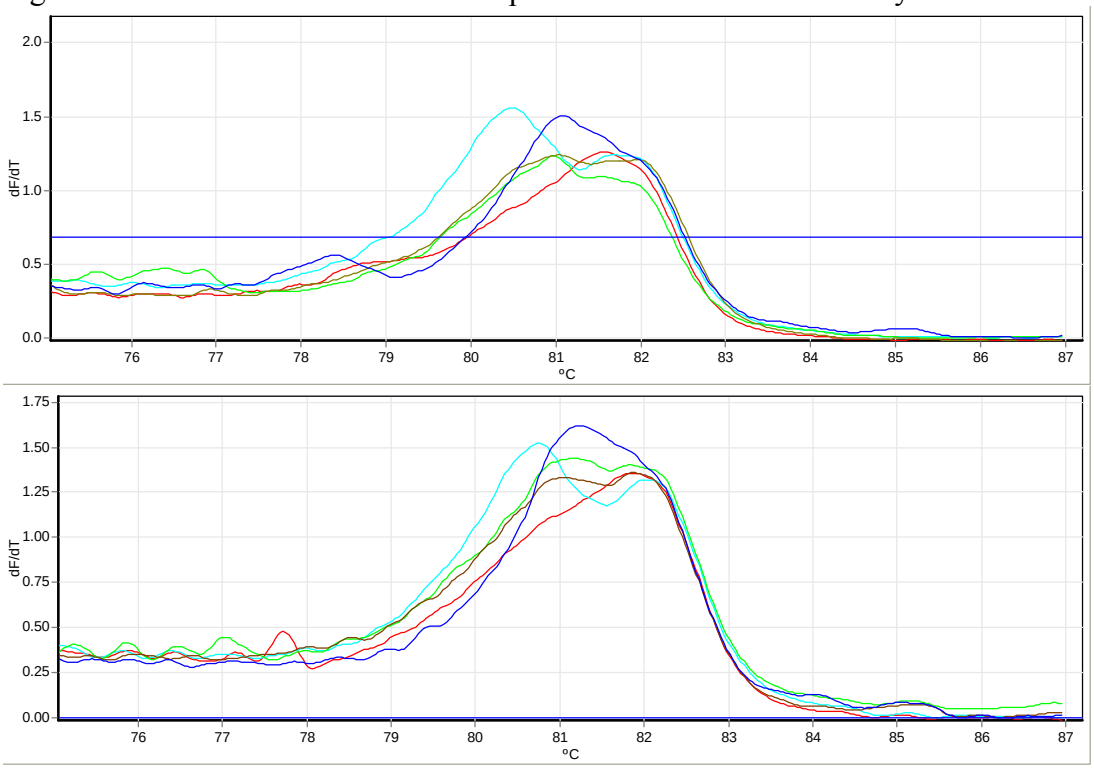


Figure 34 – Melting patterns for THO1 are constant even when a sample is diluted 1/512.

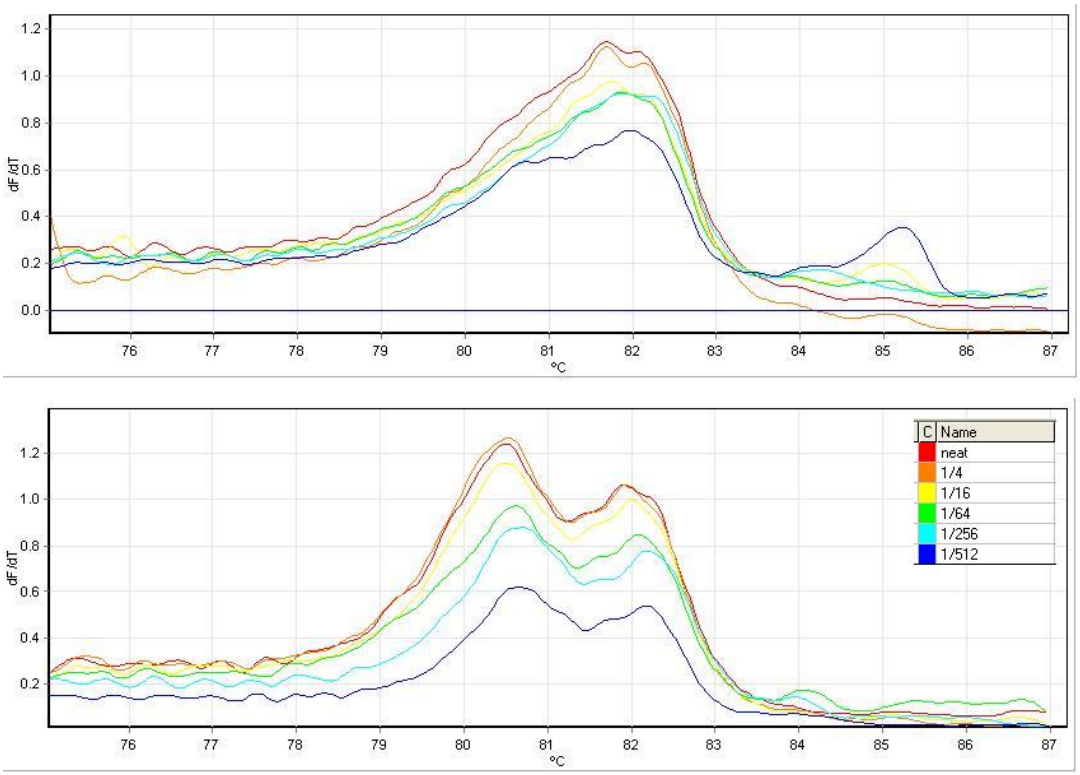


Figure 35 - Some genotypes cannot be differentiated. Genotype #1 (turquoise) appears identical to genotype #2 (brown) and genotype #3 (yellow) appears identical to genotype #4 (purple) for this STR.

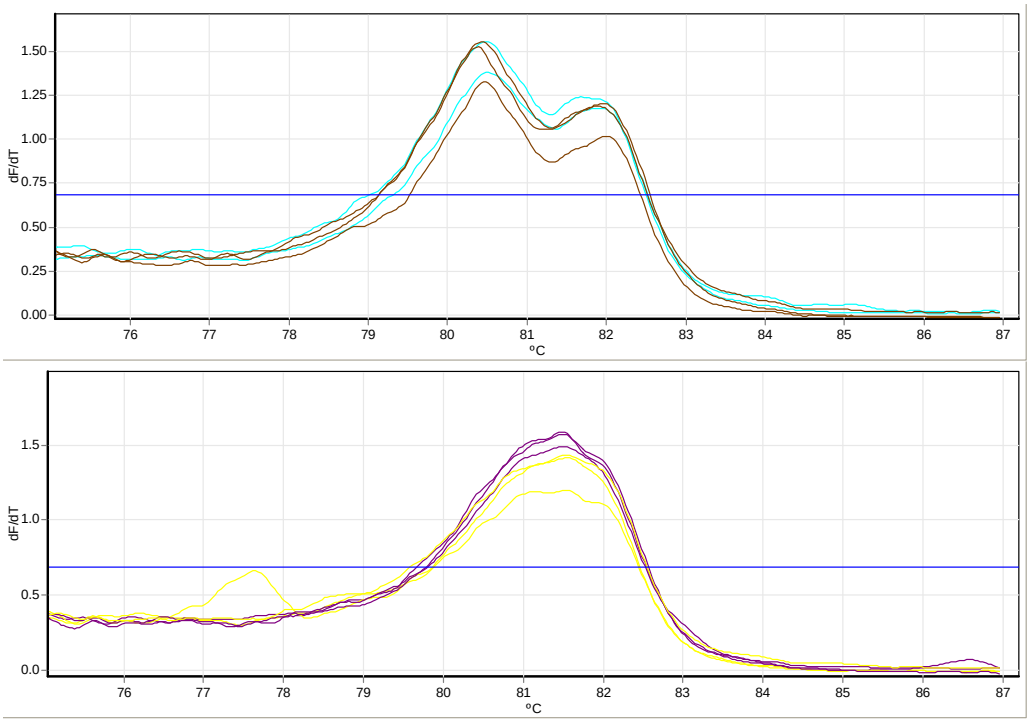


Figure 36 - Some samples with the same genotype had different melting profiles (vWA).

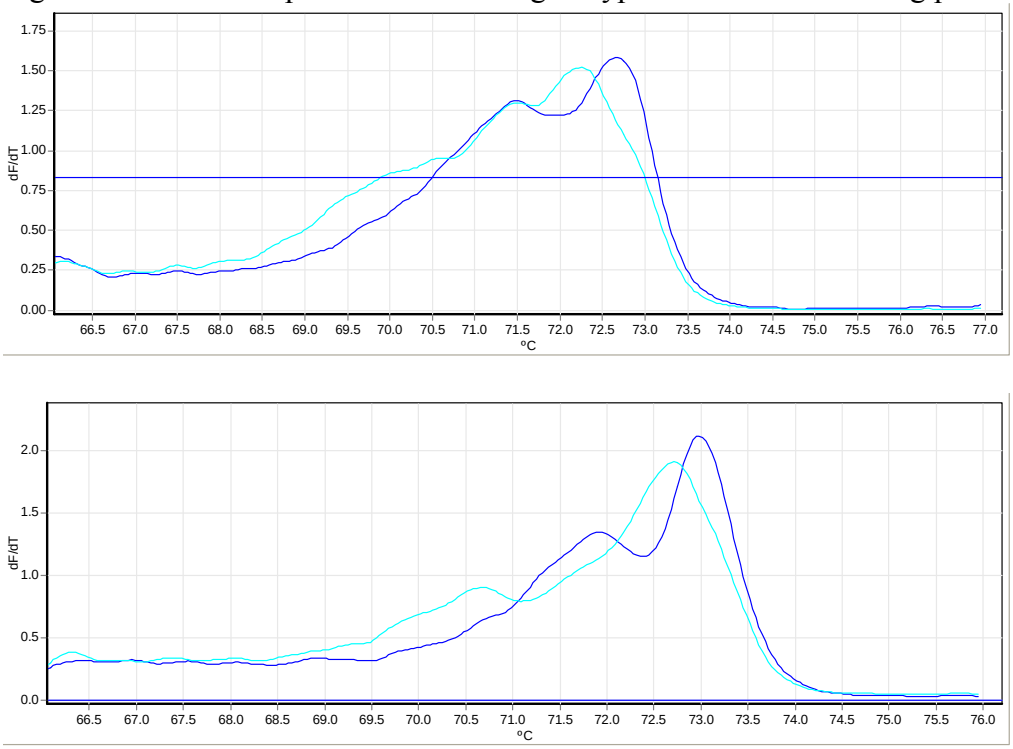


Figure 37 - Amplification curve and standard curve for a dilution series of DNA.

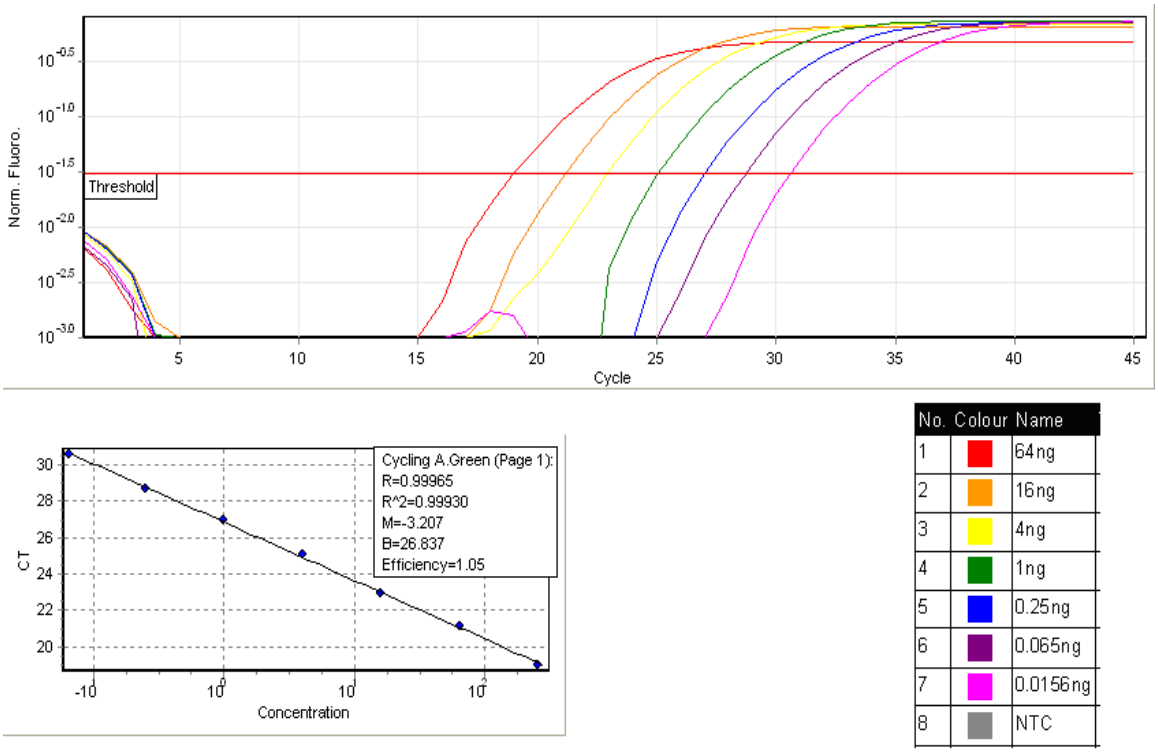
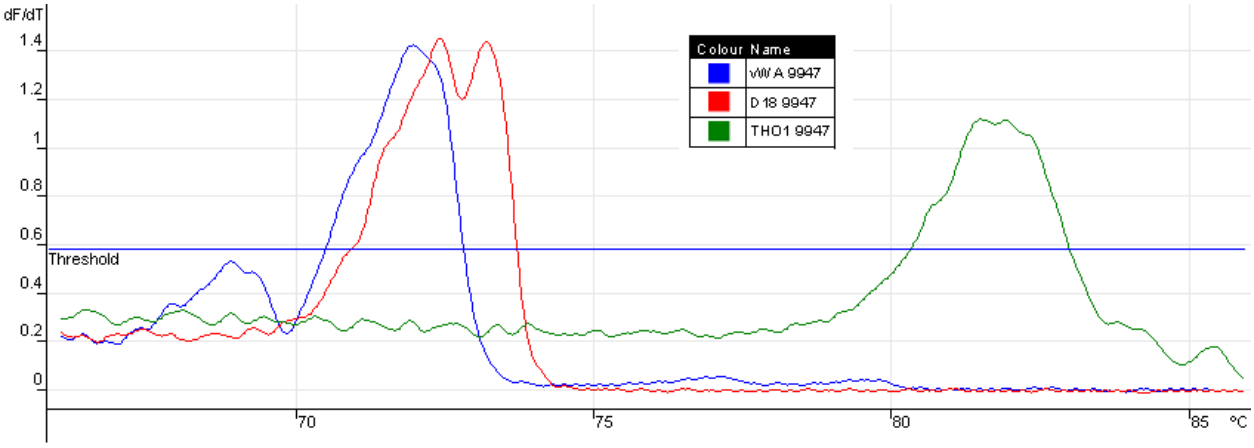
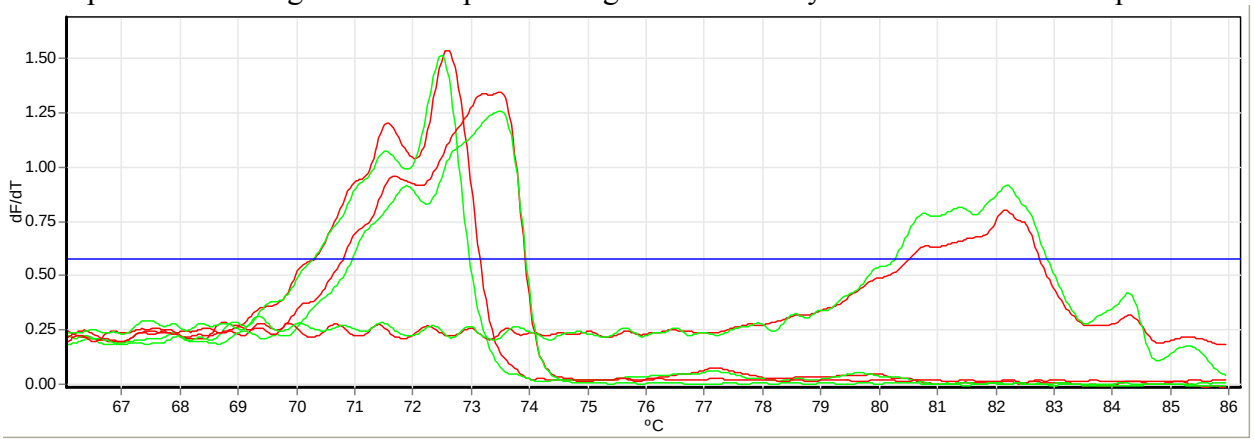


Figure 38 – New triplex STR HRM assay.

A. Results with 9947



B. Duplicates of a single DNA sample showing the consistency between identical samples.



C. Two different DNA samples showing that the profiles are quite different.

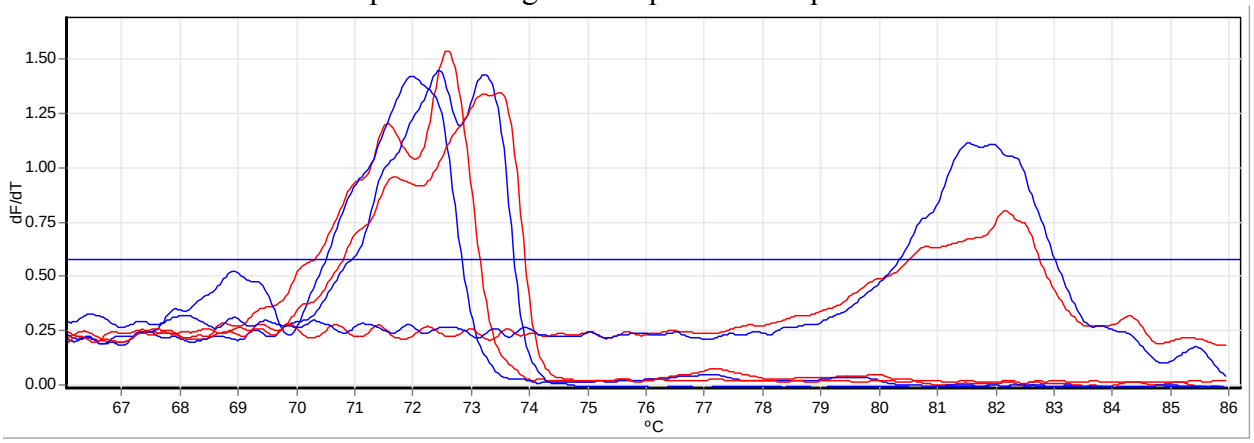


Figure 39 – Results of BSA titration. Upper effects on amplification. Lower effects on melting. Red is vWA, Green is D18S51, Blue is THO1. Darker colors have more BSA. DNA is 9947.

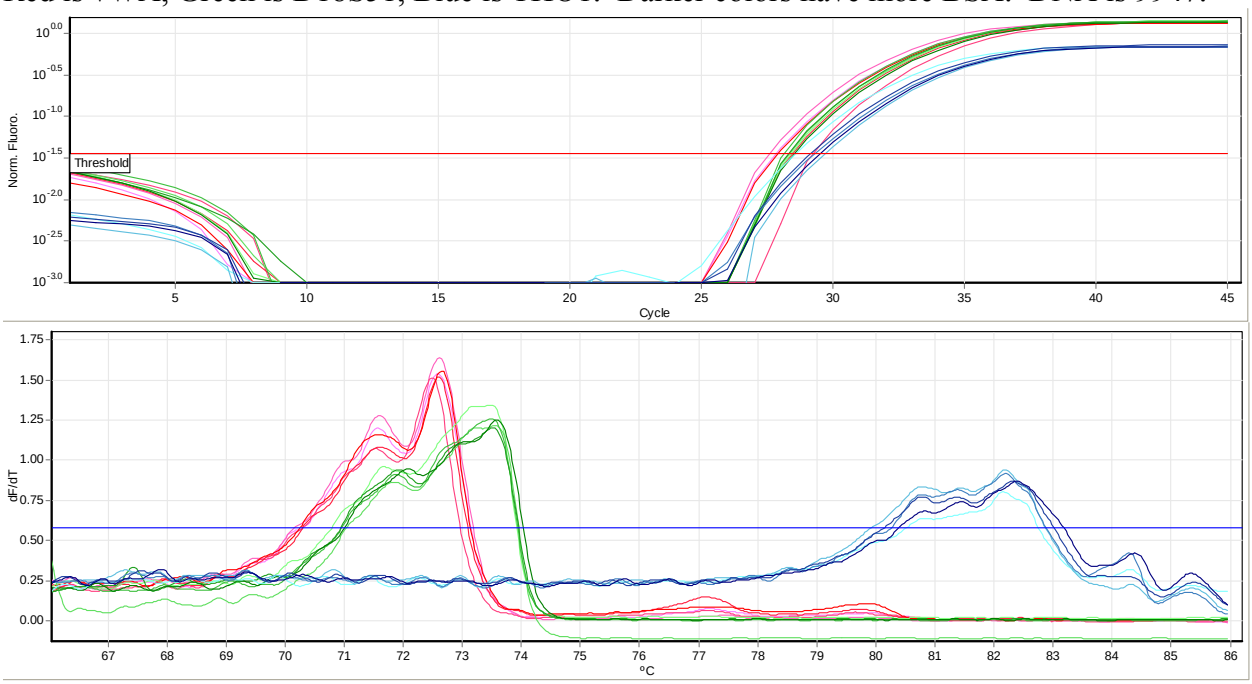


Figure 40 – DNA degradation does not affect the melting profiles. Red is vWA, Green is D18S51, Blue is THO1. The right panel shows the degraded DNA (DNase I treated) and the left panel shows the melting of the same DNA samples.

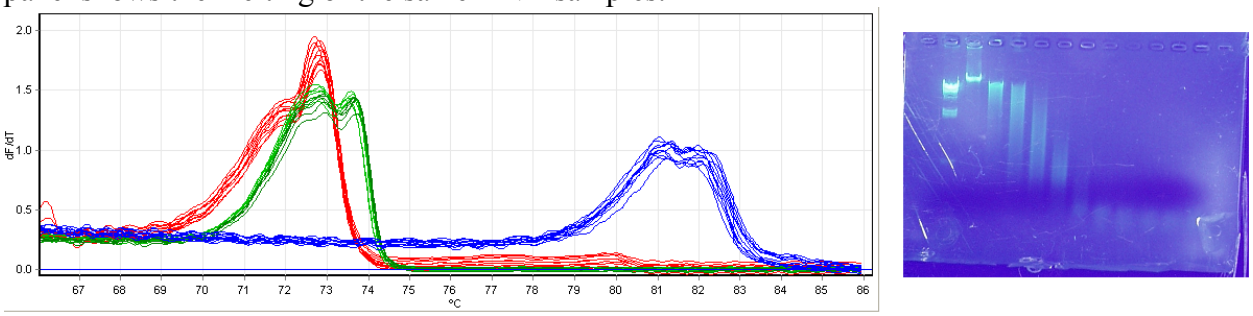


Figure 41 – Test of the Different DNA extraction methods on HRM.

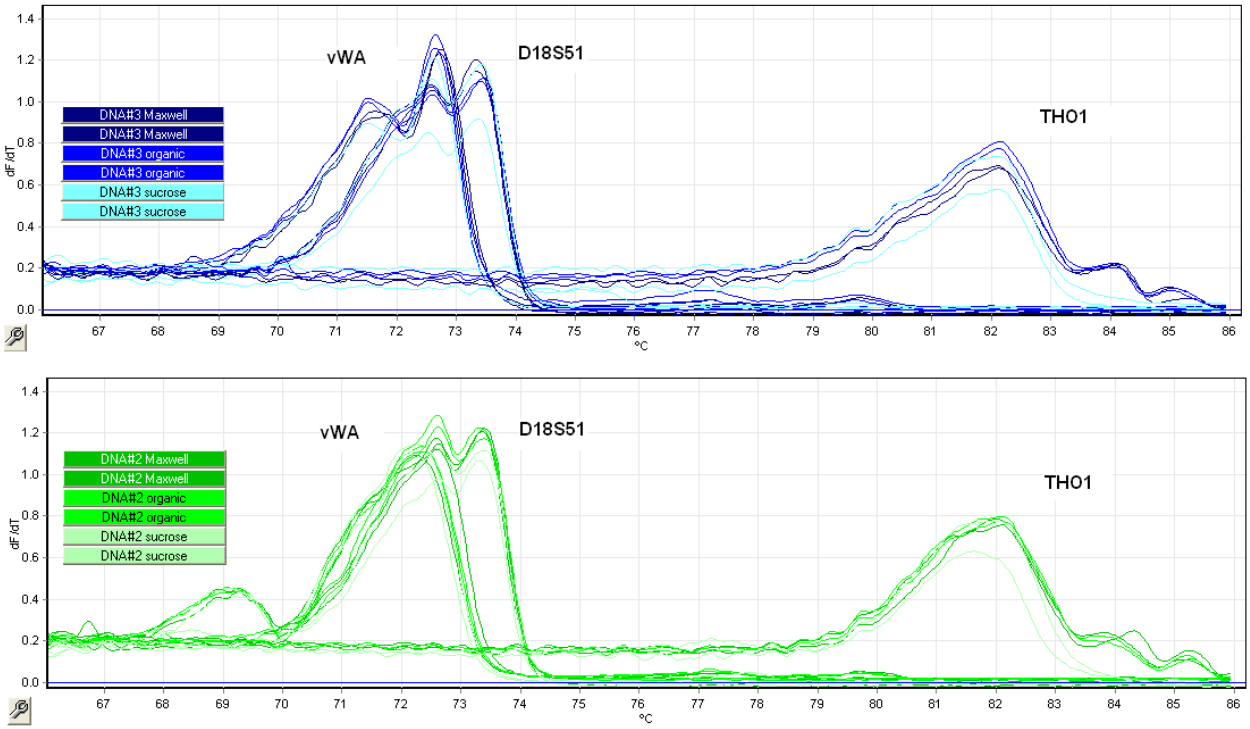
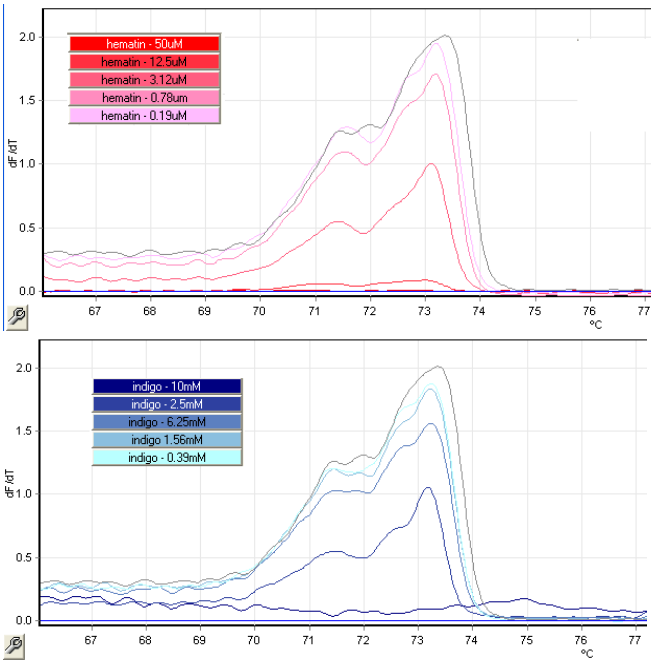
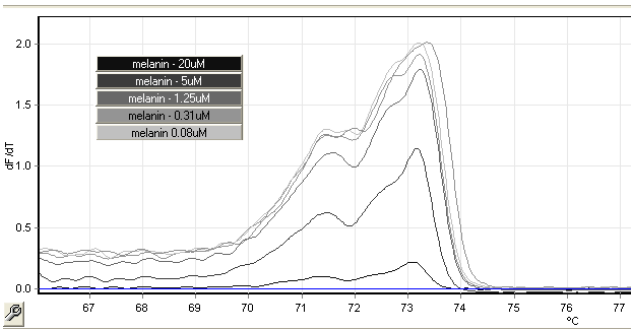
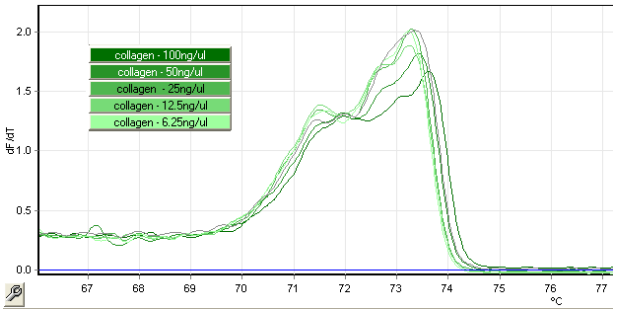
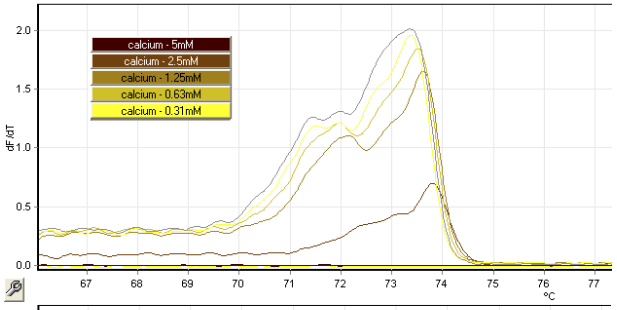


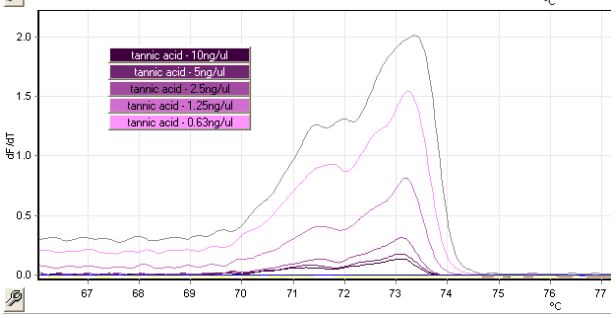
Figure 42 – Effects of Inhibitors on the HRM melting, (black line in all graphs is control with no inhibitor).











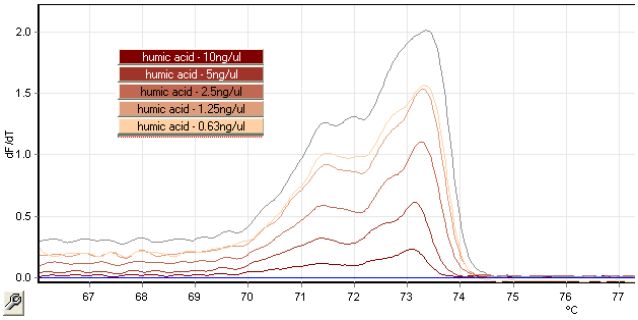


Figure 43 – Effects alone of the buffers used to dilute the inhibitors.

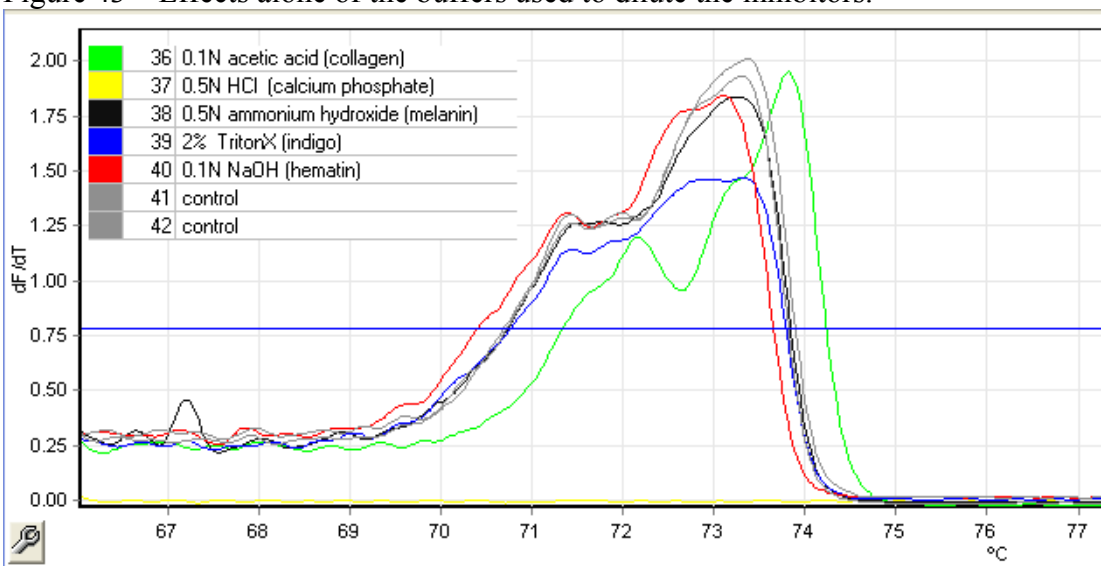
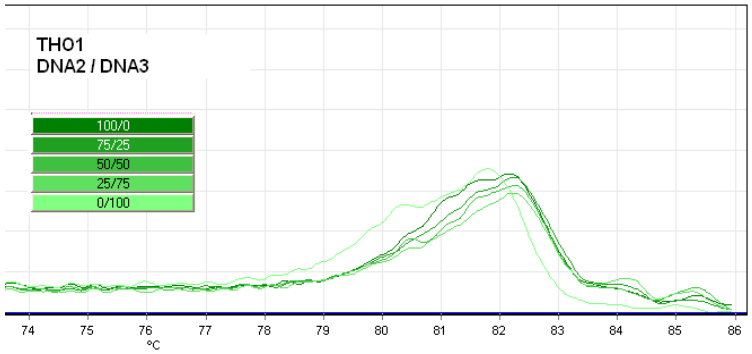
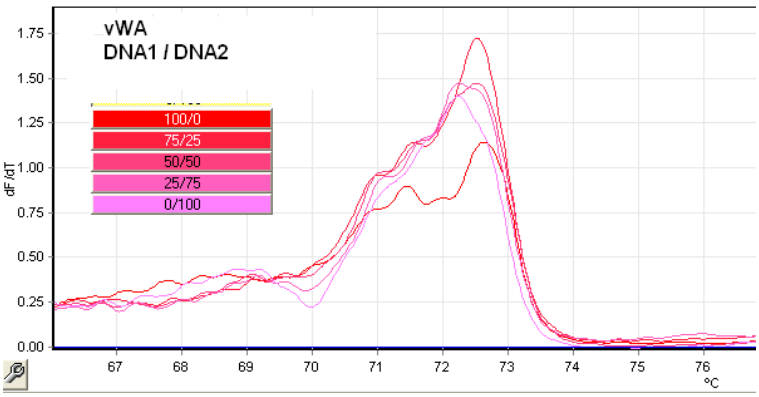


Figure 44 – Examples of HRM mixtures.

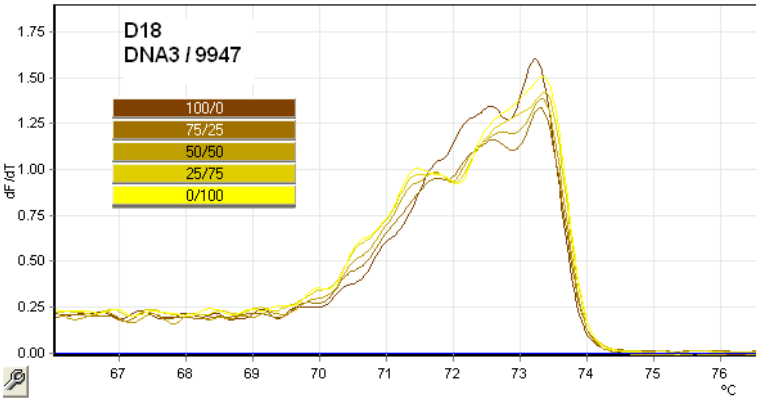
A. Mixtures like one DNA and not the other.



B. Mixtures have different pattern than individual DNAs.



C. Mixtures are a combination of the two individual DNAs.



D. Patterns are very similar.

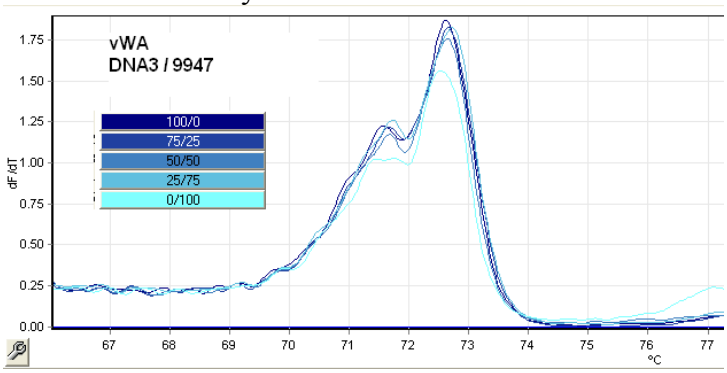


Figure 45 – Results for five samples with THO1 and vWA run separately (top) and together (bottom).

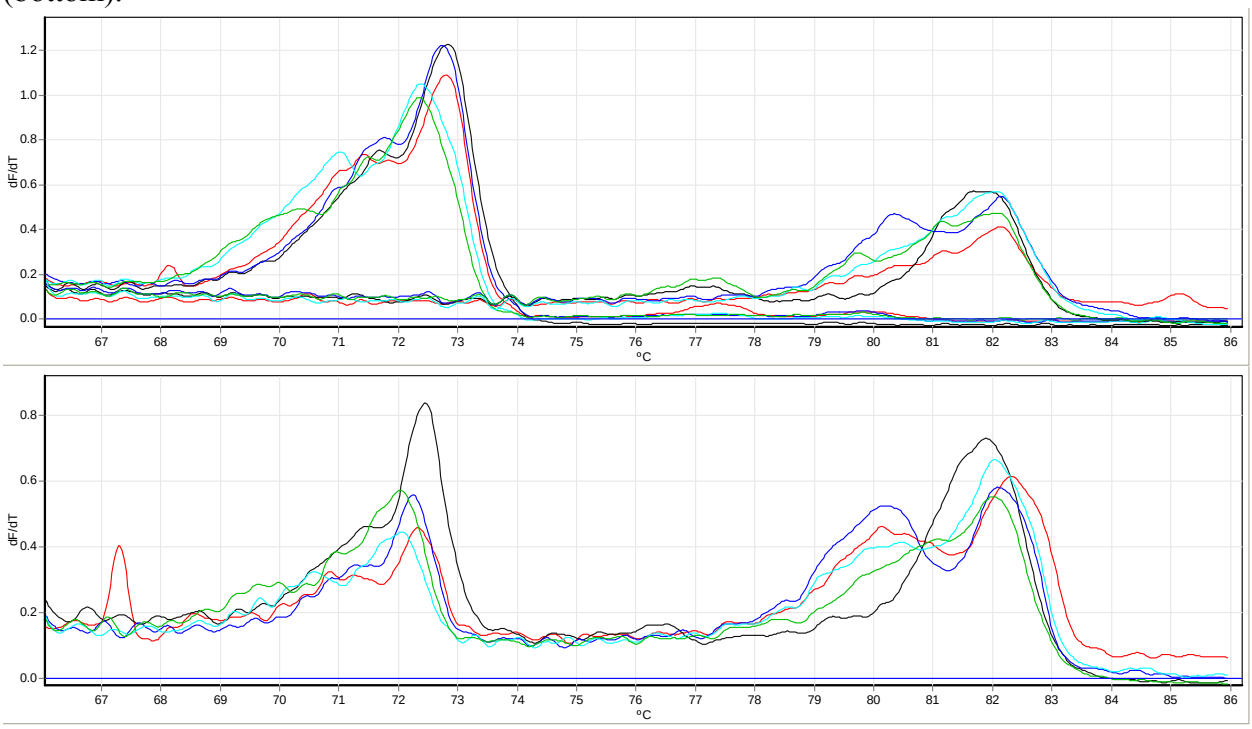


Figure 46 – Comparison of Quantace and Qiagen HRM Mastermix.

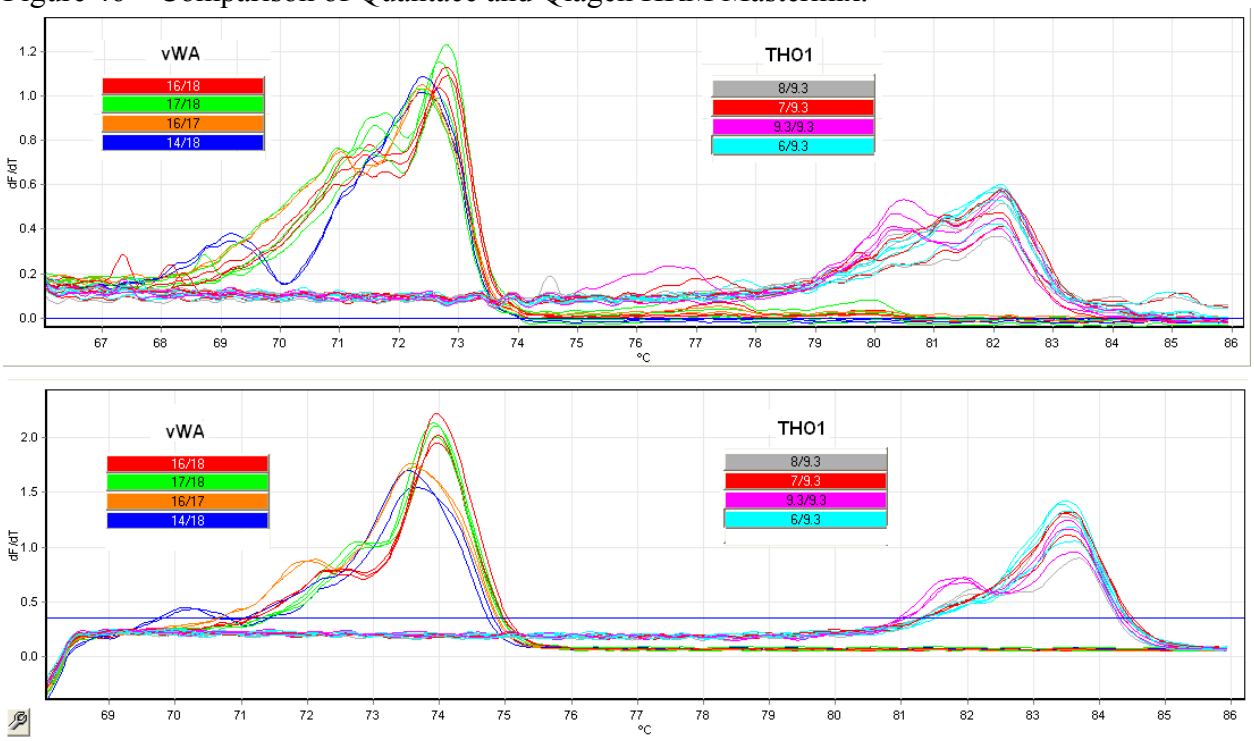
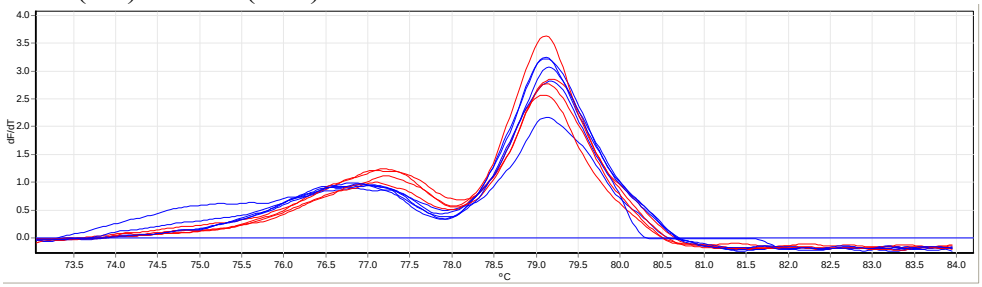
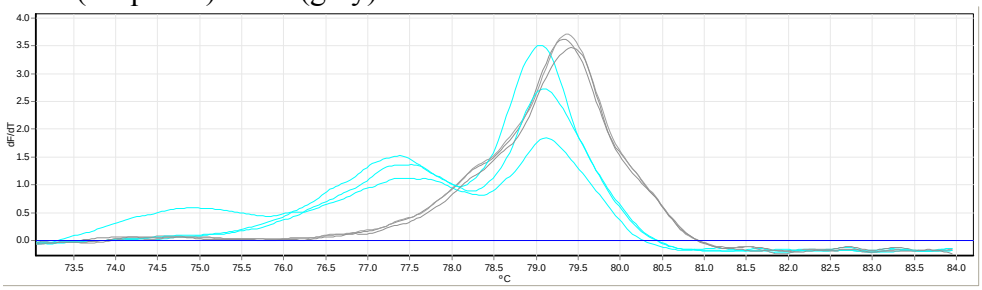


Figure 47 – STR HRM of THO1 using Plexor.

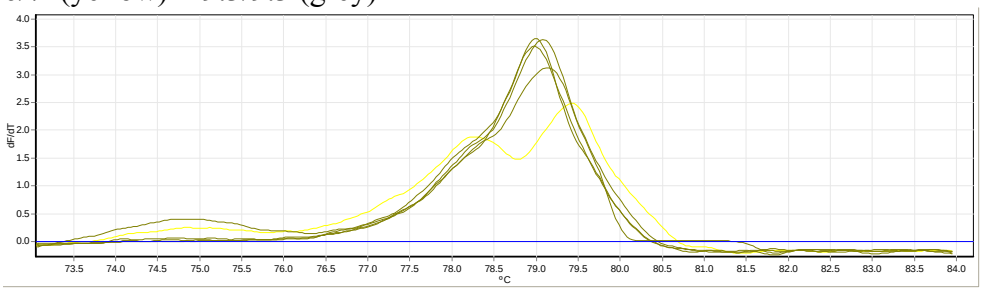
7/9.3 (red) + 6/9.3 (blue)



8/9.3 (turquoise) + 6/6 (grey)



6/7 (yellow) + 9.3/9.3 (grey)



9/9.3 (pink) + 5/9.3 (purple)

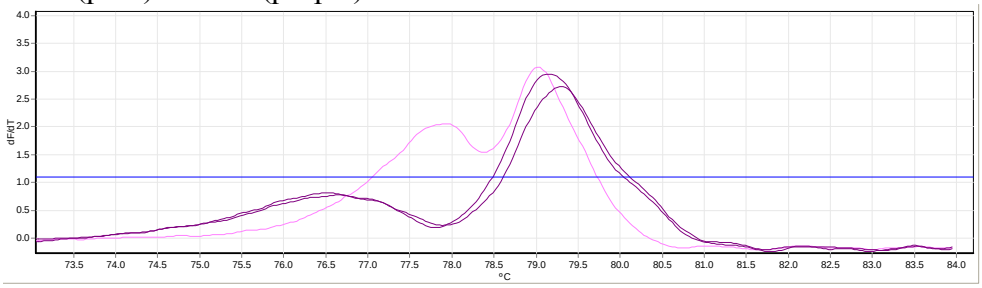
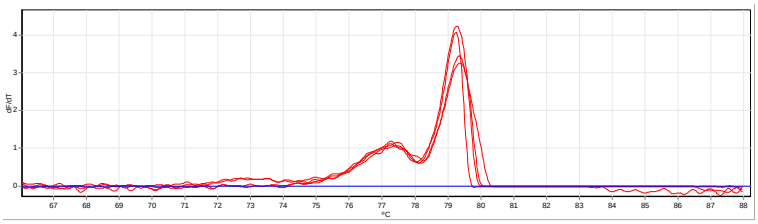
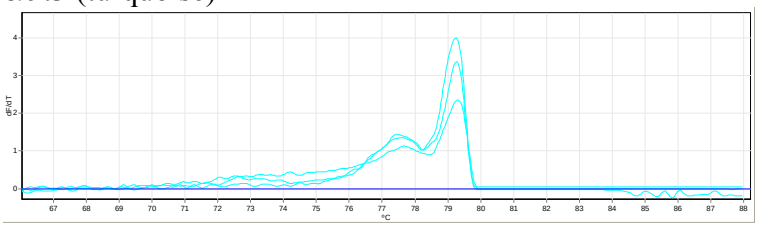


Figure 48 – Plexor THO1 melting in a non-HRM RG6000.

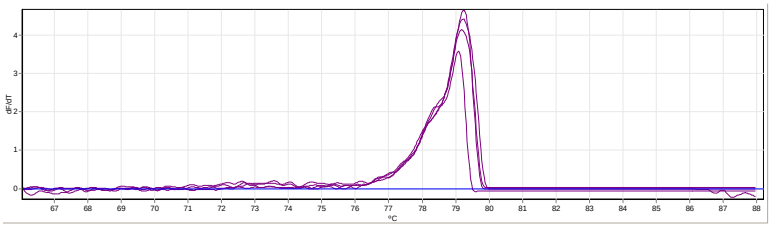
7/9.3



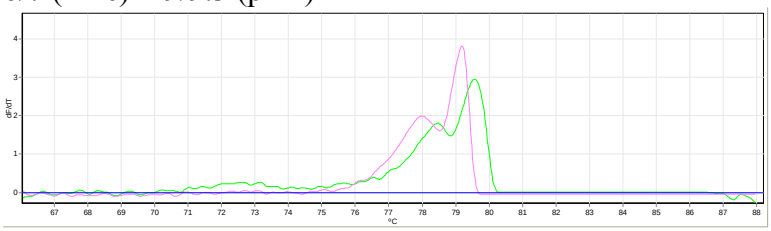
8/9.3 (turquoise)



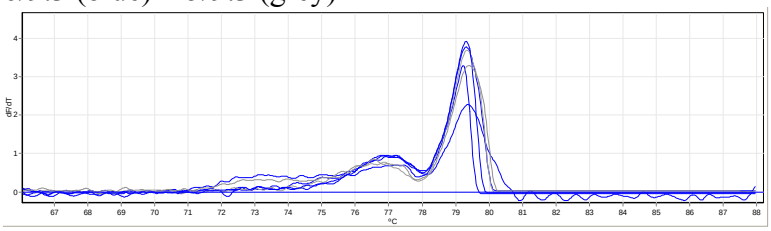
9.3/9.3



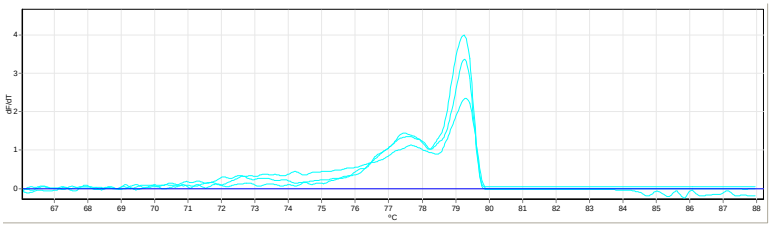
6/7 (lime) + 9/9.3 (pink)



6/9.3 (blue) + 5/9.3 (grey)



8/9.3



6/6

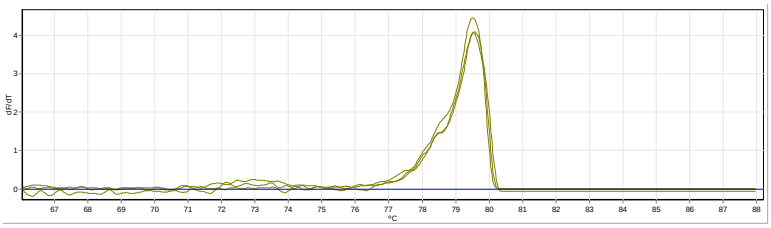


Figure 49A – Gel of Alu PCR results for degradation studies.
Lane A – ΦX HaeIII marker
Lane B – Alu forward + short reverse primers
Lane C – Alu forward + middle reverse primers
Lane D – Alu forward + long reverse primers
Lane E – Alu forward + all 3 reverse primers

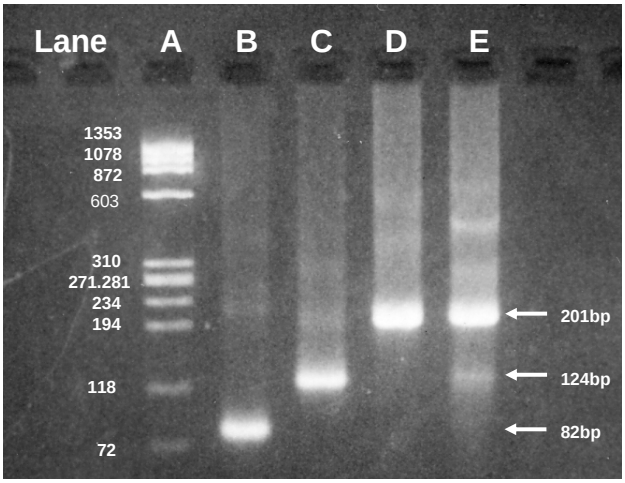
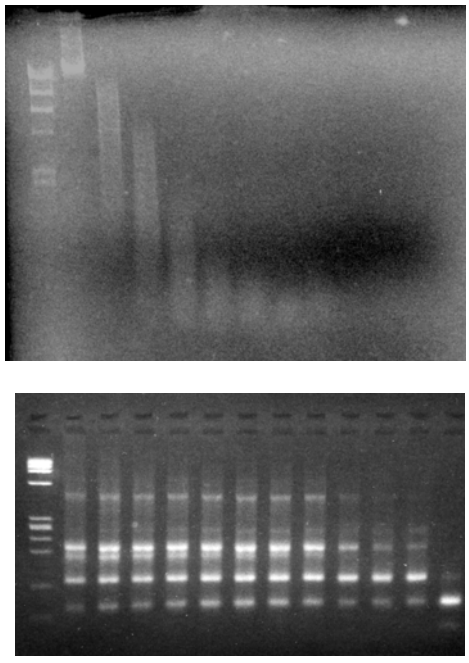


Figure 49B – Overlapping *Alu* PCR of DNAse I degraded DNA -- larger amplicons decrease with digestion time.



Upper panel shows DNAseI digested DNA

Lower panel shows *Alu* PCR results with primers for the three overlapping amplicons on same DNAs

Lane A – upper gel - λ HindIII, lower gel - ΦX HaeIII

Lane B – No digestion

Lane C – 30 sec digestion

Lane D – 1 min digestion

Lane E – 2 min digestion

Lane F – 3 min digestion

Lane G – 4 min digestion

Lane H – 8 min digestion

Lane I – 12 min digestion

Lane J – 16 min digestion

Lane K – 24 min digestion

Lane L – 32 min digestion

Lane M – 48 minute digestion

Figure 50 – Graph of difference in Ct versus DNA concentration for the triplex Plexor DNA degradation assay. Notes slopes of lines are -0.33 to -0.78 (well outside of the acceptable -0.1 to 0.1 range).

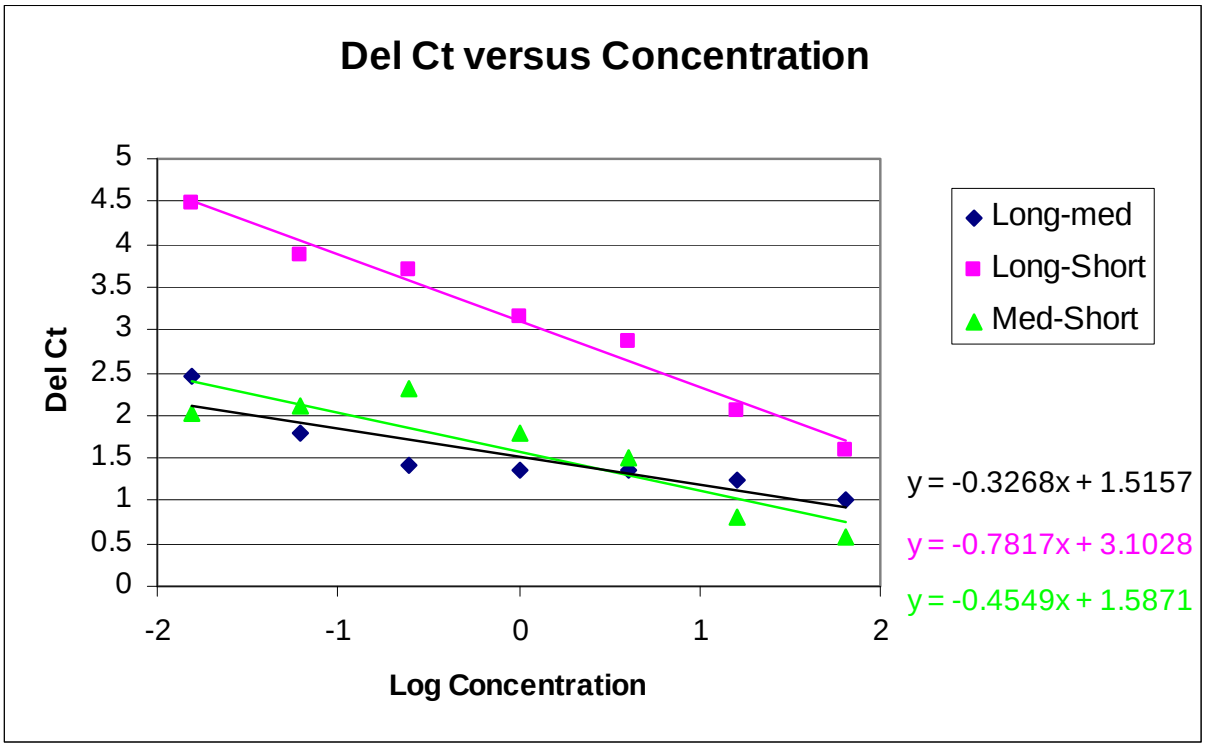


Figure 51 – New duplex degradation assay.

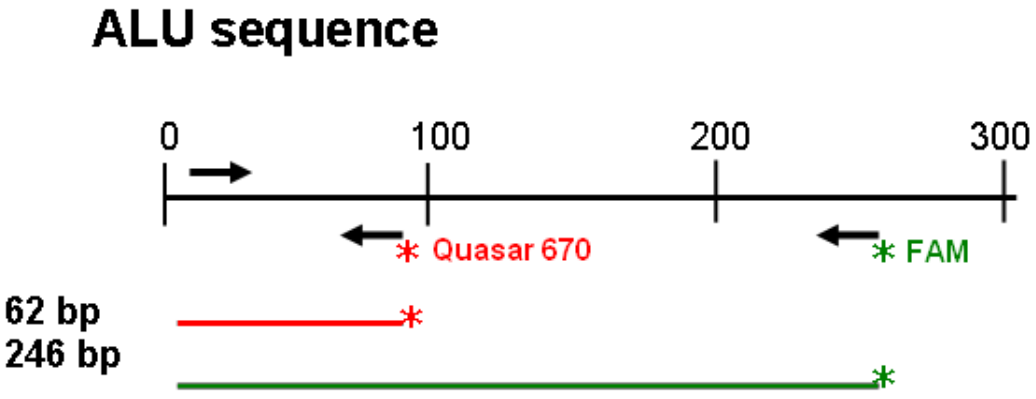


Figure 52 – Improved Del Ct for duplex assay – slopes are now between -0.1 and 0.1 (results for three different DNAs shown).

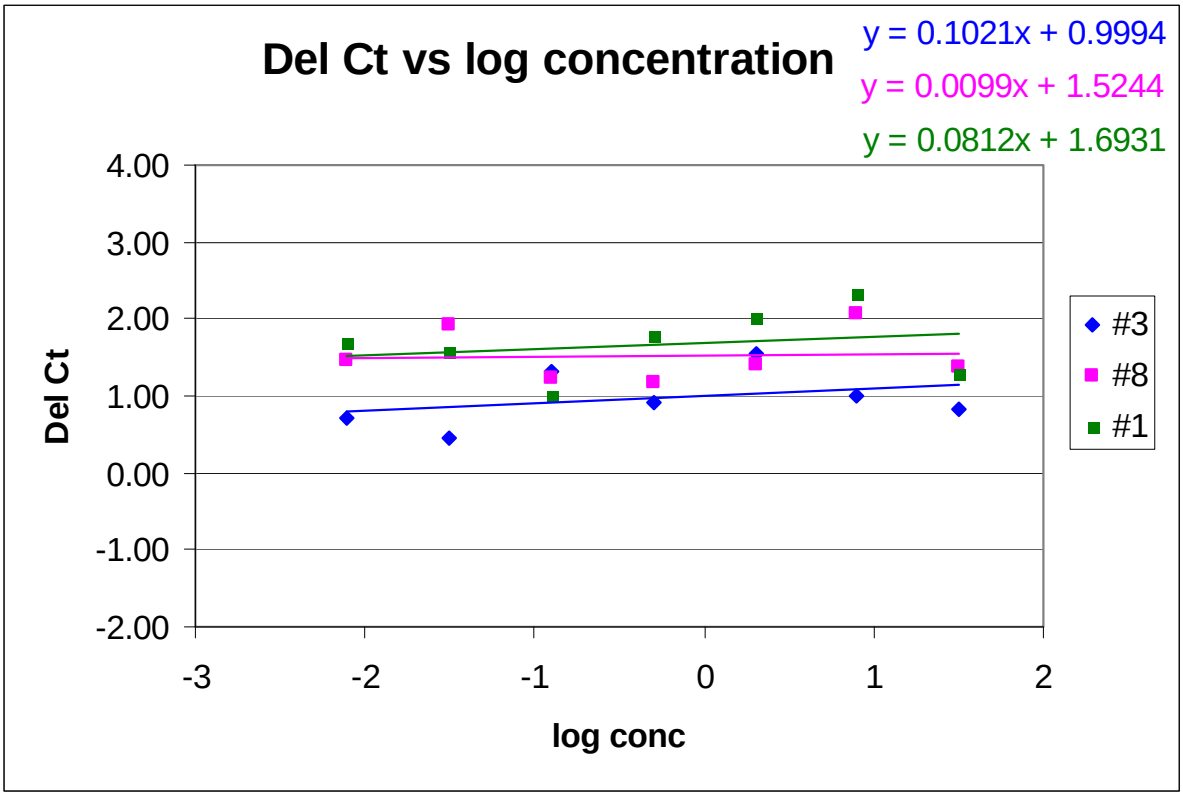


Figure 53 – Gel of two different degraded DNAs showing increased degradation with increased DNase I treatment time.

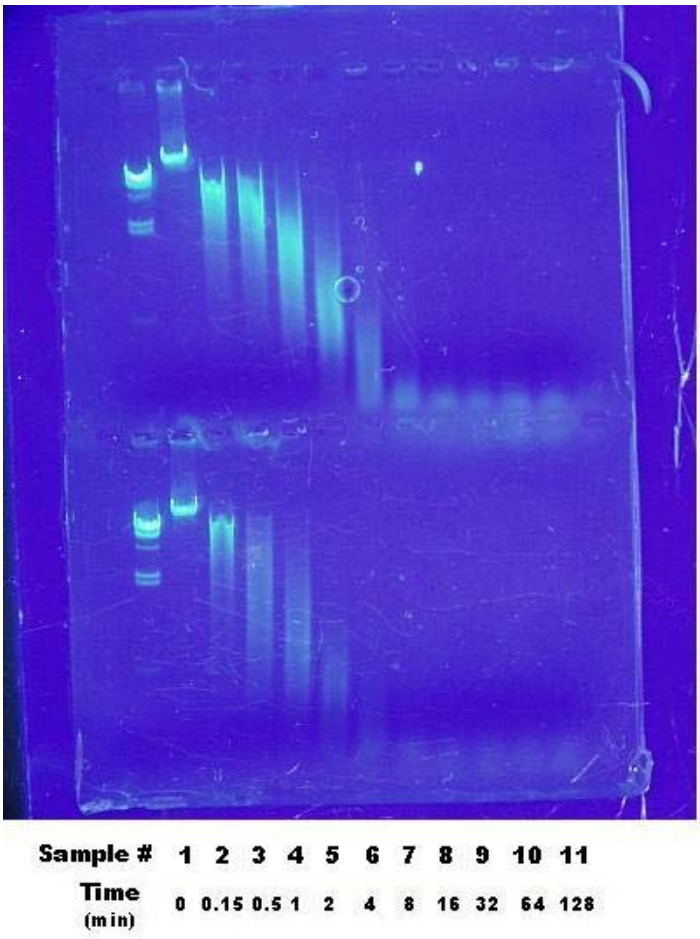


Figure 54 – Graph of ratio of the short to long concentration versus degradation time.

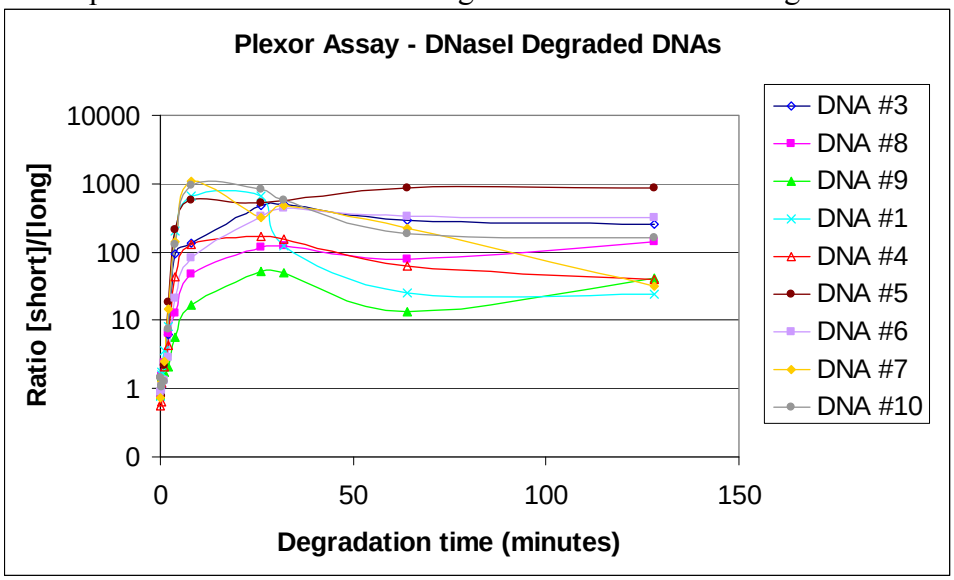


Figure 55 – COfiler electropherograms of non-degraded (upper) and highly degraded DNA (lower).

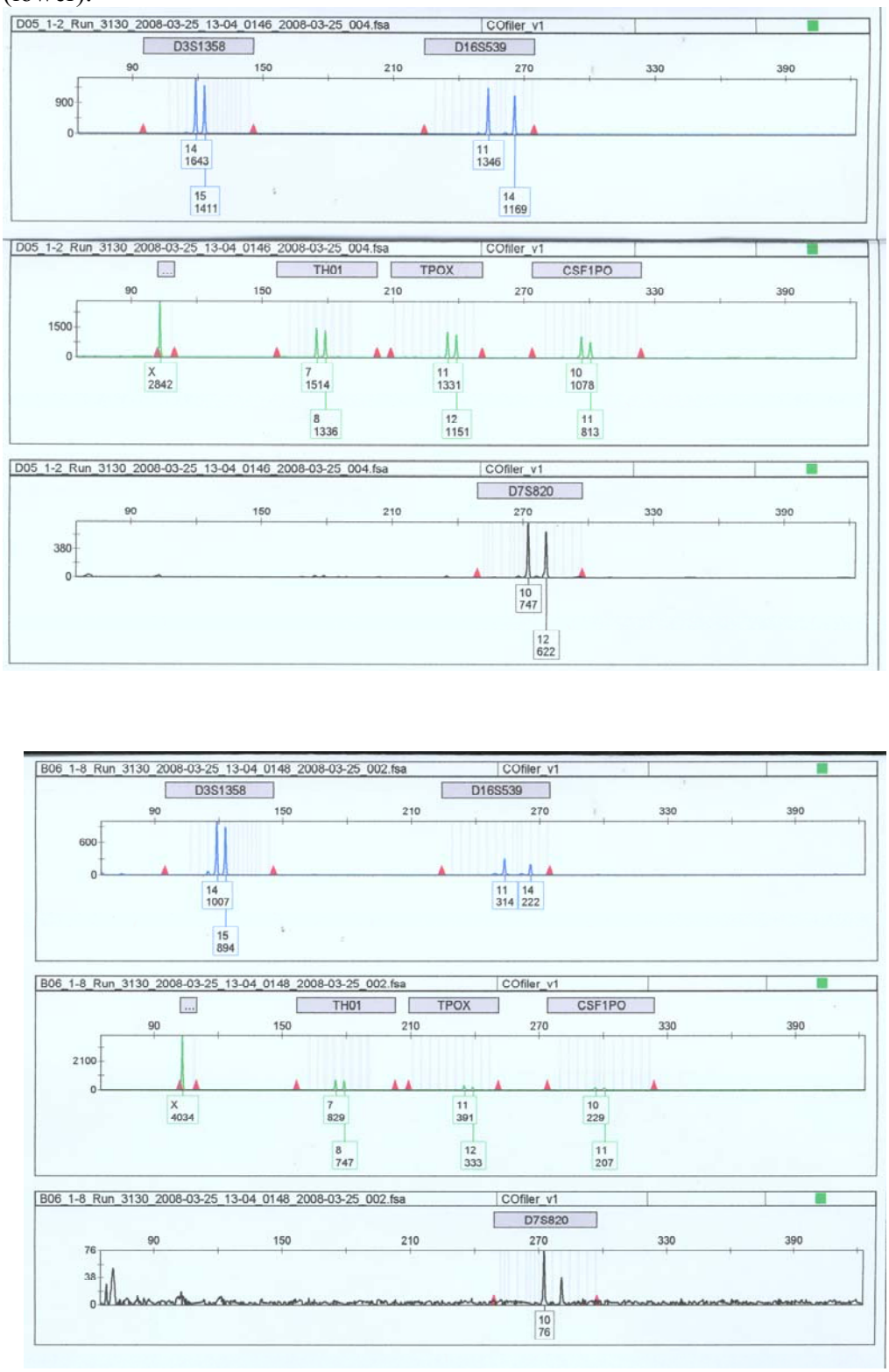


Figure 56 – Relationship of measured degradation ratio and ratio of RFUs from a short to a long STR product (D3 to CSF) from STR amplification (uses log ratios and only samples where concentration was 0.1ng/ul into STR – i.e. 1ng total input).

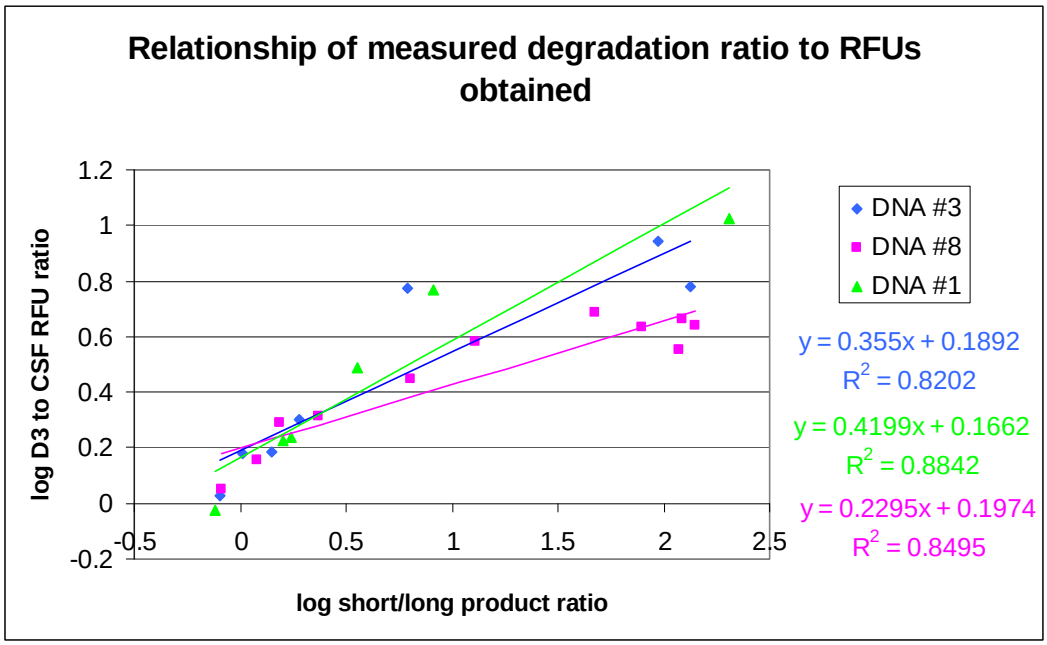
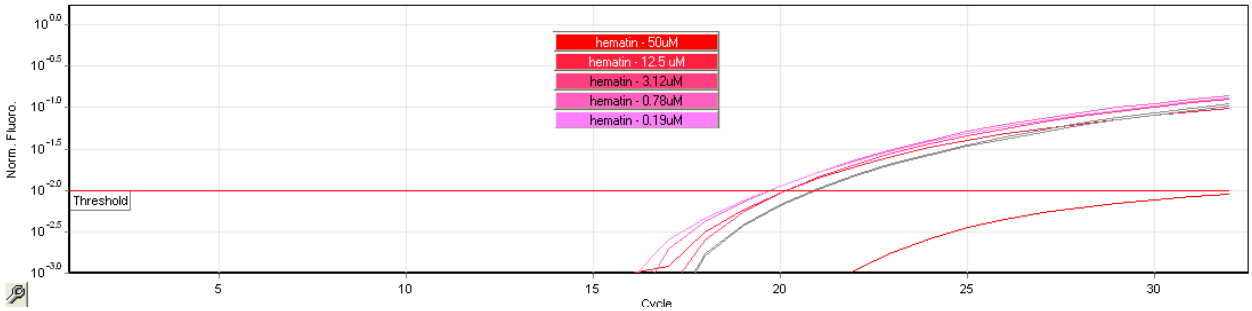
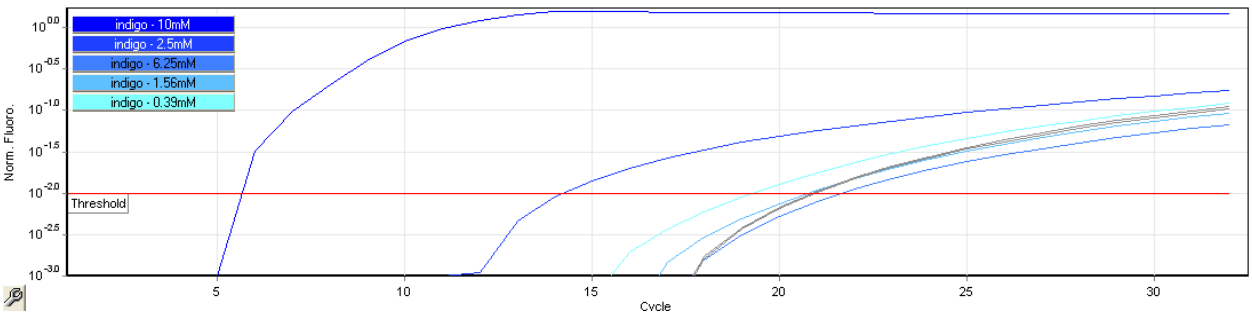


Figure 57 – Selected effects of inhibitors on DNA degradation assay (controls in grey).

A. Hematin on Long (FAM) – Inhibition at high concentrations.



B. Indigo on Long (FAM) – Dye interferes with detection and causes Ct values to be inflated.



IV. Conclusions

1. Discussions of Findings

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

These multiplex FRET assays using quenching probes can determine the genotype of an individual for five to seven SNPs plus gender determination in a fast (2 hour), accurate manner using a real-time PCR instrument. The 6-plex A assay was sensitive, giving correct typings at comparable template amounts to that required for STR analysis using standard typing kits. The assay did fail to give the correct genotype for heterozygotes at low template amounts, detecting only one or no alleles. One allele still may be helpful as a screening procedure where the data is meant only to sort between a limited number of individuals Using a single tube or well test (or two wells for the double 4-plex), the assays are effective down to approximately 50 pg of DNA.

Theoretically, using five SNPs (with p=q=0.5) plus gender, two samples coming from two different random individuals will be identical 0.37% of the time, whereas using seven SNPs and including gender this will only occur 0.05% of the time (1 in 1900). The percent identical matches for this assay will, of course, vary depending on racial group and this is only an approximation of what might be expected. These percentages are sufficient for an initial forensic screening technique and certainly compares well with not so ancient techniques. This assay could also be used in a medical setting where questions occasionally arise as to the possibility of a tissue swap or contamination.

There could be concerns that the assays may have limitations when relatives are involved. If the relatives are of different genders, then this will be immediately detected by the gender SD so the concern would be same-sex pairs. If one considers two same-sex siblings for a single SNP

with p=q=0.5, the probability of being identical at one locus is 19/32 and being non-identical is 13/32 so for a 4-plex assay with 3 SNPs and the gender assay, the probability of being identical is $(19/32)^3$ or 21%; high but still not identical >75% of the time (see footnote #2). For the 7 SNP plus gender, this falls to only 2.6% or over 97% non-identical. In the case of a same gender, parent-child pair, the chance of being identical for a single SNP is ½, for 3 SNPs this falls to 1/8 (12.5%) and for 7 SNPs this falls to 1/128 or 0.78% (see footnote #3). While obviously not as robust as for unrelated individuals, this assay still will exclude relatives at a high rate.

This assay is not designed for use with mixtures as with only two alleles at every locus, mixtures would be very hard to detect especially if the inputs were unequal. A mixture could give a different profile than either sample alone assuming near equal input from the two input DNAs, except in the case where both individuals typed identically (same probability as above). If the question was whether two samples were the same (not to be STR tested) or different (need testing), the result would be to test the sample and the mixture would become evident on STR analysis.

This SNP assay is the first report using six SNPs in one multiplex assay in a real-time instrument. Other real-time methodologies such as TaqMan™ can perform at most three assays in a multiplex because of the limit of using two colors (fluorescence channels) per SNP-one color for each allele. The melting method allows use of only one fluorophore (channel) per SNP. While a FRET assay using excitation of one fluorophore and emission of the second (as opposed to the assay used here which uses the excitation of one fluorophore and quenching by the other), could theoretically be multiplexed, it was thought that cross-talk of multiple fluorophores might become a problem and that the quenching technique should be cleaner. Although assays that

generate fluorescence are typically considered more sensitive than those that monitor its decrease, the work reported here shows that the assay works at template concentrations similar to other forensic DNA assays and the quenching approach does not limit the usefulness of the assay.

In addition to sample identification, the assay could be used simultaneously as a DNA quantitation measure if a standard curve is run at the same time. A good estimate of sample concentration could be made by taking a mean of the results for the six loci. Running a standard curve using a mixed sample also gives melt curves with both alleles at various concentrations, making interpretation of sample results easier.

Additional SNPs could be added to the assays by duplexing two SNPs in one color (Lin et al., 2004; Schutz et al., 2006; Pont-Kingdon et al., 2007; Pont-Kingdon and Lyon, 2005). This requires picking SNPs where the melting peaks of the two perfect matches and two mismatches are all distinct. Alternatively, two tightly linked SNPs under a single probe (preferably not in significant linkage disequilibrium) can give four different melting peaks for the four haplotypes (Steffensen et al., 2003). Pont-Kingdon and Lyon (2005) have also described spanning probes which span two somewhat distant SNPs by looping out the DNA in between.

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

While the FRET technique did technically work for STRs in that amplification could be detected by the system by decreased fluorescence and clear melting peaks could be visualized, insufficient discrimination in the melting of the different alleles was observed in the performed experiments. It was deemed that further attempts at optimization and use of different STRs

might improve the assay somewhat but that it was very unlikely to result in a viable assay for forensic use. Therefore, we stopped work on this technique to work on the STR HRM technique.

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

Not all individuals with the same genotype had the same melting curve. This is believed to be due to micro sequence differences – alleles can have the same length and therefore genotype by classic STR analysis but have different sequences. A classic example is the 16/16' and 17/17' alleles of D3. The different sequences can result in different melting. Sequence analysis is being performed on samples with the same genotype but different melting curves to confirm sequence differences.

This assay requires further validation and testing using different types of samples and further blind tests of the ability to discriminate samples. However, studies to date indicate that this simple, inexpensive and fast assay (using three STRs) can discriminate individuals sufficiently to be used as a screening test. [This assay is not designed to replace classic STR analysis but is to be used as a time and money saving screening test to select samples for full STR analysis.] This assay should also be able to quantitate the DNA in samples when run with a standard curve.

Very promising results were obtained by multiplexing two STRs with different melting temperatures or using Plexor in an HRM or regular fine melting program. These results could lead to the ability to multiplex and thus greatly increase the discriminatory power of the assay.

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

While the original triplex assay to determine the state of DNA degradation using three overlapping *Alu* amplicons of different sizes proved to be untenable, despite a great deal of attempts at optimization, a duplex test was developed and optimized. The ratio of the concentrations of short to long product gives a measure of DNA degradation. Non-degraded DNA gives a ratio of approximately 1.0; DNA is clearly degraded if the ratio is above 1.5. Ratios of very degraded DNA can be in the 100s. This ratio plateaus with very severe degradation because the amount of short amplification also starts to decrease.

Using the concentration of long product to determine the amount of input DNA for a degraded sample for STR analysis gives appropriate RFUs for analysis. Ski slope (lower RFUs or complete loss of the larger loci) is seen with more severe degradation. The amount of ski slope that will be seen can be predicted from the short/long degradation ratio.

The assay is not as quantitative as might be desired in that very highly degraded DNA gives ratios of between 50 and 1000 although recent results gave tighter values. Tests with inhibitors also showed most inhibitors did not greatly affect the degradation ratio.

2. Implications for Policy and Practice

The implementation of DNA analysis within the forensic laboratory has been a tremendous benefit to the criminal justice community allowing information to be obtained from seemingly innocuous items. As a result, there are more cases and more items per case that require DNA analysis; our studies have resulted in the development of assays that can reduce the number of unnecessary (duplicated) STR tests performed and reduce the time and cost involved in casework analysis. The analyst will be able to easily and quickly choose the most probative samples and to

gain more information from each analysis. The FRET assay and/or the HRM assay will allow determination of whether two crime scene samples arise from the same person; thus, eliminating the need to test duplicate samples. The state of DNA degradation assay will allow the analyst to more easily determine the correct amount of input DNA for STR analysis and/or choose the proper assay type. If the DNA is highly degraded then a SNP assay or mtDNA may be the route to follow for analysis.

3. Implications for Further Research

This research shows that multiplexing of up to six SNPs is possible with the FRET melting technique. Thus, this technique could be used to develop other assays of interest to the forensic community such as biogeographic (racial) data or phenotypic data (e.g. eye or hair color). The data from the HRM studies indicates that HRM is a powerful technique that could be used for other forensically relevant assays. The degradation assay results demonstrate that the Plexor system is a good methodology for assay development.

V. References

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VII. Dissemination of Research Findings

1. Publications

a. Nicklas JA, Buel E. A real-time multiplex SNP melting assay to discriminate individuals. J Forensic Sci. 2008 Nov;53(6):1316-24.

b. Opel KL, Fleishaker EL, Nicklas JA, Buel E, McCord BR. Evaluation and quantification of nuclear DNA from human telogen hairs. J Forensic Sci. 2008 Jul;53(4):853-7

c. We are also writing several chapters for a book (tentative citation below) summarizing workshop presentations at the American Academy of Forensics Sciences in February of 2008.

Barbisin M, Buel E, Buoncristiani MR, Conti T, Diegoli T, Fang R, Furtado MR, Hudlow WR, Kline M, Krenke B, McCord B, Nicklas JA, Niederstaetter H, Parson W, Shewale JG, Timken M, Vallone P (2008) Quantitative PCR for Forensics, Humana Press, Totowa, NJ

2. Presentations

Individual	Meeting	Date	Presentation
Janice Nicklas	NIJ DNA Grantees' meeting (Wash, DC)	6/06	Talk
Janice Nicklas	Association for Molecular Pathology (Orlando, FL)	11/06	Poster
Eric Buel	Sixth Annual Advanced DNA Technical Workshop (East Captiva Island, FL)	5/07	Talk
Janice Nicklas	The NIJ Conference (Washington, DC)	7/07	Poster
Janice Nicklas	Promega, 18th Inter Symp on Human ID (Hollywood, CA)	10/07	Poster
Janice Nicklas	Amer. Acad. Forensic Sci Annual Meet (Washington, DC)	2/08	Workshop talk on QPCR
Janice Nicklas	NE SWGDAM meeting (Maynard, MA)	3/08	Talk
Janice Nicklas	The NIJ Conference (Washington, DC)	7/08	Poster
Janice Nicklas	Green Mountain Forensic DNA conference (Burlington, VT)	8/08	Talk
Janice Nicklas	California Forensic Science institute	9/09	Two talks