IMPROVED QUANTITATION OF HUMAN DNA

USING QUANTITATIVE TEMPLATE

AMPLIFICATION TECHNOLOGY

By

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I would like to dedicate my thesis in the memory of my father Marcus Benson, my mother Beatrice Benson and my sister Lydia Benson.

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NOMENCLATURE

Alu	repetitive DNA elements in primate DNA
Amelogenin	sex determination gene
AMEL-X	amelogenin X
AMEL-Y	amelogenin Y
BSA	bovine serum albumen
bp	basepair
CCD	charge-coupled device
DNA	deoxyribonucleic acid
EDTA	ehtylenediaminetetraacetic acid
FBI	Federal Bureau of Investigation
hTERT	human telomerase reverse transcriptase gene
M	molar
MGB	minor groove binding probes
mM	milli molar
nm	nano meter
NFSTC	National Forensic Science Technology Center
ng	nanogram
NIST	National Institute of Standards and Technology
OD	optical density
PCR	polymerase chain reaction

pRL	Renila Luciferase plasmid
pg	pictogram
qPCR	real-time PCR
Q-TAT	quantitative template amplification technology
RB1	retinoblastoma gene
RFLP	restriction fragment length polymorphism
U	unit
µL	micro liter
µM	micro molar
RFU	relative florescence unit
SRY	sex-determining region of the Y chromosome
STR	short tandem repeat
THO1	STR marker within the tyrosine hydroxylase gene
TWGDAM	Technical Working Group on DNA Analysis Methods
U	units
ul	microliter
UV	ultraviolet
VNTR	variable number of tandem repeats
Y-STR	Y-chromosomal short tandem repeats

CHAPTER I

1. INTRODUCTION

Identification of the perpetrators of violent crimes, resolving questioned parentage, or identification of remains of missing persons or victims of mass disasters follow an established progression of steps including preliminary screening, presumptive and confirmatory testing, DNA extraction, DNA quantitation and STR analysis. In the early stages of processing, the technologist must choose an analytical strategy for evidence processing that will provide the greatest amount of probative information about the source of the DNA recovered. Generally, the challenge will be to decide what kind of DNA analysis method is used (Horsman et al 2006). In the field of forensic science, where most biological evidence samples are limited, nonrenewable, and may be degraded or contain PCR inhibitors, quantitation of the amount of human DNA extracted from the crime scene sample is an essential step in the typing process, where optimal results are dependent on a relatively narrow range of input DNA template quantity, and the amount of DNA extracted may be only enough for one chance at DNA analysis (Butler, 2005).

Because the efficiency of PCR amplification is influenced by the quality, purity and quantity of human DNA in a sample, the DNA Advisory Board Standard 9.3 in the Quality Assurance Standards for Forensic DNA Testing Laboratories mandates that forensic DNA typing laboratories determine the amount of human genomic DNA recovered from evidentiary samples before STR typing begins. Accreditation bodies have made this a basic requirement of all laboratories desiring accreditation (DNA Advisory Board, 2000). DNA laboratories are also advised to utilize only the required amount of DNA for analysis and retain the remainder for re-testing or independent confirmatory testing under court order (Allen 2006).

The quantitation of amplifiable DNA present in a sample is critical as a narrow range of input template works best for the PCR reaction: a very high concentration will exhibit split, off-scale or truncated allelic peaks and very low DNA concentration will result in stochastic fluctuations and / or an incomplete DNA profile (Butler, 2005). Applied Biosystems' Profiler Plus, Cofiler multiplexes and Promega STR typing kits specify the addition of 1- 2.5 ng of DNA to the PCR mixture for optimal results (Krenke at al 2002).

The primary aim of DNA quantitation is to determine the amount of amplifiable DNA in an evidentiary sample as PCR amplification may be reduced or fail due to highly degraded DNA, the presence of co-extracted inhibitors, or limited amounts of DNA (Butler 2005). Most evidentiary samples from crime scenes are exposed to harsh environmental conditions that may promote DNA degradation or introduce PCR inhibition. Heme, humic compounds from soil and indigo dye are some of the common

PCR inhibitors found in evidentiary samples (Butler, 2005). These inhibitors act by binding to the active sites of DNA polymerase, making them unavailable for the PCR reaction (Butler, 2005). DNA degradation occurs through enzymatic and chemical processes randomly breaking up DNA strands into smaller pieces. When the DNA strands break where primers bind or between forward and reverse primers, the target region fails to amplify (Butler, 2006).

If PCR inhibitors are detected in a sample, extraordinary clean-up steps can be taken to try and separate the DNA from the inhibitor. If DNA is degraded, alternate PCR primers targeting sites more proximal to the VNTR will result in smaller allele sizes but will also be less affected by degradation. For example, Whitaker and coworkers (1995), reported a correlation between allele length and successful DNA typing among samples recovered from the Waco fire disaster. Wiegand and Kleiber (2001), using new redesigned primers that were close to the STR repeat region also demonstrated that highly degraded as well as suboptimal amounts of DNA template could be more successfully amplified because of reduced size alleles than conventional larger sized STR markers.

In some evidentiary samples, the DNA content is too limited to obtain a complete profile from nuclear DNA. In these circumstances the analysis of mitochondrial DNA (mtDNA) has been used because of the high copy number of mtDNA molecules per cell (Bogenhagen et al, 1974). MtDNA analysis is very sensitive, and 10 mtDNA molecules (30fg DNA) can be used to generate a complete profile (Andreasson et al, 2002). The main drawback of mtDNA analysis is that the discriminatory power is significantly lower

than a multiplex nuclear analysis. MtDNA analysis may be useful when additional circumstantial evidence is required and for exclusions (Butler 2005).

It should be clear from the discussion above that decisions must be made by a forensic analyst before the actual DNA typing process begins. A pretest analysis method that provides accurate information on aspects of DNA quantity and quality, the presence of inhibitors and whether or not a mixture of male and female DNA exists in the sample is highly desirable.

The ideal quantitation method should be simple, sensitive, rapid, accurate, human specific, fully automatable, cost effective, and have a large dynamic range. In addition, a method that consumes limited sample, reports both the male/female and total genomic DNA and detects the presence of PCR inhibition and degradation would be an improvement over many methods in current use. Using a quantitation method close to the ideal, an analyst will not waste valuable time and resources, producing quality STR results that are easily interpreted. Each of the numerous current methods of quantitation has its advantages and disadvantages but none meets the ideal criteria.

Quantitative Template Amplification Technology (Q-TAT), an end-point PCR quantitation method developed in the OSU Human Identity laboratory by Allen and Fuller (2006) is a low cost quantitation method that targets the amelogenin genes on the X and Y chromosomes, making it a valuable quantitation method in sexual assault cases.

Research Question

Can we enhance the existing Q-TAT quantitation methodology to monitor not only total genomic concentrations and the male/female proportions of DNA present in a sample but also to detect the presence of PCR inhibitors and assess the extent of DNA degradation (Allen and Fuller, 2006)?

The specific aims of this study are:

To improve the Q-TAT multiplex reaction that amplifies the amelogenin locus on the X and Y chromosomes (AMELX 210 bp and AMELY 216 bp) through simultaneous detection of inhibition and degradation by:

Adding a second Y chromosomal target for amplification, the SRY gene (110 bp) to the Q-TAT reaction:

a) To assess DNA degradation

b) As an additional Y chromosome marker for unambiguous gender identification

Adding the Renila luciferase pRL gene cloned in the pRL-null vector (200 bp) to the Q-TAT reaction:

To detect the presence of PCR inhibitors

CHAPTER II

II. REVIEW OF LITERATURE

II.A. DNA Analysis

The deciphering of the structure of DNA (Deoxyribonucleic Acid) and the unraveling of its genetic information has revealed the fundamental concepts of inheritance. Molecular biologists continue to decode the basic structure of genes, produce new products and diagnostic tools through recombinant DNA engineering, and discover new treatments for genetic disorders. The finding that portions of DNA structure of certain genes are as unique to each individual as fingerprints by Sir Alec Jeffreys made the terms 'DNA fingerprinting' and 'DNA typing' the common parlance of forensic scientists worldwide. Through DNA typing, forensic scientists have been able to link virtually any kind of biological evidence to a single individual. This technology has evolved to new areas of forensic DNA typing such as mitochondrial and Y chromosomal DNA typing, the use of forensic science in wildlife crimes like poaching and microbial forensics in the identification of pathogens that infect humans, plants and animals (Rudin and Inman, 2002). DNA analysis has become routine in public crime laboratories and private laboratories all over the world. Multiple population groups have been studied, new technologies for rapid typing of DNA samples have been developed and standardized protocols have been validated (Butler, 2005).

Courts have admitted DNA evidence and accepted the reliability of its firm scientific foundation. The procedures and techniques used have satisfied the criteria of admissibility established by the courts and governed by Federal Rules of Evidence 702, and the Frye and Daubert standards (Frye, 1923, Daubert ,1993). Legislative and judicial reviews at state and federal levels and stringent quality assurance guidelines have contributed significantly to the evolution of DNA analysis and played an important role in its acceptance as a legal tool, making this technology one of the most robust and powerful tools used in the justice system today (Rudin and Inman, 2002; Butler, 2005).

One of the pivotal DNA analysis developments in recent years is the use of the polymerase chain reaction (PCR) to replicate DNA molecules in vitro. More than 2,000 studies have been published on the amplification of short tandem repeat (STR) markers used for forensic DNA testing, detailing the technology and allele frequencies of STR alleles in different population groups worldwide (Butler, 2005). New platforms, such as microchips, nanotechnology, automation, miniaturization, and portable expert systems are all in development and will further revolutionize the next generation of methodologies developed for DNA analysis. Costs and processing time will decrease and sensitivity and success rates will increase (Butler, 2005).

II.A.i. Human Identity Testing

The human genome consists of 22 matched pairs of autosomal chromosomes and a pair of sex determining chromosomes making a total of 23 pairs, with one chromosome in each chromosomal pair being derived from each parent at the time of conception. A normal human has one pair of sex chromosomes in each cell, females have two X

chromosomes (XX), and males have one X and one Y chromosome (XY) making the Y chromosome a distinct specific feature of the human male (Butler 2005, Jobling 1997; Carrel, 2006).

Most human identity testing is performed using short tandem repeat (STR) markers on the 22 pairs of autosomal chromosomes. The Y chromosome may also be used for the human male identification purposes in sexual assaults or in establishing relatedness to a particular male lineage within a particular family (Zerjal et al, 2003). In the past few years, there has been a substantial surge in the use of Y-STR markers in forensic laboratories, especially in cases where typing of autosomal STRs has not been possible (Mulero, 2006).

Most forensic testing laboratories today use a minimum of 13 STR loci as a standard test battery. Chakraborty and coworkers (1999) concluded that this set of STR loci was adequate for addressing most problems of human identification, including interpretations of DNA mixtures. When a complete DNA profile is obtained using the 13 autosomal STR loci, the probability of a chance match with a randomly chosen individual is usually less than one in one trillion, which provides an extremely high discriminatory power compared with an average of one in one thousand probability chance match using 17 STR loci on the Y chromosome (Butler, 2005).

II.A.ii. Amelogenin Gene

The human amelogenin gene, AMEL, is located on the short arm of the X chromosome at Xp22.1-p22.3. A homologous amelogenin gene sequence on the Y chromosome, AMEL-Y, maps to the pericentric region of the Y chromosome at Yp11 (Nakahori et al., 1991;

Rickords et al 1999) (Figure 1). The human enamel protein gene amelogenin is expressed on both the X and the Y chromosomes (Nakahori et al., 1991; Salido et al., 1992).

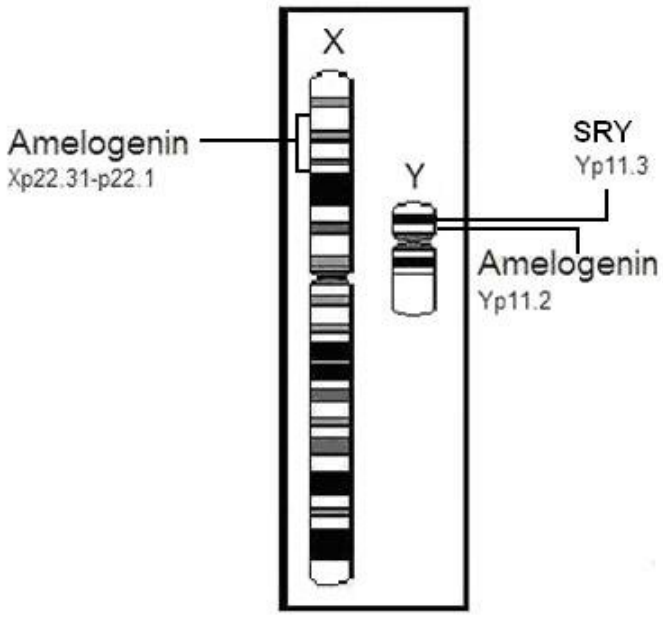

Figure 1: Location of the Amelogenin and SRY genes on the X and Y chromosomes (Stavely, 2006) (modified)

Amelogenin is a low molecular weight protein found in developing tooth enamel that belongs to a family of extra-cellular matrix (ECM) proteins. The function of amelogenin is to organize enamel rods during tooth development, by promoting the initiation and assembly of hydroxyapatite crystals during the mineralization of enamel (Nakahori et al., 1991; Salido et al., 1992).

The AMEL-X and AMEL-Y genes may also be used for the sex determination of unknown samples since amplification of the AMEL-X gene produces a 210 bp amplicon while the AMEL-Y gene produces a 216 bp amplicon. Thus the AMEL-X gene contains a 6 bp deletion in intron 1, and PCR products amplified from a male source will reveal 2 DNA fragments of 210 bp and 216 whilst a female source (XX) will show only a single amplicon of 210 bp (Thangaraj et al., 2002; Allen and Fuller, 2006; Chang et al., 2006).

Several companies manufacture multiplex STR kits that include primers for the amelogenin gene system, allowing for both individual and gender identification, and in most samples, the gender of the donor can be determined. However, studies exist that report the deletion of the amelogenin Y genes in occasional males. For example, Thangaraj and coworkers (2002), using the AmpF/STR Profiler Plus multiplex, reported a deletion of the amelogenin gene on the Y chromosome in 1.85% of East Indian males. The aberration of the Y chromosome was restricted to the amelogenin gene however, since PCR products from the SRY gene (another Y chromosome specific target) were present in all the amelogenin-deleted males. Santos and coworkers (1998), have also reported deletions of AMEL-Y in two Sri Lankan males, and Steinclechner and coworkers (2002) demonstrated the lack of amelogenin Y in 0.2% (6 out of 29,432) of Austrian males. The reliability of gender testing is of paramount importance when identifying useful forensic samples from sexual assaults, and these studies indicate that conclusions about gender based upon the amelogenin gene alone may not always be accurate.

II.A.iii. The SRY Gene

The Y chromosome also contains the master sex-determining gene, SRY, at position Yp11.3 (Figure 1). SRY codes for the testis-determining factor (TDF) that triggers the precursor of the gonad to develop as a testis (Stavely, 2006). Amplification of the SRY gene yields an 110 bp amplicon which is characteristic in its size of newer mini-STR testing methods which are designed to amplify STR markers from degraded DNA (Jobling, 1997; Alonso et al., 2003; Hartl, 2005).

II.A.iv. The Renila Luciferase Gene (pRL gene)

The sea pansy is a fleshy, leaf shaped colony of marine organisms belonging to the genus Renilla which is in the same phylum as the jellyfish. This anthozoan consists of polyps with different forms and functions. A single giant polyp about two inches in diameter forms the peduncle, the anchoring stem that is extended to anchor the colony on the substrate. The sea pansy is bioluminescent when disturbed, due to the Green Fluorescent Protein, a molecule that is used extensively as a reporter in gene expression assays in modern biological science. (Promega, 2007) The AluQuant human DNA quantitation system marketed by Promega Corp (Madison, WI) uses light production by the luciferase gene to quantitate amounts of human DNA (Mandrekar et al., 2001)

Figure 2: Sea Pansy (Bio E-lab)

The pRL family of reporters derives from the wild-type Renilla luciferase, (Rluc) control reporter vectors (Promega, 2006). The pRL vectors, which provide constitutive expression of Renilla luciferase, may be used with a firefly luciferase vector to cotransfect mammalian cells. The pRL vectors contain the cDNA encoding Rluc cloned from the anthozoan coelenterate sea pansy, Renilla reniformis. All pRL vectors are carried by an E. coli K host strain (Promega, 2006). The pRL gene of 200bp cloned in the pRL-null vector was selected for use in the Q-TAT multiplex ampification reactions due its similar size to the AMELX/Y genes and does not exhibit cross reactivity to any sequence in the human genome (Promega, 2006).

II.A.v. Variant Number of Repeats (VNTRs)

The human genome is full of repeated DNA sequences containing up to several thousands base pairs in a core repeat element (Ellegren, 2004). These regions are referred to as satellites with a core repeat of about 10-100 bases, called a minisatellite or variant

number of tandem repeats (VNTRs) (Nakamura et al., 1987; Tautz 1993, Chambers et al., 2000).

II.A.vi. Short Tandem Repeats (STRs)

Regions on chromosomes consisting of repeated elements 2-6 base pairs in length are called microsatellites or short tandem repeats, and account for 3% of the total genome, occurring about once every 10,000 nucleotides (Edwards et al., 1991, Ellegren, 2004). STRs are commonly used as DNA markers because of their ease of PCR amplification and their polymorphic nature among individuals, making them ideal for human identification. Tetranucleotide repeats have become more widely used than loci consisting of di- or tri-nucleotides and can be multiplexed with up to16 loci co-amplified in a single PCR reaction (Schumm et al., 2004) .

A common set of standardized STR markers that were initially characterized by Dr. Thomas Caskey at the Baylor College of Medicine and the Forensic Science Service in England was used extensively by the forensic community (Edwards et al., 1991, Kimpton et al., 1993, Hammond., et al 1994, Urquhart et al., 1994). In 1997, the 13 core STR loci were chosen as the basis for the CODIS national DNA database effort, and the analysis of all the 13 core loci yields an average random match probability of about one in a trillion among unrelated individuals (Chakraborty et al., 1999).

Applied Biosystems in Foster City, California and Promega Corporation in Madison, Wisconsin are the vendors of multiplex kits that cover the 13 core loci for CODIS, analyzed either by capillary or acrylamide gel electrophoresis with allele

identification based on size and fluorescent color. Manufacturers provide allelic ladders in these multiplex kits for accurate genotyping.

II.B. DNA Degradation and PCR Inhibition

II.B.i. DNA Degradation

Samples from the crime scene may have been exposed to a harsh environment that promotes degradation of DNA molecules into randomly broken pieces. In addition to degradation, UV irradiation from sunlight can also lead to the cross-linking of adjacent thymine nucleotides in a DNA molecule and prevent its amplification during PCR (Butler, 2005). DNA degradation and / or crosslinking can prevent successful amplification of conventional STR markers (Lindahl, 1993; Butler, 2005).

Studies have shown an inverse relationship between the size range for alleles of an STR locus and the probability of successful amplification from degraded DNA samples (Sparkes et al., 1996; Takahashi et al., 1997; Schneider et al., 2004). These experiments have shown that larger loci are the first to disappear from electropherograms when amplifying extremely degraded DNA. During the analysis of samples from the Waco disaster for example, the Forensic Science Service observed a correlation between successful typing at a locus and the average length of the alleles at that locus (Whitaker et al., 1995). Loci with larger alleles failed first while the short amelogenin X and Y amplicons of 106 bp and 112 bp were successfully amplified from all samples tested. The FES/FPS locus with allele size ranging from 212-240 bp yielded only 91 successful amplifications whereas the vWA locus with allele sizes of 130-169 bp had 115 successful amplifications.

II. B.ii. MiniSTRs

Based upon the observations of others working with degraded DNA, Wiegand and Kleiber (2001), redesigned STR primers by reducing the flanking sequence surrounding the STR repeats and demonstrated that degraded DNA templates could be successfully typed more often when STR alleles were small. The PCR primers for the Penta D locus, amplified in the PowerPlex 16 multiplex, have been reduced by 282 bp to produce products of 94-167 bp in a mini-STR typing multiplex, by bringing the primers to within 11 bp upstream and 19 bp downstream of the repeat region (Krenke et al., 2002; Butler et al. 2003) (Table 1, Figures 3 and 4).

Reference	Locus	MiniSTR Size Range	Size Reduction from Standard Multiplex	STR Size Range
Hellman et al (2001)	THO1	61-85 bp	-103 bp	164-188 bp
	TPOX	58-86 bp	-157 bp	215-243 bp
	FES/FPS	81-105 bp	-132 bp	213-237 bp
Tsukada et al (2002)	THO1	74-98 bp	- 90 bp	164-188 bp
	TPOX	107-135 bp	-110 bp	217-245 bp
	CSFIPO	90-122 bp	-194 bp	284-316 bp
	VWA	99-143 bp	- 53 bp	152-196 bp
Butler et al (2003)	THO1	51-98 bp	-105 bp	156-203 bp
	TPOX	65-101 bp	-148 bp	213-350 bp
	CSF1PO	89-129 bp	-191 bp	280-320 bp
	VWA	88-148 bp	- 64 bp	152-212 bp
	FGA	125- 281 bp	- 71 bp	196-352 bp
	D3S1358	72-120 bp	- 25 bp	97-145 bp
	D5S818	81-117 bp	- 53 bp	134-170 bp
	D7S820	136-176 bp	-117 bp	253-293 bp
	D8S1179	86-134 bp	- 37 bp	123-171 bp
	D13S317	88-132 bp	-105 bp	193-237 bp
	D16S539	81- 121 bp	-152 bp	233-273 bp
	D18S51	113-193 bp	-151 bp	264-344 bp
	D21S11	153-211 bp	- 33 bp	168-244 bp
	Penta D	94-167 bp	-282 bp	376-449 bp
	Penta E	80- 175 bp	-299 bp	379-474 bp
	D2S1338	90-142 bp	-198 bp	288-340 bp

Table 1: PCR product size reduction obtained with new primers in several miniSTR studies (Butler, 2005)

In experiments using DNase1 digested DNA, miniSTRs performed better than commercial STR loci (Chung et al., 2004). MiniSTRs have also been used for successful identifications of World Trade Center victims from severely burned and damaged bone samples and in telogen hair shaft nuclear DNA typing (Hellman et al., 2001, Schumm et al., 2004). Potential miniSTR systems are currently being examined to be added to the

standard CODIS markers for forensic applications in the future for typing degraded DNA specimens (Ohtaki et al., 2002; Coble and Butler 2005).

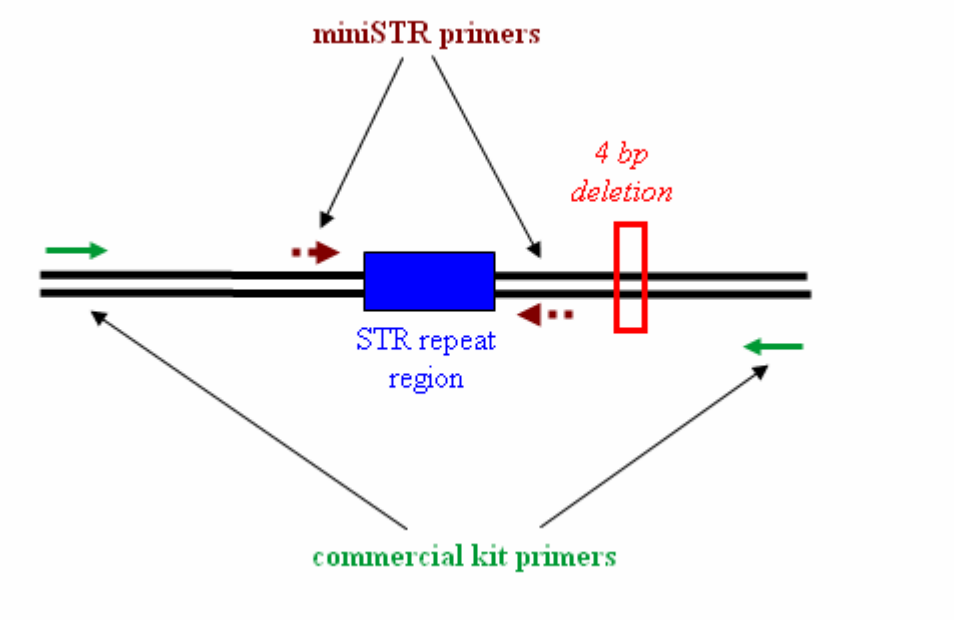

Figure 3: MiniSTRs (NIST, 2007)

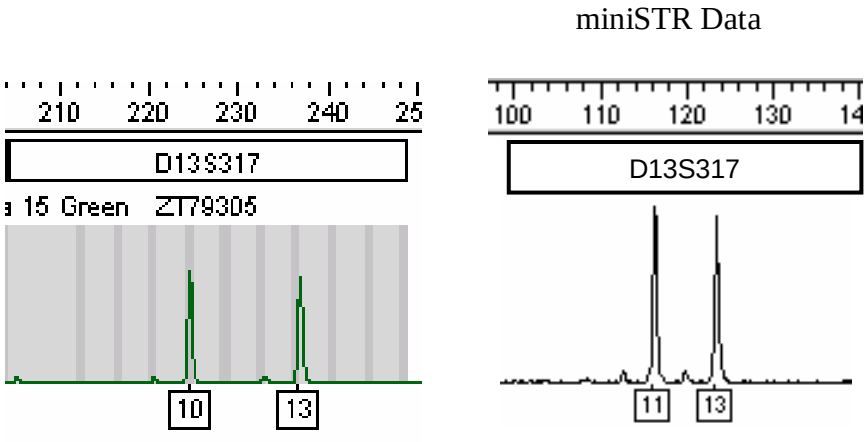

Figure 4: Standard versus MiniSTR amplicon allele size (bp) (NIST, 2007).

II.B.iii. PCR Inhibition

In addition to evidentiary DNA samples compromised due to template degradation, the substrates upon which biological evidence is deposited often contain inhibitors of the PCR reaction. PCR inhibitors may be co-extracted with DNA from crime scene samples and thus prevent or reduce the sensitivity of PCR amplification. It is important for an analyst to know if an inhibitor is present in a DNA extract and to take extraordinary steps to remove it prior to PCR amplification. The three most common chemical inhibitors found in forensic samples are hemin from hemoglobin, indigo dye from denim and humic acid from soil (Akane et al.,1994; Del Rio, 1996; Radstrom et al., 2004; Butler, 2005). Melanin in hair samples may also inhibit PCR amplification when typing mitochondrial DNA (Butler, 2005). Although less commonly encountered in evidence, polysaccharides and bile salts from feces and urea from urine may also be sources of PCR inhibitors (Butler, 2005).

Inhibitors act by binding to the active sites of the Taq DNA polymerase, thereby preventing its function and they may also interfere with cell lysis required for DNA extraction. Inhibitors may also interfere with PCR by promoting nucleic acid degradation. When a DNA sample containing an inhibitor such as hemin is amplified, larger sized alleles are lost from the profile first, resulting in a partial profile, or there may be the complete failure to amplify all loci (Applied Biosystems, 1998; Butler, 2005).

II.B.iv. Hemin

The heme compound found in DNA extracted from bloodstains is regarded as a major inhibitor of Taq DNA polymerase (Akane et al., 1994). The Fe3+ oxidation product of heme is termed hemin (King, 2006). Hemin acts as a feed-back inhibitor on ALA synthase, inhibits transport of ALA synthase from the cytosol into the mitochondria and represses the synthesis of the enzyme (King, 2006).

In experiments performed by Akane and coworkers (1994), 0.25 mM of alkaline or acid hematin inhibited amplification of the mtDNA D-loop region. Hemin inhibition of DNA polymerase was reversed by globin (molecular weight 16,500) and BSA even after 10 cycles of PCR were performed (Akane, 1994). Hemin (alkaline hematin) is also known to suppress the activities of restriction endonucleases, DNase I, RNA polymerases and reverse transcriptase (Akane, 1994).

II.B.v. **Humic Acid**

Humic acid is one of the major constituents of the organic matter of soil known as humus. It contributes to the physical and chemical qualities of soil, and is one of the precursors of fossil fuels. Humic substances are naturally occurring complex supramolecular mixtures that make up a major portion of the dark brown matter in humus, distinguished from chemical extractions such as humic acids and fulvic acids, as defined by their solubility in acid or alkaline solution (Johnson, et al., 1982; Tsai and Olson, 2007).

Humic compounds from soil are known inhibitors of PCR (Tsai and Olson, 1992). Outdoor crimes may leave body fluids on soil, which may contain humic compounds that

may be co extracted with DNA and prevent PCR amplification (Tebbe and Vahjen, 1993). The addition of aluminum ammonium sulfate to soil samples has prevented the co-purification of inhibitors with DNA (Braid et al., 2003).

II.B.vi. Indigo Dye

Indigo dye is a common dye with a distinctive blue color and is among the most common inhibitors of the PCR reaction (Butler, 2005). The basic chemical compound in indigo dye is indigotin, and it is one of the oldest dyes to be used for textile dying and printing. The natural dye is extracted from many plant species as well as Phoenician sea snail (Ferreira et al., 2004). Today, nearly all indigo dye produced is synthetic. In the United States, the primary use for indigo is as a dye for cotton work clothes and blue jeans. Over one billion pairs of jeans around the world are dyed blue with indigo annually. Indigo does not bond strongly to fiber and repeated washing slowly removes the dye. As amplifiable DNA was obtained from pre-washed denim but not from dark blue denim, the levels of indigo dye in the material may be related to PCR inhibition (Del Rio et al., 1996).

Sexual assaults and other violent crimes may leave body fluids like semen, blood, saliva and urine on blue denim clothing. The dye in blue jeans may be co- extracted with DNA and thereby prevent PCR amplification of DNA markers by inhibiting Taq polymerase activity (Shutler et al., 1999).

II.B.vii. Ethylenediaminetetraacetic Acid (EDTA)

EDTA is a chelating agent widely used to sequester di- and trivalent metal ions. It forms strong complexes with Mg^{++}, Ca^{++} and Fe^{+++}. EDTA is a known inhibitor of metal dependent enzymatic reactions, and can be removed from reaction mixtures by ultrafiltration or alcohol precipitation of DNA. It is used in chelation therapy in medicine and as an additive to detergents for industrial cleaning. EDTA, a common PCR inhibitor is a preservative and anticoagulant added to blood (Rossen et al., 1992; Al-Soud et al., 2000; Al-Soud et al., 2001). EDTA inhibits PCR reactions by chelating the divalent cation Mg^{++}, which is a required cofactor for Taq polymerase activity. In PCR inhibitory studies, Al-Soud and coworkers (2001) found that the addition of 0.25 mM EDTA to the PCR mixture reduced fluorescence to approximately 46 %. In a study of the inhibition of PCR by the components of food samples, Rossen and coworkers (1992) found that at 0.1mM EDTA, there was no inhibition of PCR products, whilst there was inhibition at 1mM concentration.

If PCR inhibitors present in a sample can be detected, extraordinary steps can be taken to try and purify the DNA in a way that will eliminate the inhibitor from the sample. Included among possible treatments are ultrafiltration, affinity chromatography, and the addition of polymers like bovine serum albumin to the PCR reaction to neutralize the inhibitors (Comey et al., 1994; Al-Soud, 1998; Moreira, 1998).

II.C. DNA Quantitation

II.C.i. Physical Methods

The principal reason for performing quantitation of human DNA prior to analysis is to assess and determine the amount of amplifiable DNA in a given sample. Determination of the amount of DNA present in a sample is important for STR analysis, as a fairly narrow range of 1-1.5 ng of template DNA will produce optimal DNA typing results (Applied Biosystems, 1998; Krenke et al., 2002).

One of the earliest methods developed for DNA quantitation was the absorbance of ultraviolet light at 260 nm. All nucleic acids quantitatively absorb ultraviolet light at 260 nm and thus spectrophotometry lacks specificity as it cannot distinguish the amounts of DNA from RNA that may be present in a sample (Molecular Diagnostic Lab Manual, 2005). In addition, DNA from all organisms absorbs UV light to the same degree. Spectrophotometry also does not distinguish between intact and degraded DNA, nor does it detect the presence of inhibitors. Due to its non-specificity, this method is not ideal for quantitating the amount of human DNA in forensic samples.

An improvement over spectrophotometry, yield gels can estimate both the quantity and the state of degradation of DNA in forensic samples. Yield gel technology quantitates DNA by estimating the amount of ethidium bromide fluorescence intercalated with genomic DNA co-electrophoresed in an agarose gel with known amounts of a well characterized DNA sample (Lambda DNA for example). Thus samples of unknown quantity are loaded in the gel with standards of known quantity allowing for comparison (Virginia Department of Forensic Science, 2006). After electrophoresis, the gel is stained

with ethidium bromide and exposed to ultraviolet light to visually compare and estimate the relative fluorescence of unknown samples with the intensity of fluorescence of the reference lamda standards.

Yield gels also provide information about the level of degradation in forensic samples. After electrophoresis and staining, large intact DNA molecules will form a compact band near the sample wells whereas degraded DNA will form a hazy smear of staining along the length of the gel track depending on the extent of degradation. Yield gels, while providing an estimate of quantity and quality, are also not specific for human DNA and will also reveal RNA present in an extract, albeit at a much lower sensitivity than double stranded DNA. Yield gels therefore provide some insight about DNA degradation but still lack sensitivity and specificity (NIST, 2004; Kline et al., 2005).

II.C.iii. DNA Quantitation- Hybridization Methods

Because ultrviolet spectrophotmetry and yield gels require close to microgram quantities of nondenatured DNA for analysis and are not specific for human DNA, a slot blot method that is specific for human and other primate DNA was developed (Walsh et al,. 1992). The “slot blot” method incorporates a 40 bp probe that is complimentary to a primate alpha satellite DNA sequence located on chromosome 17. This assay was first described with radioactive probes but has been modified for colormetric or chemiluminescent detection (Waye et al., 1989; Walsh et al., 1992). Slot blottting was at one time the most commonly used method for quantitation for human DNA in forensic laboratories. It is still widely used today though real-time PCR methods are replacing slot blotting in popularity. The Quantiblot Human DNA Quantification Kit, available from

Applied Biosystems, Foster City, CA, has a range of detection from 0.15 ng-10 ng of human DNA (Applied Biosystems, 2004).

In the slot blot technique, DNA is immobilized on a membrane and hybridized to the biotinylated oligonucleotide probe complementary to the alpha satellite target sequence, D17Z1 (Walsh et al., 1992; Applied Biosystems, 2004). The membrane is washed, and then soaked in a solution of streptavidin congujated to horseradish peroxidase; the amount of bound probe is detected by colorimetric or chemiluminescent means and compared against the signal intensity of a known DNA standard. A computerized method for DNA estimation uses chemiluminescent detection by scanning the silver grain density patterns on X-ray films (Walsh et al., 1992).

Although QuantiBlot technology represents a great improvement over ultraviolet spectrophotometry and yield gels in terms of specificity and sensitivity, the method is laborious to perform, subjective in the interpretation of result and does not give an indication as to the state of degradation nor the presence of PCR inhibitors in the sample (Timken et al., 2005). Slot blot quantitation methods may also underestimate the quantity of nuclear DNA in contaminated or degraded samples and it does not utilize PCR technology and the instrumentation which are common for genotyping in forensic laboratories. In addition, the technology is not readily amenable to extensive automation (Timken et al., 2005).

II.C.iii. DNA Quantitation- PCR Based Quantitation Methods

The forensic science community is constantly in search of faster, more automated and cheaper quantitation methods amenable to high throughput. Current identity testing

methods incorporate PCR multiplex assays that perform optimally with a narrow range of input template DNA, and thus the current development of PCR based quantitation methods are designed to be accurate enough to ensure that quality STR results are produced.

PCR-based quantitation methods can be of two types: end-point detection/ quantitation and real-time detection / quantitation. For end-point assays, a target genomic DNA sequence is amplified by PCR and the amount of amplicon produced is quantitated. Since product synthesis is generally directly proportional to input template amount, quantitation of template amounts in unknowns is possible (Allen and Fuller, 2006; Sifis et al., 2002; Nicklas and Buel, 2003a). Target genomic sequences for end-point quantitation assays have included the amelogenin gene and human Alu repeats (Allen and Fuller, 2006, Nicklas and Buel, 2003a; Sifis et al., 2002). To quantitate DNA in unknowns, a standard curve of amplicon quantity is first generated from the DNA samples of known concentrations to which the amount of amplicon produced from unknowns are compared.

Quantitative template amplification technology (Q-TAT), an end point PCR quantitation methodology developed in the OSU CHS Human ID laboratory by Allen and Fuller (2006), involves the quantitation of human genomic DNA through the amplification of the amelogenin locus using fluorescent PCR primers. Q-TAT estimates the amount of human DNA present in a sample by the comparison of the relative fluorescence in X and Y amplicons produced from unknown samples with fluorescent amplicons produced from well characterized DNA standards (Figure 5).

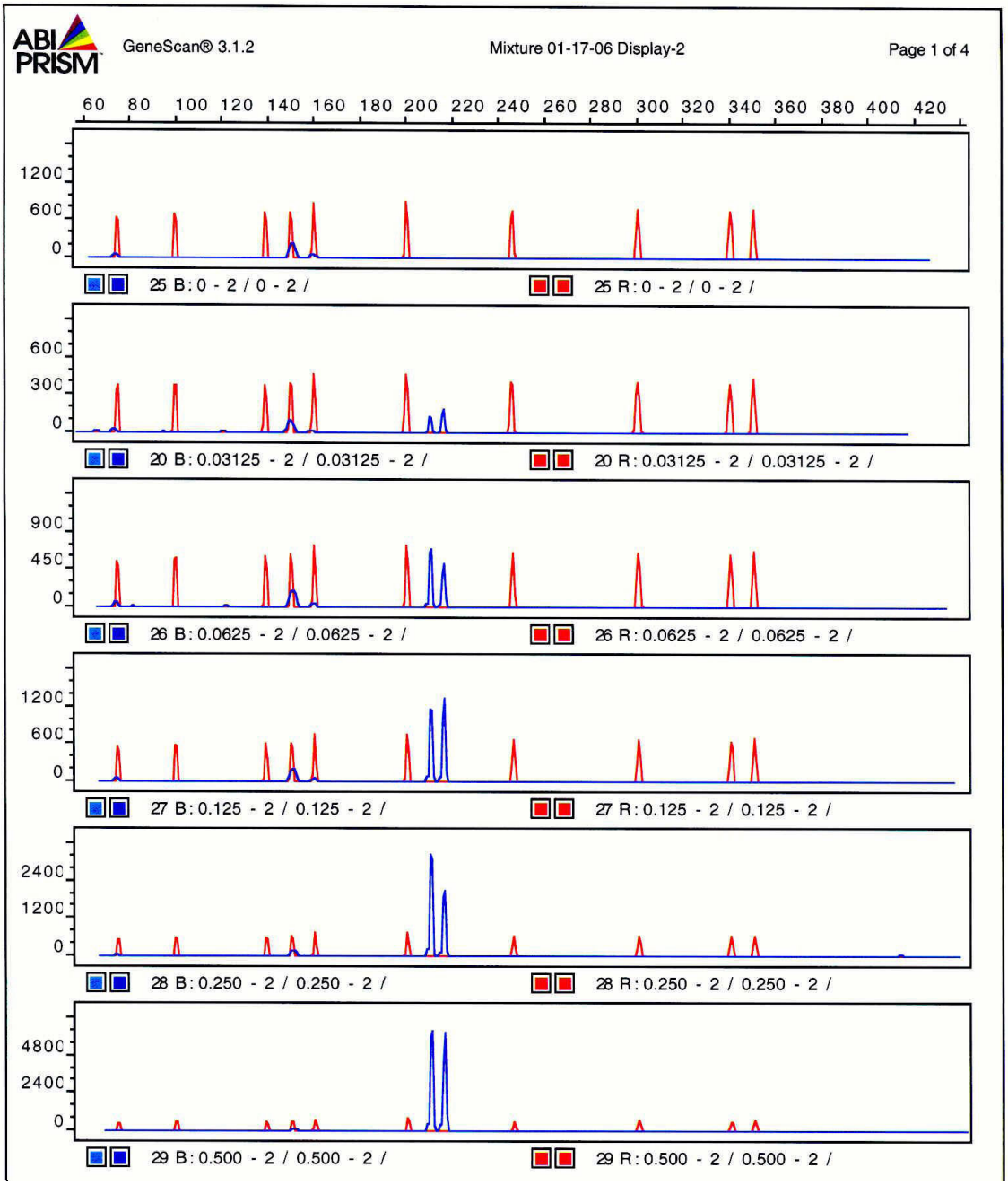

Figure 5: Electropherogram of AMEL- X and AMEL-Y Peaks for the production of a standard curve. The red peaks are the internal sizing standards, the blue peaks are the AMEL-X (210 bp) and the AMEL-Y (216 bp) amplicons. The increasing concentrations are from 0, 31.25, 62.50, 125, 250 to 500 pg with relative increase in amplicon sizes.

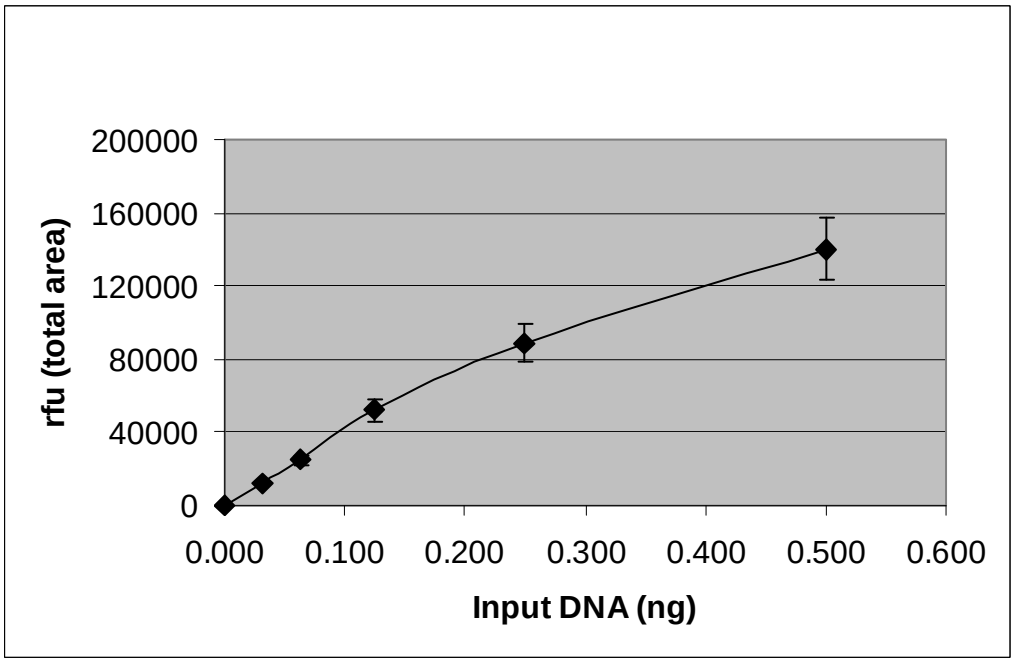

Figure 6: Q-TAT Standard Curve depicting the amount of input male reference DNA (ng) versus the total area of fluorescence incorporated into the AMEL-X and AMEL-Y amplicons (RFUs) Dilutions range from 0 pg to 500 pg (Allen and Fuller, 2006).

The AMEL-Y amplicon from the amelogenin gene on the Y chromosome is 216 bp and the AMEL-X amplicon produced from the amelogenin gene on the X chromosome is 210 bp. Because of the six basepair difference in size, the X and Y amplicons can be easily distinguished electrophoretically. Since one primer of the pair is linked to fluorescein, the amount of PCR product can be quantitated using the charge coupled device (CCD) camera, part of the Genetic Analyzer, used to produce STR profiles.

Fluorescently labeled DNA fragments are separated with a high resolution by capillary electrophoresis using the ABI 310 Genetic Analyzer from Applied Biosystems (Foster City, CA). Fluorescence from the PCR products is captured as a function of

electrophoretic migration and this is compared to the fluorescently labeled size standards present in each sample, allowing for size and quantity estimations of the unknown. The amount of fluorescence in each sample, expressed in relative fluorescent units (RFU), is calculated from the peak area of each amplicon using Genemapper ID software supplied for use with the Genetic Analyzer. Therefore, the peak areas from the AMEL- X and AMEL-Y amplicons amplified from the standards can be used to create a standard curve from which DNA quantity in an unknown sample can be estimated (Figure 6). Q-TAT is reproducible, and has a detection range of 20-500 pg of human DNA which is more sensitive than Quantiblot (Allen and Fuller, 2006). There is a reasonable agreement in the estimates of human DNA when comparing the two methods (Juroske, 2006; Allen and Fuller, 2006).

Other advantages of Q-TAT over the slot blot method are that it is performed using exactly the same methods and instrumentation as STR typing (Allen and Fuller, 2006). Q-TAT thus allows a forensic DNA typing lab to use existing technology and instrumentation for the assay. In addition, because Q-TAT amplifies the amelogenin locus on the X and Y chromosomes, it can be used to identify the sex of a sample donor and also quantitate male and female DNA that may be mixed in sexual assault samples (Allen and Fuller, 2006).

In another end-point based quantitation method, Sifis and coworkers (2002), used an Alu-based assay. Alu is the most well known primate short interspersed element (SINE), and was named for the Alu I restriction endonuclease site in its sequence. It accounts for 5-10% of the genome and was discovered in 1979 by Houck and coworkers. It has a 282-nucleotide consensus sequence, followed by a 3’ Adenosine (A) rich region,

resembling a poly (A) tail of varying length (Houck et al., 1979). Alu is found about a million times per haploid genome, most being located on the same chromosomal positions within the genome of primates and is a good target for the detection of human DNA (Sifis et al., 2002).

In the study of Sifis and coworkers (2002), a fluorescently labeled primer pair was designed enabling high efficiency amplification of the core Alu sequence within primate DNA. Quantitation was achieved by measurement of fluorescence intensity and compared to a standard curve constructed from a series of standard DNA template amounts. The detection range reported was 100 pg- 2.5 ng and the assay was compatible with the use and throughput of current forensic procedures (Sifis et al., 2002). Q-TAT's advantage over the Alu assay is its ability to determine the gender of the donor of the sample as well as the relative proportions of male and female DNA in mixed samples which are commonly encountered in sexual assaults.

II.C.iv. Real Time PCR (qPCR)

A new method of quantitation that is gaining in popularity in forensic laboratories worldwide is qPCR which can accurately reflect the quantity of DNA template present in an extracted sample during a real-time PCR procedure. Using real-time PCR negates the need for post PCR analysis quantitation as part of the process (Tringal et al., 2004). qPCR was first described by Higuchi and coworkers in the early 1990s and there are instruments and assays available now for use by forensic DNA typing laboratories (Higuchi et al., 1992, Higuchi et al., 1993). Because this quantitation is done without opening the PCR tube, it is referred to as a closed tube or homogenous detection assay.

Many detection chemistries are available for qPCR assays including SYBR Green detection, fluorogenic probes and molecular beacon technology (Holland et al., 1991; Lee et al., 1993; Tyagi et al., 1996; Wittwer et al., 1997). The two most common methods use either the fluorogenic 5' nuclease assay (TaqMan) or an intercalating dye such as SYBR Green that is specific for double stranded DNA molecules. The TaqMan technology measures change in fluorescence due to the displacement of a dual dye labeled probe within a target region and the SYBR Green assay detects formation of all PCR products simply by intercalation (Singer et al., 1997; Butler, 2005).

The three distinct phases of qPCR are: geometric amplification, linear amplification and the plateau region (Bloch, 1991) (Figure 7). During the exponential amplification phase, the reaction performs close to 100% efficiency, doubling amplicons with each cycle and producing a linear relationship between cycle number and the log scale of the DNA concentration. The linear amplification occurs after the exponential phase slows down due to an arithmetic increase in contrast to the geometric increase during the exponential phase. Some of the components, such as dNTPs or primers, will be consumed at different rates during this second phase, so this linear phase is not useful for comparison. The plateau region is the final phase as PCR product accumulation slows down to a halt, after reaching 1-7M (Bloch, 1991). For DNA quantitation by qPCR, fluorescence versus cycle number is optimally measured during the exponential phase of the reaction where there is direct proportionality between PCR product accumulation and input DNA. The cycle threshold (C_T) is used for qPCR calculations, the C_T value being the point in PCR amplification cycles when the level of fluorescence exceeds an arbitrary threshold set by the qPCR software and increases exponentially with each cycle. A plot

of the log of DNA concentrations versus the C_T value for each sample gives a linear relationship with a negative slope. The number of amplification cycles needed to attain the C_T threshold is correlated with the initial DNA template of samples of known DNA concentration, generating a standard based on C_T values. A sample with an unknown DNA concentration is once again compared to the standard curve to quantitate its initial concentration (Butler, 2005).

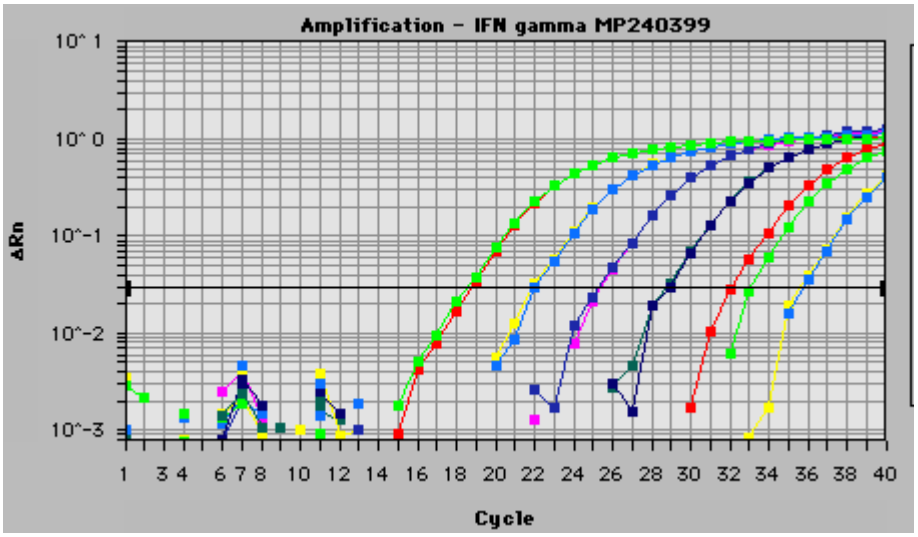

Figure 7: qPCR Output and Standard Curve (Davidson, 2003)

Several qPCR assays have been developed that include the following genomic targets: Alu assay, Amelogenin assay, CFS THO1 assay, TPOX and SRY duplex, and RB1 and mtDNA multiplex (Timken et al., 2005; Tringal et al., 2004; Richard et al., 2003, Alonso et al., 2003, 2004, 2005; Nicklas and Buel, 2003c, 2005; Horsman et al., 2006; Andreasson et al., 2002). Commercial qPCR kits such as the Quantifiler Human

DNA Quantification Kit which targets the total telomerase reverse transcriptase gene, (hTERT) for the detection of total human DNA and the Quantifiler Y Human Male DNA Quantification Kit which targets the sex determining region SRY gene on the Y-chromosome DNA present in a sample are available commercially (Applied Biosystems, 2003). These kits produce reliable and reproducible results important for providing an efficient approach to DNA typing (Sifis et al., 2002, Green et al., 2005, Applied Biosystems, 2005).

II.C.v. Quantitation of Mixed Samples

In sexual assault evidence, where detection of male DNA and quantitation in a mixed sample is crucial, selection of a DNA typing method which has a high probability of producing a highly discriminatory DNA profile of the assailant is essential to successfully identify and subsequently prosecute suspects. Samples containing large contributions of male DNA are generally best analyzed through autosomal STR markers because of their high discriminatory power. In contrast, samples containing very small amounts of male DNA in the presence of an excess of female victim DNA may be better suited to Y-STR analysis which, although less discriminatory, will only produce STR results from the limited amount of assailant Y chromosome present (Butler, 2005).

II.C.vi. Simultaneous Quantitation of Male and Female DNA

With the growing use of Y-chromosome STR typing in forensic labs, the need to quantitate male DNA in a sample is clear (Horsman et al., 2006). Of the quantitation methods described in this review, the assays targeting the amelogenin locus and the SRY

locus have the potential to characterize and identify male and female DNA present in mixed samples simultaneously (Alonso et al., 2004; Allen and Fuller 2006; Horsman et al., 2006). The QuantiBlot slot blot methodology does not have this capability, and although a Y chromosome probe could be designed for use, the method would still be laborious with a low limit of detection. Two commercially available singleplex qPCR kits are available from Applied Biosystem for total human DNA and Y-chromosome DNA quantitation, but multiplex PCR has the advantage of consuming half as much sample and being more efficient (Horsman, 2006).

Q-TAT, developed by Allen and Fuller (2006), has many advantages over qPCR methods in that although it uses the same PCR typing methodology, it does not require new instrumentation, training, additional allocation of space nor added quality assurance and thus is cost effective for labs with limited space or budgets. qPCR on the other hand uses unique instrumentation, requires training of analysts to be proficient in this new methodology, and incorporates new quality assurance measures to ensure accuracy of results (Allen and Fuller, 2006). The major advantages of qPCR over end point methods are increased sensitivity and greater dynamic range (Butler, 2005).

CHAPTER III

METHODOLOGIES

III.A. Q-TAT Genomic Targets

The main goal of this study was to enhance the Q-TAT reaction to:

- Simultaneously detect the presence of PCR inhibitors
- Assess the extent of DNA degradation
- Target multiple male specific PCR amplicons to compensate for possible Y chromosomal mutations that would prevent amplification of a single DNA target and lead to mistyping of a sample.

The strategy for achieving the goal of improving the Q-TAT assay that targets the amplification of the AMELX gene (210 bp) and the AMELY gene (216 bp), was to include two additional DNA targets into the multiplex PCR reaction:

- The Renila luciferase gene (200 bp) cloned in the pRL null vector was incorporated into the Q-TAT reaction to detect the presence of PCR inhibitors
- A second amplification target on the Y chromosome, the SRY gene (110 bp) was incorporated into the multiplex PCR reaction as an additional Y chromosome marker for unambiguous gender identification and as an indicator of template degradation.

III.A.ii. Techniques

III.A.ii.a. Q-TAT PCR Amplification

The Amelogenin locus that is the target for the Q-TAT assay is present on the X and Y chromosomes. The AMEL-X (210 bp) and AMEL-Y (216 bp) amplicons are detected through fluorescence and are differentiated by size. The X and Y amplicons were generated through PCR amplification of genomic DNA samples utilizing the following primers targeting the Amelogenin locus with the 5' end of the downstream primer labeled with the fluorescein derivative, FAM.

Upstream primer:

5'-ACCTCATCCTGGGCACCCTGG-3'

Downstream primer:

5'-FAM -AGGCTTGAGGCCAACCATCAG-3'

The upstream and downstream primers used in this study were synthesized by Invitrogen, (Chicago, IL), and are identical in sequence to the amelogenin primers included with the sex typing kit available from Promega Corp (Madison, WI). There were no significant differences observed in the quantitation characteristics for human DNA when using either a labeled upstream or a labeled downstream amelogenin primers. Labeled with FAM, the primers used are specific for primate DNA, but minor amounts of amplicons of different sizes can be produced with non primate genomic DNA (Micka et al., 1999; Pogemiller, 2006).

The Q-TAT assay incorporates the production of a standard curve with each assay (Allen and Fuller, 2006). The standard curve is prepared from a series of dilutions of a male reference DNA sample of known concentration. In this study, a reference sample of

male genomic DNA, at a concentration of 50 µg/ml concentration was diluted 1:100 with UV treated ultrapure water to produce a 500 pg/µL sample. Two fold serial dilutions of the 500 pg/µL standard were made to produce four additional dilutions of 250 pg/µL, 125 pg/µL, 62.5 pg/µL and 31.25/ µL pg concentrations. One microliter of each dilution was then amplified by PCR in a total volume of 12.5µL containing 1 µM of Amelogenin forward and reverse primers, 1 µM of SRY primers, 0.1 µM of pRL primers, 0.5 pg of pRL- null plasmid, 1.25 µL of 10X Gold ST*R buffer (Promega, Madison, WI) and 1.25 U of AmpliTaq Gold (Applied Biosystems, Foster City, CA). All samples were amplified using an ABI9700 thermal cycler, (Applied Biosystems, Foster City, CA) using the protocol below.

1 cycle	10 cycles			20 cycles			1 cycle	
Initial Incubation	Melt	Anneal	Extend	Melt	Anneal	Extend	Final Extension	Final Step
96^0 C	94^0 C	60^0 C	70^0 C	90^0 C	60^0 C	70^0 C	60^0 C	4^0 C
11mins	1min	1min	1.5min	1min	1min	1.5min	45min	Hold

Table 2: PCR conditions for Q-TAT (Allen and Fuller, 2006)

III.A.ii.b. Capillary Electrophoresis

After amplification, 1 µL of each amplified sample was added to a mixture of 24.5 µL formamide and 0.5 µL of LIZ 500 internal size standard (Applied Biosystems, Foster City, CA). A sample of AmpFISTR positive control DNA 9947A was also amplified and analyzed with each sample batch as an amplification control containing a

known amount of human DNA template. The samples were placed in order on a rack on the autosampler of an ABI 310 Genetic Analyzer (Appied Biosystems, Foster City, CA) for capillary electrophoresis and amplicon analysis (Figure 8). Each sample was electrophoresed at 60^{o}C for 20 minutes at 15 KV and 8-11 uA.

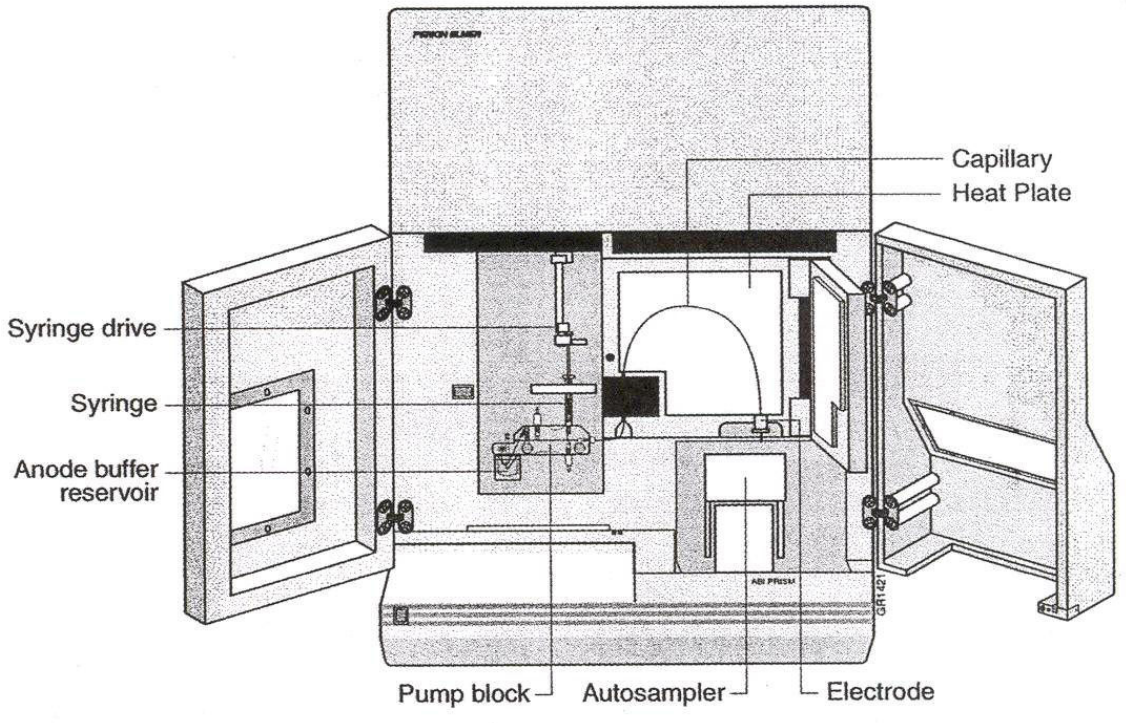

Figure 8: 310 Genetic Analyzer used for capillary electrophoresis (Budowle, 2000)

The ABI 310 Genetic Analyzer uses capillary electrophoresis and fluorescence detection technology to separate and visualize separation patterns of DNA fragments by size and color. Each sample is electro injected into a polymer filled capillary. The polymer acts as a sieve to separate DNA fragments by size. The smaller amplicons move faster and are therefore detected earlier in each run. LIZ 500, an internal size standard containing a collection of known DNA fragments labeled with the fluorescent dye, LIZ,

was run with each sample. The migration of the known markers was used by the GeneMapper ID software (Applied Biosystems, Foster City, CA) to precisely estimate the size of the unknowns to within a basepair of resolution with a local homology size algorithm (Elder and Southern, 1983).

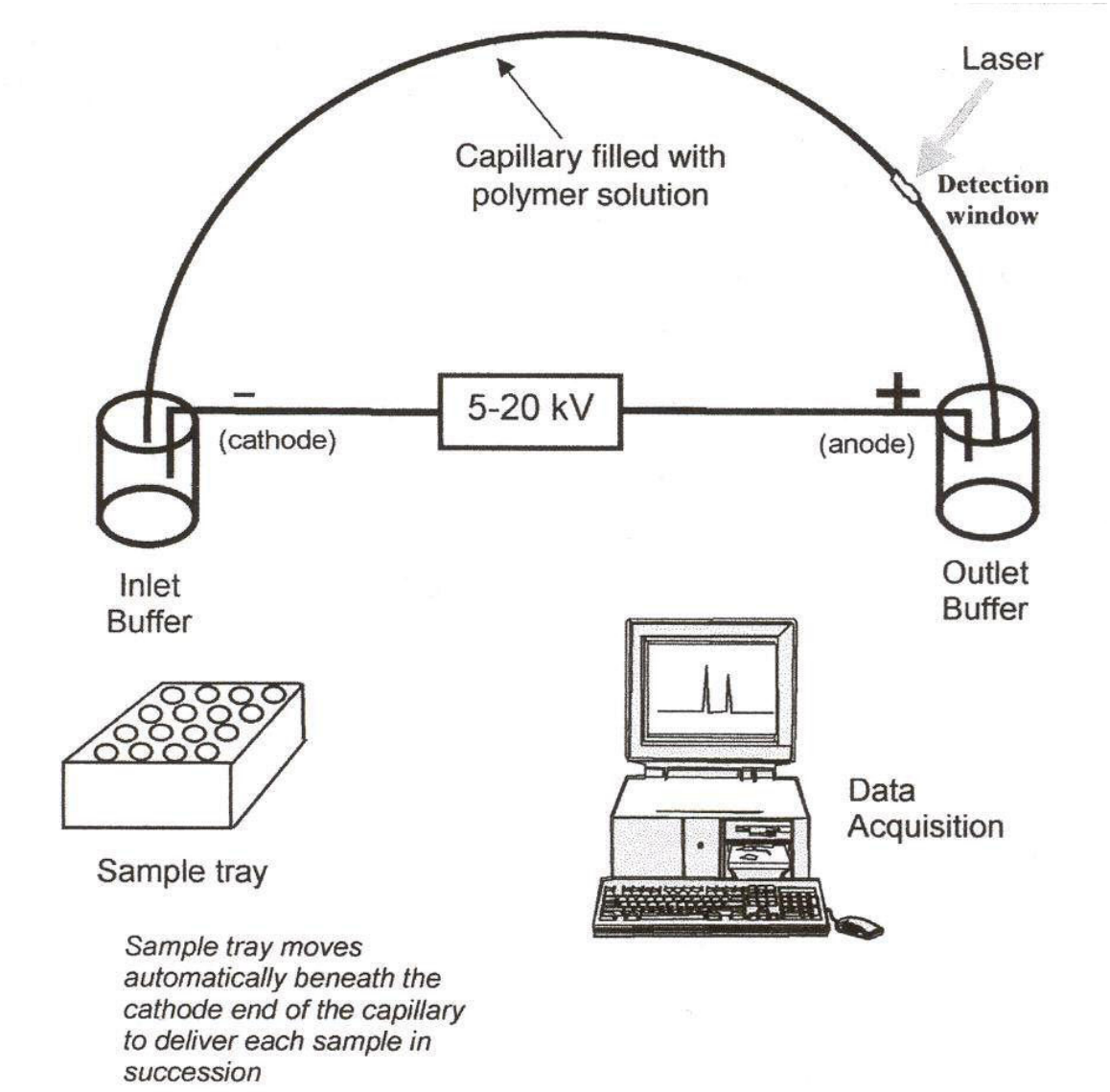

Figure 9: The process of capillary electrophoresis (Butler, 2005)

As fragments pass a capillary window, a laser beam excites the fluor and the CCD camera is able to capture and interpret its fluorescent emission. The software quantitates the relative fluorescent units (RFU) for each amplicon. The amount of fluorescence detected is directly proportional to the amount of amplicon product which, in turn, is proportional to the amount of input DNA template (Allen and Fuller, 2006).

III.A.ii.c. Internal Standards

After the establishment of a reproducible standard curve, the inhibition control for detecting PCR inhibitors (pRL) was incorporated into the basic Q-TAT reaction.

pRL –Upstream Primer (Invitrogen, Chicago)

(5' to 3') (DNA) –AAGGTGGTAAACCTGACGTTG

pRL – Reverse Primer (downstream)

(5' -3') (DNA) – Fam- TTCATCAGGTGCATCTTCTTG

For each PCR reaction, 1 µL of 10 X Gold ST*R buffer, 1 µM of amelogenin primers, 0.1 µM pRL primers, 0.5 pg of pRL plasmid, 500 pg of male DNA, and 1.25 units of Taq Gold was added to 6.25 µL of UV treated ultra pure water to make up a total volume of 12.5 µL. A master mix was made up and aliquotted into tubes, to which the diluted series of template DNA was added and amplified. Following amplification, 1 µL of each was added to 24.5 µL of formamide and 0.5 µL of LIZ 500 and placed in the ABI 310 Genetic Analyzer (Applied Biosystems, Foster City, CA) for electrophoresis and analysis.

A second internal control, the SRY gene was then added to the Q-TAT reaction.

SRY Forward Primer (Invitrogen, Chicago)

(5' to 3'): (DNA) - ACGAAAGCCACACACTCAAGAAT

SRY Reverse Primer

(5’ to 3’): (DNA) – FAM- CTACAGCTTTGTCCAGTGGC

For each PCR reaction, 1 µL of 10X Gold ST*R buffer, 1 µM of amelogenin primer, 1 µM of SRY primer, 0.1 µM of pRL primer, 1.25 units Taq Gold polymerase, 500 pg male DNA and 0.5 pg of pRL DNA was added to 5.25 µL of ultra pure water to make up a total volume of 12.5 µL.

Each PCR reaction mix was amplified in the GeneAmp PCR System 9700 thermocycler and 1 µL of each amplified product was added to 24.5 µL of formamide and 0.5 µL of LIZ 500 and electrophoresed in the ABI 310 Genetic Analyzer (Applied Biosystems Foster City, CA)

III.A.ii.d. Multi Primer Mix

A multi primer master mix, containing 10 µM of forward and reverse primer pairs of the amelogenin gene, 10 µM of forward and reverse the SRY primer pairs, 1 µM of the pRL primer pairs and 79 µL of ultrapure UV treated ultrapure water was mixed up in one tube. 1.25 µL of this 10 X concentrated multi primer mix was used for each 12.5 µL PCR reaction.

III.B. Experiments

III.B.i. Controlled DNA Degradation Studies

Degradation of genomic DNA template was performed over specific time periods to test the effectiveness of the modified Q-TAT assay to assess the degree of degradation in forensic samples.

DNA samples were randomly fragmented by treatment with RNase-Free DNase 1 (1 U/µL, Promega Corporation). A reaction mix of 18 µL (40 ng/µL) of DNA, 12.5 µL of 10 X buffer (provided with the DNase1), 89.5 µL UV treated ultrapure water and 5.0 µL of DNase I at various concentrations was created, with the DNase 1 added last. Immediately, 2.5 µL of Stop solution was added to a 25 µL aliquot of the reaction mix (time 0 sample) which was then heated at 65 degrees for 10 minutes to halt enzyme activity. The remaining digestion was incubated for increasing times at 37^0C. At 5, 10, 20, 40 and 60 minute time points. 25 µL of digest was removed and enzyme activity was halted with stop solution and heat as described above.

1 µL of each DNA sample (corresponding to 500 pg of genomic template) was then added to 11.5 µL of Q-TAT multiplex PCR mix and amplified in the GeneAmp PCR System 9700 thermocycler. After amplification, 1 µL of each amplified product was added to 24.5 µL of formamide and 0.5 µL of LIZ 500 and placed on the autosampler of an ABI 310 Genetic Analyzer (Applied Biosystems, Foster City, CA) for electrophoresis and amplicon analysis. Relative fluorescence in the AMEl-X and AMEL-Y, SRY and pRL amplicons was quantitated and used to investigate the integrity of the DNA template.

III.B.ii. Inhibition Studies

EDTA, hemin, humic acid and indigo dye which are known inhibitors of PCR, were added to the Q-TAT multiplex to assess the sensitivity of the pRL internal control to detect PCR inhibition using 500pg of male DNA as template.

III.B.ii.a. EDTA

The ability of the pRL plasmid to detect inhibition by PCR was assessed in the following way: 0.5 M EDTA was diluted with dH_2O to 0.1 mM, 0.25mM, 0.5 mM, 0.75 mM, and 1 mM concentrations. One µL of each EDTA dilution was then added to 11.5 µL of Q-TAT multiplex PCR reaction mix containing 500 pg male DNA and amplified in the normal way. 1 µL of each PCR product was then analyzed on the ABI 310.

III.B.ii.b. Hemin

The ability of the pRL plasmid to detect the inhibitory effect of hemin on PCR was investigated in the following way: 35 mg of Hemin ($C_{34}H_{32}ClFeN_4O_4$; molecular weight 651.94, SIGMA, EC No. 240-1140-1) was dissolved in 53.7 ml of 10 mM of Sodium Hydroxide (NaOH) to form a solution of 1 mM concentration of alkaline hematin (ferriprotoporphyrin hydroxide). Four further dilutions of 0.75 mM, 0.5 mM, 0.25 mM and 0.1 mM were made from the 1 mM stock. 1 µL of each hemin concentration was then added to11.5 µL of multiplex Q-TAT PCR reaction mix containing 500 pg of male DNA. It should be noted that the pH of the final PCR reaction mix was unchanged after adding the small volume of alkaline hemin (not shown). One microliter of each PCR reaction was then analyzed on an ABI 310 Genetic Analyzer (Applied Biosystems, Foster City, CA).

III.B.ii.c. Humic Acid

Detection of humic acid inhibition on PCR amplification was studied by dissolving 1.0 gm of humic acid (sodium salt 68131-04-4, H16752-5G, SIGMA, EC

No.268-608-0) in 100 ml of UV treated ultrapure water, to make a 0.1 % solution. Six dilutions of 0.05 %, 0.025 %, 0.020 %, 0.015%, 0.010 %, and 0.005 % were prepared from the stock and 1 µL of each dilution was added to to11.5 µL of multiplex PCR reaction mix. All the samples were amplified and 1 µL of each PCR product was then analyzed on the ABI 310 Genetic Analyzer (Applied Biosystems, Foster City, CA) for electrophoresis and amplicon analysis.

III.B.ii.d. Indigo Dye

Liquid blue denim indigo dye was purchased from Walmart Super Center and diluted into the following factors: 1: 10, 1: 50, 1: 65, 1: 85, and 1: 100. 1 µL of each dilution was added to11.5 µL of multiplex Q-TAT PCR reaction mix containing 500 pg of male DNA. 1 µL aliquot of each PCR amplification product was then analyzed on the ABI 310 Genetic Analyzer (Applied Biosystems, Foster City, CA).

III.C. Comparison of Quantitation Methodologies

Q-TAT has been shown to produce comparable DNA concentration estimates with other DNA quantitation methods used in forensic laboratories (Allen and Fuller, 2006). In a comparison study with shared samples, Q-TAT was found to be more sensitive than the widely used slot blot method, but less sensitive than qPCR (Allen and Fuller, 2006).

Fourteen samples previously quantitated by qPCR were quantitated using UV spectrophometry and using the modified Q-TAT method.

III.D. Data Analysis and Statistics

The Genemapper ID software quantitates the relative florescence units (RFU) for each amplicon, the amount of fluorescence detected is proportional to the amount of amplicon product which, in turn, is proportional to the amount of input DNA template (Allen and Fuller, 2006). The SRY, pRL, AMEL-X and AMEL-Y peaks were identified and their respective RFUs were recorded on an Excel spreadsheet. The peak areas under the SRY, pRL, AMEL-X and AMEL-Y curves in each dilution was normalized for the different injections through comparison with the total RFU contained within the 200 bp standard present in the LIZ 500 size standards. The size standard is present in constant amount in the 25 µL aliquot in each sample tube, from a master mix of formamide and LIZ500, and thus variation in the fluorescence from injection fluctuations and laser CCD camera variability that affect LIZ 500 fluorescence and the amplicon fluorescence could be normalized across the entire run. Normalized fluorescence peak areas from SRY, pRL, X and Y amplicons per picogram input of reference DNA were then computed from the slope of the standard curve (Allen and Fuller, 2006).

The standard error of the mean (SEM) was calculated for each sample using the formula below. The standard error of the mean measures how far the sample mean is likely to be from the true population mean.

$$SEM = \frac{\sqrt{\sum (Yi-Ymean)^2 / (N-1)}}{\sqrt{N}}$$

CHAPTER IV

RESULTS

IV.A. Internal Control for Inhibition- pRL plasmid

The first modification to the basic Q-TAT assay was the addition of the Renila Luciferase pRL gene (200 bp) to the PCR reaction, as an internal inhibition control. The pRL gene was chosen because it is similar in size to the AMEL-X and AMEL-Y amplicons but can be distinguished from those amplicons by size.

As a first step to optimize pRL amplification, four dilutions of 1 µM, 0.5 µM, 0.2 µM, and 0.1µM pRL primers were each added to the PCR mixture with 1 ng of pRL plasmid, amplified in the GeneAmp PCR System 9700 thermocycler and electrophoresed in the ABI 310 Genetic Analyzer (Applied Biosystems, Foster City, CA).

At the same time, the amount of input pRL gene template was also varied. Four dilutions of 5.0 pg, 2.50 pg, 1.25 pg and 0.5 pg were amplified with the four pRL primer dilutions listed above to determine the best ratio of primer concentration to template for the assay. Too much pRL gene resulted in split and truncated peaks, and too little pRL DNA template resulted in non-reproducibility (Data not shown).

Ultimately, the final concentrations of 0.5 pg of pRL DNA per reaction containing 0.1 µM pRL primer pair were found to be optimal for PCR, and multiple runs produced robust, reliable and reproducible amplifications, showing the pRL gene at 200 bp, the AMEL-X gene at 210 bp and AMEL-Y gene at 216 bp (Figure 10).

IV.B. Internal Control for Degradation- the SRY gene

Incorporating an additional PCR target on the Y chromosome was investigated for two reasons: first, mutations in the amelogenin locus have been described that prevent amplification of the locus and can lead to the false conclusion that male DNA is not present in the sample. The incorporation of a second DNA target on the Y chromosome would therefore be expected to compensate for possible mutations in the amelogenin locus and vice versa resulting in curate gender determination of forensic samples. Secondly, a second target that is also smaller in size than the AMEL-X and AMEL-Y amplicons could be useful as an indicator of template integrity (i.e., degradation).

For each PCR reaction, 1 µM of amelogenin primer pair, 1 µM of SRY primer pair, 0.1 µM of pRL primer pair, 0.5 pg of pRL DNA and 500 pg of male DNA were combined in a 12.5 µL PCR reaction mix and amplified. Multiple runs on the ABI 310 (Applied Biosystems, Foster City, CA) produced robust, reliable and reproducible amplicons with the SRY gene at 110 bp, the pRL gene at 200 bp, the AMEL-X gene at 210 bp and the AMEL-Y gene at 216 bp (Figure 10). Because the SRY primers annealed to the SRY locus on the Y chromosome, fluorescence in the SRY amplicon increased proportionately to the input DNA from 31.25 pg-500 pg (Figure 10). The electropherogram also showed a constant peak area for the pRL gene at all the serial dilutions of the male DNA as equal amounts of template and primer were aliquoted into each PCR tube from the master mix (Figure 10).

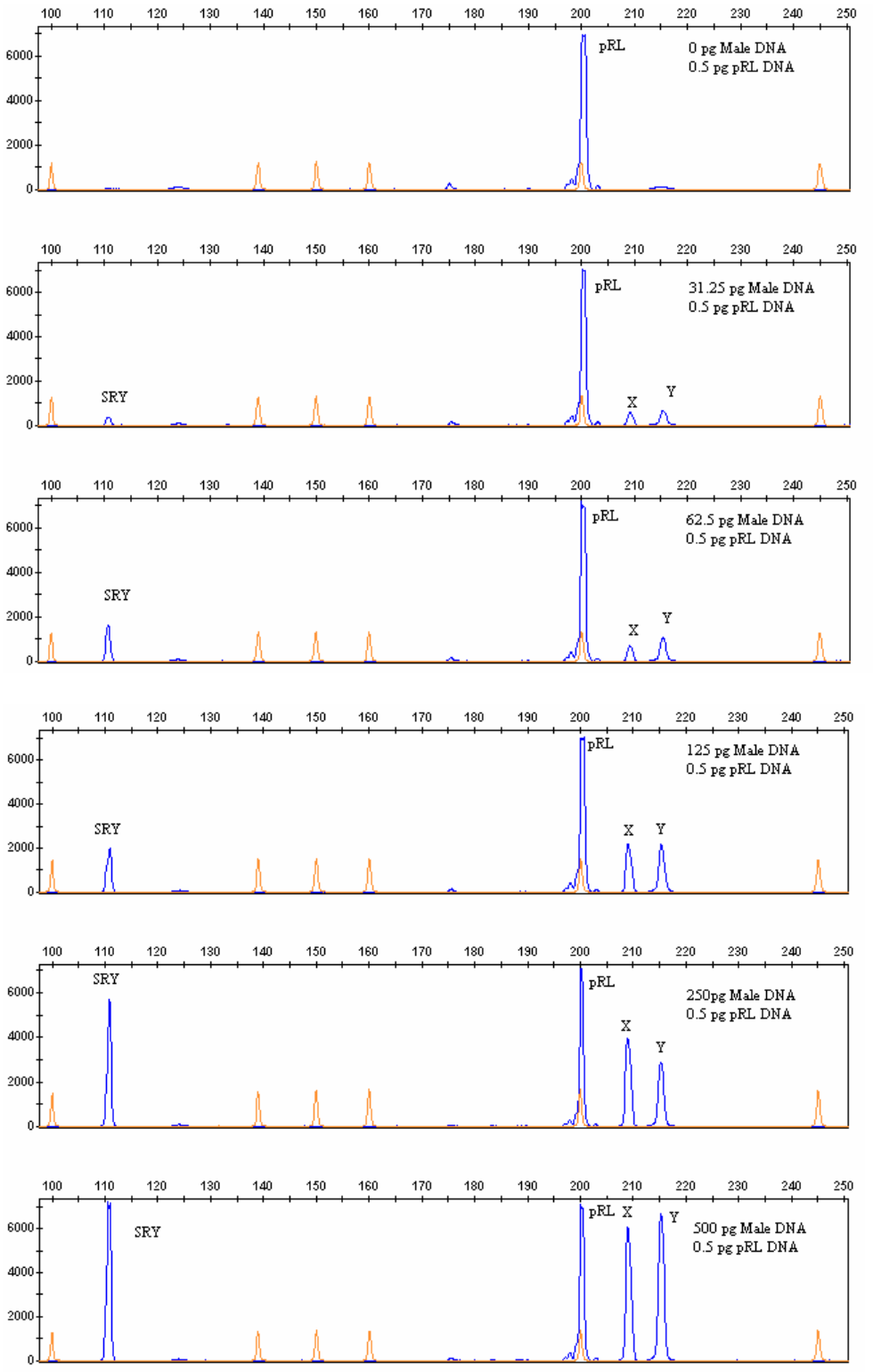

Figure10: Electropherogram of SRY, pRL, AMEL-X and AMEL-Y Peaks

IV.C. Multi Primer Mix

A master mix containing appropriate amounts of all the primer pairs, SRY, pRL and amlogenin was mixed in one tube and dispensed simultaneously into each PCR tube required for amplification. The ease of setting up a reaction with the multi-primer mix is more efficient and resulted in fewer pipetting fluctuations than the alternative of adding each individual primer separately and was found to give results comparable to those produced with each reactant added separately. The multi-primer mix was used for all subsequent degradation and inhibition experiments.

IV.D. Q-TAT Standard Curves

Highly reproducible standard curves were produced from multiple runs, using serially diluted male DNA as the quantitation standard, including a 100 pg aliquot positive control DNA contained within the Profiler Plus STR typing kit, (Applied Biosysytems, Foster City,CA) to serve as an internal quantitation control (data not shown). The average slope of AMEL-X +Y was 308.95x with an R^2 value of 0.9879. The peak areas under the SRY, AME-X and AMEL-Y curves amplified from each dilution of human DNA was normalized among the different injections through comparison with the total RFU contained within the 200 bp standard present in the LIZ 500 standards.

	SRY/LIZ		PRL/LIZ		X+Y/LIZ		Y/LIZ	
pg	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
0.00	0.00	0.00	84971	7097	0.00	0.00	0.00	0.00
31.25	5374	1470	75441	12766	10625	3693	4968	2768
62.50	14524	2404	80386	9922	31915	14783	10778	2573
125.0	24271	2157	87707	6009	49946	5453	23667	4147
250.0	38907	6542	72146	2390	71218	5026	34090	1045
500.0	62148	2446	74061	4459	130089	10207	66225	6279

Table 3: Average Data for Multiplex Standard Curve (RFU)

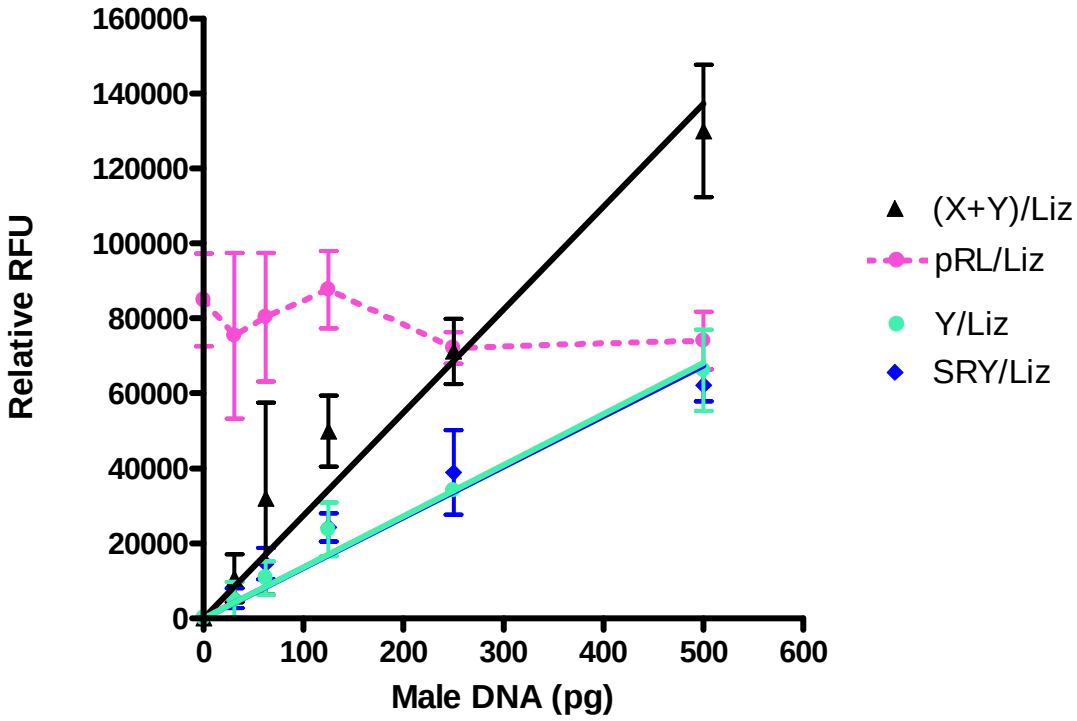

Figure 11: Average standard curves for multiplex quantitation.

The 200 bp size standard was present in a constant amount in each 25 uL aliquot of formamide –Liz dye as each sample was prepared using a single mastermix. Thus variation in the fluorescence from injection fluctuations and laser CCD camera variability was normalized across the entire run. Normalized fluorescence peak areas from the SRY, pRL, AMEL-x and AMEL-Y amplicons per picogram input of reference DNA were then compared from the slope of the standard curve.

IV.E. Analysis of Standard Curves

The standard curves produced for AMEL-X, AMEL-Y, AMEL-X+Y, SRY and pRL using the multi-primer mix in the Q-TAT reaction is shown in Figure 11. The normalized values for fluorescence in amplicons in each quantitation standard sample, was divided by the known amount of input template DNA. The results were averaged for all standard curve points and a constant was produced. The RFU in the pRL amplicon hovers around a constant range between 72,000 and 88,000 RFU. The AMEL-X and AMEL-Y have a combined peak area of about 130,000 RFU at 500pg of input DNA.

IV.F. Comparison of SRY and AMEL-Y Quantitations

Because the SRY and AMEL-Y primers both target the Y chromosome, similar fluorescence in each amplicon was expected, and was observed (Figure 11). Thus fluorescence and hence quantification estimates from the SRY and AMEL-Y amplicons are similar and well within the range of variation. The SRY and Y amplicons show essentially equal peak areas in the range of 62,000 - 66,000 RFU at 500pg input DNA

and also have similar slopes. For the SRY amplicon, y is equal to 149.1 x and R^2 is equal to 0.9109. For the AMEL-Y amplicon, y is equal to 156.37 x and R^2 is equal to 0.969. Thus intact undegraded DNA yields essentially equal quantities of AMEL-Y and SRY amplicons, forming an SRY to AMEL-Y ratio of 1.0.

IV.G. Detection of Degraded DNA with Internal Control using DNase1

Sometimes age and exposure to the elements can compromise the integrity of DNA in forensic evidence. An assay that would alert an analyst to the existence of degraded DNA in a sample would facilitate choosing the type of subsequent DNA analysis performed on the sample. One feature of the Q-TAT assay is the amplification of two loci on the Y chromosome (AMEL-Y and SRY) whose size differs by a factor of two (216 bp and 110 bp respectively). In as much as intact DNA yields essentially equal quantities of AMEL-Y and SRY amplicons, significant deviations of an SR : AMEL-Y ratio from 1.0 could reflect degradation of the genomic template. To test this hypothesis, intact male DNA was digested with DNase1 to varying degrees.

Amplification of enzymatically degraded DNA was assessed with Q-TAT , and RFU in AMEL-X, AMEL-Y and SRY amplicons were quantitated. Chung and coworkers (2004), using DNase degraded DNA, reported that minimum amplicon size provides an effective tool for the analysis of degraded forensic samples.

This DNase 1 is suited for applications such as nick translation, production of random fragments and cleavage of genomic DNA for foot printing. In the presence of Mg2+, DNase 1 attacks each strand of DNA independently, and the sites of cleavage are distributed in a statistically random fashion. In the presence of Mn2+, DNase1 cleaves

both strands of DNA at approximately the same site to yield fragments with blunt ends or protruding termini of one or two nucleotides (Promega catalog, 2007).

DNase 1 was used to degrade DNA and the degree of degradation over time was assessed using the relative fluorescence in the AMEL-Y and SRY amplicons. The degree of fragmentation induced by different amounts of DNase 1 was first assessed by agarose gel electrophoresis. Four dilutions of 1 U/µL, 0.50 U/µL, 0.1 U/µL and 0.05 U/µL of DNase1 were made from the enzyme stock and incubated with 5 ng of male DNA. 1 µL of each DNase1 dilution was added to 500 pg of DNA in 21.5 µL and 2.5 µL of 10 X buffer to make up a 25 µL solution. A control tube without DNase1 was included and the 5 tubes were incubated at 37^0 C for 30 minutes. 2.5 µL of Stop solution was added to each tube which was then heated at 65^0 C for 10 minutes to halt digestion. 5 µL of 5 X gel buffer was added to each sample and 25 µL was separated on a 0.8 % agarose gel.

The degree of fragmentation was assessed following ethidium bromide staining and UV radiation, using a Hind III digest of lambda DNA (Invitrogen) as size markers. The DNase dilution of 0.1 U/µL was selected as the optimum enzyme concentration to be used in the degradation experiments as it resulted in a partial digest of the DNA.

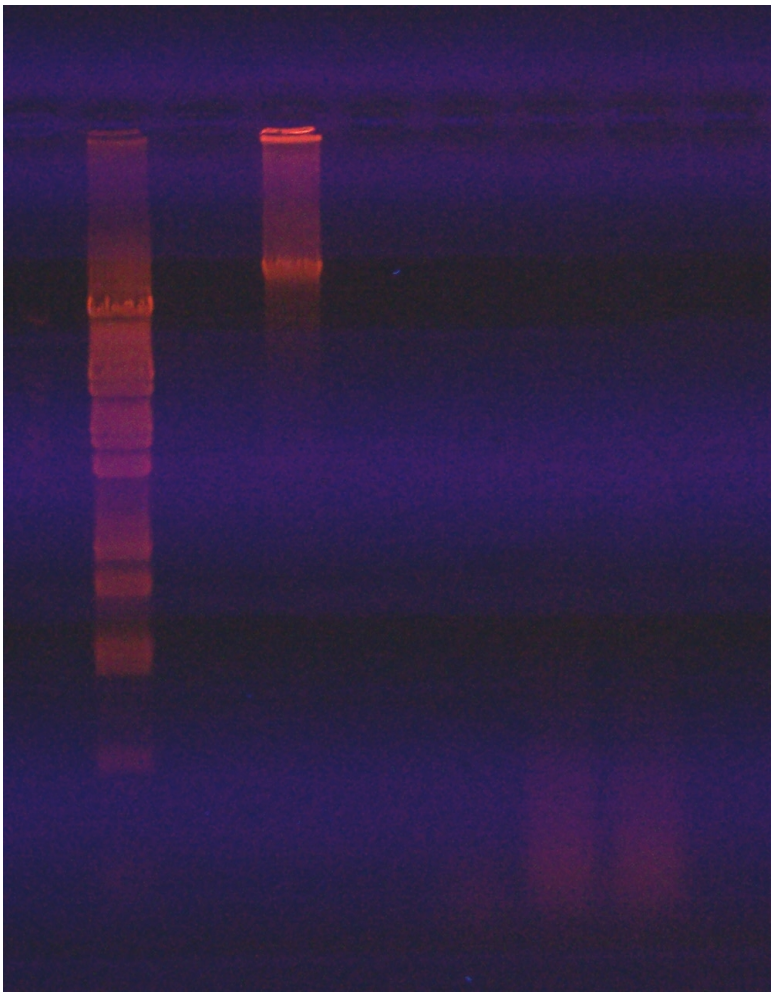

Figure 12: Ladder DNA 0.04 0.02, 0.004, 0.002/U DNase 1

The ladder is on the left, the next lane is the undigested DNA. The DNA is completely digested in the next two lanes (0.04, 0.02 U) and partial digestion in the last two lanes (0.004, 0.002 U)

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	SRY/LIZ		pRL/LIZ		X+Y/LIZ		Y/LIZ	
DNase mins	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
0	77484	5131	63751	8252	117816	9044	59938	4759
5	39943	2372	63526	4485	75731	5794	39036	6498
10	54373	3122	70086	14445	71667	6259	36366	4702
20	50523	3790	84471	10140	66081	8184	29875	3451
40	36533	808	79082	9554	52348	4349	28116	1677
60	37092	6753	67418	22958	54583	12882	19934	3216

Table 4: Average Data for DNase 1 Degradation of Male DNA (RFU)

Having established 0.004 U as the optimal enzyme concentration for the partial degradation of chromosomal DNA, 500 pg aliquots were digested with DNase1 for varying amounts of time, enzyme activity was halted with stop buffer and heat, and then the template was amplified using Q-TAT.

Analysis of results showed that PCR product yield for digested male DNA samples began to decrease in proportion to amplicon size. However, the pRL amplicon RFU remained unchanged with DNase 1 digestion demonstrating that inactivation of DNase 1 was effective.

After 60 minutes of digestion with 0.004U of DNase 1, there were still PCR products for all four targets in the samples; the AMEL-Y amplicon decreased the most from an initial fluorescence of 60,000 RFU down to 20,000 RFU after 60 minutes of

digestion (Table 4). Following DNase1 digestion, the SRY product decreased least (Table 4). These results suggest that since the SRY template is smaller, it is less affected by DNase 1 digestion and hence is more intact as a target for amplification. Shown in Figure 13 are the RFU in amplicons for AMEL-X, AMEL-Yand SRY. An initial sudden drop in the RFU for all amplicons at 5 minutes of digestion time is observed, followed by a more gradual decay in template integrity.

Throughout the DNA degradation period, it is noted that the fluorescence in the SRY amplicon was greater than that of the AMEL-Y amplicon. At 60 minutes the SRY amplicon was 37092 RFU, twice the fluorescence of the AMEL-Y amplicon at 19934 RFU. Thus the inclusion of the SRY marker in the Q-TAT assay gives an indication of DNA degradation in forensic samples.

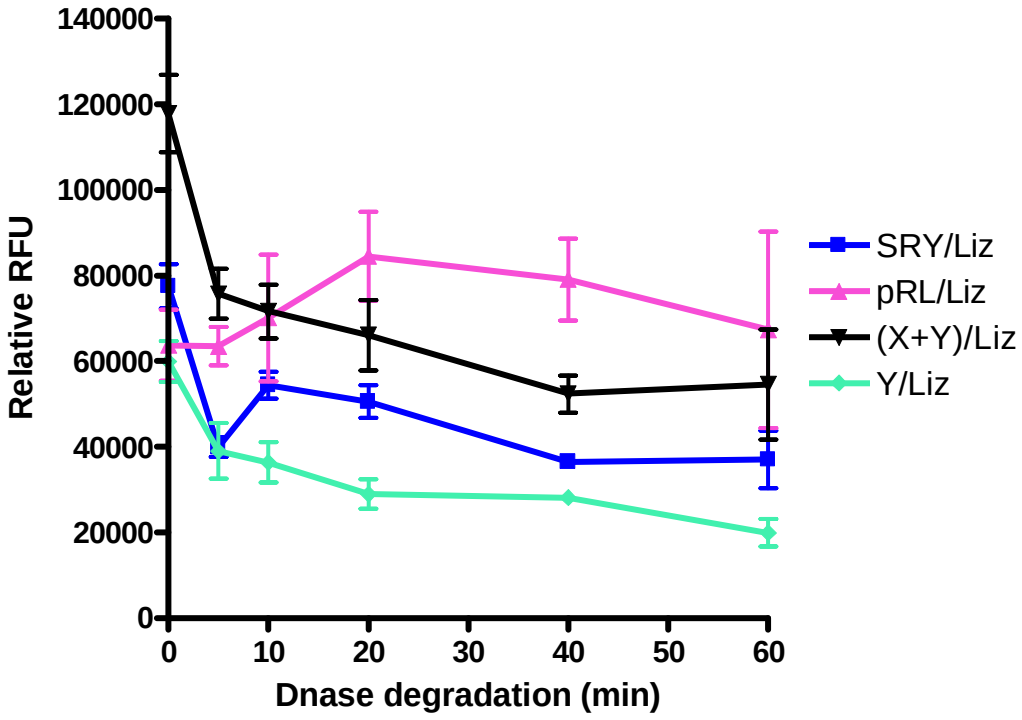

Figure 13: DNase 1 Degradation of Male DNA

IV.H. Detection of Inhibition

IV.H.i. EDTA

The successful production of a DNA profile can be prevented in part by the presence of inhibitory substances in complex biological compounds that may be co-extracted with DNA. EDTA, a common PCR inhibitor is a preservative and anticoagulant added to blood. EDTA inhibits PCR reaction by chelating divalent cations needed as cofactors by Taq DNA polymerase. To evaluate the detection of EDTA inhibition, varying amounts of the compound were added to Q-TAT reactions and the fluorescence of the control pRL product was quantitated. (Figures 14, 15 and Table 5)

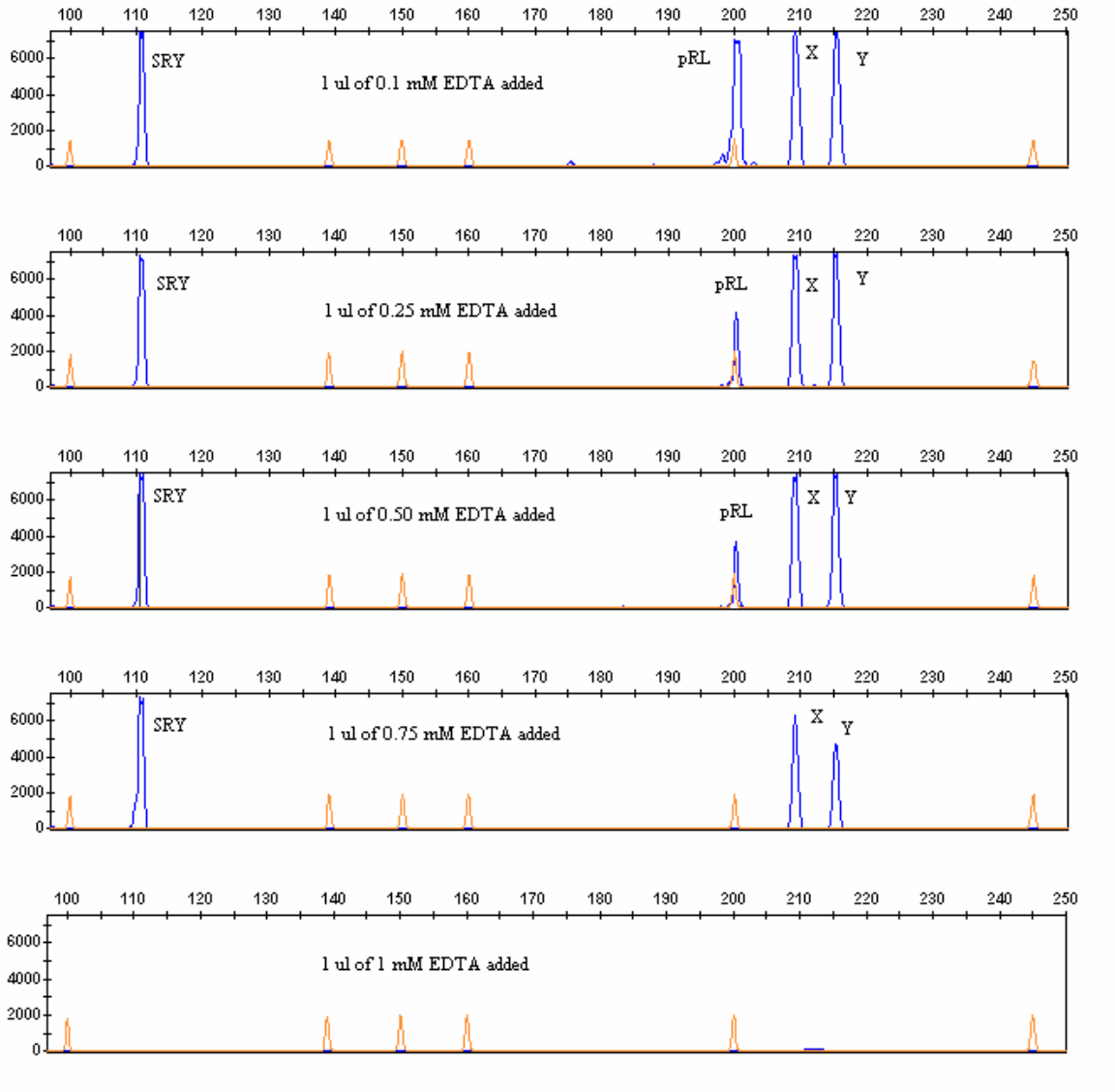

Figure 14: Electropherogram for EDTA Inhibition

The electropherogram shown in Figure 14 and the standard curves in Figure 15 demonstrate that there is no inhibitory effect of 0.008 mM EDTA on PCR amplification. At 0.020mM and 0.040mM mM EDTA concentrations, the pRL amplicon RFU begins to decrease, with no significant effect yet observed on the SRY, AMEL-X and AMEL-Y amplicons. These results confirm that the pRL control is a sensitive indicator of PCR inhibition by EDTA. At low concentrations of EDTA, the AMEL-X, AMEL-Y

mM	SRY/LIZ		pRL/LIZ		X+Y/LIZ		Y/LIZ	
EDTA	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
0.008	68514	4391	88395	5465	148731	10997	74152	6275
0.020	57499	1043	48205	13066	119827	3693	59910	1173
0.040	64178	5367	10203	5280	114054	8853	56355	6570
0.060	62296	1898	1018	586	106076	11705	52958	8853
0.080	0	0	0	0	0	0	0	0

Table 5: Average Data for EDTA inhibition (RFU)

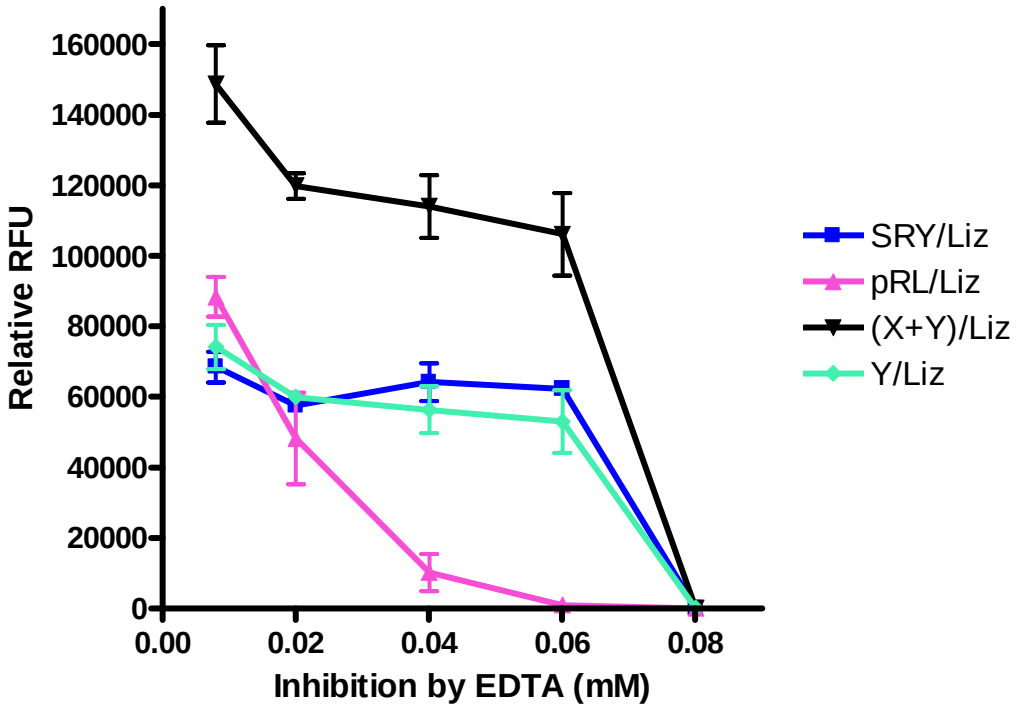

Figure 15: Standard Curves for EDTA Inhibition

and SRY amplicons show no evidence of inhibition while there is a 90% reduction in RFU in the pRL amplicon at 0.0.040 mM. At 0.060 mM EDTA, the pRL product completely disappears, and the RFU of the AMEL-X and AMEL-Y amplicons begin to decrease with no significant effect on the smaller SRY amplicon. At 0.080 mM, there is complete inhibition of all four loci.

IV.H.ii. Hemin

Studies have shown that the compound heme in blood is a PCR inhibitor (Akane 1994). For example, Al-Soud and Radstrom (1998) reported that a 0.004% (v/v) mixture of human blood added to a PCR reaction will totally inhibit the amplification process. Of course, the largest repository of heme in the human body is in red blood cells. The FE^{+++} oxidation product of heme is termed hemin.

To evaluate the detection of hemin inhibition by the pRL amplicon, varying amounts of the compound were added to Q-TAT reactions and the fluorescence of the control pRL product was quantitated (Figures 16, 17 and Table 7). The pRL amplicon is reduced in fluorescence from an average of 65305 RFU at 0.008 mM hemin to 50221 RFU at 0.040mM hemin. At these concentrations, the SRY fluorescence is unaffected but partial inhibition of the AMEL-Y amplicon fluorescence at 0.060 mM is evident. At the highest hemin concentration of 0.080mM, the SRY shows only limited inhibition with a 15 % decrease in fluorescence to 41684 RFU from an initial value of about 49509 RFU and amplification of the pRL product was completely inhibited from an initial fluorescence value of 65305 RFU to 852 RFU (Figures 16, 17 and Table 6).

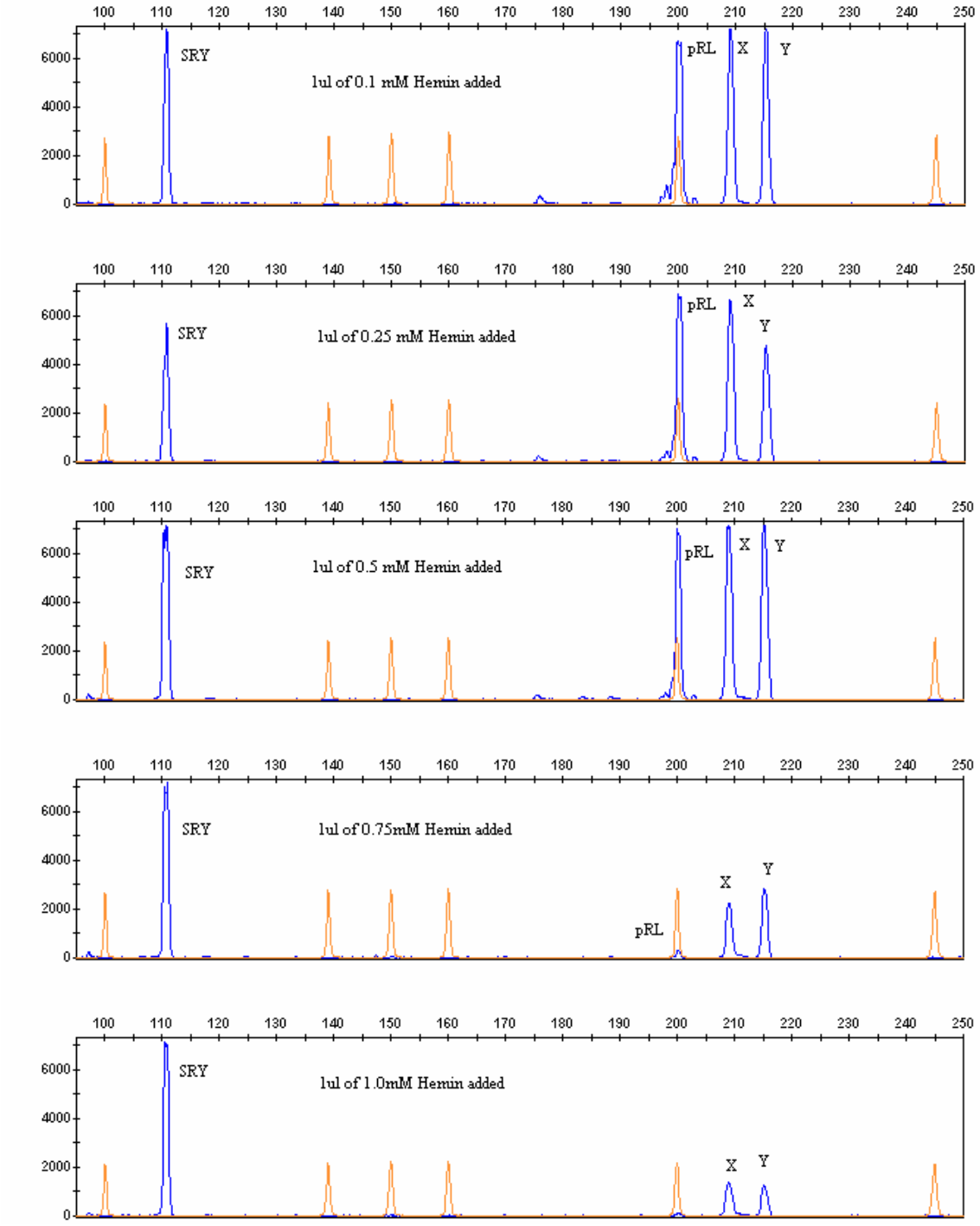

Figure 16: Electropherogram of Hemin Inhibition

mM	SRY/LIZ		pRL/LIZ		X+Y/LIZ		Y/LIZ	
hemin	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
0.008	49509	6062	65305	6894	97076	12123	45681	5736
0.020	51828	7636	65630	12434	96499	6445	45434	4121
0.040	51495	4478	50221	8269	86812	2556	41667	1677
0.060	50019	4195	4036	1506	49582	7731	25240	3375
0.080	41684	1607	853	405	25546	6029	11905	3357

Table 6: Average Data for Hemin Inhibition (RFU)

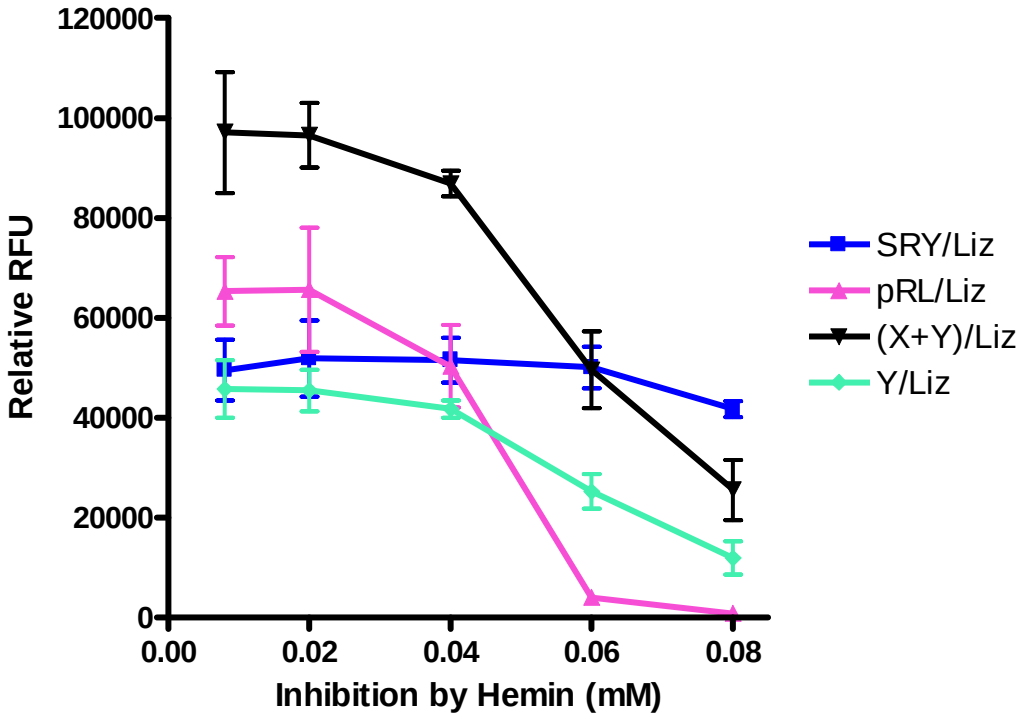

Figure 17: Standard curves for Hemin Inhibition

IV.H.iii. Humic Acid

Humic acid is one of the major components of humic substances that are dark brown and the major constituents of soil organic matter. Humus is a common source of PCR inhibitor in forensic samples obtained from outdoor forensic crime scenes (Tsai and Olson 1992). DNA from a forensic sample retrieved from soil may be contaminated with humic acid and thus cause PCR inhibition.

The effects of different concentrations of humic acid on PCR amplification were studied to evaluate the detection of humic acid inhibition by the pRL plasmid. Six dilutions of 0.004 mM, 0.008 mM, 0.012 mM, 0.016 mM, 0.020 mM, and 0.040 mM were amplified with the Q-TAT assay and the fluorescence of the control pRL product was quantitated (Figures 18, 19 and Table 7).

All humic acid inhibition curves are tri-phasic in nature (Figure 19). The pRL amplicon exhibited extreme sensitivity to increasing concentrations of humic acid. At 0.008 mM humic acid, the pRL locus shows about 80% inhibition and complete inhibition occurs at 0.016 mM humic acid. The SRY allele is the last to drop out at 0.040 mM humic acid. At 0.016 mM, the SRY: Y ratio had increased from an initial value of 1 to a final ratio of 188. There was complete amplification failure of all PCR products at the highest concentration of 0.040mM humic acid.

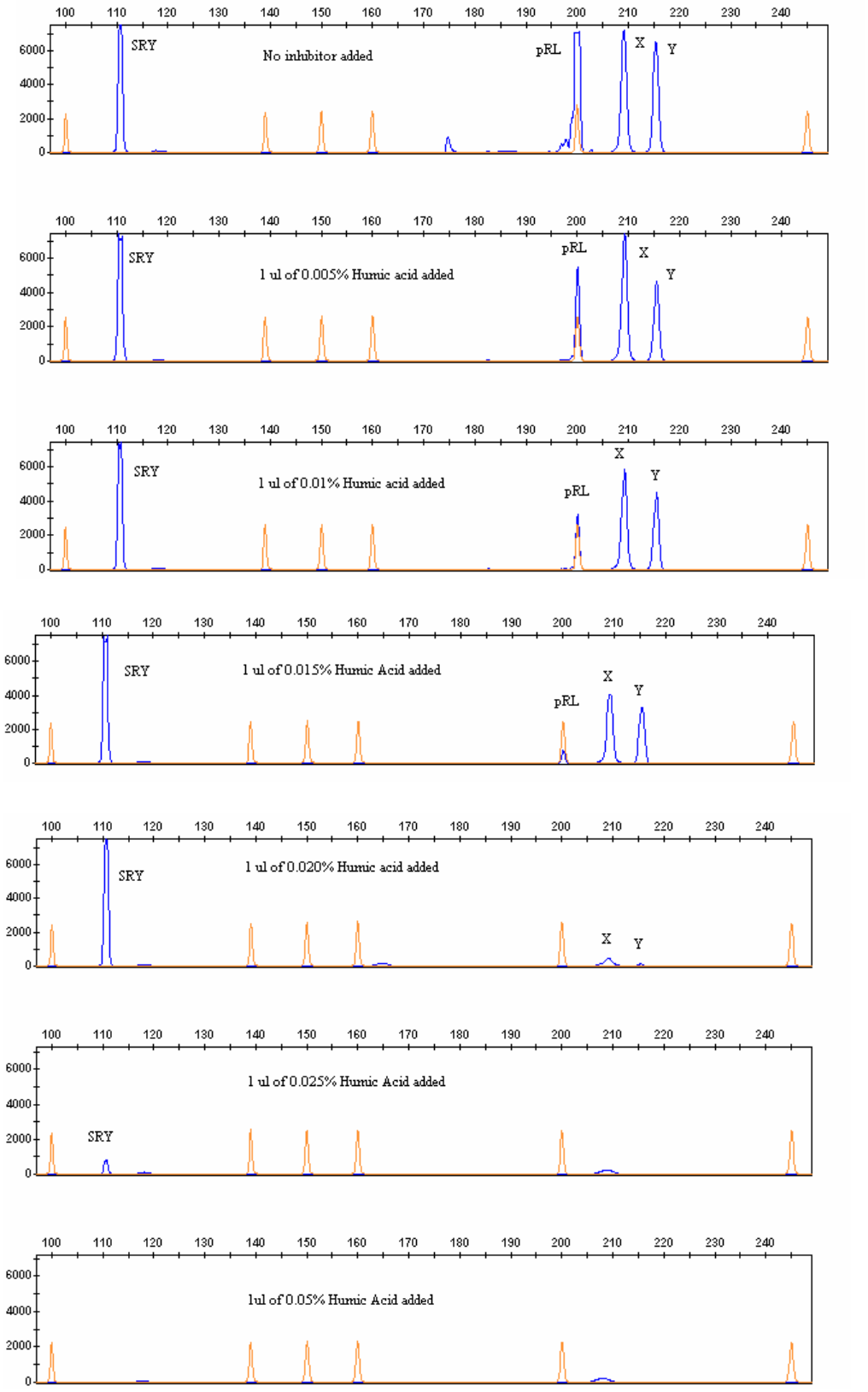

Figure 18: Electropherogram for Humic Acid Inhibition

mM	SRY/LIZ		PRL/LIZ		X+Y/LIZ		Y/LIZ	
HA	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
0.000	44121	888	58000	885	85085	4757	40009	2855
0.004	42046	575	14653	2473	69244	4228	30137	3162
0.008	43365	1102	8724	1651	70695	2468	31638	1799
0.012	41481	5306	1062	647	35525	12212	15512	5226
0.016	35437	3522	0	0	1426	1426	188	188
0.020	2799	978	0	0	0	0	0	0
0.040	0	0	0	0	0	0	0	0

Table 7: Average Data for Humic Acid (RFU)

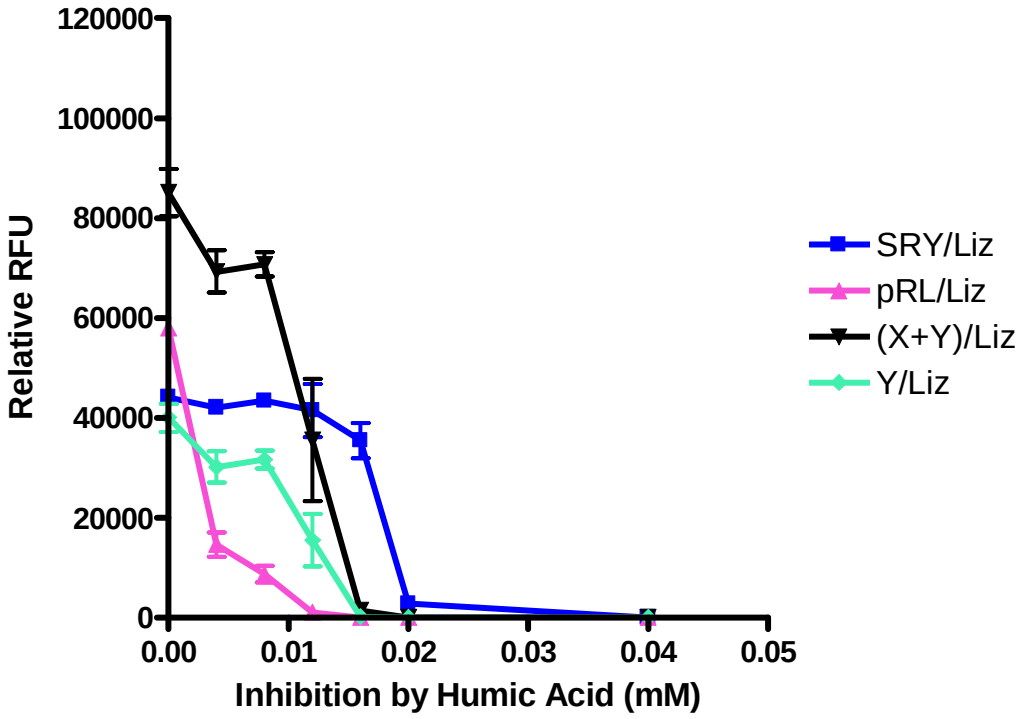

Figure 19: Standard Curves for Humic Acid Inhibition

IV.H.iv. Indigo Dye

Indigo dye from denim is also a common PCR inhibitor encountered in biological evidence recovered from denim fabric. Indigo may be synthetically manufactured in a number of different ways. In the modern synthesis of indigo, which is credited to Pfleger in 1901, phenylglycine is treated with an alkaline melt of sodium and potassium hydroxides containing sodamide. This produces indoxyl, which is subsequently oxidized in air to form indigo and used to dye blue denim fabric (Pfleger,1901; Ferreira et al., 2004). Forensic samples may be deposited on denim with the dye co-extracted with the target DNA. Indigo dye's effect on PCR amplification was studied in the following experiment.

To evaluate the detection of indigo inhibition, five dilutions of 1: 10, 1: 50, 1: 65, 1:85, and 1:100 of the liquid indigo compound were amplified in the Q-TAT assay and the fluorescence of the control pRL product was quantitated (Figures 20, 21 and Table 8).

At the highest concentration of indigo (1:10 dilution), there is a complete inhibition of all four loci (Figures 20, 21 and Table 8). The control pRL product at 1:50 dilution was still the most sensitive and still exhibited a complete inhibition, whilst the SRY allele was essentially unaffected by the inhibitor. The SRY: Y ratio had increased from 1.4 to 39. The larger Y loci had decreased from 30367 at 1250 dilution to 1226 at 1:50 dilution.

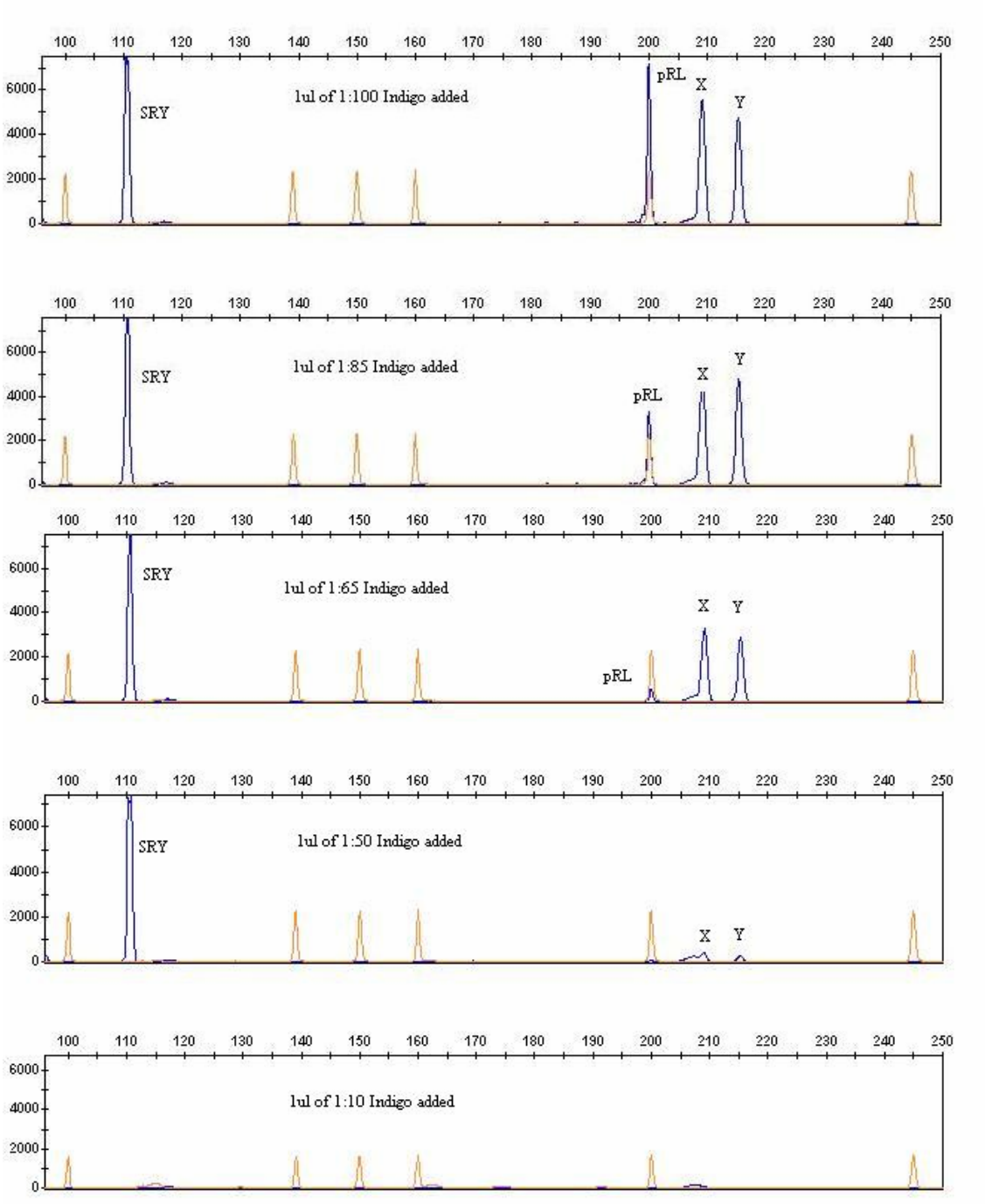

Figure 20: Electropherogram of Indigo Dye Inhibition

Dilution	SRY/LIZ		pRL/LIZ		X+Y/LIZ		Y/LIZ	
Indigo	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
1: 10	0	0	0	0	0	0	0	0
1: 50	47955	2174	0	0	3819	2503	1226	801
1: 65	45186	1827	1472	893	21591	9539	9846	4394
1:85	47075	1869	17212	7514	76222	5223	36328	2315
1:100	44790	1379	15393	5668	68097	3923	30367	1654

Table 8: Indigo: Means with SEM (RFUs)

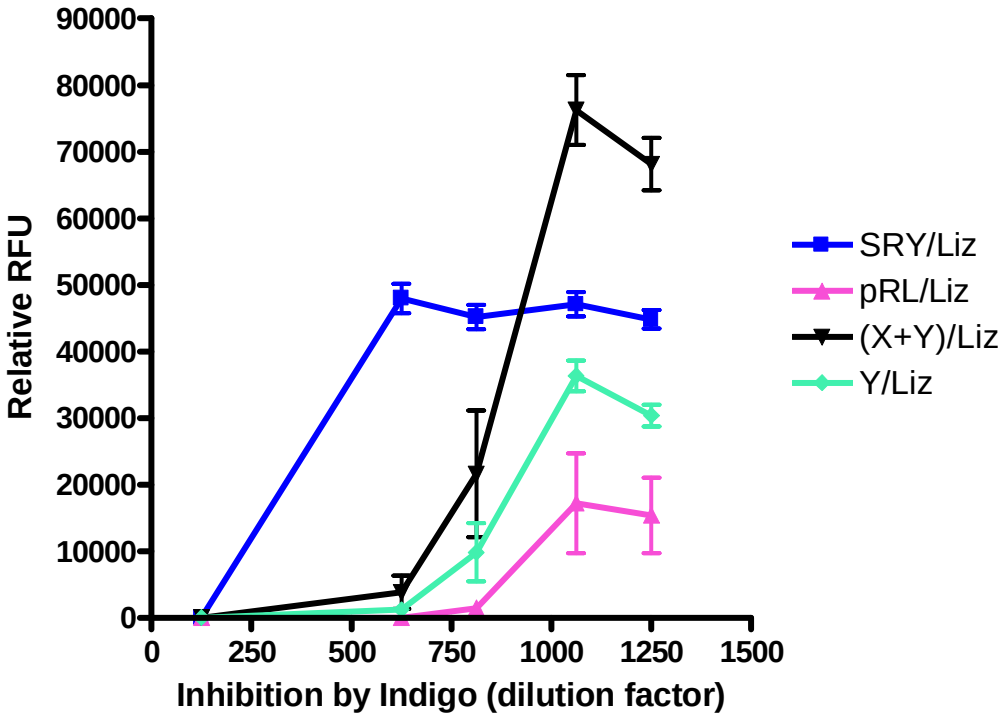

Figure 21 : Standard Curves for Indigo Dye Inhibition (final concentrations)

IV.I. Analysis of DNA Quantitations Methodologies

The true test of any DNA quantitation assay s how well it performs in the quantitation of DNA from actual forensic samples. A group of fourteen forensic samples, previously quantitated by qPCR , were subjected to analysis using the Q-TAT assay.

Of the fourteen samples quantitated with Q-TAT, six were typed as male since the SRY and AMEL-Y amplicons were detected. There could be some degree of DNA degradation for samples 8, 12 and 13, based on their respective SRY/AMEL-Y ratios of 2.4, 3.4 and 4.1 respectively (Table 9), indicating more integrity of the SRY locus than the AMEL-Y locus

Sample #	SRY	Y	SRY/Y	DNase1 minutes	SRY	Y	SRY/Y
5	47538	40528	1.2	0	77484	59938	1.20
7	28016	18270	1.5	5	39943	39036	1.02
8	10248	4232	2.4	10	54373	36366	1.50
11	8036	5452	1.5	20	50523	29875	1.70
12	23490	7010	3.4	40	36533	28116	1.30
13	11887	2872	4.1	60	37092	19934	1.90

Table 9: Comparison of SRY and Y Ratios in Evidentiary Samples and DNase1 Experiments.

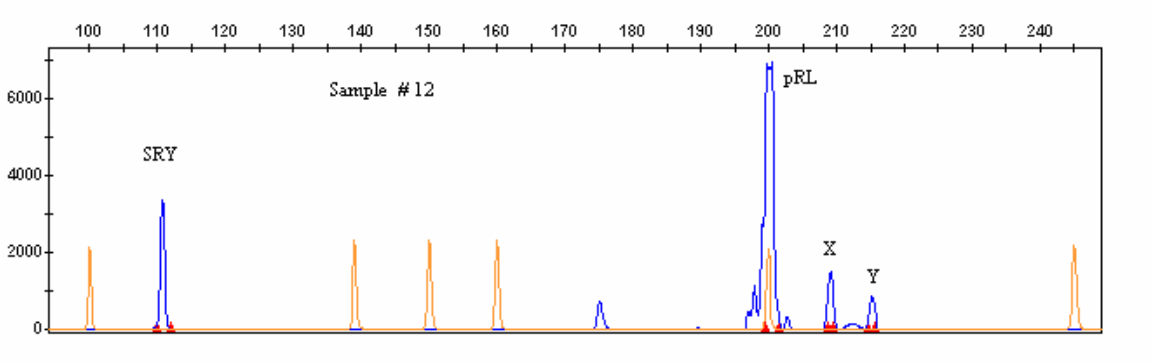

Figure 22: Potential Degradation of DNA template in Evidentiary Sample # 12

The ratio of absorption of UV light at 260 nm and 280 nm is used to evaluate the purity of a DNA sample. A ratio of 260/280 close to 1.8 indicates that the DNA is reasonably pure and the concentration can be accurately determined from the absorbance value. (Maniatis et al.,1982). Spectrophotmetric analysis of the 14 crime scene samples revealed an abnormally high 260/280 nm ratio, probably due to residual phenol or other chemicals used in the extraction process. The samples were therefore "cleaned" free of potential contaminants by binding the DNA to silica. "Clean" DNA samples had A260/A280 ratio more characteristic of pure DNA (Data not shown).

Four of the samples were then quantitated by spectrophotometry and Q-TAT and the results were compared with the qPCR quantitation (Table 10). A threefold range of concentrations were seen with these three techniques of quantitation. Q-TAT and qPCR produced results that agreed reasonably well, although the DNA estimates from qPCR quantitations were consistently higher. The higher Spectrophotometer quantitations obtained for samples 1 and 9 compared to Q-TAT and qPCR may indicate the presence of non-human DNA.

	Q-TAT	Spectrophotometer	qPCR
Sample 1	0.03	10.97	0.046
Sample 6	3.95	14.0	10.9
Sample 9	2.78	10.8	4.91
Sample 14	8.44	14.0	30.28

Table 10: Comparison of Quantitation Estimates using Three Methodologies (ng).

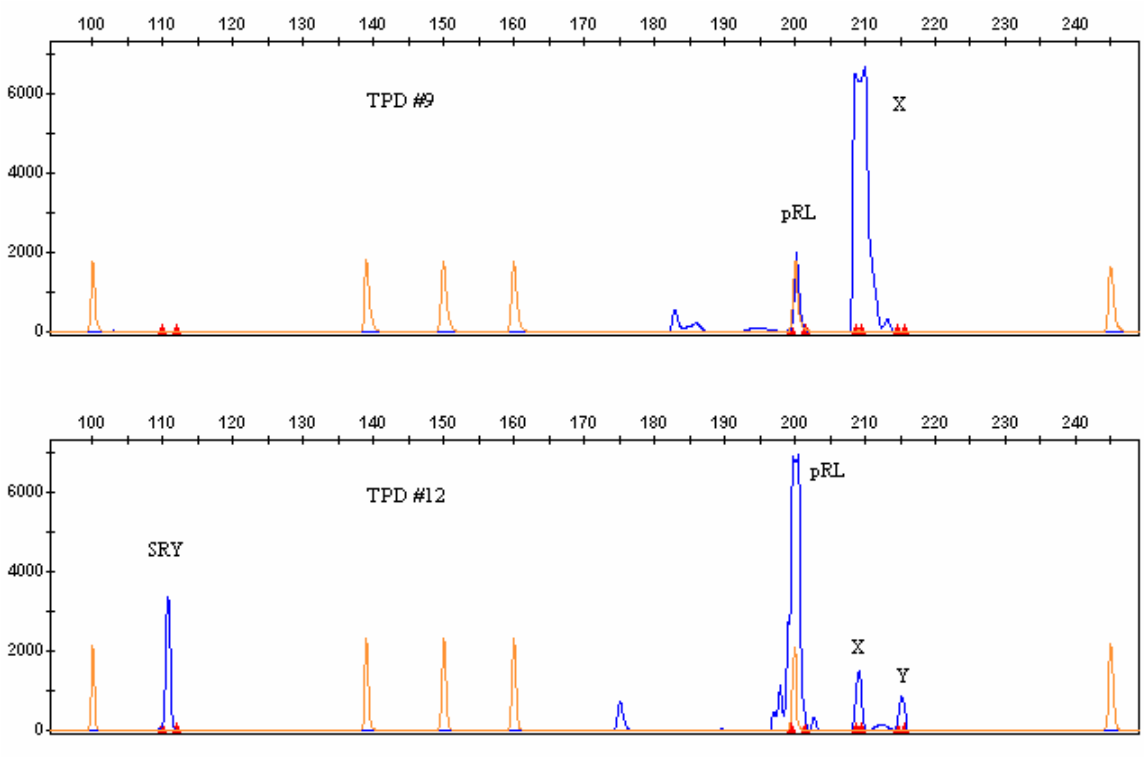

Figure 23: Inhibition in Evidentiary Sample # 9

Out of the fourteen evidentiary samples analyzed with the Q-TAT assay, only sample 9 showed a decrease in the pRL amplicon fluorescence from the average peak area of about 100,000 RFU for all 13 evidentiary samples to about 14,000 RFU in sample 9 (Figure 23). This may be an indication of the presence of an inhibitor in sample 9. Two control experiments were set up to confirm that in the presence of extremely large amounts of female or male DNA (5ng) there was no effect on pRL amplification (data not shown).

CHAPTER V

DISCUSSION

V.A. DNA Degradation

Samples recovered from crime scenes are often degraded and contaminated with inhibitors, leading to poor amplification and preventing the production of STR profiles. It is therefore of great importance in forensic DNA analysis to ensure that the limited amount of DNA found in evidence materials are used in an efficient and effective manner that is likely to produce reliable results.

DNA quantitation methods that are available may not:

- be specific for human DNA
- be PCR based
- be discriminatory enough to assess the quality of DNA as to the extent of degradation or the presence of PCR inhibitors
- be able to reveal the presence of a male: female mixture
- reveal the relative proportions of male and female DNA

At a minimum, a quantitation method must be sensitive and human specific. An ideal quantitation method can also alert an analyst to the quality of the DNA template recovered from a sample both in terms of the integrity of the DNA and the presence of inhibitors. Another desirable feature of a quantitation assay would be the ability to detect the presence of DNA from more than one donor. It is clear from Table 11 below that none of the quantitation methods listed possess all the ideal criteria.

Quantitation Methods

Method	Det	Sensit	Spec	Range	M/F	Deg	Inh	Auto	Ref
A260	UV	poor	N	Estimate	N	N	N	N	Maniatis 1982
Yield G	Fl	poor	N	Estimate	N	Y	N	N	VDFSci(2006)
QuantiB	Hyb	good	Y	>150pg	N	N	N	N	Walsh (1992)
P-green	Fl	good	N	>250pg	N	N	N	Y	Hopwood (1997)
A-Quant	Hyb	good	Y	>100pg	N	N	N	N	Mandrekar (2001)
Alu	ePCR	good	Y	>100pg	N	N	N	N	Sifis (2002)
BodeQ	ePCR	good	Y	>100pg	N	N	Y	N	Fox (2003)
THO1	ePCR	good	Y	>100pg	N	N	Y	N	Nicklas (2003a)
Q-TAT	ePCR	good	Y	>500pg	Y	N	N	N	Allen (2006)
Q-filer	qPCR	good	Y	>500pg	N	N	Y	N	A-Biosystems
Q-filerY	qPCR	good	Y	>500pg	Y	N	Y	N	A-Biosystems
Alu	qPCR	good	Y	>500pg	N	N	Y	N	Nicklas (2003c)
AMEL	qPCR	good	Y	>500pg	Y	N	Y	N	Alonso (2004)
THO1	qPCR	good	Y	>500pg	N	N	Y	N	Richard (2003)
THO1	qPCR	good	Y	>500pg	N	N	N	N	Timken (2005)
RBmtDNA	qPCR	good	Y	>500pg	N	Y	Y	Y	Andreasson (2002)

Table 11: Summary of various DNA quantitation methods (Butler 2005) (modified)

Det (detection), Sensit (sensitivity), Spec (human specific), M/F (male/female) Deg (degradation), Inh (inhibition), Auto (automation), Ref (references), Fl (fluorescence), Hyb (hybridization), ePCR (end point PCR), qPCR (real time PCR)

The Q-TAT assay was developed to quantitate the total amount of human DNA in a sample through the amplification of the amelogenin locus on the X and Y chromosomes (Allen and Fuller, 2006). The original Q-TAT assay not only provided the quantity of DNA in sample, but also detected the presence of male: female mixtures of DNA, especially valuable when investigating sexual assaults (Juroske, 2006).

The goal of this study was to enhance the Q-TAT assay to detect PCR inhibitors and the presence of degraded DNA. The strategy for the desired enhancement was to incorporate two additional PCR targets, the SRY gene and the pRL gene, into the Q-TAT assay along with AMEL-X and AMEL-Y.

Several reports have appeared confirming the validity of using miniSTR primer pairs that direct the amplification of small STR locus amplicons of around 100 base pairs in size. Ricci et al (1999) decreased amplified fragment sizes of D12S391from 253 bp to 125 bp using primer pairs located closer to the VNTR and increased the success rate of typing degraded DNA. Using new redesigned PCR primer pairs for THO1, D10S2325, DYS319, DYS19 and CSF1PO that were closer to the STR repeat, resulted in successful production of DNA profiles from highly degraded DNA (Wiegand and Kleiber 2001). STR loci in commercial kits that generate longer amplicons do so because primers have been purposely moved away from the repeat region so that alleles from the different loci amplified in the multiplex did not overlap, to give predictable size variability for different STR loci in multiplex assays. When the primers for the Penta D locus in the Powerplex 16 multiplex are brought very close to the core repeat, amplicon sizes can be reduced from about 400 bp to 100 bp, resulting in a higher success in typing degraded DNA than the conventional multiplex kit (Krenke et al., 2002; Butler et al., 2003).

Many studies have also shown an inverse relationship between the size of the locus and successful PCR amplification from degraded DNA that may be collected from a crime scene. In the Q-TAT assay, amplification of the amelogenin locus on the X and Y chromosomes results in product sizes of 210 bp and 216 bp respectively. Because these two amplicon differ by 6 bp, they can be separated by capillary electrophoresis. The fluorescence in each amelogenin amplicon is proportional to the amount of input DNA template, allowing for the estimation of total human DNA, and studies by Juroske (2006) have shown the fluorescence in AMEL-X and AMEL-Y amplicons can be used to detect mixtures of male and female DNA and estimate the relative contributions of DNA from each contributor. Equal efficiency of PCR amplification of the amelogenin locus on the X and Y chromosomes should produce an X: Y ratio of 1.0. Repeated analysis among single source male samples shows no significant difference in the amplification of AMEL-X versus AMEL-Y except at low levels of input DNA (Juroske, 2006). Below an input of 62.5 pg of template DNA, "stochastic" effects can adversely affect PCR amplification such that the proportionality of input template to amplicon product is disrupted. Work from Juroske (2006), with male: female mixtures also showed that reliably estimating the quantity of male DNA when male DNA is less than 10-20% of the total is not possible. This is likely the result of the excess AMEL-X target which more effectively competes during PCR for amelogenin primer. The inclusion of the SRY target in the Q-TAT assay (which is not present on the X chromosome) will eliminate competition for primers during amplification and therefore should be better able to accurately estimate minor contributions of male DNA in the presence of excess female DNA.

Alonso and coworkers (2004), also targeted the amelogenin locus on the X and Y chromosome using the 5' nuclease Taqman assay, making their assay suitable for DNA quantification and sex determination simultaneously. They used primers that amplified AMEL-X (106 bp) and AMEL-Y (112 bp) which is close to STR alleles produced using miniSTR typing. Their study confirmed the inverse correlation between amplicon length and amplification efficiency in the quantification of degraded DNA. An allele dropout was observed for samples containing DNA fragments less than 100 bp.

The Quantifiler Y Human Male DNA Quantification Kit (Applied Biosystems, Foster City, CA) is a qPCR assay that targets the SRY locus on the Y chromosome for the estimation of the male DNA in a sample (Applied Biosystems, 2005). However, this assay provides no simultaneous information about any possible female contribution DNA to the sample. Two qPCR reactions therefore must to be performed in order to compare to the Q-TAT assay.

The Forensic Science Service in Great Britain first demonstrated the value of miniSTR typing of degraded samples during their involvement with investigating the Waco fire disaster. A correlation was observed between successful typing and the average length of alleles. The miniSTR VWA locus (130-169 bp) for example yielded 115 successful DNA profiles whilst the conventional FES/FPS locus (212-240 bp) only yielded 91 successful profiles, confirming that with degraded DNA, amplification of larger alleles failed first. Amelogenin amplicons (106 and 112 bp) yielded a 100 % successful typing on all samples.

Chung and coworkers (2004), studied newly developed miniSTR markers present in miniplexes that generate STR profiles consisting of alleles of smaller size. The

effectiveness of primer sets in the miniplex to produce STR profiles were tested with DNA, enzymatically degraded using DNase 1 as well as degraded DNA from bone samples, and compared profiles produced with miniplexes with those generated using the traditional Powerplex 16 commercial multiplex STR typing kit. The results from both types of degraded DNA showed that the miniplex primer sets produced more complete profiles when compared to larger sized amplicons from the Powerplex 16 kit.
Their data confirmed that the redesigned primers yielded successful amplifications where the standard commercial kit with larger sized STR loci failed. Immediately following DNA degradation with DNase 1, the Penta D and Penta E loci, with allele sizes between 365-480 bp, dropped out first followed by D18S11, CSF1PO and FGA , (350-250 bp), and then finally D16S539 and TPOX alleles (179-222 bp). Only the alleles form the smallest loci (THO1, D5S818, and vWA) were detectable when the DNA sample were degraded to an average size of 126 bp. These results unequivocally confirmed the effect of template size and amplification efficiency.

In the present study, degradation experiments with DNase 1 followed a similar trend demonstrated in the study by Chung and coworkers (2004). When degraded template was amplified with Q-TAT, the SRY amplicon (110 bp) was more successfully amplified than the larger AMEL-Y locus. After 60 minutes of DNase1 digestion, even the smaller SRY locus became affected by DNase1 with amplicons dropping from the initial fluorescence of 80,000 RFU to about 40,000 RFU (a drop of 2X), whereas the larger the AMEL-Y amplicon had dropped from 60,000 RFUto 20,000 RFU (a drop of 3X) under the same conditions. In addition, the initial drop in fluorescence in AMELY after 5 minutes was steeper than the SRY amplicon. After the initial speedy degradation for the

AMEL-X, AMEL-Y and the SRY DNA, there was a slight rise and then a steady gradual degradation over the 60 minute period. In contrast, the pRL amplicon fluorescence remained steady over the entire one hour incubation.

V.B. Multiple Male Specific PCR Targets

In addition to serving as an indicator for degradation, the SRY gene target is an additional indicator for maleness. The amelogenin gene has been shown to be an effective method for gender typing of biological samples from crime scenes, especially in sexual assault cases. However, results are not full proof as a rare deletion of the amelogenin gene on the Y chromosome has been described that eliminates the amplification of AMEL-Y from the male DNA present in the sample leading to a failure to overlooking that sample because of the erroneous opinion of the analyst that it does not contain male DNA (Thangaraj et al., 2002).

Thangaraj and coworkers (2002) demonstrated the deletion of the amelogenin gene on the Y chromosome in 1.85% of East Indian males. Among these donors, the PCR amplicons from the SRY gene were still present and would therefore indicate a male sample. Santos and coworkers (1998) also reported AMEL-Y deletions in two Sri Lankan males and Steinlechner and coworkers (2002) revealed this deletion in 6 out of 29,432 (0.2%) of Austrian males. Among Indians and Malays, Chang and coworkers (2006) observed amelogenin mutations rates of 3.2 and 0.6% respectively. This study also found that the individuals harboring amelogenin mutations predominantly belonged to the J2e lineage of Y-STR haplotypes, suggesting a common ancestor for some of these chromosomes.

One of the most important databases of DNA information obtained from samples of biological evidence from crime scenes is the Combined DNA Index System (CODIS). The original purpose of CODIS was to track sex offenders because of the high recidivism of those convicted of these kinds of charges. 46,325 investigations have resulted in hits in the CODIS system in unsolved rapes and murders using the 13 CODIS loci (CODIS, 2007). Though the number of hits is high and impressive, reliance on the AMEL-Y gene amplicon to identify suitable evidence for STR typing, with a possible 1.85 % mistyping rate due to amelogenin mutation, could have eliminated many of the samples linked to sex offenders because those samples would be ignored thinking they contained only female DNA. The reliability of gender testing especially in sexual assault cases is therefore of paramount importance, and a quantitation assay like Q-TAT that incorporates both the AMEL-Y and SRY genes products simultaneously for gender determination, will be more reliable and efficient in detecting and quantitating male DNA present in sexual assault evidence.

AMEL-X amplicon drop out has also been reported in males, with only the presence of the AMELY amplicon in 3 out of 7,000 males (Shewale et al., 2000). This is due to a rare polymorphism at the amelogenin primer binding sites in commercial STR kits, but when different primers targeting the 6 bp deletion are used on the X chromosome, both the amelogenin X and Y loci are amplified (Shewale et al., 2000).

V.C. PCR Inhibition

The presence of PCR inhibitors can completely inhibit the amplification of DNA markers from chromosomal DNA or reduce the sensitivity of detection, usually for larger PCR amplicons (Butler, 2005). PCR inhibitors typically originate from the substrate harboring biological evidence, the sample itself, or from chemicals co-extracted with DNA using typical extraction methods. Three PCR inhibitors most commonly found in forensic samples are hemoglobin, indigo dyes from denim and humic acid from soil (Butler, 2005). EDTA, another common PCR inhibitor is used as blood preservative and anticoagulant in purple topped blood collection tubes. EDTA will also chelate Mg^{2+} which is required as a cofactor for Taq polymerase during PCR. EDTA is also a common component in buffers used in DNA laboratories. In high enough doses, EDTA may act as a PCR inhibitor preventing amplification.

Three mechanisms can be proposed by which inhibitors interfere with PCR amplification: (1) inactivation of Taq DNA polymerase, (2) degradation and /or sequestration of nucleic acids, and (3) interference with cell lysis during DNA extraction, but the identities and biochemical mechanisms of many inhibitors still remain unclear (Wilson, 1997).

PCR inhibition can most easily be studied using dose-response PCR assays with defined amplification templates. Adding different concentrations of the inhibitor to a well characterized PCR reaction assesses the strength of the inhibitor on the amplification capacity of template (Radstrom et al, 2004). The Q-TAT assay incorporates a plasmid vector harboring the luciferase gene from the marine coelenterate known as the sea pansy (Promega Catalog). This template (known as pRL) was added to the Q-TAT multiplex as

a potential indicator of the presence of PCR inhibitors. Thus, different concentrations of known or suspected PCR inhibitors were used to evaluate the sensitivity of the pRL template as an indicator of PCR inhibitors.

The ability of the modified Q-TAT assay to reveal the presence of EDTA, which chelates the cofactor Mg++ in PCR reaction was studied by adding different concentrations of EDTA to Q-TAT master mix containing 500pg of male DNA. The pRL plasmid revealed the presence of inhibitor at 0.020 mM EDTA with an almost complete inhibition at 0.060mM EDTA. In contrast, the AMEL-X, AMEL-Y and the SRY amplicons resisted inhibition until 0.060 mM EDTA. At 0.080 mM EDTA all four loci were completely inhibited. The AMEL-Y locus was inhibited more readily by EDTA than the SRY locus, possibly because the AMEL-Y amplicon is about twice the size of SRY. The general observation that larger STR markers disappear first in degraded DNA may also apply to the PCR inhibitors simply due to the efficiency of amplification of the smaller SRY target (Whitaker, 1995; Alonso, 2002). Regardless of the varying sensitivities of the two human loci to inhibitors, the pRL inhibition indicator is exquisitely sensitive to inhibition by EDTA.

Some PCR inhibitors may interfere with amplification even at lower concentrations. The heme compound found in DNA extracted from bloodstains is regarded as a major inhibitor of Taq polymerase in 0.004% (v/v) of human blood (Al-Soud and Radstrom 1998). This contaminant is sometimes co-purified with DNA by phenol/chloroform treatment and ethanol precipitation. In a chromatographic study, hemoglobin and lactoferrin were found to be the major PCR inhibitors derived from erythrocytes and leukocytes respectively. These compounds contain iron and the

mechanism of inhibition may be due to the release of iron ions into the PCR reaction mixture, and iron interferes with DNA synthesis. The derivatives of hemoglobin such as bilirubin, bile salts and hemin have also been found to inhibit PCR amplification (Al-Soud and Radstrom, 2001). In a study by Byrnes et al., (1975), hemin was found to be a competitive inhibitor with target DNA and a noncompetitive inhibitor for the nucleotides through direct action with DNA polymerase. Heme was also found to regulate DNA polymerase activity and coordinate the synthesis of hemoglobin by feedback inhibition.

The effect of alkaline hemin on PCR inhibition was assessed in five concentrations of 0.008 mM, 0.020 mM, 0.040 mM, 0.060 mM and 0.080 mM. Again, the pRL locus showed the greatest sensitivity, beginning at 0.020 Mm hemin, and decreased steadily, with complete amplification failure at 0.80 mM hemin. The AMEL-X and AMEL-Y loci also showed a gradual loss of amplification with amplicon fluorescence decreasing from about 110,000 RFU at 0.008 mM to 30,000 RFU at 0.080 mM hemin. Amplification of the SRY locus was less affected by hemin inhibition than AMELX and AMELY, possibly due to the smaller size of the amplicon product. The SRY locus being a smaller size, maintained a constant RFU from 0.008mM to0.080 mm hemin, whislt the larger AMEL-Y locus showed a steady loss of amplification from a high of 50,000RFu at 0.008mM hemin to 15,000 RFU at 0.08 mM hemin

Amplification of a DNA sample containing an inhibitor such as hemin can prevent amplification of alleles from larger sized STR loci or even completely inhibit producing any STR results whatsoever (Butler, 2005). In this study, there was a complete failure to amplify the pRL target in the presence of 0.080 mM hemin, and clear inhibition

of AMEL-Y and AMEL-X amplification as well, confirming pRL as a very sensitive indicator of PCR inhibition.

Humic substances are also major concern for inhibition of amplification of target DNA extracted from evidence in contact with soil. Humic compounds have been reported to inhibit PCR amplification of STR loci and sometimes prevent STR results form being produced (Tsai and Olson, 1992). While it is uncertain how the inhibitor exerts its effect, Taq polymerase may be degraded or denatured, or have reduced enzymatic activity in the presence of humic acid present in biological samples, recovered from crime scenes (Al-Soud and Radstrom, 2001). Taq DNA polymerase from different commercial sources is inhibited to different extent by humic substances in soil extracts (Tebbe and Vahjen, 1993). Inhibition by humic acids may also result from the chelation of magnesium ions required by Taq polymerase as a cofactor (Tsai and Olson, 1992).

Consistent with other inhibitors tested, the pRL target was the most sensitive indicator of humic acid inhibition, with amplification inhibited to almost 50 % at the lowest concentration of inhibitor of 0.004 mM. The SRY target was not appreciably inhibited by increasing concentrations of humic acid, until a high concentration of 0.020 mM was achieved. In contrast, the AMELY amplicon, being larger, failed to amplify at 0.025 mM humic acid. At 0.040 mM which was the highest concentration used in this study, all four amplicons exhibited complete inhibition. The failure to amplify larger sized STR loci for a forensic sample can be due to either template degradation or to a sufficient concentration of PCR inhibitor that leads to the reduced activity of the Taq polymerase. In such samples, the use of miniSTRs in DNA typing may help in the

recovery of information since smaller PCR products have a higher success rate for amplification than larger amplicons.

Fabrics stained with body fluids are common evidentiary materials for DNA analysis found at crime scenes. In most cases extracted DNA products produces robust PCR products for typing, but sometimes inhibition of amplification occurred in some substrates that contained the indigo dye (Del Rio et al., 1996, Shortsleeve et al., 1996). These inhibitors present in the indigo dye occupy the active sites of Taq polymerase preventing its proper function. In the study of Del Rio et al., (1996), they found that amplifiable DNA was obtained only when pre-washed light blue denim was the substrate, but not dark denim, therefore the levels of indigo in the material may be directly related to PCR inhibition. This may suggest that residual indigo dye compounds or its degradation products and derivatives may inhibit PCR when present in concentrated levels. Indigo may not bond strongly to the fiber as pre-washing may remove the concentrated dye from dark blue denim (Del Rio et al., 1996).

In the present study, high concentrations of indigo dye at 1: 10 completely inhibited amplification of all loci during PCR. At 1:50 dilution of indigo dye, there was no amplification product for the pRL allele and minimum amplification for AMEL-X and AMEL-Y. In contrast the SRY amplicon appeared unaffected by indigo at this dilution. The pRL amplicon was the most sensitive to indigo dye inhibition even at 1: 65 dilution of dye. Conversely the SRY amplicon was the least inhibited. These results agree with Del Rio's 1996 study that showed the correlation between the concentration of indigo and PCR inhibition. The sensitivity of the pRL marker to the inhibitor indigo dye mirrored

our experience with the other compounds in that the pRL locus was the most sensitive indicator of the presence of inhibitor in the Q-TAT reaction.

Del Rio et al., (1996), also demonstrated in their study that chelex extraction, but not organic extraction of DNA, was able to yield typable results from DNA deposited on dark blue denim that contained higher concentrations of the indigo dye. Thus the chelex extraction method may be more suited for extracting DNA on blue denim. Rubin, 1998 demonstrated that when proteinase K treated samples were incubated for 10 minutes with the cationic detergent CTAB and the amount of Taq polymerase was doubled, there was robust PCR amplification in previously weak samples.

V.D. Comparison of Quantitation Methodologies

The U.S. National Institute of Standards and Technology (NIST) has conducted several inter –laboratory tests to evaluate DNA quantification methods (Duewer et al, 2001, Kline et al 2003). Although most DNA quantitation assays are precise to within an acceptable factor of two, a ten-fold range of reported concentration was reported for a 1ng DNA sample supplied to 74 laboratories that reported using the Quantiblot technology (Kline et al 2003). Reasons for the wide variation in DNA concentration estimates include the nature of the Quantiblot technology itself which relies on quantitative blotting and hybridization to a labeled probe as well as the rather subjective interpretation of color intensity on the blots which is used to estimate the quantity of DNA in unknowns (Walsh et al., 1992).

As stated in a recent publication by Allen and Fuller (2006), the absolute accuracy of a DNA quantitation method is not as important to the DNA typing laboratory as is the

validity of a method to give consistent results that are close to the true value. Once the method has been validated and revealed the quantity of DNA needed to produce an STR profile of high quality, methods used in the lab can target that ideal quantity of input DNA for case work. The Q-TAT assay meets the requirements for an assay suitable for DNA quantitation in the DNA typing laboratory (Allen and Fuller, 2006). The Q-TAT assay modifications reported here offer enhancements to the basic assay developed by Allen and Fuller (2006) that include detecting the presence of inhibitors in DNA samples, detecting male DNA in samples from males with Y-chromosome mutations that prevent amplification of either AMELY or SRY, but not both, and indicating possible degradation of a male DNA template. It is also likely that the SRY target incorporated into the Q-TAT multiplex may be a better quantitative indicator of small amounts of male DNA in male: female mixtures since, unlike AMELY, the SRY target amplification will not be competing against a counterpart on the X chromosome like the amelogenin locus.

Allen and Fuller (2006) reported reasonable agreement in DNA concentrations between Q-TAT and qPCR in their study, though qPCR consistently yielded higher estimates than Q-TAT. Higher estimates from qPCR were also observed in this study (Table 11). This could be due to the higher dynamic range of qPCR and the fact that accurate estimation of template DNA could be made after the early rounds of the PCR cycles. Because Q-TAT is an end-point PCR technology, DNA template quantitation can be made only on the completion of all cycles, at which time some of the reactants may be limiting. When Q-TAT DNA concentrations are high, a 1:25 dilution of the original concentrate may yield a more accurate estimate of template DNA.

V.E. The Improved Q-TAT Assay

The main goal of this study was to incorporate two additional PCR targets into the existing Q-TAT reaction to detect inhibition and to assess degradation. These two additional PCR targets would enhance the Q-TAT assay and assess the overall quanlity as well as quantity of DNA in a forensic sample. The pRL gene has proven a very sensitive indicator of inhibitory compounds including EDTA, hemin, humic acid and indigo dye. The most important function of an inhibition control is to reveal to an analyst the presence of inhibitory substances in a DNA sample early during testing so that extended clean up measures can be applied to the template before the STR amplification step in the process.

An inverse correlation between amplicon size and amplification efficiency was observed in the degradation and all inhibition experiments. Florescence in the SRY amplicon was the last to disappear in inhibited or degraded DNA samples, confirming the observations of Butler (2005) that the loss of larger STR loci amplicons can be either due to degraded DNA or due to the presence of PCR inhibitors.

In summary, the advantages of the improved Q-TAT assay over the assay reported by Allen and Fuller (2006), as well as other quantitation methods includes:

- Quantitates of total human DNA in a sample
- A PCR method with good dynamic range
- Accurate estimates of the relative proportions of male and female DNA in mixed samples
- Low cost, easily incorporated in a laboratory setting as a routine DNA quantitation method

- Detects common PCR inhibitors from crime scene samples
- Provides some indication of DNA degradation
- Evaluates samples not amenable to differential extraction
- Successful amplification of SRY (110 bp) when there is preferential dropout of the AMEL-Y (216 bp) allele in samples containing minor amount of male DNA
- Multiple targeting of male DNA (SRY and AMEL-Y)
- Produces DNA concentration estimates comparable to other quantitation methods

The future development of quantitation methods will expand in response to the growing demand for rapid, robust and more discriminating protocols integrating screening and quantitation with automated operations.

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VITA

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Scope and Method of Study: The purpose of this study was to enhance the Q-TAT assay to detect the presence of PCR inhibitors and to assess the extent of DNA degradation in a forensic sample. The specific aim was approached experimentally through the simultaneous detection of inhibition and degradation by adding a): the SRY gene (110bp) to the Q-TAT reaction to assess DNA degradation and as an additional marker for the unambiguous gender identification, and b): the Renila luciferase pRL gene cloned in the pRL-null vector (200bp) to detect the presence of PCR inhibitors. Q-TAT was used to evaluate enzymatically degraded DNA and to evaluate the detection of EDTA, hemin, humic acid and indigo inhibition in PCR reactions. DNA estimations using the modified Q-TAT were then compared to qPCR and UV spectrophotmetry using evidentiary samples. All amplicons were analyzed on the310 Genetic Analyzer, and quantitated using Genescan software and Excel spreadsheets. STR analysis was used to ensure the quality of results from Q-TAT estimations.

Findings and Conclusions: Two internal standards were successfully incorporated into the basic Q-TAT assay, the SRY amplicon at 110 bp to assess degradation, and the pRL amplicon at 200 bp to detect inhibition. An inverse correlation between amplicon size and amplification efficiency was observed in the DNA degradation and PCR inhibition studies. Deviations from the expected SRY: AMEL-Y ratio of 1.0, observed in the modified Q-TAT assay for intact DNA, could reflect on possible degradation of the genomic template. The pRL amplicon proved in all four inhibition studies using EDTA, hemin, humic acid and indigo dye to be a very sensitive detector of PCR inhibition. Comparison studies confirmed that the improved Q-TAT assay produced concentration estimates comparable to other quantitation methods, and could also be utilized to assess DNA degradation and PCR inhibition in evidentiary samples.

ADVISER'S APPROVAL: Dr. Robert Allen