EVALUATING DNA SAMPLE DEGRADATION WITH A QUANTITATIVE GENDER TYPING END-POINT PCR MULTIPLEX

By

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END-POINT PCR MULTIPLEX

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NOMENCLATURE

AB	Applied Biosystems
bp	basepair
CCD	Charge-Coupled Device
CE	Capillary Electrophoresis
CODIS	Combined DNA Index System
DAB	DNA Advisory Board
DNA	Deoxyribonucleic Acid
ePCR	End-Point PCR
FBI	Federal Bureau of Investigation
HID	Human Identification
IPC	Internal Positive Control
LDIS	Local DNA Index System
mL	Milliliter
NDIS	National DNA Index System
ng	Nanogram
NIST	National Institute of Standards and Technology
PCR	Polymerase Chain Reaction
pg	Picogram

QAS	Quality Assurance Standards
qPCR	Quantitative PCR
RFLP	Restriction Fragment Length Polymorphism
RFU	Relative Fluorescence Units
RVG	Revised Validation Guidelines
SDIS	State DNA Index System
SRM	Standard Reference Material
SRY	Sex-Determining Region of the Y Chromosome
STR	Short Tandem Repeat
SWGDAM	Scientific Working Group on DNA Analysis Methods
µL	Microliter
UV	Ultraviolet
Y-STR	Y-chromosomal Short Tandem Repeat

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CHAPTER I

INTRODUCTION

Forensic DNA analysts encounter a variety of samples in casework. The myriad of evidence subjected to DNA typing includes samples of differing cellular quantity, as well as quality. With the increased demands for DNA results involving investigative, judicial, and human identification applications, developing technologies continue to provide high-throughput solutions for the numerous steps required in the DNA testing process. However, the external demands for faster processing are further complicated by the nature of samples often submitted to DNA laboratories for analysis. Many evidentiary samples contain low-quantity DNA, and are previously exposed to varying degrees of environmental insults prior to collection and storage. Once an item, stain, or swab is selected for testing, its cellular quantity and quality is unknown prior to the time-consuming and expensive analysis. This issue becomes further exacerbated when items or surfaces contain multiple stains of interest, of which the ability to obtain useful information from cellular material remains unknown.

Although the DNA Advisory Board (DAB) Quality Assurance Standards require CODIS-participating forensic laboratories to quantify the amount of human DNA prior to HID profiling, present standardization committees do not require forensic laboratories to evaluate the quality of a biological sample prior to analysis, (FBI DNA Quality Assurance Audit Document, 2009, Standard 9.4). This lack of qualitative measurement limits prior knowledge of the biological

sample's cellular integrity, which may contain degraded template DNA. As a result, examiners confronted with a backlog of casework samples must utilize presumptive serological tests and their laboratory's standard operating procedures in an attempt to select the sample(s) that will yield useful typing results (Figure 1). This sampling practice, coupled with turnaround-time demands, requires forensic laboratories to invest significant time and money processing samples that may not produce DNA information eligible for upload into the CODIS database. As a result, backlogs develop that could be averted if both the quantity and quality of a DNA sample are known prior to HID typing.

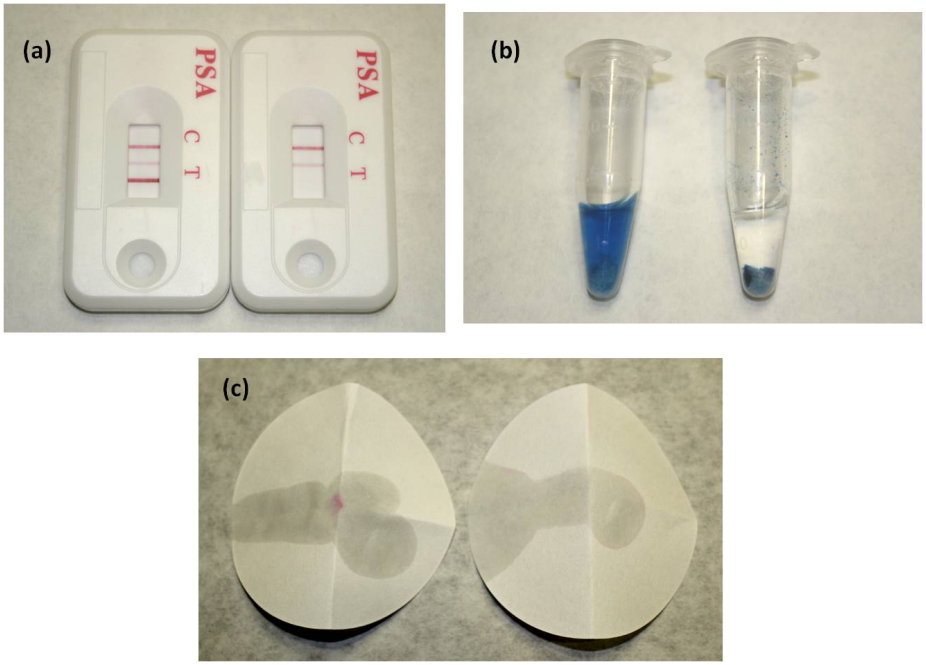

Figure 1: Common types of serological tests available to forensic laboratories. The results of these presumptive tests direct analysts to stains that may yield probative DNA information. (a) positive and negative results obtained from the Seratec® PSA Semiquant test for the detection of the prostate specific antigen (PSA), a component of seminal fluid. (b) positive and negative results obtained from the Phadebas® Amylase test for the detection of alpha-amylase activity, a component of saliva. (c) positive and negative results obtained from the Kastle-Meyer test for the detection of hemoglobin peroxidase activity in blood.

Current quantitative DNA methods validated for use in forensic laboratories include real-time PCR techniques and end-point PCR (ePCR) techniques. Both quantitative methods simultaneously estimate the quantity of human-genomic, male-genomic, and male Y-chromosomal DNA, as well as assess sample inhibition. In addition, these quantitative methods are sensitive and capable of detecting small quantities of DNA in a sample extract within the range of one-trillionth grams per microliter. However, neither of these quantitative methods can detect DNA sample degradation prior to analysis with an HID typing kit. Although literature suggests that prior knowledge of both template DNA quantity and quality are important factors in determining how to process casework samples, no current method exists that can simultaneously quantify and measure the integrity of extracted DNA (Whitaker et al., 1995).

CODIS-participating forensic laboratories may utilize several types of commercially available HID typing kits developed for characterizing short tandem repeat (STR) loci as a triage for DNA profiling needs. These individual STR kits are manufactured to amplify and detect one of three types of STRs: human genomic, shortened-length "mini-" human genomic, and male Y-chromosomal. Prior to the development of mini-STR and Y-STR typing kits, implementation of the CODIS database in 1998 restricted analysts to upload qualified profiles containing STR information obtained from the FBI's 13 core loci. The recent development of mini-STR kits may be used to obtain information from large core loci sequences, which are often susceptible to amplification drop-out in traditional genomic STR kits. In response to the technological developments of HID testing, the CODIS database now accepts entry of Y-chromosomal profiles, albeit this information is limited to searches under the missing person indexes (FBI CODIS and NDIS fact sheet). DNA profiles generated from these STR typing kits may be suitable for upload onto the CODIS database for search at the local (LDIS), state (SDIS), and national (NDIS) indexing systems as long as a minimum threshold of STR information is obtained, and the evidentiary sample is deemed eligible as regulated by state and national legislation.

With the 2011 release of Promega's PowerPlex® 18D System, genomic STR typing kits now have the ability to target a maximum of 18 different locations of human DNA in a single PCR reaction. Each STR locus amplified in an HID kit is comprised of a series of alleles, and each allele is comprised of differing lengths that correspond to the number of sequence repeats in the tandem array. The total range of allele lengths from an 18-locus STR kit spans approximately 100 – 500 base pairs (bp). Although genomic STR kits provide a high power of discrimination, their success is dependent upon the amount and quality of template DNA, which may be limited if the integrity of the DNA molecule is disrupted. In particular, degradation of template DNA may result in a loss of STR locus information that will hinder the success of profiling analysis (Butler, 2005). Although the 100 – 500 bp STR typing kits target DNA sequences significantly smaller than previous RFLP methods, STR loci are still susceptible to the effects of degradation, and once the average size of template sequences is reduced to less than 300 bp, a significant loss of information occurs (Bender et al., 2004). Currently, there is no way to predict which STR typing kit will be most successful at generating a complete DNA profile for comparative purposes and CODIS entry.

Figure 2: Sample quantity is not equivalent to sample quality. This photograph depicts two unopened bags of cereal removed from their respective boxes. Both boxes contain 27 grams of cereal. However, the physical integrity of the cereal on the right was modified so the pieces are no longer whole. This representation demonstrates the difference between DNA quantity and DNA quality. Although the DNA sample extract may contain a large amount of template DNA, if the physical integrity of the DNA is degraded then successful STR amplification may not occur.

The following research utilized a developmentally validated ePCR quantitative assay that amplifies DNA targets of differing size to assess DNA degradation while simultaneously quantifying the amount of total-human and the amount of male-only DNA. A small male-specific haploid SRY target (110 bp) is used to quantify the amount of male-only DNA within a sample, and a large diploid Amelogenin target [X (210 bp) and Y (216 bp)] is used to quantify the amount of total-human DNA. The ability to evaluate DNA template degradation by comparing targets of differing size is not a novel concept, and the literature supports this approach as applied to HID

amplification (Alaeddini et al., 2010). However, few studies suggest that this type of qualitative comparison has been performed. The purpose of this research was to examine if a gender typing ePCR quantitative assay is capable of detecting DNA degradation in male samples by comparing the two independent male-only and total-human quantitative estimates, which are derived from DNA targets of differing size. Utilization of this assay may benefit practitioners by simultaneously determining the quantity of human DNA and evaluating template DNA degradation prior to HID analysis so informed decisions can be made regarding the best approach to producing probative STR information from available evidence, which would otherwise yield problematic or incomplete data.

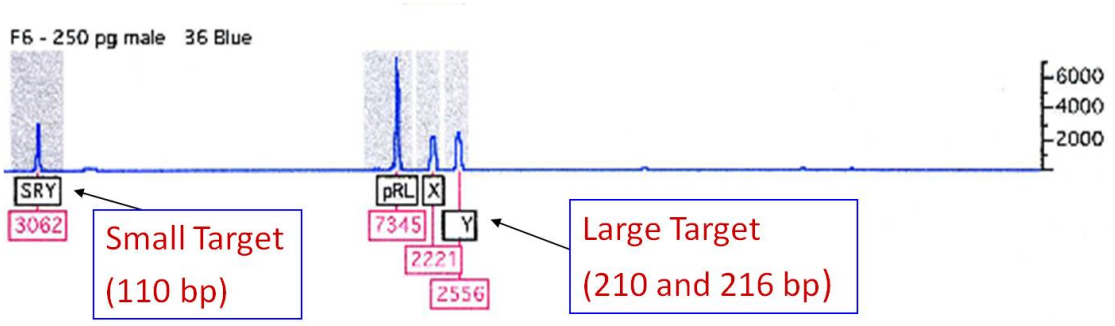

Figure 3: The ePCR quantitative assay detects human-specific DNA targets of differing size. The small haploid male-specific SRY target is used to quantitate the amount of male-only DNA for Y-STR HID profiling. The large diploid Amelogenin target is used to quantitate the amount of total-human DNA for autosomal STR HID profiling. Note that detection of the pRL amplicon (Renila Luciferase plasmid) serves as an internal positive control for the amplification reaction.

CHAPTER II

REVIEW OF LITERATURE

II.A. DNA Degradation

The inability to produce a complete STR profile from a degraded DNA sample is a common issue addressed in the literature. In numerous articles, authors discuss problems encountered with profiling highly-degraded DNA samples recovered from the scenes of mass disasters (Butler et al., 2003; Coble and Butler, 2005; Graham, 2006; Leclair et al., 2004; Mundorff et al., 2009; Olaisen et al., 1997; Whitaker et al., 1995). Biological samples recovered from these scenes are exposed to the extremes of thermal, physical, and chemical insult, which creates a challenge for the forensic DNA laboratory to select the best sample for HID testing (Whitaker et al., 1995). However, DNA degradation is not solely limited to instances of mass disasters, but may also be encountered in routine casework. One of the earliest articles describing the presence of degraded DNA in casework samples involved a study of 100 substrata containing bloodstained evidence submitted to the Office of the Chief Medical Examiner (OCME) of the City of New York (McNally et al., 1989). Analysts evaluated the quantity and quality of each sample with agarose gel electrophoresis, and categorized the DNA integrity as: high-molecular weight, high-molecular weight plus degradation, degraded, or not detected. Based on the yield gels, 12 of the samples were classified as degraded and 11 were classified as containing partial degradation. These results indicate almost one quarter of the bloodstained samples submitted to

the New York City OCME as a part of this study exhibited some degree of degradation (McNally et al., 1989). Given the results of the New York City OCME study, most forensic DNA laboratories will encounter evidentiary samples containing degraded DNA. Outside of functioning repair systems, "DNA is a relatively weak molecule that degrades rapidly in an environment- and time-dependent manner" (Jobling et al., 2004). Exposure to dynamic conditions such as "light, humidity, elevated temperatures as well as bacterial and fungal contamination followed by the growth of these microorganisms lead to physical, chemical, and biochemical degradation of genomic DNA" (Schneider et al. 2004). The variety of conditions that degrade unprotected DNA may be classified as either an enzymatic process or a non-enzymatic process. The enzymatic process is the first to affect the physical integrity of the DNA molecule as cellular proteases degrade protein structures that are complexed with and protect genomic DNA. The absence of protecting structures exposes the phosphodiester backbone of DNA to random digestion by endonuclease activity (Alaeddini et al., 2010). The progression of cell death also releases nutrient-rich fluids that stimulate the growth of environmental microorganisms, which exacerbate the decomposition of cellular material (Alaeddini et al., 2010).

Without functioning cellular repair systems, non-enzymatic processes also contribute to DNA degradation. These non-enzymatic processes include hydrolytic and oxidative reactions, crosslinkage, and radiation, all of which proceed at a slower rate than the enzymatic degradation process. Hydrolytic reactions are pH and temperature dependent, and cleave the glycosidic sugar-base bond resulting in the loss of a base (Alaeddini et al., 2010). In addition, free-radical oxygen and peroxides released from ionization reactions may modify the sugar structure, of which pyrimidines are particularly vulnerable. Despite the numerous sources of DNA degradation, research in ancient studies suggests that certain stable conditions may protect the integrity of the DNA molecule, such as "temperature, pH, humidity, and salt concentration" (Jobling et al., 2004).

II.B. PCR Amplification

PCR amplification of STR loci is dependent upon the physical integrity of the template DNA strands. If either the STR template strand or primer-binding regions of both the forward and reverse primers are not intact, then priming and elongation cannot occur and amplification is halted. Template samples containing partial degradation may yield successful PCR results if the majority of template strands remain intact. However, the more prolonged a biological sample is exposed to degradation events, the more widespread damage will occur to the majority of template DNA. Consequently, the loss of STR strands containing the full length needed for successful PCR amplification will result in a decrease in PCR product (AmpFℓSTR® Identifiler® User's Manual, 2011).

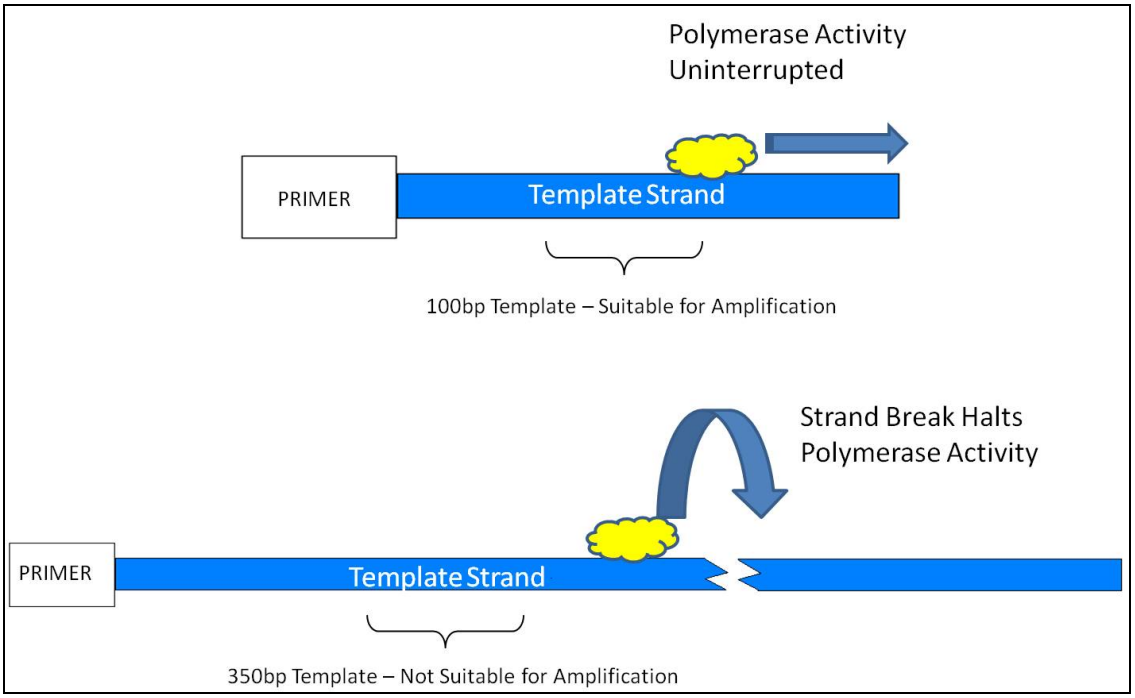

Figure 4: Manufactured STR kits amplify target sequences of differing size. Larger targets are more susceptible to DNA degradation, which causes breaks in the template strand. Although primers may bind to the complimentary template sequence, polymerase extension is interrupted due to strand fragmentation. The inability to amplify a fragmented template strand may result in a loss of data for the affected STR locus.

The correlation between the success of STR typing and the length of template DNA was recognized as early as 1995 when researchers, utilizing a four-locus STR multiplex ranging in sizes between 130 – 240 bp, observed partial or complete dropout of larger loci occurring before the loss of smaller loci (Whitaker et al., 1995). This preferential amplification of smaller DNA target sequences over larger DNA target sequences produced a curve in the data such that the signal intensity was inversely proportional to the amplicon size (Alaeddini et al., 2010). Practitioners often refer to this effect as a "wedge" or "ski slope" that is apparent in data generated by capillary electrophoresis (CE) fluorescent detection.

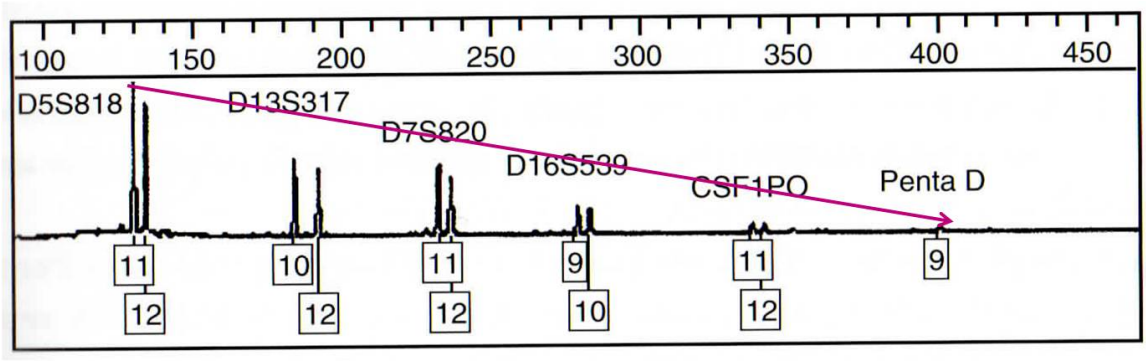

Figure 5: An example of a degraded STR profile displaying the loss of signal intensity with larger amplicon products. Note the visual slope appearance within the data that occurs between these STR loci, which encompass the range of 100 – 400 bp (image from Butler, 2005; modified).

The primary advantage of multiplexed STR kits is the ability to amplify multiple STR targets in a single reaction. Manufactured primer sequences contain fluorescent dyes that are incorporated into either the forward or reverse STR primers. When a fluorescently labeled primer anneals to a complementary template sequence during amplification, the resulting product retains this fluorescent tag. After amplification the STR amplicon products can be separated by both fluorescent color and size using a CE detection platform. Multiplex technology eliminates the

need to perform separate PCR reactions and enhances the power of discrimination of HID kits with the addition of more STR loci that contain overlapping sequence lengths (AmpFℓSTR® Profiler Plus® User's Manual, 2006).

The effects of degradation on the success of STR amplification warrant additional discussion regarding the theoretical limits of PCR. In 1996 Dimitrov and Apostolova published an article classifying the different types of DNA products formed during the initial cycles of PCR involving the amplification of an optimal template sequence. These classifications include long DNA (lDNA), medium DNA (mDNA), and short DNA (sDNA) molecules (Figure 6). The desired product of PCR is the single-strand form of sDNA, which is recognized with a fluorescent tag and is within the size range necessary for CE detection. In order to obtain sDNA molecules mDNA molecules must be synthesized from the successful amplification of the template strand. However, without an intact template the mDNA molecule cannot be generated, resulting in no production of sDNA molecules. According to Dimitrov and Apostolova, the initial cycle of PCR amplification with a template (lDNA) strand results in the generation of two lDNA/mDNA molecules. The lDNA molecule never multiples during PCR because it is the template strand, of which there can only be a forward and reverse sequence. The second cycle of PCR produces two new mDNA molecules in conjunction with the template strand. Although the number of lDNA molecules remains fixed at two during the duration of PCR, mDNA molecules will regenerate at a linear rate, increasing by two during each subsequent cycle. The second cycle also produces two mDNA/sDNA molecules from the mDNA generated at the end of cycle one. In the third cycle of PCR the newly generated sDNA molecules will serve as template strands for amplification of more sDNA copies in addition to the mDNA sequences that also serve as a template for sDNA. From this point forward sDNA molecules are exponentially produced from the regeneration of previous sDNA, and the subsequent production of mDNA molecules. Although the initial amount of sDNA molecules lags behind the sum quantity of lDNA and mDNA molecules, by the fourth

amplification cycle sDNA will reach equivalency, and by the 11th cycle sDNA will comprise the majority of amplification product (achieving 99% of the total product at the end of the reaction).

The importance of Dimitrov and Apostolova's theory regarding the limit of PCR cannot be understated because the ability to amplify and detect multiplexed STR loci is dependent upon production of the three PCR products. Moreover, this theory supports the explanation of why preferential amplification may occur in smaller sequences within a multiplexed STR typing kit containing targets of differing size. The smaller sequences of template DNA are more likely to amplify at an optimal rate according to Dimitrov and Apostolova's theory especially in samples compromised by DNA degradation. The larger sequences that contain degraded template strands will always lag behind in their production of PCR product. This disruption in PCR efficacy is observed by the loss of signal intensity within electropherogram data as displayed in Figure 5.

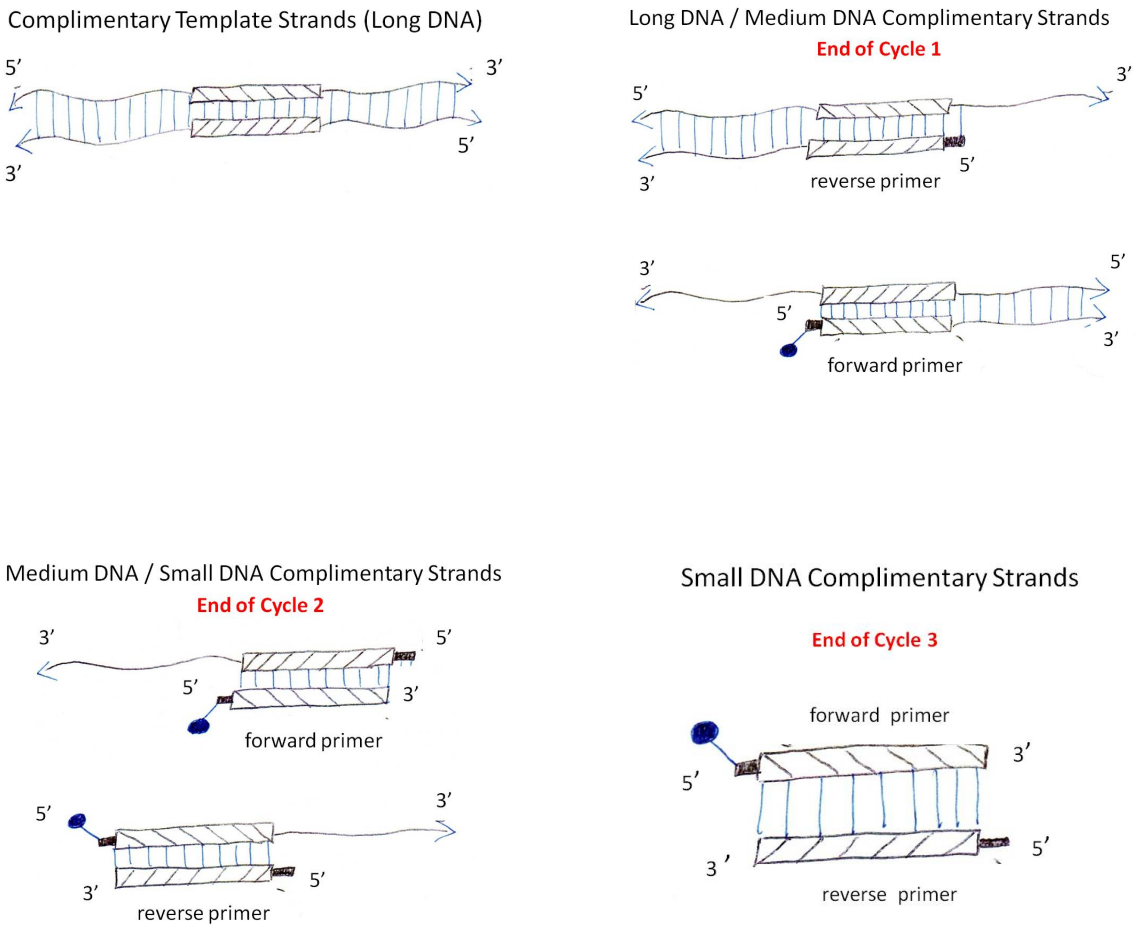

Figure 6: The different classes of PCR products according to Dimitrov and Apostolova's theory on the limit of PCR amplification. Note that the fluorescent tag is only incorporated into the forward primers within the quantitative assay.

II.C. Assessment of Degradation – Past and Current Methods

Although yield gels are not a human-specific method for quantitation, they can be used to assess DNA quantity and evaluate the state of template DNA degradation (Rudin and Inman, 2002). Samples are separated by size using gel electrophoresis and visualized with ethidium bromide staining under UV light. The migration pattern of sample DNA is compared against the migration patterns of a DNA ladder containing fragments of known size to estimate sample quantity. Samples exhibiting effects of degradation will appear as an elongated smear throughout the gel track. This smearing is the visual result of numerous template fragments that are degraded in a random assortment of sizes.

Due to the DAB Quality Assurance Standard 9.4, forensic DNA laboratories are required to evaluate the quantity of human-specific DNA (FBI DNA Quality Assurance Audit Document, 2009). Therefore, yield gels are no longer used in common practice in forensic laboratories, and their unique qualitative assessment for degradation is of little practical value. Nevertheless, manufacturers of commercial DNA typing kits still acknowledge the usefulness of yield gel analysis as a means to assess the molecular weight of samples suspected of degradation due to STR locus dropout (AmpFℓSTR® Identifiler® User's Manual, 2011).

Current quantitative DNA methods used in accredited forensic laboratories include commercially available real-time PCR quantitative systems. Although these real-time systems can estimate the quantity of total-human DNA and male-specific Y-chromosomal DNA, and assess inhibitors to the PCR reaction, these methods currently cannot detect DNA degradation prior to HID profiling. End-point PCR (ePCR) is another commercially available quantitative DNA method that meets current national standards. As with real-time systems, ePCR quantitative assays also estimate the quantity of total-human DNA and male-specific Y-chromosomal DNA,

and assess inhibitors to the PCR reaction. Currently, no published ePCR quantitative method has the ability to detect DNA degradation.

Although commercial manufacturers of DNA quantitation kits offer multiplexes capable of examining both male and female DNA, no current method exists that claims to assess degradation. Nevertheless, these quantitative methods are intended to provide accurate estimates for successful HID profiling within the range of 100–500 bp. Since the majority of quantitative kits obtain information from target sequences less than 150bp, the quantitative results may misrepresent the true amount of intact template DNA for large STR loci, which are more susceptible to degradation. If, however, quantitative kits contain targets within the mid-size range of STR loci, this may help better determine the quantity of template DNA is suitable for successful HID testing.

Swango et al. (2006) published their validation of a quantitative real-time PCR multiplex containing two amplicon targets of differing size. The small amplification target was nuTHO1 (67 bp) from the THO1 STR locus, and the large amplification target was nuCSF (170–190 bp) from the CSF1PO STR locus. An AB 7000 SDS instrument was used as a detection platform for their method (Applied Biosystems, Foster City, CA). In their study, Swango et al. digested high-molecular weight HL60 cell line DNA with DNase I over specified amounts of time. DNA fragments from DNase I activity were separated with gel electrophoresis and visualized using ethidium bromide staining. The authors assessed the degree of degradation by constructing a "qPCR degradation ratio" between quantitative estimates obtained from the nuTH01 and nuCSF targets. These timecourse samples were also amplified using the AmpFℓSTR Identifiler® STR kit (Applied Biosystems, Foster City, CA) to confirm the presence of locus-to-locus preferential amplification commonly associated with a "ski-slope" effect. Although the experimental results confirm that targets of differing size may be used to assess degradation, a critical limitation of the Swango et al. assay for quantitative purposes is that it does not decipher gender-specific

characteristics of samples such as male and female contributors to mixtures encountered in sexual assault evidence. The detection of gender-specific characteristics is necessary to estimate male Y-chromosomal DNA in a sample for either autosomal or Y-STR profiling.

II.D. The Gender Typing End-Point PCR Quantitative Assay

Allen and Fuller (2006) published the previously discussed ePCR quantitative method (Figure 3), which at the time only contained Amelogenin primers for the amplification of the X and Y sex chromosomes. In their article, the fluorescently labeled Amelogenin primers produced amplicons that were detected using the charge-coupled device within an AB 310 Genetic Analyzer (Applied Biosystems, Foster City, CA). The quantity of human DNA within a sample could be evaluated by comparing the sum relative-fluorescent units (RFUs) of the X and Y amplicons to the RFU values generated from a NIST-traceable standard curve produced from known quantities of human DNA. Unlike current real-time PCR quantitative methods, this ePCR assay did not require the purchase of additional equipment other than a thermal cycler and CE genetic analyzer already used for HID testing.

Ensuing graduate research enhanced the capabilities of this ePCR quantitative assay. In 2007, Benson added the male-specific SRY primer sequence and a non-human target to serve as an internal positive control (Benson, 2007). Benson's multiplex enabled the ePCR quantitative assay to estimate the quantity of male-only DNA for Y-chromosomal STR amplification, and provided a mechanism to detect inhibitors in the sample extract that may interfere with PCR amplification. In 2008, Wilson completed a developmental validation of this ePCR assay for use in forensic DNA laboratories based on criteria outlined by the Scientific Working Group on DNA Analysis Methods (SWGDAM) Revised Validation Guidelines (RVG).

.*II.E. In Vitro Degradation Methods*

Several in vitro degradation methods were considered for this study based on the literature and practitioner communications. One popular DNA degradation method discussed in the literature involves time-dependent exposure of DNA to the activated DNase I enzyme (Bender et al., 2004; Benson, 2007; Chung et al., 2004; Swango et al., 2006; Timken et al., 2005). Exposure to DNase I endonuclease activity in the presence of Mg^{2+} yields a random selection of DNA fragments generated from both double-stranded and single-stranded DNA (Promega Corporation, 2005). Although the DNase I treatment appears well suited for this study, this method of degradation requires the purchase of additional reagents, is labor intensive, and difficult to control.

Sonication is another method of DNA degradation discussed in the literature. Sonication utilizes high-frequency sound waves to irradiate DNA, which results in a random assortment of fragments of differing size. Bender et al. (2004) utilized sonication as part of a two-step method of controlled degradation, which was coupled with DNase I treatment. According to their study, the rate of DNA fragmentation was monitored empirically using agarose gel electrophoresis in intervals of 30-second ultrasound exposure. They determined that sonication for 5 minutes produced DNA fragments no smaller than 200 bp in length, and extended sonication had no additional effect on fragment size. Although sonication could produce DNA fragments within the mid-size locus range of HID amplification kits, this method would require the purchasing of additional equipment, such as a Branson Sonifier® ultrasonic cell disrupter (Branson Ultrasonic Corporation, Danbury, CT), and associated training to become familiar with this technique.

A third method of degradation discussed within the literature involves exposure of DNA samples to ultraviolet (UV) light. Several previous studies have utilized this approach by mimicking environmental scenarios that might affect the integrity of forensic samples. In two

independent studies, Krenke et al. (2002) and Micka et al. (1999) exposed bloodstained samples to summertime light and temperature in Pennsylvania and Florida respectively. In both studies the samples were collected over intervals of days, weeks, and even months. After environmental exposure, Micka et al. (1999) amplified the samples using PowerPlex® 1.1/Amelogenin and FFFL Multiplex Systems (Promega Corporation, Madison, WI) and reported "After 14 days of environment exposure, decreased yield was observed for the larger amplification products, suggesting degradation or damage to the DNA derived from these samples" (Micka et al., 1999). However, Krenke et al. (2002) reported no loss of allelic information from their samples, which were amplified using the PowerPlex® 16 HID System (Promega Corporation, Madison, WI).

Even if a reproduction of these environmental studies was performed, or if samples were exposed to a lab-generated UV light source, the physical changes that occur to DNA may not actually mimic the traditional definition of degradation involving strand breakage. The mutagenic effects of UV light are caused by the absorption of energy, which covalently joins pyrimidine molecules. This physical change is defined as pyrimidine dimers (also called thymine dimers) because UV light distorts the double-helix strand causing bases to move closer together (Hartl and Jones, 2009). This UV-induced change to the template strand may cause insertions, deletions, or other forms of "DNA damage that prevent subsequent replication of the genome" (Brown, 2002). Because UV exposure does not cause strand fragmentation, it could be argued that UV exposure should be classified only as a mutagen as opposed to a source of DNA degradation. Therefore, this method was not further considered as a source of in vitro degradation for this research.

It is interesting to note one final study portrayed in the literature that utilized degraded DNA for the validation of a mini-STR multiplex. In this article, the authors obtained 92 aged bloodstain samples that were stored at room temperature for up to 15 years prior to extraction (Butler et al., 2003). Although no mention of allelic drop-out was reported in their study, the

authors did notice reduced signal intensity from alleles present in the larger loci of the PowerPlex® 16 HID System (Promega Corporation, Madison, WI). Although the implications of a replicate study as described by Butler et al. (2003) may promote practitioner discussions regarding the most appropriate method for the long-term evidence storage, such as dry, frozen, or refrigerated environments, a set of samples maintained for this length of time could not be obtained for this research. In addition, the degree of degradation would likely be insignificant for samples stored at room temperature as indicated in Butler et al., (2003).

One method that appeared well suited for a controlled degradation experiment involves incubating DNA in water at high temperature. Previous experimentation with this method was performed using genomic DNA from the K562 cell line, and appeared to degrade DNA in a very controlled manner over time (Bruce McCord and Jing Wang, personal communications, May, 2010). Degradation induced by heating can be halted by simply placing samples on ice. Therefore, the exact volume and concentration of input DNA would remain constant throughout the course of this procedure barring evaporation, which can be monitored before and after high-temperature exposure. Furthermore, this procedure requires no additional reagents or equipment with the exception of nuclease-free water and a thermal cycler capable of maintaining high temperature for an extended period of time.

CHAPTER III

METHODOLOGY

III.A. Experimental DNA Samples

Two independent sets of samples were utilized in this research: A high-molecular weight human male DNA sample generated from an OSU faculty member, and a series of casework male reference samples previously quantitated using the ePCR assay and profiled using Applied Biosystems' Identifiler® HID amplification kit (Applied Biosystems, Foster City, CA) at the Tulsa Police Department Forensic Laboratory. These samples were extracted using two different techniques. The OSU sample was extracted with a Proteinase K solution, and concentrated and de-salted with an ethanol wash to obtain a high-molecular weight sample. This sample was stored at -20ºC until the high-temperature degradation step was performed. The casework buccal swabs were extracted using Promega's Tissue and Hair Extraction Kit, and purified using Promega's manual DNA IQ™ System (Promega Corporation, Madison, WI). After extraction each sample was stored at -20ºC until quantitative ePCR and HID amplifications were performed.

III.B. In Vitro Degradation Protocol

A protocol was developed for this research to degrade the high-molecular weight DNA sample in water using high temperature as described by McCord and Wang (Bruce McCord and Jing Wang, personal communications, May, 2010). This high-molecular weight sample was previously determined to contain a concentration of 199 ng/μL based on UV spectrophotometry. For this degradation protocol the high-molecular weight sample was diluted to 52.4 ng/μL in a final volume of 380 μL using nuclease-free PCR quality water (Teknova Corporation, Hollister, CA). This concentration was selected because the male NIST SRM 2372A contains a comparable DNA concentration. Aliquots of 20 μL were dispensed into individual microAmp tubes for each degradation timepoint. An AB 9700 thermal cycler was programmed to maintain a temperature of 95ºC for the high-temperature incubation (Applied Biosystems, Foster City, CA).

Three separate degradation experiments were performed using this protocol, and the summaries are described in Table 1. The t=0 timecourse sample contained a 20 μL aliquot of the 52.4 ng/μL dilution, and was stored at -20ºC. All other timecourse samples were placed on the thermal cycler set at 95ºC for their respective times. At completion of each time interval, the sample was removed from the thermal cycler, placed on a chilled micro-tube rack, and stabilized at -20ºC. The samples were then thawed, and the volume measured to monitor any loss of sample due to evaporation. No instance of evaporation was ever observed in any of the samples prepared for this research. At the conclusion of the degradation protocol all samples were either stored in the freezer, or immediately subjected to agarose gel electrophoresis.

Sample Preparation for the In Vitro Degradation Experiments

Date of Analysis	Starting Sample Concentration	Dilution Target (final volume 380 µL)	Total Aliquots (20 µL each)	Degradation Timepoints
06/28/10	199 ng/µL	52.4 ng/µL	11	0, 1, 2, 3, 4, 5, 10, 20, 30, 40, 60
08/05/10	199 ng/µL	52.4 ng/µL	19	0, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90
05/17/11	199 ng/µL	52.4 ng/µL	11	0, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90

Table 1: Three in vitro degradation experiments were performed during this study. All three experiments utilized the same starting tube of the high-molecular weight DNA sample. Aliquots of the original starting material were diluted to 52.4 ng/µL prior to high-temperature incubation, and were exposed to 95ºC over the course of several timepoints extending from t=0 minutes to t=90 minutes.

III.C. Yield Gel Detection

Yield gel electrophoresis was performed on the timecourse samples as a means to visually assess degradation. A 1% agarose gel was prepared using 0.60 grams of powdered agarose in 60 mL of 1X TAE solution. Each lane contained a total input volume of 12 µL (containing approximately 500 ng of DNA) consisting of 9.5 µL of each timecourse sample and 2.5 µL of 5X loading dye. Samples were electrophoresed at 75 volts for approximately 2 hours for fragment separation. Samples were visually assessed using ethidium bromide staining, and photographed under UV light. An Axygen Biosciences ladder containing a fragment range of 100 bp – 3,000 bp was co-electrophoresed with the degradation samples (Axygen Biosciences, Union City, CA).

III.D. Quantitative ePCR Amplification

Casework male reference samples were quantitated using a "homebrewed" version of the ePCR assay. Each amplification included 1 μL of input DNA into a reaction mix containing 2.65 μL of Teknova PCR-quality water (Teknova Corporation, Hollister, CA), 1.25 μL of 1X triple primer mix, 7.5 μL of 1.2X GoTaq® Colorless Mastermix (Promega Corporation, Madison, WI), and 0.1 μL of 5 pg/μL pRL plasmid (Wilson, 2008). The high-molecular weight timecourse samples were quantitated using the manufactured Maven QST™ quantitation kit (Maven Analytical, LLC, Milton, WV) following the formulation described above.

All samples were co-amplified with a serial dilution of NIST SRM 2372 Standard A, which is a single source sample containing 52.4 ng/μL of human male DNA. The NIST SRM was diluted using nuclease-free PCR-quality water (Teknova Corporation, Hollister, CA) to make a standard curve series that includes the following concentrations: 1000 pg/μL, 500 pg/μL, 250 pg/μL, 125 pg/μL, 62.5 pg/μL, and 31.25 pg/μL. All samples were amplified using an AB 9700 thermal cycler (Applied Biosystems, Foster City, CA). Each amplification underwent a total of 30 PCR cycles using the following pre-programmed parameters: 98ºC for 10 seconds, 55ºC for 60 seconds, 72ºC for 30 seconds, a 60ºC 10-minute hold, and an infinite hold of 25ºC (Wilson et al., 2010).

III.E. Quantitative ePCR Electrophoretic Detection

Post PCR amplification both the casework male reference samples and the high-molecular weight timecourse samples were analyzed using either an AB 310 genetic analyzer, or an AB 3130xL genetic analyzer (Applied Biosystems, Foster City, CA). The following sampling conditions were met when using an AB 310 genetic analyzer: 1 μL of DNA product was mixed into a 25 μL solution containing 24.5 μL of HiDi formamide (Applied Biosystems, Foster City,

CA) and 0.5 μL of GeneScan-500 LIZ internal size standard (Applied Biosystems, Foster City, CA). The AB 310 genetic analyzer electro-kinetically injected each sample for one second, and electrophoresed the product for 21 minutes. The following sampling conditions were met when using an AB 3130xL genetic analyzer: 1 μL of DNA product was mixed into a 10 μL solution containing 9.5 μL of HiDi formamide (Applied Biosystems, Foster City, CA) and 0.5 μL of Internal Lane Standard 600 (Promega Corporation, Madison, WI). The AB 3130xL genetic analyzer electro-kinetically injected each sample for one second, and electrophoresed the product for 21 minutes.

III.F. Quantitative ePCR Data Normalization

After electrophoresis, Genemapper ID software (version 3.2, Applied Biosystems, Foster City, CA) was used to spectrally resolve fluorescent dye colors, size-call amplicon products, and measure the amplicon peak-height in relative fluorescence units (hRFU). A peak-height detection threshold was set at 50 hRFU. The total-human quantitative standard curve was determined by plotting the averaged hRFU values of the Amelogenin X and Amelogenin Y amplicons from each NIST sample serial dilution. The R^2 value of the total-human standard curve was evaluated for linearity and compared to the averaged X and Y hRFU values of each degradation sample to determine respective total-human DNA estimates. The male-only quantitative standard curve was determined by plotting the hRFU values of the SRY amplicon from each NIST sample serial dilution. The R^2 value of the male-only standard curve was evaluated for linearity and compared to the SRY hRFU values of each degradation sample to determine respective male-only DNA estimates.

III.G. Qualitative Assessment of Samples via a Degradation Ratio

A degradation ratio was determined for each sample by dividing the male-only quantitative estimate (pg/μL) by the total-human quantitative estimate (pg/μL). As previously described, the male-only quantitative estimate is derived from a small haploid SRY target (110 bp), and the total-human quantitative estimate is derived from a large diploid Amelogenin target (X, 210 bp; Y, 216 bp). A non-degraded male sample would be expected to achieve a theoretical ratio of 1.0 because the sample should render comparable male-only and total-human quantitative estimates.

III.H. HID Amplification

Casework male reference samples were subsequently HID amplified using Applied Biosystems' Identifiler® STR typing kit (Applied Biosystems, Foster City, CA). The quantitative estimates for each sample were determined using the "homebrewed" version of the ePCR assay. Each Identifiler® reaction was amplified according to manufacturer recommendations using a 25 μL total-volume reaction containing10 μL of 1 ng input DNA and 15 μL of Identifiler® reagents. Samples were detected with an AB 310 genetic analyzer using 1.5 μL of DNA product mixed into a 25 μL solution containing 24.5 μL of HiDi formamide (Applied Biosystems, Foster City, CA) and 0.5 μL of GeneScan-500 LIZ internal size standard (Applied Biosystems, Foster City, CA). The AB 310 genetic analyzer electro-kinetically injected each sample for five seconds, and electrophoresed the product for 28 minutes. GeneScan Analysis data collection software (version 3.1.2; Applied Biosystems, Foster City, CA) was used to resolve fluorescent dye colors and size-call amplicon product. Genotyper software (version 2.5.2; Applied Biosystems, Foster City, CA) was used to measure hRFU values and determine the sample genotype based on comparison to a co-electrophoresed allelic ladder.

The high-molecular weight timecourse samples were HID amplified using either Applied Biosystem's Profiler Plus® STR typing kit (Applied Biosystems, Foster City, CA), or Promega's PowerPlex® 16 Hot Start STR typing kit (Promega Corporation, Madison, WI). In both instances the quantitative estimates for each sample were determined using the commercially manufactured Maven QST™ ePCR quantitation kit (Maven Analytical, LLC, Milton, WV). HID amplifications using the Profiler Plus® STR typing kit targeted two separate reaction concentrations: 1) targeting an optimal 300 pg concentration of input DNA, and 2) targeting 1,200 pg, which is four-times the optimal concentration of input DNA. The four-times optimal input concentration was selected to intentionally over-amplify the DNA product as a means to assess the effects of degradation upon larger STR loci. Both types of Profiler Plus® reactions were amplified according to manufacturer recommendations using an 8 μL total-volume reaction containing 0.5 μL of input DNA and 7.5 μL of Profiler Plus® reagents. Samples were detected with an AB 310 genetic analyzer using 0.5 μL of DNA product mixed into a 25.25 μL solution containing 25 μL of HiDi formamide (Applied Biosystems, Foster City, CA) and 0.25 μL of GeneScan-500 ROX internal size standard (Applied Biosystems, Foster City, CA). An AB 310 genetic analyzer electro-kinetically injected each sample for five seconds, and electrophoresed the product for 28 minutes. Genemapper ID software (version 3.2; Applied Biosystems, Foster City, CA) was used to resolve fluorescent dye colors, size-call allelic targets, measure hRFU values, and determine the sample genotype based on comparison to a co-electrophoresed allelic ladder.

HID amplifications of the high-molecular weight timecourse samples using Promega's PowerPlex® 16 Hot Start STR typing kit (Promega Corporation, Madison, WI) also targeted two separate reaction concentrations: 1) targeting an optimal 500 pg concentration of input DNA, and 2) targeting 2,000 pg, which is four-times the optimal concentration of input DNA. The four-times optimal input concentration was selected to intentionally over-amplify the DNA product as a means to assess the effects of degradation upon larger STR loci. Both types of PowerPlex® 16

Hot Start reactions were amplified according to manufacturer recommendations using a 25 μL total-volume reaction containing 17.5 μL of input DNA and 7.5 μL of PowerPlex® 16 Hot Start reagents. Samples were detected with an AB 3130xL genetic analyzer using 1 μL of DNA product mixed into a 10 μL solution containing 9.5 μL of HiDi formamide (Applied Biosystems, Foster City, CA) and 0.5 μL of Internal Lane Standard 600 (Promega Corporation, Madison, WI). The AB 3130xL genetic analyzer electro-kinetically injected each sample for five seconds, and electrophoresed the product for 28 minutes. Genemapper ID software (version 3.2; Applied Biosystems, Foster City, CA) was used to resolve fluorescent dye colors, size-call allelic targets, measure hRFU values, and determine the sample genotype based on comparison to a co-electrophoresed allelic ladder.

CHAPTER IV

RESULTS & DISCUSSION

IV. A. Former Method of Degradation Assessment: Detection via Yield Gel Electrophoresis

The first opportunity to observe the effects of the degradation protocol on the high-molecular weight samples occurred with yield gel electrophoresis. The summaries of all yield gel experiments are described in Table 2. It is important to note that each yield gel produced data suggesting the successful fragmentation of samples over the duration of the timecourse. Moreover, this degradation appeared to be controlled in that each sample with an extended exposure time contained progressive fragmentation of template DNA into smaller and smaller sizes (Figure 7). This fragmentation is demonstrated by sample smearing within the yield gel, which appears to progress at a linear rate over time.

Yield Gel Results with Degraded DNA

Experiment	Starting Sample	Total Concentration Per Lane	Timepoints (Time Intervals)	Visual Result of Degradation
Initial	52.4 ng/μL	498 ng	0 – 5, (1 min), 10– 40 (10min), 60	Success
Second	52.4 ng/μL	498 ng	0 – 90 (5min)	Success
Third	52.4 ng/μL	498 ng	0, 5, 10 – 90 (10min)	Success

Table 2: Three separate yield gels were performed during the course of this research. Each yield gel corresponds to a high-temperature degradation experiment performed on the high-molecular weight samples. See Table 1 for a corresponding summary of the respective in vitro degradation experiments.

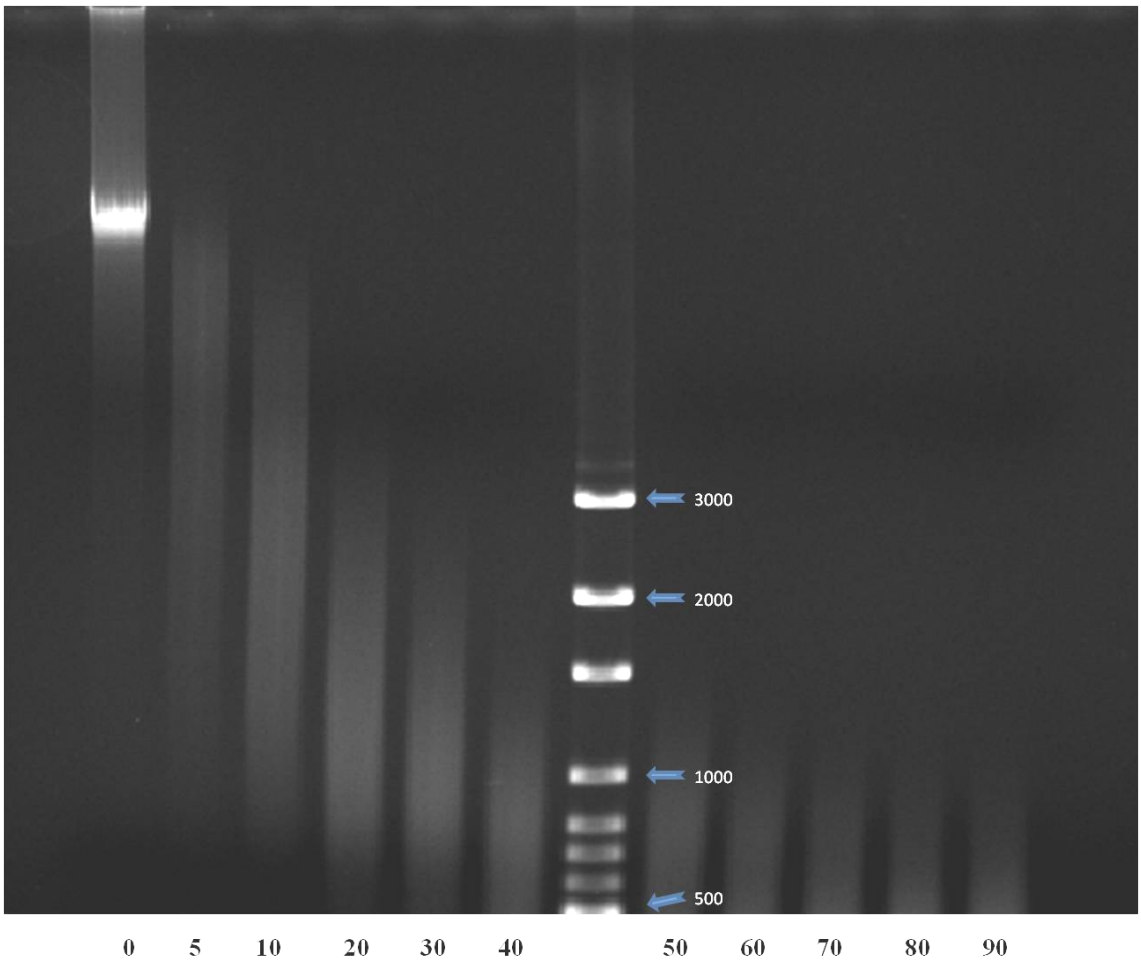

Figure 7: Yield gel detection as a means to assess degradation within the high-molecular weight samples. This yield gel was produced from the third experiment, and contains 11 timecourse samples exposed in minutes to high-temperature degradation. Lane 1 contains the t=0 timepoint sample. Notice the high-quality intactness of this sample, which extends well above the upper 3,000 bp fragment within the ladder (Lane 7).

IV. B. Quantitative ePCR Amplification Results

The second opportunity to observe the effects of the degradation protocol on the high-molecular weight samples occurred with the ePCR quantitative assay. Quantitation of all high-molecular weight samples from the three high-temperature degradation experiments was conducted both to evaluate the quantity of template DNA for HID amplification, as well as to assess a ratio between the quantitative estimates produced from Amelogenin or SRY for each sample. A total of 11 sets of individual amplifications were performed for each timecourse sample using the manufactured Maven QST™ quantitation kit (Maven Analytical, LLC, Milton, WV). Note that all timecourse samples associated with the third degradation experiment were amplified in triplicate on three separate occasions. A summary of all quantitative amplifications is provided in Table 3.

Data analysis of all quantitative amplifications was performed by comparing the high-molecular weight samples to a co-amplified NIST standard curve of known quantities. Comparison of the hRFU values from the timecourse sample to the NIST standard curve resulted in two independent quantitative estimates: 1) a total-human quantitative estimate produced from the averaged hRFU values of the large diploid Amelogenin target, and 2) a male-only quantitative estimate produced from the hRFU value of the small haploid SRY target. In all instances, the two quantitative estimates were calculated in a pg/μL format using a standardized excel worksheet.

Quantitative Amplifications of Timecourse Samples
from Three Degradation Experiments

Experiment	Date	Number of Samples	Timepoints (Time Intervals)	Amplifications per Timepoint	Detection Instrument
Initial	07/02/10	11	0 – 5, (1 min), 10– 40 (10min), 60	1	310
Second	09/26/10	19	0 – 90 (5min)	1	310
Third	05/26/11	11	0, 5, 10 – 90 (10min)	3	3130xL
Third	06/03/11	11	0, 5, 10 – 90 (10min)	3	3130xL
Third	06/12/11	11	0, 5, 10 – 90 (10min)	3	3130xL

Table 3: Summary of quantitative ePCR amplifications performed with the manufactured Maven QST™ quantitation kit (Maven Analytical, LLC, Milton, WV). Note that quantitative amplification was performed in triplicate on the timecourse samples from the third experiment on three separate occasions.

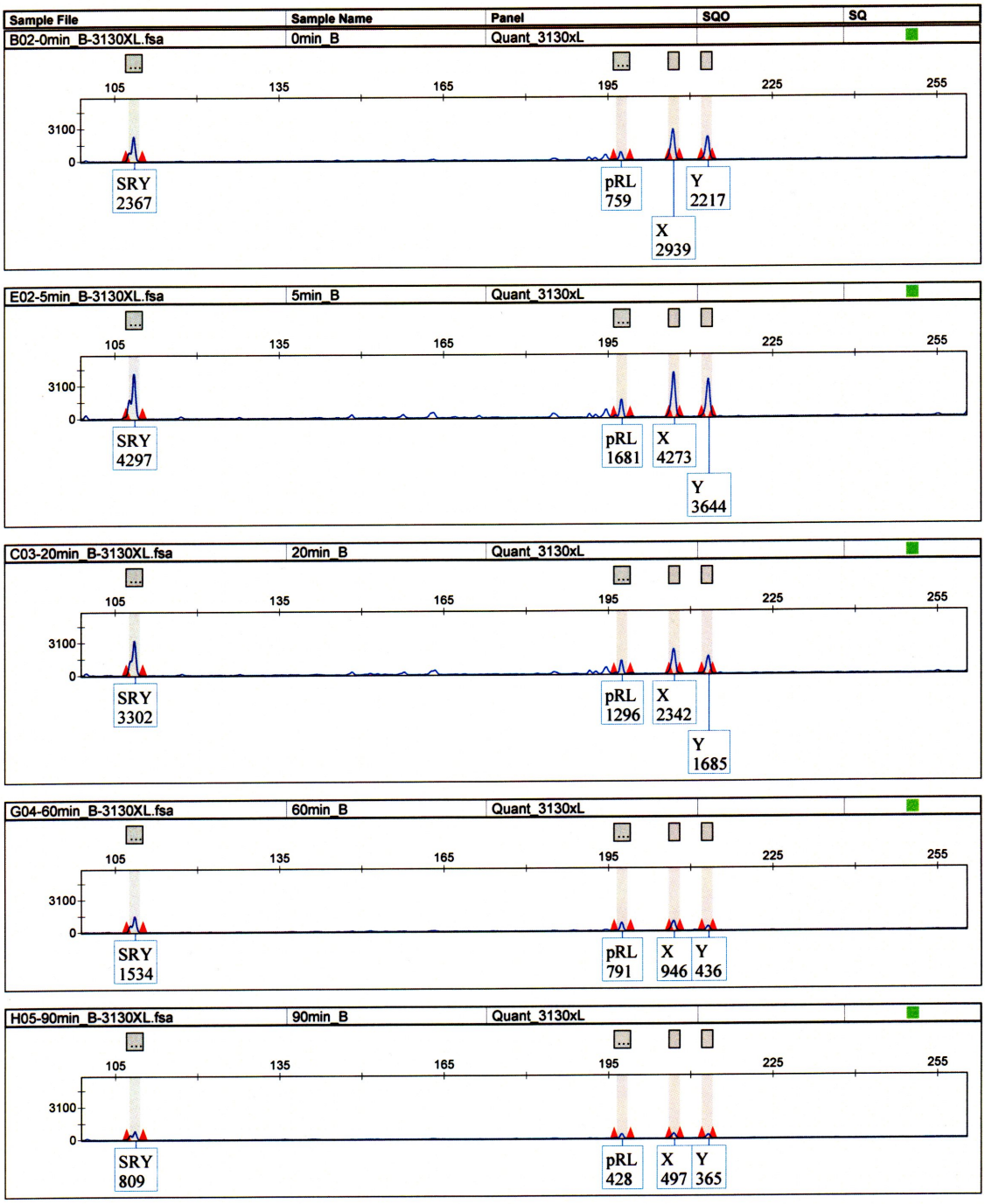

Figure 8: Quantitative electropherograms produced from the Maven QST™ quantitation kit (Maven Analytical, LLC, Milton, WV). Electropherograms displayed include: t=0, t=5, t=20, t=60, and t=90 –minute samples arranged from top to bottom. Recall that the associated peak heights of each sample are used to calculate both quantitative estimates. Notice the increase in hRFU between the t=0 and t=5 samples. HRFU values then decrease during the duration of the timecourse.

All timecourse estimates were normalized to a ng/µL value to evaluate the ePCR quantitative performance in comparison to the initial 199 ng/µL value obtained from UV spectrophotometry. Because different timepoints were targeted within each experiment, a consensus of timepoint samples was selected to further evaluate the degradation ratios from as many datapoints as possible. The following 11 timepoints from each degradation experiment were selected for this analysis: t=0, t=5, t=10, t=20, t=30, t=40, t=50, t=60, t=70, t=80, and t=90. Note that the total-human and male-only quantitative estimates were derived from 11 separate quantitative amplifications with the exception of the t=50, t=70, t=80, and t=90 timepoints, which contained 10 separate quantitative amplifications due to the limited time range of the initial experiment.

The mean total-human and male-only quantitative estimates (ng/µL) were graphed individually to observe the effects of the high-temperature degradation protocol. Figure 9 represents the mean total-human quantitative estimates, and Figure 10 represents the mean male-only quantitative estimates. Both figures also include associated error bars that represent the variability observed in amplification and detection of the high-molecular weight DNA with the quantitative assay. The literature indicates an expected variance rate of ±30% in the assay's performance (Wilson et al., 2010). Interestingly, the variability of both quantitative estimates gradually decreases over the timecourse as the high-molecular weight DNA sample degrades. It is also noteworthy to mention that the mean total-human quantitative estimate obtained from the non-degraded t=0 sample is 206 ng/µL, which is a difference of only 7 ng/µL from the original quantitative estimate derived from UV spectrophotometry. However, the mean male-only quantitative estimate from the same sample is 123 ng/µL. In addition, both quantitative estimates increase in quantity between the t=0 and t=5 timepoints, then gradually decrease during subsequent exposure times, and finally approach 50 ng/µL at or around the t=90 timepoint.

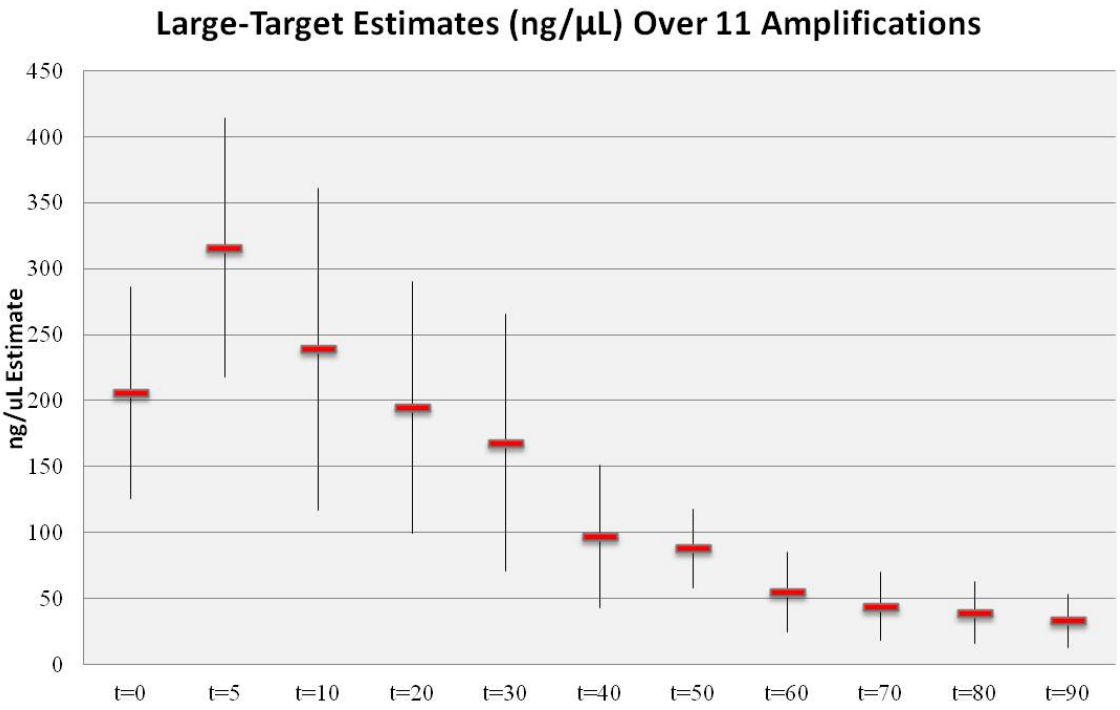

Figure 9: Average total-human quantitative estimates (ng/µL) of each timecourse sample derived from the hRFU values of the large diploid Amelogenin target. Each averaged estimate was based on all quantitative amplifications produced from the timepoint samples generated in this research. Associated error bars depict the variability resulting from the in vitro degradation method, PCR amplification, and the day-to-day performance of genetic analyzer instruments.

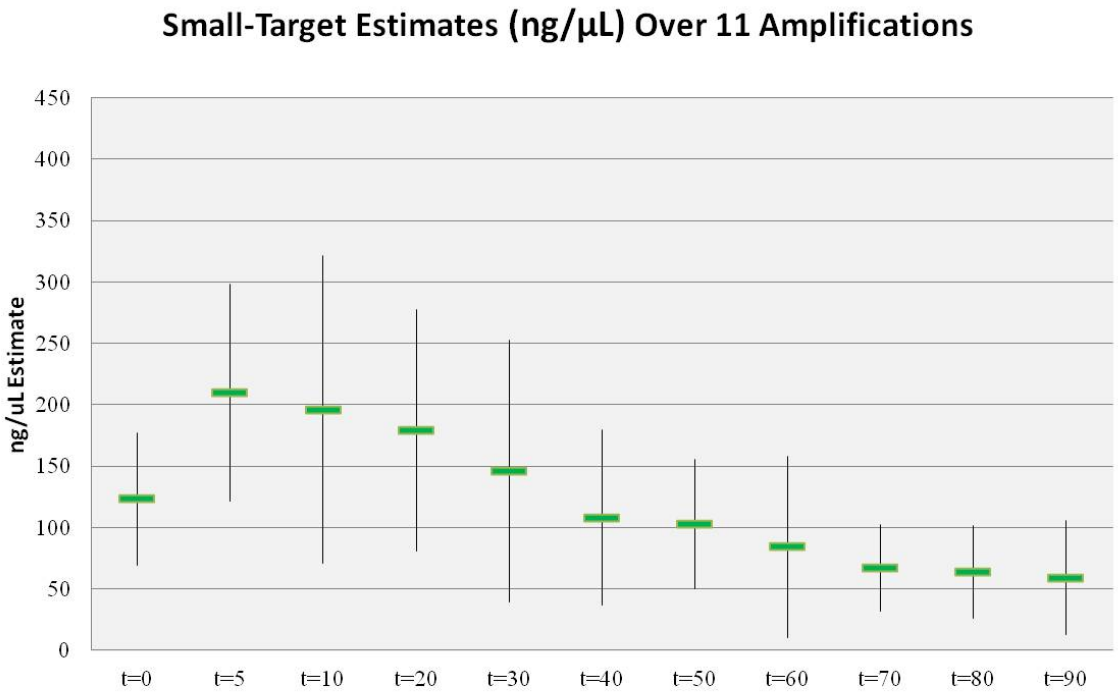

Figure 10: Average male-only quantitative estimates (ng/µL) of each timecourse sample derived from the hRFU values of the small haploid SRY target. Each averaged estimate was based on all quantitative amplifications produced from the timepoint samples generated in this research. Associated error bars depict the variability resulting from the in vitro degradation method, PCR amplification, and the day-to-day performance of genetic analyzer instruments.

The total-human and male-only quantitative estimates are directly compared in Figure 11 to display trends and overlap that occurred throughout the degradation timecourse. As previously discussed, a disparity exists between the two quantitative estimates derived from the t=0 sample. The total-human quantitative estimate is 206 ng/µL and the male-only quantitative estimate is 123 ng/µL. A possible explanation for this disparity can be attributed to the Dimitrov and Apostolova theory regarding the limit of PCR amplification as it relates to primer-binding opportunity (Dimitrov and Apostolova, 1996).

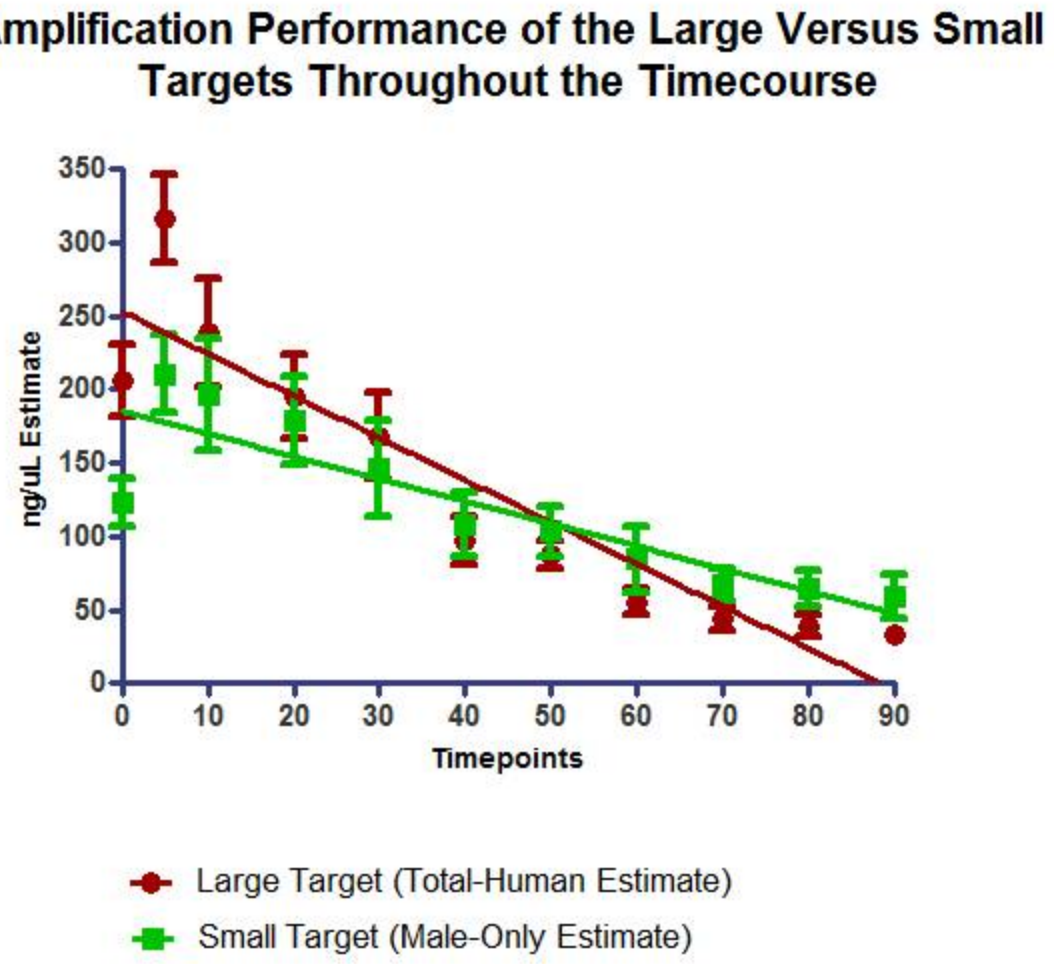

Figure 11: Comparison of the two quantitative estimates (ng/µL) derived from the high-molecular weight sample over the course of the degradation experiment. Each datapoint represents the mean values obtained from 11 independent amplifications with the exception of the t=50, t=70, t=80, and t=90 datapoints, which contain mean values from 10 independent amplifications. A significant difference was observed between the slopes of each target using linear regression ($p < 0.001$) indicating that the small target outperforms the large target over the duration of the experiment.

Another interesting trend displayed in Figure 11 is the increase in both mean quantitative estimates between the t=0 and t=5 timepoints. The t=5 timepoint represents the first exposure of the high-molecular weight sample to heat. This initial exposure results in an increase for both the total-human and male-only quantitative estimates, which were 206 ng/μL and 123 ng/μL respectively at the t=0 timepoint. After the initial five-minute exposure the mean total-human estimate becomes 316 ng/μL and the mean male-only estimate becomes 210 ng/μL. A possible explanation for this increase in both quantitative estimates could be attributed to the physical intactness of the high-molecular weight sample, which begins to open as a result of high-temperature exposure. Recall that the yield gel displayed in Figure 7 depicts a tightly bound DNA molecule at the t=0 timepoint, which may be thought of as a large ball of yarn. Initially, this intact ball of yarn contains a reduced number of primer-binding sites accessible for primer annealing due to the limited surface area exposed. However, the high-temperature incubation causes the tightly bound DNA macromolecule to uncoil so more primer-binding sites become available to participate in PCR (Figure 12). This uncoiling effect of the high-molecular weight DNA sample is a component of the high-temperature degradation process. Both the total-human and male-only quantitative estimates increase in quantity because more template primer-binding sites participate in PCR.

Although high-molecular weight DNA is well suited for RFLP analysis, which employs yield gel electrophoresis as a quantitative technique, it appears that high-molecular weight samples may not be well suited for PCR-based methods. Whereas RFLP analysis utilizes a variety of restriction enzymes that are capable of physically manipulating the structure of highly-coiled DNA, PCR-based primers cannot manipulate the physical intactness of the DNA macromolecule. Rather, PCR primers are limited to the amount of target sequences readily exposed for primer binding during each PCR cycle. The less intact a DNA macromolecule is at the beginning of each cycle, the greater the chance that primers will locate and bind to their

respective target sequences so amplification may occur. The results of this research indicate that the external exposure to heat uncoils the DNA macromolecule, which aids in primer binding efficacy of high-molecular weight samples.

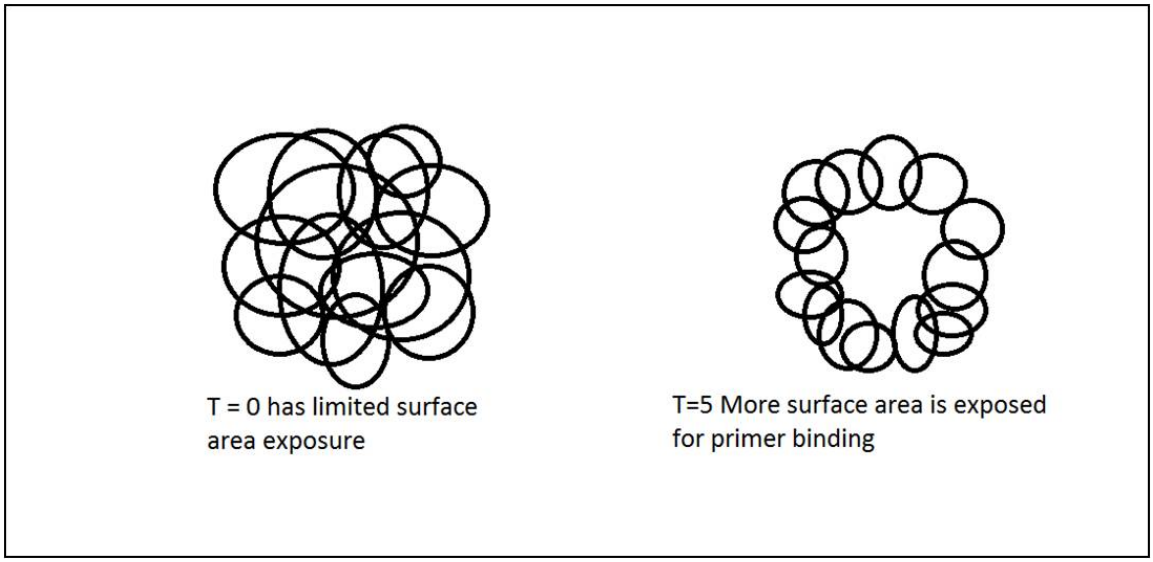

Figure 12: Surface area exposure increases primer-binding efficacy. As high temperature uncoils the high-molecular weight DNA molecule, more targets become available for primer binding.

Following the trends observed in Figure 11 through the duration of the timecourse, a negative slope is observed with both quantitative estimates. At the beginning of the experiment through the t=40 timepoint the large-target estimate contains values that are greater than the small-target estimate. However, the successive fragmentation of template DNA eventually results in small target outperforming the large target. A significant difference was observed between the two slopes over the duration of the timecourse using linear regression ($p < 0.001$). The statistical difference between the two slopes supports the ability to compare the individual performance of each target as a means to assess degradation. Once the male-only quantitative estimate attains a value larger than the total-human quantitative estimate the two estimates may be compared as a predictor for STR degradation.

IV. C. Degradation Ratio Results

A degradation ratio was constructed to evaluate the amplification efficacy of the SRY and Amelogenin primers by comparing the total-human and male-only quantitative estimates (Figure 13). The small target estimate represents the quantity predicted from the small haploid SRY target, and the large target estimate represents the quantity predicted from the large diploid Amelogenin target. The ratio compares the quantitative estimates as opposed to signal hRFU values because each target is normalized against a standard curve. Recall that, in theory, the ratio for a non-degraded male sample is expected to be about 1.0 because both quantitative estimates should render comparable quantities.

$$\text{RATIO} = \frac{\text{small-target estimate (SRY)}}{\text{large-target estimate (AMEL)}}$$

Figure 13: The degradation ratio derived from the comparison of both quantitative estimates from the high-molecular weight male sample amplified with the ePCR quantitative assay. Note that the quantitative estimates were calculated using a standardized excel worksheet.

The degradation ratios obtained from each timepoint of the high-molecular weight sample are presented in Figure 14. Notice that the degradation ratios obtained from the t=0 through t=30 timepoint samples are below 1.0. The first observation of a degradation ratio greater than 1.0 occurs at the t=40 timepoint. The largest degradation ratio obtained was 1.78 at the t=90 timepoint. This gradual increase in the degradation ratios throughout the timecourse appears to follow a linear function. The degradation ratio becomes very significantly different ($p < 0.01$) from t=0 at the t=60 timepoint and extremely significantly different ($p < 0.001$) from t=0 with

successive timepoints based on one-way ANOVA with Tukey's multiple comparison test. The t=60 timepoint corresponds to a ratio just above 1.5, and samples that have experienced degradation according to this research model would be expected to display significance between the amplification of the large and small targets in the high-molecular weight sample if a value of 1.5 were obtained in the ePCR assay.

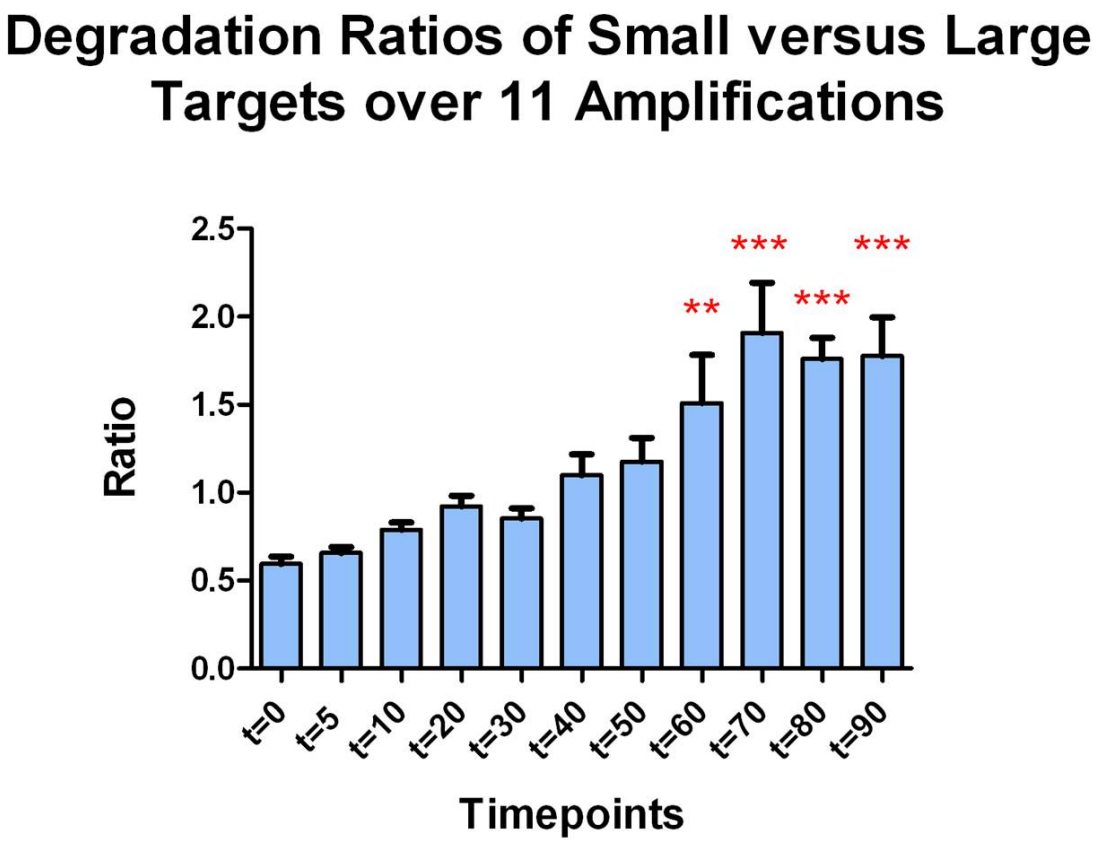

Figure 14: Degradation ratios obtained from each timepoint of the high-molecular weight sample throughout the degradation experiment. Significance tests were performed on the data using one-way ANOVA with Tukey's multiple comparison test. Notice that at the t=60 timepoint the ratios become very significant ($p < 0.01$), and successive timepoints indicate extreme significance ($p < 0.001$) in the degradation ratios.

IV. D. HID Amplification Results

A selection of timecourse samples from each degradation experiment were amplified with either of two HID typing kits using the total-human quantitative estimates obtained from the ePCR quantitative assay. Two sets of HID amplifications were performed with each kit targeting different input concentrations. The first amplification targeted an optimal concentration of input DNA within the amplification reaction based on manufacturer recommendations. The second amplification targeted four-times the optimal concentration of input DNA within the amplification reaction. The intentional excess of input DNA was performed to include a maximum quantity of high-molecular weight template sequences that may participate in the PCR reaction, and thus gauge the effects of degradation on large STR loci that would otherwise fail to amplify. The resulting genotype peaks were labeled with their associated allele-call and hRFU value using Genemapper ID software (version 3.2, Applied Biosystems, Foster City, CA). Two heterozygous loci encompassing the small and large bp size range were selected from the same fluorescent dye group in order to evaluate the performance of each HID kit using the high-molecular weight DNA exposed to the degradation protocol. Table 4 describes general manufacturer information regarding each HID typing kit, as well as the heterozygous loci in the high-molecular weight sample that were selected to evaluate HID kit performance.

(a) HID Typing Kits Amplified with Degraded DNA				
Kit Name	**Manufacturer**	**Date of Release**	**Locus Count**	**Size Range (bp)**
Profiler Plus®	Applied Biosystems	2006	10	107 – 341
PowerPlex® 16 Hot Start	Promega	2009	16	106 – 474

(b) Heterozygous Loci Selected for Comparison				
Kit Name	**Small Locus**	**Size Range (bp)**	**Large Locus**	**Size Rage (bp)**
Profiler Plus®	D8S1179	128 – 168	D18S51	273 – 341
PowerPlex® 16 Hot Start	D5S818	119 – 155	D16S539	264 – 304

Table 4: (a) Manufacturer information from the two HID typing kits amplified with the degraded high-molecular weight sample. Note the amount of loci multiplexed in each kit, and that both HID typing kits detect DNA targets significantly larger in bp size than the targets associated with the ePCR quantitative assay.

(b) The small and large heterozygous loci selected from each HID typing kit that are detected in the same fluorescent color group. These loci were studied to observe the effects of the high-temperature degradation experiment on the success of HID amplification.

The Profiler Plus® HID typing kit contains primer sequences that multiplex a total of 10 loci. The Amelogenin locus and nine of the FBI's 13 core STR loci are included in the Profiler Plus® HID typing kit. Figure 15 displays the arrangement of each locus multiplexed in the Profiler Plus® HID typing kit by fluorescent color and bp size. These loci range in size from 107 – 341 bp, and are separated with three dye colors as a part of a four-dye multiplex kit (AmpFℓSTR® Profiler Plus® PCR Amplification Kit User's Manual, 2006).

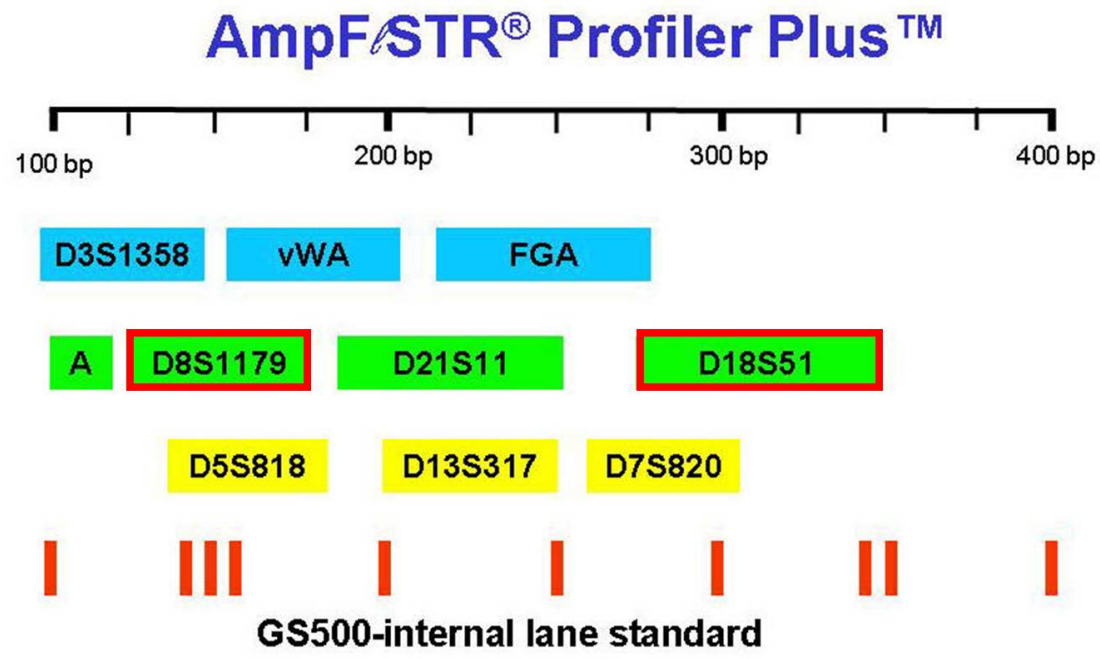

Figure 15: Base pair size range of loci amplified within the 10-locus AmpFℓSTR® Profiler Plus™ PCR Amplification Kit manufactured by Applied Biosystems. The outlined heterozygous loci in the high-molecular weight sample were selected to evaluate the amplification performance of this kit with degraded DNA (image from Butler and Reeder; modified).

The D8S1179 locus and the D18S51 locus were selected from the Profiler Plus® HID typing kit to compare the STR amplification performance of the high-molecular weight timecourse samples. These particular loci were selected because both are a part of the FBI's CODIS core loci, each locus represents a small and large target within the Profiler Plus® HID typing kit, and both loci amplify a heterozygous genotype in the high-molecular weight sample. Results of the 300 pg timecourse amplification displayed numerous instances where either partial information or complete drop-out was observed in the D8S1179 and D18S51 loci. The results from the 300 pg amplification of the degraded high-molecular weight samples were not further

assessed due to the limited amount of detectable information obtained. However, the results of the 1,200 pg timecourse amplification did produce information that could be further evaluated for each timecourse sample. Figure 16 compares the sum hRFU values detected in the small versus large loci. Figure 17 depicts the "ski-slope" effect present in data as observed in the electropherograms of the 1,200 pg Profiler Plus® amplification.

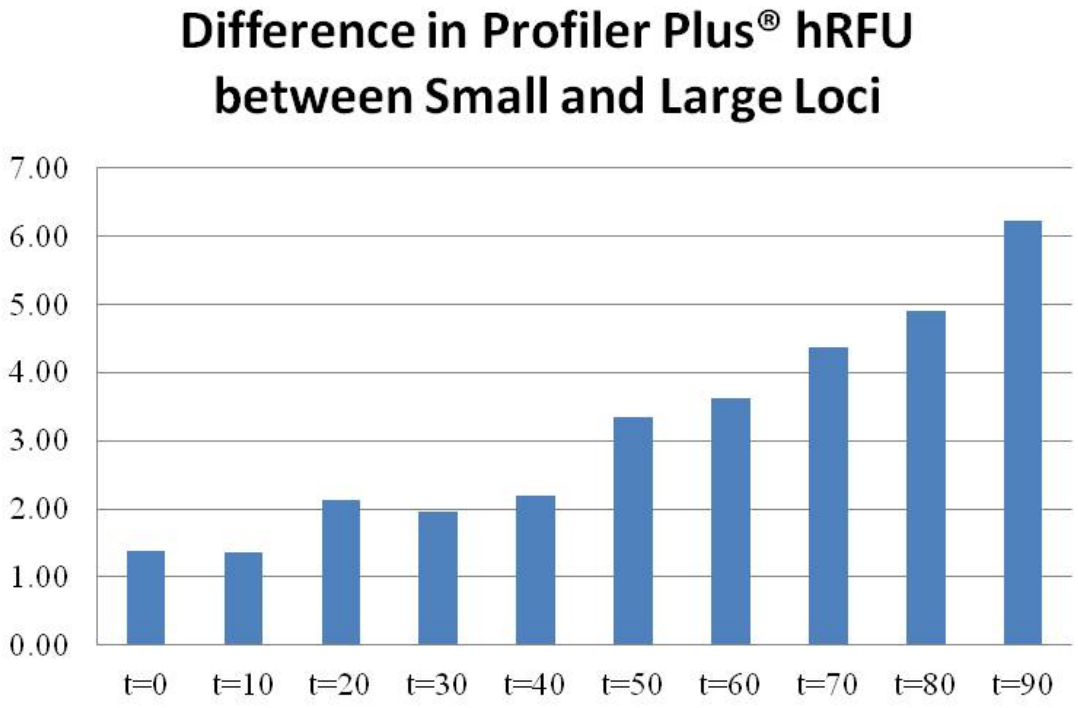

Figure 16: Ratios obtained from the single amplification of select timepoint samples targeting 1,200 pg of input DNA with the Profiler Plus® HID typing kit. The ratios represent the comparison of the hRFU values obtained from a small locus (D8S1179) versus the hRFU values obtained for a large locus (D18S51). Note that the t=0 sample was only amplified at the 300 pg concentration in this research. The progressive increase in the ratios of the small versus large loci over the duration of the timecourse mimic the trend also observed in the quantitative assay.

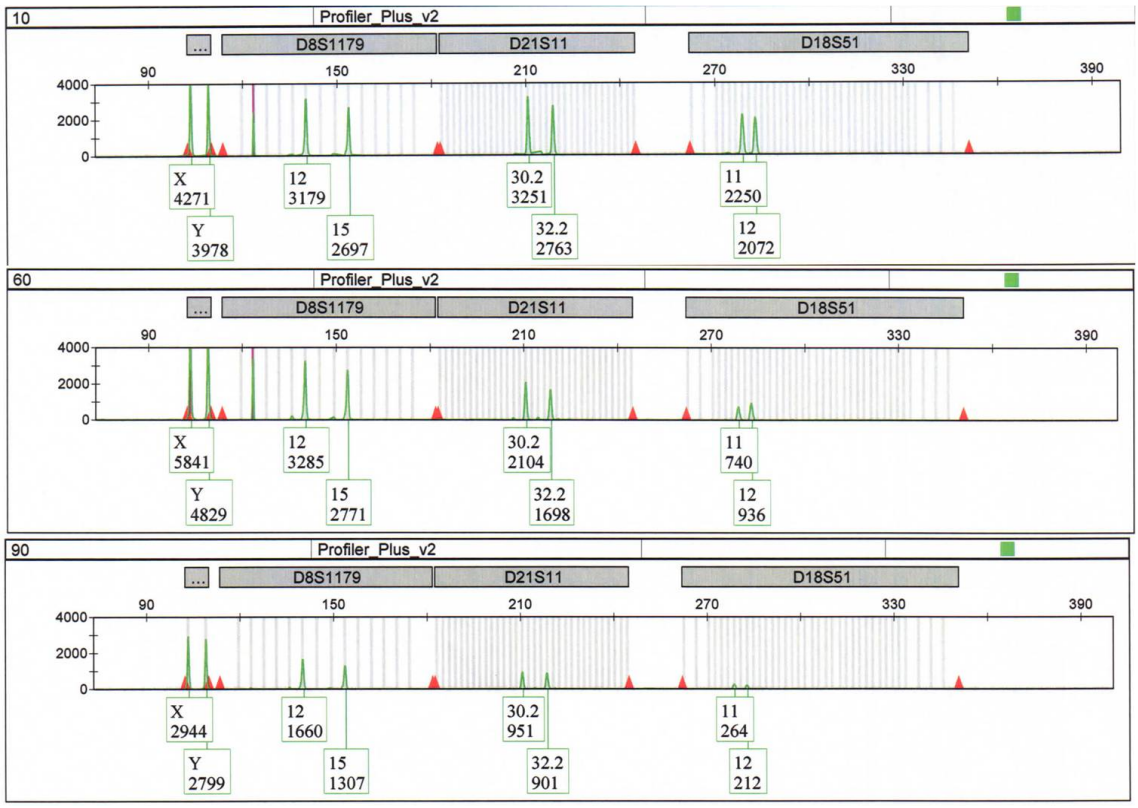

Figure 17: Loci detected in the green (JOE) dye color from the 1,200 pg Profiler Plus® amplification. Displayed electropherograms include the t=10 (top), t=60 (middle), and t=90 (bottom) timepoint samples. The t=0 sample was not included in this presentation because it was only amplified at the 300 pg concentration for this research. Each peak is labeled with its associated allele-call and hRFU value. Note the appearance of the "ski-slope" effect associated with the hRFU intensity across the four loci in each electropherogram.

The PowerPlex® 16 HID typing kit was the second of two HID kits used to amplify the high-molecular weight timecourse samples. The PowerPlex® 16 HID typing kit contains primer sequences that multiplex a total of 16 loci. All 13 of the FBI's core STR loci are included in this kit, as well as Amelogenin and two penta-repeat STR loci. Figure 18 displays the arrangement of each locus multiplexed in the PowerPlex® 16 HID typing kit by fluorescent color and bp size. These loci range in size from 106 – 474 bp, and are separated with three dye colors as a part of a four-dye multiplex kit (Powerplex® 16 HS System Technical Manual, 2011).

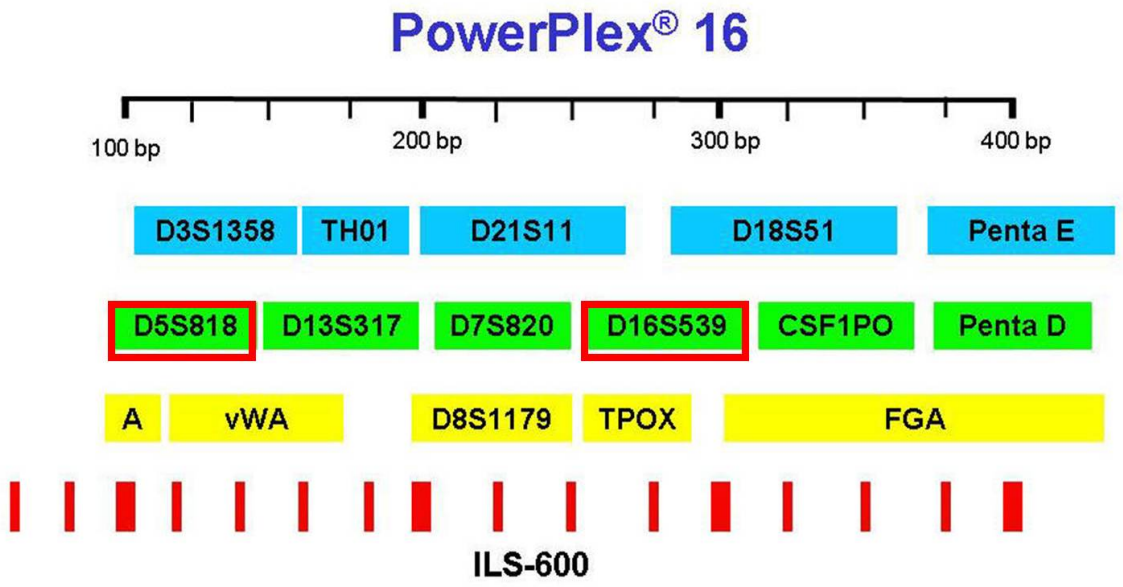

Figure 18: Base pair size range of loci amplified within the 16-locus PowerPlex® 16 Hot Start STR typing kit manufactured by Promega. The outlined heterozygous loci in the high-molecular weight sample were selected to evaluate the amplification performance of this kit with degraded DNA (image from Butler and Reeder; modified).

The D5S818 locus and the D16S539 locus were selected from the PowerPlex® 16 Hot Start HID typing kit to compare the STR amplification performance of the high-molecular weight timecourse samples. These particular loci were selected because both are a part of the FBI's

CODIS core loci, each locus represents a small and large target within the PowerPlex® 16 HID typing kit, and both loci amplify a heterozygous genotype in the high-molecular weight sample. Results of the 500 pg PowerPlex® 16 amplification displayed numerous instances where either partial information or complete drop-out were observed in the D5S818 and D16S539 loci. The results from the 500 pg amplification of the degraded high-molecular weight samples were not further assessed due to the limited amount of detectable information obtained. However, the results of the 2,000 pg amplification did produce information that could be further evaluated for each timecourse sample. Figure 19 compares the sum hRFU values detected in the small versus large loci. Figure 20 depicts the "ski-slope" effect present in data as observed in the electropherograms from the 2,000 PowerPlex® 16 amplification.

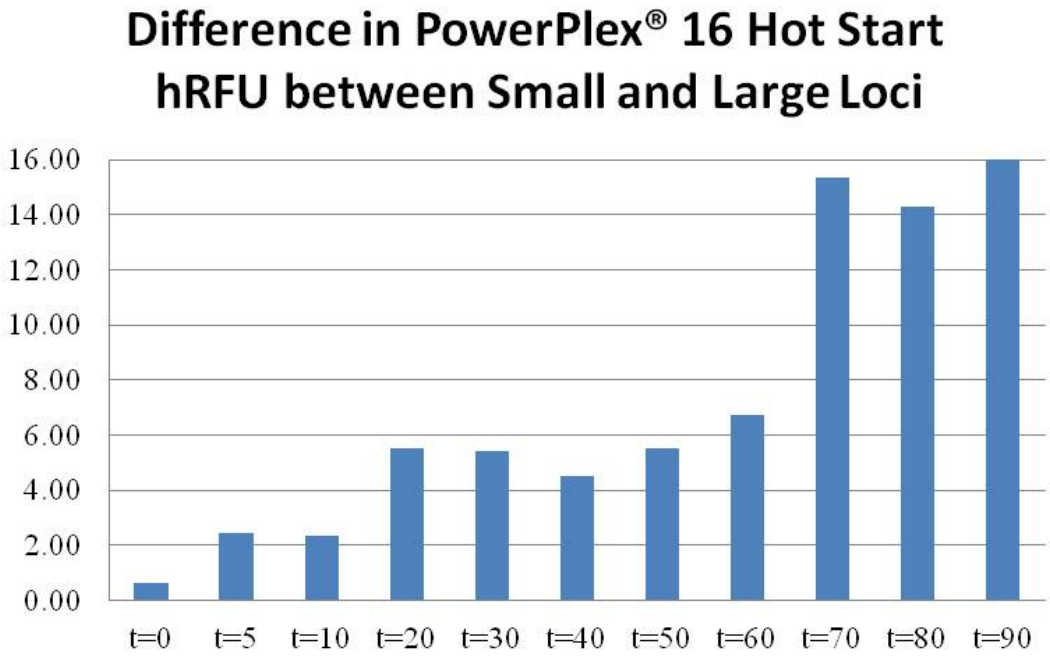

Figure 19: Ratios obtained from the single amplification of select timepoint samples targeting 2,000 pg of input DNA with the PowerPlex® 16 Hot Start HID typing kit. The ratios represent the comparison of the hRFU values obtained from a small locus (D5S818) versus the hRFU values obtained from a large locus (D16S539). The increase in ratios throughout the duration of the timecourse mimics the trend also observed in the quantitative assay.

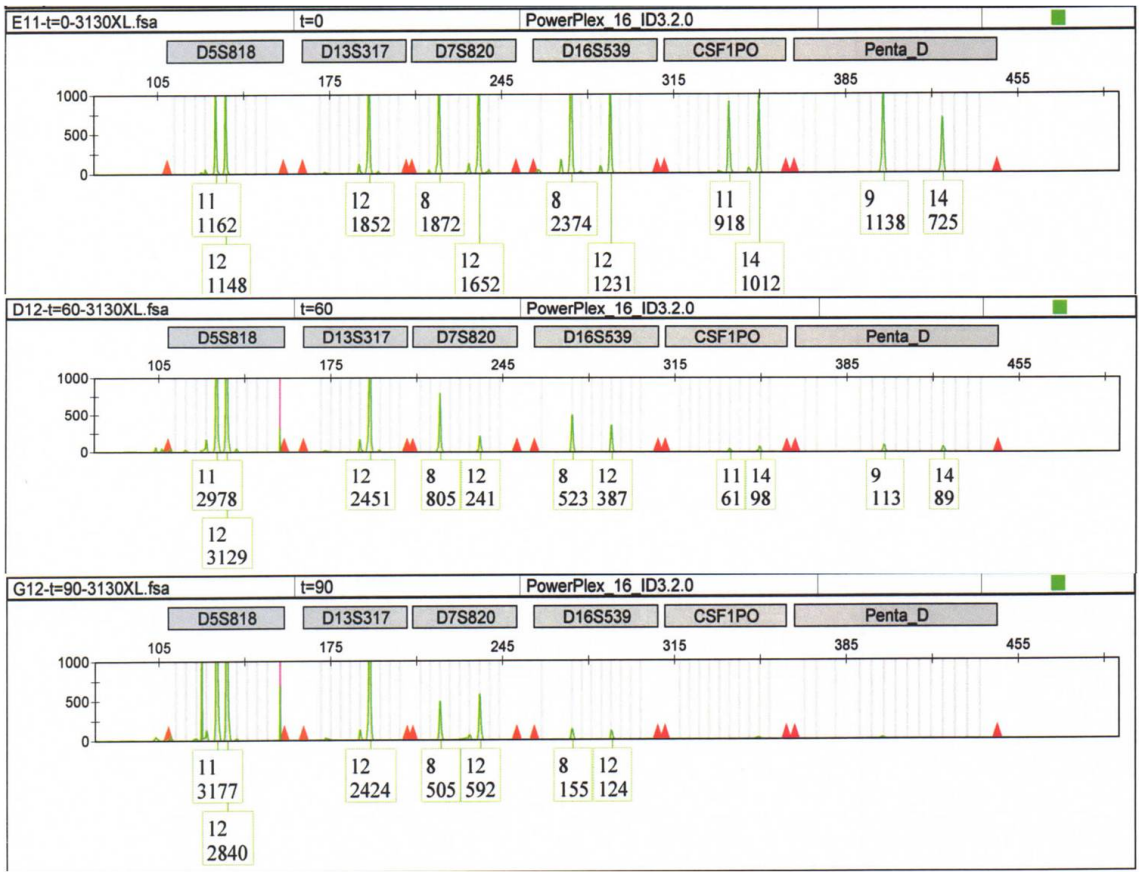

Figure 20: Loci detected in the green (JOE) dye color from the 2,000 pg PowerPlex® 16 Hot Start amplification. Displayed electropherograms include the t=0 (top), t=60 (middle), and t=90 (bottom) timepoints. Each peak within the locus range is labeled with its associated allele-call and hRFU value. Note the appearance of the "ski-slope" effect associated with a decrease in hRFU intensity across the six loci in the t=60 and t=90 sample electropherograms. Complete drop-out of STR information is observed in both the CSF1PO and Penta D loci in the t=90 timepoint.

The ability to obtain complete STR information from the high-molecular weight timecourse samples was examined over the duration of the degradation experiment by comparing STR alleles from the D5S818 locus, and STR alleles from the five largest core loci containing heterozygous genotypes in the high-molecular weight sample. Recall that the initial amplification of the high-molecular weight DNA using both the Profiler Plus® and the PowerPlex® 16 Hot Start HID typing kits resulted in minimal amplification STR loci. The intentional input of excess template DNA in both HID typing kits ensured the successful amplification of core loci targets, but artificially decreased the actual instances of locus dropout in the timecourse samples. Complete profiles were obtained for both the t=0 and t=5 timepoints. However, locus dropout was first observed in the t=10 sample at the CSF1PO and FGA loci (Table 5). The remaining timepoint samples examined in the study resulted in sporadic dropout within the five largest core loci in the PowerPlex® 16 Hot Start HID typing kit. Interestingly, the overall performance of the CSF1PO locus (green dye) was worse than the performance of the larger FGA locus (yellow dye). This disparity in performance indicates that some STR loci are more susceptible to dropout than others regardless of size.

95°C Treatment (t=min)	Quantitative Ratio	Instances of Locus Dropout	Percent of Alleles in Core Loci ≥200 RFU (n=23)	119–155 bp		262–290 bp		264–304 bp		290–366 bp		321–357 bp		322–444 bp	
				D5S818		TPOX		D16S539		D18S51		CSF1PO		FGA	
				11	12	8	11	8	12	11	12	11	14	20	21
t=0	0.59	0	100												
t=5	0.66	0	83												
t=10	0.79	2	52												
t=20	0.92	2	39												
t=30	0.85	3	22												
t=40	1.10	1	70												
t=50	1.18	4	30												
t=60	1.51	2	48												
t=70	1.91	1	57												
t=80	1.76	1	61												
t=90	1.78	4	48												

≥200 RFU ≤199 RFU Not Detected (<50 RFU)

Table 5: Summary of the PowerPlex® 16 Hot Start amplifications targeting 2,000 pg of input DNA. Alleles were categorized according to a ≥ 200 hRFU threshold, a ≤ 199 hRFU threshold, and a < 50 hRFU threshold (limit of detection). The performance of a small heterozygous locus (D5S818) is compared to the five largest CODIS core loci that also exhibit a heterozygous genotype in the high-molecular weight sample. Notice the sporadic dropout of locus information observed throughout the timecourse. In addition, the larger FGA locus always outperforms the smaller CSF1PO locus in this study with the high-molecular weight DNA sample.

IV. E. Degradation Ratio Results Observed in Casework Samples

In 2008 the Tulsa Police Department Forensic Laboratory developmentally validated this ePCR quantitative assay for use in casework. This developmental validation is documented in Wilson's thesis, which follows the SWGDAM Revised Validation Guidelines (Wilson, 2008; SWGDAM, 2004). It is important to note that the decision to complete a developmental validation was made in response to Applied Biosystems' 2007 announcement that the manufactured Quantiblot® quantitative hybridization assay would be discontinued. Applied Biosystems' decision, coupled with a lack of other commercially available quantitative hybridization methods forced CODIS-participating forensic laboratories to select a new type of human-specific quantitation method to satisfy the DAB standards. The most popular option available to forensic labs was to "upgrade" to a real-time qPCR platform. This "upgrade" included the purchase of new equipment, expendables, and additional quality assurance measures to operate a real-time qPCR method.

Within the inaugural year of using the ePCR quantitative assay in casework, the total-human and male-only quantitative estimates of 91 male reference samples were assessed in the form of the degradation ratio (Figure 13). These ratio results were then compared to the corresponding HID profiles amplified using the AmpFℓSTR® Identifiler® HID typing kit (Applied Biosystems, Foster City, CA). It was observed that the degradation ratios appeared to predict the loss of peak signal intensity among the larger STR loci, as is associated with the "ski-slope" effect. This empirical study indicated a need for further research into the ability of the ePCR assay to detect degradation, and these results were presented at a regional practitioner conference (Smith, 2008). Figure 21 depicts the spread of degradation ratios obtained from the 91 male reference samples that were HID amplified based on the quantitative total-human results of this ePCR assay using the Identifiler® HID typing kit.

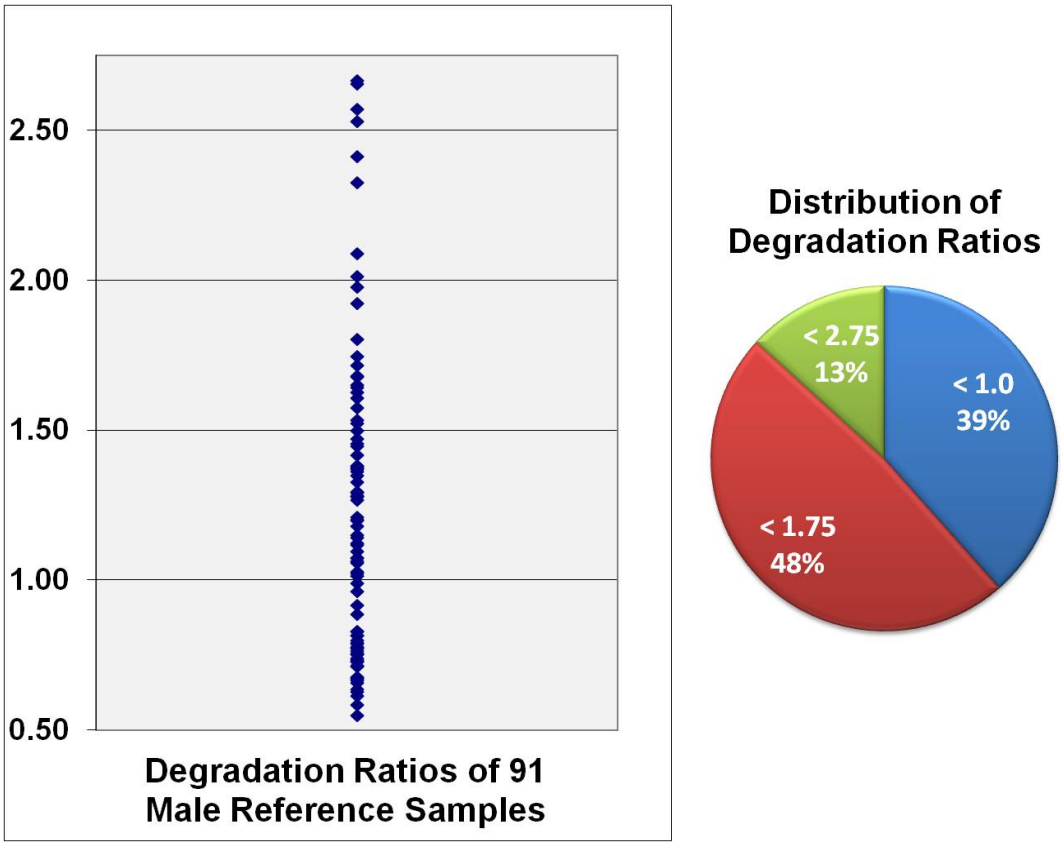

Figure 21: (a) Spread of degradation ratios obtained from the empirical male reference sample study. (b) Distribution of the 91 male reference samples.

Similar to the degradation ratios observed in the high-molecular weight timecourse samples, several ratios were also less than 1.0 in the casework reference samples. However, unlike the high-molecular weight samples, degradation ratios that were less than 1.0 in the casework samples could be attributed to peak truncation of the SRY amplicon as observed in Figure 22. This peak truncation caused the male-only quantitative estimate to under predict the actual amount of SRY amplicons, which produced a ratio where the total-human quantitative estimate was a larger value than the male-only quantitative estimate.

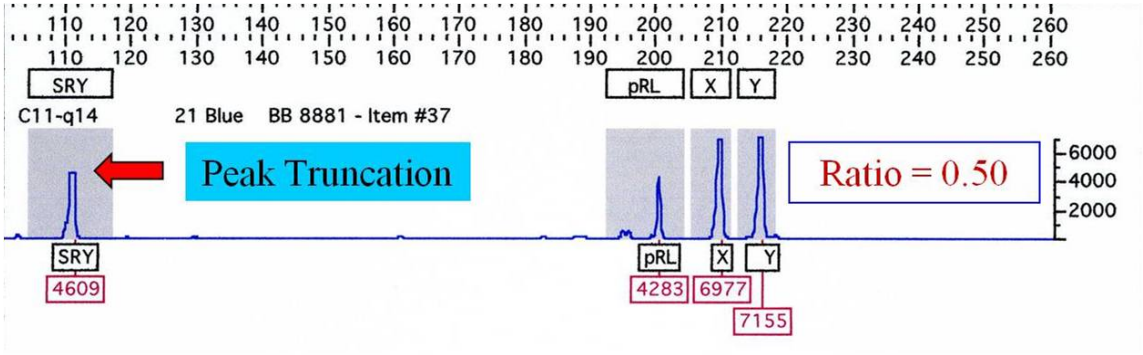

Figure 22: Quant electropherogram of a high-quantity reference sample where SRY fluorescence has exceeded the CCD camera's limit of linearity. This excess of fluorescence results in peak truncation so not all of the information is recorded.

The samples containing ratio values between 0.5 and 1.67 produced optimally amplified HID data in which all the loci contained peaks of near equal signal intensity throughout the locus-to-locus size range. However, as the samples approached quantitative degradation ratios above 1.70, a noticeable decrease in signal intensity of the larger loci become apparent in the HID data. Figure 23 depicts an Identifiler® electropherogram from a casework male reference sample that contained a degradation ratio of 2.67.

One possibility for the difference in degradation ratios obtained in the high-molecular weight sample versus the casework reference samples may be attributed to the difference in extraction techniques. The OSU sample utilized an extraction method that is suited for RFLP analysis, which was necessary in order to obtain yield gel results. However, the extraction technique used in PCR-based casework samples does not require high-molecular weight DNA. Rather, high-molecular weight DNA in a PCR-based system may actually hinder the ability to obtain successful HID results, as was observed in the initial Profiler Plus® and PowerPlex® 16 Hot Start HID results, which targeted optimal amounts of sample input. Unlike the high-

molecular weight extraction methods, PCR-based extraction methods utilized in forensic laboratories contain incubation periods and numerous vortexing steps, which may further disrupt the structure of the DNA macromolecule. As was observed in the high-temperature degradation model developed for this research, degradation uncoils high-molecular weight DNA. Thus, the extraction process may physically disrupt well preserved DNA molecules in a manner that is well suited for the PCR-based quantitation and HID amplification methods. However, those samples that exhibit endogenous degradation, such as environmental or enzymatic processes, may already contain unprotected and exposed template strands, that may be further disrupted due to the incubation and vortexing steps associated with common extraction methods. Future study on the effects of sample degradation might include concordance studies on the results degraded DNA profiles that are obtained with a common extraction method, and the results of the same sample that are obtained by skipping the extraction step, and instead immediately purifying the sample for ePCR quantitation.

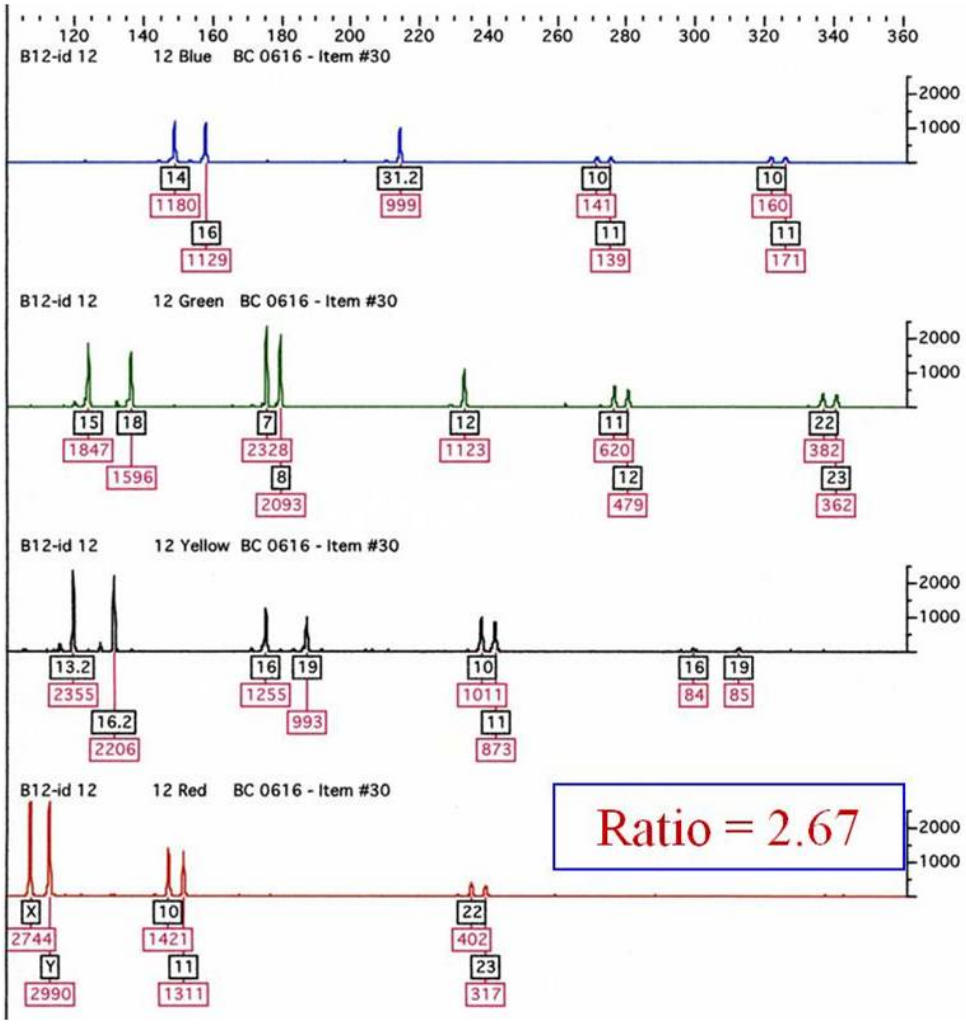

Figure 23: Identifiler® electropherogram from a male reference sample containing the uppermost degradation ratio (2.67) with the assay in the 91-sample study.

IV. F. Predicting HID Amplification Success

A goal of this research was to determine if the quantitative assay can predict the amplification success or failure of large-target STR loci in HID typing kits. To this end the performance of the small and large targets in the high-molecular weight timecourse samples serves as a model that can be applied to actual casework. The quantitative amplification performance from the timecourse samples produced ratios of significant difference throughout the degradation study. As degradation increased there were fewer intact large-target sequences available to amplify, which resulted in a larger ratio. To test this trend between the assay degradation ratios and HID ratios obtained in casework, a selection of readily available male reference samples from the original 91-sample study were further evaluated to determine if the assay degradation ratios did predict a "ski-slope" trend in the HID data. As in the previous HID studies, two loci were selected from the Identifiler® HID typing kit that are a part of the CODIS core loci and represent a small and large target in the HID kit. The D3S1358 locus and the D16S539 locus from the green dye color were selected for this evaluation. Only casework male reference samples that contained heterozygous genotypes at these loci were selected for evaluation, which reduced the sample size to 37 profiles. The statistical assessment of the 37 samples indicated a strong correlation ($r = 0.7478$, $p < 0.001$) between the assay degradation ratios and the HID ratios (Figure 24). This confirms the predictive ability of the quantitative assay to assess large-target amplification performance via a ratio, which indicates the "ski-slope" trend of sample degradation in HID typing.

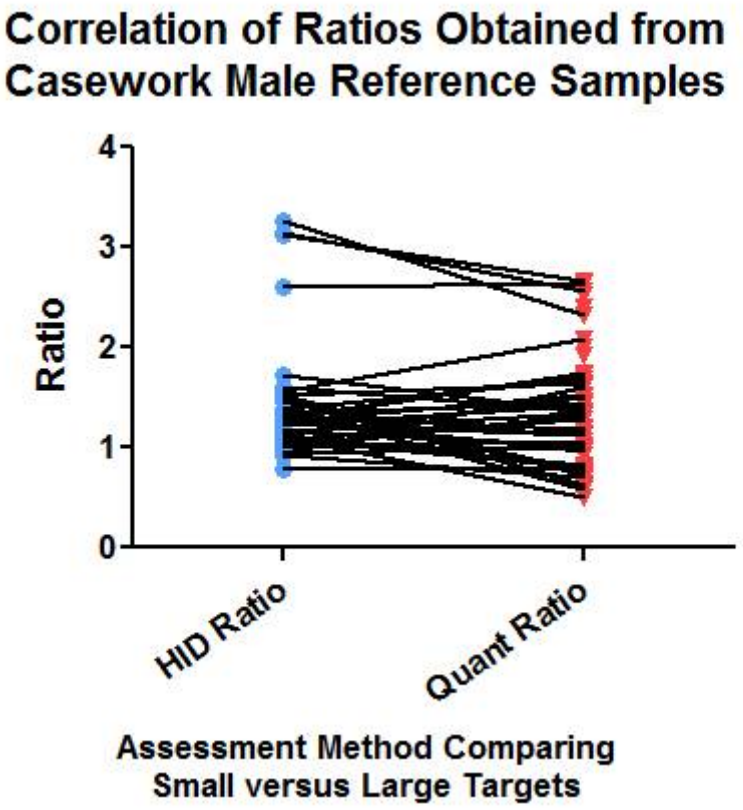

Figure 24: Statistical correlation between the Identifiler® HID ratios of the D3S1358 (small) versus the D16S539 (large) loci and the ratios obtained from the quantitative assay in 37 casework male reference samples. Correlation was performed using one-way ANOVA with Tukey's multiple comparison test. This analysis suggests a strong correlation (r 0.7478, $p < 0.001$) in the ability of the quantitative assay to predict the amplification performance of large targets in the Identifiler® HID typing kit. A decrease in large target performance is visually displayed as a "ski-slope" trend in electropherogram data.

Although the D16S539 locus within JOE (green) dye of the Identifiler® HID typing kit behaved in a predictable manner, the same could not be said about the large core loci targets labeled with 6-FAM (blue) dye. The two large targets with 6-FAM dye, D7S820 and CSF1PO, appeared to have an initial peak-height imbalance compared to the smaller core loci targets in the blue dye regardless of the sample's degradation ratio. However, the severity of peak-height

imbalance of the large 6-FAM labeled targets did follow the same trend in signal reduction as observed in the JOE labeled targets, albeit with a more intense reduction of signal.

The “ski-slope” trend observed in the STR loci results from the reduction of signal in large targets compared to small targets. The amount and availability of intact target sequences within the large STR loci will determine the success of HID amplification. As large target sequences degrade, fewer template strands are suitable to participate in PCR. The decrease of intact target sequences may result in locus dropout if successful amplification does not occur within the initial cycles of PCR. Although no instance of locus dropout was observed in the casework reference samples, the signal reduction in large loci was predicted by the sample’s degradation ratio. The lack of observed locus dropout is not surprising considering the 65 samples readily available from the empirical study originated from high-quantity reference samples. If this assay were applied to low-quantity casework samples it is probable that the assay’s degradation ratio would predict locus dropout based on the observed trends in this research. Based on this research the ePCR quantitative assay can distinguish the amplification performance of targets of differing size. This unique ability can be used as a diagnostic tool to predict sample degradation in HID typing kits.

CHAPTER V

CONCLUSIONS

V.A Increase Efficiency with Degradation Assessment

Many forensic DNA laboratories are confronted with backlogs, limited resources, and external demands that hamper expeditious sample processing. In addition, the physical integrity of DNA evidence often remains unknown until laboratories invest a significant amount of time and money examining the evidence. To overcome these constraints laboratories may consider the use of sampling plans to triage the analysis of evidence expected to yield DNA information suitable for entry into the CODIS database. The results of this study suggest that the ePCR quantitative assay is a method that can simultaneously quantify the amount of template DNA for HID testing, and predict the amplification performance of large STR loci by detecting template-strand degradation. Currently, no other gender typing assay is reported to predict the performance of PCR-based profiling methods prior to the utilization of an HID kit. This diagnostic ability could direct sample processing so laboratories select typing methods, such as mini-STR kits, which are suited for samples suspected of yielding locus dropout with traditional HID methods.

In this study the degradation assessment of the quantitative assay was evaluated using a high-molecular weight DNA sample that was artificially degraded in a controlled manner. This research model was applied to actual male reference samples from casework, and correlates with the performance of associated HID profiling results. In the instances where the assay detected a

significant difference between the large and small targets (degradation ratio > 1.5) a visual "ski-slope" trend could be observed in the HID data. This effect of template degradation is the result of signal loss in the large target sequences, which may drop below a laboratory's set signal threshold, or result in sample dropout. Although few instances of sample dropout were observed in the empirical study, the further assessment of low-quality evidence is certain to detect large-target dropout following the "ski-slope" trend. This trend is the result of degradation, which is the inability to amplify and detect core STR loci. The effects of degradation are exacerbated with the loss of multiple core loci in which a sample does not satisfy the FBI's eligibility requirements for CODIS entry.

V.B Areas of Future Research

This assay is well suited for the multiplex of additional markers. These markers should incorporate a haploid female-specific target to observe the effects of degradation within single-source female samples and sample mixtures, which are an inherent part of forensic DNA testing. Ultimately, this assay may be used as a roadmap to determine when particular targets of an HID typing kit experience reduced amplification performance and are susceptible to dropout. In addition, future studies should incorporate a revised degradation ratio where the estimates of the large Amelogenin target are compared to the estimates of the small SRY target to avoid the inability of calculating an undefined number should the large target fail to amplify.

Predicting DNA degradation prior to HID profiling is just another tool of this assay. However, the assay's ability to evaluate amplification performance between targets of differing size could be applied to fields outside of forensic DNA analysis. Further research may include additional diagnostic applications of this assay beyond the scope of forensic testing.

V.C Immediate Application of Degradation Assessment

The predictive ability to detect DNA degradation can be applied to the processing of biological evidence recovered from the scenes of mass disasters, in which samples are often exposed to physical, chemical, and biological stressors that may damage template DNA. In many instances the ability to yield useful profiling results from suspected high-quality evidence is compromised due to sample degradation. This quantitative assay could provide a quick screening method to eliminate the further processing of tissue samples that are not expected to yield complete profiling results, thereby directing HID testing for remains identification. In addition, high-volume database laboratories that process reference samples may utilize this assay as a reliable method to assess the amplification performance of male reference samples.

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VITA

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Completed the requirements for the Master of Science in Forensic Science at the Oklahoma State University Center for Health Sciences, Tulsa, Oklahoma in December, 2011.

Completed the requirements for the Bachelor of Science in Forensic Science at Baylor University, Waco, Texas in 2005.

Professional Employment:

Tulsa Police Department Forensic Laboratory (Tulsa, Oklahoma)
December 2011 – Present: Forensic Technical Leader – Biology Section
December 2010 – December 2011: Senior Forensic Scientist – Biology Section
January 2008 – Present: Biology Section Quality Assurance Leader
December 2007 – November 2010: Forensic Scientist II – Biology Section
August 2005 – November 2007: Forensic Scientist I – Biology Section

Professional Memberships:
Member of the Midwestern Association of Forensic Scientists (MAFS).

Name: Byron Cody Smith Date of Degree: December, 2011

Institution: Oklahoma State University Center for Health Sciences Location: Tulsa, OK

Title of Study: EVALUATING DNA SAMPLE DEGRADATION WITH A QUANTITATIVE GENDER TYPING END-POINT PCR MULTIPLEX

Pages in Study: 68 Candidate for the Degree of Master of Science

Major Field: Forensic Science

Scope and Method of Study:

DNA sample degradation is a common problem encountered in forensic DNA casework. In some instances the severity of sample degradation prevents the inclusion of an evidentiary profile into the FBI CODIS database. In this study an end-point PCR quantitative assay is assessed to determine if human DNA targets of differing size can be used to predict DNA sample degradation in STR profiling. Primer sequences from a small male-specific target (SRY) and a large human target (Amelogenin) are multiplexed. These targets estimate the respective total-human and male-only quantities of sample DNA based on comparison to a standard curve prepared from known quantities of human DNA. The quantitative estimate of the small target versus the quantitative estimate of the large target can be compared in male samples to form a ratio. This ratio may indicate the probable amplification performance of large-target STR loci multiplexed in HID typing kits. A method was developed here to degrade DNA in a controlled manner for analysis with this quantitative assay. The resulting quantitative ratios were compared to HID ratios developed from the amplification performance of small and large CODIS core loci. In addition, a selection of male reference samples was also examined to evaluate the correlations of quantitative ratios and HID ratios obtained in actual casework.

Findings and Conclusions:

Regression analysis of the performance of the small and large targets amplified in the quantitative assay demonstrated a significant difference ($p < 0.001$) between both targets through the course of the in vitro degradation experiment. These results indicate that in degraded samples the small target will outperform the large target in the quantitative assay. Assessment of the quantitative ratios demonstrated significant degradation ($p < 0.001$) at and above the t=60 minute timepoint in the successive degradation of samples. This experimental model was applied to the quantitative and HID typing results obtained from male reference samples in actual casework. Statistical assessment of these samples indicates a strong correlation ($r = 0.7478$, $p < 0.001$) between the quantitative ratios and the HID ratios. This confirms the ability of the quantitative assay to assess large-target amplification performance via a ratio, which predicts the trend of sample degradation observed in HID profiling.

ADVISER'S APPROVAL: Dr. Robert Allen